(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 November 2014 (27.11.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/190138 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2014/039110

(22) International Filing Date:
22 May 2014 (22.05.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/826,484 22 May 2013 (22.05.2013) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/190138 A2

(54) Title: MEASURES OF SHORT TELOMERE ABUNDANCE

(57) Abstract: This invention provides methods and materials for measuring telomere abundance from chromosomes in a sample having telomeres within a pre-determined length range, e.g., short telomeres up to a certain length. The methods can involve a first step of performing a time- limited extension reaction calibrated to produce extension products from a double-stranded chromosomal DNA template of no more than a defined length, and a second step of amplifying, from the extension products, sequences bounded by a sub-telomeric sequence and the anchor sequence, to produce a length-limited telomere sequence product. The abundance of telomeric sequences in this product can be measured, and the measures can be correlated to a variety of indices.

MEASURES OF SHORT TELOMERE ABUNDANCE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This Application claims the benefit of U.S. Provisional Application No. 61/826,484, filed on May 22, 2013, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The statements in the Background are not necessarily meant to endorse the characterization in the cited references.

[0003] Telomeres, the tips of eukaryotic chromosomes, protect the chromosomes from nucleolytic degradation, end-to-end fusion, and recombination. Telomeres are structures at the ends of chromosomes characterized by repeats of the nucleotide sequence $(5'\text{-TTAGGG-}3')_n$. Telomeres shorten as a consequence of normal cell division and critically short telomeres lead to cellular senescence or apoptosis. A rich body of epidemiological and clinical studies in humans in the past decade has linked short telomere length to high risks of aging-related disease and all-cause mortality (Puterman, E. and E. Epel, Soc Personal Psychol Compass, 2012. **6**(11) 807-825; Zhu, H., M. Belcher, and P. van der Harst, Clin Sci (Lond), 2011. **120**(10) 427-40; and Fyhrquist, F. and O. Saijonmaa. Ann Med, 2012. **44 Suppl 1** S138-42). Genetic, environment, lifestyle, and behavioral factors collectively impact telomere length. Therefore, telomere length has become an index for overall health, disease, and mortality risk.

[0004] While average telomere length was measured in almost all the clinical studies published and has demonstrated utility in stratifying patient disease and mortality risk, recent work in mice has also shown that the population of short telomeres is the triggering signal to senescence or apoptosis (Hemann, M.T., *et al.*Cell, 2001. **107**(1) 67-77), and thus disease and mortality risk. In a study reported by Hemann et al, 6th generation telomerase RNA knockout mice (mTR-/- G6) with short telomeres were crossed with mice heterozygous for telomerase (mTR+/-) with long telomeres. The phenotype of the telomerase null offspring mirrors that of the mTR-/- parent despite the fact that half of their telomeres are long, suggesting that the quantity of short telomeres, and not average telomere length, is critical for cell viability and chromosome stability. In people taking a natural product-derived telomerase activator (TA-65®), a significant reduction in the percentage of short (<3 or <4 kbp) telomeres (as measured by a quantitative FISH technology; see (Canela, A., *et al.* Proc Natl Acad Sci U S A, 2007. 104(13) 5300-5) was detected in the leukocytes, although no change in mean telomere length was seen (Harley, C.B., *et al.,* Rejuvenation Res. 2011. 14(1)

45–56). Changes in the percentage of short telomere abundance therefore is expected to be a more sensitive measurement of the effects of lifestyle and pharmacological or other interventions on telomeres. Another study (Vera *et al.*, "The Rate of Increase of Short Telomeres Predicts Longevity in Mammals", Cell Reports (2012), world wide web URL: dx.doi.org/10.1016/].celrep.2012.08.023) found that "the rate of increase in the abundance of short telomeres was a predictor of lifespan".

[0005] Various methods have been developed for the measurement of telomere length in genomic DNA, including Southern blotting (Kimura, M. *et al.*, *Nature Protocols*, 2010, **5**:1596-1607), Q-FISH (Rufer, N. *et al.*, *Nat. Biotechnol.*, 1998, **16**:743-747), flow FISH (Baerlocher, G. M. *et al.*, *Cytometry*, 2002, **47**:89-99), and qPCR (Cawthon, R. M., *Nucleic. Acids Res.*, 2002, **30**(10):e47). All of these methods can be used in a clinical setting to monitor health status and permit physicians to prescribe prophylactic or therapeutic intervention tailored to the needs of the individual patient.

[0006] To measure the population of short telomeres, quantitative fluorescent in situ hybridization (Q-FISH) of metaphase-spread cells has been used to generate histograms of telomere signal intensities which represent the length of individual telomeres (Poon, S.S., *et al.*, Cytometry, 1999. **36**(4) 267-78). Limitations of this method are that live cells are needed, costs are high, and throughput is low. A higher throughput modification of the Q-FISH assay (HTQ-FISH; see Canela, A., *et al.* Proc Natl Acad Sci U S A, 2007. **104**(13) 5300-5) was recently championed by the company Life Length (Spain) to measure percentage of short telomeres. Despite the claim, unfortunately, with current technology, this assay cannot be accurate, due to clustering of telomeres, especially short telomeres, in single spots (telomeric associations; see Paeschke, K., K.R. McDonald, and V.A. Zakian. FEBS Lett, 2010. **584**(17) 3760-72). Confounding this issue is the fact that short telomeres tend to associate with one another more frequently than long telomeres. In addition, FISH technologies are known to suffer from non-specific binding of the probe to macromolecules in live or fixed cells. A high throughput method to measure percentage of short telomeres that is low-cost and does not require live cells will be much easier to be adapted in both epidemiological and clinical settings, and will have better analytical performance than Q-FISH.

[0007] U.S. Patent No. 5,741,677 (Kozlowski *et al.*) refers to methods for measuring telomere length. One method involves contacting the telomere with a linker sequence under conditions in which the linker sequence is ligated or otherwise covalently bonded to the 3′ end of the telomere. The telomere sequences are amplified by long PCR amplification with a

first primer specific for the linker sequence and a second primer specific for a subtelomeric region of the chromosome. Another method involves preparing DNA extracts of cells, incubating the extract with an oligonucleotide probe complementary to a telomere repeat sequence, and determining amount of probe bound as a measure of telomere length. In addition, a method of measuring telomere length by binding the genomic DNA to a solid phase, and hybridizing the bound DNA with a labeled probe is described.

[0008] U.S. Patent Publication No. 2004/0265815 (Baird *et al.*) refers to a method for measuring telomere length. Baird *et al.* describes the following steps to detect the length of a population of telomeres: a) annealing the 3′ end of a single-stranded oligonucleotide (hereinafter referred to as a telorette) to a single-stranded overhang of the telomere comprising the G-rich telomere strand (comprising TTAGGG repeat sequences) and covalently binding the telorette to the 5′ end of the C-rich telomeric strand (having CCCTAA repeat sequences), b) amplifying the ligation product formed in step (a) to form a primer extension product; and (c) detecting the length of the primer extension product(s) of step (b). (See also Baird, D.M., *et al.*, *Nat Genet.*, 2003, **33**(2):203-7; and Baird DM, Rowson J, Wynford-Thomas D, Kipling D.; *Nat Genet.*, 2003, **33**(2):203-7. Epub 2003 Jan 21. PMID: 12539050)

[0009] U.S. Patent No. 6,514,693 (Lansdorp) refers to a method for detecting multiple copies of a repeat sequence in a nucleic acid molecule in morphologically intact chromosomes, cells, or tissue sections comprising: (a) treating the nucleic acid molecule with a PNA probe which hybridizes to a repeat sequence in the nucleic acid molecule and which is labeled with a detectable substance, under denaturing conditions utilizing a denaturing agent, permitting the probe to hybridize in situ to the repeat sequence in the nucleic acid molecule; and (b) identifying said probe hybridized to the repeat sequence in the nucleic acid molecule by directly or indirectly detecting the detectable substance, thereby detecting the multiple copies of a repeat sequence in a nucleic acid molecule.

[0010] Methods of determining short telomere abundance include Southern blot analysis, quantitative fluorescence in situ hybridization (Q-FISH) (Poon, S.S., *et al.*, *Cytometry*, 1999, **36**(4):267-78) and a modified high throughput version of Q-FISH (HT-Q-FISH) (Canela, A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 2007., **104**(13):5300-5).

[0011] U.S. Patent No. 7,695,904 (Cawthon) describes methods for amplifying target nucleic acids using nucleic acid primers designed to limit non-target nucleic acid dependent priming events. The methods permit amplifying and quantitating the number of repetitive units in a repetitive region, such as the number of telomere repetitive units. The patent also

refers to determining average telomere length of an organism by qPCR method.

[0012] Thus, despite advances in materials and methods pertaining to telomeres, there remains a need for improved methods and materials for determining measures of short telomere abundance in a population of chromosomes and the use of these measures to determine measures of health and effects of interventions that increase or decrease telomere length and, hence, increase or decrease health, or conversely decrease or increase risk of future disease or death, respectively. These needs and other needs are addressed by the present invention.

SUMMARY OF THE INVENTION

[0013] In one aspect, this invention provides a method of making a nucleic acid extension product comprising: i) hybridizing an extension primer to a telomeric repeat sequence in a 3′ overhang of double stranded chromosomal DNA, wherein: (1) the double stranded chromosomal DNA has a telomeric region comprising telomeric repeat sequences and a sub-telomeric region comprising sub-telomeric sequences; and (2) the extension primer comprises: (A) a 3′ portion that hybridizes to a telomeric repeat sequence in the 3′ overhang under annealing conditions, and (B) a 5′ portion having an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3′ overhang under the annealing conditions; and ii) performing a time-limited extension reaction to extend the extension primer towards the sub-telomeric region of the double stranded chromosomal DNA, wherein the extension reaction is timed to produce an extension product comprising both telomeric repeat sequences and sub-telomeric sequences only from double stranded chromosomal DNA having a telomeric region within a pre-determined length range. In one aspect the double-stranded chromosomal DNA comprises chromosomes molecules having telomeres of different lengths. In a further aspect, the anchor sequence does not hybridize under the annealing conditions to: (1) a sequence in the sub-telomeric region of the strand of chromosomal DNA having the 3′ overhang; (2) a sequence in the G-strand of the chromosomal DNA within 20 kb of the 3′ overhang; (3) a sequence in the G-strand of the chromosomal DNA within 50 kb or within 20 kb of the 3′ overhang; or (4) a sequence in the double stranded chromosomal DNA. In a further aspect, the extension reaction is timed to no more than 30 minutes, no more than 10 minutes, no more than 5 minutes, no more than 4 minutes, no more than 3 minutes, no more than 2 minutes, no more than 1 minute, no more than 30 seconds, no more than 20 sec, no more than 10 sec, no more than 5 sec, or no more than 2 sec. Since the rate to primer extension can range from high (400 nucleotides per sec) to very low (e.g. 50 nucleotides per second), a broad range of extension time allows assessment of a broad range of telomere

lengths (theoretically from about 100 nucleotides, to many thousands of nucleotides. In a further aspect, the extension reaction is timed to at least 30 minutes, at least 10 minutes, at least 5 minutes, at least 4 minutes, at least 3 minutes, at least 2 minutes, at least 1 minute or at least 30 seconds. In a further aspect, the extension reaction is timed to at least 30 minutes, at least 10 minutes, at least 5 minutes, at least 4 minutes, at least 3 minutes, at least 2 minutes, at least 1 minute or at least 30 seconds. In a further aspect, the double-stranded chromosomal DNA is provided from a solid, fluid, semisolid or gaseous sample. In a further aspect, the chromosomal DNA is provided from a liquid sample selected from blood, saliva, urine, plasma, serum, cerebrospinal fluid ("CSF") or bronchoalveolar lavage fluid. In a further aspect, the chromosomal DNA is provided from a solid sample selected from lung, muscle or skin. In a further aspect, the chromosomal DNA is provided from a semi-solid sample comprising bone marrow. In a further aspect, the chromosomal DNA is provided from a gaseous sample comprising breath. In a further aspect, the double-stranded chromosomal DNA is vertebrate DNA, mammalian DNA or human DNA. In a further aspect, the 3′ portion of the extension primer hybridizes to a human telomeric repeat sequence. In a further aspect, the 3′ portion of the extension primer comprises the sequence 5′-(CCCTAA)n -3′ or its same order permutations, wherein n is at least 1. In a further aspect, n is at least 2. In a further aspect, the 5′ portion of the extension primer comprises the sequence: 5′-TGCTCGGCCGATCTGGCATC- 3′ [SEQ ID NO:8]. In a further aspect, the extension primer comprises the sequence: 5′-TGCTCGGCCGATCTGGCATCCCTAACC-3′ [SEQ ID NO: 7]. In a further aspect, the time-limited extension reaction employs a DNA polymerase possessing strand-displacement activity, exonuclease activity or strand degradation activity. In a further aspect, the DNA polymerase is selected from T7 polymerase (e.g., Sequenase), exonuclease-deficient Klenow fragment of E. coli DNA polymerase I, and Bst DNA polymerase large fragment and Deep VentR (exo-nuclease). In a further aspect, the first reaction is performed with a helicase, in combination with a DNA polymerase, or a DNA polymerase with 5′-3′ exonuclease activity.

[0014] In another aspect, this invention provides a method of amplifying telomeric repeat sequences and sub-telomeric sequences of a chromosome comprising: a) making a nucleic acid extension product by: i) hybridizing an extension primer to a telomeric repeat sequence in a 3′ overhang of double stranded chromosomal DNA, wherein: (1) the double stranded chromosomal DNA has a telomeric region comprising telomeric repeat sequences and a sub-telomeric region comprising sub-telomeric sequences; and (2) the extension primer comprises: (A) a 3′ portion that hybridizes to a telomeric repeat sequence in the 3′ overhang

under annealing conditions, and (B) a 5′ portion having an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3′ overhang under the annealing conditions; and ii) performing a time-limited extension reaction to extend the extension primer towards the sub-telomeric region of the double stranded chromosomal DNA, wherein the extension reaction is timed to produce an extension product comprising both telomeric repeat sequences and sub-telomeric sequences only from double stranded chromosomal DNA having a telomeric region within a pre-determined length range; and b) amplifying sequences of the extension product that are bounded by a sub-telomeric sequence and the anchor sequence; thereby producing a length-limited amplification product comprising nucleic acids having telomeric repeat sequences and sub-telomeric sequences. In one aspect the sequences are amplified using: (1) a first amplification primer that hybridizes to a sequence unique to the sub-telomeric region in the extension product under annealing conditions; and (2) a second amplification primer that hybridizes to the anchor sequence under the annealing conditions. In a further aspect, the first amplification primer comprises a sequence selected from: 5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO: 2], 5′-CGGGCCGGCTGAGGGTACCGCGA-3′ [SEQ ID NO: 10] (chromosome 1), 5′-GCTAATGCACTCCCTCAATAC-3′ [SEQ ID NO: 11] (chromosome 5) and 5′-CATTCCTAATGCACACATGATACC-3′ [SEQ ID NO: 12] (chromosome 9). In a further aspect, the first amplification primer comprises the sequence 5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO: 2] and the second primer comprises the sequence 5′-TGCTCGGCCGATCTGGCATC-3′ [SEQ ID NO: 8]. The length range of the amplified telomere products can be determined by using a time-limited extension time in the PCR reaction. In a further aspect, the method further comprising in co-amplifying a control sequence. In a further aspect, the control sequence comprises a plurality of non-telomeric repeat sequences. In a further aspect, the control sequence is synthesized in vitro or produced *in vivo* (e.g., in a bacterial or fungal clone).

[0015] In another aspect, this invention provides a method for determining of short telomere abundance comprising: a) providing a sample comprising double-stranded chromosomal DNA comprising a 3′ overhang from a subject; b) producing a length-limited amplification product from the double-stranded chromosomal DNA using a method of amplifying telomeric repeat sequences and sub-telomeric sequences of a chromosome of this invention as described herein (e.g., above); and c) determining of short telomere abundance from the length-limited amplified product. In one aspect the method further comprises: d) comparing short telomere abundance with total telomere abundance from the sample. In a

further aspect, short telomeres are telomeres having length no more than about than about 0.5 kb, than about 1 kb, than about 2 kb, than about 3 kb, than about 4 kb or than about 5 kb. In a further aspect, comparing comprises determining short telomere abundance as a function of total telomere abundance, e.g., a ratio of short telomere abundance to total telomere abundance. In a further aspect, determining of short telomere abundance is performed using qPCR. In a further aspect, qPCR is performed using a first and a second primer, (i) wherein said first primer hybridizes to at least one repetitive unit of said first strand and said second primer hybridizes to at least one repetitive unit of said second strand, (ii) wherein said hybridized primers are capable of primer extension when hybridized to their respective strands, and wherein at least one nucleotide of said first primer produces an internal base pair mismatch between said first primer and a nucleotide of said repetitive unit when said first primer is hybridized to at least one repetitive unit of said first strand, (iii) wherein said first primer also produces a mismatch with the 3′ terminal nucleotide of said second primer when first and second primers hybridize to each other, (iv) wherein at least one nucleotide of said second primer produces an internal base pair mismatch between said second primer and a nucleotide of said repetitive unit when said second primer is hybridized to at least one repetitive unit of said second strand. In a further aspect, determining of short telomere abundance comprises measuring average telomere length in the sample by Southern blot, dot blot, slot blot, immunochemistry, nucleic acid sequencing or digital PCR. In a further aspect, the short telomere abundance is a measure of relative abundance. In a further aspect, the total telomere abundance is measured relative to abundance of a genomic reference sequence. In a further aspect, the genomic reference sequence comprises a single copy reference nucleotide sequence (e.g., human beta-globin) or abundance of non-telomere repetitive DNA (e.g., Alu repeats or centromeric repeats).

[0016] In another aspect, this invention provides a method comprising: a) determining of short telomere abundance in a sample from a subject; and b) correlating the short telomere abundance with a condition or disease. In one aspect the measure of short telomere abundance is determined by comparing the short telomere abundance with total telomere abundance from the sample. In a further aspect, the short telomere abundance is determined using a method described herein (e.g., above). In a further aspect, the condition or disease is mortality risk. In a further aspect, the telomere abundance is absolute abundance. In a further aspect, absolute abundance is measured as length of telomeric sequences. In a further aspect, the determining of telomere abundance comprises measuring average telomere length in the sample by qPCR Southern blot, dot blot, slot blot, immunochemistry, nucleic acid

sequencing and digital PCR. In a further aspect, the correlation of the condition or disease is associated with the Health Status Survey Score of Perceived Stress (see, for example, Cohen, S; Kamarck T, and Mermelstein R (1983) J. Health Social Behav. 24(4) 385–396). In a further aspect, the risk of a pathological condition is a risk of disease, e.g., cardiovascular disease, diabetes, cancer, liver fibrosis, and depression. In a further aspect, the disease is a disease of aging. In a further aspect, the disease of aging is cardiovascular disease and wherein a measure lower than average in a population correlates with increased risk of cardiovascular disease. In a further aspect, the method comprises correlating a measure of telomere abundance in the lowest two or three tertiles of a population with significantly higher risk for cardiovascular disease compared with a measure in a top tertile of the population. In a further aspect, the method comprises correlating the measure with a telomeric disease. A telomeric disease can include, but is not limited to dyskeratosis congenita, pulmonary fibrosis, aplastic anemia and interstitial pneumonia. In a further aspect, the method comprises the measure with drug responsiveness. For example, the method can comprise the measure with drug responsiveness to a statin (wherein short average telomere length in an individual's normal white blood cells is positively correlated with drug responsiveness) or adverse response to imetelstat (GRN163L, a cancer drug) (wherein short telomere length in normal white blood cells is correlated with adverse effects such as thrombocytopenia or neutropenia). In a further aspect, the method comprises correlating the measure with disease progression and treatment outcome in chronic infections, such as HIV, HCV HBV, and CMV. In a further aspect, the method further comprises reporting the correlation to the subject. In a further aspect, the method further comprises providing the subject with a diagnosis or a prognosis based on the correlation. In a further aspect, the method further comprises treating the subject based on the correlation.

[0017] In another aspect, this invention provides a method for monitoring the status of a subject comprising: determining measures of short telomere abundance from cells in each of a plurality of subject samples taken over a period of time; determining differences in the measures; and correlating the differences with progression of a telomeric disease, wherein decreases in the measures indicates progression of the disease. In one aspect the measure of short telomere abundance is determined by comparing the measure of short telomere abundance with a measure of total telomere abundance from the sample.

[0018] In another aspect, this invention provides a method comprising: determining a rate of change in a measure of short telomere abundance in cells from a plurality of subject samples, each sample taken at different times; and correlating the rate of change with: (1) a

measure of health; (2) a risk of a pathological condition; (3) a telomeric disease or (4) drug responsiveness. In one aspect the measure of short telomere abundance is determined by comparing the measure of short telomere abundance with a measure of total telomere abundance from the sample.

[0019] In another aspect, this invention provides a kit comprising: (1) a first amplification primer comprising: (A) a 3′ portion that hybridizes to a telomeric repeat sequence in a 3′ overhang of double-stranded chromosomal DNA under annealing conditions, and (B) a 5′ portion having an anchor sequence that does not hybridize under the annealing conditions to a sequence in the telomeric region or to a sequence in the sub-telomeric region in the chromosomal DNA; and (2) a second amplification primer that hybridizes to a sub-telomeric sequence under annealing conditions. In one aspect the kit further comprises: (3) a third amplification primer that hybridizes to a complement of the anchor sequence under annealing conditions. In a further aspect, the kit comprises: (3) reagents to carry out the hybridization, extension, amplification and quantification steps of the short telomere measurement. In a further aspect, the kit further comprises: (3) a control sample and a reference sample comprising chromosomal DNA with known telomere lengths, or synthetic oligonucleotides with telomeric repeats with known amount of mass.

INCORPORATION BY REFERENCE

[0020] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Figure 1 shows overall scheme of a short telomere assay (STA).

[0022] Figure 2 shows confirmation of short telomere amplification during strand displacement by TELOTEST, a test for measuring average telomere length that uses mismatched primers that hybridize to telomeric repeat sequences in a qPCR assay. An assay of similar type is described in U.S. Patent No. 7,695,904 (Cawthon).

[0023] Figure 3 shows Southern blot analysis of strand-displacement products. M: molecular weight markers; Lane 1: total genomic DNA from the bladder cancer cell-line, UM-UC3, amplified by modified STELA (Single Telomere Elongation Length Analysis) protocol (Baird *et al.*, 2003); Lanes 2-5: PCR products using SUS and TeloAnchor as primers and the strand-displacement products are template. Strand displacement reaction times are indicated (30 seconds, 1, 3, and 5 minutes). Strand lengths are: 30 sec = ~ 1 kb; 1 minute =

~2 kb; 3 minutes = ~ 5 kb; 5 minutes = ~8 kb.

[0024] Figure 4 shows a proof-of-concept calculation for short telomere quantification in DNA samples with different average telomere length. UM-UC3 is a cell population with short average telomeres, and UM-UC3/hTER is the identical cell population but with extended (elongated) telomeres due to over-expression of telomerase (with hTER). As shown, the relative abundance of short telomeres is much greater in UM-UC3 than in UM-UC3/hTER.

[0025] Figure 5 is a table that compares the relative abundance of short telomeres in UM-UC3 and UM-UC3/hTER by Southern Blot (where the relative signal of terminal restriction fragments in the 0.2-5kbp range is shown), and by a short telomere assay of this invention, wherein the percentage of short telomeres is the ratio of short and total telomere measurements. In both cases, there are roughly 3-fold more short telomeres in UM-UC3 than in UM-UC3/hTER.

[0026] Figure 6 shows high-throughput steps for a short telomere assay.

[0027] Figure 7A and Figure 7B show time-controlled primer extension on chromosomes A, B and C, each having telomeres of different length. Chromatin is represented by dashed line. The subtelomeric unique sequence is represented by "SUS". Telomeres are represented by the solid line. The extension product is represented by the dashed line. Figure 7B shows second strand synthesis of the three primer extension products. In Figure 7A, the time for primer extension is selected so that the primer extends past the subtelomeric region (having a subtelomeric unique sequence "SUS") in chromosomes A and B, but not C. In Figure 7B, a primer "SUS" having a subtelomeric unique sequence can hybridize to extension products A and B, but not to extension product C, which, due to long telomere length in the original chromosome, did not extend into the subtelomeric region. Thus, second strand synthesis can proceed from the SUS primer hybridized to extension products A and B, but not from C. The second strand is represented by the solid line. This product, then, represents a "short telomere" fraction of the original chromosomal DNA.

DETAILED DESCRIPTION OF THE INVENTION

[0028] **Definitions**

[0029] It is to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting.

[0030] As used in the specification and in the claims, the term "comprising" can include the aspects "consisting of" and "consisting essentially of."

[0031] As used herein, nomenclature for compounds, including organic compounds, can

be given using common names, IUPAC, IUBMB, or CAS recommendations for nomenclature. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined herein.

[0032] As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell," "a nucleotide," or "a primer" includes mixtures of two or more such cells, nucleotides, or primers, and the like.

[0033] Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, a further aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms a further aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0034] As used herein, the terms "about" and "at or about" mean that the amount or value in question can be the value designated some other value approximately or about the same. It is generally understood, as used herein, that it is the nominal value indicated ±10% variation unless otherwise indicated or inferred. The term is intended to convey that similar values promote equivalent results or effects recited in the claims. That is, it is understood that amounts, sizes, formulations, parameters, and other quantities and characteristics are not and need not be exact, but can be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art. In general, an amount, size, formulation, parameter or other quantity or characteristic is "about" or "approximate" whether or not expressly stated to be such. It is understood that where "about" is used before a quantitative value, the parameter also includes the specific quantitative value itself, unless specifically stated otherwise.

[0001] References in the specification and concluding claims to parts by weight of a particular element or component in a composition denotes the weight relationship between

the element or component and any other elements or components in the composition or article for which a part by weight is expressed. Thus, in a compound containing 2 parts by weight of component X and 5 parts by weight component Y, X and Y are present at a weight ratio of 2:5, and are present in such ratio regardless of whether additional components are contained in the compound.

[0002] A weight percent (wt. %) of a component, unless specifically stated to the contrary, is based on the total weight of the formulation or composition in which the component is included.

[0003] As used herein, the terms "optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

[0035] As used herein, the term "effective amount" refers to an amount that is sufficient to achieve the desired modification of a physical, chemical, or biological property of the composition or method.

[0036] As used herein, "kit" means a collection of at least two components constituting the kit. Together, the components constitute a functional unit for a given purpose. Individual member components may be physically packaged together or separately. For example, a kit comprising an instruction for using the kit may or may not physically include the instruction with other individual member components. Instead, the instruction can be supplied as a separate member component, either in a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation.

[0037] As used herein, "instruction(s)" means documents describing relevant materials or methodologies pertaining to a kit. These materials may include any combination of the following: background information, list of components and their availability information (purchase information, etc.), brief or detailed protocols for using the kit, trouble-shooting, references, technical support, and any other related documents. Instructions can be supplied with the kit or as a separate member component, either as a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation. Instructions can comprise one or multiple documents, and are meant to include future updates.

[0038] As used herein, the term "subject" can be a vertebrate, such as a mammal, a fish, a bird, a reptile, or an amphibian. Thus, the subject of the herein disclosed methods can be a human, non-human primate, horse, pig, rabbit, dog, sheep, goat, cow, cat, guinea pig or

rodent. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. In one aspect, the subject is a mammal. A patient refers to a subject afflicted with a condition, disease or disorder. The term "patient" includes human and veterinary subjects. In some aspects of the disclosed methods, the subject has been diagnosed with a need for treatment of one or more conditions or diseases associated with a dysfunction in short telomere abundance.

[0039] As used herein, the term "treatment" refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder. In various aspects, the term covers any treatment of a subject, including a mammal (*e.g.*, a human), and includes: (i) preventing the disease from occurring in a subject that can be predisposed to the disease but has not yet been diagnosed as having it; (ii) inhibiting the disease, i.e., arresting its development; or (iii) relieving the disease, i.e., causing regression of the disease. In one aspect, the subject is a mammal such as a primate, and, in a further aspect, the subject is a human. The term "subject" also includes domesticated animals (*e.g.*, cats, dogs, etc.), livestock (*e.g.*, cattle, horses, pigs, sheep, goats, etc.), and laboratory animals (*e.g.*, mouse, rabbit, rat, guinea pig, fruit fly, etc.).

[0040] As used herein, the term "prevent" or "preventing" refers to precluding, averting, obviating, forestalling, stopping, or hindering something from happening, especially by advance action. It is understood that where reduce, inhibit or prevent are used herein, unless specifically indicated otherwise, the use of the other two words is also expressly disclosed.

[0041] As used herein, the terms "administering" and "administration" refer to any method of providing a pharmaceutical preparation to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, oral administration, transdermal administration, administration by inhalation, nasal administration, topical

administration, intravaginal administration, ophthalmic administration, intraaural administration, intracerebral administration, rectal administration, sublingual administration, buccal administration, and parenteral administration, including injectable such as intravenous administration, intra-arterial administration, intramuscular administration, and subcutaneous administration. Administration can be continuous or intermittent. In various aspects, a preparation can be administered therapeutically; that is, administered to treat an existing disease or condition. In further various aspects, a preparation can be administered prophylactically; that is, administered for prevention of a disease or condition.

[0042] As used herein, the terms "effective amount" and "amount effective" refer to an amount that is sufficient to achieve the desired result or to have an effect on an undesired condition. For example, a "therapeutically effective amount" refers to an amount that is sufficient to achieve the desired therapeutic result or to have an effect on undesired symptoms, but is generally insufficient to cause adverse side effects. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of a compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose can be divided into multiple doses for purposes of administration. Consequently, single dose compositions can contain such amounts or submultiples thereof to make up the daily dose. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. In further various aspects, a preparation can be administered in a "prophylactically effective amount"; that is, an amount effective for prevention of a disease or condition.

[0043] As used herein, "extension primer" means an oligonucleotide primer used to perform the time-limited extension reaction step carried out by a DNA polymerase. The extension primer can comprises a 3′ portion and a 5′ portion. For example, the 3′ portion can hybridize to a telomeric repeat sequence in the 3′ overhang under annealing conditions, and a

5′ portion can have an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3′ overhang under the annealing conditions.

[0044] As used herein, "telomeric region" means the DNA segment at the ends of a chromosome with repeat telomeric sequence. In the case of vertebrates, it can be the (TTAGGG)n repeat sequence at the ends of chromosomes.

[0045] As used herein, "sub-telomeric region" means the segment of DNA immediately adjacent to telomere at the centromeric end of telomeres. Subtelomeric region often contains degenerate telomeric repeats. In the case of humans, repeats of TGAGGG and TCAGGG can be present in subtelomeric region.

[0046] As used herein, "time-limited extension reaction" means an enzymatic reaction carried out by a DNA polymerase wherein the size of the product of the reaction, the time-limited extension product, in nucleotides is a function of both the intrinsic extension rate of the DNA polymerase utilized in the reaction and the reaction time.

[0047] As used herein, "anchor sequence" means a unique sequence segment within a primer that is not present in the template genome that can be used in the PCR reaction or present within 20 kb of the intended amplicon. For example, the 5′ portion of an extension primer can be an anchor sequence that is configured not to hybridize under annealing conditions to a telomeric repeat sequence in the G-strand to which the 3′ portion of the extension primer hybridizes.

[0048] As used herein, "G-strand of the chromosomal DNA" means the strand of the telomere having the 3′ overhang, and includes the telomeric repeat sequence 5′-TTAGGG-3′. For example, "G-strand of the chromosomal DNA" can refer to the DNA strand in a chromosome comprising the $(TTAGGG)_n$ telomeric sequence in humans and other vertebrates.

[0049] As used herein, a “polymerase” refers to an enzyme that catalyzes the polymerization of nucleotides. Generally, the enzyme will initiate synthesis at the 3′-end of the primer annealed to a nucleic acid template sequence. “DNA polymerase” catalyzes the polymerization of deoxyribonucleotides. Known DNA polymerases include, for example, Pyrococcus furiosus (Pfu) DNA polymerase (Lundberg *et al.*, (1991) Gene 108:1), E. coli DNA polymerase I (Lecomte and Doubleday (1983) Nucleic Acids Res. 11:7505), T7 DNA polymerase (Nordstrom *et al.* (1981) J. Biol. Chem. 256:3112), Thermus thermophilus (Tth) DNA polymerase (Myers and Gelfand (1991) Biochemistry 30:7661), Bacillus stearothermophilus DNA polymerase (Stenesh and McGowan (1977) Biochim Biophys Acta 475:32), Thermococcus litoralis (Tli) DNA polymerase (also referred to as Vent DNA

polymerase, Cariello *et al.* (1991) Nucleic Acids Res 19:4193), Thermotoga maritima (Tma) DNA polymerase (Diaz and Sabino (1998) Braz J. Med. Res 31:1239), and Thermus aquaticus (Taq) DNA polymerase (Chien *et al.*, (1976) J. Bacteoriol 127:1550). The polymerase activity of any of the above enzymes can be determined by means well known in the art.

[0050] As used herein, "thermostable" DNA polymerase activity means DNA polymerase activity which is relatively stable to heat and functions at high temperatures, for example 45-100 °C., preferably 55-100 °C, 65-100 °C, 75-100 °C, 85-100 °C or 95-100 °C, as compared, for example, to a non-thermostable form of DNA polymerase.

[0051] Strand displacement activity of a DNA polymerase describes the ability to displace downstream DNA encountered during synthesis. For example, Strand displacement activity of a DNA polymerase can refer to the polymerases ability to separate a double strand of DNA into two single strands. Examples of DNA polymerases with strand displacement activity are holoenzymes or parts of replicases from viruses, prokaryotes, eukaryotes or archaea, the phi29 DNA polymerases, Klenow DNA polymerase exo$^-$ and DNA polymerase from Bacillus stearothermophilus designated as Bst exo$^-$. "Exo$^-$" signifies that the corresponding enzyme does not have 5'-3' exonuclease activity. A well known example of a phi29 DNA polymerase is the bacteriophage phi29 DNA polymerase. Further suitable DNA polymerases with strand displacement activity useful in the methods of the present invention are well known to the person skilled in the art, and include DNA polymerases such as a modified T7 polymerase, (e.g, Sequenase), exonuclease-deficient Klenow fragment of E. coli DNA polymerase I, and Bst DNA polymerase Large fragment and Deep VentR (exo-).

[0052] Alternatively, also understood to be DNA polymerases with strand displacement activity are those without strand displacement activity provided a catalyst is used in addition to a respective DNA polymerase, e.g. a protein or enzyme, which enables a double strand of DNA to be separated or a single strand of DNA to be stabilized. These proteins include, for example, the helicases, SSB proteins and recombinant proteins, which can be present as components of larger enzyme complexes, such as replicases. In this case, a polymerase with strand displacement activity is produced with components in addition to the polymerase itself. The polymerases with strand displacement activity can be either heat-unstable or heat-stable.

[0053] As used herein, "primer" refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, e.g., in the presence of four

different nucleoside triphosphates and an agent for extension (e.g., a DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer need not reflect the exact sequence of the template nucleic acid, but must be sufficiently complementary to hybridize with the template. The design of suitable primers for the amplification of a given target sequence is well known in the art and described in the literature cited herein.

[0054] The terms "target, "target sequence", "target region", and "target nucleic acid," as used herein, are synonymous and refer to a region or subsequence of a nucleic acid which is to be amplified or detected.

[0055] The term "hybridization," as used herein, refers to the formation of a duplex structure by two single-stranded nucleic acids due to complementary base pairing. Hybridization can occur between fully complementary nucleic acid strands or between "substantially complementary" nucleic acid strands that contain minor regions of mismatch. Conditions under which only fully complementary nucleic acid strands will hybridize are referred to as "stringent hybridization conditions" or "sequence-specific hybridization conditions". Stable duplexes of substantially complementary sequences can be achieved under less stringent hybridization conditions; the degree of mismatch tolerated can be controlled by suitable adjustment of the hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair composition of the oligonucleotides, ionic strength, and incidence of mismatched base pairs, following the guidance provided by the art (see, e.g., Sambrook *et al.*, (1989) Molecular Cloning—A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); and Wetmur (1991) Critical Review in Biochem. and Mol. Biol. 26 (3/4):227-259; both incorporated herein by reference).

[0056] The term "amplification reaction" refers to any chemical reaction, including an enzymatic reaction, which results in increased copies of a template nucleic acid sequence or results in transcription of a template nucleic acid.

[0057] Polymerase chain reaction (PCR) is a method that allows exponential amplification of DNA sequences within a longer double stranded DNA molecule. PCR entails the use of a pair of primers that are complementary to a defined sequence on each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round

of synthesis. This leads to logarithmic amplification. Since it is necessary to raise the temperature to separate the two strands of the double strand DNA in each round of the amplification process, a major step forward was the discovery of a thermo-stable DNA polymerase (Taq polymerase) that was isolated from Thermus aquaticus, a bacterium that grows in hot pools; as a result it is not necessary to add new polymerase in every round of amplification. After several (often about 40) rounds of amplification, the PCR product is analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide stain.

[0058] It is understood that real-time PCR, also called quantitative real time PCR (qRT-PCR), quantitative PCR (Q-PCR/qPCR), or kinetic polymerase chain reaction, is a laboratory technique based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. qPCR enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.

[0059] As used herein, a primer is "specific," for a target sequence if, when used in an under sufficiently stringent conditions, the primer hybridizes primarily only to the target nucleic acid. Typically, a primer is specific for a target sequence if the primer-target duplex stability is greater than the stability of a duplex formed between the primer and any other sequence found in the sample. One of skill in the art will recognize that various factors, such as salt conditions as well as base composition of the primer and the location of the mismatches, will affect the specificity of the primer, and that routine experimental confirmation of the primer specificity will be needed in most cases. Hybridization conditions can be chosen under which the primer can form stable duplexes only with a target sequence. Thus, the use of target-specific primers under suitably stringent amplification conditions enables the specific amplification of those target sequences which contain the target primer binding sites. The use of sequence-specific amplification conditions enables the specific amplification of those target sequences which contain the exactly complementary primer binding sites.

[0060] As used herein, "complementary" refers to a nucleic acid molecule that can form hydrogen bond(s) with another nucleic acid molecule by either traditional Watson-Crick base pairing or other non-traditional types of pairing (e.g., Hoogsteen or reversed Hoogsteen hydrogen bonding) between complementary nucleosides or nucleotides.

[0061] It is understood in the art that a nucleic acid molecule need not be 100% complementary to a target nucleic acid sequence to be specifically hybridizable. That is, two

or more nucleic acid molecules may be less than fully complementary and is indicated by a percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds with a second nucleic acid molecule. For example, if a first nucleic acid molecule has 10 nucleotides and a second nucleic acid molecule has 10 nucleotides, then base pairing of 5, 6, 7, 8, 9, or 10 nucleotides between the first and second nucleic acid molecules represents 50%, 60%, 70%, 80%, 90%, and 100% complementarity, respectively. "Perfectly" or "fully" complementary nucleic acid molecules means those in which all the contiguous residues of a first nucleic acid molecule will hydrogen bond with the same number of contiguous residues in a second nucleic acid molecule, wherein the nucleic acid molecules either both have the same number of nucleotides (i.e., have the same length) or the two molecules have different lengths.

[0062] The term "non-specific amplification," as used herein, refers to the amplification of nucleic acid sequences other than the target sequence which results from primers hybridizing to sequences other than the target sequence and then serving as a substrate for primer extension. The hybridization of a primer to a non-target sequence is referred to as "non-specific hybridization" and is apt to occur especially during the lower temperature, reduced stringency, pre-amplification conditions.

[0063] The term "primer dimer," as used herein, refers to a template-independent non-specific amplification product, which is believed to result from primer extensions wherein another primer serves as a template. Although primer dimers frequently appear to be a concatamer of two primers, i.e., a dimer, concatamers of more than two primers also occur. The term "primer dimer" is used herein generically to encompass a template-independent non-specific amplification product.

[0064] The term "reaction mixture," as used herein, refers to a solution containing reagents necessary to carry out a given reaction. An "amplification reaction mixture", which refers to a solution containing reagents necessary to carry out an amplification reaction, typically contains oligonucleotide primers and a DNA polymerase or ligase in a suitable buffer. A "PCR reaction mixture" typically contains oligonucleotide primers, a DNA polymerase (most typically a thermostable DNA polymerase), dNTPs, and a divalent metal cation in a suitable buffer. A reaction mixture is referred to as complete if it contains all reagents necessary to enable the reaction, and incomplete if it contains only a subset of the necessary reagents. It will be understood by one of skill in the art that reaction components are routinely stored as separate solutions, each containing a subset of the total components, for reasons of convenience, storage stability, or to allow for application-dependent

adjustment of the component concentrations, and that reaction components are combined prior to the reaction to create a complete reaction mixture. Furthermore, it will be understood by one of skill in the art that reaction components are packaged separately for commercialization and that useful commercial kits may contain any subset of the reaction components which includes the blocked primers of the disclosure.

[0065] 1. Introduction

[0066] This disclosure provides methods and materials for determining measures of short telomere abundance in a population of chromosomes and of using these measures for determining measures of health and effects of interventions that increase or decrease telomere length and, hence, increase or decrease health, or conversely decrease or increase risk of future disease or death, respectively. The methods involve producing a population of copies of chromosomal fragments only from chromosomes having telomeres within a pre-defined length range (e.g., all telomeres no longer than a certain length, e.g., shorter than about 5 kbp). This assay can be used to represent the relative abundance of short telomere telomeres in a set of samples, or the assay can be used in combination with a measure of total telomere abundance in order to generate an absolute percentage of short telomeres.

[0067] In one aspect, the method of measuring the abundance of short telomere products includes two steps. A first step involves generating extension products from a double-stranded chromosomal DNA template using an extension primer. The extension primer has at its 3′ end a sequence complementary to telomeric repeat sequences in the G-strand of the telomere, and an "anchor" sequence at its 5′ end. In this way, the extension primer is adapted to hybridize to a telomeric repeat sequence in the 3′ overhang of chromosomes in a sample, and utilizes a 5′-anchor sequence for priming a subsequent PCR reaction. The extension products are generated in a time-limited extension reaction. Because it is time limited, the extension reaction can be configured to produce extension products of no more than a certain length. Because the extension products are length limited, they will extend into the sub-telomeric region only in chromosomes that are sufficiently short. A second step involves amplifying, from the extension products, sequences bounded by a sub-telomeric sequence and the anchor sequence.

[0068] The length of the extension products produced by defined reaction times can be estimated by at least three different methods: (a) by the polymerization rate (R) of the strand displacement enzyme times the extension time: (b) by analyzing the size of PCR products on a Southern gel; and (c) by analyzing the size of the pure TTAGGG region by sequencing. The sequencing method is limited by the ability to accurately sequence long stretches of

repetitive DNA.

[0069] Because the extension reaction is time-limited, sub-telomeric sequences will be present only in extension products from chromosomes in which the distance from the location in the 3′ overhang where the extension primer hybridized to the sub-telomeric region is within a pre-determined length range of extension. The pre-determined length range and can be up to any length chosen by the practitioner. For example, the length range can be a length up to a pre-determined length. In certain aspects, the length range embraces the length of short telomeres, e.g., up to 5 kb. In other aspects, the length range is a length shorter than a pre-determined distance. So, for example, in chromosomes in which the length of the telomere is no longer than the defined extension length (e.g., chromosomes with short telomeres), the time-limited extension reaction will extend the primer into the sub-telomeric region of the chromosome. In chromosomes in which the length of the telomere is longer than the pre-determined extension length (e.g., chromosomes with long telomeres), the time-limited extension reaction will not extend the primer into the sub-telomeric region of the chromosome, and the extension product will not have the sub-telomeric sequences necessary for the second amplification step. Accordingly, the population of extension products can be controlled to include telomeric sequences only from chromosomes having telomeres shorter than a certain size. If the length range is, for example, 4 kb, the extension products include, for example, products in which the telomeres are 4 kb, 3 kb, 2 kb, 1 kb, etc.

[0070] When the extension products are amplified using a pair of amplification primers adapted to amplify sequences bounded by a sub-telomeric sequence and the anchor sequence, only extension products having sub-telomeric sequences are amplified. Those are the extension products generated from chromosomes having telomeres shorter than the defined extension length, e.g., chromosomes with short telomeres. Accordingly, the extended product is not amplified from total telomeric sequences from chromosomes in the sample, but only from telomeric sequences from chromosomes having telomeres no greater than the predefined length. Such an amplified product is sometimes referred to herein as a "length-limited telomere amplification product" or, depending on context, a "short telomere amplification product". By using limited extension time in this PCR amplification step, the short telomeres will be further enriched. This limited extension time PCR step therefore will increase the specificity of the assay to amplify only the short telomere population.

[0071] The amount or abundance of telomeric sequences in a length-limited telomere amplification product is sometimes referred to herein as "length-limited telomere abundance" or, depending on context, "short telomere abundance". The abundance of telomeric

sequences in a length-limited telomere amplification product can be measured by any method used to determine abundance of telomeric sequences in a product of total telomeric sequences.

[0072] Methods of this disclosure begin with double stranded DNA, e.g., chromosomes in their native state. In comparison methods that measure total telomeric sequences, particularly this use qPCR, can involve providing a sample of single-stranded, or denatured, nucleic acid.

[0073] 2. Length-limited Telomere Amplification Product

[0074] 2.1 Sample

[0075] Chromosomal DNA in its native, double-stranded state, can be obtained from a solid, fluid, semisolid or gaseous sample containing nucleic acid, e.g., from liquid tissues such as blood, saliva, urine, plasma, serum, CSF, bronchoalveolar lavage fluid; from solid tissues such as lung, muscle, skin; from semi-solid tissues such as bone marrow; and from gaseous sample such as exhaled breath. The organisms from which the chromosomal DNA is obtained can be any organism with linear chromosomes with a 3′ overhang. The template double stranded chromosomal DNA can be obtained using any DNA purification method which yields high molecular weight genomic DNA (greater than 20 kb) including phenol/chloroform extraction, cesium chloride gradient, and commercial kits that use silicone membrane binding technology, selective detergent-mediated DNA precipitation method. Examples of DNA purification commercial kits include Agencourt DNAdvance and Agencourt Genfind (Beckman Coulter), QIAamp kit (QIAGEN, Valencia, California), QIAamp blood kit (QIAGEN), QIAamp FFPE tissue kit QIAGEN), AHPrep kit (QIAGEN), Puregene kit (QIAGEN), PureLink and GeneCatcher (Invitrogen) and Wizard (Promega).

[0076] A sample for use in the methods of this disclosure can be any genomic DNA with 3′ end single-stranded overhang. In certain aspects, the sample comprises high molecular weight genomic DNA (e.g., >20 kb). Any method that yields high molecular weight native genomic DNA can be used.

[0077] In double-stranded chromosomes possessing telomeres, the single DNA strand having a 3′ terminus with the telomeric repeat sequence (the "3′ overhang") extends beyond the terminus of the paired single strand having the 5′ terminus. The strand of the telomere having the 3′ overhang is referred to as the "G strand", and includes the telomeric repeat sequence 5′-TTAGGG-3′. Same order permutations are permutations in which the letters are not scrambled, but begin at a different point in the same sequence, e.g., inversions (e.g., XYZ, YZX, ZXY rather than YXZ). Same order permutations of this sequence include: 5′-TAGGGT-3′, 5′-AGGGTT-3′, 5′-GGGTTA-3′, 5′-GGTTAG-3′ and 5′-GTTAGG-3′. The

strand having the 5′ terminus is referred to as the "C strand", and includes the telomeric repeat sequence 5′-CCCTAA-3′.

[0078] The length of a telomere can be the distance, e.g., in kilobases, from the end of the chromosome to the sub-telomeric region. In cells of normal human adults, telomeres can range from less than 1 kb to 12 kb or, in some cases, to >20 kb in length. Telomere length is known to vary in different cell types (Lin *et al.*, *J Immunol Methods*, 2010, **31**:352(1-2):71 - 80). For these reasons, the useful length ranges of the short telomere population can be broad, based on clinical utility. Accordingly, in certain aspects, a short telomere has a length no more than about 5 kb, no more than 4 kb, no more than 3 kb, no more than 2 kb, no more than 1 kb or no more than about 0.5 kb. Methods of this disclosure can be configured to detect telomeres up to each of these lengths.

[0079] Short telomere products can be generated from a single telomere, a single chromosome, a population of chromosomes from a single cell or a population of chromosomes from a plurality of cells.

[0080] 2.2 Producing the Extension Product

[0081] In the extension reaction, a primer is annealed to the 3′ overhang of the double stranded chromosomal DNA under annealing conditions. Appropriate annealing conditions are known to those skilled in the art, such as those typically used to hybridize nucleic acid strands for strand extension or for PCR. Such conditions include, without limitation, incubation at 65 °C for 10 min in a heating block and then cooling down to room temperature over the period of one hour. Other conditions may include incubation at temperature ranging from 37 °C to 65 °C for at 5 minutes to 30 minutes and then cooling down to room temperature over the period of one hour to three hours.

[0082] 2.2.1 Extension Primer

[0083] The extension primer comprises a 3′ portion and a 5′ portion.

[0084] 2.2.1.1 3′ Portion

[0085] The 3′ portion has a sequence adapted to hybridize a telomeric repeat sequence in the G-strand of a telomere. The sequence in the 3′ portion can be complementary to the telomeric repeat, or it can have certain mismatches as described above, as long as the mismatches allow for hybridization under annealing conditions for primer extension. For example, the 3′ portion can have at least 8 consecutive nucleotides of a telomeric repeat sequence (i.e., the sequence of the C-strand of the telomere). The consecutive nucleotides can be in any permutation of the telomeric repeat sequence. In other aspects, the sequence of the 3′ potion can have at least 9 consecutive nucleotides, at least 10 consecutive nucleotides,

at least 11 consecutive nucleotides or at least 12 consecutive nucleotides of a telomeric repeat sequence. In other aspects, the sequence of the 3′ potion can have two or more, three or more, or four or more telomeric repeat units in any same-order permutation.

[0086] 2.2.1.1 5′ Portion

[0087] The 5′ portion of the extension primer (also referred to as an "anchor sequence") is configured not to hybridize under annealing conditions to a telomeric repeat sequence in the G-strand to which the 3′ portion hybridizes. Preferably, the anchor sequence does not hybridize under annealing conditions to a sequence in the sub-telomeric region of the G-strand. The anchor sequence also can be configured not to hybridize to any sequence of the target G-strand within 10 kb, within 20 kb or within 50 kb of the terminus of the 3′ overhang of the G-strand, or to any unique sequence in the target chromosome. The anchor sequence can be configured such that its complement does not hybridize to any sequence in the C-strand of the chromosome in the telomeric or sub-telomeric region, or within 10 kb, within 20 kb or within 50 kb of the terminus of the C-strand of the telomere. For example, the anchor sequence can be a unique sequence not found in the chromosomes being tested.

[0088] 2.2.2 Extension Reaction

[0089] After annealing the extension primer to the chromosomal DNA, an extension reaction is performed using a polymerase with strand displacement activity and/or exonuclease activity. Examples of strand displacement polymerases include, but are not limited to T7 polymerase (e.g., Sequenase), exonuclease-deficient Klenow fragment of E. coli DNA polymerase I, and Bst DNA polymerase Large fragment and Deep VentR (exo-). In addition, polymerases with 5′-3′ exonuclease activity can also be used.

[0090] The extension reaction is time limited. That is, the extension reaction is allowed to proceed for a pre-determined amount of time. The time is calibrated to produce extension products having an average of no more than a pre-determined length. The time used to produce the extension product by the strand-displacement enzyme can be determined empirically under the chosen conditions and reactants to produce extension products of pre-determined length. The extensions rates of various polymerases useful in the present invention have been previously determined, and the extension rates can be used to calculate the approximate time necessary to achieve the desired extension product. The extension rates for exemplary polymerases are given in the table below.

DNA Polymerase	Extension Rate*	Reference
Klenow	13.5 nucleotides/sec	Maier B, Bensimon D, and Croquette V. *Proc Natl Acad Sci U S A.* (2000) 2497(22):12002-7.
T7	75.9 nucleotides/sec	Tanner, N. A. et al. *Nuc. Acids Res.* (2009) 37, e27.
Taq	35-100 nucleotides/sec (75 ˚C) 0.9-2.55 nucleotides/sec (37 ˚C)	Wittwer, C.T. and Garling, D.J., BioTechniques (1991) 10(1), 76-83.
phi29	25 nucleotides/sec	Blanco, L., et al. J Biol Chem (1989) 264, 8935-8940
Bst	50-100 nucleotides/sec	New England Biolabs

* Under conditions specified in the associated reference or standard reaction conditions in the absence of other information.

[0091] Initiation of the extension reaction is achieved by adding the strand displacement enzyme to the reaction tube. Reaction can be stopped by placing the reaction tube to 80 °C for 20 minutes or by adding EDTA. Furthermore, the reaction can be controlled (slowed down or sped up) by incubating at a lower or higher temperature, e.g. 25 °C or 30 °C. For instance, the displacement rate of the strand displacement polymerase Sequenase at 37 °C is about 28 bp per second. At 30 °C it is about 3-fold slower, based on Southern blot analysis. At 37 °C, Sequenase can produce an extension product of ~ 1 kb in 30 sec; ~2 kb in 1 minute; ~ 5 kb in 3 minutes; and ~8 kb in 5 minutes. The timing necessary to produce extension products of pre-determined lengths with various polymerase systems and various sample sources can be determined empirically, e.g., a similar time course experiment followed by Southern blot analysis can be performed to calibrate the extension time. Accordingly, the extension reaction can be timed to no more than 30 minutes, no more than 10 minutes, no more than 5 minutes, no more than 4 minutes, no more than 3 minutes, no more than 2 minutes, no more than 1 minute or no more than 30 seconds.

[0092] **2.3 Amplification Reaction**

[0093] Sequences in the time-limited extension product are then amplified, for example, by PCR. More specifically, sequences bounded by the anchor sequence on one side and a sub-telomeric sequence on the other side are amplified. The primer can be exactly complementary or have mismatched sequences, as long as it allows annealing to its target (anchor sequence or sub-telomeric sequence) and extension.

[0094] Anchor sequences can vary. In certain aspects, the anchor sequence is a specific sequence not present in the genome of the organism for which telomere quantity is being

measured, or if the anchor sequence is found elsewhere in the genome, it could still be useful if that sequence is a significant distance from the SUS sequence, so that no amplification occurs during the PCR step except between the telomeric anchor sequence and the telomeric SUS sequence.

[0095] PCR conditions are optimized to yield best analytic performance. The extension time in the PCR condition is predetermined by examining the product profile on Southern blots to ensure that the intended size range of short telomeres are enriched.

[0096] The subtelomeric primer used in the method of the disclosure can contain sequences on the G strand, found in all or most chromosomes, e.g. variants of the telomeric sequence $(TGAGGG)_{3-6}$ (Xu and Blackburn, *Mol. Cell*, **28**:315-327, 2007) or $(TTGGGG)_{3-6}$ (Allshire *et al.*, *Nucleic Acid Research*, **17**:4611-4627,1989), or $(TCAGGG)_{3-6}$ (Baird *et al.*, *EMBO J.*, **14**(21):5433-5443, 1995). Alternatively, the sub-telomeric primer could be a segment found on specific chromosome(s), e.g. the XpYpE2 primer described in Xu et al (5′-GTTGTCTC AGGGTCCTAGTG-3′ [SEQ ID NO:1]) (Xu and Blackburn, *Mol Cell*, **28**:315-327, 2007). In one aspect, the primer for the sub-telomeric sequence comprises, consists essentially of or consists of:

5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO: 2]

5′-CGGGCCGGCTGAGGGTACCGCGA-3′ [SEQ ID NO: 10] (chromosome 1)

5′-GCTAATGCACTCCCTCAATAC-3′ [SEQ ID NO: 11] (chromosome 5)

5′-CATTCCTAATGCACACATGATACC-3′ [SEQ ID NO: 12] (Chromosome 9)

[0097] When a short telomere assay is applied in organisms other than human using the methods of the disclosure, the specific sequence of the subtelomeric primer can be designed based on the genome sequence of the organism. The sequence of the sub-telomeric primer should match with the strand with the 3′ overhang.

[0098] The amplification product comprises a population of nucleic acids having telomeric repeat sequences within a certain length range, and excludes sequences longer than this range. Thus, the population can be configured to include sequences only from chromosomes having telomeres shorter than a threshold length.

[0099] An internal control sequence to control for the extension reaction efficiency can be included in the extension reaction step. This internal control sequence can be a double strand DNA with subtelomeric primer sequence at one end and a 3′ overhang with the G strand of telomeric sequence. Between the subtelomeric primer sequence and telomeric sequence can be a stretch of unique non-telomeric sequence. One such sequence can be, for example, the hTERT gene, or the RNase P gene. The efficiency of the extension reaction for this internal

control can be measured by a Taqman based assay to quantify, for example the hTERT gene or the RNase P gene.

[0100] 3. Methods of Measuring Telomere Abundance

[0101] Any method used to measure telomere abundance in a sample comprising telomeric sequences can be used to measure telomere abundance in a length-limited telomeric sample.

[0102] Measures of telomere abundance can be absolute or relative. Absolute measures of telomere abundance include, for example, total length of telomere sequences in a genome measured, for example, by number of nucleotides. More typically, telomere abundance is measured relative to a reference. Detection of telomere sequences can be measured in terms of signal strength produced in an assay. This signal strength can be compared with the signal strength produced by a reference sequence in the assay. The relative signal strength can function as a method of standardization. Standardized methods can be compared more easily between assays. For example, the signal produced by detection of telomere sequences can be compared with the signal produced by the measure of a control sequence. The control sequence can be, for example, a portion of the beta-globin gene. Thus, regardless of the assay method used, the relative signals of telomere sequences to reference sequences can be expressed, for example, as a ratio. This ratio can be used to compare results of telomere sequence abundance measurements.

[0103] In other aspects, measures of short telomere abundance are compared with total telomere abundance. The total telomere abundance can be measured by a long extension time in the strand displacement reaction and in the amplification reaction, followed by qPCR method as described in U.S. Patent No. 7,695,904 (Cawthon) and Lin *et al.* (Lin et al,, 2010, **352**(1-2):71-80). To determine the percentage of short telomeres, the ratio of short telomeres to total telomeres can be determined. For example, the signal strength of the short telomere measurement can be divided by the signal strength of the total telomere measurement.

[0104] 3.1 qPCR

[0105] One method of quantifying the abundance of the short telomeres is quantitative PCR, as described in by Cawthon (*Nucleic. Acids Res.*, 2002, **30**(10):e47; in U.S. Patent No. 7,695,904; Lin *et al.*, *J. Immunol. Methods*, 2010, **352**(1-2):71-80); or Cawthon 2009 (Nucleic Acids Res. 2009 **37**(3):e21.).

[0106] A variety of methods known in the art can be used in the present disclosure to determine average telomere length or telomere abundance. Preferably, the real time kinetic quantitative polymerase chain reaction (qPCR) is utilized as specifically modified for

telomere length detection by Cawthon (*Nucleic. Acids Res.*, 2002, **30**(10):e47; U.S. Patent No. 7,695,904). The method is simple and allows for rapid high throughput processing of large numbers of DNA samples. The qPCR method is based on the detection of the fluorescence produced by a reporter molecule which increases as the polymerase chain reaction proceeds. This increase in fluorescence occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (for example, SYBR Green or ethidium bromide) or sequence specific probes (for example, Molecular Beacons or TAQMAN Probes). Alternatively, the qPCR method described by Cawthon (*Nucleic Acids Res.* 2009, **37**(3):e21) can be used The method allows determination of T/S ratios in a single sample via multiplexing, including experimental contexts when the telomere repeats are the high abundance species and a single copy gene is the low abundance species.

[0107] In the method of the present disclosure, primer probes specific to the repeated telomere sequence $(TTAGGG)_n$ are used. The size of the primer may vary, in general, from 5 to 500 nucleotides in length, between 10 and 100 nucleotides, between 12 and 75 nucleotides, or between 15 to 50 nucleotides, depending on the use, required specificity, and the amplification technique. In the present disclosure, one aspect utilizes a first primer which hybridizes to a first single strand of the target telomere sequence and a second primer which hybridizes to a second single strand of the target telomere sequence, where the first and second strands are substantially complementary. In this aspect, for example, the paired primer set consisting of tel1 (5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′) [SEQ ID NO: 3] and tel2 (5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′) [SEQ ID NO: 4] can be used. In one aspect, at least one of the primers comprises at least one altered or mutated nucleotide residue, which produces a mismatch between the altered residue and the 3′ terminal nucleotide of the other primer when the primers hybridize to each other. The presence of a mismatch at the 3′ terminal nucleotide blocks extension by polymerase, thus limiting non-target nucleic acid dependent extension reactions. In this aspect, for example, the paired primer set consisting of tel 1b 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′ [SEQ ID No.: 5]; and tel 2b 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′ [SEQ ID No.: 6] can be used. In a further aspect, for example, the paired primer set consisting of telg 5′-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3′ [SEQ ID No.: 13]; and telc 5′-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3′ [SEQ ID No.:

14] can be used. One skilled in the art will appreciate that other substantially complementary or mismatched sets of primers may be employed in this disclosure. Such primers are described in U.S. Patent No. 7,695,904 (Cawthon *et al.*).

[0108] Amplification reactions are carried out according to procedures well known in the art. Procedures for PCR are widely used and described (see for example, U.S. Patent Nos. 4,683,195 and 4,683,202). In brief, a double stranded target nucleic acid is denatured, generally by incubating at a temperature high enough to denature the strands, and then incubated in the presence of excess primers, which hybridize (anneal) to the single-stranded target nucleic acids. A DNA polymerase extends the hybridized primer, generating a new copy of the target nucleic acid. The resulting duplex is denatured and the hybridization and extension steps are repeated. By reiterating the steps of denaturation, annealing, and extension in the presence of a second primer for the complementary target strand, the target nucleic acid encompassed by the two primers is exponentially amplified. The time and temperature of the primer extension step will depend on the polymerase, length of target nucleic acid being amplified, and primer sequence employed for the amplification. The number of reiterative steps required to sufficiently amplify the target nucleic acid will depend on the efficiency of the amplification. One skilled in the art will understand that the present disclosure is not limited by variations in times, temperatures, buffer conditions, and amplification cycles applied in the amplification process.

[0109] The products of the amplification are detected and analyzed by methods well known in the art. Amplified products may be analyzed following separation and/or purification of the products, or by direct measurement of product formed in the amplification reaction. For detection, the product may be identified indirectly with fluorescent compounds, for example, with ethidium bromide or SYBR Green, or by hybridization with labeled nucleic acid probes. Alternatively, labeled primers or labeled nucleotides are used in the amplification reaction to label the amplification product. The label comprises any detectable moiety, including fluorescent labels, radioactive labels, electronic labels, and indirect labels such as biotin or digoxigenin.

[0110] Instrumentation suitable for conducting the qPCR reactions of the present disclosure are available from a number of commercial sources (ABI Prism 7700, Applied Biosystems, Carlsbad, CA; LIGHTCYCLER 480, Roche Applied Science, Indianapolis, IN; Eco Real-Time PCR System, Illumina, Inc., San Diego, CA; RoboCycler 40, Stratagene, Cedar Creek, TX).

[0111] When real time quantitative PCR is used to detect and measure the amplification products, various algorithms are used to calculate the number of target telomeres in the samples. (For example, see ABI Prism 7700 Software Version 1.7; Lightcycler Software Version 3). Quantitation may involve use of standard samples with known copy number of the telomere nucleic acids and generation of standard curves from the logarithms of the standards and the cycle of threshold (C_t). In general, C_t is the PCR cycle or fractional PCR cycle where the fluorescence generated by the amplification product is several deviations above the baseline fluorescence.

[0112] **3.2 Other Methods**

[0113] The abundance of short telomeres from the amplification reaction may be measured by other methods known in the art. Such methods include, but are not limited to, direct nucleic acid sequencing and Southern blotting, and dot blot, or slot blot hybridization and digital PCR.

[0114] Conventional techniques for the direct determination of nucleic acid sequences in isolated DNA may be employed in the present disclosure. For example, see: "DNA Sequencing," *The Encyclopedia of Molecular Biology*, J. Kendrew, ed., Blackwell Science Ltd., Oxford, UK, 1995, pp. 283-286. Dye-terminator automated sequencing is now most commonly used for nucleic acid sequencing ("DNA Sequencing", *Lab Manager*, at world wide web URL labmanager.com/?articles.view/articleNo/3364/article/DNA-Sequencing). Automated sequencing equipment is conveniently used and may be purchased from companies such as Applied Biosystems, Roche Applied Science, and Illumina Inc. Once the sequence of a DNA sample is determined, the number of copies of the telomere nucleotide sequence (TTAGGG) at either end can then be counted. This method of the present disclosure provides a measure of absolute telomere abundance by direct measurement of telomeric sequences.

[0115] Southern blotting (Southern, E. M., *J. Mol. Biol.*, 1975, **98**(3): 503-517) may also be utilized in the present disclosure to determine telomere abundance by detecting the specific presence of the human telomere nucleotide sequence $(TTAGGG)_n$. In the present disclosure, Terminal Restriction Fragment (TFR) Southern blotting combines the transfer of DNA fragments separated by electrophoresis to a filter membrane, followed by detection of the fragments by hybridization to probes specific for the (TTAGGG) sequence (Allshire, R. C. *et al.*, *Nucleic Acids Res.*, 1989, **17**, 4611-4627). Such probes have sequences complementary to the telomere sequence. For ease of detection, probes are radioactively labeled or tagged with a fluorescent or chromogenic dye. The amount of radioactivity or

fluorescence present may then be quantified to give the telomere abundance in the sample. M. Kimura *et al.* (*Nature Protocols*, 2010, 5:1596-1607) describes an appropriate Southern blot procedure for determining telomere length.

[0116] Variations of the Southern blotting method include dot blot, or slot blot, where the DNA is spotted as dots or slots on a filter membrane, followed by detection of the fragments by hybridization to probes specific for the (TTAGGG) sequence (Kimura M, Aviv A, *Nucleic Acids Res.*, 2011, **39**(12):e84. doi: 10.1093/nar/gkr235. Epub 2011 Apr 27).

[0117] In the above aspects of the present disclosure, fluorescence may be measured in relative fluorescence units (RFU). Fluorescence is detected using a charged coupled device (CCD) array, when the labeled fragments, which are separated within a capillary by using electrophoresis, are energized by laser light and travel across the detection window. A computer program measures the results, determining the quantity or size of the telomere-containing fragments, at each data point, from the level of fluorescence intensity ("Relative fluorescence unit (RFU)", DNA.gov: Glossary, April 2011, world wide web URL dna.gov/glossary/).

[0118] Measures of telomere abundance can be measured using DNA sequencing methodologies. Such methods can involve sequencing molecules in a sample that comprise telomeric repeats, and determining the abundance of telomeric repeat sequences. DNA sequencing methods can include any known method of sequencing including, for example, classical sequencing methods, such as Sanger sequencing or Maxam Gilbert sequencing, and next generation sequencing methods, such as ligation sequencing, nanopore sequencing, pyrosequencing, superpyro sequencing, sequencing by proton detection, sequencing-by-synthesis and, single-molecule sequencing. Short telomere abundance can be also measured by digital PCR. Such technique platforms include, but not limited to, the digital PCR, including, for example, the RAINDROP Digital PCR System (Raindance Technologies, Billerica, Massachusetts) or the QX200 DROPLET DIGITAL PCR System (Bio-Rad Laboratories, Hercules, California), microfluidic digital PCR (Fluidigm Corporation, South San Francisco, California), or the OPENARRAY Real-Time PCR System (APPLIED BIOSYSTEMS division of Thermo Fisher Scientific, Inc., Waltham, Massachussetts). In various further aspects, the abundance of short telomeres can be measured by a suitable hybridization technology, e.g. digital color-coded barcode methods such as NCOUNTER Analysis System (Nanostring Technologies, Seattle, Washington).

[0119] 4. Kits

[0120] This disclosure also provides kits useful in the methods of this disclosure. Such kits can include an extension primer of this disclosure and an amplification primer that hybridizes to a sub-telomeric sequence or to the complement of an anchor sequence of the extension primer. Alternatively, the kit can include an extension primer of this disclosure and a pair of amplification primers adapted to amplify a sequence in a nucleic acid bounded by a sub-telomeric sequence and an anchor sequence in the extension primer. The kit can include a container that contains the extension primer and another container that contains one or both of the amplification primers. The kit also can include reagents for primer extension and reagents for nucleic acid amplification, e.g., for PCR. The kit can also include control and reference samples with known values. In certain aspects, the kit includes an extension primer and a pair of amplification primers. In certain aspects, the extension primer comprises a 3′ portion that hybridizes to a telomeric repeat sequence in a 3′ overhang of double-stranded chromosomal DNA under annealing conditions, and a 5′ anchor portion that does not hybridize to sequences in the telomeric or sub-telomeric regions.

[0121] 5. Conditions Correlated with Telomere Abundance, Abundance of Short Telomeres, and Rate of Change in Telomere Abundance or Rate of Change in Abundance of Short Telomeres

[0122] In general, conditions associated with telomere abundance have typically been derived using a measure of average telomere length, as described herein. However, as discussed above, emerging data suggest that the measuring the abundance of short telomeres, and or the rate of change in abundance of short telomeres may be a more sensitive and potentially a more accurate predictor of clinically meaningful outcomes (e.g. disease or mortality risk) than is the measurement of average telomere length or the rate of change in average telomere length. Hence it is important to have sensitive and accurate measurement of the abundance of short telomeres. It has been reported that the presence of critically short telomeres caused loss of cell viability and tissue function, indicating that there is a causal relationship between very short telomeres and cell senescence (Hemann, M.T., *et al.* Cell, 2001. **107**(1) 67-77).

[0123] Although short average telomere length (as discussed below) is an established, good predictor of clinical outcomes, it is expected that accurate measurement of the abundance of short telomeres may be a more sensitive measurement for the effects of intervention on telomere dynamics (Canela, A., *et al.* Proc Natl Acad Sci U S A, 2007. **104**(13) 5300-5). Harley *et al.* (Rejuvenation Res. 2011. 14(1) 45–56) showed that humans

taking a relatively weak activator of telomerase, TA-65®, showed a reduction in percentage of short telomeres, but there was not a significant shift in average telomere length. In Vera *et al.* supra., the rate of increase in abundance of short telomeres predicted increased longevity in two separate cohorts of mice "The Rate of Increase of Short Telomeres Predicts Longevity in Mammals, Cell Reports (2012), world wide web URL: dx.doi.org/10.1016/].celrep.2012.08.023). Due to the lack of an accurate, cost effective, high throughput method for measuring the abundance of short telomeres, there has until now been no large human clinical studies using abundance of short telomeres or the rate of change in abundance of short telomeres to establish the clinical utility of measuring the abundance of short telomeres.

[0124] Proof of concept in animal studies from Vera *et al.*, supra also indicate that mice with a high rate of increase over time in the percentage of short telomeres have reduced survival compared to mice with a low rate of increase in percentage of short telomeres over time. In wild type mice, the difference in survival of mice that had a low rate of increase (e.g., 0.4%/month) compared to mice with a high rate of increase (e.g. 1%/month) was about 100 weeks, or nearly two thirds of the maximum survival in the wild type mice.

[0125] Accordingly, humans with a high percentage of short telomeres as measured by an accurate and precise assay as described herein, will be more prone to disease or mortality risk compared to humans with a low percentage of short telomeres. Quantitative assessment of % short telomeres will have significant diagnostic utility in not only health monitoring but disease diagnostics, prognosis, and companion diagnostics.

[0126] Monitoring the abundance of short telomeres has immediate utility for health monitoring since it is known that short telomeres (typically less than 1kbp or less then 2kpb or less than 3kbp) trigger cellular senescence (Hemann, M.T., *et al.*Cell, 2001. **107**(1) 67-77), and cellular senescence is known to lead to loss of tissue function and ultimately disease and mortality. Thus, an individual with a greater than average abundance of short telomeres compared to an age-matched reference population of normal individuals is at increased risk of morbidity or mortality. Such a person would be motivated to change their behavior towards a better lifestyle and lower abundance of short telomeres, while an individual with a lower than average abundance of short telomeres compared to the reference population would know that their cellular health was likely better than average, and hence would be motivated to at least to maintain, if not further improve, their lifestyle habits to maintain their good health.

[0127] Average telomere length per chromosome end determined from genomic DNA is a measure of overall telomere abundance, and this has been shown to correlate with several important biological indices. These indices include, for example, risk of various disease conditions, e.g., cardiovascular risk, cancer risk, pulmonary fibrosis risk, infectious disease risk, and risk of mortality. Abundance of telomeres also correlates with chronological age, body-mass index, hip/weight ratio, and perceived stress. One measurement of telomere length is the telomere/single copy ("T/S") ratio. Such ratios in a given population can be divided into quantiles, for example, into tertiles. It has been found that individuals with telomere abundance by T/S ratios in the lower two tertiles are at significantly higher risk for cardiovascular disease than those in the top fertile for telomere length.

[0128] In general, percentile value of measure of telomere abundance, e.g., T/S values represented as a percentage of the reference population (typically the highest fertile or quartile of telomere lengths), in a population correlates negatively with risk of disease, i.e. shorter average telomere length is associated with improved measures of health, while lower percentile scores are associated with decreased measures of health, increased disease risk or presence of telomere disease.

[0129] In a population, telomere length decreases with age. Accordingly, measures of telomere length for an individual can be compared with measures for persons in the same age range in the population, that is, an age-matched population. For example, a person at age 30 might have a measure of telomere abundance about equal to the population average for age 30, or equal to the population average for age 20 or age 40. Correlations of a measure of telomere abundance with measures of health are more accurate when compared with the measure for an age-matched population. The range for an age matched population can be, for example, one year, two years, three years, four years, 5 years, 7 years or 10 years.

[0130] 5.1 Measures of Health

[0131] Short Telomere abundance determined from subject samples by the method of the present disclosure can be correlated with measures of health. Of particular interest are measures of health involving perceived stress. Telomere shortening can be accelerated by genetic and environmental factors, including multiple forms of stress such as oxidative damage, biochemical stressors, chronic inflammation and viral infections (Epel, E.S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 2004, **49**:17312-15). A convenient measure of general health status is the SF-36® Health Survey developed by John Ware (see, e.g., world wide web URL sf-36.org/tools/SF36.shtml). The SF-36 is a multi-purpose, short-form health survey with only 36 questions to be posed to patients, preferably by trained individuals. It provides an 8-

scale profile of functional health and well-being scores as well as psychometrically-based physical and mental health summary measures and a preference-based health utility index. The SF-36 survey is used to estimate disease burden and compare disease-specific benchmarks with general population norms. The most frequently studied diseases and conditions include arthritis, back pain, cancer, cardiovascular disease, chronic obstructive pulmonary disease, depression, diabetes, gastro-intestinal disease, migraine headache, HIV/aids, hypertension, irritable bowel syndrome, kidney disease, low back pain, multiple sclerosis, musculoskeletal conditions, neuromuscular conditions, osteoarthritis, psychiatric diagnoses, rheumatoid arthritis, sleep disorders, spinal injuries, stroke, substance abuse, surgical procedures, transplantation and trauma (Turner-Bowker *et al.*, *SF-36® Health Survey & "SF" Bibliography: Third Edition* (1988-2000), QualityMetric Incorporated, Lincoln, RI, 2002). One skilled in the art will appreciate that other survey methods of general health status, for example, the RAND-36, may find use in the present disclosure.

[0132] In one aspect of the present disclosure, subject samples are collected over time and measurements of short telomere abundance are determined from the samples. Appropriate time periods for collection of a plurality of samples include, but are not limited to, 1 month, 3 months, 6 months, 1 year, 2 years, 5 years and 10 years (for example, the time between the earliest and the last sample can be about these time periods). This method allows for monitoring of patient efforts to improve their general health status and/or to monitor their health status and/or disease risk. Since short telomeres trigger cell death, a finding that the percentage of short telomere length is lowered or maintained with time within an individual indicates a health improvement, while increase of percentage of short telomeres overtime represents a decrease or worsening in health.

[0133] 5.2 Risk of a Pathological Condition

[0134] 5.2.1 Diseases

[0135] Measuring the number of repetitive units of telomeres has a wide variety of applications in medical diagnosis, e.g., for disease risk, disease prognosis, and therapeutics. In particular, measurement of telomere length finds application in assessing pathological conditions leading to the risk of disease. In one aspect of the disclosure, the disease is one associated with aging, for example but not limited to, cardiovascular disease, diabetes, cancer, liver fibrosis, and depression.

[0136] In one aspect, the present disclosure finds use in the assessment and monitoring of cardiovascular disease. Telomere length in white blood cells has been shown to be shorter in patients with severe triple vessel coronary artery disease than it is in individuals with normal

coronary arteries as determined by angiography (Samani, N. J. *et al.*, *Lancet*, 2001, **358**:472-73), and also in patients who experiencing a premature myocardial infarction before age 50 years as compared with age- and sex-matched individuals without such a history (Brouilette S. *et al.*, *Arterioscler. Thromb. Vase. Biol.*, 2003, **23**:842-46). Brouilette *et al.* (*Lancet*, 2007, **369**:107-14) has suggested that shorter leucocyte telomeres in people prone to coronary heart disease could indicate the cumulative effect of other cardiovascular risk factors on telomere length. Increased oxidative stress also contributes to atherosclerosis, and increased oxidant stress has been shown to increase rates of telomere attrition *in vitro* (Harrison, D., *Can. J. Cardiol.*, 1998, **14**(suppl D):30D-32D; von Zglinicki, T., *Ann. N. Y. Acad. Sci.*, 2000, **908**:99-110). In cross-sectional studies, smoking, body-mass index, and type 1 diabetes mellitus have also been reported to be associated with shorter leucocyte telomere length (Valdes, A., *et al.*, *Lancet*, 2005, **366**:662-64; Jeanclos, E. *et al.*, *Diabetes*, 1998, **47**:482-86). Increased life stress, a factor known to increase the risk of coronary heart disease, has been shown to be associated with shorter telomeres, possibly as a consequence of increased oxidative stress (Epel, 2004, *ibid.*). Thus, smokers and patients with a high body-mass index, diabetes and/or increased life stress would all benefit from determination and continued monitoring of their telomere abundance according to the method of the disclosure.

[0137] Type 2 diabetes is characterized by shorter telomeres (Salpea, K. and Humphries, S. E., *Atherosclerosis*, 2010, **209**(1):35-38). Shorter telomeres have also been observed in type 1 diabetes patients (Uziel O. *et al.*, *Exper. Gerontology*, 2007, **42**:971-978). The etiology of the disease in type 1 diabetes is somewhat different from that in type 2, although in both cases, beta cell failure is the final trigger for full-blown disease. Telomere length is thus a useful marker for diabetes since it is associated with the disease's progression. Adaikalakoteswari *et al.* (*Atherosclerosis*, 2007, **195**:83-89) have shown that telomeres are shorter in patients with pre-diabetic impaired glucose tolerance compared to controls. In addition, telomere shortening has been linked to diabetes complications, such as diabetic nephropathy (Verzola D. *et al.*, *Am. J. Physiol.*, 2008, **295**:F1563-1573), microalbuminuria (Tentolouris, N. *et al.*, *Diabetes Care*, 2007, **30**:2909-2915), and epithelial cancers (Sampson, M. J. *et al.*, *Diabetologia*, 2006, **49**:1726-1731) while telomere shortening seems to be attenuated in patients with well-controlled diabetes (Uziel, 2007, *ibid.*). The method of the present disclosure is particularly useful in monitoring the status of pre-diabetic and diabetic patients to provide an early warning for these complications and others.

[0138] The present disclosure is useful for determining telomere lengths of various types of cancer cells because activation of telomerase activity is associated with immortalization of

cells. While normal human somatic cells do not or only transiently express telomerase and therefore shorten their telomeres with each cell division, most human cancer cells typically express high levels of telomerase and show unlimited cell proliferation. High telomerase expression allows cells to proliferate and expand long term and therefore supports tumor growth (Roth, A. *et al.*, in *Small Molecules in Oncology, Recent Results in Cancer Research*, U. M. Martens (ed.), Springer Verlag, 2010, pp. 221-234). Shorter telomeres are significantly associated with risk of cancer, especially cancers of the bladder and lung, smoking-related, the digestive system and the urogenital system. Excessive telomere shortening likely plays a role in accelerating tumor onset and progression (Ma H. *et al.*, *PLo SONE*, 2011, **6**(6): e20466. doi:10.1371/journal.pone.0020466). Studies have further shown that the effect of shortened telomeres on breast cancer risk is significant for certain population subgroups, such as premenopausal women and women with a poor antioxidative capacity (Shen J., *et al.*, *Int. J. Cancer*, 2009, **124**:1637-1643). In addition to the assessing and monitoring cancers in general, the present disclosure is particularly useful for the monitoring of oral cancers if genomic DNA derived from saliva samples is utilized.

[0139] Cirrhosis of the liver is characterized by increasing fibrosis of the organ often associated with significant inflammatory infiltration. Wiemann *et al.* (*FASEB Journal*, 2002, **16**(9):935-982) have shown that telomere shortening is a disease- and age-independent sign of liver cirrhosis in humans. Telomere shortening is present in cirrhosis induced by viral hepatitis (chronic hepatitis A and B), toxic liver damage (alcoholism), autoimmunity, and cholestasis (PBC and PSC); telomeres are uniformly short in cirrhosis independent of the age of the patients. Telomere shortening and senescence specifically affect hepatocytes in the cirrhotic liver and both parameters strongly correlate with progression of fibrosis during cirrhosis. Thus, the method of the present disclosure finds use in diagnosing and monitoring liver fibrosis.

[0140] Depression has been likened to a state of "accelerated aging," and depressed individuals have a higher incidence of various diseases of aging, such as cardiovascular and cerebrovascular diseases, metabolic syndrome, and dementia. People with recurrent depressions or those exposed to chronic stress exhibit shorter telomeres in white blood cells. Shorter telomere length is associated with both recurrent depression and cortisol levels indicative of exposure to chronic stress (Wikgren, M. *et al.*, *Biol. Psych.*, 2011, DOI: 10.1016/j.biopsych.2011.09.015). However, not all depressed individuals show shortened telomeres equally because of a large variance in depressive episodes over a lifetime. Those who suffered from depression for long durations have significantly shorter telomeres due to

longer exposure to oxidative stress and inflammation induced by psychological stress when compared with control populations (Wolkowitz *et al.*, *PLos One*, 2011, **6**(3):e17837). Thus, the method of the present disclosure may find use in monitoring depression.

[0141] Chronic infection

[0142] Abnormal telomere length is associated with chronic infection including HIV (Effros RB et al, AIDS. 1996 Jul;10(8):F17-22, Pommier et al Virology. 1997, 231(1):148-54), and HBV, HCV and CMV (Telomere/telomerase dynamics within the human immune system: effect of chronic infection and stress. (Effros RB, Exp Gerontol. 2011 Feb-Mar;46(2-3):135-40. Rejuvenation Res. 2011 Feb;14(1):45-56. doi: 10.1089rej.2010.1085. Epub 2010 Sep 7.)

[0143] In Harley *et al.* ("A natural product telomerase activator as part of a health maintenance program", Harley CB, Liu W, Blasco M, Vera E, Andrews WH, Briggs LA, Raffaele JM, Rejuvenation Res. 2011 Feb;14(1):45-56), it was found that individuals who were CMV seropositive had shorter telomeres than those who were CMV negative, and moreover, the CMV positive subjects were more likely to respond to a nutrional supplement program of TA-65, a natural product-derived telomerase activator along with other supplements, in reducing the abundance of senescent CD8+/CD28- cells, suggesting a companion diagnostics application for measuring average telomere length or abundance of short telomeres, in conjunction with administration of telomerase activators.

[0144] Measurement of short telomere population can be used as indicator of prognosis disease progression and treatment outcome.

[0145] One study reported that telomere length in CD4+ cells is related to inflammatory grade, fibrosis stage, laboratory indices of severity, subsequent hepatic decompensation and treatment outcome in patients with chronic HCV infection (Hoare et al, *J. Hepatol.*, 2010, **53**(2):252-260).

[0146] In another report, longer leukocyte telomere length predicts increased risk of hepatitis B virus-related hepatocellular carcinoma (Liu et al, 2011, **117**(18):4247-56.)

[0147] In the case of HIV, telomere shortening is caused by viral infection. In addition, the nucleoside analog reverse-transcriptase inhibitors used to treat HIV are telomerase inhibitors (Strahl and Blackburn, *Mol Cell Biol.*, 1996, **16**(1):53-65; Hukezalie et al, *PLoS One*, 2012, **7**(11):e47505). Measurement of short telomere abundance might help determine the side effects and efficacy of HAART treatment.

[0148] 5.2.2 Other Pathological Conditions

[0149] The present disclosure also finds use in diagnosis of diseases related to early onset of aging. For example, individuals with Hutchinson Gilford progeria disease show premature aging and reduction in proliferative potential in fibroblasts associated with loss of telomeric length (Allsopp, R. C. et al, *Proc. Natl. Acad. Sci. USA*, 1992, **89**:10114-10118). Amplification and quantitation of the number of telomeric repeats according to the method of this disclosure is useful for determining disease risk or prognosis and taking appropriate interventional steps as described above.

[0150] 5.3 Telomere Diseases

[0151] In one aspect of the present disclosure, both the presence and the progress of telomeric-specific diseases may be determined using samples. Telomeric diseases are associated with an abnormal or premature shortening of telomeres, which can, for example, result from defects in telomerase activity. Telomerase is a ribonucleoprotein complex required for the replication and protection of telomeric DNA in eukaryotes. Cells lacking telomerase undergo a progressive loss of telomeric DNA that results in loss of viability and a concomitant increase in genome instability. These diseases may be inherited and include certain forms of congenital aplastic anemia, in which insufficient cell divisions in the stem cells of the bone marrow lead to severe anemia. Certain inherited diseases of the skin and the lungs are also caused by telomerase defects. For telomere diseases, a threshold for T/S<0.5 is appropriate for some conditions. Also, a commonly used metric is an age-adjusted percentile telomere score less than <10% or preferably <1% relative to a normal population.

[0152] Dyskeratosis congenita (DKC), also known as Zinsser-Engman-Cole syndrome, is a rare, progressive bone marrow failure syndrome characterized by mucocutaneous abnormalities: reticulated skin hyperpigmentation, nail dystrophy, and oral leukoplakia (Jyonouchi S. *et al.*, *Pediatr. Allergy Immunol.*, 2011, **22**(3):313-9; Bessler M., *et al.*, *Haematologica*, 2007, **92**(8):1009-12). Evidence exists for telomerase dysfunction, ribosome deficiency, and protein synthesis dysfunction in this disorder. Early mortality is often associated with bone marrow failure, infections, fatal pulmonary complications, or malignancy. The disease is inherited in one of three types: autosomal dominant, autosomal recessive, or the most common form, X-linked recessive (where the gene responsible for DC is carried on the X-chromosome). Early diagnosis and measurement of disease progress using the method of this disclosure is very beneficial for families with these genetic characteristics so that early treatment with anabolic steroids or bone-marrow-stimulating drugs can help prevent bone marrow failure. The non-invasive, patient friendly saliva-testing

method of the present disclosure is particularly useful for DKC because babies and small children need testing and continued monitoring.

[0153] Idiopathic interstitial pneumonias are characterized by damage to the lung parenchyma by a combination of fibrosis and inflammation. Idiopathic pulmonary fibrosis (IPF) is an example of these diseases that causes progressive scarring of the lungs. Fibrous scar tissue builds up in the lungs over time, affecting their ability to provide the body with enough oxygen. Heterozygous mutations in the coding regions of the telomerase genes, TERT and TERC, have been found in familial and sporadic cases of idiopathic interstitial pneumonia. All affected patients with mutations have short telomeres. A significant fraction of individuals with IPF have short telomere lengths that cannot be explained by coding mutations in telomerase (Cronkhite, J. T., *et al.*, *Am. J. Resp. Crit. Care Med.*, 2008, **178**:729-737). Thus, telomere shortening can be used as a marker for an increased predisposition toward this age-associated disease (Alder, J. K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 2008, **105**(35):13051-13056). Further, the course of IPF varies from person to person. For some, the disease may progress slowly and gradually over years, while for others it may progress rapidly. The method of the present may be conveniently used to monitor the course of pulmonary fibrosis and taking appropriate interventional steps as described above.

[0154] Aplastic anemia is a disease in which bone marrow stops making enough red blood cells, white blood cells and platelets for the body. Any blood cells that the marrow does make are normal, but there are not enough of them. Aplastic anemia can be moderate, severe or very severe. People with severe or very severe aplastic anemia are at risk for life-threatening infections or bleeding. Patients with aplastic anemia carrying telomerase mutations have an increased risk of developing myelodysplasia. Telomerase deficiency may cause variable degrees of telomere shortening in hematopoietic stem cells and lead to chromosomal instability and malignant transformation (Calado, R. T. and Young, N. S., *The Hematologist*, 2010 world wide web URL hematology.org/Publications/Hematologist/2010/4849.aspx). Aplastic anemia patients with shorter telomeres have a lower survival rate and are much more likely to relapse after immunotherapy than those with longer telomeres. Scheinberg *et al.* (*JAMA*, 2010, **304**(12):1358-1364) found that relapse rates dropped as telomere lengths increased. The group of patients with the shortest telomeres was also at greater risk for a conversion to bone marrow cancer and had the lowest overall survival rates. The method of the present disclosure can be used in aplastic anemia patients to monitor the risk of developing major complications so that the clinical management of an individual may be tailored accordingly.

[0155] 5.4 Drug Responsiveness

[0156] In another aspect, the present disclosure is useful in monitoring effectiveness of therapeutics or in screening for drug candidates affecting telomere length or telomerase activity. The ability to monitor telomere characteristics can provide a window for examining the effectiveness of particular therapies and pharmacological agents. The drug responsiveness of a disease state to a particular therapy in an individual may be determined by the method of the present disclosure. For example, the present disclosure finds use in monitoring the effectiveness of cancer therapy since the proliferative potential of cells is related to the maintenance of telomere integrity. As described above, while normal human somatic cells do not or only transiently express telomerase and therefore shorten their telomeres with each cell division, most human cancer cells typically express high levels of telomerase and show unlimited cell proliferation. Roth *et al.*, (ibid., 2010) have suggested that individuals with cancer who have very short telomeres in their tumors (in which the shortest telomeres in most cells are near to telomere dysfunction) and high telomerase activity might benefit the most from anti-cancer telomerase-inhibiting drugs. Because telomerase is either not expressed or expressed transiently and at very low levels in most normal cells, telomerase inhibition therapies may be less toxic to normal cells than conventional chemotherapy. An example of such drugs is the short oligonucleotide-based telomerase inhibitor imetelstat (previously named GRN163L). Imetelstat is a novel lipid-based conjugate of the first-generation oligonucleotide GRN163 (Asai, A. *et al.*, *Cancer Res.*, 2003, **63**:3931-3939). However, cancer patients having very short telomeres in normal blood cells (particularly their granulocytes) are at higher risk of adverse effects of imetelstat on proliferative tissues such as the bone marrow. Rattain *et al.* (2008) found that such subjects with short granulocyte telomere length were more likely to have bone marrow failure symptoms such as neutropenia or thrombocytopenia. In this situation, a doctor might prescribe a lower dose of imetelstat, a different drug, or more frequent monitoring for bone marrow problems.

[0157] In other aspects, drug efficacy in the treatment of diseases of aging, for example but not limited to, cardiovascular disease, diabetes, pulmonary fibrosis, liver fibrosis, interstitial pneumonia and depression. In the case of cardiovascular disease, Brouilette *et al.* reported that middle-aged men with shorter telomere lengths than control groups benefit the most from lipid-lowering therapy with pravastatin (Brouilette, S. W. *et al.*, *Lancet*, 2007, **369**:107-114). Satoh *et al.* (*Clin. Sci.*, 2009, **116**:827-835) indicating that intensive lipid-lowering therapy protected telomeres from erosion better in patients treated with atorvastatin

when compared with patients treated with moderate pravastatin therapy. The method of the present disclosure can be used to monitor the efficacy of statins in treated patients, wherein shorter telomere length correlates with better drug efficacy. Since subjects with the longest telomeres did not on average benefit from prophylactic statins, a doctor might suggest that the patient be especially compliant with good lifestyle habits as part of their treatment program. Conversely, patients with short telomeres who fear side effects of chronic statin usage might be persuaded to take statins based on their higher probability of benefiting from statins. Examples of statins that can be used include niacin (ADVICOR, SIMCOR), lovastatin (ALTOPREV, MEVACOR), amolopidine (CADUET), rosuvastatin (CRESTOR), sitagliptin/simvastatin (JUVISYNC), fluvastatin (LESCOL), pravastatin (PRAVACHOL), atorvastatin (LIPITOR), pitavastatin (LIVALO), and ezetimibe/simvastatin (VYTORIN).

[0158] In further aspects, drug effectiveness in the treatment of telomeric diseases, for example but not limited to, Dyskeratosis congenita, pulmonary fibrosis, and aplastic anemia, may be measured. For example, dyskeratosis congenita and pulmonary fibrosis are both treated with high-dose steroids, which are well known to have numerous deleterious side effects. Use of the lowest possible steroid dose is thus highly desirable, making the method of the present disclosure a valuable tool for monitoring these patients.

[0159] **5.5 Drug Candidate Screening**

[0160] In another aspect, the present disclosure finds use as a general method of screening for candidate drugs, dietary supplements, and other interventions including lifestyle changes which affect biological pathways regulating telomere length, such as telomerase activity. Ability to rapidly and specifically amplify telomere repeats in a quantitative manner provides a high throughput screening method for identifying small molecules, candidate nucleic acids, and peptides agents and other products or interventions affecting telomere dynamics in a cell. Drug or other product candidates that have a positive, telomere lengthening effect on normal cells would be preferred in the treatment of degenerative, or cell senescence related conditions over those with telomere shortening (or telomerase inhibiting) effects, everything else being equal. In the case of treatment of cancer, drugs that have a negative, telomere shortening effect, especially in cancer cells would be preferred.

EXAMPLES

[0161] **Example 1 – Amplification of Short Telomere Sequences**

[0162] Here we describe a quantitative PCR based method using purified genomic DNA to measure percentage of short telomeres. This method incorporates non-covalent binding of a non-human primer 'TeloPrimer' to the 3′-overhang of vertebrate genomic DNA, time-

controlled extension of the TeloPrimer towards a subtelomere unique sequence 'SUS' with an enzyme that displaces or degrades the C-strand of the telomere during the extension reaction, followed by an amplification reaction utilizing SUS and the non-human sequence portion of TeloPrimer. Only telomeres that are short enough such that the extended TeloPrimer product reaches the SUS sequence are amplified. The controlled timing of the strand displacement reaction thus allows detection of telomeres shorter than a specified threshold of telomere length. In addition, by controlling the extension time in the PCR step by SUS and TeloAnchor primers, the short telomere population will be selectively amplified, further increasing the specificity of this assay to measure short telomeres. The abundance of short telomeres is quantified in the T-run of TeloTest.

[0163] Materials and Methods

[0164] Primers

[0165] All primers were purchased from Integrated DNA Technologies in standard desalted or HPLC purified form. Sequences of the primers are listed below:

TeloPrimer: 5′- TGCTCGGCCGATCTGGCATCCCTAACC- 3′ [SEQ ID NO: 7]

TeloAnchor: 5′-TGCTCGGCCGATCTGGCATC-3′ [SEQ ID NO:8]

SUS (HPLC purified): 5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO:2]

TeloProbe (HPLC purified): 5′-CCCTAACCCTAACCCTAACCCTAA- 3′ [SEQ ID NO:9]

[0166] Annealing of TeloPrimer to genomic DNA

[0167] Human genomic DNA was mixed with TeloPrimer at final concentration of 20 ng/ul DNA and 1uM TeloPrimer in a 50 ul reaction, incubated at 65 °C for 10 min in a heating block and then cooled down to room temperature over the period of one hour. The annealed samples are kept on ice until strand displacement reaction.

[0168] Strand displacement

[0169] Strand displacement reaction was carried out in 5 ul volume, with 50 ng of the annealed DNA, 40 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 50 mM NaCl, 5 mM DTT (PN 70726, Affymetrix, Santa Clara, CA, USA), 100 ug/ml BSA (Cat# B9001S, New England Biolabs, Ipswich, MA), 500 uM dNTP (Cat# 1708874, Bio-Rad, Hercules, CA), 5 uM Single Strand Binding protein (SSB, Cat#70032Y 100 UG, Affymetrix, Santa Clara, CA) and 400 nM Sequenase 2.0 (cat # 70775Y 200 UN, Affymetrix, Santa Clara, CA) (Concentration of Sequenase is 13 U/ul). The mixture without Sequenase 2.0 is pre-warmed at 37 °C for one

minute, and then incubated at 37 °C for 30 sec to 10 min after sequenase 2.0 was added to the mixture. The reaction is stopped by heat inactivation at 80 °C for 20 min.

[0170] PCR Amplification of strand displacement product

[0171] The product from the strand displacement DNA reaction was diluted 50-fold in DNA suspension buffer (Cat# T0227, Teknova, Hollister, CA). PCR was carried out in 40 ul reaction volume that contains 1.5 mM MgCl2, 300 uM dNTP (Cat# 1708874, Bio-Rad, Hercules, CA), 0.5 uM SUS primer, 0.5 uM TeloAnchor primer, 2 ul diluted DNA, 5 units PLATINUM Taq polymerase (Cat # 10966-083, Life Technologies, Grand Island, NY).

[0172] The cycling program is as the following: 2 min at 94 °C, 35 cycles of 15 sec at 94 °C, 30 sec at 65 °C, 30 seconds to 10 min at 72 °C, followed by 10 min extension at 72 °C. The PCR reaction is carried in a Bio-Rad C1000 Thermocycler (Bio-Rad, Hercules, CA).

[0173] Quantification of amplified PCR product by TeloTest

[0174] PCR products were diluted 10 to 1000 fold in DNA suspension buffer. The T-run of TELOTEST was used to quantify the PCR products. PCR fragments containing between 60-600 bp perfect telomere repeats are used as standard to calculate DNA concentration of each sample. An internal standard of ~2kb DNA fragment with TTAGGG 3′ overhang and SUS sequence near 5′ will be included in each sample from displacement reaction to qPCR steps. Reaction efficiency of each sample will be estimated by qPCR of a non-human sequence within the internal standard. A normalizing factor for each sample will be generated based on the reaction efficiency.

[0175] Southern Blot

[0176] Southern blot analysis was carried out to confirm the size of displaced telomere DNA. Strand displacement products amplified by SUS and TeloAnchor primers were run on a 0.5% agarose gel, transferred to a positively charged Nylon membrane and hybridized overnight at 37 °C to a DIG labeled oligo probe with four TTAGGG repeats. Chemiluminescent detection was used for signal detection.

[0177] Results

[0178] Overall scheme

[0179] The short telomere assay (STA) described here quantifies the amount of short telomeres in human genomic DNA samples by strand-displacement or strand degradation followed by quantitative PCR. The overall scheme of the assay is illustrated in Figure 1.

[0180] The assay is carried out by first annealing TeloPrimer to human genomic DNA. TeloPrimer contains 19 nucleotide of non-human sequence at the 5′ end and 8 nucleotides of telomeric sequence at the 3′ end. Using native genomic DNA ensures that TeloPrimer will

only anneal to the 3′ single-strand tail (overhang) of telomeres, not the double strand region. Extension of the TeloPrimer is achieved by a strand displacement or strand degradation reaction. With limited time, the reaction will only be able to reach to the telomere variance region on those chromosomes with short telomeres, while on the chromosomes with long telomeres, the strand-displaced products will stop within the perfect telomere repeat region. Displaced short telomeres are then enriched by PCR with SUS primer and TeloAnchor primer. SUS primer contains 3 repeats of the TGAGGG sequence that are found on most chromosomes in the telomeric variant region, at or near the junction between the subtelomeric region and true telomere repeats. TeloAnchor primer shares the same non-human sequence as TeloPrimer and does not contain telomere sequence. The pair of SUS and TeloAnchor is designed to specifically amplify the strand-displaced short telomeres. Finally, TELOTEST T-run is used to quantify the amount of short telomeres.

[0181] Quantification of strand displacement products

[0182] Strand displacement amplification is a DNA synthesis reaction where the downstream DNA encountered is displaced by the polymerase during synthesis. Many protocols including whole genome amplification and forensic analysis use strand displacement to amplify minute quantities of template DNA. Because of the strand-displacing activity of the DNA polymerase, DNA with nicks or single strand tails can be used as templates without a denaturation step. We chose to use a strand-displacing enzyme in our experimental design because the single-strand tail of telomeres serves as a natural template for strand-displacing enzymes and is less likely than a 5′-3′ exonuclease (degradative enzyme) to create gaps in genome that might cause non-specific amplification of interstial telomere-like sequences. Annealing of TELOPRIMER to the single-strand tails of telomeres on native genomic DNA places TeloPrimer adjacent to the 5′ end of the C-strand (Figure 1). In this design, the length of strand-displaced products will accurately reflect the length of the telomeres. By controlling the strand-displacement time, the reaction will only be able to reach to the telomere variance region on those chromosomes with short telomeres. We chose to use Sequenase 2.0, a genetically engineered form of T7 DNA polymerase with no exonuclease activity, because it is reported to synthesize DNA on a linear template with high specificity and processivity (Joneja, A. and X. Huang, Anal Biochem, 2011. 414(1) 58-69). We performed a time course experiment where the strand displacement reaction took from 30 seconds to 5 minutes. Genomic DNA from the bladder cancer cell line UM-UC3 was annealed to TeloPrimer and strand-displacement reaction was performed as detailed in Materials and Methods.

[0183] Figure 2 showed increased telomere products measured by T-run of TELOTEST qPCR with increased strand displacement reaction time. The linear regression line showed a strong relationship between time abundance of the amplicon (R^2= 0.99). Furthermore, in the negative control, where no Sequenase 2.0 was added, no PCR product was detected in the TELOTEST T-run (Crossing point (Cp)= 25, calculated concentration=1.85E-07) which is 159-fold lower than the lowest measured concentration of the reactions containing Sequenase 2.0. A parallel reaction using the single copy gene (beta-globin) primers in the TELOTEST S-run also showed no amplification product, further confirming that the telomeric products measured in T-run are derived from the strand-displacement reaction, not from total genomic DNA.

[0184] The experimental design we propose here predicted that with short displacement reaction time, only products from short telomeres will be amplified with SUS and TeloAnchor primers. We sought to confirm this by Southern blot analysis. Strand displacement products amplified by SUS and TeloAnchor primers were run on a 0.5% agarose gel, transferred to a positively charged Nylon membrane and hybridized to a DIG labeled oligo probe with four TTAGGG repeats. Figure 3 shows that the length of the amplified PCR products increases with increasing strand displacement time. The estimated modes (peak of intensity) of the PCR products are approximately 0.6 kb, 0.9kb. 1.2 and 1.4kb with 0.5 minute, 1 minute, 3 minute and 5 minute displacement time respectively. With 1 minute displacement time, the majority of the products are below 2 kb. In an independent comparison test, we used a modified single telomere elongation length analysis (STELA) (Baird, D.M., *et al.*, Nat Genet., 2003. 33(2) 203-7) protocol to analyze the telomere length profile of the total genomic DNA from the same cancer cell line UM-UC3. TeloPrimer was ligated to the 5′ end of the C strand and XpYpE2 and TeloAnchor primers were used to amplify telomeres. The PCR product was run on the gel (lane 1 in Figure 3). This revealed that the mode of UM-UC3 telomeres is around 1.8 kb, consistent with previous reports (Xu, L. and E.H. Blackburn, Mol Cell, 2007. 28(2) 315-27).

[0185] To further validate the short telomere assay, we carried out this assay with two different genomic DNA samples. In addition to the UM-UC3 DNA, we used genomic DNA from UM-UC3 infected by a lentivirus vector that expresses the RNA gene of telomerase hTER, resulting in extension of telomeres (Xu, L. and E.H. Blackburn, Mol Cell, 2007. 28(2) 315-27). The average telomere length measured by qPCR in UM-UC3/hTER is 2.1 compared to 0.56 for UM-UC3 (Telome Health Inc. data). UM-UC3/hTER cells have a lower percentage of short telomeres, as judged by Southern blot analysis (Figure 5).

Consistent with this, the calculated amount of short telomeres in UM-UC3 is 3-fold higher compared to UM-UC3/hTER (Figure 4).

[0186] We conclude that the short telomere assay specifically measures the relative percentage of short telomeres. Furthermore, by controlling the strand displacement reaction time, the telomere length cutoff that will be measured in this assay can be varied and predetermined.

[0187] The above mentioned short telomere assay can be easily adapted to a high-throughput automated format. Figure 6 shows individual steps in such a format.

[0188] While preferred aspects of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such aspects are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the disclosure. It should be understood that various alternatives to the aspects of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of making a nucleic acid extension product comprising:

i) hybridizing an extension primer to a telomeric repeat sequence in a 3′ overhang of double stranded chromosomal DNA, wherein:

(1) the double stranded chromosomal DNA has a telomeric region comprising telomeric repeat sequences and a sub-telomeric region comprising sub-telomeric sequences; and

(2) the extension primer comprises:

(A) a 3′ portion that hybridizes to a telomeric repeat sequence in the 3′ overhang under annealing conditions, and

(B) a 5′ portion having an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3′ overhang under the annealing conditions; and

ii) performing a time-limited extension reaction to extend the extension primer towards the sub-telomeric region of the double stranded chromosomal DNA, wherein the extension reaction is timed to produce an extension product comprising both telomeric repeat sequences and sub-telomeric sequences only from double stranded chromosomal DNA having a telomeric region within a pre-determined length range.

2. The method of claim 1, wherein the double-stranded chromosomal DNA comprises chromosomes molecules having telomeres of different lengths.

3. The method of claim 1, wherein the anchor sequence does not hybridize under the annealing conditions to a sequence in:

(1) the sub-telomeric region of the strand of chromosomal DNA having the 3′ overhang;

(2) a G-strand of the chromosomal DNA within 20 kb of the 3′ overhang;

(3) a G-strand of the chromosomal DNA within 50 kb or within 20 kb of the 3′ overhang; or

(4) the double stranded chromosomal DNA.

4. The method of claim 1, wherein the pre-determined length range from 0-0.5 kb, 0-1 kb, 0-2 kb, 0-3 kb, 0-4 kb, or 0-5 kb.

5. The method of claim 1, wherein the extension reaction is timed to be 0.1-30 minutes, 0.1-10 minutes, 0.1-5 minutes, 0.1-4 minutes, 0.1-3 minutes, 0.1-2 minutes, no more 0.1-1 minute, or 0.1-0.5 minute.

6. The method of claim 1, wherein the extension reaction is timed to be 60 minutes, 45 minutes, 30 minutes, 20 minutes, 15 minutes, 10 minutes, 9 minutes, 8 minutes, 7 minutes, 6 minutes, 5 minutes, 4 minutes, 3 minutes, 2 minutes, 1 minute or 0.5 minute.

7. The method of claim 1, wherein the double-stranded chromosomal DNA is provided from a solid, fluid, semisolid or gaseous sample.

8. The method of claim 7, wherein the chromosomal DNA is from a liquid sample selected from blood, saliva, urine, plasma, serum, cerebrospinal fluid ("CSF") or bronchoalveolar lavage fluid.

9. The method of claim 7, wherein the chromosomal DNA is from a solid sample selected from lung, muscle or skin.

10. The method of claim 7, wherein the chromosomal DNA is from a semi-solid sample comprising bone marrow.

11. The method of claim 7, wherein the chromosomal DNA is provided from a gaseous sample comprising breath.

12. The method of claim 1, wherein the double-stranded chromosomal DNA is vertebrate DNA, mammalian DNA, or human DNA.

13. The method of claim 1, wherein the 3′ portion of the extension primer hybridizes to a human telomeric repeat sequence.

14. The method of claim 1, wherein the 3′ portion of the extension primer comprises the sequence 5′-(CCCTAA)n -3′ or its same order permutations, wherein n is at least 1.

15. The method of claim 14, wherein n is at least 2.

16. The method of claim 1, wherein the 5′ portion of the extension primer comprises the sequence: 5′- TGCTCGGCCGATCTGGCATC- 3′ [SEQ ID NO:8].

17. The method of claim 1, wherein the extension primer comprises the sequence: 5′-TGCTCGGCCGATCTGGCATCCCTAACC-3′ [SEQ ID NO: 7].

18. The method of claim 1, wherein the time-limited extension reaction employs a DNA polymerase possessing strand-displacement activity, exonuclease activity or strand degradation activity.

19. The method of claim 18, wherein the DNA polymerase is selected from a modified T7 polymerase, an exonuclease-deficient Klenow fragment of E. coli DNA polymerase I, and Bst DNA polymerase.

20. The method of claim 18, wherein the first reaction is performed with in combination with a DNA polymerase a helicase or with a DNA polymerase with 5′-3′ exonuclease activity.

21. A method of amplifying telomeric repeat sequences and sub-telomeric sequences of a chromosome comprising:

a) making a nucleic acid extension product by:

i) hybridizing an extension primer to a telomeric repeat sequence in a 3′ overhang of double stranded chromosomal DNA, wherein:

(1) the double stranded chromosomal DNA has a telomeric region comprising telomeric repeat sequences and a sub-telomeric region comprising sub-telomeric sequences; and

(2) the extension primer comprises:

(A) a 3′ portion that hybridizes to a telomeric repeat sequence in the 3′ overhang under annealing conditions, and

(B) a 5′ portion having an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3′ overhang under the annealing conditions; and

ii) performing a time-limited extension reaction to extend the extension primer towards the sub-telomeric region of the double stranded chromosomal DNA, wherein the extension reaction is timed to produce an extension product comprising both telomeric repeat sequences and sub-telomeric sequences only from double stranded chromosomal DNA having a telomeric region within a pre-determined length range; and

b) amplifying sequences of the extension product;

thereby producing a length-limited amplification product comprising nucleic acids having telomeric repeat sequences and sub-telomeric sequences.

22. The method of claim 21, wherein the sequences are amplified using:

(1) a first amplification primer that hybridizes to a sequence unique to the sub-telomeric region in the extension product under annealing conditions; and

(2) a second amplification primer that hybridizes to the anchor sequence under the annealing conditions.

23. The method of claim 22, wherein the first amplification primer comprises the sequence 5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO: 2], 5′-CGGGCCGGCTGAGGGTACCGCGA-3′ [SEQ ID NO: 10] (chromosome 1), 5′-GCTAATGCACTCCCTCAATAC-3′ [SEQ ID NO: 11] (chromosome 5), or 5′-CATTCCTAATGCACACATGATACC-3′ [SEQ ID NO: 12] (Chromosome 9)

24. The method of claim 22, wherein the first amplification primer comprises the sequence 5′-GATGGATCCTGAGGGTGAGGGTGAGGG-3′ [SEQ ID NO: 2] and the second primer comprises the sequence 5′-TGCTCGGCCGATCTGGCATC-3′ [SEQ ID NO: 8].

25. The method of claim 21, wherein the length range of the amplified telomere products can be determined by using a time-limited extension time in the PCR reaction.

26. The method of claim 21, further comprising co-amplifying a control sequence.

27. The method of claim 26, wherein the control sequence comprises a plurality of non-telomeric repeat sequences.

28. The method of claim 27, wherein the control sequence is synthesized *in vitro* or produced *in vivo*.

29. A method for determining short telomere abundance comprising:

a) providing a sample comprising double-stranded chromosomal DNA comprising a 3′ overhang from a subject;

b) producing a length-limited amplification product from the double-stranded chromosomal DNA using the method of claim **21**; and

c) determining short telomere abundance from the length-limited amplified product.

30. The method of claim 29, further comprising:

d) comparing the short telomere abundance with a measure of total telomere abundance from the sample.

31. The method of claim 29, wherein short telomeres are telomeres having length of 0.1-0.5 kb, 0.1-1 kb, 0.1-2 kb, 0.1-3 kb, 0.1-4 kb, or 0.1-5 kb.

32. The method of claim 30, wherein step d) comprises determining short telomere abundance as a function of total telomere abundance.

33. The method of claim 29, wherein determining short telomere abundance is performed using qPCR.

34. The method of claim 33, wherein qPCR is performed using a first and a second primer,

i) wherein said first primer hybridizes to at least one repetitive unit of said first strand and said second primer hybridizes to at least one repetitive unit of said second strand,

ii) wherein said hybridized primers are capable of primer extension when hybridized to their respective strands, and wherein at least one nucleotide of said first primer produces an internal base pair mismatch between said first primer and a nucleotide of said repetitive unit when said first primer is hybridized to at least one repetitive unit of said first strand,

iii) wherein said first primer also produces a mismatch with the 3′ terminal nucleotide of said second primer when first and second primers hybridize to each other,

iv) wherein at least one nucleotide of said second primer produces an internal base pair mismatch between said second primer and a nucleotide of said repetitive unit when said second primer is hybridized to at least one repetitive unit of said second strand.

35. The method of claim 29, wherein determining short telomere abundance comprises measuring average telomere length in the sample by Southern blot, dot blot, slot blot, immunochemistry, nucleic acid sequencing, or digital PCR.

36. The method of claim 29, wherein the short telomere abundance is a measure of relative abundance.

37. The method of claim 30, wherein the total telomere abundance is measured relative to abundance of a genomic reference sequence.

38. The method of claim 37, wherein the genomic reference sequence comprises a single copy reference nucleotide sequence or a non-telomere repetitive DNA sequence.

39. The method of claim 38, wherein the single copy reference nucleotide sequence is human beta-globin.

40. The method of claim 38, wherein the non-telomere repetitive DNA sequence is an Alu repeat sequence or centromeric repeat sequence.

41. A method comprising:

a) obtaining a sample from a subject;

b) determining short telomere abundance using the method of any of claims 29-38; and

c) correlating short telomere abundance with a condition or disease.

42. The method of claim 41, wherein the measure of short telomere abundance is determined by comparing short telomere abundance with total telomere abundance from the sample.

43. The method of claim 41, wherein the condition or disease is mortality risk.

44. The method of claim 41, wherein the telomere abundance is absolute abundance.

45. The method of claim 44, wherein the absolute abundance is measured as length of telomeric sequences.

46. The method of claim 41, wherein determining telomere abundance comprises measuring average telomere length in the sample by qPCR Southern blot, dot blot, slot blot, immunochemistry, nucleic acid sequencing, or digital PCR.

47. The method of claim 41, wherein the condition or disease is a Health Status Survey Score of Perceived Stress.

48. The method of claim 41, wherein the condition or disease is a risk of disease.

49. The method of claim 48, wherein the risk of disease is an age-associated disease.

50. The method of claim 49, wherein the age-associated disease is cardiovascular disease; and wherein a measure lower than average in a population correlates with increased risk of cardiovascular disease.

51. The method of claim 50, wherein determining the short telomere abundance comprises correlating short telomere abundance in the lowest two or three tertiles of a population with a significantly higher risk for cardiovascular disease compared with a measure in a top tertile of the population.

52. The method of claim 48, wherein correlating short telomere abundance comprises correlating with a telomeric disease.

53. The method of claim 52, wherein the telomeric disease is dyskeratosis congenita, pulmonary fibrosis, aplastic anemia, or interstitial pneumonia.

54. The method of claim 48, wherein correlating the short telomere abundance comprises correlating with drug responsiveness.

55. The method of claim 54, wherein the drug responsiveness is responsiveness to a statin; and wherein short telomere length is positively correlated with drug responsiveness.

56. The method of claim 54, wherein the drug responsiveness is responsiveness to imetelstat (GRN163L); and wherein long telomere length is positively correlated with drug responsiveness.

57. The method of claim 48, wherein correlating the measure comprises correlating with disease progression and treatment outcome in chronic infections,

58. The method of claim 57, wherein the chronic infection is selected from HIV, HCV, HBV, and CMV.

59. The method of any of claims 39-58, further comprising reporting the correlation to the subject.

60. The method of any of claims 39-59, further comprising providing the subject with a diagnosis or a prognosis based on the correlation.

61. The method of any of claims 39-60, further comprising treating the subject based on the correlation.

62. A method for monitoring the status of a subject comprising:

a) obtaining a plurality of samples from a subject; wherein the samples are taken over a period of time; and wherein each sample is obtained at a different time;

b) determining short telomere abundance in each of the plurality of samples using the method of any of claims any of claims 29-38;

c) determining a rate of change in the measures of short telomere abundance; and

d) correlating the differences with progression of a telomeric disease, wherein decreases in the measures indicates progression of the disease.

63. The method of claim 62, wherein the short telomere abundance is determined by comparing the short telomere abundance with total telomere abundance from the sample.

64. A method comprising:

a) obtaining a plurality of samples from a subject; wherein the samples are taken over a period of time; and wherein each sample is obtained at a different time;

b) determining short telomere abundance in each of the plurality of samples using the method of any of claims any of claims 29-38;

c) determining a rate of change in the measures of short telomere abundance; and

d) correlating the rate of change with: (1) a measure of health; (2) a risk of a pathological condition; (3) a telomeric disease or (4) drug responsiveness.

65. The method of claim 64, wherein the short telomere abundance is determined by comparing the short telomere abundance with total telomere abundance from the sample.

66. A kit comprising:

(1) a first amplification primer comprising:

(A) a 3′ portion that hybridizes to a telomeric repeat sequence in a 3′ overhang of double-stranded chromosomal DNA under annealing conditions, and

(B) a 5′ portion having an anchor sequence that does not hybridize under the annealing conditions to a sequence in the telomeric region or to a sequence in the sub-telomeric region in the chromosomal DNA; and

(2) a second amplification primer that hybridizes to a sub-telomeric sequence under annealing conditions.

67. The kit of claim 66, further comprising:

(3) a third amplification primer that hybridizes to a complement of the anchor sequence under annealing conditions.

68. The kit of claim 66, further comprising:

(3) reagents to carry out the hybridization, extension, amplification and quantification steps of the short telomere measurement.

69. The kit of claim 66, further comprising:

(3) a control sample and a reference sample comprising chromosomal DNA with known telomere lengths.

FIG. 1

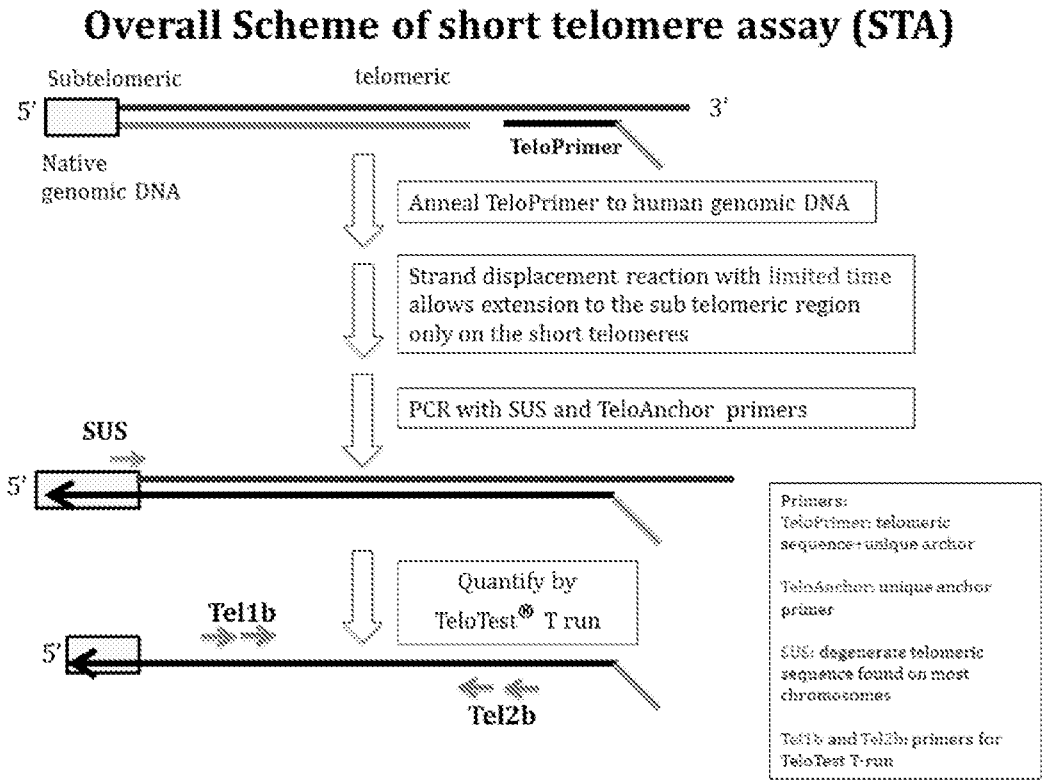

Overall Scheme of short telomere assay (STA)
Subtelomeric
telomeric
5'
3'
TeloPrimer
Native
genomic DNA
Anneal TeloPrimer to human genomic DNA
Strand displacement reaction with limited time allows extension to the sub telomeric region only on the short telomeres
PCR with SUS and TeloAnchor primers
SUS
5'
Quantify by
TeloTest® T run
Tel1b
5'
Tel2b
Primers:
TeloPrimer: telomeric sequence+unique anchor
TeloAnchor: unique anchor primer
SUS: degenerate telomeric sequence found on most chromosomes
Tel1b and Tel2b: primers for TeloTest T-run

FIG. 2

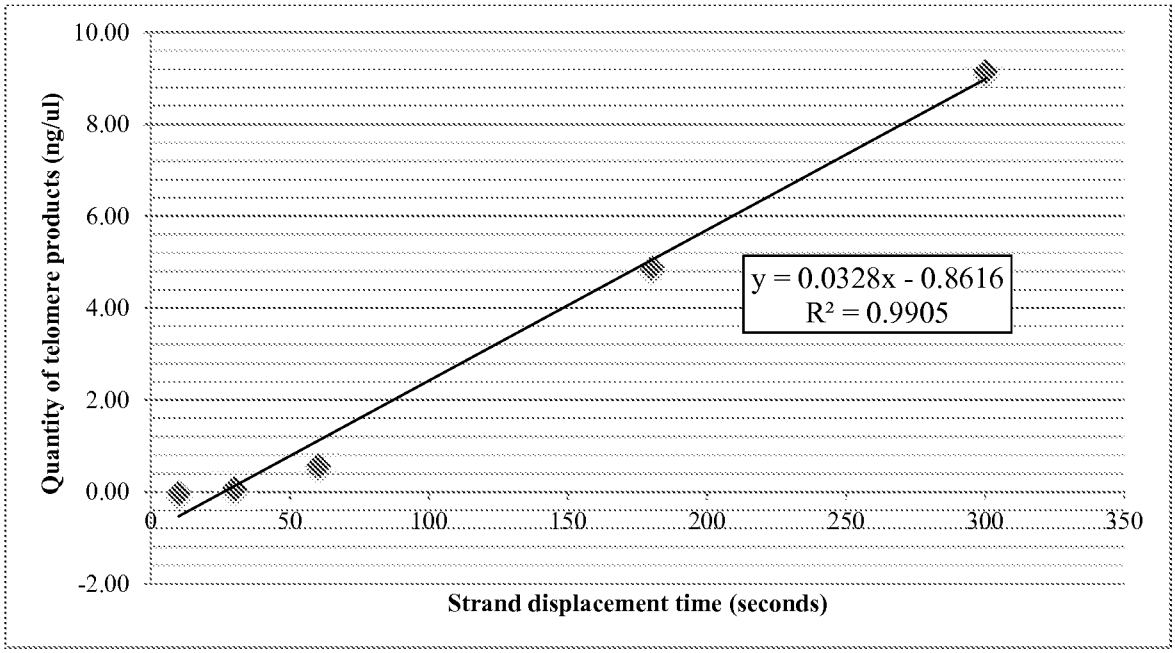

10.00
8.00
6.00
4.00
2.00
0.00
-2.00
Quantity of telomere products (ng/ul)
0
50
100
150
200
250
300
350
Strand displacement time (seconds)
y = 0.0328x - 0.8616
R² = 0.9905

FIG. 3

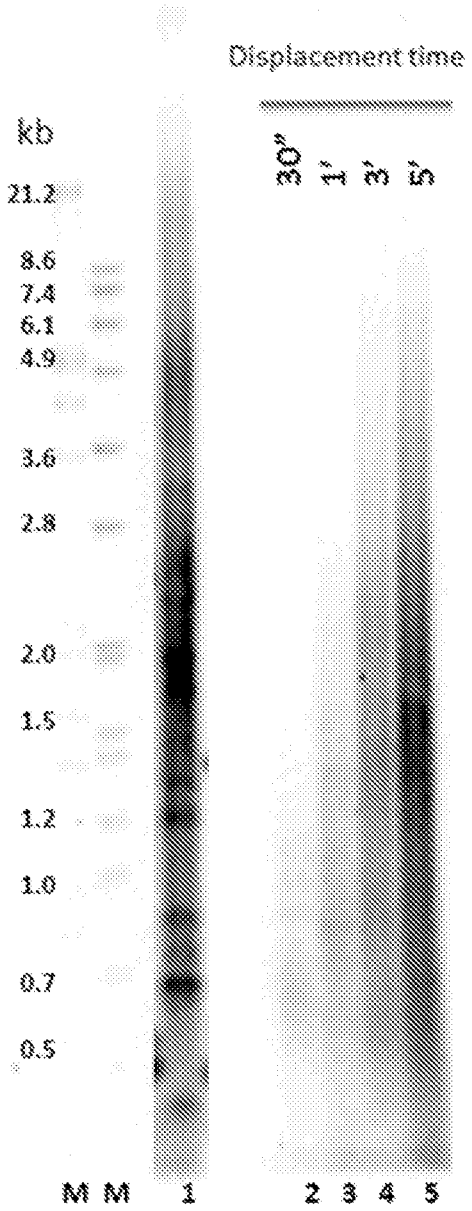

Displacement time
30'
1'
3'
5'
kb
21.2
8.6
7.4
6.1
4.9
3.6
2.8
2.0
1.5
1.2
1.0
0.7
0.5
M M 1 2 3 4 5

FIG. 4

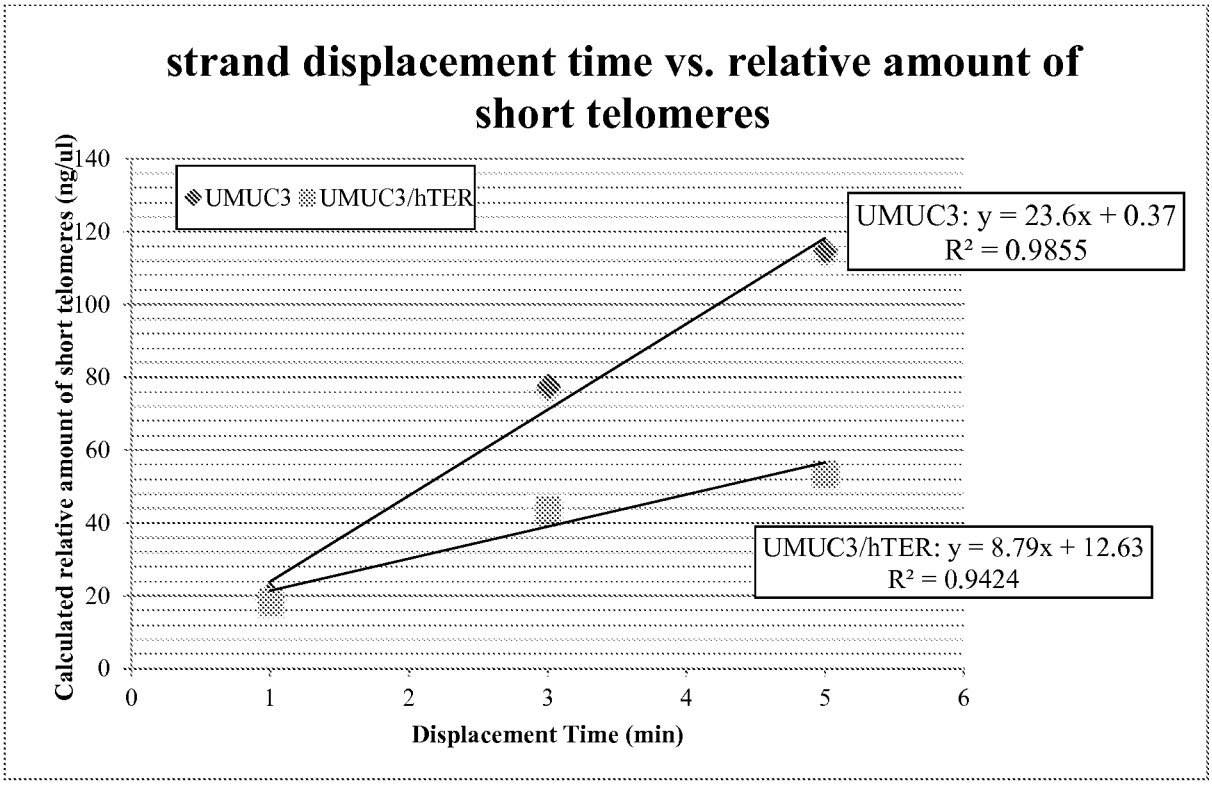

strand displacement time vs. relative amount of short telomeres
Calculated relative amount of short telomeres (ng/ul)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Displacement Time (min)
UMUC3
UMUC3/hTER
UMUC3: y = 23.6x + 0.37
R² = 0.9855
UMUC3/hTER: y = 8.79x + 12.63
R² = 0.9424

FIG. 5

percentage of short telomeres	UMUC3	UMUC3/hTER
Southern blot (0.2-5Kb)	75%	26%
short telomere assay	100%	33%

FIG. 6

STEP	DESCRIPTION	AUTOMATION OR MANUAL
Annealing	1.1. pipette gDNA and mix with TeloPrimer and water	Automated
	1.2. incubate at 65 °C for 10 min, cool down slowly	
Strand displacement	2.1. pipette master mix with Sequenase 2.0/SSB/buffer	Automated
	2.2. pipette gDNA and mix	Automated
	2.3. seal plate	Manual
	2.4. incubate at 37 °C for 3 minutes	Manual
	2.5. transfer plate to 80 °C for 20 minutes	Manual
PCR with SUS and TELOANCHOR	3.1. Dilute samples from step 2.5 with low EDTA Tris	Automated
	3.2. pipette PCR master mix into a new 96 well plate	Automated
	3.3. pipette diluted DNA from step 3.1 to 96 well plate from step 3.2	Automated
	3.4. seal both plate	Manual
	3.5. transfer the PCR plate to Thermocycler	Manual
	3.6. PCR	
TELOTEST	4.1.Dilute PCR products from step 3.6 with low EDTA Tris	Automated
	4.2. Perform the T-run of TeloTest® with diluted samples from 4.1.	Automated

FIG. 7A

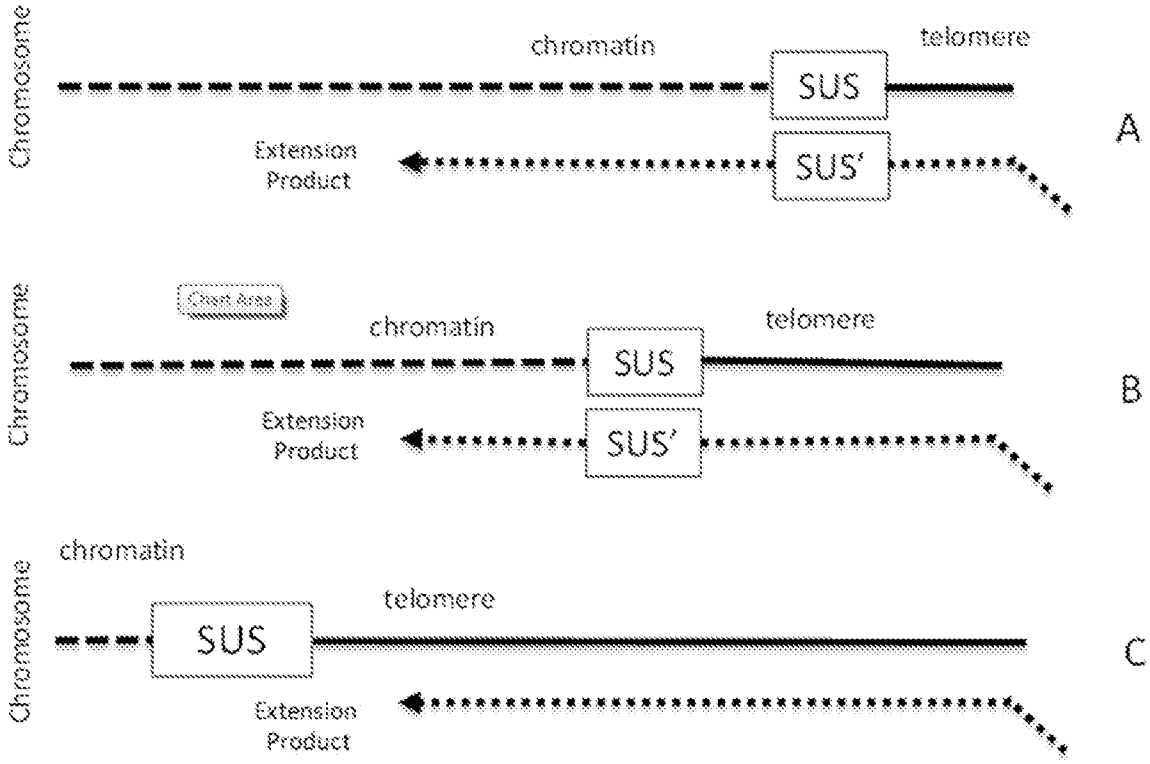
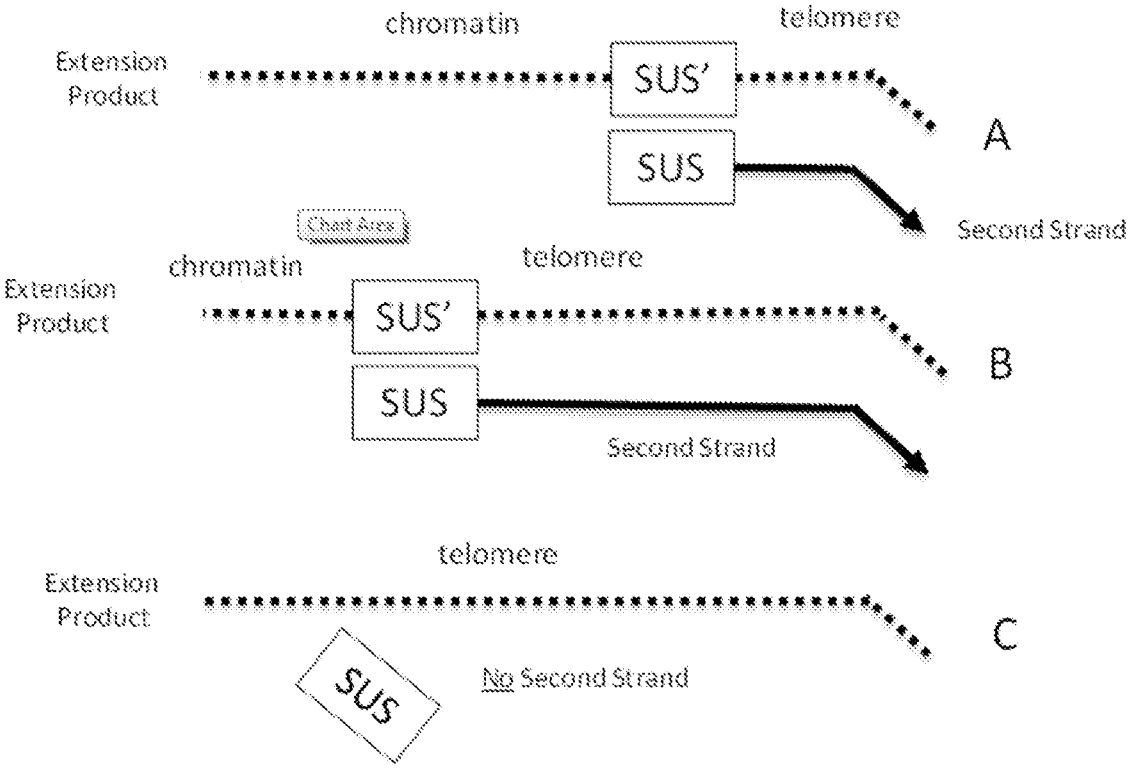

Chromosome
chromatin
telomere
SUS
SUS'
Extension Product
A
Chromosome
chromatin
telomere
SUS
SUS'
Extension Product
B
Chromosome
chromatin
telomere
SUS
Extension Product
C

FIG. 7B


chromatin
telomere
Extension Product
SUS'
SUS
A
Second Strand
Chart Area
chromatin
telomere
Extension Product
SUS'
SUS
B
Second Strand
telomere
Extension Product
C
SUS
No Second Strand

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 November 2014 (27.11.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/190138 A3

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2014/039110

(22) International Filing Date:
22 May 2014 (22.05.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/826,484 22 May 2013 (22.05.2013) US

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(74) Agents: **MARTY, Scott, D.** et al.; Ballard Spahr LLP, 999 Peachtree Street, Suite 1000, Atlanta, GA 30309 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
26 February 2015

WO 2014/190138 A3

(54) Title: MEASURES OF SHORT TELOMERE ABUNDANCE

(57) Abstract: This invention provides methods and materials for measuring telomere abundance from chromosomes in a sample having telomeres within a pre-determined length range, e.g., short telomeres up to a certain length. The methods can involve a first step of performing a time- limited extension reaction calibrated to produce extension products from a double-stranded chromosomal DNA template of no more than a defined length, and a second step of amplifying, from the extension products, sequences bounded by a sub-telomeric sequence and the anchor sequence, to produce a length-limited telomere sequence product. The abundance of telomeric sequences in this product can be measured, and the measures can be correlated to a variety of indices.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/39110

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2014.01)
CPC - C12Q 1/6883; C12Q 1/6827
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
CPC: C12Q 1/6883; C12Q 1/6827

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C12Q 1/6883; C12Q 1/6827 (text search)
USPC: 435/6.11, 6.12 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase, Google Scholar, Google Patents, GenCore sequence search (NT)
Search terms: telomere, subtelomere region, G-strand 3' overhang,quantify short telomere abundance or percentage, primer extension, amplification, qPCR, disease, drug response, hexamer repeat 5'-TTAGGG-3', measurement

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	HEACOCK et al. "Molecular analysis of telomere fusions in Arabidopsis: multiple pathways for chromosome end-joining" EMBO J 2 June 2004 Vol 23 No 11 Pages 2304-2313. Especially pg 2305 col 2 para 1-2, pg 2305 fig 1A,C; pg 2306 col 1 para 1, pg 2306 col 2 para 2, pg 2312 col 1 para 3 and 5,	1-15, 18-23, 25-58, 62-65 ---------- 16, 17, 24
Y --- A	US 5,834,193 A (KOZLOWSKI et al.) 10 November 1998 (10.11.1998). Especially col 5 ln 16-20, col 8 ln 24-34, col 11 ln 59-63, col 13 ln 20-22, col 14 ln 49-54, col 21 ln 17-21	1-15, 18-23, 25-58, 62-65 ---------- 16, 17, 24
Y	VINCENT et al. Helicase-dependent isothermal DNA amplification. EMBO Rep August 2004 Vol 5 No 8 Pages 795-800. Especially abstract.	20
Y	US 2007/0161031 A1 (TRINKLEIN et al.) 12 July 2007 (12.07.2011). SEQ ID NO: 1	23
Y	MARULLO et al. Expressed Alu repeats as a novel, reliable tool for normalization of real-time quantitative RT-PCR data. Genome Biol 28 January 2010 Vol 1 R9 Pages 1-12. Especially abstract.	27, 28, 40
Y	VERA et al. The rate of increase of short telomeres predicts longevity in mammals. Cell Rep 25 October 2012 Vol 2 No 4 Pages 732-737. Especially pg 733 fig 1C.	29-58, 62-65

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2014 (26.11.2014)	30 DEC 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/39110

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0151477 A1 (CAWTHON) 17 June 2010 (17.06.2010). Especially para [0015], [0063], [0078], [0079], [0081].	33, 34, 37-40, (41-58, 62-65)/(33,34,37,38)
Y	COHEN et al. A global measure of perceived stress. J Health Soc Behav December 1983 Vol 24 No 4 Pages 385-396. Especially abstract, pg 394 appendix A.	47
Y	BROUILETTE et al. Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. Lancet 13 January 2007 Vol 369 No 9556 Pages 107-114. Especially abstract.	51, 55
Y	CALADO et al. Telomere dynamics in mice and humans. Semin Hematol April 2013 Vol 50 No 2 Pages 165-174. Especially pg 5 para 2.	52, 53, 62, 63
Y	US 2010/0010064 A1 (MOORE et al.) 14 January 2010 (14.01.2010). Especially para [0105].	56
A	US 2011/0296543 A1 (CHANG et al.) 01 December 2011 (01.12.2011). SEQ ID NO: 36843	16, 24
A	US 2011/0244462 A1 (BENDIX et al.) 06 October 2011 (06.10.2011). SEQ ID NO: 11	17
A	US 2011/0182862 A1 (GREEN et al.) 28 July 2011 (28.07.2011). SEQ ID NO: 24935	24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/39110

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.4.1 SEQ ID NOs: 2, 7, 8, 10, 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/39110

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 59-61
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-----go to extra sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-58 and 62-65

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/39110

Continuation of Box III (Lack of Unity of Invention)

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-58 and 62-65, drawn to a method of making a nucleic acid extension product comprising:
i) hybridizing an extension primer to a telomeric repeat sequence in a 3' overhang of double stranded chromosomal DNA, wherein:
(1) the double stranded chromosomal DNA has a telomeric region comprising telomeric repeat sequences and a subtelomeric region comprising sub-telomeric sequences; and
(2) the extension primer comprises: (A) a 3' portion that hybridizes to a telomeric repeat sequence in the 3' overhang under annealing conditions, and
(B) a 5' portion having an anchor sequence that does not hybridize to a telomeric repeat sequence in the 3' overhang under the annealing conditions; and
ii) performing a time-limited extension reaction to extend the extension primer towards the sub-telomeric region of the double stranded chromosomal DNA, wherein the extension reaction is timed to produce an extension product comprising both telomeric repeat sequences and sub-telomeric sequences only from double stranded chromosomal DNA having a telomeric region within a pre-determined length range. Group I may also include the step of amplifying the extension product beyond limitation (ii) indicated above.

Group II: Claims 66-69 drawn to a kit/composition comprising:
(1) a first amplification primer comprising:
(A) a 3' portion that hybridizes to a telomeric repeat sequence in a 3' overhang of double-stranded chromosomal DNA under annealing conditions, and
(B) a 5' portion having an anchor sequence that does not hybridize under the annealing conditions to a sequence in the telomeric region or to a sequence in the sub-telomeric region in the chromosomal DNA; and
(2) a second amplification primer that hybridizes to a sub-telomeric sequence under annealing conditions.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I has the special technical features of method steps including hybridizing an extension primer and performing a time-limited extension reaction to extend the extension primer, not required by Group II.

Group II has the special technical feature of being a kit/composition, not required by Group I.

The common technical feature of Groups I and II are (1) a primer whose 3' portion that hybridizes to a telomeric repeat sequence in a 3' overhang of double-stranded chromosomal DNA under annealing conditions, and a 5' portion having an anchor sequence that does not hybridize under the annealing conditions to a sequence in the telomeric region or to a sequence in the sub-telomeric region in the chromosomal DNA; and [implicitly] (2) a second amplification primer, (because independent claim 21 teaches amplifying an extension product of the first primer).

Group II is related to Group I as a composition that could be used in practicing the methods of Group I.

However, said common technical features do not represent a contribution over the prior art, and is anticipated by the publication titled "Molecular analysis of telomere fusions in Arabidopsis: multiple pathways for chromosome end-joining" by HEACOCK et al. (hereinafter "Heacock") [EMBO J 2004 2 June 2004 Vol 23 No 11 Pages 2304-2313].
Heacock teaches a primer whose 3' portion hybridizes to a telomeric repeat sequence in a 3' overhang of double-stranded chromosomal DNA under annealing conditions, and a 5' portion having an anchor sequence that does not hybridize under the annealing conditions to a sequence in the telomeric region or to a sequence in the sub-telomeric region in the chromosomal DNA (pg 2305, col 2 para 1-2; "We devised a PCR-based technique called primer extension telomere repeat amplification (PETRA) that requires the G-overhang, a key structural element necessary for telomere function, and can accurately determine telomere length at multiple chromosome ends from a single plant. The principle of PETRA is outlined in Figure 1A. An adaptor primer (PETRA-T) is hybridized to the 3' G-rich overhang at the chromosome terminus. PETRA-T consists of 12 nucleotides complementary to the telomeric G-strand at its 3' end, with a 5' end that bears a unique sequence tag. Once annealed, the PETRA-T primer is extended by DNA Pol 1; the formation of PETRA products is dependent upon the action of DNA Pol1 (Figure 1C). We previously showed that this primer extension reaction requires the presence of an intact G-overhang (Riha et al, 2000)"; pg 2305 Fig 1A)), and a second primer (pg 2305 col 2 para 2-pg 2306 col 1 para 1; "In the next step of PETRA, telomeres of specific chromosome arms are amplified by PCR using a unique subtelomeric primer").

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 105283559 A
(43)申请公布日 2016.01.27

(21)申请号 201480029826.9
(22)申请日 2014.05.22
(30)优先权数据
61/826,484 2013.05.22 US
(85)PCT国际申请进入国家阶段日
2015.11.23
(86)PCT国际申请的申请数据
PCT/US2014/039110 2014.05.22
(87)PCT国际申请的公布数据
WO2014/190138 EN 2014.11.27
(71)申请人 端粒诊断公司
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(51)Int.Cl.
C12Q 1/68(2006.01)

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(54)发明名称

短端粒丰度的量度

(57)摘要

本发明提供了用于从具有预定长度范围内的端粒（例如最多为一定长度的短端粒）的样本中的染色体测量端粒丰度的方法和材料。所述方法可以涉及进行被校准以从不超过限定长度的双链染色体DNA模板产生延伸产物的限时延伸反应的第一步骤；和从所述延伸产物扩增以亚端粒序列和锚定序列为边界的序列，以产生限长的端粒序列产物的第二步骤。可以测量这种产物中的端粒序列的丰度，并且可以使所述量度与各种指标相关联。

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1. 一种制备核酸延伸产物的方法，其包括：

i) 使延伸引物与双链染色体 DNA 的 3’突出中的端粒重复序列杂交，其中：

(1) 所述双链染色体 DNA 具有包含端粒重复序列的端粒区域和包含亚端粒序列的亚端粒区域；且

(2) 所述延伸引物包含：

(A)3’部分，其在退火条件下与所述 3’突出中的端粒重复序列杂交，和

(B) 具有锚定序列的 5’部分，其在所述退火条件下不与所述 3’突出中的端粒重复序列杂交；以及

ii) 进行限时延伸反应，以使所述延伸引物向所述双链染色体 DNA 的所述亚端粒区域延伸，其中所述延伸反应被定时为仅从具有在预定长度范围内的端粒区域的双链染色体 DNA 产生包含端粒重复序列和亚端粒序列的延伸产物。

2. 根据权利要求 1 所述的方法，其中所述双链染色体 DNA 包括具有不同长度的端粒的染色体分子。

3. 根据权利要求 1 所述的方法，其中所述锚定序列在所述退火条件下不与以下中的序列杂交：

(1) 具有所述 3’突出的所述染色体 DNA 的链的所述亚端粒区域；

(2) 在所述 3’突出的 20kb 内的所述染色体 DNA 的 G 链；

(3) 在所述 3’突出的 50kb 内或 20kb 内的所述染色体 DNA 的 G 链；或

(4) 所述双链染色体 DNA。

4. 根据权利要求 1 所述的方法，其中所述预定长度范围为 0-0.5kb、0-1kb、0-2kb、0-3kb、0-4kb 或 0-5kb。

5. 根据权利要求 1 所述的方法，其中所述延伸反应被定时为 0.1-30 分钟、0.1-10 分钟、0.1-5 分钟、0.1-4 分钟、0.1-3 分钟、0.1-2 分钟、不超过 0.1-1 分钟或 0.1-0.5 分钟。

6. 根据权利要求 1 所述的方法，其中所述延伸反应被定时为 60 分钟、45 分钟、30 分钟、20 分钟、15 分钟、10 分钟、9 分钟、8 分钟、7 分钟、6 分钟、5 分钟、4 分钟、3 分钟、2 分钟、1 分钟或 0.5 分钟。

7. 根据权利要求 1 所述的方法，其中所述双链染色体 DNA 由固体、流体、半固体或气体样本提供。

8. 根据权利要求 7 所述的方法，其中所述染色体 DNA 来自液体样本，所述液体样本选自血液、唾液、尿液、血浆、血清、脑脊液（“CSF”）或支气管肺泡灌洗液。

9. 根据权利要求 7 所述的方法，其中所述染色体 DNA 来自选自肺、肌肉或皮肤的固体样本。

10. 根据权利要求 7 所述的方法，其中所述染色体 DNA 来自包括骨髓的半固体样本。

11. 根据权利要求 7 所述的方法，其中所述染色体 DNA 由包括呼气的气体样本提供。

12. 根据权利要求 1 所述的方法，其中所述双链染色体 DNA 是脊椎动物 DNA、哺乳动物 DNA 或人 DNA。

13. 根据权利要求 1 所述的方法，其中所述延伸引物的所述 3’部分与人端粒重复序列杂交。

14. 根据权利要求 1 所述的方法，其中所述延伸引物的所述 3’部分包括序列

5'-(CCCTAA)n-3' 或其同阶排列，其中 n 为至少 1。

15. 根据权利要求 14 所述的方法，其中 n 为至少 2。

16. 根据权利要求 1 所述的方法，其中所述延伸引物的所述 5' 部分包括以下序列：5'-TGCTCGGCCGATCTGGCATC-3'[SEQ ID NO:8]。

17. 根据权利要求 1 所述的方法，其中所述延伸引物包括以下序列 :5'-TGCTCGGCCGATCTGGCATCCCTAACC-3'[SEQ ID NO:7]。

18. 根据权利要求 1 所述的方法，其中所述限时延伸反应采用具有链置换活性、外切核酸酶活性或链降解活性的 DNA 聚合酶。

19. 根据权利要求 18 所述的方法，其中所述 DNA 聚合酶选自改性的 T7 聚合酶、大肠杆菌 DNA 聚合酶 I 的外切核酸酶缺乏型 Klenow 片段以及 Bst DNA 聚合酶。

20. 根据权利要求 18 所述的方法，其中第一反应是利用与 DNA 聚合酶组合的解旋酶或具有 5'-3' 外切核酸酶活性的 DNA 聚合酶进行。

21. 一种扩增染色体的端粒重复序列和亚端粒序列的方法，其包括：

a) 通过以下方式制备核酸延伸产物：

i) 使延伸引物与双链染色体 DNA 的 3' 突出中的端粒重复序列杂交，其中：

(1) 所述双链染色体 DNA 具有包含端粒重复序列的端粒区域和包含亚端粒序列的亚端粒区域；且

(2) 所述延伸引物包含：

(A)3' 部分，其在退火条件下与所述 3' 突出中的端粒重复序列杂交，和

(B) 具有锚定序列的 5' 部分，其在所述退火条件下不与所述 3' 突出中的端粒重复序列杂交；以及

ii) 进行限时延伸反应，以使所述延伸引物向所述双链染色体 DNA 的所述亚端粒区域延伸，其中所述延伸反应被定时为仅从具有在预定长度范围内的端粒区域的双链染色体 DNA 产生包含端粒重复序列和亚端粒序列的延伸产物；以及

b) 扩增所述延伸产物的序列；

从而产生包含具有端粒重复序列和亚端粒序列的核酸的限长扩增产物。

22. 根据权利要求 21 所述的方法，其中使用以下各者来扩增序列：

(1) 第一扩增引物，其在退火条件下与所述延伸产物中的所述亚端粒区域所独有的序列杂交；和

(2) 第二扩增引物，其在所述退火条件下与所述锚定序列杂交。

23. 根据权利要求 22 所述的方法，其中所述第一扩增引物包括以下序列 :5'-GATGGATCCTGAGGGTGAGGGTGAGGG-3'[SEQ IDNO:2]、5'-CGGGCCGGCTGAGGGTACCGCGA-3'[SEQ ID NO:10](染色体 1)、5'-GCTAATGCACTCCCTCAATAC-3'[SEQ ID NO:11](染色体 5) 或 5'-CATTCCTAATGCACACATGATACC-3'[SEQ ID NO:12](染色体 9)。

24. 根据权利要求 22 所述的方法，其中所述第一扩增引物包括序列 5'-GATGGATCCTGAGGGTGAGGGTGAGGG-3'[SEQ ID NO:2] 且所述第二引物包括序列 5'-TGCTCGGCCGATCTGGCATC-3'[SEQID NO:8]。

25. 根据权利要求 21 所述的方法，其中所述扩增端粒产物的长度范围可以通过在 PCR 反应中使用限时的延伸时间来确定。

26. 根据权利要求 21 所述的方法，其进一步包括共扩增对照序列。

27. 根据权利要求 26 所述的方法，其中所述对照序列包括多个非端粒重复序列。

28. 根据权利要求 27 所述的方法，其中所述对照序列为体外合成或体内产生。

29. 一种测定短端粒丰度的方法，其包括 ：

a) 从受试者提供包含双链染色体 DNA 的样本，所述双链染色体 DNA 包含 3’ 突出 ；

b) 使用根据权利要求 21 所述的方法从所述双链染色体 DNA 产生限长扩增产物 ；和

c) 测定所述限长扩增产物的短端粒丰度。

30. 根据权利要求 29 所述的方法，其进一步包括 ：

d) 比较所述样本的所述短端粒丰度与总端粒丰度的量度。

31. 根据权利要求 29 所述的方法，其中短端粒是长度为 0.1-0.5kb、0.1-1kb、0.1-2kb、0.1-3kb、0.1-4kb 或 0.1-5kb 的端粒。

32. 根据权利要求 30 所述的方法，其中步骤 d) 包括测定作为总端粒丰度的函数的短端粒丰度。

33. 根据权利要求 29 所述的方法，其中测定短端粒丰度是使用 qPCR 进行。

34. 根据权利要求 33 所述的方法，其中 qPCR 是使用第一引物和第二引物进行，

i) 其中所述第一引物与所述第一链的至少一个重复单元杂交，且所述第二引物与所述第二链的至少一个重复单元杂交，

ii) 其中所述杂交引物在与其相应的链杂交时能够进行引物延伸，且其中当所述第一引物与所述第一链的至少一个重复单元杂交时，所述第一引物的至少一个核苷酸在所述第一引物与所述重复单元的核苷酸之间产生内部碱基对错配，

iii) 其中当第一引物和第二引物互相杂交时，所述第一引物还与所述第二引物的 3’ 末端核苷酸产生错配，

iv) 其中当所述第二引物与所述第二链的至少一个重复单元杂交时，所述第二引物的至少一个核苷酸在所述第二引物与所述重复单元的核苷酸之间产生内部碱基对错配。

35. 根据权利要求 29 所述的方法，其中测定短端粒丰度包括通过 DNA 印迹、斑点印迹、狭缝印迹、免疫化学法、核酸测序或数字 PCR 测量所述样本中的平均端粒长度。

36. 根据权利要求 29 所述的方法，其中所述短端粒丰度是相对丰度的量度。

37. 根据权利要求 30 所述的方法，其中所述总端粒丰度是相对于基因组参考序列的丰度来测量。

38. 根据权利要求 37 所述的方法，其中所述基因组参考序列包括单拷贝参考核苷酸序列或非端粒重复 DNA 序列。

39. 根据权利要求 38 所述的方法，其中所述单拷贝参考核苷酸序列是人 β- 球蛋白。

40. 根据权利要求 38 所述的方法，其中所述非端粒重复 DNA 序列是 Alu 重复序列或着丝粒重复序列。

41. 一种方法，其包括 ：

a) 从受试者获取样本 ；

b) 使用根据权利要求 29-38 中任一项所述的方法测定短端粒丰度 ；和

c) 使短端粒丰度与病状或疾病相关联。

42. 根据权利要求 41 所述的方法，其中通过比较所述样本的短端粒丰度与总端粒丰度

来测定所述短端粒丰度的量度。

43. 根据权利要求 41 所述的方法，其中所述病状或疾病是死亡风险。

44. 根据权利要求 41 所述的方法，其中所述端粒丰度是绝对丰度。

45. 根据权利要求 44 所述的方法，其中所述绝对丰度被测量为端粒序列的长度。

46. 根据权利要求 41 所述的方法，其中测定端粒丰度包括通过 qPCR DNA 印迹、斑点印迹、狭缝印迹、免疫化学法、核酸测序或数字 PCR 测量所述样本中的平均端粒长度。

47. 根据权利要求 41 所述的方法，其中所述病状或疾病是知觉应激的健康状况调查评分。

48. 根据权利要求 41 所述的方法，其中所述病状或疾病是疾病风险。

49. 根据权利要求 48 所述的方法，其中所述疾病风险是年龄相关疾病。

50. 根据权利要求 49 所述的方法，其中所述年龄相关疾病是心血管疾病；且其中低于群体平均值的量度与增加的心血管疾病风险相关联。

51. 根据权利要求 50 所述的方法，其中测定所述短端粒丰度包括使群体的最低的两个或三个三分位数中的短端粒丰度与相较于所述群体的最高三分位数中的量度显著较高的心血管疾病风险相关联。

52. 根据权利要求 48 所述的方法，其中关联短端粒丰度包括与端粒疾病相关联。

53. 根据权利要求 52 所述的方法，其中所述端粒疾病为先天性角化不良、肺纤维化、再生障碍性贫血或间质性肺炎。

54. 根据权利要求 48 所述的方法，其中关联所述短端粒丰度包括与药物响应性相关联。

55. 根据权利要求 54 所述的方法，其中所述药物响应性是对他汀类的响应性；且其中短端粒长度与药物响应性正相关。

56. 根据权利要求 54 所述的方法，其中所述药物响应性是对伊美司他 (GRN163L) 的响应性；且其中长端粒长度与药物响应性正相关。

57. 根据权利要求 48 所述的方法，其中关联所述量度包括与慢性感染中的疾病进展和治疗结果相关联。

58. 根据权利要求 57 所述的方法，其中所述慢性感染选自 HIV、HCV、HBV 和 CMV。

59. 根据权利要求 39-58 中任一项所述的方法，其进一步包括向所述受试者报告所述相关性。

60. 根据权利要求 39-59 中任一项所述的方法，其进一步包括基于所述相关性向所述受试者提供诊断或预后。

61. 根据权利要求 39-60 中任一项所述的方法，其进一步包括基于所述相关性治疗所述受试者。

62. 一种用于监测受试者的状况的方法，其包括：

a) 从受试者获取多个样本；其中所述样本是在一段时间内获取；且其中每个样本是在不同的时间获取；

b) 使用根据权利要求 29-38 中任一项所述的方法来测定所述多个样本中的每一个中的短端粒丰度；

c) 测定短端粒丰度的量度的变化率；以及

d) 使所述差异与端粒疾病的进展相关联，其中所述量度的降低指示所述疾病的进展。

63. 根据权利要求 62 所述的方法，其中通过比较所述样本的所述短端粒丰度与总端粒丰度来测定所述短端粒丰度。

64. 一种方法，其包括：

a) 从受试者获取多个样本；其中所述样本是在一段时间内获取；且其中每个样本是在不同的时间获取；

b) 使用根据权利要求 29-38 中任一项所述的方法来测定所述多个样本中的每一个中的短端粒丰度；

c) 测定短端粒丰度的量度的变化率；以及

d) 使所述变化率与以下各者相关联：(1) 健康量度；(2) 病理状况的风险；(3) 端粒疾病或 (4) 药物响应性。

65. 根据权利要求 64 所述的方法，其中通过比较所述样本的所述短端粒丰度与总端粒丰度来测定所述短端粒丰度。

66. 一种试剂盒，其包括：

(1) 第一扩增引物，其包含：

(A)3’ 部分，其在退火条件下与双链染色体 DNA 的 3’ 突出中的端粒重复序列杂交，和

(B) 具有锚定序列的 5’ 部分，其在所述退火条件下不与所述染色体 DNA 中的端粒区域中的序列或亚端粒区域中的序列杂交；以及

(2) 第二扩增引物，其在退火条件下与亚端粒序列杂交。

67. 根据权利要求 66 所述的试剂盒，其进一步包括：

(3) 第三扩增引物，其在退火条件下与所述锚定序列的补体杂交。

68. 根据权利要求 66 所述的试剂盒，其进一步包括：

(3) 用于进行所述短端粒测量的杂交、延伸、扩增和定量步骤的试剂。

69. 根据权利要求 66 所述的试剂盒，其进一步包括：

(3) 包含具有已知端粒长度的染色体 DNA 的对照样本和参考样本。

短端粒丰度的量度

[0001] 相关申请的交叉引用

[0002] 本申请要求在2013年5月22日提交的美国临时申请号61/826,484的权益，该申请以引用方式整体并入本文中。

[0003] 发明背景

[0004] 背景中的陈述不一定意味着认可所引用的参考文献中的特征描述。

[0005] 端粒是真核生物染色体的尖端，其保护染色体免于溶核退化、端与端融合和重组。端粒是染色体末端的结构，其特征为核苷酸序列 $(5'\text{-}TTAGGG\text{-}3')_n$ 的重复。端粒由于正常细胞分裂而缩短并且极短的端粒导致细胞衰老或凋亡。过去十年在人类中的丰富的流行病学和临床研究已经将短端粒长度与老龄化相关疾病和全因死亡的高风险联系起来(Puterman,E. 和 E.Epel,Soc Personal Psychol Compass,2012.6(11)807-825；Zhu,H.、M.Belcher 和 P.van der Harst,Clin Sci(Lond),2011.120(10)427-40；和 Fyhrquist,F. 和 O.Saijonmaa.Ann Med,2012.44 增刊 1S138-42)。遗传、环境、生活方式和行为因素共同影响端粒长度。因此，端粒长度已成为总体健康、疾病和死亡风险的指标。

[0006] 虽然平均端粒长度在几乎所有公开的临床研究中被测量并且已经在患者疾病和死亡风险的分层中显示效用，但最近在小鼠中的工作也已显示短端粒群体是衰老或凋亡的触发信号 (Hemann,M.T. 等，Cell,2001,107(1)67-77)，且因此是疾病和死亡风险的触发信号。在由 Hemann 等报道的研究中，将具有短端粒的第 6 代端粒酶 RNA 敲除小鼠 (mTR-/-G6) 与具有长端粒的端粒酶杂合型小鼠 (mTR+/-) 进行杂交。尽管端粒酶缺失后代的端粒中的一半是长的，但是其表型反映 mTR-/- 母本的表型，这表明短端粒的数量而非平均端粒长度对于细胞活力和染色体稳定性是关键的。在服用由天然产品衍生的端粒酶激活剂(TA-65®)的人中，在白细胞中检测到显著降低的短 (<3 或 <4kbp) 端粒的百分比 (如通过定量 FISH 技术所测量；参见 (Canela,A. 等，Proc Natl Acad Sci USA,2007,104(13)5300-5)，然而没有观察到平均端粒长度的变化 (Harley,C.B. 等，Rejuvenation Res.2011,14(1)45-56)。因此短端粒丰度的百分比的变化被预期为生活方式和药理干预或其它干预对端粒的影响的更灵敏量度。另一项研究 (Vera 等，“The Rate of Increase of Short Telomeres Predicts Longevity in Mammals”，Cell Reports(2012)，万维网 URL:dx.doi.org/10.1016/].celrep.2012.08.023) 发现“短端粒丰度的增加速率是寿命的预测指标”。

[0007] 已经开发了用于测量基因组 DNA 中的端粒长度的多种方法，包括 DNA 印迹 (Kimura,M. 等，Nature Protocols,2010,5:1596-1607)、Q-FISH(Rufer,N. 等，Nat.Biotechnol.,1998,16:743-747)、流式 FISH(Baerlocher,G.M. 等，Cytometry,2002,47:89-99) 和 qPCR(Cawthon,R.M.,Nucleic.Acids Res.,2002,30(10):e47)。所有这些方法均可以在临床环境中被用来监测健康状况并允许医师根据个体患者的需求规定预防性或治疗性干预。

[0008] 为了测量短端粒的群体，中期扩散细胞的定量荧光原位杂交 (Q-FISH) 已经被用来生成表示个别端粒的长度的端粒信号强度的直方图 (Poon,S.S. 等，

Cytometry,1999.36(4)267-78)。这种方法的限制为需要活细胞,成本高,且通量低。Q-FISH测定的高通量改良型(HTQ-FISH;参见Canela,A.等,Proc Natl Acad Sci USA,2007,104(13)5300-5)最近被Life Length公司(西班牙)支持用于测量短端粒的百分比。尽管这样声称,然而可惜的是,利用当前技术,这种测定无法准确,原因是端粒尤其短端粒在单一斑点中的聚簇(端粒联合;参见Paeschke,K.、K.R.McDonald和V.A.Zakian.FEBS Lett,2010.584(17)3760-72)。困扰这个问题的事实是短端粒相比长端粒倾向于更频繁地彼此联合。另外,已知FISH技术遭受探针与活细胞或固定细胞中的大分子的非特异性结合。低成本且不需要活细胞的用于测量短端粒百分比的高通量方法将更容易适应流行病学和临床环境,并且相比Q-FISH将具有更好的分析性能。

[0009] 美国专利第5,741,677号(Kozlowski等)涉及用于测量端粒长度的方法。一种方法涉及使端粒与接头序列接触,这是在该接头序列与端粒的3'末端连接或以另外方式共价结合的条件下进行。端粒序列是通过长PCR扩增,其中第一引物对接头序列具有特异性并且第二引物对染色体的亚端粒区域具有特异性。另一种方法涉及制备细胞的DNA提取物,使所述提取物与和端粒重复序列互补的寡核苷酸探针孵育,以及测定结合的探针的量作为端粒长度的量度。另外,描述了一种通过使基因组DNA与固相结合并使结合的DNA与标记探针杂交来测量端粒长度的方法。

[0010] 美国专利公开第2004/0265815号(Baird等)涉及一种用于测量端粒长度的方法。Baird等描述了以下步骤来检测端粒群体的长度:a)使单链寡核苷酸(下文称作telorette)的3'末端与包含富G端粒链(包含TTAGGG重复序列)的端粒的单链突出退火并使telorette共价结合至富C端粒链(具有CCCTAA重复序列)的5'末端;b)扩增在步骤(a)中所形成的连接产物以形成引物延伸产物;和(c)检测步骤(b)的引物延伸产物的长度。(还参见Baird,D.M.等,Nat Genet.,2003,33(2):203-7;和Baird DM、Rowson J、Wynford-Thomas D、Kipling D.;Nat Genet.,2003,33(2):203-7.Epub 2003Jan 21.PMID:12539050)

[0011] 美国专利第6,514,693号(Lansdorp)涉及一种用于检测形态完整的染色体、细胞或组织切片中的核酸分子中的重复序列的多个拷贝的方法,其包括:(a)在利用变性剂的变性条件下,用与核酸分子中的重复序列杂交且被可检测物质标记的PNA探针处理核酸分子,以允许该探针与核酸分子中的重复序列原位杂交;和(b)通过直接或间接检测可检测物质来鉴定与核酸分子中的重复序列杂交的所述探针,从而检测核酸分子中的重复序列的多个拷贝。

[0012] 测定短端粒丰度的方法包括DNA印迹分析、定量荧光原位杂交(Q-FISH)(Poon,S.S.等,Cytometry,1999,36(4):267-78)和改良型高通量形式的Q-FISH(HT-Q-FISH)(Canela,A.等,Proc.Natl.Acad.Sci.USA,2007,104(13):5300-5)。

[0013] 美国专利第7,695,904号(Cawthon)描述了使用被设计成限制非靶核酸依赖性引发事件的核酸引物扩增靶核酸的方法。这些方法允许扩增和定量重复区域中的重复单元数,诸如端粒重复单元数。所述专利还涉及通过qPCR方法测定生物体的平均端粒长度。

[0014] 因此,尽管关于端粒的材料和方法有进步,但仍然需要用于测定染色体群体中的短端粒丰度的量度的改良方法和材料以及使用这些量度来测定健康量度和干预的效果,这些干预增加或减小端粒长度且因此增加或减少健康,或相反地分别减少或增加未来疾病或

死亡的风险。本发明解决了这些需求和其它需求。

发明概要

[0015] 在一个方面，本发明提供一种制备核酸延伸产物的方法，其包括：i) 使延伸引物与双链染色体DNA的3’突出中的端粒重复序列杂交，其中：(1) 该双链染色体DNA具有包含端粒重复序列的端粒区域和包含亚端粒序列的亚端粒区域；且 (2) 该延伸引物包含：(A)3’部分，其在退火条件下与3’突出中的端粒重复序列杂交，和 (B) 具有锚定序列的5’部分，其在退火条件下不与3’突出中的端粒重复序列杂交；以及 ii) 进行限时延伸反应，以使延伸引物向双链染色体DNA的亚端粒区域延伸，其中该延伸反应被定时为仅从具有在预定长度范围内的端粒区域的双链染色体DNA产生包含端粒重复序列和亚端粒序列的延伸产物。在一个方面，双链染色体DNA包括具有不同长度的端粒的染色体分子。在另一方面，锚定序列在退火条件下不与以下序列杂交：(1) 具有3’突出的染色体DNA的链的亚端粒区域中的序列；(2) 在3’突出的20kb内的染色体DNA的G链中的序列；(3) 在3’突出的50kb内或20kb内的染色体DNA的G链中的序列；或 (4) 在双链染色体DNA中的序列。在另一方面，延伸反应被定时为不超过30分钟、不超过10分钟、不超过5分钟、不超过4分钟、不超过3分钟、不超过2分钟、不超过1分钟、不超过30秒、不超过20秒、不超过10秒、不超过5秒或不超过2秒。因为引物延伸的速率可以从高（400个核苷酸/秒）变化至极低（例如50个核苷酸/秒），所以宽泛范围的延伸时间允许评估宽泛范围的端粒长度（理论上从约100个核苷酸至数千个核苷酸。在另一方面，延伸反应被定时为至少30分钟、至少10分钟、至少5分钟、至少4分钟、至少3分钟、至少2分钟、至少1分钟或至少30秒。在另一方面，延伸反应被定时为至少30分钟、至少10分钟、至少5分钟、至少4分钟、至少3分钟、至少2分钟、至少1分钟或至少30秒。在另一方面，双链染色体DNA由固体、流体、半固体或气体样本提供。在另一方面，染色体DNA由液体样本提供，该液体样本选自血液、唾液、尿液、血浆、血清、脑脊液（“CSF”）或支气管肺泡灌洗液。在另一方面，染色体DNA由选自肺、肌肉或皮肤的固体样本提供。在另一方面，染色体DNA由包括骨髓的半固体样本提供。在另一方面，染色体DNA由包括呼气的气体样本提供。在另一方面，双链染色体DNA是脊椎动物DNA、哺乳动物DNA或人DNA。在另一方面，延伸引物的3’部分与人端粒重复序列杂交。在另一方面，延伸引物的3’部分包括序列5’-(CCCTAA)n-3’或其同阶排列，其中n为至少1。在另一方面，n为至少2。在另一方面，延伸引物的5’部分包括序列：5’-TGCTCGGCCGATCTGGCATC-3’[SEQ ID NO:8]。在另一方面，延伸引物包括序列：5’-TGCTCGGCCGATCTGGCATCCCTAACC-3’[SEQ ID NO:7]。在另一方面，限时延伸反应采用具有链置换活性、外切核酸酶活性或链降解活性的DNA聚合酶。在另一方面，DNA聚合酶选自T7聚合酶（例如，测序酶 (Sequenase))、大肠杆菌 (E.coli)DNA聚合酶I的外切核酸酶缺乏型Klenow片段以及Bst DNA聚合酶大片段和Deep VentR(外切核酸酶)。在另一方面，第一反应利用与DNA聚合酶组合的解旋酶或具有5’-3’外切核酸酶活性的DNA聚合酶进行。

[0016] 在另一方面，本发明提供一种扩增染色体的端粒重复序列和亚端粒序列的方法，其包括：a) 通过以下方式制备核酸延伸产物：i) 使延伸引物与双链染色体DNA的3’突出中的端粒重复序列杂交，其中：(1) 该双链染色体DNA具有包含端粒重复序列的端粒区域和包含亚端粒序列的亚端粒区域；且 (2) 该延伸引物包含：(A)3’部分，其在退火条件

下与 3’ 突出中的端粒重复序列杂交，和 (B) 具有锚定序列的 5’ 部分，其在退火条件下不与 3’ 突出中的端粒重复序列杂交 ；和 ii) 进行限时延伸反应，以使延伸引物向双链染色体 DNA 的亚端粒区域延伸，其中该延伸反应被定时为仅从具有在预定长度范围内的端粒区域的双链染色体 DNA 产生包含端粒重复序列和亚端粒序列的延伸产物 ；以及 b) 扩增该延伸产物的以亚端粒序列和锚定序列为边界的序列 ；从而产生包含具有端粒重复序列和亚端粒序列的核酸的限长扩增产物。在一个方面，使用以下各者来扩增序列 ：(1) 第一扩增引物，其在退火条件下与延伸产物中的亚端粒区域所独有的序列杂交 ；和 (2) 第二扩增引物，其在退火条件下与锚定序列杂交。在另一方面，第一扩增引物包括选自以下的序列 ：5’-GATGGATCCTGAGGGTGAGGGTGAGGG-3’[SEQ ID NO:2]、5’-CGGGCCGGCTGAGGGTACCGCGA-3’[SEQ I D NO:10](染色体 1)、5’-GCTAATGCACTCCCTCAATAC-3’[SEQ ID NO:11](染色体 5) 和 5’-CATTCCTAATGCACACATGATACC-3’[SEQ ID NO:12](染色体 9)。在另一方面，第一扩增引物包括序列 5’-GAT GGATCCTGAGGGTGAGGGTGAGGG-3’[SEQ ID NO:2] 且第二引物包括序列 5’-TGCTCGGCCGATCTGGCATC-3’[SEQ ID NO:8]。扩增的端粒产物的长度范围可以通过在 PCR 反应中使用限时的延伸时间来确定。在另一方面，所述方法进一步包括共扩增对照序列。在另一方面，对照序列包括多个非端粒重复序列。在另一方面，对照序列是在体外合成或在体内产生 (例如，在细菌或真菌克隆中)。

[0017] 在另一方面，本发明提供一种用于测定短端粒丰度的方法，其包括 ：a) 从受试者提供包含双链染色体 DNA 的样本，该 DNA 包含 3’ 突出 ；b) 使用如本文 (例如，上文) 所描述的本发明的扩增染色体的端粒重复序列和亚端粒序列的方法从双链染色体 DNA 产生限长扩增产物 ；和 c) 测定限长扩增产物的短端粒丰度。在一个方面，所述方法进一步包括 ：d) 比较短端粒丰度和样本的总端粒丰度。在另一方面，短端粒是长度不超过约 0.5kb、约 1kb、约 2kb、约 3kb、约 4kb 或约 5kb 的端粒。在另一方面，比较包括测定作为总端粒丰度的函数的短端粒丰度，例如短端粒丰度与总端粒丰度的比率。在另一方面，使用 qPCR 进行短端粒丰度的测定。在另一方面，使用第一引物和第二引物进行 qPCR，(i) 其中所述第一引物与所述第一链的至少一个重复单元杂交，且所述第二引物与所述第二链的至少一个重复单元杂交 ；(ii) 其中所述杂交引物在与其相应的链杂交时能够进行引物延伸，且其中当所述第一引物与所述第一链的至少一个重复单元杂交时，所述第一引物的至少一个核苷酸在所述第一引物与所述重复单元的核苷酸之间产生内部碱基对错配 ；(iii) 其中当第一引物和第二引物互相杂交时，所述第一引物还与所述第二引物的 3’ 末端核苷酸产生错配 ；(iv) 其中当所述第二引物与所述第二链的至少一个重复单元杂交时，所述第二引物的至少一个核苷酸在所述第二引物与所述重复单元的核苷酸之间产生内部碱基对错配。在另一方面，测定短端粒丰度包括通过 DNA 印迹、斑点印迹、狭缝印迹、免疫化学法、核酸测序或数字 PCR 测量样本中的平均端粒长度。在另一方面，短端粒丰度是相对丰度的量度。在另一方面，总端粒丰度相对于基因组参考序列的丰度来测量。在另一方面，基因组参考序列包括单拷贝参考核苷酸序列 (例如，人 β- 球蛋白) 或非端粒重复 DNA(例如，Alu 重复或着丝粒重复) 的丰度。

[0018] 在另一方面，本发明提供一种方法，其包括 ：a) 测定来自受试者的样本中的短端粒丰度 ；和 b) 使短端粒丰度与病状或疾病相关联。在一个方面，通过比较样本的短端粒丰度与总端粒丰度来测定短端粒丰度的量度。在另一方面，使用本文 (例如，上文) 所描述的

方法测定短端粒丰度。在另一方面，病状或疾病是死亡风险。在另一方面，端粒丰度是绝对丰度。在另一方面，绝对丰度被测量为端粒序列的长度。在另一方面，测定端粒丰度包括通过 qPCR DNA 印迹、斑点印迹、狭缝印迹、免疫化学法、核酸测序和数字 PCR 测量样本中的平均端粒长度。在另一方面，病状或疾病的关联与知觉应激的健康状况调查评分 (Health Status Survey Score of Perceived Stress) 相关（参见例如 Cohen, S ;Kamarck T 和 Mermelstein R(1983) J. Health Social Behav. 24(4)385-396)。在另一方面，病理状况的风险是疾病例如心血管疾病、糖尿病、癌症、肝纤维化和抑郁的风险。在另一方面，疾病是老龄化疾病。在另一方面，老龄化疾病是心血管疾病且其中低于群体平均值的量度与增加的心血管疾病风险相关联。在另一方面，所述方法包括使群体的最低的两个或三个三分位数中的端粒丰度的量度与相较于该群体的最高三分位数中的量度显著较高的心血管疾病风险相关联。在另一方面，所述方法包括使所述量度与端粒疾病相关联。端粒疾病可以包括但不限于先天性角化不良、肺纤维化、再生障碍性贫血和间质性肺炎。在另一方面，所述方法包括使所述量度与药物响应性相关联。例如，所述方法可以包括使所述量度与对他汀类的药物响应性（其中个体的正常白血球中的短平均端粒长度与药物响应性正相关）或对伊美司他 (imetelstat)(GRN163L，癌症药物）的不良响应（其中正常白血球中的短端粒长度与不良反应诸如血小板减少症或中性粒细胞减少症相关）相关联。在另一方面，所述方法包括使所述量度与慢性感染诸如 HIV、HCV HBV 和 CMV 中的疾病进展和治疗结果相关联。在另一方面，所述方法进一步包括向受试者报告相关性。在另一方面，所述方法进一步包括基于相关性向受试者提供诊断或预后。在另一方面，所述方法进一步包括基于相关性治疗受试者。

[0019] 在另一方面，本发明提供一种监测受试者的状况的方法，其包括 ：从在一段时间内获取的多个受试者样本中的每个样本中的细胞测定短端粒丰度的量度 ；测定所述量度的差异 ；以及使所述差异与端粒疾病的进展相关联，其中所述量度的降低指示疾病的进展。在一个方面，通过比较样本的短端粒丰度的量度与总端粒丰度的量度来测定短端粒丰度的量度。

[0020] 在另一方面，本发明提供一种方法，其包括 ：测定来自多个受试者样本的细胞中的短端粒丰度的量度的变化速率，每个样本在不同时间获取 ；以及使该变化速率与以下各者相关联 ：(1) 健康量度 ；(2) 病理状况的风险 ；(3) 端粒疾病或 (4) 药物响应性。在一个方面，通过比较样本的短端粒丰度的量度与总端粒丰度的量度来测定短端粒丰度的量度。

[0021] 在另一方面，本发明提供一种试剂盒，其包括 ：(1) 第一扩增引物，包含 ：(A)3’ 部分，其在退火条件下与双链染色体 DNA 的 3’ 突出中的端粒重复序列杂交，和 (B) 具有锚定序列的 5’ 部分，其在退火条件下不与染色体 DNA 中的端粒区域中的序列或亚端粒区域中的序列杂交 ；以及 (2) 第二扩增引物，其在退火条件下与亚端粒序列杂交。在一个方面，所述试剂盒进一步包括 ：(3) 第三扩增引物，其在退火条件下与锚定序列的补体杂交。在另一方面，所述试剂盒包括 ：(3) 用于进行短端粒测量的杂交、延伸、扩增和定量步骤的试剂。在另一方面，所述试剂盒进一步包括 ：(3) 包含具有已知端粒长度的染色体 DNA 的对照样本和参考样本，或具有已知质量的端粒重复的合成寡核苷酸。

[0022] 通过引用并入

[0023] 本说明书中所提及的所有出版物、专利和专利申请均通过引用并入本文中，其程

度就如同每个个别的出版物、专利或专利申请被具体和单独地指示通过引用并入。

[0024] 附图简述

[0025] 图 1 示出了短端粒测定 (STA) 的总体方案。

[0026] 图 2 示出了通过 TELOTEST 对链置换期间的短端粒扩增的确认，TELOTEST 是一种用于在 qPCR 测定中使用与端粒重复序列杂交的错配引物测量平均端粒长度的测试。相似类型的测定描述于美国专利第 7,695,904 号 (Cawthon) 中。

[0027] 图 3 示出了链置换产物的 DNA 印迹分析。M：分子量标准；泳道 1：通过改良 STELA（单端粒延长长度分析）方案 (Baird 等，2003) 扩增的源自膀胱癌细胞系 UM-UC3 的总基因组 DNA；泳道 2-5：使用 SUS 和 TeloAnchor 作为引物的 PCR 产物且链置换产物为模板。指出了链置换反应时间 (30 秒、1、3 和 5 分钟）。链长度为：30 秒＝约 1kb；1 分钟＝约 2kb；3 分钟＝约 5kb；5 分钟＝约 8kb。

[0028] 图 4 示出了针对具有不同平均端粒长度的 DNA 样本中的短端粒定量的概念验证计算。UM-UC3 是具有短平均端粒的细胞群体，且 UM-UC3/hTER 是相同的细胞群体，但是由于端粒酶（具有 hTER) 的过度表达而具有延伸（延长）的端粒。如所示出，短端粒的相对丰度在 UM-UC3 中比在 UM-UC3/hTER 中大得多。

[0029] 图 5 是通过 DNA 印迹（其中示出了在 0.2-5kbp 范围内的末端限制性片段的相对信号）和通过本发明的短端粒测定比较在 UM-UC3 和 UM-UC3/hTER 中的短端粒的相对丰度的表格，其中短端粒的百分比是短端粒和总端粒测量值的比率。在两种情况下，UM-UC3 中的短端粒比在 UM-UC3/hTER 中多大约 3 倍。

[0030] 图 6 示出了针对短端粒测定的高通量步骤。

[0031] 图 7A 和图 7B 示出了在染色体 A、B 和 C（各自具有不同长度的端粒）上的时控引物延伸。核染色质由虚线表示。亚端粒单一序列由“SUS”表示。端粒由实线表示。延伸产物由虚线表示。图 7B 示出了三种引物延伸产物的第二链合成。在图 7A 中，选择引物延伸的时间以使得引物延伸越过染色体 A 和 B 中，但不越过染色体 C 中的亚端粒区域（具有亚端粒单一序列“SUS”）。在图 7B 中，具有亚端粒单一序列的引物“SUS”可以与延伸产物 A 和 B 杂交，但不与延伸产物 C 杂交，延伸产物 C 由于原始染色体中的长端粒长度而不延伸至亚端粒区域中。因此，第二链合成可以从与延伸产物 A 和 B 杂交的 SUS 引物开始，但不从 C 开始。第二链由实线表示。这种产物然后表示原始染色体 DNA 的“短端粒”部分。

具体实施方式

[0032] 定义

[0033] 应该理解本文所使用的术语仅用于描述特定方面的目的且无意具有限制性。

[0034] 如在本说明书和权利要求书中所使用，术语“包含”可以包括“由其组成”和“基本上由其组成”的方面。

[0035] 如本文所使用，针对化合物包括有机化合物的命名可以使用普通名称、IUPAC、IUBMB 或 CAS 推荐命名给出。除非另有限定，否则本文所使用的所有技术和科学术语具有如本发明所属技术领域的一般技术人员通常所理解的相同含义。在本说明书和随附的权利要求书中，将参考可以在本文中定义的许多术语。

[0036] 如在本说明书和随附权利要求书中所使用，单数形式“一个 / 种 (a)”和“一个 / 种

(an)”和“该/所述(the)”包括复数的指示对象，除非上下文另有明确说明。因此，例如，提及“细胞”、“核苷酸”或“引物”包括两种或多种此类细胞、核苷酸或引物等的混合物。

[0037] 范围在本文中可以表示为从“约”一个具体数值和/或至“约”另一个具体数值。当表示这样的范围时，另一方面包括从所述一个具体数值和/或至所述另一个具体数值。类似地，当通过使用先行词“约”将值表达为近似值时，应该理解具体值形成另一方面。应该进一步理解，每个范围的端点在与另一端点有关以及与另一端点无关时均是有意义的。还应该理解本文中公开了很多值，并且除了该值本身之外，每个数值在本文中还被公开为″约″该特定值。例如，如果公开了值“10”，那么也公开了“约10”。还应该理解，两个特定单位之间的每个单位也被公开。例如，如果公开了10和15，那么也公开了11、12、13和14。

[0038] 如本文所使用，术语“约”和“在或约”意指所讨论的量或值可以是指定近似或约相同的一些其它值的值。一般应该理解，如本文所使用，除非另外指示或推测，否则标称值指示±10％的变化。该术语旨在传达类似的值促进权利要求书中所叙述的相等结果或作用。即，应该理解，量、大小、公式、参数和其它数量和特征不是且不必是确切的，而是可以是近似值和/或根据需要更大或更小，以反映容差、转换因子、四舍五入、测量误差等和本领域技术人员已知的其它因素。一般来说，量、大小、公式、参数或其它数量或特征为“约”或“近似”，无论是否明确说明是如此。应该理解，除非另外明确指出，否则当“约”用在定量值前时，该参数还包括特定的定量值本身。

[0039] 在本说明书和最后的权利要求书中提及的组合物中特定元素或组分的重量份表示该元素或组分与针对其表示重量份的组合物或制品中的任何其它元素或组分之间的重量关系。因此，在含有2重量份的组分X和5重量份的组分Y的化合物中，X和Y以2:5的重量比存在，并且不管该化合物中是否含有额外组分都以这样的比例存在。

[0040] 除非明确有相反的说明，否则组分的重量百分比（重量％）基于包含该组分的制剂或组合物的总重量。

[0041] 如本文所使用，术语“可选的”或“可选地”意指随后描述的事件或情形可以发生或不发生，且该描述包括其中所述事件或情形发生的例子和其中它没有发生的例子。

[0042] 如本文所使用，术语“有效量”是指足以实现组合物或方法的物理、化学或生物性质的所需改变的量。

[0043] 如本文所使用，“试剂盒”意指构成该试剂盒的至少两种组分的集合。所述组分共同构成用于给定目的的功能单位。个别成员组分可以物理方式包装在一起或分开包装。例如，包括试剂盒的使用说明书的试剂盒可以物理方式包括或不包括与其它个别成员组分一起的说明书。相反地，说明书可以作为单独的成员组分，以纸张形式或可以提供在计算机可读存储装置上或从互联网网站下载的电子形式，或作为记录演示来提供。

[0044] 如本文所使用，“说明书”意指描述关于试剂盒的相关材料或方法的文件。这些材料可以包括以下各者的任何组合：背景信息、组分清单和其可用性信息（购买信息等）、试剂盒的简明或详细使用方案、问题解决、参考、技术支持和任何其它相关文件。说明书可以与试剂盒一起提供或作为单独的成员组分，以纸张形式或可以提供在计算机可读存储装置上或从互联网网站下载的电子形式或作为记录演示来提供。说明书可以包括一个或多个文件，且意图包括未来的更新。

[0045] 如本文所使用，术语“受试者”可以是脊椎动物，例如哺乳动物、鱼、鸟、爬行动物或

两栖动物。因此，本文所公开的方法的受试者可以是人、非人灵长类动物、马、猪、兔、狗、绵羊、山羊、奶牛、猫、豚鼠或啮齿动物。该术语不表示特定年龄或性别。因此，旨在涵盖成人和新生受试者以及胎儿，无论男性或女性。在一个方面，受试者为哺乳动物。患者是指患有病状、疾病或病症的受试者。术语“患者”包括人和兽医受试者。在公开的方法的一些方面，受试者已经被诊断为需要治疗与短端粒丰度的功能障碍有关的一种或多种病状或疾病。

[0046] 如本文所使用，术语“治疗”是指意图治愈、改善、稳定或预防疾病、病理状况或病症的患者的医疗管理。该术语包括积极治疗，即具体针对改善疾病、病理状况或病症的治疗，并且还包括病因性治疗，即针对去除相关疾病、病理状况或病症的病因的治疗。另外，该术语包括姑息治疗，即设计成用于缓解症状而不是治愈疾病、病理状况或病症的治疗；预防性治疗，即涉及最小化或部分或完全抑制相关疾病、病理状况或病症的发展的治疗；和支持治疗，即用于补充针对改善相关疾病、病理状况或病症的另一特定疗法的治疗。在各个方面，所述术语涵盖受试者包括哺乳动物（例如人）的任何治疗且包括：(i) 预防疾病在可以易患该疾病但尚未被诊断为患病的受试者中发生；(ii) 抑制疾病，即阻止其发展；或 (iii) 缓解疾病，即使疾病消退。在一个方面，受试者为哺乳动物例如灵长类动物，且在另一个方面，受试者为人。术语“受试者”还包括家养动物（例如，猫、狗等）、牲畜（例如，牛、马、猪、绵羊、山羊等）和实验动物（例如，小鼠、兔、大鼠、豚鼠、果蝇等）。

[0047] 如本文所使用，术语“预防 (prevent_)”或“预防 (preventing)”是指阻止、避免、消除、预先制止、停止或阻碍某些事情发生，特别是通过提前动作。应该理解的是，当在本文中使用减少、抑制或预防时，除非另外具体说明，否则也明确地公开了使用其它两个词。

[0048] 如本文所使用，术语“施用 (administering)”和“施用 (administration)”是指将药物制剂提供给受试者的任何方法。此类方法是本领域技术人员所熟知的且包括但不限于：口服施用、经皮施用、吸入施用、鼻腔施用、局部施用、阴道内施用、眼部施用、耳内施用、脑内施用、直肠施用、舌下施用、颊部施用和肠胃外施用，包括可注射施用例如静脉内施用、动脉内施用、肌肉内施用和皮下施用。施用可以是连续或间歇的。在各个方面，制剂可以治疗性施用；即，被施用来治疗现有疾病或病状。在进一步的各个方面，制剂可以预防性施用；即，被施用以预防疾病或病状。

[0049] 如本文所使用，术语“有效量”和“有效的量”是指足以实现期望的结果或对不期望的病状具有效果的量。例如，“治疗有效量”是指足以实现期望的治疗结果或对不期望的症状具有效果，但一般不足以引起不良副作用的量。针对任何特定患者的具体治疗有效剂量水平将取决于多种因素，包括所治疗的病症和病症的严重度；所用的具体组合物；患者的年龄、体重、一般健康状况、性别和饮食；施用时间；施用途径；所用的具体化合物的排泄速率；治疗的持续时间；与所用的具体化合物组合或同时使用的药物以及医学领域中熟知的类似因素。例如，本领域技术人员已知，化合物的剂量水平一开始比实现所需治疗效果所需要的剂量低，并且逐渐增加该剂量直至实现所需效果。如果需要，有效日剂量可以分成多个剂量用于施用。因此，单剂量组合物可以含有这样的量或其约数，以构成日剂量。如果有任何禁忌症，剂量可以由个别医师进行调整。剂量可以变化，并且可以每天以一个或多个施用剂量来施用，持续一天或几天。在文献中可以找到关于给定类别的药品的适当剂量的指导。在其它各个方面，制剂可以以“预防有效量”，即有效预防疾病或病状的量施用。

[0050] 如本文所使用，“延伸引物”意指用于进行由 DNA 聚合酶进行的限时延伸反应步骤

的寡核苷酸引物。延伸引物可以包含 3’ 部分和 5’ 部分。例如,3’ 部分可以在退火条件下与 3’ 突出中的端粒重复序列杂交,且 5’ 部分可以具有在退火条件下不与 3’ 突出中的端粒重复序列杂交的锚定序列。

[0051] 如本文所使用,“端粒区域”意指位于染色体末端的具有重复端粒序列的 DNA 区段。在脊椎动物的情况下,它可以是位于染色体末端的 (TTAGGG)n 重复序列。

[0052] 如本文所使用,“亚端粒区域”意指位于端粒的着丝粒末端处的紧邻端粒的 DNA 区段。亚端粒区域经常包含退化的端粒重复。在人的情况下,TGAGGG 和 TCAGGG 的重复可以存在于亚端粒区域中。

[0053] 如本文所使用,“限时延伸反应”意指由 DNA 聚合酶进行的酶促反应,其中该反应的产物(限时延伸产物)的大小(按核苷酸数计)是该反应中所用的 DNA 聚合酶的固有延伸速率和反应时间的函数。

[0054] 如本文所使用,“锚定序列”意指引物中的单一序列区段,其不存在于可以用于 PCR 反应的模板基因组中或存在于预期扩增子的 20kb 内。例如,延伸引物的 5’ 部分可以是锚定序列,其被配置成在退火条件下不与 G 链中的端粒重复序列(其与延伸引物的 3’ 部分杂交)杂交。

[0055] 如本文所使用,“染色体 DNA 的 G 链”意指具有 3’ 突出的端粒的链,且包括端粒重复序列 5’-TTAGGG-3’。例如,“染色体 DNA 的 G 链”可以指包含人和其它脊椎动物中的 $(TTAGGG)_n$ 端粒序列的染色体中的 DNA 链。

[0056] 如本文所使用,“聚合酶”是指催化核苷酸的聚合的酶。一般来说,该酶将在与核酸模板序列退火的引物的 3’ 末端处引发合成。“DNA 聚合酶”催化脱氧核糖核苷酸的聚合。已知的 DNA 聚合酶包括例如强烈火球菌 (Pfu)DNA 聚合酶 (Lundberg 等,(1991)Gene 108:1)、大肠杆菌 DNA 聚合酶 I(Lecomte 和 Doubleday(1983)Nucleic Acids Res.11:7505)、T7DNA 聚合酶 (Nordstrom 等 (1981)J.Biol.Chem.256:3112)、嗜热栖热菌 (Tth)DNA 聚合酶 (Myers 和 Gelfand(1991)Biochemistry30:7661)、嗜热脂肪芽孢杆菌 (Bacillus stearothermophilus)DNA 聚合酶 (Stenesh 和 McGowan(1977)Biochim Biophys Acta 475:32)、嗜热高温球菌 (Tli)DNA 聚合酶(又称作 Vent DNA 聚合酶,Cariello 等 (1991) Nucleic Acids Res 19:4193)、海栖热袍菌 (Tma)DNA 聚合酶 (Di az 和 Sabino(1998)Braz J.Med.Res 31:1239) 和水生栖热菌 (Taq)DN A 聚合酶 (Chien 等,(1976)J.Bacteoriol 127:1550)。以上酶中的任一个的聚合酶活性可以通过本领域中公知的方法来测定。

[0057] 如本文所使用,“热稳定性”DNA 聚合酶活性意指相较于例如 DNA 聚合酶的非热稳定形式,对热相对稳定且在高温例如 45-100℃,优选 55-100℃、65-100℃、75-100℃、85-100℃或 95-100℃下发挥作用的 DNA 聚合酶活性。

[0058] DNA 聚合酶的链置换活性描述置换在合成期间所遇到的下游 DNA 的能力。例如,DNA 聚合酶的链置换活性可以指将 DNA 的双链分离成两条单链的聚合酶能力。具有链置换活性的 DNA 聚合酶的实例为全酶或源自病毒、原核生物、真核生物或古生菌的复制酶的部分、phi29DNA 聚合酶、Klenow DNA 聚合酶 exo 和源自嗜热脂肪芽孢杆菌的 DNA 聚合酶(被命名为 Bst exo)。"Exo " 表示相应的酶不具有 5’-3’ 外切核酸酶活性。phi29DNA 聚合酶的公知实例为噬菌体 phi29DNA 聚合酶。可用于本发明方法中的更合适的具有链置换活性的 DNA 聚合酶为本领域技术人员所公知,且包括 DNA 聚合酶诸如改性的 T7 聚合酶(例如测

序酶）、大肠杆菌 DNA 聚合酶 I 的外切核酸酶缺乏型 Klenow 片段以及 Bst DNA 聚合酶大片段和 Deep VentR(exo-)。

[0059] 或者，没有链置换活性的那些 DNA 聚合酶也应该被理解为具有链置换活性的 DNA 聚合酶，条件是除了相应的 DNA 聚合酶以外使用催化剂，例如使 DNA 的双链能够分离或使 DNA 的单链能够稳定的蛋白质或酶。这些蛋白质包括例如解旋酶、SSB 蛋白和重组蛋白，它们可以作为较大酶复合物例如复制酶的组分存在。在这种情况下，具有链置换活性的聚合酶是利用除聚合酶本身以外的组分而产生。具有链置换活性的聚合酶可以是热不稳定或热稳定的。

[0060] 如本文所使用，“引物”是指在诱导与核酸链互补的引物延伸产物的合成的条件下，例如在四种不同的三磷酸核苷和延伸用试剂（例如，DNA 聚合酶或逆转录酶）的存在下，在适当的缓冲液中和合适的温度下能够作为 DNA 合成的起始点的寡核苷酸。引物不必反映模板核酸的确切序列，但是必须充分互补以与模板杂交。适用于扩增给定靶序列的引物的设计在本领域中是公知的并且描述于本文所引用的文献中。

[0061] 如本文所使用，术语“靶标、“靶序列”、“靶区域”和“靶核酸”是同义的并且是指待扩增或检测的核酸的区域或子序列。

[0062] 如本文所使用，术语“杂交”是指两个单链核酸由于互补碱基配对而形成双螺旋结构。杂交可以在完全互补的核酸链之间或在含有少数错配区域的“大体上互补”的核酸链之间发生。使得只有完全互补的核酸链才会杂交的条件被称作“严格杂交条件条件”或“序列特异性杂交条件”。大体上互补的序列的稳定双螺旋可以在不太严格的杂交条件下实现；可以通过适当地调节杂交条件来控制可接受的错配度。核酸技术领域的技术人员可以根据由本领域所提供的指导凭经验确定双螺旋稳定性，这考虑到许多变量包括例如寡核苷酸的长度和碱基对组成、离子强度和错配碱基对的发生率（参见例如，Sambrook 等，(1989)Molecular Cloning—A Laboratory Manual(Cold Spring Harbor Laboratory,Cold Spring Harbor,N.Y.)；和 Wetmur(1991)Critical Review in Biochem. and Mol.Biol.26(3/4):227-259，它们均通过引用并入本文中）。

[0063] 术语“扩增反应”是指导致模板核酸序列的拷贝的增加或导致模板核酸的转录的任何化学反应，包括酶促反应。

[0064] 聚合酶链式反应 (PCR) 是允许较长双链 DNA 分子内的 DNA 序列的指数扩增的方法。PCR 需要使用一对引物，它们与 DNA 的两条链中的每一条上的限定序列互补。这些引物通过 DNA 聚合酶延伸，使得拷贝由指定序列组成。在形成这种拷贝后，可以再次使用相同的引物，不仅形成 input DNA 链的另一个拷贝，而且形成在第一轮合成中形成的短拷贝的拷贝。这导致对数扩增。因为需要在每一轮的扩增过程中提高温度以分离双链 DNA 的两条链，所以一个重要的进步是发现了从水生栖热菌 (Thermus aquaticus)（一种在热水池中生长的细菌）分离出的热稳定性 DNA 聚合酶 (Taq 聚合酶)；因此不需要在每一轮扩增中加入新的聚合酶。在若干（经常为约 40）轮扩增后，将 PCR 产物在琼脂凝胶上加以分析且其丰度足以利用溴化乙锭染色剂检测。

[0065] 应该理解，实时 PCR（又称作定量实时 PCR(qRT-PCR)）、定量 PCR(Q-PCR/qPCR) 或动态聚合酶链式反应是基于 PCR 的实验室技术，用于扩增和同时定量靶 DNA 分子。qPCR 允许检测和定量 DNA 样本中的特定序列（作为拷贝的绝对数或当归一化至 DNA input 或其它归

一化基因时作为相对量）。

[0066] 如本文所使用，如果当在足够严格的条件下使用时引物主要只与靶核酸杂交，则引物对靶序列具有“特异性”。通常，如果引物－标靶双螺旋稳定性大于在引物与样本中发现的任何其它序列之间形成的双螺旋的稳定性，则引物对靶序列具有特异性。本领域技术人员将认识到各种因素诸如盐条件以及引物的碱基组成和错配的位置将影响引物的特异性，并且在大多数情况下将需要引物特异性的常规实验确定。可以选择使得引物可以只与靶序列形成稳定双螺旋的杂交条件。因此，在适当严格的扩增条件下使用靶特异性引物使得能够进行包含靶引物结合位点的那些靶序列的特异性扩增。使用序列特异性扩增条件使得包含完全互补的引物结合位点的那些靶序列的特异性扩增。

[0067] 如本文所使用，“互补”是指一个核酸分子可以通过互补核苷或核苷酸之间的传统的沃森－克里克（Watson-Crick）碱基配对或其它非传统类型的配对（例如，Hoogsteen 或逆向 Hoogsteen 氢键结合）与另一核酸分子形成氢键。

[0068] 在本领域中应该理解，核酸分子不需要与可特异性杂交的靶核酸序列 100％互补。即，两个或多个核酸分子可以小于完全互补并且由可以与另一核酸分子形成氢键的核酸分子中的连续残基的百分比指示。例如，如果第一核酸分子具有 10 个核苷酸且第二核酸分子具有 10 个核苷酸，则第一核酸分子和第二核酸分子之间的 5、6、7、8、9 或 10 个核苷酸的碱基配对分别表示 50％、60％、70％、80％、90％和 100％的互补性。“完美”或“完全”互补的核酸分子意指其中第一核酸分子的所有连续残基将与第二核酸分子中的相同数量的连续残基氢键结合的那些，其中两个核酸分子具有相同数量的核苷酸（即，具有相同长度）或两个分子具有不同长度。

[0069] 如本文所使用，术语“非特异性扩增”是指非靶序列的核酸序列的扩增，这是由于引物与非靶序列的序列杂交且然后作为引物延伸的底物所致。引物与非靶序列的杂交被称作“非特异性杂交”且尤其易于在较低温度、低严格度的预扩增条件期间出现。

[0070] 如本文所使用，术语“引物二聚体”是指与模板无关的非特异性扩增产物，据信它源于其中另一引物作为模板的引物延伸。尽管引物二聚体经常显现为两个引物的串联体即二聚体，但是还存在多于两个引物的串联体。术语“引物二聚体”在本文中被一般地用来包括与模板无关的非特异性扩增产物。

[0071] 如本文所使用，术语“反应混合物”是指含有进行给定反应所必需的试剂的溶液。“扩增反应混合物”是指含有进行扩增反应所必需的试剂的溶液，其通常含有于合适缓冲液中的寡核苷酸引物和 DNA 聚合酶或连接酶。“PCR 反应混合物”通常含有于合适缓冲液中的寡核苷酸引物、DNA 聚合酶（最典型为热稳定性 DNA 聚合酶）、dNTP 和二价金属阳离子。如果反应混合物含有使反应能够进行所必需的所有试剂，则称之为完全反应混合物，且如果它只含有必需试剂的一部分，则称之为不完全反应混合物。本领域技术人员将理解，反应组分通常是分开储存的溶液，它们各自含有总组分的一部分，为的是方便、储存稳定性或允许根据应用调节组分浓度，且反应组分在反应前组合以形成完全反应混合物。此外，本领域技术人员将理解，反应组分为了商品化而分开包装且可用的商品试剂盒可以含有反应组分的任何子集，其包括本公开的封闭引物。

[0072] 1. 引言

[0073] 本公开提供了用于测定染色体群体中的短端粒丰度的量度和使用这些量度来测

定健康量度和干预效果的方法和材料，这些干预增加或减小端粒长度且因此增加或减少健康，或相反地分别减少或增加未来疾病或死亡的风险。所述方法涉及仅从具有在预定长度范围内的端粒（例如，所有端粒不长于一定长度，例如短于约 5kbp）的染色体产生染色体片段的拷贝的群体。该测定可以用于表示一组样本中的短端粒的相对丰度，或该测定可以与总端粒丰度的量度联用以产生短端粒的绝对百分比。

[0074] 在一个方面，测量短端粒产物的丰度的方法包括两个步骤。第一步骤包括使用延伸引物从双链染色体 DNA 模板生成延伸产物。延伸引物的 3’末端具有与端粒的 G 链中的端粒重复序列互补的序列，且它的 5’末端具有“锚定”序列。依此方式，延伸引物适于与样本中的染色体的 3’突出中的端粒重复序列杂交，并利用 5’-锚定序列来引发随后的 PCR 反应。在限时延伸反应中产生延伸产物。由于延伸反应是限时的，因此它可以被配置来产生不超过特定长度的延伸产物。因为延伸产物是限长的，所以它们将仅在足够短的染色体中延伸至亚端粒区域。第二步骤包括从延伸产物扩增以亚端粒序列和锚定序列为界的序列。

[0075] 可以通过至少三种不同的方法来估算由限定的反应时间产生的延伸产物的长度：(a) 通过链置换酶的聚合速率 (R) 乘以延伸时间；(b) 通过在 DNA 印迹凝胶 (Southern gel) 上分析 PCR 产物的大小；和 (c) 通过测序分析纯 TTAGGG 区域的大小。测序方法受到对长段重复 DNA 进行准确测序的能力的限制。

[0076] 因为延伸反应是限时的，所以亚端粒序列将仅存在于来自这样的染色体的延伸产物中：其中从 3’突出中延伸引物杂交的位置到亚端粒区域的距离在延伸的预定长度范围内。预定长度范围可以多至为从业人员选择的任何长度。例如，长度范围可以是最多为预定长度的长度。在某些方面，长度范围包括短端粒的长度，例如最多为 5kb。在其它方面，长度范围是短于预定距离的长度。因此，例如，在其中端粒长度不长于限定延伸长度的染色体（例如，具有短端粒的染色体）中，限时延伸反应将使引物延伸至染色体的亚端粒区域。在其中端粒长度长于预定延伸长度的染色体（例如，具有长端粒的染色体）中，限时延伸反应将不会使引物延伸至染色体的亚端粒区域，且延伸产物将不具有第二扩增步骤所必需的亚端粒序列。因此，可以控制延伸产物的群体以包括仅来自具有短于一定大小的端粒的染色体的端粒序列。如果长度范围为例如 4kb，则延伸产物包括例如其中端粒为 4kb、3kb、2kb、1kb 等的产物。

[0077] 当使用适于扩增以亚端粒序列和锚定序列为界的序列的一对扩增引物来扩增延伸产物时，仅扩增具有亚端粒序列的延伸产物。那些是从具有短于限定延伸长度的端粒的染色体（例如具有短端粒的染色体）生成的延伸产物。因此，延伸产物不从源自样本中的染色体的总端粒序列扩增，而仅从源自具有不大于预定长度的端粒的染色体的端粒序列扩增。这种扩增产物在本文中有时被称作“限长端粒扩增产物”或根据上下文称作“短端粒扩增产物”。通过在此 PCR 扩增步骤中使用有限的延伸时间，短端粒将进一步富集。这种有限延伸时间的 PCR 步骤因此将增加测定的特异性以仅扩增短端粒群体。

[0078] 限长端粒扩增产物中的端粒序列的量或丰度在本文中有时被称作“限长端粒丰度”或根据上下文称作“短端粒丰度”。限长端粒扩增产物中的端粒序列的丰度可以通过用于测定总端粒序列的产物中的端粒序列丰度的任何方法来测量。

[0079] 本公开的方法开始于双链 DNA，例如处于其天然状态的染色体。在测量总端粒序列的比较方法中，具体来说，这使用 qPCR，可以涉及提供单链或变性核酸的样本。

[0080] 2. 限长端粒扩增产物

[0081] 2.1 样本

[0082] 天然双链状态下的染色体 DNA 可以从含有核酸的固体、流体、半固体或气体样本，例如从液体组织诸如血液、唾液、尿液、血浆、血清、CSF、支气管肺泡灌洗液；从固体组织诸如肺、肌肉、皮肤；从半固体组织诸如骨髓；以及从气体样本诸如呼出气体获得。染色体 DNA 获自其中的生物体可以是具有带 3' 突出的线性染色体的任何生物体。可以使用产生高分子量基因组 DNA(大于 20kb) 的任何 DNA 纯化方法包括苯酚 / 氯仿萃取、氯化铯梯度和使用硅胶膜结合技术的商品试剂盒、选择性洗涤剂介导 DNA 沉淀方法来获得模板双链染色体 DNA。DNA 纯化商品试剂盒的实例包括 Agencourt DNAdvance 和 Agencourt Genfind(Beckman Coulter)、QIAamp 试剂盒 (QIAGEN, Valencia, California)、QIAamp 血液试剂盒 (QIAGEN)、QIAamp FFPE 组织试剂盒 QIAGEN)、AHPrep 试剂盒 (QIAGEN)、Puregene 试剂盒 (QIAGEN)、PureLink 和 GeneCatcher(Invitrogen) 和 Wizard(Promega)。

[0083] 用于本公开的方法中的样本可以是具有 3' 末端单链突出的任何基因组 DNA。在某些方面，样本包括高分子量基因组 DNA(例如，>20kb)。可以使用产生高分子量天然基因组 DNA 的任何方法。

[0084] 在具有端粒的双链染色体中，具有含有端粒重复序列的 3' 末端 (“3' 突出”) 的单一 DNA 链延伸超出具有 5' 末端的配对单链的末端。具有 3' 突出的端粒的链被称作“G 链”，且包括端粒重复序列 5'-TTAGGG-3'。同阶排列为其中字母未被扰乱，而是开始于相同序列中的不同点，例如倒位 (例如，XYZ、YZX、ZXY 而非 YXZ) 的排列。这种序列的同阶排列包括 :5'-TAGGGT-3'、5'-AGGGTT-3'、5'-GGGTTA-3'、5'-GGTTAG-3' 和 5'-GTTAGG-3'。具有 5' 末端的链被称作“C 链”且包括端粒重复序列 5'-CCCTAA-3'。

[0085] 端粒的长度可以是例如从染色体的末端至亚端粒区域的距离，例如以千碱基计。在正常成人的细胞中，端粒的长度可以在从小于 1kb 至 12kb 或在一些情况下至 >20kb 的范围内。已知端粒长度在不同的细胞类型中有所不同 (Lin 等，JImmunol Methods, 2010, 31:352(1-2):71-80)。出于这些原因，短端粒群体的可用长度范围基于临床效用可以是广泛的。因此，在某些方面，短端粒具有不超过约 5kb、不超过 4kb、不超过 3kb、不超过 2kb、不超过 1kb 或不超过约 0.5kb 的长度。本公开的方法可以被配置来检测最多为这些长度中的每一个的端粒。

[0086] 短端粒产物可以从单一端粒、单一染色体、源自单个细胞的染色体群体或源自多个细胞的染色体群体生成。

[0087] 2.2 产生延伸产物

[0088] 在延伸反应中，在退火条件下使引物与双链染色体 DNA 的 3' 突出退火。适当的退火条件为本领域技术人员已知，诸如通常用于杂交核酸链以用于链延伸或用于 PCR 的那些退火条件。此类条件包括但不限于在加热器中于 65℃下孵育 10 分钟且然后历时 1 小时的时间冷却至室温。其它条件可以包括在从 37℃至 65℃范围内的温度下孵育 5 分钟至 30 分钟且然后历时 1 小时至 3 小时的时间冷却至室温。

[0089] 2.2.1 延伸引物

[0090] 延伸引物包含 3' 部分和 5' 部分。

[0091] 2.2.1.1 3' 部分

[0092] 3’部分具有适于与端粒的G链中的端粒重复序列杂交的序列。3’部分中的序列可以与端粒重复互补，或者它可以如上所述具有某些错配，只要这些错配允许在退火条件下进行用于引物延伸的杂交即可。例如，3’部分可以具有端粒重复序列（即，端粒的C链的序列）的至少8个连续核苷酸。连续核苷酸可以呈端粒重复序列的任何排列方式。在其它方面，3’部分的序列可以具有端粒重复序列的至少9个连续核苷酸、至少10个连续核苷酸、至少11个连续核苷酸或至少12个连续核苷酸。在其它方面，3’部分的序列可以具有呈任何同阶排列的两个或多个、三个或多个、或四个或多个端粒重复单元。

[0093] 2.2.1.1 5’部分

[0094] 延伸引物的5’部分（又称作“锚定序列”）被配置成在退火条件下不与G链中的端粒重复序列（其与3’部分杂交）杂交。优选地，锚定序列在退火条件下不与G链的亚端粒区域中的序列杂交。锚定序列还可以被配置成不与G链的3’突出的末端的10kb内、20kb内或50kb内的靶G链的任何序列或靶染色体中的任何单一序列杂交。锚定序列可以被配置以使得它的补体不与染色体的C链的端粒区域或亚端粒区域中的任何序列或端粒的C链末端的10kb内、20kb内或50kb内的任何序列杂交。例如，锚定序列可以是未在测试染色体中发现的单一序列。

[0095] 2.2.2 延伸反应

[0096] 在将延伸引物与染色体DNA退火后，使用具有链置换活性和/或外切核酸酶活性的聚合酶进行延伸反应。链置换聚合酶的实例包括但不限于T7聚合酶（例如，测序酶）、大肠杆菌DNA聚合酶I的外切核酸酶缺乏型Klenow片段以及Bst DNA聚合酶大片段和Deep VentR(exo)。另外，还可以使用具有5’-3’外切核酸酶活性的聚合酶。

[0097] 延伸反应是限时的。即，允许延伸反应进行预定量的时间。时间被校准来产生具有不超过预定长度的平均值的延伸产物。可以凭经验在选定的条件和反应物下测定用于通过链置换酶产生延伸产物的时间，以产生预定长度的延伸产物。适用于本发明的各种聚合酶的延伸速率之前已经被测定，且延伸速率可以用于计算获得所需延伸产物所必需的近似时间。下表中给出了示例性聚合酶的延伸速率。

[0098]

DNA聚合酶	延伸速率*	参考文献
Klenow	13.5个核苷酸/秒	Maier B、Bensimon D和Croquette V. *Proc Natl Acad Sci USA.*(2000) 2497(22):12002-7。
T7	75.9个核苷酸/秒	Tanner, N. A.等 *Nuc.Acids Res.*(2009) 37, e27.
Taq	35-100个核苷酸/秒(75℃) 0.9-2.55个核苷酸/秒(37℃)	Wittwer, C.T.和Garling, D.J., BioTechniques (1991) 10(1), 76-83。
phi29	25个核苷酸/秒	Blanco, F.等J Biol Chem (1989) 264,8935-8940
Bst	50-100个核苷酸/秒	New England Biolabs

[0099] *在相关参考文献中指定的条件下或不存在其它信息的标准反应条件下。

[0100] 通过将链置换酶加入反应管中实现延伸反应的引发。反应可以通过将反应管置于80℃下达20分钟或通过加入EDTA而停止。此外，可以通过在更低或更高温度例如25℃或

30℃下孵育来控制（减缓或加速）反应。例如，链置换聚合酶测序酶在37℃下的置换速率为约28bp/秒。在30℃下，基于DNA印迹分析，该置换速率约慢3倍。在37℃下，测序酶可以产生30秒内约1kb、1分钟内约2kb、3分钟内约5kb以及5分钟内约8kb的延伸产物。利用各种聚合酶系统和各种样本源产生预定长度的延伸产物所必需的时间安排可以凭经验来测定，例如，可以先后进行类似的时程实验和DNA印迹分析以校准延伸时间。因此，延伸反应可以被定时为不超过30分钟、不超过10分钟、不超过5分钟、不超过4分钟、不超过3分钟、不超过2分钟、不超过1分钟或不超过30秒。

[0101] 2.3 扩增反应

[0102] 然后例如通过PCR扩增限时延伸产物中的序列。更具体地说，扩增一侧以锚定序列为界且另一侧以亚端粒序列为界的序列。引物可以是完全互补的或具有错配序列，只要它允许与其靶标（锚定序列或亚端粒序列）退火和延伸即可。

[0103] 锚定序列可以变化。在某些方面，锚定序列是被测量端粒数量的生物体的基因组中不存在的特异性序列，或者如果在基因组中的别处中发现了锚定序列，则如果该序列明显远离SUS序列，其仍可以是适用的，从而使得在PCR步骤期间除了端粒锚定序列与端粒SUS序列之间以外不发生扩增。

[0104] 优化PCR条件以产生最佳的分析性能。通过在DNA印迹上检查产物分布以确保使短端粒的预期大小范围富集来预先确定PCR条件中的延伸时间。

[0105] 用于本公开的方法中的亚端粒引物可以含有在所有或大多数染色体发现的G链上的序列，例如端粒序列的变体$(TGAGGG)_{3\ 6}$(Xu和Blackburn,Mol.Cell,28:315-327,2007)或$(TTGGGG)_{3\ 6}$(Allshire等，Nucleic Acid Research,17:4611-4627,1989)或$(TCAGGG)_{3\ 6}$(Baird等，EMBO J.,14(21):5433-5443,1995)。或者，亚端粒引物可以是在特定染色体上发现的区段，例如描述于Xu等中的XpYpE2引物(5'-GTTGTCTCAGGGTCCTAGTG-3'[SEQ ID NO:1])(Xu和Blackburn,Mol Cell,28:315-327,2007)。在一个方面，针对亚端粒序列的引物包括以下各者，基本上由它们组成或由它们组成：

[0106] 5'-GATGGATCCTGAGGGTGAGGGTGAGGG-3'[SEQ ID NO:2]

[0107] 5'-CGGGCCGGCTGAGGGTACCGCGA-3'[SEQ ID NO:10](染色体1)

[0108] 5'-GCTAATGCACTCCCTCAATAC-3'[SEQ ID NO:11](染色体5)

[0109] 5'-CATTCCTAATGCACACATGATACC-3'[SEQ ID NO:12](染色体9)

[0110] 当使用本公开的方法在非人的生物体中运用短端粒测定时，可以基于该生物体的基因组序列设计亚端粒引物的特定序列。亚端粒引物的序列应该与具有3'突出的链相配。

[0111] 扩增产物包括具有在一定长度范围内的端粒重复序列的核酸群体，且不包括长于该范围的序列。因此，所述群体可以被配置成包括仅来自具有短于临界长度的端粒的染色体的序列。

[0112] 可以在延伸反应步骤中包括用于控制延伸反应效率的内部对照序列。该内部对照序列可以是一端具有亚端粒引物序列和具有端粒序列的G链的3'突出的双链DNA。亚端粒引物序列和端粒序列之间可以是一段独特非端粒序列。一种此类序列可以是例如hTERT基因或RNA酶P基因。可以通过基于Taqman的测定测量针对该内部对照的延伸反应的效率，以定量例如hTERT基因或RNA酶P基因。

[0113] 3.测量端粒丰度的方法

[0114] 用于测量包含端粒序列的样本中的端粒丰度的任何方法均可以用于测量限长端粒样本中的端粒丰度。

[0115] 端粒丰度的量度可以是绝对或相对的。端粒丰度的绝对量度包括例如通过例如核苷酸的数目所测量的基因组中端粒序列的总长度。更通常地，端粒丰度是相对于参照物来测量的。端粒序列的检测可以根据测定中所产生的信号强度来测量。可以将这个信号强度与由测定中的参考序列产生的信号强度作比较。相对信号强度可以起到标准化方法的作用。标准化的方法可以更容易地在测定之间进行比较。例如，可以将通过检测端粒序列所产生的信号与通过测量对照序列所产生的信号作比较。该对照序列可以是例如 β-球蛋白基因的一部分。因此，与所使用的测定方法无关，端粒序列与参考序列的相对信号可以例如用比率表示。这个比率可以用于比较端粒序列丰度测量的结果。

[0116] 在其它方面，将短端粒丰度的量度与总端粒丰度作比较。总端粒丰度可以通过链置换反应和扩增反应中的长延伸时间和随后的如美国专利第 7,695,904 号 (Cawthon) 和 Lin 等 (Lin 等，2010,352(1-2):71-80) 中所描述的 qPCR 方法来测量。为了测定短端粒的百分比，可以测定短端粒与总端粒的比率。例如，可以用短端粒测量的信号强度除以总端粒测量的信号强度。

[0117] 3.1 qPCR

[0118] 一种定量短端粒丰度的方法是定量 PCR，如 Cawthon(Nucleic. Acids Res.,2002,30(10):e47；美国专利第 7,695,904 号；Lin 等，J.Immunol. Methods,2010,352(1-2):71-80) 或 Cawthon 2009(Nucleic Acids Res.200937(3):e21.) 中所描述。

[0119] 在本公开中可以使用本领域中已知的各种方法来测定平均端粒长度或端粒丰度。优选地，如由 Cawthon(Nucleic.Acids Res.,2002,30(10):e47；美国专利第 7,695,904 号) 特定地针对端粒长度检测所修改，利用实时动态定量聚合酶链式反应 (qPCR)。这种方法是简单的且允许大量 DNA 样本的快速高通量处理。qPCR 方法基于由报道分子所产生的荧光的检测，该荧光随着聚合酶链式反应的进行而增加。这种荧光增加是由于每个扩增周期的 PCR 产物的累积而发生。这些荧光报道分子包括结合至双链 DNA 的染料 (例如，SYBR Green 或溴化乙锭) 或序列特异性探针 (例如，分子信标 (Molecular Beacons) 或 TAQMAN 探针)。或者，可以使用由 Cawthon(Nucleic Acids Res.2009,37(3):e21) 所描述的 qPCR 方法。该方法允许经由多重扩增 (multiplexing)(包括当端粒重复为高丰度种类且单拷贝基因为低丰度种类时的实验环境) 测定单个样本中的 T/S 比。

[0120] 在本公开的方法中，使用对重复的端粒序列 $(TTAGGG)_n$ 具有特异性的引物探针。引物的大小可以一般在 5 个至 500 个核苷酸长度之间变化，介于 10 个与 100 个核苷酸之间，介于 12 个与 75 个核苷酸之间，或介于 15 个至 50 个核苷酸之间，这取决于用途、所需特异性和扩增技术。在本公开中，一个方面利用与靶端粒序列的第一单链杂交的第一引物以及与靶端粒序列的第二单链杂交的第二引物，其中第一链和第二链大体上互补。在这个方面，例如，可以使用由以下组成的成对引物组：tel1(5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGG TGAGGGT-3')[SEQ ID NO:3] 和 tel2(5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3')[SEQ ID NO:4]。在一个方面，这些引物中的至少一个包含至少一个改变或突变的核苷酸残基，这使得当引物彼此杂交时，在改变的残基与另一引物的

3’末端核苷酸之间产生错配。在3’末端核苷酸处存在错配会阻碍通过聚合酶进行延伸，由此限制非靶核酸依赖性延伸反应。在这个方面，例如，可以使用由以下组成的成对引物组：tel 1b 5’-CGGTTTGTTTGGGTTTGGGTTTGGG TTTGGGTTTGGGTT-3’[SEQ ID No.:5]；和 tel 2b 5’-GGCTTGCCT TACCCTTACCCTTACCCTTACCCTTACCCT-3’[SEQ ID No.:6]。在另一方面，例如，可以使用由以下组成的成对引物组：telg 5’-ACACT AAGGTTTGGGTTTGGGTTTGGGTTT GGGTTAGTGT-3’[SEQ ID N o.:13]；和 telc 5’-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATC CCTAACA-3’[SEQ ID No.:14]。本领域技术人员应该了解，在本公开中可以采用其它大体上互补或错配的引物组。此类引物描述于美国专利第7,695,904号（Cawthon等）中。

[0121] 根据本领域中公知的程序来进行扩增反应。用于PCR的程序被广泛使用和描述（参见例如美国专利第4,683,195和4,683,202号）。简单地说，双链靶核酸一般是通过在高到足以使这些链变性的温度下孵育而变性，然后在过量引物的存在下孵育，这些引物与单链靶核酸杂交（退火）。DNA聚合酶延伸杂交的引物，从而产生靶核酸的新拷贝。使所得双螺旋变性，并且重复杂交和延伸步骤。通过在用于互补靶链的第二引物的存在下反复进行变性、退火和延伸的步骤，被两个引物包围的靶核酸以指数方式扩增。引物延伸步骤的时间和温度将取决于聚合酶、被扩增的靶核酸的长度以及用于扩增的引物序列。充分扩增靶核酸所需的反复步骤的数量将取决于扩增的效率。本领域技术人员应该理解，本公开不受扩增过程中所应用的时间、温度、缓冲条件和扩增周期的变化所限制。

[0122] 通过本领域中熟知的方法来检测和分析扩增的产物。扩增产物可以在分离和/或纯化产物之后加以分析，或通过直接测量扩增反应中所形成的产物来分析。对于检测来说，可以使用荧光化合物例如使用溴化乙锭或SYBR Green，或通过与标记的核酸探针杂交来间接地鉴定产物。或者，在扩增反应中使用标记的引物或标记的核苷酸来标记扩增产物。所述标记包括任何可检测的部分，包括荧光标记、放射性标记、电子标记以及间接标记诸如生物素或地高辛(digoxigenin)。

[0123] 适于进行本公开的qPCR反应的仪器可获自许多商业来源(ABI Prism 7700,Applied Biosystems,Carlsbad,CA；LIGHTCYCLER 480,Roche Applied Science,Indianapolis,IN；Eco Real-Time PCR System,Illumina,Inc.,San Diego,CA；RoboCycler 40,Stratagene,Cedar Creek,TX)。

[0124] 当使用实时定量PCR来检测和测量扩增产物时，各种算法被用来计算样本中的靶端粒的数目。（例如，参见ABI Prism 7700软件版本1.7；Lightcycler软件版本3)。定量可以涉及使用具有已知拷贝数的端粒核酸的标准样本以及从标准和阈值周期(C_t)的对数生成的标准曲线。一般来说，C_t是PCR循环或部分PCR循环，其中由扩增产物产生的荧光高于基线荧光若干个偏差。

[0125] 3.2 其它方法

[0126] 来自扩增反应的短端粒的丰度可以通过本领域中已知的其它方法来测量。此类方法包括但不限于直接核酸测序和DNA印迹，以及斑点印迹或狭缝印迹杂交和数字PCR。

[0127] 在本公开中可以采用用于直接测定分离的DNA中的核酸序列的常规技术。例如，参见：“DNA Sequencing,”The Encyclopedia of Molecular Biology,J.Kendrew编辑,Blackwell Science Ltd.,Oxford,UK,1995,第283-286页。染料-终止子自动化测序现在最常被用于核酸测序（“DNA Sequencing”,Lab Manager,在万维网URL labmanager.

com/？articles.view/articleNo/3364/article/DNA-Sequencing 上）。自动化测序设备被方便地使用并且可以购自诸如 Applied Biosystems、Roche Applied Science 和 Illumina Inc. 的公司。一旦测定了 DNA 样本的序列，然后即可对任一端的端粒核苷酸序列 (TTAGGG) 的拷贝数进行计数。本公开的这种方法通过直接测量端粒序列提供了绝对端粒丰度的量度。

[0128] 在本公开中还可以利用 DNA 印迹 (Southern, E. M., J. Mol. Biol., 1975, 98(3):503-517) 以通过检测人端粒核苷酸序列 $(TTAGGG)_n$ 的特定存在来测定端粒丰度。在本公开中，末端限制性片段 (TFR)DNA 印迹组合了将通过电泳分离的 DNA 片段转移至滤膜，接着通过与对 (TTAGGG) 序列具有特异性的探针杂交来检测这些片段 (Allshire, R. C. 等，Nucleic Acids Res., 1989, 17, 4611-4627)。此类探针具有与端粒序列互补的序列。为了便于检测，将探针进行放射性标记或用荧光染料或显色染料加标签。然后，可以定量所存在的放射性或荧光的量以得到样本中的端粒丰度。M. Kimura 等 (Nature Protocols, 2010, 5:1596-1607) 描述了一种用于测定端粒长度的适当 DNA 印迹程序。

[0129] DNA 印迹的变型包括斑点印迹或狭缝印迹，其中将 DNA 作为斑点或狭缝点样于滤膜上，接着通过与对 (TTAGGG) 序列具有特异性的探针杂交来检测片段 (Kimura M、Aviv A, Nucleic Acids Res., 2011, 39(12):e84. doi:10.1093/nar/gkr235. Epub 2011Apr 27)。

[0130] 在本公开的上述方面，荧光可以相对荧光单位 (RFU) 来测量。当在毛细管内通过使用电泳而分离的标记片段受激光激励并穿过检测窗口时，使用电荷耦合装置 (CCD) 阵列来检测荧光。计算机程序测量结果，从而在每个数据点处从荧光强度水平测定含端粒的片段的数量或大小（“Relative fluorescence unit(RFU)”, DNA.gov:Glossary, 2011 年 4 月，万维网 URL dna.gov/glossary/）。

[0131] 可以使用 DNA 测序方法来测量端粒丰度的量度。此类方法可以涉及对包含端粒重复的样本中的分子进行测序以及测定端粒重复序列的丰度。DNA 测序方法可以包括任何已知的测序方法，包括例如经典的测序方法，诸如 Sanger 测序或 Maxam Gilbert 测序；以及新一代测序方法，诸如连接测序、纳米孔测序、焦磷酸测序、超级焦磷酸测序 (superpyrosequencing)、通过质子检测来测序、通过合成测序和单分子测序。还可以通过数字 PCR 来测量短端粒丰度。此类技术平台包括但不限于数字 PCR，包括例如 RAINDROP 数字 PCR 系统 (Raindance Technologies, Billerica, Massachusetts) 或 QX200DROPLET DIGITAL PCR 系统 (Bio-Rad Laboratories, Hercules, California)、微流体数字 PCR(Fluidigm Corporation, South San Francisco, California) 或 OPENARRAY 实时 PCR 系统 (Thermo Fisher Scientific, Inc., Waltham, Massachussetts 的 APPLIED BIOSYSTEMS 部门）。在各个其它方面，短端粒的丰度可以通过合适的杂交技术来测量，例如数字颜色编码条形码方法如 NCOUNTER 分析系统 (Nanostring Technologies, Seattle, Washington)。

[0132] 4. 试剂盒

[0133] 本公开还提供了可用于本公开的方法中的试剂盒。此类试剂盒可以包括本公开的延伸引物以及与亚端粒序列或与延伸引物的锚定序列的补体杂交的扩增引物。或者，试剂盒可以包括本公开的延伸引物和一对扩增引物，所述扩增引物适于扩增核酸中以亚端粒序列和延伸引物中的锚定序列为边界的序列。试剂盒可以包括含有延伸引物的容器和含有扩增引物中的一个或两个的另一个容器。试剂盒还可以包括用于引物延伸的试剂和用于核酸

扩增例如用于PCR的试剂。试剂盒还可以包括具有已知值的对照和参考样本。在某些方面，试剂盒包括延伸引物和一对扩增引物。在某些方面，延伸引物包括在退火条件下与双链染色体DNA的3’突出中的端粒重复序列杂交的3’部分，以及不与端粒或亚端粒区域中的序列杂交的5’锚定部分。

[0134] 5. 与端粒丰度、短端粒丰度和端粒丰度的变化率或短端粒丰度的变化率相关的病状

[0135] 一般来说，与端粒丰度有关的病状通常是已使用如本文所描述的平均端粒长度的量度来推导。然而，如上所讨论，新兴数据表明相比于测量平均端粒长度或平均端粒长度的变化率，测量短端粒丰度和或短端粒丰度的变化率可以是临床上有意义的结果（例如疾病或死亡风险）的更灵敏且可能更准确的预测指标。因此，重要的是灵敏和准确地测量短端粒的丰度。已经报道了极短端粒的存在会导致失去细胞活力和组织功能，这表明极短的端粒与细胞衰老之间存在因果关系 (Hemann, M. T. 等 Cell, 2001. 107(1)67-77)。

[0136] 尽管短平均端粒长度（如下所讨论）是临床结果的确立的良好预测指标，然而预期短端粒丰度的准确测量可以更灵敏地测量干预对端粒动力学的影响 (Canela, A. 等 Proc Natl Acad Sci USA, 2007. 104(13)5300-5)。Harley 等 (Rejuvenation Res. 2011. 14(1)45-56) 显示服用端粒酶的相对弱激活剂TA-65®的人显示出短端粒百分比的降低，但是平均端粒长度没有明显改变。在Vera等（同上）中，短端粒丰度的增加速率预测了小鼠的两个不同群体中的寿命增加“The Rate of Increase of Short Telomeres Predicts Longevity in Mammals, Cell Reports(2012), 万维网URL:dx.doi.org/10.1016/].celrep.2012.08.023)。由于缺少用于测量短端粒丰度的准确、有成本效益的高通量方法，直到现在还没有使用短端粒丰度或短端粒丰度的变化率来建立测量短端粒丰度的临床效用的大型人临床研究。

[0137] 在Vera等（同上）的动物研究中的概念验证还指示，具有短端粒的百分比随着时间的高增加速率的小鼠相较于具有短端粒的百分比随着时间的低增加速率的小鼠具有减少的生存。在野生型小鼠中，具有低增加速率（例如，0.4%/月）的小鼠相较于具有高增加速率（例如，1%/月）的小鼠的生存差异为约100周，或野生型小鼠中的最大生存的将近三分之二。

[0138] 因此，相较于具有低短端粒百分比的人，具有如通过本文所描述的准确和精确的测定所测量的高短端粒百分比的人将更容易有疾病或死亡风险。短端粒%的定量评估将具有显著的诊断效用，这不仅在于健康监测，而且在于疾病诊断、预后和伴随诊断。

[0139] 监测短端粒的丰度具有用于健康监测的直接效用，因为已知短端粒（通常小于1kbp或小于2kpb或小于3kbp）引发细胞衰老 (Hemann, M. T. 等 Cell, 2001, 107(1)67-77)，且已知细胞衰老会导致组织功能的丧失且最终导致疾病和死亡。因此，具有大于短端粒的平均丰度的个体与年龄匹配的正常个体的参考群体相比处于增加的发病或死亡风险下。此类人将被激励朝着较好的生活方式和较低的短端粒丰度改变其行为，而具有低于短端粒的平均丰度的个体相较于参考群体将知道其细胞健康可能优于平均水平，并且因此将被激励如果不进一步改善则至少保持其生活方式习惯以维持其良好的健康。

[0140] 从基因组DNA测定的每个染色体末端的平均端粒长度是总端粒丰度的量度，并且已经显示这与若干个重要的生物指标相关联。这些指标包括例如各种疾病状况的风险，例

如心血管风险、癌症风险、肺纤维化风险、传染病风险和死亡风险。端粒丰度还与实足年龄、体质指数、臀部/重量比和知觉应激相关联。端粒长度的一个量度是端粒/单拷贝("T/S")比。给定群体中的此类比率可以分成分位数例如三分位数。已经发现,由T/S比表示的端粒丰度在较低两个三分位数中的个体比在端粒长度的最高三分位数中的那些个体处于显著更高的心血管疾病风险下。

[0141] 一般来说,在群体中的端粒丰度的量度(例如表示为参考群体的百分比的T/S值)的百分位数值(通常为端粒长度的最高三分位数或四分位数)与疾病风险负相关,即较短的平均端粒长度与改善的健康量度相关,而较低的百分位数得分与降低的健康量度、增加的疾病风险或端粒疾病的存在相关。

[0142] 在群体中,端粒长度随着年龄而减小。因此,个体的端粒长度的量度可以与群体中相同年龄范围内的个人(即,年龄匹配的群体)的量度相比较。例如,30岁的个人的端粒丰度的量度可能约等于30岁的群体平均值,或等于20岁或40岁的群体平均值。端粒丰度的量度与健康量度的相关性当与年龄匹配的群体的量度相比较时更加准确。年龄匹配的群体的范围可以是例如一年、两年、三年、四年、5年、7年或10年。

[0143] 5.1 健康量度

[0144] 通过本公开的方法从受试者样本中测定的短端粒丰度可以与健康量度相关联。特别受关注的是涉及知觉应激的健康量度。端粒缩短可以由遗传和环境因素来加速,这些因素包括多种形式的应激诸如氧化损伤、生化应激源、慢性发炎和病毒感染(Epel,E.S. 等,Proc.Natl.Acad.Sci.USA,2004,49:17312-15)。一般健康状况的方便量度是由John Ware开发的SF-36®健康调查(参见例如万维网URL sf-36.org/tools/SF36.shtml)。SF-36是多用途的简短健康调查,其中优选地由经过培训的个体向患者提出仅仅36个问题。这个健康调查提供了功能性健康和幸福感得分的8等级概况以及基于心理测量学的身心健康综合量度和基于偏好的健康效用指数。SF-36调查被用来估计疾病负担并且比较疾病特异性基准与一般群体标准。最频繁研究的疾病和病状包括关节炎、背痛、癌症、心血管疾病、慢性阻塞性肺病、抑郁、糖尿病、胃肠病、偏头痛、HIV/艾滋病、高血压、肠道易激综合症、肾病、下背痛、多发性硬化、肌肉骨骼病状、神经肌肉病状、骨关节炎、精神病诊断、类风湿性关节炎、睡眠障碍、脊髓损伤、中风、物质滥用、手术程序、移植和创伤(Tumer-Bowker等,*SF-36*® Health Survey&"SF"Bibliography:第三版(1988-2000),QualityMetric Incorporated,Lincoln,RI,2002)。本领域技术人员应该了解,一般健康状况的其它调查方法例如RAND-36可以在本公开中得到应用。

[0145] 在本公开的一个方面,随时间的推移收集受试者样本,并且从这些样本中测定短端粒丰度的量度。用于收集多个样本的适当时间段包括但不限于1个月、3个月、6个月、1年、2年、5年和10年(例如,最早的样本与最后的样本之间的时间可以是大约这些时间段)。这种方法允许监测患者为了改善其一般健康状况和/或监测其健康状况和/或疾病风险所作的努力。因为短端粒引发细胞死亡,所以短端粒长度的百分比在个体内随时间降低或维持的发现指示健康改善,而短端粒的百分比随时间增加表示健康减退或恶化。

[0146] 5.2 病理状况的风险

[0147] 5.2.1 疾病

[0148] 测量端粒的重复单元数在医疗诊断(例如关于疾病风险的诊断)、疾病预后和治

疗中具有多种应用。具体来说，端粒长度的测量在评估导致疾病风险的病理状况中得到应用。在本公开的一个方面，疾病是与老龄化相关的疾病，例如但不限于心血管疾病、糖尿病、癌症、肝纤维化和抑郁。

[0149] 在一个方面，本公开在评估和监测心血管疾病中得到应用。已经显示，如通过血管造影术所测定，白血球中的端粒长度在患有严重三支冠状动脉疾病的患者中比其在具有正常冠状动脉的个体中更短 (Samani, N. J. 等，Lancet, 2001, 358:472-73)，并且在 50 岁之前经历过早心肌梗塞的患者中的端粒长度与没有这种历史的年龄和性别匹配的个体相比更短 (Brouilette S. 等，Arterioscler. Thromb. Vase. Biol., 2003, 23:842-46)。Brouilette 等 (Lancet, 2007, 369:107-14) 已经提出，在易患冠心病的人中较短的白细胞端粒可以指示其它心血管风险因子对端粒长度的累积影响。增加的氧化应激也造成动脉粥样硬化，并且已经显示增加的氧化剂应激会增加体外的端粒耗损速率 (Harrison, D., Can. J. Cardiol., 1998, 14(增 刊 D):30D-32D ; von Zglinicki, T., Ann. N. Y. Acad. Sci., 2000, 908:99-110)。在横向研究中，也已经报道了吸烟、体质指数和 1 型糖尿病与较短的白细胞端粒长度相关 (Valdes, A. 等，Lancet, 2005, 366:662-64 ; Jeanclos, E. 等，Diabetes, 1998, 47:482-86)。已经显示增加的生活应激（一种已知会增加冠心病风险的因素）与较短的端粒相关，这可能是由于增加的氧化应激 (Epel, 2004, 同上）。因此，具有高体质指数、糖尿病和 / 或增加的生活应激的吸烟者和患者将全部受益于根据本公开的方法对其端粒丰度进行的测定和连续监测。

[0150] 2 型糖尿病的特征为较短的端粒 (Salpea, K. 和 Humphries, S. E., Atherosclerosis, 2010, 209(1):35-38)。较短的端粒在 1 型糖尿病患者中也已经被观察到 (Uziel O. 等，Exper. Gerontology, 2007, 42:971-978)。1 型糖尿病中的疾病病原学与 2 型糖尿病略有不同，但是在这两种情况下，β 细胞衰竭是全面爆发的疾病的最终触发因子。端粒长度因此是糖尿病的有用标记物，因为其与疾病的进展相关。Adaikalakoteswari 等 (Atherosclerosis, 2007, 195:83-89) 已经显示，在具有糖尿病前期葡萄糖耐受受损的患者中的端粒与对照相比更短。另外，端粒缩短已经与糖尿病并发症诸如糖尿病性肾病 (Verzola D. 等，Am. J. Physiol., 2008, 295:F 1563-1573)、微量白蛋白尿 (Tentolouris, N. 等，Diabetes Care, 2007, 30:2909-2915) 和上皮癌 (Sampson, M. J. 等，Diabetologia, 2006, 49:1726-1731) 有关联，而端粒缩短似乎在糖尿病得到良好控制的患者中有所减弱 (Uziel, 2007，同上）。本公开的方法特别适用于监测糖尿病前期和糖尿病患者的状况，以提供这些并发症和其它疾病的预警。

[0151] 本公开适用于测定各种类型的癌细胞的端粒长度，因为端粒酶活性的活化与细胞的永生化相关。虽然正常人体细胞不表达或仅短暂表达端粒酶且因此随着每次细胞分裂缩短其端粒，但大多数人癌细胞通常表达高水平的端粒酶并且显示出不受限制的细胞增殖。高端粒酶表达使得细胞长期增殖并扩张且因此支持肿瘤生长 (Roth, A. 等，Small Molecules in Oncology, Recent Results in Cancer Research, U. M. Martens（编）, Springer Verlag, 2010, 第 221-234 页）。较短的端粒与癌症风险显著相关，尤其是膀胱癌和肺癌、与吸烟有关的癌症、消化系统和泌尿生殖系统的癌症。过度的端粒缩短可能在加速肿瘤发作和进展方面起作用 (Ma H. 等，PLo SONE, 2011, 6(6):e20466. doi:10.1371/journal.pone.0020466)。研究已经进一步显示，缩短的端粒对乳癌风险的

影响对于某些群体子组诸如绝经前女性和具有较差抗氧化能力的女性来说是显著的 (Shen J. 等，Int. J. Cancer, 2009, 124:1637-1643)。除了一般评估和监测癌症以外，如果利用源自唾液样本的基因组 DNA，则本公开特别用于监测口腔癌。

[0152] 肝硬化的特征为器官的纤维化渐增，其常常与显著的炎性浸润相关。Wiemann 等 (FASEB Journal, 2002, 16(9):935-982) 已经显示，端粒缩短是人肝硬化的与疾病和年龄无关的征象。端粒缩短存在于由病毒性肝炎（慢性肝炎 A 和 B)、毒性肝损伤（酒精中毒）、自体免疫和胆汁郁积 (PBC 和 PSC) 诱发的硬化中；端粒在与患者年龄无关的硬化中一律很短。端粒缩短和衰老特异性地影响硬化的肝中的肝细胞，并且这两个参数与硬化期间的纤维化的进展强烈相关。因此，本公开的方法在诊断和监测肝纤维化方面得到应用。

[0153] 抑郁已经被比作“加速老龄化”的状态，并且抑郁的个体具有各种老龄化疾病诸如心血管和脑血管疾病、代谢综合症和痴呆的较高发病率。患有复发性抑郁的人或暴露于长期压力的人展现出白血球中的较短端粒。较短的端粒长度与复发性抑郁和指示暴露于长期压力的皮质醇水平均相关 (Wikgren, M. 等，Biol. Psych., 2011, DOI:10.1016/j.bio psych. 2011.09.015)。然而，并不是所有抑郁的个体都显示同等缩短的端粒，因为抑郁发作在一生中有很大变化。长期遭受抑郁的人与对照群体相比具有显著更短的端粒，这是由于他们较长时间暴露于氧化应激和由心理应激诱发的炎症 (Wolkowitz 等，PLos One, 2011, 6(3):e17837)。因此，本公开的方法可以在监测抑郁方面得到应用。

[0154] 慢性感染

[0155] 异常端粒长度与慢性感染有关，其包括 HIV(Effros RB 等，AID S. 1996 年 7 月；10(8):F17-22；Pommier 等，Virology. 1997, 231(1):148-54) 以及 HBV、HCV 和 CMV(Telomere/telomerase dynamics within t he human immune system:effect of chronic infection and stress(Ef fros RB, Exp Gerontol. 2011 年 2 月 -3 月；46(2-3):135-40. Rejuvenation Res. 2011 年 2 月；14(1):45-56. doi:10.1089rej. 2010. 1085. Epub 2010 年 9 月 7 日。)

[0156] 在Harley等(“A natural product telomerase activator as part of a health maintenance program”, Harley CB、Liu W、Blasco M、Vera E、Andrews WH、Briggs LA、Raffaele JM, Rejuvenation Res. 2011 年 2 月；14(1):45-56) 中，发现了 CMV 血清反应阳性的个体相比于 CMV 血清反应阴性的个体具有更短的端粒，而且，CMV 阳性受试者更可能响应于 TA-65 的营养补充方案（由天然产物衍生的端粒酶激活剂连同其它补充物）以降低衰老 CD8+/CD28- 细胞的丰度，这表明了结合施用端粒酶激活剂用于测量平均端粒长度或短端粒丰度的伴随诊断应用。

[0157] 短端粒群体的测量可以被用作预后疾病进展和治疗结果的指标。

[0158] 一项研究报道了 CD4+ 细胞中的端粒长度与患有慢性 HCV 感染的患者中的炎症等级、纤维化阶段、实验室严重度指数、随后的肝功能代偿不全和治疗结果有关 (Hoare 等，J. Hepatol., 2010, 53(2):252-260)。

[0159] 在另一个报道中，较长的白细胞端粒长度预测乙型肝炎病毒相关性肝细胞癌的风险增加 (Liu 等，2011, 117(18):4247-56)。

[0160] 在 HIV 的情况下，端粒缩短是由病毒性感染引起。另外，用于治疗 HIV 的核苷类似物逆转录酶抑制剂是端粒酶抑制剂 (Strahl 和 Blackburn, Mol Cell

Biol.,1996,16(l):53-65;Hukezalie 等，PLoS One,2012,7(11):e47505)。短端粒丰度的测量可能有助于测定 HAART 治疗的副作用和功效。

[0161] 5.2.2 其它病理状况

[0162] 本公开还在诊断与老龄化的早发有关的疾病方面得到了应用。例如，患有早衰病 (Hutchinson Gilford progeria disease) 的个体显示出过早老龄化以及与端粒长度损失有关的成纤维细胞的增殖潜力下降 (Allsopp,R.C. 等，Proc.Natl.Acad.Sci.USA,1992,89:10114-10118)。根据本公开的方法对端粒重复数进行扩增和定量可用于确定疾病风险或预后并采取如上所述的适当干预步骤。

[0163] 5.3 端粒疾病

[0164] 在本公开的一个方面，端粒特异性疾病的存在和进展都可以使用样本来测定。端粒疾病与端粒的异常或过早缩短有关，这可以例如起因于端粒酶活性的缺陷。端粒酶是真核细胞中端粒 DNA 的复制和保护所需要的核糖核蛋白复合物。缺乏端粒酶的细胞经历端粒 DNA 的渐进性损失，这导致活力损失并伴随有基因组不稳定性的增加。这些疾病可能是遗传的并且包括某些形式的先天性再生障碍性贫血，其中骨髓干细胞的细胞分裂不足导致严重贫血。皮肤和肺的某些遗传性疾病也是由端粒酶缺陷引起。对于端粒疾病来说，针对 T/S<0.5 的阈值适于一些病状。此外，常用的度量是针对年龄调整的百分位数端粒得分相对于正常群体小于 <10% 或优选 <1%。

[0165] 先天性角化不良 (DKC)，又称为津 - 恩 - 科三氏综合症 (Zinsser-Engman-Cole syndrome)，是一种罕见的进行性骨髓衰竭综合症，其特征为粘膜皮肤异常：网状皮肤色素沉着过度、指甲营养不良和口腔粘膜白斑病 (Jyonouchi S. 等，Pediatr.Allergy Immunol.,2011,22(3):313-9;Bessler M. 等，Haematologica,2007,92(8):1009-12)。有证据表明这种病症中存在端粒酶功能障碍、核糖体缺乏和蛋白质合成功能障碍。早期死亡常常与骨髓衰竭、感染、致命性肺并发症或恶性肿瘤有关。这种疾病在以下三种类型之一中是遗传的：常染色体显性、常染色体隐性或最常见形式 X 连锁隐性 (其中导致 DC 的基因由 X 染色体携带)。使用本公开的方法对疾病进展进行早期诊断和测量对于具有这些遗传特征的家族而言非常有益，从而使得利用合成代谢类固醇或骨髓刺激药物进行早期治疗可以有助于防止骨髓衰竭。本公开的非侵入性、对患者友好的唾液测试方法特别适用于 DKC，因为婴儿和小孩需要测试和连续监测。

[0166] 特发性间质性肺炎的特征为纤维化和炎症的组合对肺实质的损伤。特发性肺纤维化 (IPF) 是这些疾病的一个实例，其导致肺的渐进性疤痕形成。纤维性疤痕组织在肺中随着时间而累积，影响了其向身体提供足够氧的能力。端粒酶基因 TERT 和 TERC 的编码区中的杂合子突变已经在特发性间质性肺炎的家族性和散发性病例中被发现。带有突变的所有受影响患者都具有短端粒。患有 IPF 的个体中的大部分具有短的端粒长度，这无法由端粒酶中的编码突变来解释 (Cronkhite,J.T. 等，Am.J.Resp.Crit.Care Med.,2008,178:729-737)。因此，端粒缩短可以被用作对这种年龄相关疾病的倾向增加的标志 (Alder,J.K. 等，Proc.Natl.Acad.Sci USA,2008,105(35):13051-13056)。此外，IPF 的过程因人而异。对于一些人来说，这种疾病可以历经多年缓慢和逐渐地进展，而对于其它人来说，其可以快速地进展。本发明的方法可以方便地用于监测肺纤维化的过程并且采取如上所述的适当干预步骤。

[0167] 再生障碍性贫血是其中骨髓停止为身体制造足够的红血球、白血球和血小板的疾病。骨髓所制造的任何血细胞是正常的，但是并不足够。再生障碍性贫血可以是中度、严重或极严重的。患有严重或极严重的再生障碍性贫血的人处于威胁生命的感染或出血的风险下。患有携带端粒酶突变的再生障碍性贫血的患者发展脊髓发育不良的风险增加。端粒酶缺乏可以引起造血干细胞中不同程度的端粒缩短并且导致染色体不稳定和恶性转化 (Calado, R. T. 和 Young, N. S., The Hem atologist, 2010 万维网 URL hematology.org/Publications/Hematologist/2010/4849.aspx)。具有较短端粒的再生障碍性贫血患者具有较低生存率，并且在免疫疗法后比具有较长端粒的那些患者复发的可能性大得多。Scheinberg 等 (JAMA, 2010, 304(12):1358-1364) 发现，复发率随着端粒长度的增加而下降。具有最短端粒的患者组还处于转变成骨髓癌的较高风险下，并且具有最低的总体生存率。本公开的方法可以用于再生障碍性贫血患者中以监测发展主要并发症的风险，使得可以相应地调整个体的临床管理。

[0168] 5.4 药物响应性

[0169] 在另一个方面，本公开可用于监测治疗剂的有效性或筛选影响端粒长度或端粒酶活性的候选药物。监测端粒特征的能力可以提供用于检查特定疗法和药理学药剂的有效性的窗口。个体中的疾病状态对特定疗法的药物响应性可以通过本公开的方法来测定。例如，本公开在监测癌症疗法的有效性方面得到应用，因为细胞的增殖潜力与端粒完整性的维持有关。如上所述，虽然正常人类体细胞不表达或仅短暂表达端粒酶且因此随着每次细胞分裂缩短其端粒，但大多数人癌细胞通常表达高水平的端粒酶并且显示出不受限制的细胞增殖。Roth 等（同上，2010）已经提出，在肿瘤中具有极短端粒（其中大多数细胞中的最短端粒接近于端粒功能障碍）且具有高端粒酶活性的癌症个体可能最得益于抗癌端粒酶抑制药物。因为端粒酶在大多数正常细胞中不被表达或被短暂表达并且处于极低水平下，所以端粒酶抑制疗法相比常规的化学疗法可能对正常细胞的毒性更小。此类药物的一个实例是基于短寡核苷酸的端粒酶抑制剂伊美司他（先前名为GRN163L）。伊美司他是第一代寡核苷酸 GRN163 的新型基于脂质的缀合物 (Asai, A. 等，Cancer Res., 2003, 63:3931-3939)。然而，在正常血细胞（特别是粒细胞）中具有极短端粒的癌症患者处于伊美司他对增生组织诸如骨髓产生不良影响的较高风险下。Rattain 等 (2008) 发现，具有短粒细胞端粒长度的此类受试者更可能患上骨髓衰竭综合症，诸如中性粒细胞减少症或血小板减少症。在这种情况下，医生可能开出更低剂量的伊美司他、不同的药物，或更频繁地监测骨髓问题。

[0170] 在其它方面，药物功效在于治疗老龄化疾病，例如但不限于心血管疾病、糖尿病、肺纤维化、肝纤维化、间质性肺炎和抑郁。在心血管疾病的情况下，Brouilette 等报告，相比对照组具有更短端粒长度的中年男性最得益于使用普伐他汀 (pravastatin) 的降脂疗法 (Brouilette, S. W. 等，Lancet, 2007, 369:107-114)。Satoh 等 (Clin. Sci., 2009, 116:827-835) 指示，当与经温和普伐他汀疗法治疗的患者相比，经阿托伐他汀 (atorvastatin) 治疗的患者中集中降脂疗法更好地保护端粒免遭侵蚀。本公开的方法可以用于监测他汀类在经治疗的患者中的功效，其中较短的端粒长度与较好的药物功效相关联。因为具有最长端粒的受试者平均来说未得益于预防性他汀类，所以医生可能建议患者要尤其顺应良好的生活方式习惯以作为其治疗方案的一部分。相反地，担心长期使用他汀类的副作用的具有短端粒的患者可能基于其得益于他汀类的较高可能性而被说服服用

他汀类。可以使用的他汀类的实例包括烟酸(ADVICOR、SIMCOR)、洛伐他汀(lovastatin)(ALTOPREV、M EVACOR)、氨氯地平(amolopidine)(CADUET)、罗苏伐他汀(rosuvasta tin)(CRESTOR)、西他列汀/辛伐他汀(sitagliptin/simvastatin)(JUVISY NC)、氟伐他汀(fluvastatin)(LESCOL)、普伐他汀(PRAVACHOL)、阿托伐他汀(LIPITOR)、匹伐他汀(pitavastatin)(LIVALO)和依泽替米贝/辛伐他汀(ezetimibe/simvastatin)(VYTORIN)。

[0171] 在其它方面,可以测量在端粒疾病(例如但不限于先天性角化不良、肺纤维化和再生障碍性贫血)治疗中的药物有效性。例如,先天性角化不良和肺纤维化都用高剂量的类固醇来治疗,众所周知,这些类固醇具有众多有害的副作用。因此非常期望使用最低可能的类固醇剂量,这使得本公开的方法成为用于监测这些患者的有价值工具。

[0172] 5.5 候选药物筛选

[0173] 在另一方面,本公开被用作筛选影响调节端粒长度(诸如端粒酶活性)的生物途径的候选药物、膳食补充剂和其它干预(包括生活方式改变)的一般方法。以定量方式快速和特异性地扩增端粒重复的能力提供了一种用于鉴定影响细胞中的端粒动力学的小分子、候选核酸和肽剂及其它产品或干预的高通量筛选法。对正常细胞具有正向端粒延长作用的候选药物或其它候选产品在治疗退行性病状或细胞衰老相关病状方面将优于具有端粒缩短(或端粒酶抑制)作用而其它方面都等同的那些药物或产品。在治疗癌症的情况下,尤其在癌细胞中具有负向的端粒缩短作用的药物将是优选的。

[0174] 实施例

[0175] 实施例1-短端粒序列的扩增

[0176] 在此,我们描述了一种利用纯化基因组DNA来测量短端粒的百分比的基于定量PCR的方法。这种方法包括将非人引物'TeloPrimer'非共价结合至脊椎动物基因组DNA的3'-突出,使用在延伸反应期间置换或降解端粒C链的酶使TeloPrimer向亚端粒单一序列'SUS'进行时控延伸,随后利用SUS及TeloPrimer的非人序列部分进行扩增反应。只有足够短以使得延伸的TeloPrimer产物到达SUS序列的端粒才被扩增。因此,控制链置换反应的时间允许检测短于指定端粒长度阈值的端粒。另外,通过控制以SUS和TeloAnchor引物进行的PCR步骤的延伸时间,短端粒群体将被选择性扩增,从而进一步提高这种测定测量短端粒的特异性。在TeloTest的T-运行中定量短端粒的丰度。

[0177] 材料与方法

[0178] 引物

[0179] 所有引物都是以标准脱盐形式或HPLC纯化形式购自Integrated DNA Technologies。下面列出这些引物的序列:

[0180] TeloPrimer:5'-TGCTCGGCCGATCTGGCATCCCTAACC-3'[SEQ ID NO:7]

[0181] TeloAnchor:5'-TGCTCGGCCGATCTGGCATC-3'[SEQ ID NO:8]

[0182] SUS(HPLC纯化):5'-GATGGATCCTGAGGGTGAGGGTGAGG G-3'[SEQ ID NO:2]

[0183] TeloProbe(HPLC纯化):5'-CCCTAACCCTAACCCTAACCCTAA-3'[SEQ ID NO:9]

[0184] 使TeloPrimer与基因组DNA退火

[0185] 将人基因组DNA与TeloPrimer以20ng/ul DNA和1uM TeloPrimer的最终浓度混合在50ul反应中,在65℃下于加热器中孵育10分钟,且然后历时一小时时间冷却至室温。将退火的样本保持在冰上,直到进行链置换反应。

[0186] 链置换

[0187] 使用50ng退火DNA、40mM Tris-HCl(pH 8.0)、10mM MgCl2、50mM NaCl、5mM DTT(PN 70726,Affymetrix,Santa Clara,CA,USA)、100ug/ml BSA(目录号B9001S,New England Biolabs,Ipswich,MA)、500uM dNTP(目录号1708874,Bio-Rad,Hercules,CA)、5uM单链结合蛋白(SSB,目录号70032Y 100UG,Affymetrix,Santa Clara,CA)和400nM测序酶2.0(目录号70775Y 200UN,Affymetrix,Santa Clara,CA)(测序酶浓度为13U/ul),以5ul体积进行链置换反应。不含测序酶2.0的混合物在37℃下预热一分钟,且然后在向混合物中添加测序酶2.0后在37℃下孵育30秒至10分钟。通过在80℃下热钝化20分钟使反应停止。

[0188] 链置换产物的PCR扩增

[0189] 将链置换DNA反应的产物在DNA悬浮缓冲液(目录号T0227,Teknova,Hollister,CA)中稀释50倍。以40ul反应体积进行PCR,该体积包含1.5mM MgCl2、300uM dNTP(目录号1708874,Bio-Rad,Hercules,CA)、0.5uM SUS引物、0.5uM TeloAnchor引物、2ul稀释DNA、5单位PLATINUM Taq聚合酶(目录号10966-083,Life Technologies,Grand Island,NY)。

[0190] 循环程序如下:在94℃下2分钟;在94℃下15秒,在65℃下30秒、在72℃下30秒至10分钟,然后在72℃下延伸10分钟,循环35次。在Bio-Rad C1000热循环仪(Bio-Rad C1000Thermocycler)(Bio-Rad,Hercules,CA)中进行PCR反应。

[0191] 通过TeloTest对扩增的PCR产物进行定量

[0192] 将PCR产物在DNA悬浮缓冲液中稀释10至1000倍。利用TELOTEST的T-运行对PCR产物进行定量。使用含有60-600bp之间的完整端粒重复的PCR片段作为标准物来计算每个样本的DNA浓度。从置换反应到qPCR步骤,每个样本中将包括具有TTAGGG 3'突出和靠近5'的SUS序列的约2kb DNA片段内标物。每个样本的反应效率将通过内标物中的非人序列的qPCR来估算。针对每个样本的归一化因子将基于反应效率而生成。

[0193] DNA印迹

[0194] 进行DNA印迹分析,以确定置换的端粒DNA的大小。使通过SUS和TeloAnchor引物扩增得到的链置换产物在0.5%琼脂糖凝胶上电泳,转移至带正电荷的尼龙膜上,并在37℃下与具有四个TTAGGG重复的DIG标记的寡核苷酸探针杂交过夜。使用化学发光检测来检测信号。

[0195] 结果

[0196] 总体方案

[0197] 本文所描述的短端粒测定(STA)通过链置换或链降解以及随后的定量PCR来定量人基因组DNA样本中的短端粒的量。这种测定的总体方案示于图1中。

[0198] 这种测定通过首先使TeloPrimer与人基因组DNA退火来进行。TeloPrimer在5'端包含19个核苷酸的非人序列,且在3'端包含8个核苷酸的端粒序列。利用天然基因组DNA确保了TeloPrimer将只会与端粒的3'单链尾部(突出)退火,而不与双链区域退火。TeloPrimer的延伸是通过链置换或链降解反应实现。由于有限的时间,反应将只能够到达那些具有短端粒的染色体上的端粒变异区,而在具有长端粒的染色体上,链置换产物将止于完整端粒重复区域内。然后通过使用SUS引物和TeloAnchor引物进行PCR使置换的短端粒富集。SUS引物包含3个重复的TGAGGG序列,这被发现在大多数染色体上的位于或靠近

亚端粒区与真正端粒重复之间的接合处的端粒变异区中。TeloAnchor 引物与 TeloPrimer 共用相同的非人序列并且不含端粒序列。将 SUS 与 TeloAnchor 对设计成特异性地扩增链置换短端粒。最后，利用 TELOTEST T- 运行定量短端粒的量。

[0199] 链置换产物的定量

[0200] 链置换扩增是 DNA 合成反应，其中所遇到的下游 DNA 在合成期间被聚合酶置换。包括全基因组扩增的许多方案和法医分析使用链置换来扩增极少量的模板 DNA。由于 DNA 聚合酶具有链置换活性，具有缺口 (nick) 或单链尾部的 DNA 可以用作模板，而无需变性步骤。我们选择在实验设计中使用链置换酶，因为端粒的单链尾部充当链置换酶的天然模板，且相比 5'-3' 外切核酸酶（降解酶）不太可能在基因组中产生可能引起中间端粒样序列的非特异性扩增的缺口。将 TELOPRIMER 与天然基因组 DNA 上的端粒的单链尾部退火使得 TeloPrimer 邻近 C 链的 5' 末端（图 1）。在这个设计中，链置换产物的长度将准确地反映端粒长度。通过控制链置换时间，反应将只能够到达那些具有短端粒的染色体上的端粒变异区。我们选择使用测序酶 2.0（一种没有外切核酸酶活性的 T7DNA 聚合酶的基因工程化形式），因为据报道，它在线性模板上以高特异性和持续合成性合成 DNA(Joneja,A. 和 X.Huang,Anal Biochem,2011.414(1)58-69)。我们进行时程实验，其中链置换反应耗时 30 秒至 5 分钟。将来自膀胱癌细胞系 UM-UC3 的基因组 DNA 与 TeloPrimer 退火，并且如材料与方法中所详述进行链置换反应。

[0201] 图 2 示出了端粒产物随着链置换反应时间的增加而增加，端粒产物是通过 TELOTEST qPCR 的 T- 运行测量。线性回归线显示时间与扩增子丰度之间的强烈关系 (R^2 = 0.99)。此外，在未添加测序酶 2.0 的阴性对照中，未在 TELOTEST T- 运行中检测到 PCR 产物（交叉点 (Cp) = 25，计算浓度 = 1.85E-07），这比含测序酶 2.0 的反应的最低测量浓度低 159 倍。在 TELOTEST S- 运行中利用单拷贝基因（β- 球蛋白）引物的平行反应也没有显示扩增产物，这进一步证实了 T- 运行中测量的端粒产物衍生自链置换反应，而不是全基因组 DNA。

[0202] 我们在此提出的实验设计预测，在短的置换反应时间下，利用 SUS 和 TeloAnchor 引物将只扩增来自短端粒的产物。我们试图通过 DNA 印迹分析证实该预测。使通过 SUS 和 TeloAnchor 引物扩增得到的链置换产物在 0.5％琼脂糖凝胶上进行电泳，转移至带正电荷的尼龙膜上，并与具有四个 TTAGGG 重复的 DIG 标记的寡核苷酸探针杂交。图 3 显示，扩增的 PCR 产物的长度随着链置换时间的增加而增加。使用 0.5 分钟、1 分钟、3 分钟和 5 分钟的置换时间，PCR 产物的估算众数（峰强度）分别为近似 0.6kb、0.9kb、1.2 和 1.4kb。使用 1 分钟的置换时间，大多数产物低于 2kb。在独立的比较试验中，我们使用改良的单端粒延伸长度分析 (STELA)(Baird,D.M. 等，Nat Genet.,2003.33(2)203-7) 方案来分析来自相同癌症细胞系 UM-UC3 的全基因组 DNA 的端粒长度分布。将 TeloPrimer 连接至 C 链的 5' 末端，并且使用 XpYpE2 和 TeloAnchor 引物来扩增端粒。使 PCR 产物在凝胶上进行电泳（图 3 中第 1 泳道）。这表明 UM-UC3 端粒的众数为约 1.8kb，与先前的报道 (Xu,L. 和 E.H.Blackburn,Mol Cell,2007.28(2)315-27) 一致。

[0203] 为了进一步验证短端粒测定，我们用两种不同的基因组 DNA 样本进行这种测定。除 UM-UC3DNA 以外，我们使用感染了慢病毒载体的 UM-UC3 的基因组 DNA，该慢病毒载体表达端粒酶的 RNA 基因 hTER，从而延伸端粒 (Xu,L. 和 E.H.Blackburn,Mol

Cell,2007.28(2)315-27)。通过 qPCR 测量的 UM-UC3/hTER 中的平均端粒长度为 2.1,与之相比,UM-UC3 为 0.56(Telome Health Inc. 数据)。如通过 DNA 印迹分析(图 5)所判断,UM-UC3/hTER 细胞具有较低的短端粒百分比。与此一致的是,UM-UC3 中的短端粒的计算量比 UM-UC3/hTER 高 3 倍(图 4)。

[0204] 我们的结论是,短端粒测定特异性地测量短端粒的相对百分比。此外,通过控制链置换反应时间,将在这个测定中测量的端粒长度截止值可以变化,且可以预定。

[0205] 上述短端粒测定可以容易地适于高通量自动化模式。图 6 示出了这种模式中的个别步骤。

[0206] 尽管本文中已经显示和描述了本发明的优选方面,但对于本领域技术人员将显而易见的是,这些方面仅作为实例而提供。众多变更、变化和替代在不脱离本公开的情况下现在将被本领域技术人员所想到。应该理解,在实施本发明时可以采用本文所描述的本发明的方面的各种替代物。希望以下权利要求限定本发明的范围并且在这些权利要求和其等效物的范围内的方法和结构由此被涵盖在内。

[0001]

序列表

<110> Telome Health, Inc.
Harley, Calvin
Lin, Jue
Hu, Yajing

<120> 短端粒丰度的量度

<130> 37502.0001P1-

<160> 14

<170> PatentIn 3.5 版

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[0003]

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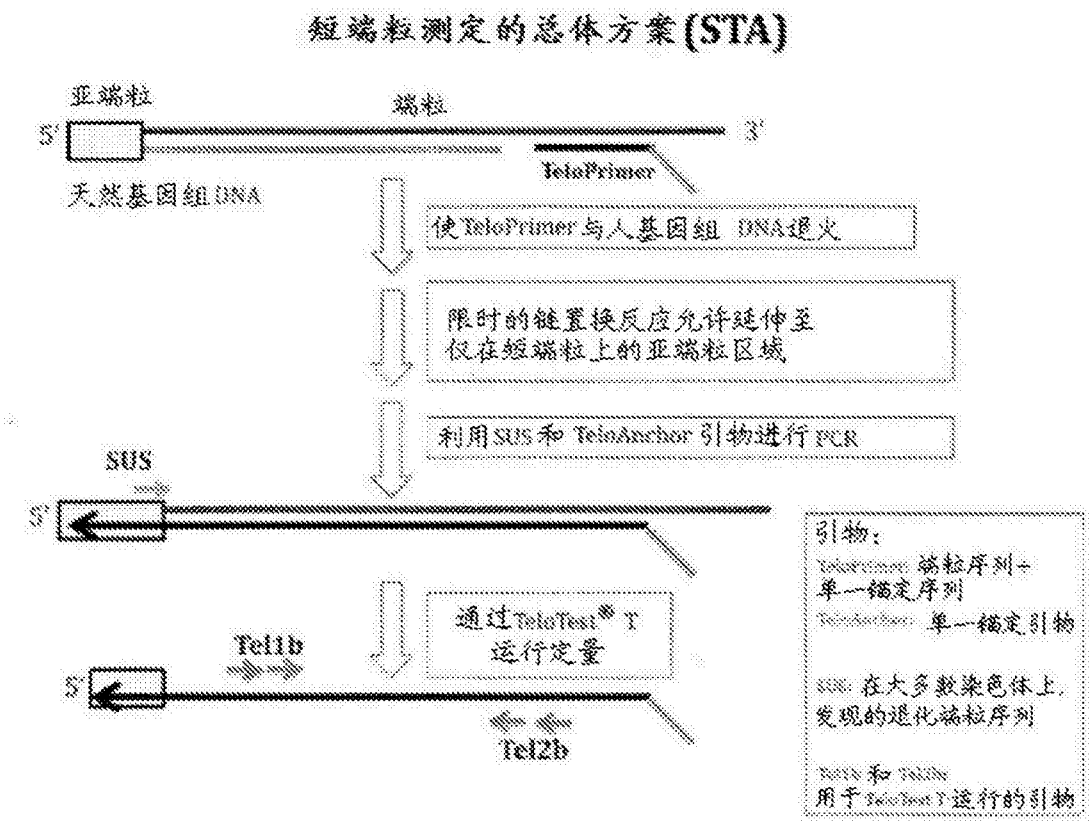
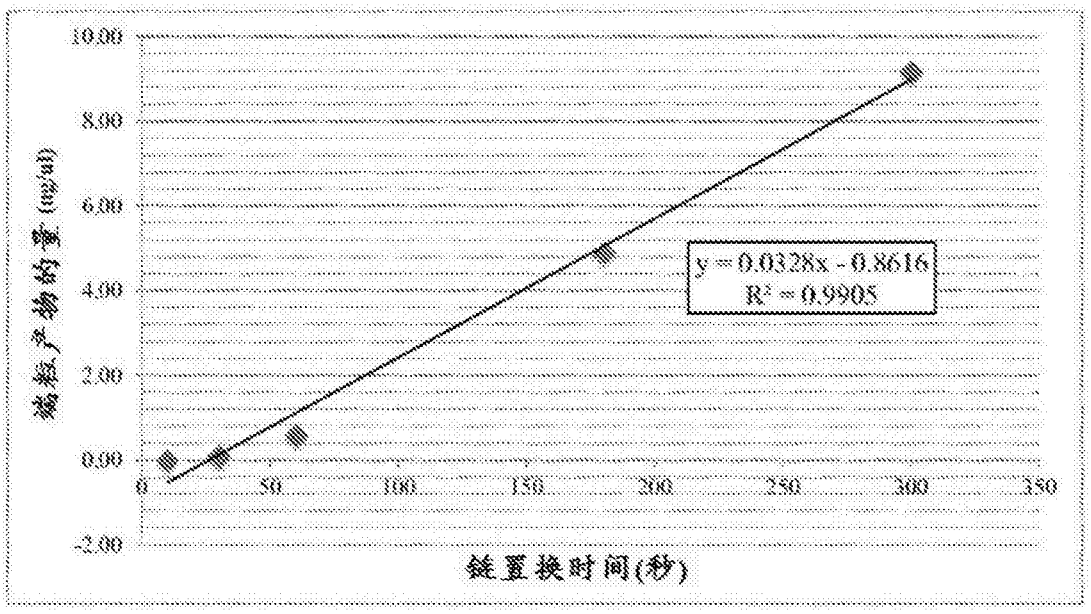

短端粒测定的总体方案(STA)
亚端粒
端粒
5'
3'
TeloPrimer
天然基因组DNA
使TeloPrimer与人基因组 DNA退火
限时的链置换反应允许延伸至
仅在短端粒上的亚端粒区域
利用SUS和 TeloAnchor 引物进行 PCR
SUS
5'
引物：
端粒序列+
单一锚定序列
单一锚定引物
在大多数染色体上，
发现的退化端粒序列
和
用于 运行的引物
通过TeloTest® T
运行定量
Tel1b
5'
Tel2b

图 1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8.00
6.00
4.00
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端粒产物的量 (ng/ul)
y = 0.0328x - 0.8616
R² = 0.9905
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50
100
150
200
250
300
350
链置换时间(秒)

图 2

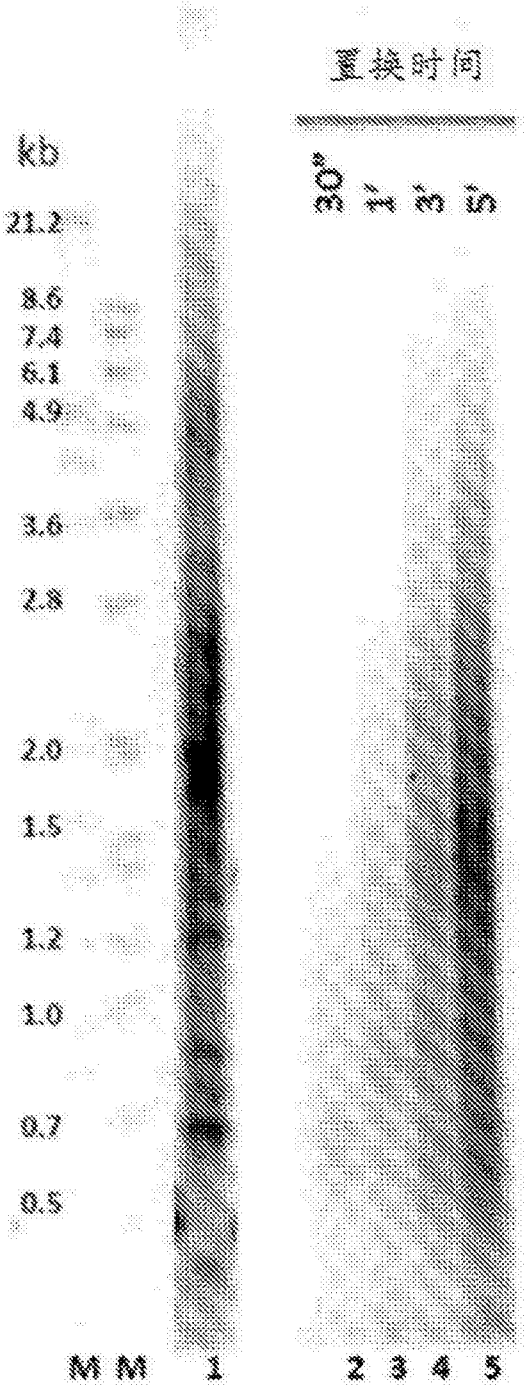

图 3

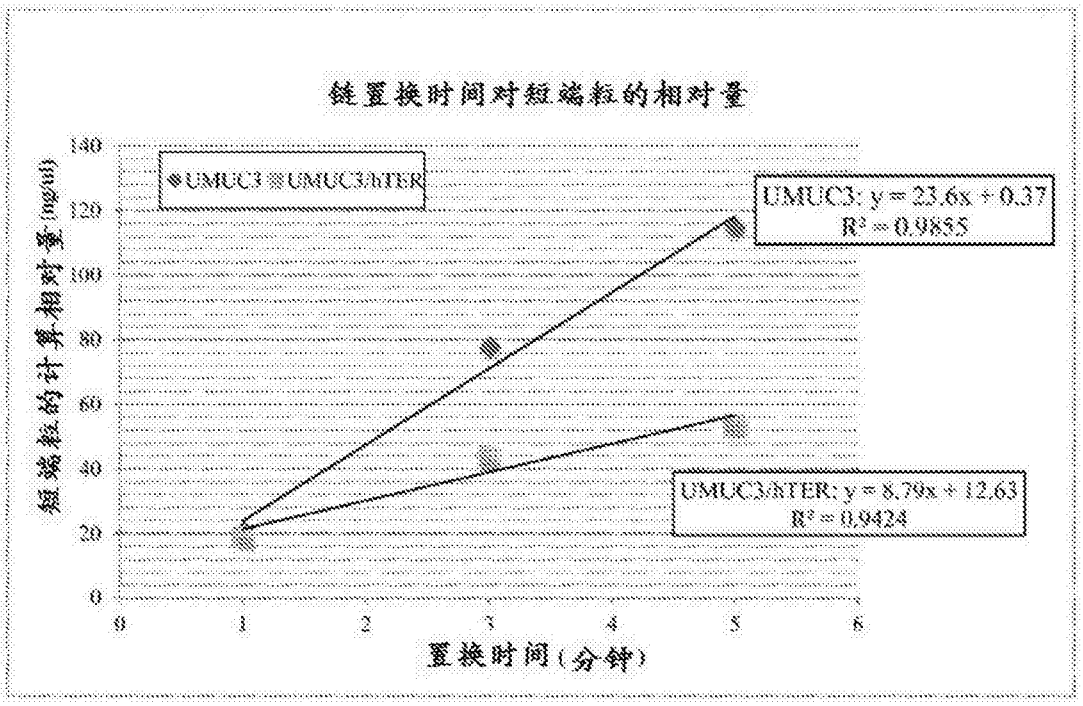

图 4

短端粒的百分比	UMUC3	UMUC3/hTER
DNA 印迹 (0.2-5Kb)	75%	26%
短端粒测定	100%	33%

图 5

步骤	描述	自动化或手动
退火	1.1. 用移液管吸取 gDNA 并与TeloPrimer 和水混合	自动
	1.2. 在 65 °C 下孵育10分钟，缓慢冷却	
链置换	2.1. 用移液管吸取具有测序酶 2.0/SSB/ 缓冲剂的主体混合物(master mix)	自动
	2.2. 用移液管吸取 gDNA 并混合	自动
	2.3. 密封平板	手动
	2.4. 在37 °C 下孵育 3 分钟	手动
	2.5. 将平板转移至 80 °C 下，保持20 分钟	手动
利用 SUS和 TELOANCHOR进行 PCR	3.1. 用低 EDTA Tris 稀释来自步骤 2.5 的样本	自动
	3.2. 用移液管将PCR主体混合物吸取到新的96孔板中	自动
	3.3. 用移液管将来自步骤 3.1 的稀释 DNA 吸取到来自步骤 3.2 的96孔板中	自动
	3.4. 密封两个板	手动
	3.5. 将PCR板转移至热循环仪中	手动
	3.6. PCR	
TELOTEST	4.1. 用低 EDTA Tris稀释来自步骤3.6 的PCR产物	自动
	4.2. 利用来自 4.1 的稀释样本进行 TeloTest® 的T-运行	自动

图 6

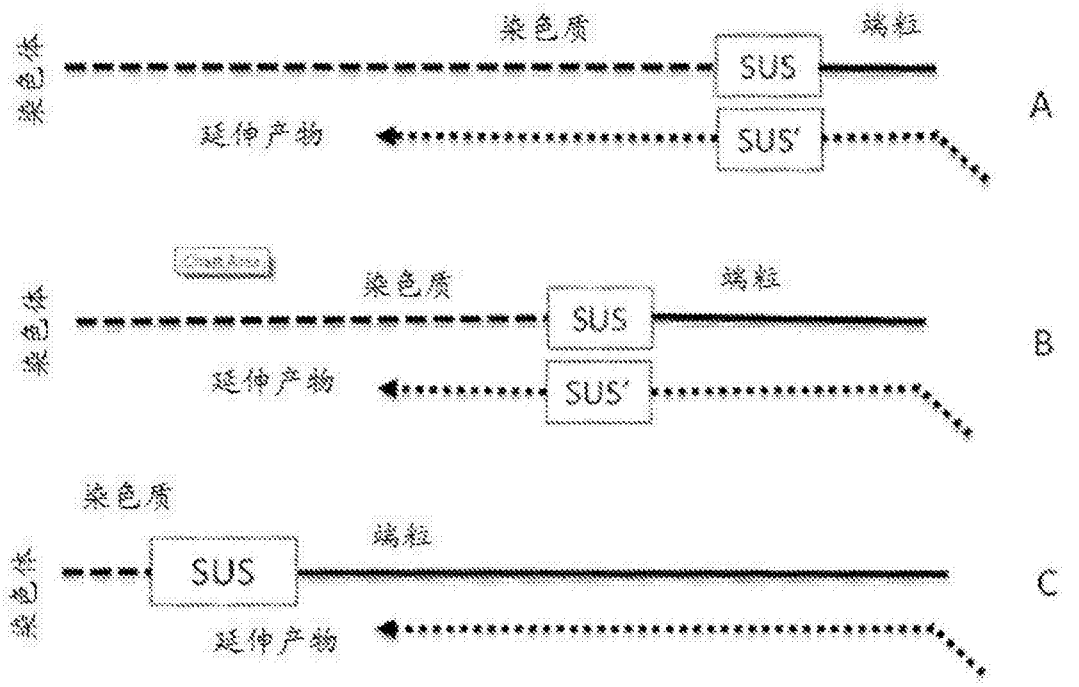
染色质
端粒
SUS
SUS'
延伸产物
A
染色质
端粒
SUS
SUS'
延伸产物
B
染色质
SUS
端粒
延伸产物
C

图 7A

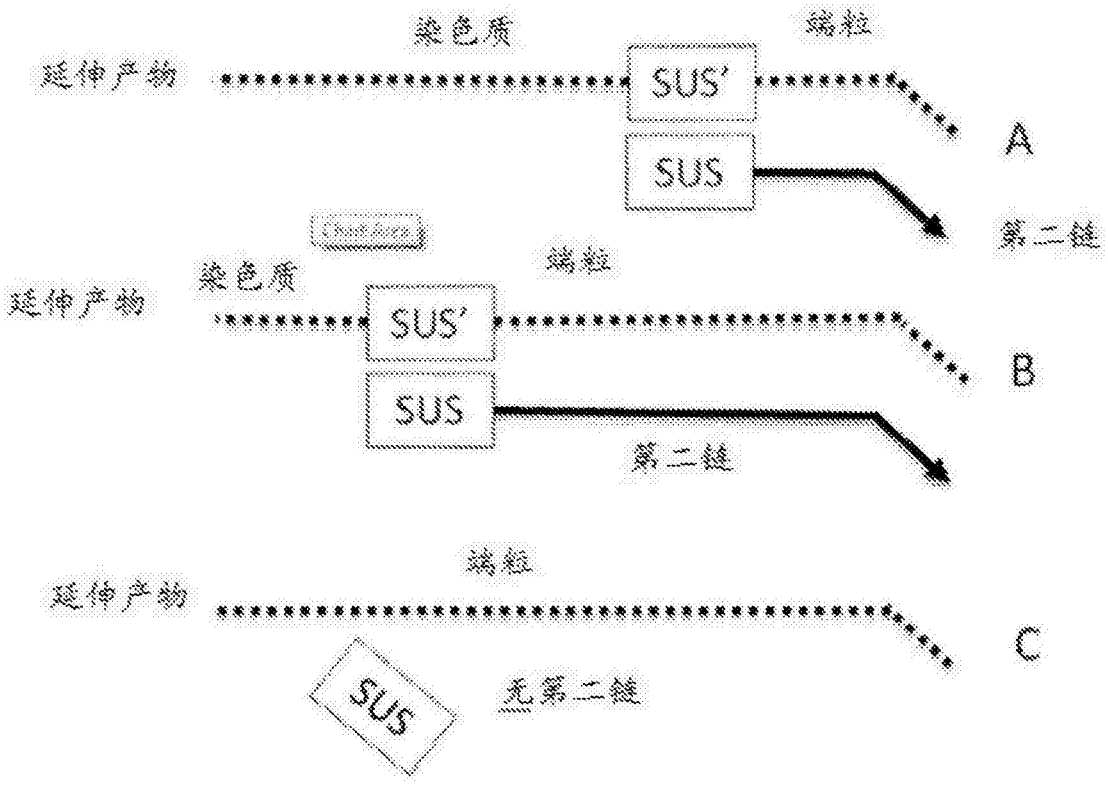
染色质
端粒
延伸产物
SUS'
SUS
A
第二链
染色质
端粒
延伸产物
SUS'
SUS
B
第二链
端粒
延伸产物
C
SUS
无第二链

图 7B