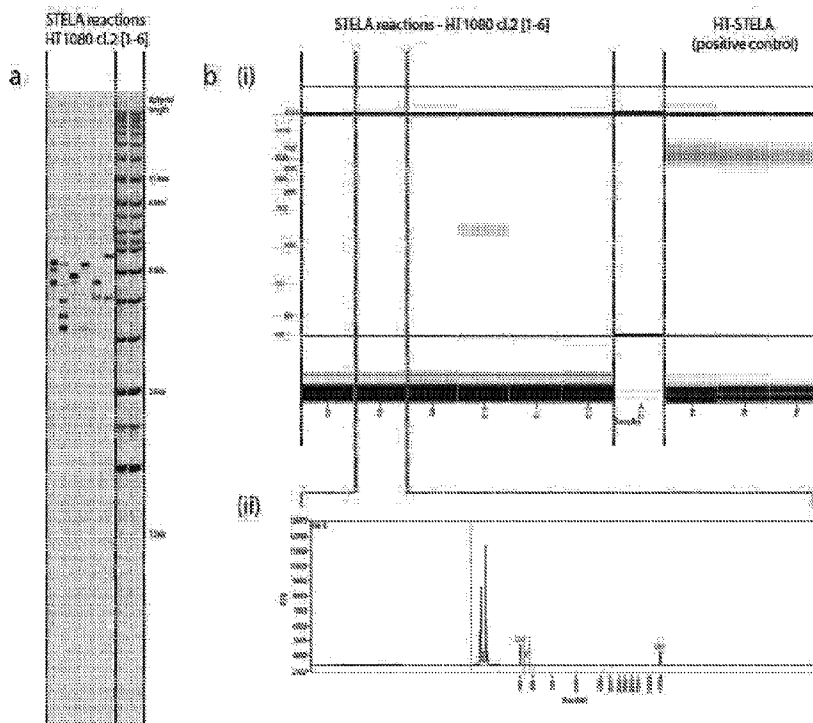




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(54) **Titre : SEQUENCAGE A CADENCE ELEVEE**
(54) **Title: HIGH THROUGHPUT SEQUENCING**



(57) **Abrégé/Abstract:**

The invention relates to a high throughput method for determining telomere length of mammalian chromosomal DNA; primers for use in said method; a kit comprising said primers; use of said method to diagnose or prognose or to determine the risk of

(57) Abrégé(suite)/Abstract(continued):

developing a telomere shortening disease such as cancer, ageing, neurological disorders including Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyeraal-Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis. Additionally, the invention also has application in assessing an individual's suitability to be a transplantation donor, for example a bone marrow donor.

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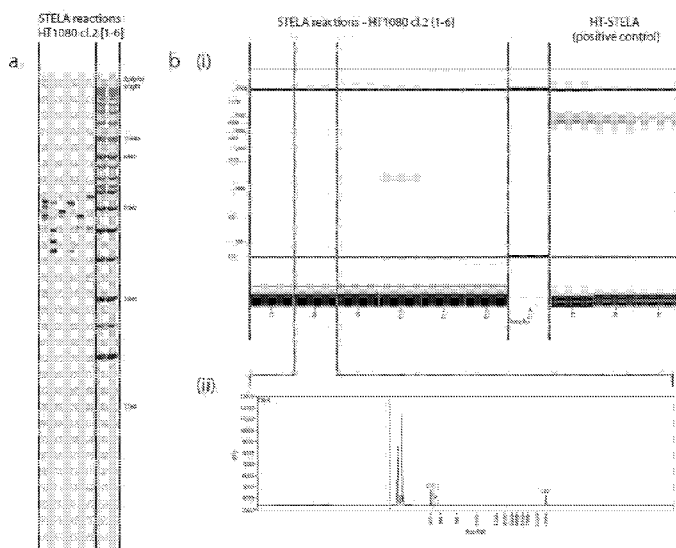


Figure-1.

(57) Abstract: The invention relates to a high throughput method for determining telomere length of mammalian chromosomal DNA; primers for use in said method; a kit comprising said primers; use of said method to diagnose or prognose or to determine the risk of developing a telomere shortening disease such as cancer, ageing, neurological disorders including Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyerall-Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis. Additionally, the invention also has application in assessing an individual's suitability to be a transplantation donor, for example a bone marrow donor.

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High throughput sequencing

The invention relates to a high throughput method for determining telomere length of mammalian chromosomal DNA; primers for use in said method; a kit comprising said primers; use of said method to diagnose or prognose or to determine the risk of developing a telomere shortening disease such as cancer, ageing, neurological disorders including Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyeraal-Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis. Additionally, the invention also has application in assessing an individual's suitability to be a transplantation donor, for example a bone marrow donor.

Background of Invention

Telomeres are nucleoprotein structures composed of repetitive DNA sequences (up to 25kb of predominantly the tandem repeat sequence TTAGGG, termed the telomere repeat array) that cap the ends of linear eukaryotic chromosomes, protecting them from deterioration or fusion with adjacent chromosomes. In addition to TTAGGG repeats, the proximal 1-2kb of human telomere repeat arrays also contain telomere variant repeats (TVRs) that include amongst others TCAGGG and TGAGGG (e.g. Baird et al EMBO 1995 and Letsolo et al NAR 2010). During DNA replication telomere sequences are lost from the chromosome ends. The enzyme telomerase compensates for this by synthesizing new telomere repeats but in somatic cells this enzyme is inactive and so, over time, the telomere shortens. Telomere ends are, however, maintained in certain cell types such as germ cells, stem cells and certain white blood cells, by telomerase catalysing the RNA templated addition of telomere repeats.

Telomere length is a key determinant of telomeric function and it has been shown that short dysfunctional telomeres can drive genomic instability and tumourigenesis in

mouse models. Furthermore, deregulation of telomerase has been shown to drive oncogenesis. Additionally, the loss of telomeres in somatic cells has been linked to replicative senescence which typically would provide genomic stability preventing cancer. Conversely, it has also been shown that malignant cells can bypass this senescence and become immortalised by the aberrant activation of telomerase. Numerous diseases are known to exist as a consequence of genetic abnormalities and, specifically telomere shortening. These diseases include Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyeraal–Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis. Additionally, a determination of telomere length has application in assessing an individual's suitability to be a transplantation donor, for example a bone marrow donor.

Consistent with the role of telomere biology in tumour progression, there is now a substantial body of evidence indicating that telomere length can provide prognostic information in many human malignancies including chronic lymphoid leukemia (CLL). However, there is a lack of resolution in the currently available technologies for detecting telomere length and this has hampered progress in translating telomeric assays into clinical practice. For example, a putative role of telomere dysfunction during the progression of breast cancer has been shown, and low-resolution telomere length analysis has been shown to provide limited prognostic information. A key problem with these technologies is that they are based on hybridisation of DNA probes to telomere repeat units. Consequently, as telomeres get shorter there is less probe target, and this means the shortened telomeres are not detectable. This is significant because it is the shortened telomeres that become dysfunctional and are subject to fusion, causing the genomic instability that can drive the progression of human cancers. Q-PCR-based methods have also been described for the estimation of telomere repeat content (WO 2004068110US). These methods allow for high

throughput analysis however their linearity for the detection of short telomeres (< 4 kb) has not been established¹⁸. This, coupled with the reported high co-efficient of variance (CV) values of up to 28%, renders Q-PCR methods inappropriate for the detection of short telomeres and so unreliable as a prognostic tool for clinical decision making¹⁹. In summary, hitherto, telomere analysis using existing low-resolution techniques is not a sufficiently informative prognostic tool.

To address this problem, we have previously developed single-molecule technologies that allow us to detect the presence of critically shortened telomeres^{20,21} and to characterise telomere end-end fusions^{16,17}. Single telomere length analysis (STELA) allows complete resolution of telomere lengths at specific chromosome ends, including telomere lengths at which telomere end-end fusions can occur^{16,20}. It therefore permits detection of short telomeres that are potentially dysfunctional and capable of fusion; these telomeres cannot reliably be detected with any other method.

Using STELA and telomere fusion analysis^{22, 23} we have defined the length of telomeres in tumour cells at which telomere fusion can be detected^{24, 25}. We have used STELA together with a fusogenic threshold to stratify patients with CLL based on telomere length. These data show that telomere lengths below the fusogenic threshold are highly prognostic and that the mean of the fusogenic range (2.26 kb) provides an optimum prognostic resolution. Telomere length below the fusion threshold was the most powerful predictor of survival in CLL and this was particularly prognostic in early-stage patients²⁴. Telomere dysfunction has been implicated in the progression of many tumour types and we have now established that the same thresholds are prognostic in breast cancer and myelodysplastic syndromes . We therefore consider that our threshold may provide clinical utility in many different tumour types.

Given the prognostic significance of our findings, we sort to establish robust high throughput methods, to determine telomere lengths. The complexity of the original STELA method makes it labour intensive and time consuming. Furthermore it utilises Southern hybridisation with radioactively labelled DNA probes, which render the method low throughput. The original STELA method is therefore most suitable for the research laboratory environment where low throughput, but high resolution, is

required. However it is not suited for routine high throughput assessment of cancer patients.

To resolve these issues we herein disclose a novel variation of the STELA method - High Throughput STELA (HT-STELA). Like the original STELA method, HT-STELA is a PCR based technique that requires a linker oligonucleotide and amplifies specific human telomeres. However, it differs in that the resolution and detection of telomere length distributions is not achieved through gel electrophoresis and Southern hybridisation. Instead, the PCR products generated during the STELA PCR are resolved via capillary electrophoresis (using a Fragment Analyzer™ Advanced Analytical) and detected by a camera that monitors the incorporation of a fluorescent intercalating dye into the PCR product. This altered resolution and detection step significantly decreases the amount of time taken to generate a telomere length measurement. However, the use of these different resolution and detection techniques require process modification if they are to work with the requisite sensitivity needed to reliably measure telomere length, thus, a number of steps have been devised to ensure the sensitivity of the method.

Statements of Invention

According to a first aspect of the invention there is provided a high throughput method for determining telomere length of mammalian chromosomal DNA comprising:

- i) annealing a primer sequence to a region adjacent a telomere repeat array of chromosomal DNA wherein said region is between 3843bp-30bp from said telomere repeat array;
- ii) PCR amplifying 20ng-35ng of said chromosomal DNA using 21-23 cycles to generate an amplification product; and
- iii) detecting the length of the amplification product.

As mentioned, the telomere is a region of repetitive nucleotide sequences predominantly composed of (TTAGGG)_n at each end of a chromosome, thus reference to a region 3843bp-30bp from said telomere repeat array is reference to a region upstream from the start of the array (characterized predominantly by TTAGGG repeats) that caps the chromosome end.

Surprisingly, we have discovered that the method works most effectively if the method produces, for analysis, a telomere length distribution 'smear' as opposed to a banding pattern. Through subsequent deduction we have established that there are a number of reasons why a telomere length distribution smear is beneficial: it allows a more rapid analysis of mean telomere length as it requires less human intervention in the analyses of data within the (PROSize™) software; it requires less reactions per sample; it increases the total amount of product and it ensures that non-specific background bands present are not incorporated into the sizing analysis. To achieve this telomere length distribution 'smear' we have discovered that we need to use an optimum amount of DNA and an optimum number of PCR cycles. With these two parameters defined we find we can reliably produce a telomere length distribution 'smear' for subsequent analysis. A typical telomere length distribution band is shown in Figure 1a, in contrast a typical telomere length distribution smear is shown in Figure 4.

Moreover, we have come to realize that these optimized parameters are consistent with specific amplification of the telomere length distributions that are representative of telomere shortening. Thus the selection of an optimised cycle number and amount of DNA provides a better representation of the telomere length distributions. Indeed, an optimised PCR cycle number results in less artefactual amplification, less heterogeneity and so a more accurate representation of the telomere length distribution.

Using a fluorescent signal or label which intercalates into the PCR amplification product we have discovered that PCR cycle numbers 21 and 23 produce a robust signal (between 27-65 RFU) and a reliable estimation of telomere length (fig. 11) in a system where the lowest signal to provide a reliable telomere length estimate was 25 RFUs or 25% (Tel Peak area LM peak area, where Tel Peak area is the area under the curve of the telomere-specific products, and the LM area is the area under the curve for the lower marker). Both are determined using the PROSize™ software. Most ideally, PCR cycle number of 23 is used.

In yet a further preferred method of the invention the amount of chromosomal DNA used for PCR amplification is between 25-30ng.

More ideally the amount of chromosomal DNA used for PCR amplification is selected from the group comprising: 25ng, 26ng, 27ng, 28ng, 29ng and 30ng. Most ideally, 25ng of chromosomal DNA are used and 22 PCR cycles are executed to provide the amplification product. More preferably still, 30ng of chromosomal DNA was subjected to 23 PCR cycles.

In yet a further preferred method of the invention said primer is provided at a concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 μ M, but most ideally at 0.1 or 0.2 μ M.

In yet a further preferred method of the invention, said chromosomal DNA is extracted from at least one cell, and ideally a plurality of cells, using an automated extraction method with a view to providing an equal amount of DNA into the PCR reaction each time the reaction is undertaken. An example of a suitable DNA extraction system is the Promega® DNA IQ™ system. Using this system, DNA was extracted from cell suspensions and subsequently diluted to the single molecule level to provide an equal number of amplifiable molecules with the original STELA method. We then further optimized the method to ensure the production of a telomere length distribution smear for use in HT-STELA, this was done by determining the optimal elution volumes from the Promega® DNA IQ™ system and subsequent volume of DNA solution added to the HT-STELA reactions. It was found that this provided a reliable telomere length distribution smear without the need to quantify input DNA. This increases the speed and reliability of the STELA process and allows for automation of DNA extraction, dilution and PCR setup. It also increases the ranges of samples that can be analysed, allowing for example, the use of FTA DNA cards with fingersticks or buccal swabs. Advantageously, this technology also allows individuals to provide the samples at home and send them in the standard post for telomere testing.

For the routine assessment of samples from patients, the isolation of cells to be tested (e.g. CD19+ CLL) and the subsequent extraction of DNA from these cells, HT-STELA PCR, resolution and detection of generated PCR products and the measurement of mean telomere length (e.g. using XpYp chromosome) from these products could all be achieved within one working day: whereas with the original STELA method this

would have taken 7 working days to achieve. Thus HT-STELA provides a powerful tool allowing a rapid, accurate prognostic evaluation of cancer patients.

In a preferred embodiment of the method, said cell can be extracted from any biological sample. Reference herein to biological sample refers to any sample, or processed derivative thereof, taken from a subject whose telomere length is to be analysed. This includes but is not limited to complex samples, such as whole blood, tissue samples or tumour samples, or purified cell samples thereof.

Alternatively, a processed derivative of a biological sample may be used such as, but not limited to, cells harvested previously isolated and extracted from a biological sample and, if necessary, re-suspended in an appropriate medium. For example, FTA cards are a commonly used format for the convenient sampling and storage of blood samples for subsequent downstream DNA analysis. It provides a quick method for patients that may require a telomere test, to provide a sample at home, without the requirement of a clinician or phlebotomist. A small blood sample is spotted onto an FTA card then returned to the testing facility by standard mail services.

In a preferred embodiment of the invention, the DNA IQ™ system appears to be saturated at 4×10^5 cells and so the preferred method involves the use of between 2×10^5 and 6×10^5 and preferably 4×10^5 cells.

It has been found that occasionally telomere lengths can display considerable heterogeneity, for example in samples derived from peripheral blood of normal individuals, which can lead to unfavourable PCR amplification products. In these circumstances, it has been found that pre-digesting the chromosomal DNA prior to performing HT-STELA reduces the complexity of sample DNA providing a simpler target for amplification.

Therefore, in a further preferred embodiment of the invention said chromosomal DNA is digested with a restriction endonuclease that cleaves non-telomeric DNA. As will be appreciated by those skilled in the art, any enzyme that cleaves the DNA phosphodiester bond within the polynucleotide chain can be used provided it does not cleave within the telomere to be amplified.

However, given that we are assessing telomere length it is advisable to use a restriction endonuclease that does not cleave the telomeric region and so, more ideally, said restriction enzyme is selected on the basis it does not cleave between the 5' end of said primer and the telomere repeat array or chromosome telomere end.

In yet a further preferred embodiment of the invention said detecting the length of the amplified product involves the use of markers: a lower marker that was larger than the size of the larger primer complexes and primer-dimers (200bp) but smaller than the smallest fragment size of a telomere generated with HT-STELA (such as 882bp or 415bp); and an upper marker that was larger than the longest mean telomere length observed in our cohorts of CLL. Thus, ideally, a lower marker less than 882bp or 415bp was chosen. Ideally, the marker is selected from the group comprising: 882bp, 881bp, 880bp, 879bp, 878bp, 877bp, 876bp, 875bp, 874bp, 873bp, 872bp, 871bp, 870bp, 410bp, 409bp, 408bp, 407bp, 406bp, 405bp, 404bp, 403bp, 402bp, 401bp, 400bp, 399bp, 398bp, 397bp, 396bp, 395bp, 394bp, 393bp, 392bp, 391bp, and 390bp. Ideally, a commercially available 400bp DNA fragment was obtained for this purpose (NoLimits™, Thermo Scientific®). Similarly, an upper marker greater than 10kb was chosen such as a marker selected from the group comprising: 11kb, 12kb, 13kb, 14kb, 15kb, 16kb, 17kb, 18kb, 19kb and 20kb, including all 0.1kb integers therebetween.

In yet a further preferred method of the invention to achieve specificity for a particular chromosome, primers are designed to anneal with a region adjacent a telomere repeat array of chromosomal DNA of chromosomes XpYp, 7q, 12q and 17p. More ideally still, telomere-adjacent primers are designed incorporating telomere specific nucleotides at the 3' end.

Preferably, the 7q chromosome primers are designed around i.e. to be complementary to the 7q-specific nucleotides (with respect to the beginning of the telomere repeat array, or hg38 human genome reference sequence) 1115T (chr7:159334755 hg38), 551A (chr7:159335319 hg38), 195G (chr7:159335675 hg38) and, to a lesser extent, 29G (chr7:159335841 hg38). These primers produced robust telomere length distribution smears.

Preferably, the 12q chromosome primers are designed around i.e. to be complementary to the 12q specific nucleotides (with respect to the beginning of the telomere repeat array, or hg38 human genome reference sequence) 350G (chr12:133264524 hg38) and 550T (chr12:133264324 hg38). These primers generated robust telomere length distribution smears.

Preferably, the XpYp chromosome primers are designed around i.e. to be complementary to the XpYp specific nucleotides (with respect to the beginning of the telomere repeat array, or hg38 human genome reference sequence) 397C (XpYpE5)(chrX:10432 hg38) and 861C (XpYpC)(chrX:10896 hg38). These primers generated robust telomere length distribution smears.

Preferably, the 17p chromosome primers are designed around i.e. to be complementary to the 17p specific nucleotides 292C (17pseq1rev), 1018T (17pseq2rev), 1837C (17pseq4rev), 2559C (17p2) and 3018G (17p7) (with respect to the beginning of the telomere repeat array). These primers generated robust telomere length distribution smears.

Preferably, primers designed to amplify other chromosomal telomeres, including 2p, 4q, 4p, 5p, 11q, 16p and 18q, that also generate telomere-specific products with the original STELA method, may also provide utility with HT-STELA. These primers include: 2p2 (chr2: 10755 hg38), 4qK1 (chr4:190122098 hg38), 4p4 (chr4:10457 hg38), 5p3 (chr5:12240 hg38), 11q13B (chr11:135076467 hg38), 16prev1 (chr16:10241 hg38) and 18qrev4M (chr18:80262147 hg38).

In fact, we have found that primers that anneal up to at least 3.843kb from the start of the telomere repeat array yield robust telomere profiles. However, it was also apparent that primers that anneal within 30bp of the telomere repeat array did not produce efficient or specific amplification. Accordingly, when working the invention we use primers that anneal to a region of the chromosome that is between 3843bp-30bp from the start of the telomere repeats TTAGGG.

As used herein, the term "primer(s)" describes an oligonucleotide that hybridizes under physiological or reaction conditions (see **HT-STELA PCR**, page 20) to end

chromosome DNA. Those skilled in the art will recognize that the exact length of the oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence, notwithstanding the fact that ideally the oligonucleotide primers are designed to be complementary to at least one and ideally a plurality of said chromosome end specific nucleotides.

It is preferred that the oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological/reaction conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological/reaction conditions.

In order to be sufficiently selective and potent the oligonucleotides should comprise at least 7²⁶ and more preferably, at least 15 consecutive bases which are complementary to the target. Most preferably, the oligonucleotides comprise a complementary sequence of 20-30 bases.

In yet a further preferred embodiment of the invention detecting said amplification product(s) involves a resolution (or separation) process using capillary electrophoresis, an example of a suitable apparatus for undertaking this work is the Fragment Analyzer™ (available from Advanced Analytical). Other capillary gel systems are available from Agilent® and Lifetechnologies®. Moreover, once the amplification product(s) have been separated using capillary electrophoresis it/they is/are detected using an appropriate signal or label and we prefer to use a fluorescent dye, ideally one that intercalates into the amplification product. Using the method of the invention we have discovered that a fluorescent signal or label which intercalates into the PCR amplification product and produces a signal between 27-65 RFU, ideally at least 25 RFU, is robust and reliable enough for an estimation of telomere length (fig. 11) in a system where the lowest signal to provide a reliable telomere length estimate was 25 RFUs, this equates to, or can be expressed as 25% of (the area under the curve for the HT-STELA signal divided by the area under the curve for lower marker as shown in Figure 11 i.e. Tel Peak area/LM peak area). Please see page 35, Quality Control.

In yet a further embodiment of the invention said method is undertaken to diagnose or prognose or to determine the risk of developing a condition selected from the group comprising: cancer, ageing, neurological disorders including Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyeraal-Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis.

In yet a further embodiment of the invention said method is undertaken to assess an individual's suitability to be a transplantation donor, for example a bone marrow donor.

According to a further aspect of the invention there is provided a kit for determining telomere length of mammalian chromosomal DNA comprising:

at least one oligonucleotide primer able to anneal to a region adjacent a telomere repeat array of chromosomal DNA wherein said region is between 3843bp-30bp from said telomere repeat array.

In a preferred kit of the invention a plurality of primers are provided and ideally said primers are able to anneal to at least said region in one of the following chromosomes 7q, 12q, 17p, XpYp, 2p, 4q, 4p, 5p, 11q, 16p and 18q.

More preferably still, said kit comprises reagents for carrying out said method.

In yet a further aspect of the invention there is provided a primer selected from the group comprising:

Chromosome end	Primer name	Genomic Coordinates (HG38)	Sequence
2p	2p2	chr2: 10755	GAGCTGCGTTTTGCTGAGCAC (SEQ ID No: 1)

Chromosome end	Primer name	Genomic Coordinates (HG38)	Sequence
4q	4qK1	chr4:190122098	ACGGTGGCATGCCTCTCTC (SEQ ID No: 2)
4p	4p4	chr4:10457	TCTGCGCCTGCGATGGCGCTATG (SEQ ID No: 3)
5p	5p3	chr5:12240	GCATTCTCTTCACCACAGATGTTG (SEQ ID No: 4)
7q	7q1533C 7q1115T 7q843T 7q551A 7q195G 7q29G	chr7:159334337 chr7:159334755 chr7:159335027 chr7:159335319 chr7:159335675 chr7:159335841	CCCACACAGTCATCTATTGTT (SEQ ID No: 5) GAGGTGCAGTAGTGGGGATCTAACT (SEQ ID No: 6) GGGACAGCATATTCTGGTTT (SEQ ID No: 7) GCACAGCCTTTTGGGGTACCA (SEQ ID No: 8) AGTGGGAGATCCACACCGTAGCGTG (SEQ ID No: 9) CCaTGCAGTGCTAAGACAGCAATGAG (SEQ ID No: 10)
11q	11q13B	chr11:135076467	CAGACCTTGGAGGCACGGCCTTCG (SEQ ID No: 11)
12q	12q1511C 12q1387G 12q1036C 12q550T 12q350G 12q319G	chr12:133263363 chr12:133263487 chr12:133263838 chr12:133264324 chr12:133264524 chr12:133264555	CCTCTGGTCATTATGAATAGGGCTTC (SEQ ID No: 12) GGGGAAAAAATGCCCAAG (SEQ ID No: 13) CCTTCTCTTCTTGATGTC (SEQ ID No: 14) ACAGCCTTTTGGGGTACCGT (SEQ ID No: 15) GGCTTCATTGATGGTGAATACAATCG (SEQ ID No: 16) GCAGCGCTGAATATTCAGGGTG (SEQ ID No: 17)
16p	16prev1	chr16:10241	CACTTATTAGTTCCAGTCTCTG (SEQ ID No: 18)
17p	17p8 17p7 17p2 17pseq4rev 17pseq2rev 17pseq1rev 17pseq1B		AGAAGCAGCGAGGAGCTTCA (SEQ ID No: 19) CCTGGCATGGTATTGACATG (SEQ ID No: 20) GCTAGGAATGGAATCATTGACTC (SEQ ID No: 21) GATACTGGGAGGATCATATCTGGC (SEQ ID No: 22) CCATTAGCCTGTGGGGTCTGAT (SEQ ID No: 23) GAATCCACGGATTGCTTTGTGTAC (SEQ ID No: 24) AAGCAGGTTGAGAGGCTGAGG (SEQ ID No: 25)

Chromosome end	Primer name	Genomic Coordinates (HG38)	Sequence
18q	18qrev4M	chr18:80262147	CACAGGGATGGTTAGGTATCTC (SEQ ID No: 26)
XpYp	XpYpB XpYpM XpYpO XpYpC XpYpP XpYpE2 XpYpE3 XpYpE4 XpYpE5 XpYpE6 XpYpE7 XpYpJ	chrX:12573 hg38 chrX:11688 chrX:11660 chrX:10896 chrX:10575 chrX:10427 chrX:10425 chrX:10429 chrX:10432 chrX:10437 chrX:10439 chrX:10064	CGAGCAAGCATCGGAACGTGACT (SEQ ID No: 27) ACCAGGTTTTCCAGTGTGTT (SEQ ID No: 28) CCTGTAACGCTGTTAGGTAC (SEQ ID No: 29) CAGGGACCGGGACAAATAGAC (SEQ ID No: 30) ACCAGGGGCTGATGTAACG (SEQ ID No: 31) TGTCTCAGGGTCCTAGTG (SEQ ID No: 32) TCTCAGGGTCCTAGTGTG (SEQ ID No: 33) GTTGTCTCAGGGTCCTAG (SEQ ID No: 34) GGGGTTGTCTCAGGGTCC (SEQ ID No: 35) TTCTAGGGGTTGTCTCAG (SEQ ID No: 36) TCTTCTAGGGGTTGTCTC (SEQ ID No: 37) CTAATCTGCTCCCWCCCAC (SEQ ID No: 38)

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprises”, or variations such as “comprises” or “comprising” is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

The invention will now be described by way of example only with reference to the following tables and figures:

Figure-1. The electrophoresis and detection of PCR products generated by STELA on the Fragment Analyzer™. 250pg HT1080 cl.2 DNA was added to 30µl STELA PCR reactions and cycled for 22 PCR cycles with an annealing temperature of 65°C. (a) 4µl from each reaction was subsequently taken and analysed by the STELA protocol. (b) (i) The remainder of the STELA PCR product was electrophoresed and detected using the Fragment Analyzer™ along with a positive control PCR from which PCR products can be detected using the Fragment Analyzer™. (ii) Example of an electropherogram generated from one capillary through which one of the PCR reactions was resolved LM=lower marker, UM=upper marker.

Figure-2. Increased number of PCR cycles increases the amount of HT-STELA PCR product generated to allow their visualisation on the Fragment Analyzer™ 250pg MRC5 cl.13 DNA was added to 30µl STELA PCR reactions and subject to 22, 24, 26 or 28 PCR cycles with an annealing temperature of 65°C. 4µl PCR products generated were taken for (a) conventional STELA analysis and the remaining 26µl was loaded onto a 96 well plate for capillary electrophoresis and detection by the Fragment Analyzer™ (b).

Figure-3. Improving HT-STELA PCR product yield through the design and use of alternative XpYp telomere adjacent primers. (a) A number of alternative XpYp

telomere adjacent primers were designed around the same region as XpYpE2. (b) All five of the XpYp telomere-adjacent primers were used in STELA PCR and primer performance was compared between the five primers and also to XpYpE2 to determine whether there were any increases in PCR efficiency and the generation of more PCR product. (c) Triplicate HT-STELA PCR reactions containing either XpYpE2 or XpYpE5 were loaded into a 96 well plate for analysis using the Fragment Analyzer™. Relative fluorescent units (RFU) were used to indicate the amount of PCR product produced by each individual PCR reaction. The average RFU of the each reaction set was then used to determine the quantity of PCR product generated using either primer.

Figure-4. Increasing the amount of input DNA per reaction results in the formation of a telomere length distribution smear as opposed to a banding pattern. (a) 1.25ng, 6.25ng or 12.5ng HT1080 cl.5 DNA was added to duplicate 30µl HT-STELA PCR reactions containing XpYpE5 and subject to 28 PCR cycles. The resulting PCR products generated were resolved and detected using the Fragment Analyzer™. (b) The process in (a) was repeated but with DNA taken from a CLL patient (patient id - 4408) displaying a more heterogeneous telomere length distribution. 1.25ng, 3.125ng, 6.25ng, 12.5ng, 25ng and 47.5ng 4408 DNA was added to multiple 30µl HT-STELA PCR reactions which were then subject to 28 PCR fragments. Generated PCR products were resolved and detected using the Fragment Analyzer™.

Figure-5. Decreasing XpYpE5 and teltail primer concentration increases the amount of HT-STELA PCR product generated. (a) 30ng HT1080 cl.5 DNA was added to multiple reactions containing either 0.2µM, 0.5µM or 0.8µM telomere adjacent XpYpE5 and teltail primers. Resulting PCR products generated from the reactions were then resolved and detected using the Fragment Analyzer™. The Relative Fluorescence Units (RFU) was given for each reaction. Within each reaction set the individual reaction RFUs were averaged giving an indication of the quantity of PCR product generated by a particular primer concentration. (b) The process in (a) was repeated but with HT STELA PCR reactions containing different primer concentrations. PCR products generated from reaction sets containing 0.05µM, 0.1µM or 0.2µM were resolved and detected on the Fragment Analyzer™ and average RFU for a reaction set was calculated

Figure-6. Comparison of mean telomere length measured by conventional Single Telomere Length Analysis (STELA) and High Throughput STELA (HT-STELA) (a) Mean XpYp telomere length from a panel of CLL samples along with clonal populations of the HT1080 and MRC5 was analysed by conventional STELA. Mean telomere length was measured by band quantification using Phoretix software (NonLinear dynamics) and taking a mean of all the quantified bands. (b) XpYp telomere length from the same panel of samples was also measured by HT-STELA using the newly optimised conditions. For HT-STELA mean telomere length was calculated for each individual lane by the smear analysis function on the ProSize™ software (Advanced Analytical) and calculating the average mean telomere length across all the reactions for a particular sample. (c) comparison of MTL estimate generated with STELA and HT-STELA.

Figure-7. Reducing the amount of PCR cycles reduces the amplification of artefactual short molecules and preferential amplification of shorter telomeres in HT-STELA and results in an accurate mean XpYp telomere length measurement (a) 1 µl (10.06ng), 2 µl (20.12ng) and 3 µl (30.18ng) HT1080 cl.5 DNA was added to multiple HT-STELA PCR reactions and subject to 22, 24, 26 or 28 PCR cycles. Generated PCR product was resolved and detected on the Fragment Analyzer™. (b) The same process as (a) was performed but with 1 µl (6.6ng), 2 µl (13.2ng) or 3 µl (19.9ng) MRC5 cl.5 DNA. (c) 30ng HT1080 cl.2 DNA was added to multiple HT-STELA PCR reactions and subject to either 22 or 23 PCR cycles. Generated PCR products were resolved and detected on the Fragment Analyzer™. Average RFU (Relative fluorescent units) is used to indicate the average amount of PCR product produced by each PCR reaction set. The mean telomere length (MTL) for each reaction set was calculated using the smear analysis on the ProSize™ software. UA/LA=The ratio in peak height between the upper telomeric allele and the lower telomeric allele.

Figure-8. Improving the visibility of the lower marker for optimal capillary-to-capillary normalisation (a) Example of an electropherogram of HT-STELA PCR products which have not been purified resolved within a single capillary along with 0.5ng/µl lower (75bp) and upper (20kb) markers provided within the DNF-930 dsDNA reagent kit (Advanced Analytical). 'UM' indicates the location of the upper marker however the presence of PCR artefacts less than 200bp masks the position of the 75bp lower

marker 'LM'. (b) Electropherogram showing the same as (a) but after PCR purification of HT-STELA PCR products. (c) Electropherogram of resolved HT-STELA PCR products within a capillary along with new 400bp and 15kb NoLimits™ DNA fragments (0.5ng/μl). The new 400bp lower marker results in a single clear peak separate from the unwanted PCR artefacts.

Figure-9. Reanalysis of mean telomere length measured by High Throughput STELA (HT-STELA) after reducing PCR cycle number (a) XpYp HT-STELA PCR was performed on a panel of CLL samples as well as on clonal populations of HT1080 and MRC5. 20-30ng DNA of each DNA was added to 30μl HT-STELA PCR reactions which were then subject to 22 PCR cycles. Resulting PCR products generated were resolved and detected on the Fragment Analyzer™. Mean XpYp telomere length was calculated for each individual lane by the smear analysis function on the ProSize™ software (Advanced Analytical) and calculating the average mean telomere length across all the reactions for a particular sample. (b) comparison of MTL estimate generated with STELA and HT-STELA.

Figure-10. Analysis of the coefficient of variance of HT-STELA (a, b & c). 30ng MRC5 cl.5 DNA was added to 95 x 30μl replicate HT-STELA PCR reactions containing the telomere-adjacent primer XpYpE5 and subject to 23 PCR cycles. The generated PCR products were loaded onto a 96 well plate before being resolved and detected by the Fragment Analyzer™. The resulting output gels shown are from three independent runs on the Fragment Analyzer™ using the same PCR reactions. Telomere length for both telomeric alleles was measured using the smear analysis tool in ProSize™ (Advanced Analytical). The mean telomere length was then calculated for all 95 HT-STELA PCR reactions. Coefficient of variance (%CV) was calculated using the formula $\%CV = (SD/MTL) * 100$ and was calculated between PCR reactions within the same 96 well plate (stated underneath each output gel) or for the same PCR reaction resolved and detected in three independent Fragment Analyzer™ runs (table 1).

Figure-11. Peak height analysis to determine what RFU value gives inaccurate mean telomere length measurement. 30ng HT1080 cl.2 DNA was added to HT-STELA PCR reactions. These reactions were then subject to 18, 19, 20, 21, 22 or 23 PCR cycles.

Generated PCR products were resolved and detected on the Fragment Analyzer™. Relative Fluorescence Units (RFU) represent the PCR product amount. Mean telomere length was calculated for each lane using the smear analysis tool within the ProSize™ software (Advanced Analytical). Tel peak area= area under a telomere length distribution smear peak on an electropherogram in ProSize™ software. LM peak area=peak area under lower marker peak on electropherogram in ProSize™. Tel peak area was designated as a percentage of the LM peak area using the peak area values given by Prosize™ software in the formula (Tel peak area/LM peak area)*100. TL=telomere length, MTL=mean telomere length, %CV=coefficient of variation.

Figure-12. Optimisation of automated DNA extraction on the Maxwell® 16 Instrument using the DNA IQ™ protocol to generate telomere length distribution smears. (a) STELA was performed on isolated CLL cells. (b) Differing amounts of the same cell population as in (a) were lysed for 1 hour before being loaded into the Maxwell® 16 instrument for automated DNA extraction using the DNA IQ™ protocol. DNA was eluted into 80µl. After adding tel2 oligonucleotide (250pM) this DNA/tel2 mix was analysed by HT-STELA. Generated PCR products were resolved and detected on the Fragment Analyzer™ (c) DNA was extracted from eight replicates of 4X10⁵ isolated CLL cells using the DNA IQ™ protocol. Four of the replicates were eluted into 40µl elution buffer (40-1 – 40-4) and the remaining four were eluted into 80µl (80-1 - 80-4). DNA concentration was measured using the Nanodrop™ 3300 -fluorospectrometer (Thermo Scientific®). (d) Increasing amounts of DNA (1µl, 3µl & 5µl) extracted from replicate 80-4 were added to multiple HT-STELA PCR reactions to achieve a telomere length distribution smear. (e) Process in (c) was repeated but with 3µl eluted DNA added to HT-STELA PCR reactions instead of 1µl.

Figure-13. Analysis of mean XpYp telomere length of CD19+ cells isolated from whole blood of Chronic Lymphoid Leukaemia (CLL) patients CD19+ cells were isolated from whole blood using MACS® whole blood CD19 microbeads and the AutoMACS® Pro separator™ (Miltenyi Biotech). DNA was extracted from 4X10⁵ cells CCD19+cells using the DNA IQ™ protocol on the Maxwell® 16 instrument. Eluted DNA was then used to analyse the mean XpYp telomere length in the CLL patients by (a) STELA or by (b) HT-STELA. (c) Comparison of the mean XpYp telomere length measured by the two techniques.

Figure-14. Development of HT-STELA for 7q, 12q and 17p chromosome ends. Numerous chromosome-specific primers were designed within the telomere-adjacent region of (a) 7q and (b) 12q. Primers have previously been designed within the telomere-adjacent region of (c) 17p and (d) XpYp. HT-STELA PCR was performed with these alternative telomere adjacent primers and generated PCR products were resolved and detected on the Fragment Analyzer™. Average RFU (Relative Fluorescence Units) is the calculated mean of the RFU given by each PCR reaction for a given telomere adjacent primer. The identification and position (relative to the TTAGGG repeat start) of the telomere specific nucleotide which is incorporated at the 3' end of the primer is stated for each primer. Distance from the start of the TTAGGG repeats (including the length of the primer) for each primer is subtracted from the molecular weights produced by the Fragment Analyzer™ giving the actual telomere length corrected for telomere adjacent DNA. STELA was also performed for each chromosome end. Again, actual telomere length was calculated by subtracting telomere adjacent DNA.

Figure-15. Optimisation of HT-STELA PCR to improve PCR product yield. A number of different optimisation experiments were performed to improve HT-STELA PCR product yield. For each optimisation experiment generated PCR products were resolved and detected on the Fragment Analyzer™ (a) 30ng HT1080 cl.2 DNA/tel2 mix was added to HT-STELA PCR reactions containing either 2mM, 4mM or 6mM MgCl₂. (b) The linker oligonucleotide tel2 was added to HT1080 cl.2 DNA (10ng/μl) to either 250pM, 25pM or 5pM final concentration. 30ng of each DNA/tel2 mix was added to HT-STELA PCR reaction. After electrophoresis and detection RFU (Relative Fluorescence Units) were measured by the ProSize™ software. RFU are indicative of how much PCR product was detected by the Fragment Analyzer™. (c) 30ng HT1080 cl.2 DNA/tel2 mix was added to HT-STELA PCR reactions containing either Red Hot Taq polymerase (Thermo Scientific®) or Titanium Taq (Clontech®). (d) A 30μl HT-STELA PCR reaction was either undiluted or diluted 2X, 5X and 10X. The amount of PCR product detected was measured by RFU analysis. (e) 30ng HT1080 cl.2/tel2 mix was added to HT-STELA PCR reactions with a volume of either 30μl or 60μl. The amount of PCR product detected was measured by RFU analysis.

Figure-16. STELA analysis of 7q, 12q and 17p chromosome ends in HT1080 cl.2 using newly designed primers. Traditional STELA was using HT1080 cl.2 DNA at 7q and 12q chromosome ends using primers designed around the stated telomere-specific nucleotides. 17p STELA was performed using the previously designed 17pseq1rev. PCR product size was quantified using Phoretix software (Nonlinear Dynamics) and mean telomere length was subsequently calculated.

Figure-17. Digestion of DNA with EcoR1 prior to HT-STELA PCR results in reduced background noise and a cleaner HT-STELA electropherogram (a) DNA extracted from eight peripheral blood samples was digested with EcoR1 for 2 hours. Digested DNA was then added to triplicate HT-STELA PCR reactions and cycled for 23 PCR cycles. Parallel reactions were also constructed which contained undigested DNA of the same samples. The resulting PCR products were then separated by capillary electrophoresis using the Fragment AnalyzerTM. (b) Comparison of the electropherogram generated from one HT-STELA PCR reaction amplifying the (i) undigested or (ii) digested DNA of sample 3 from (a).

Figure-18. Optimising PCR cycle number and DNA input amount. DNA extracted from three peripheral blood samples (1, 9 & 11) was digested with EcoR1 for 2 hours. Following digestion DNA was quantified and either 2ul or 3ul of digested DNA was added to three sets of triplicate HT-STELA reactions. Each set of triplicate reactions were cycled for 21, 22 or 23 PCR cycles. Resulting PCR products were then resolved by capillary electrophoresis using the Fragment AnalyzerTM. Mean telomere length was calculated for each lane using the smear analysis tool within the ProSizeTM software (Advanced Analytical). Telomere adjacent DNA (distance between the PCR assay start and the start of the telomere repeat array) was removed giving a final mean telomere length. Norm peak area % was calculated by using the formula $(\text{Tel peak area} / \text{LM peak area}) * 100$ where Tel peak area = area under a telomere length distribution smear peak on an electropherogram in ProSizeTM software. LM peak area = peak area under lower marker peak on electropherogram in ProSizeTM.

Figure-19. Optimisation of the DNA IQTM system to clean up and normalize DNA input samples to achieve HT-STELA telomere length distribution smears with consistent intensity. 200, 500, 1000 and 2000ng HT1080 cl.2 DNA was added to DNA IQTM

casework pro kit cartridges (CW) or DNA IQ™ reference sample cartridges (RS). DNA was eluted into either 50ul (CW) or 300ul (RS). For the CW purified samples either 3, 6 or 9ul eluted DNA was added to individual HT-STELA reactions. For the RS purified samples 3, 10 or 20ul eluted DNA was added to each HT-STELA reaction. HT-STELA reactions were then subject to 23 PCR cycles and generated PCR product was resolved and detected on the Fragment Analyzer™. DNA concentration was measured in triplicate with picogreen™ using the nanodrop™ 3300 fluorospectrometer. $(\text{Tel peak area/LM peak area})\% = (\text{area under a telomere length distribution smear peak on an electropherogram} / \text{peak area under lower marker peak on electropherogram}) * 100$. Mean telomere length (MTL) was calculated for each HT-STELA reaction triplicate using the smear analysis tool within the ProSize™ software (Advanced Analytical).

Figure-20. Testing the reproducibility of the DNA clean up and normalization procedure using the DNA IQ™ Casework Pro kit (a) 200, 500 or 1000ng DNA was added to DNA IQ™ casework pro kit cartridges. The purification of each DNA quantity was performed four times. DNA was eluted into 80ul elution buffer. 12ul of eluted DNA was added to triplicate HT-STELA reactions and subject to 23 PCR cycles. PCR products were resolved and detected on the Fragment Analyzer™. DNA concentration was measured in triplicate with picogreen™ using the nanodrop™ 3300 fluorospectrometer. $(\text{Tel peak area/LM peak area})\% = (\text{area under a telomere length distribution smear peak on an electropherogram} / \text{peak area under lower marker peak on electropherogram}) * 100$. Mean telomere length (MTL) was calculated for each HT-STELA reaction triplicate using the smear analysis tool within the ProSize™ software (Advanced Analytical). Coefficient of variance (%CV) was calculated using the formula $\%CV = (SD/MTL) * 100$. (b) Comparison of mean telomere length calculated from the telomere length distribution smears generated from individual HT-STELA reactions. Coefficient of variation was calculated in the same manner as in (a)

Figure-21. FTA STELA analysis of DNA extracted from the dried blood of CLL patients (a) DNA was extracted from two 3mm punches of an FTA card sampling area saturated with 125ul blood for each patient or containing no blood (negative control). The punches were incubated in Casework Extraction buffer (Promega®) for 1 hour at 65°C with shaking (1400rpm) after which DNA was extracted from the eluted cell lysate

using the LEV blood cartridges. DNA concentration was measured using the NanoDrop™ 3300 fluorospectrometer. HT-STELA PCR was performed using 3ul DNA per reaction. Generated PCR products were resolved by capillary electrophoresis using the Fragment Analyzer™. Mean telomere length was calculated for each lane using the smear analysis tool within the ProSize™ software (Advanced Analytical). Normalised tel peak area % was calculated using the formula (tel peak area/lower marker peak area)X100. Peak areas are stated in the ProSize™ software. (b) The process in (a) was repeated however the DNA from the eluted cell lysate was extracted using the DNA IQ™ casework pro cartridges. (c) The process in (b) was repeated for 26 CLL patient blood samples collected. STELA was also performed for these patients and the MTL calculated by the two methods of analysis was compared.

Table-1. List of oligonucleotides.

Table-2. Coefficient of variation analysis for HT-STELA. 30ng MRC5 cl.5 DNA was added to 95 HT-STELA PCR reactions and subject to 23 PCR cycles. The resulting PCR products were electrophoresed and detected on the Fragment Analyzer™ on three independent occasions. Mean XpYp telomere length was measured for lower and upper allele after each allele. For each of the 95 PCR reactions the variation between the mean XpYp telomere length measurement after each assay on the Fragment Analyzer™ was determined using the formula %CV=SD/average MTL. This was performed for both the lower and upper allele.

Materials and Methods

Oligonucleotides

Oligonucleotides were designed based on human DNA sequences obtained from H. Reithman at the Wistar institute (<http://www.wistar.upenn.edu/Reithman>) and National Centre for Biotechnology Information (NCBI). The oligonucleotides were synthesised by MWG-Biotech AG (Ebersberg, Germany). All primers used during this study are listed in table 1.

CLL cell isolation

CD19⁺ CLL cells were isolated using MACS® whole blood CD19 microbeads. A volume of whole blood containing 2×10^6 white blood cells was taken and brought up to 1000ul with 1XPBS. 20ul MACS® whole blood microbeads were then added before mixing thoroughly and incubating at room temperature for 15 minutes. After incubation blood samples were loaded onto AutoMACS® Pro separator (Miltenyi Biotech) and CD19⁺ cells were positively selected using the posselwb program.

DNA extraction

Maxwell®

Genomic DNA was extracted from cell pellets using the Maxwell® 16 LEV DNA-IQ™ DNA kit (Promega®) along with the Maxwell® 16 instrument. Cell lysis was achieved through incubation of the cell pellet with 400ul lysis buffer and 10ul proteinase K at 56°C for at least one hour. Cell lysates were then loaded into the cartridges and placed in the Maxwell® 16 instrument. The forensic mode with LEV hardware was ran to extract DNA from the cell lysates. Extracted DNA was eluted into 40ul elution buffer.

Phenol/Chloroform

Genomic DNA from cell pellets containing over 3×10^6 cells was extracted by standard phenol/chloroform extraction (Sambrook et al., 1989). Briefly, cells were lysed overnight at 45°C in 300-500ul lysis buffer (10mM Tris-Hcl pH8, 100mM NaCl, 5mM EDTA pH8, 0.5% SDS) containing 30ug RNase A (Sigma; stock 10mg/ml) and 60ug proteinase K (Sigma; stock 20mg/ml). After brief centrifugation 300-500ul phenol/chloroform was added to the cell lysate and rotated for 20mins at room temperature. The mixture was centrifuged at 13000rpm for 5mins to separate the phases, the aqueous and interphase phases were removed and added to an eppendorf containing 300ul phenol/chloroform. This was rotated again for 20mins and then centrifuged (13000rpm) for 5 minutes. The aqueous phase was removed and to this was added 30ul 3M sodium acetate pH5.3 (Sigma) and 900ul ice-cold 100% ethanol to precipitate the DNA. The mix was left at -20°C for at least an hour. After brief centrifugation (13000rpm, 1min) the DNA pellet was washed in 70% ice-cold ethanol and air dried in the micro-flow hood. DNA was then resuspended in 10mM Tris-Hcl pH8.

DNA quantification

DNA concentrations were determined in triplicate either by Hoechst 33258 fluometry as described previously (Baird et al., 2003) or using a Nanodrop™ 3300 fluorospectrometer (Thermo Scientific®). Briefly an equal amount of a 2X dilution of Quant-iT™ Picogreen™ dsDNA reagent (Life Technologies) and DNA were mixed and incubated at room temperature for 5 minutes. 2ul was loaded onto the pedestal to measure the fluorescence. The fluorescence measurement was then converted to DNA concentration using a standard curve constructed using DNA of known quantity.

STELA PCR

DNA was diluted to 250pg/μl in 10mM Tris-Hcl (pH 8) containing 250pM Tel2 linker. Multiple 10μl reactions were set up per sample (typically 6) each containing 1X Taq buffer (75mM Tris-HCl (pH8.8), 20mM (NH₄)SO₄, 0.01% Tween-20) (Abgene™), 2mM MgCl₂, 1.2mM dNTPs, telomere-specific primer (0.5μM), teltail primer (0.5μM), and 1U Taq/PWO (Abgene™/Roche®) at a ratio of 10:1. 1μl DNA/tel2 mix (250pg) was added to each reaction. These reactions were then cycled in a Bio-Rad® DNA Engine Tetrad® Thermal Cycler using the following conditions: 94°C (20 sec), 65°C (30 sec), 68°C (8 mins) for 22 cycles.

HT-STELA PCR

DNA was diluted to 10ng/μl in 10mM Tris-Hcl (pH8) containing 250pM Tel2 linker. Multiple 30ul reactions were set up per sample (typically 3-5) each containing 1X Taq buffer (75mM Tris-HCl (pH8.8), 20mM (NH₄)SO₄, 0.01% Tween-20) (Abgene™), 2mM MgCl₂, 1.2mM dNTPs, telomere-specific primer (0.2μM), teltail primer (0.2μM), and 1U Taq/PWO (Abgene™/Roche®) at a ratio of 10:1. 3μl DNA/tel2 mix (30ng) was added to each reaction. These reactions were then cycled in a Bio-Rad® DNA Engine Tetrad® Thermal Cycler using the following conditions: 94°C (20 sec), 65°C (30 sec), 68°C (5 mins) for 23 cycles

Gel Electrophoresis

DNA fragments were resolved using a 40cm long, 0.5% Tris-acetate-EDTA agarose gel submerged in 1XTAE cooled to 4°C by a circulating cooling system. 4ul STELA PCR reactions containing 1X ficol-based loading dye (5% bromophenol blue, 5%

xylene, 15% ficol) were loaded in the gel and ran through the length of the gel at 120V for 16 hours.

Capillary Electrophoresis

30ul HT-STELA PCR reactions were loaded onto a 96 well plate (sample plate) along with a 75bp-15kb ladder in one well. Another 96 well plate (marker plate) was loaded which contained 22ul of the combined lower (400bp) and upper (15kb) markers in every well. Both plates were placed into Fragment Analyzer™ along with a deep well 96 well plate containing 1.1ml 1X inlet buffer. Both marker DNA fragments and HT-STELA PCR fragments were then resolved through DNF-930 dsDNA gel containing 0.001X intercalating gel using the following conditions: prerun (6kV, 30 seconds), marker injection (1kV, 10 seconds), sample injection (4kV, 50 seconds), separation 6kV, 120 minutes).

Southern Blotting

The resolved STELA PCR products were depurinated by washing the gel twice in depurination buffer (0.25M HCl) for 6 minutes. After rinsing, the gel was then washed in denaturation buffer (1.5M NaCl/0.5MNaOH) for 15 minutes. The DNA was then transferred onto a positively charged membrane (Hybond™ XL, Amersham™) by alkaline Southern blotting with denaturation buffer for 4-6 hours.

Probe labelling and hybridisation

Probe synthesis

25ng probe DNA and ladder (1:1 of 1kb:2.5kb) in TE buffer (10mM Tris-HCl and 1mM EDTA) was labelled using Ready-To-Go™ DNA labelling beads (GE Healthcare). This kit generates labelled probes using random hexaprime labelling with [α -33P] dCTP.

Hybridisation

After blotting the membranes were rinsed in H₂O before undergoing pre-hybridization for 15 mins in church buffer (0.5M sodium phosphate buffer (1M disodium hydrogen phosphate and 1M sodium dihydrogen phosphate), 1mM EDTA, 1% BSA, 7% SDS,

pH 7.2). 25µl of radioactively labelled probe was added to the hybridisation bottles which were then left to hybridize at 60°C overnight.

Removing unbound probe

To remove unbound probes the membrane was washed with 0.1X sodium chloride sodium citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) several times at 60°C. The washed blots were then dried in the hybridization oven at 60°C for ~30mins.

Visualisation of Radiolabelled Blots

Radiolabelled southern blots were placed in a cassette with a phosphorimager screen (Amersham™) for 24 hours. The phosphorimager screen was then scanned using the Typhoon 9410 biomolecular imager (GE healthcare).

DNA template Digestion

Peripheral blood was obtained from healthy individuals using contact-activated lancets (BD). DNA was extracted directly from peripheral blood samples (300µl) using DNA IQ™ casework pro kit cartridges and eluted into 50µl elution buffer (Promega®). Extracted DNA was digested using EcoR1. Briefly, 17.3µl DNA was added to 20µl reactions containing RE 1X buffer, acetylated BSA (1ug/µl) and EcoR1 restriction enzyme (5U). Reactions were then incubated at 37°C for 2 hours followed by a 5 min incubation at 65°C to heat inactivate the restriction enzyme. Following digestion was then quantified using NanoDrop™ 3300 fluorospectrophotometer (Thermo Scientific®) and diluted to ~10ng/ul.

HT-STELA

250pM Tel2 linker was added to each digested DNA. Three 30ul reactions were set up per sample each containing 1X Taq buffer (75mM Tris-HCl (pH8.8), 20mM (NH₄)SO₄, 0.01% Tween-20) (Abgene™), 2mM MgCl₂, 1.2mM dNTPs, XpYpC primer (0.2µM), teltail primer (0.2µM), and 1U Taq/PWO (Abgene™/Roche®) at a ratio of 10:1. Either 2µl or 3µl DNA/tel2 mix was added to each reaction. These reactions were then cycled in a Bio-Rad® DNA Engine Tetrad® Thermal Cycler using the following conditions: 94°C (20 sec), 65°C (30 sec), 68°C (5 mins) for 21, 22 or 23 cycles.

Capillary Electrophoresis

30µl HT-STELA PCR reactions were loaded onto a 96 well plate (sample plate) along with a 75bp-15kb ladder in one well. Another 96 well plate (marker plate) was loaded which contained 22ul of combined lower (400bp) and upper (15 or 20kb) markers. Both plates were placed into the Fragment Analyzer™ (AATI) along with a deep well 96 well plate containing 1.1ml 1X inlet buffer. Both marker DNA fragments and HT-STELA PCR fragments were then resolved through DNF-930 dsDNA gel containing 0.001X intercalating gel using the following conditions: prerun (6kV, 30 seconds), marker injection (1kV, 10 seconds), sample injection (4kV, 75 seconds), separation 6kV, 120 minutes).

DNA normalisation using the DNA IQ™ system

Genomic DNA was added directly to 400µl lysis buffer which was then loaded into DNA IQ™ casework pro kit cartridges or DNA IQ™ reference sample kit cartridges (Promega®) and placed in the Maxwell® 16 instrument. For the DNA IQ™ casework pro kit DNA was extracted using the forensic mode with low elution volume (LEV) hardware. DNA was eluted into 80µl elution buffer. For the reference sample kit DNA was extracted using the forensic mode with the standard elution volume (SEV) hardware. For this kit extracted DNA was eluted into 300µl elution buffer.

DNA extraction from dried blood spots

125µl blood was pipetted onto an FTA card sample target and left to air-dry overnight. Two 3mm punches were cut from each dried blood spot and incubated at 65°C with shaking (1400rpm) with 40µl 10mg/ml proteinase K and 360µl casework extraction buffer (Promega®). The eluted cell lysate was removed from the stripped FTA card punches by adding both the punches and supernatant to spin baskets (Promega®) inserted into Eppendorf tubes that were centrifuged at 12000rpm for two minutes. Cell lysates were then loaded into DNA IQ™ casework pro kit cartridges (Promega®) and placed in the Maxwell® 16 instrument. The forensic mode with LEV hardware was ran to extract DNA from the cell lysates. Extracted DNA was eluted into 50µl elution buffer.

HT-STELA and FTA STELA

250pM Tel2 linker was added to each extracted DNA. Three 30µl reactions were set up per sample each containing 1X Taq buffer (75mM Tris-HCl (pH8.8), 20mM (NH₄)SO₄, 0.01% Tween-20) (Abgene™), 2mM MgCl₂, 1.2mM dNTPs, XpYpC primer (0.2µM), teltail primer (0.2µM), and 1U Taq/PWO (Abgene™/Roche®) at a ratio of 10:1. 4µl DNA/tel2 mix was added to each reaction. These reactions were then cycled in a Bio-Rad® DNA Engine Tetrad® Thermal Cycler using the following conditions: 94°C (20 sec), 65°C (30 sec), 68°C (5 mins) for 23 cycles.

Capillary Electrophoresis

30µl HT-STELA PCR reactions were loaded onto a 96 well plate (sample plate) along with a 75bp-15kb ladder in one well. Another 96 well plate (marker plate) was loaded which contained 22µl of the combined lower (400bp) and upper (15kb) markers in every well. Both plates were placed into Fragment Analyzer™ along with a deep well 96 well plate containing 1.1ml 1X inlet buffer. Both marker DNA fragments and HT-STELA PCR fragments were then resolved through DNF-930 dsDNA gel containing 0.001X intercalating gel using the following conditions: prerun (6kV, 30 seconds), marker injection (1kV, 10 seconds), sample injection (4kV, 75 seconds), separation 6kV, 120 minutes).

Statistical Analysis

STELA band quantification

Gels scanned using the Typhoon 9410 were subsequently analysed using Molecular dynamics ImageQuant® 5.0 (GE). The molecular weights of individual telomeres on each STELA Southern blot were calculated using Phoretix 1D software (Nonlinear dynamics). Mean telomere length measurements were then calculated.

HT-STELA - Data interpretation and Statistical Analysis

The output data from the Fragment Analyzer™ was viewed in ProSize™ software (Advanced Analytical). The following peak analysis settings were applied when analysing the data: peak width – 50 seconds, minimum peak height - 25 RFU with no valley to valley baseline. Baseline setpoints were determined manually for each

sample. Mean telomere length was calculated using the smear analysis function within the ProSize™ software. The smear range was manually set for each sample. The mean telomere length was calculated from the smear analysis result for all reactions within each sample reaction set.

RESULTS

STELA product detection with a Fragment Analyzer™

STELA PCR was performed using DNA extracted from a clonal population of the HT1080 fibrosarcoma cell line (HT1080 cl.2). This DNA was used as it displays a homogenous telomere length profile at XpYp that is similar to what is observed in CLL patients. Based on the reaction conditions for the original STELA method for the XpYp telomere, 250pg DNA was added to 30µl STELA PCR reactions which were then subject to 22 PCR cycles using an annealing temperature of 65°C. 4µl of each reaction was used for the standard STELA analysis and the remainder of the PCR products resolved and detected using the Fragment Analyzer™ (Advanced Analytical; fig. 1). With the original methodology the STELA PCR products are readily detected by the annealing of a radioactively labelled telomere repeat containing probe (fig. 1a). However, as can be seen from the digital gel image generated with the PROSize™ software (Advanced Analytical), these same PCR products are not abundant enough to be detected fluorescently with the Fragment Analyzer™ (fig. 1b(i)). An electropherogram of one of the capillaries through which the generated PCR products were electrophoresed further confirms the lack of any fluorescent signal generated from the STELA PCR products (fig. 1b(ii)). The lower and upper markers are clearly visible as are large amounts of artefacts <200bp formed in the STELA PCR. The STELA PCR conditions therefore required modification to ensure that telomere length distributions could be detected by capillary electrophoresis (using the Fragment Analyzer™).

Improving conditions to detect STELA PCR products directly on the Fragment Analyzer™

As the original STELA conditions resulted in insufficient PCR product to be detected by the Fragment Analyzer™, a number of factors were optimised to increase the amount of PCR product generated and hence increase the signal intensity measured by the Fragment Analyzer™.

Increased cycle number

The first attempt to increase the amount of STELA PCR product generated was to increase the number of PCR cycles. 250pg DNA extracted from a clonal population of MRC5 fibroblasts was added to multiple 30µl PCR reactions and subject to either 22, 24, 26 or 28 cycles (fig. 2). A small number of STELA-PCR products were generated when the reactions were subject to either 22 or 24 PCR cycles however the intensity of these products was not sufficient to result in their detection using the Fragment Analyzer™ (fig. 2a&b). However when STELA PCR reactions were cycled 26 times a larger amount of PCR product was generated, resulting in more intensely hybridising bands on the STELA Southern blot. This was sufficient to allow the detection of the majority of the bands with the Fragment Analyzer™ (fig. 2a&b). Reactions subject to 28 cycles showed an even greater intensity on both the STELA blot and the Fragment Analyzer™ output gel (fig. 2a&b) and all the DNA fragments detected with the original method could be detected. Therefore subsequent HT-STELA PCR reactions were cycled 28 times as opposed to 22 cycles.

Alternative XpYp telomere-adjacent primers

Another approach taken to increase the amount of PCR product generated during STELA PCR was to increase the efficiency PCR amplification. To do this we focused on the telomere adjacent primer used to amplify telomeric DNA in the original STELA method. XpYpE2 resides 408 nucleotides from the start of the XpYp telomere. A number of similar primers were designed around this region and used in STELA PCR to replace XpYpE2, in an attempt to increase the PCR efficiency and thereby increase the amount of PCR product generated. Five additional primers were designed (E3, E4, E5, E6 and E7) (fig. 3a). The original STELA method was performed using each of these telomere adjacent primers and revealed that XpYpE5 resulted in the most intense banding pattern out of all the telomere adjacent primers, suggesting that this primer gave the greatest PCR efficiency (fig.3b). To confirm whether this increase in PCR efficiency was also detected by the Fragment Analyzer™ a similar experiment was performed in which the difference in PCR product generated by XpYpE2 and XpYpE5 was compared. The relative fluorescence units (RFU) given by the PROSize™ software reflects the signal intensity of the PCR products and thereby the amount of PCR product generated. As observed with the original STELA method the XpYpE5 primer results in a more efficient PCR and the generation of more PCR

product compared to XpYpE2 (fig. 3c). When XpYpE5 was used the average relative fluorescence was 430.3 units, approximately double the relative fluorescence achieved by the STELA PCR reaction containing XpYpE2 (244.3 RFU). Therefore subsequent HT STELA PCR was performed using XpYpE5 as opposed to XpYpE2.

Increasing the input DNA quantity

In addition to increasing the cycle number and changing the telomere adjacent primers another approach which was taken to increase PCR product signal intensity was to increase the amount of input DNA within each STELA-PCR reaction. The purpose of this approach was two-fold: not only was it to result in more PCR product being generated allowing easier fluorescent detection, it was also an attempt to produce a telomere length distribution 'smear' as opposed to a banding pattern. There are a number of reasons why a telomere length distribution smear would be beneficial: it would allow a more rapid analysis of mean telomere length as it would require less human intervention in the analyses of data within the PROSize™ software; it would require less reactions per sample; it would increase the total amount of product and it would ensure that non-specific background bands present are not incorporated into the sizing analysis.

A DNA quantity titration was used to determine the optimum amount of DNA to add to the STELA PCR reactions. In the original STELA method 10ng/μl dilutions are made from the concentrated stock DNA samples. 1μl (10ng) were taken from this dilution and added to 40μl Tris-HCl (pH8) containing tel2 linker oligonucleotide. 1μl (250pg) of this DNA/tel2 mix was then added to each PCR reaction. On the Fragment Analyzer™ this approach produced a faint banding pattern only after 28 PCR cycles (fig. 2b). To allow the detection of telomere length distributions as smears using the Fragment Analyzer™, increasing amounts of DNA was added to the DNA tel2 mix. Specifically, a 50ng/μl dilution was made from stock HT1080 cl.2 DNA. 1μl (50ng), 5μl (250ng) or 10μl (500ng) of this dilution was added to DNA/tel2 mixes. 1μl of these DNA/tel2 mixes were added to multiple 30μl HT-STELA reactions giving a final concentration of 1.25, 6.25 and 12.5ng/reaction respectively. When the generated STELA PCR products were resolved and detected by the Fragment Analyzer™ all three input DNA quantities resulted in visible telomere length distribution smears (fig. 4a)

The HT1080 cl.2 XpYp telomere length distribution is homogenous. Although XpYp telomere length distributions from CLL patients are also expected to be homogenous previous work in which the XpYp telomere length distribution was studied in a panel of CLL patients by STELA revealed that the telomere length heterogeneity varies somewhat between patients. Hence the amount of input DNA that would create a telomere length distribution smear for a homogenous telomere length distribution such as HT1080 cl.2 will not necessarily produce a smear in a sample that displays a more heterogeneous XpYp telomere length distribution. To test this, a DNA sample from a CLL patient (4408) was selected that displayed a heterogeneous XpYp telomere length distribution. DNA from this patient was used for an input DNA titration ranging from 1.25–47.5ng/reaction (fig. 4b). The input DNA amounts that produced a telomere length distribution smear in the HT1080 cl.2 cell population, did not produce a similar smear for patient 4408, instead a clear banding pattern was observed. Once the amount of DNA in the reactions was increased to 25ng DNA and above, the telomere length distribution was resolved as a smear in this CLL sample (fig. 4b); the smear was robust with a strong signal and was reproducible.

Reducing primer concentration within HT-STELA PCR reactions

A number of other factors were altered in an attempt to boost the signal intensity of the HT-STELA PCR products. These including further increasing the PCR reaction volume, diluting the PCR reactions, altering the $MgCl_2$ concentration and changing the Taq polymerase (fig. 15). None of these modifications increased the relative fluorescence of the PCR products on the Fragment Analyzer™, however reducing the primer concentration within the PCR reactions did increase PCR product formation. In the original STELA reaction conditions the final primer concentration is 0.5 μ M. A primer titration was performed using primer concentrations of 0.2, 0.5 and 0.8 μ M. A comparison of the average relative fluorescence given by each reaction set revealed that the reactions containing 0.2 μ M XpYpE5 and teltail generated more PCR product than the reactions containing 0.5 and 0.8 μ M primers (fig.5a). Because of the increased PCR product formation observed when primer concentration was reduced, the primer titration was extended further to incorporate even lower primer concentrations (0.05–0.2 μ M). However, reducing the primer concentration further did not result in an increase in PCR product formation. PCR reactions which contained 0.1 μ M XpYpE5 and teltail generated PCR products to level as the reactions containing 0.2 μ M primers.

PCR product formation began to recede as primer concentration was reduced below 0.1 μ M (fig. 5b). Therefore subsequent HT-STELA PCR reactions were performed with XpYpE5 and teltail concentrations of 0.2 μ M.

Optimizing the mean telomere length sizing accuracy using the Fragment Analyzer™

Once the conditions were optimised to give a robust and reproducible telomere length distribution smear detected with the Fragment Analyzer™, the sizing accuracy of the HT-STELA technique was compared to the original STELA method. HT-STELA and STELA was performed on a panel of CLL samples, as well as on clonal populations of the HT1080 (cl.5) and MRC5 (cl.5) cell lines (fig.6). The original STELA method revealed the expected high degree of variability in both the mean telomere length, as well the levels of heterogeneity of the XpYp telomere length distributions between samples (fig. 6a). A useful feature of the MRC5 cl.5 DNA was that it displayed a bimodal XpYp telomere length distribution (Baird et al., 2003), providing a test of the resolution for the Fragment Analyzer™ in determining whether it is able to successfully amplify and resolve the two allelic telomere length distributions separately (fig. 6a).

Quantification of the STELA PCR products was performed using Phoretix software (fig.6c; NonLinear Dynamics) and calculation of mean telomere length revealed that it varied from 1.29kb (CLL sample 4065) to 8.598kb (CLL sample 4521) (fig.6c). When the same panel of samples was analysed by HT-STELA using the newly optimised conditions a telomere length distribution smear was produced for all samples. However the intensity of the telomere length distribution smears differed between samples, suggesting differences in PCR efficiency between reaction sets (fig. 6b). One explanation for this could have been that this variation in PCR product formation could be due to differences in the input DNA quantity for each reaction set. However when comparing the input DNA quantity per reaction this does not correlate with the differences in signal intensity observed on the Fragment Analyzer™ output gel (fig. 6b). Another explanation could be that the DNA samples of the panel may contain differing levels of PCR inhibitors which could affect PCR product formation.

The correlation between HT-STELA and the original STELA method was not absolute, indeed the optimal relationship was not linear ($R^2=0.83$; fig 6c). The results are

summarised in fig. 6c. It was clear that large discrepancies in mean telomere length measurement were present between STELA and HT-STELA, particularly for longer telomeres such as those in CLL sample 4521 (fig. 6). The original STELA method revealed that HT1080 cl5 XpYp telomeres exhibit a homogenous telomere length distribution, however when this same DNA is analysed by HT-STELA a weak but considerably heterogeneous distribution is present (fig. 6a-b). Moreover the MRC5 cl5 exhibits a clear bimodal XpYp telomere length distribution (fig. 6a). However analysis by HT-STELA results in the detection of only the lower allele telomere allele on the output gel. Therefore, when the mean telomere length was measured for this sample, only the lower allele was represented. Mean telomere length was measured by the smear analysis function within the ProSize™ software (Advanced Analytical). Overall these discrepancies resulted in an underestimation in telomere length by a mean of 892bp when measured by HT-STELA. This will be problematic when using the technology in a prognostic setting.

Whilst there was a considerable improvement in the ability to detect telomere length distributions using HT-STELA with the Fragment Analyzer™ the loss of the longer telomere length distributions need to be addressed. Determination of mean telomere length rapidly, but also accurately, was required for accurate prognostication, therefore steps were taken to improve the accuracy of HT-STELA.

Cycle titration

A cycle titration was performed in which HT-STELA PCR reactions containing 10.06ng, 20.12ng or 30.18ng of HT1080 cl5 DNA were cycled for between 22-28 cycles (fig. 7a); it was apparent that the heterogeneity of the product sizes generated was dependent on the number of PCR cycles. When all three sets of HT-STELA PCR reactions were subject to 22 PCR cycles, a homogenous telomere length distribution was observed with a mean telomere length very similar to the mean telomere length measured by the original STELA method (5.822kb) (fig. 6a). 24 cycles of PCR resulted in a reduction in the mean fragment length of 263bp, 264bp and 365bp for the 10.06ng, 20.12ng or 30.18ng reaction sets respectively (fig. 7a). Further increasing the PCR cycle number results in an even greater reduction in mean telomere length to the point where after 28 PCR cycles the 1µl, 2µl and 3µl reaction sets had a mean telomere length measurement 1.741kb, 1.659kb and 2.079kb lower than the PCR reactions

cycled 22 times. From the Fragment Analyzer™ output gel it was clear that for all three sets of reactions the telomere length distribution smear extended downwards as an increased number of smaller PCR products are generated and it is this that caused such dramatic underestimations in mean telomere length: The mean telomere length measurements after 28 PCR cycles observed in fig. 7 was very similar to the HT1080 cl.5 mean telomere length measurement made when measured along with the CLL4 panel (3.618kb) (fig. 6c), suggesting this may be the cause of the mean telomere length measurement inaccuracies observed in the panel CLL of samples previously tested (fig.6). These data are consistent with amplification of non-telomeric products with high cycle numbers.

Additionally we noted that the amount of variation in mean telomere length between the different reaction sets containing different DNA quantities increased the more the reactions are cycled. After 22 PCR cycles the difference between the HT-STELA PCR reactions containing 10.06ng DNA (fig. 7ai) and 30.18ng DNA (fig. 7aiii) is 254bp. However after 28 cycles this difference was 592bp. Such differences in the concentration of the input DNA into the HT-STELA reaction may sometimes occur when this process is tested on CLL patient DNA. Using 22 cycles as opposed to 28 reduced the variation within the system and provided a more reliable comparison of mean telomere length between samples.

A cycle titration was also undertaken using DNA from a clonal population of the MRC5 fibroblast cell line (clone 5), this clone displays a bi-allelic telomere length distribution however when analysed using HT-STELA a single telomere length distributions could be detected (fig. 6). Three reaction sets were constructed containing either 6.6ng, 13.2ng or 19.8ng MRC5 cl.5 DNA. These reactions were then subject to 22, 24, 26 or 28 PCR cycles (fig. 7b). It was clear from these experiments that both the number of cycles and the amount of input DNA influenced the distributions obtained. With the 2µl and 3µl reaction sets, a reduction in the cycle numbers from 28 to 22 resulted in an improvement in the ratio of the peak height between the upper telomeric allele (UA) and the lower telomeric allele (LA), such that at 22 cycles a ratio close to expected 1:1 allelic ratio was obtained, whereas at 28 cycles the ratio was 1:10. The UA/LA ratio was also improved by reducing the cycle number from 28 to 22 in the 1µl reaction set. At 22 cycles the UA/LA ratio was 1:1.282 (fig. 7b(i)). After 28 cycles the ratio was

1:2.522 which is not as high as the 2 μ l and 3 μ l reaction sets indicating that the ratio between alleles was also influenced by the amount of input DNA, with a reduction in the amount of input DNA improving the ratio of allelic amplification. These data are consistent with a preferential amplification of shorter telomeres that becomes apparent with increasing cycle number and DNA concentration. Thus a reduction in both the cycle number and input DNA provides a better representation of the telomere length distributions.

Whilst it was clear that 22 cycles yielded a robust telomere length profile, the total signal was relatively low and close the limit below which telomere sizing becomes inaccurate (as defined later in (fig. 11)). The cycle number was therefore increased to 23 as this increased the amount of PCR product generated with no effect on mean telomere length measurement (fig. 7c).

Taken together these data demonstrate that a reduction in the PCR cycle number results in less artefactual amplification, less heterogeneity and a more accurate representation of the telomere length distribution. It also demonstrated that this could be improved still further by the optimisation of the amount of input DNA.

Marker optimisation

Another potential source of sizing inaccuracies within the HT-STELA method is the lower (75bp) and upper (20kb) markers provided with the DNF-930 dsDNA kit (Advanced Analytical) that are used for inter-capillary normalisation. During STELA-PCR a large number of fragments less than 200bp in size are generated, these are composed of primer complexes and primer-dimers. The intensity of these fragments on the Fragment Analyzer™ masked the position of the lower 75bp marker (fig. 8a) making it difficult to identify this marker. This led to incorrect normalisation of the STELA products and subsequently inaccurate telomere length measurements. A number of strategies were taken to resolve this issue. Firstly PCR purification was performed using the 96 well plate QIAquick® columns (Qiagen®) in an attempt to remove these smaller DNA fragments. Although this purification removed a significant amount of the fragments <200bp it was not sufficient to completely eliminate them and the same problem remained (fig.8b). Additionally, the PCR purification was inconsistent between samples and also removed some of the telomere derived PCR

products thereby reducing their signal intensity. It was therefore not possible to purify away the fragments that prevented detection of the lower mark and this strategy was not pursued further.

An alternative approach was to customise the markers to suit HT-STELA on the Fragment Analyzer™. We used a lower marker that was larger than the size of the larger primer complexes and primer-dimers 200bp but smaller than smallest fragment size of a telomere generated with STELA using the XpYpE5 (415bp) or the XpYpC (882bp) primers. Thus a commercially available 400bp DNA fragment was obtained for this purpose (NoLimits™, Thermo Scientific®). The upper marker was also customised for STELA: 15kb was chosen which was over 5kb larger than the longest mean telomere length observed in our cohorts of CLL or colorectal cancer tumour samples^{24, 27}. As this 15kb marker was smaller than the 20kb marker supplied with the DNF-930 kit, it will be better resolved in the gel matrix and thus sizing will be improved. These marker DNA fragments were mixed, diluted to the same concentration as the 75bp and 20kb markers supplied within the DNF-930 kit (0.5ng/μl) and then resolved on the Fragment Analyzer™ along with STELA PCR reactions (fig. 8c). The electropherogram revealed that the new 400bp marker is represented as a clear single peak that is distinct from the primer complexes and primer-dimers (fig. 6c). The resolution of the 15kb fragment was improved compared to the 20kb marker provided with the DNF-930 kit. Together these custom markers allow inter-capillary normalisation on the Fragment Analyzer™ when used for resolving HT-STELA products.

Reanalysis of mean telomere length using optimised DNA input, cycle number and the custom markers

Once HT-STELA PCR conditions were altered to improve the sizing accuracies of the method the mean telomere length of an additional panel of CLL samples as well as the clonal populations of MRC5 (cl.5) and HT1080 (cl.5) was analysed (fig.9). Strong, robust telomere length distribution smears were obtained for the samples. MRC5 cl.5 displayed a clear bi-allelic telomere length distribution and HT18080 XpYp telomeres formed a homogenous distribution similar to what is seen with the original STELA method (fig. 9a). The mean telomere length determined with HT-STELA was compared with the original STELA method. This revealed far less variation between

the two methods with a highly significant linear correlation between STELA and HT-STELA ($p < 0.0001$; $R^2 = 0.97$; fig. 9b). Before adjusting the HT-STELA conditions the relationship between STELA and HT-STELA was not linear (fig. 6c) and accurate mean telomere length measurements over 2.5kb was not possible. However the modified conditions provided a linear relationship between STELA and HT-STELA allowing accurate determination of telomere length within the full telomere length ranges analysed.

Coefficient of variance

We determined the measurement error within the HT-STELA system by calculating the inter- and intra-assay coefficient of variation. To do this we applied HT-STELA to the same MRC5 cl.5 DNA sample. Each reaction contained 30ng DNA and was subjected to 22 PCR cycles, across one 96 well plate containing 95 separate STELA reactions and one molecular weight marker. The plate was analysed three times using the Fragment Analyzer™ (fig. 10). The intra-assay coefficient of variance was determined for each of the three runs of the Fragment Analyzer™ and ranged from 1.04 to 1.51% with a mean of 1.29% (fig. 10). The inter-assay coefficient of variance was determined as 1.23% (table 2). Overall the inherent variation within the system is very low and means that robust, reproducible telomere length measurements can be made with confidence using this approach.

Quality control

It was important to incorporate a quality control measure into the HT-STELA assay to ensure that the technique maintains the high level of telomere length measurement accuracy observed in the original STELA method. In this regard an important aspect of the output data generated by HT-STELA was the total signal generated as defined by the relative fluorescence units (RFU) detectable with Fragment Analyzer™ given for each telomere length distribution smear.

To determine the lower RFU limit at which the mean telomere length measurement becomes unreliable a PCR cycle titration was performed: HT1080 cl.2 DNA was added to HT-STELA reactions which were then subject to 18-23 PCR cycles. The rationale behind this experiment was that as PCR cycle number decreases the amount of PCR product generated and hence the RFU value also decreases. The point at which

telomere length measurement becomes inaccurate can then be monitored. The signal intensity of the HT1080 cl.2 telomere smears decreased as a function of decreasing cycle numbers, such that after 18 cycles no telomere signal could be detected. Between cycle numbers 21 and 23 a robust signal was obtained (between 27-65 RFU) and the estimation of telomere length was consistent (fig. 11). The signal decreased still further at 19-20 cycles to 8-12 RFU. The telomere smear could be detected however the peak intensity of the smear was similar to that of the background and the mean telomere length estimate had decreased (fig. 11). We therefore considered that the lowest signal to provide a reliable telomere length estimate was 25 RFUs.

Although the peak RFU value is indicative of the amount of PCR product generated and detected this will not always be consistent, for several reasons: Firstly RFU will be subject to variation between different Fragment AnalyzerTM machines. Secondly the RFU measurement is taken from the highest point of a peak on the electropherogram. The heterogeneity of a telomere length distribution and hence the width of a telomere smear peak on the electropherogram is not taken into account. Therefore robust heterogeneous telomere length distributions whose RFU signal is more dispersed may be discarded as their peak height is lower than for homogenous distributions. Although this may not pose too much of a problem in CLL patients as XpYp telomere length in this disease has been shown to be homogenous it is more pertinent when applying the same criteria to other cancers which show more telomere length heterogeneity.

It was necessary to identify a unit which could be used to determine the lower limit of acceptable PCR product formation during HT-STELA without discriminating against heterogeneous telomere length distributions and would be consistent between different Fragment AnalyzersTM. The area under a telomere length distribution peak (Tel peak area) may provide a more appropriate measure of PCR product formation as it encapsulates all HT-STELA PCR products detected by the fragment analyzerTM. However the same machine-to-machine variability problem still existed and required normalization. One constant in the electrophoresis and detection process were the markers. Normalizing the telomere smear peak area value by a marker peak area value such as the lower marker peak area (LM peak area), although any marker may be selected and used consistently, would negate any such variation as differences in

signal detection of a sample plate between fragment analyzers™ would be mirrored by the same detection differences between the lower marker on a marker plate. Additionally slight capillary-to-capillary PCR product detection variation within the same assay would also be resolved. This approach was applied to the cycle titration experiment (fig.11) and as mentioned earlier a consistent estimation of telomere length was obtained when HT-STELA PCR reactions were subject to 21 -23 PCR cycles. A high Tel peak area/LM peak area percentage was also obtained for these PCR reactions (between 25.87-61.02%. Reducing the cycle number to less than 20 cycles resulted in decreased telomere length estimation and a large reduction in Tel peak area/LM peak area percentage (<8%) (fig.11). Therefore when used as a quality control measure we considered that the lowest Tel peak area/LM peak area percentage that provides a reliable telomere length estimate to be 25% when the lower marker used in the system is at a concentration of 0.5ng/μl and marker injection of 1kV for 10 seconds.

Maxwell® DNA IQ™ – DNA extraction

The use of the Fragment Analyzer™ provides a fast high-throughput method to measure mean telomere length in a large number of CLL patients. One key aspect of the STELA is the extraction and accurate quantification of DNA. Standard protocols based on phenol/chloroform extraction are time consuming, however this has largely been overcome by various methods that have been developed to allow rapid automated extraction of DNA. However one limiting factor is the requirement for quantification of each extracted DNA followed by subsequent dilution to a standard normalised concentration prior to the PCR steps of the process. The quantification steps can be overcome by extraction systems designed to extract defined amounts of DNA. We tested the DNA IQ™ Casework Pro kit (Promega®) that is run on the Maxwell® 16 automated DNA extraction system (Promega®). This system utilises paramagnetic beads that are calibrated to absorb 100ng of DNA from a cell preparation; therefore as long as the system is saturated, an equal amount of DNA should be extracted from each sample.

We first determined the minimum number of CLL B-cells from which 100ng DNA can be extracted and provide a consistent HT-STELA profile. Differing numbers of CLL B-cells (5×10^3 , 1×10^4 , 1.6×10^4 , 5×10^4 , 2×10^5 , 5×10^5 , 1×10^6 and 2×10^6) isolated from a

blood sample of a CLL patient (fig. 12a) were collected before being lysed for 1 hour and loaded into DNA IQ™ Casework Pro kits on the Maxwell® instrument for DNA extraction. DNA was eluted into 80µl of elution buffer and the tel2 oligonucleotide was added directly to the elution buffer at a final concentration of 250pM. HT-STELA was performed on the DNA/tel2 mixes and the PCR products resolved and detected with the Fragment Analyzer™ (fig. 12b). Fewer PCR products were generated from the DNA extracted from 1×10^4 and 5×10^4 cells compared to DNA extracted from greater cell numbers suggesting that this number of cells does not contain enough DNA (100ng) to saturate the DNA IQ™ system. When XpYp HT-STELA was performed on DNA extracted from 2×10^5 to 6×10^5 cells a relatively consistent telomere length profile was obtained (fig. 12b), however the number of bands and therefore the amount of DNA extracted from 8×10^5 and above appeared to decrease suggesting that this cell number is too great for the DNA IQ™ system and actually inhibits the extraction of 100ng DNA. The DNA IQ™ system appears to be saturated at 4×10^5 cells. As sufficient DNA is extracted from this amount of cells to provide a robust HT-STELA profile, this number cell was chosen for subsequent use. Furthermore this cell number is relatively small and thus small samples can be reliably analysed.

Once the cell number at which the DNA IQ™ system was saturated had been determined, the reproducibility of the HT-STELA process using DNA extracted from this cell number was assessed. DNA was extracted from eight independent replicates of 4×10^5 CLL cell pellets. DNA from four of the eight replicates were eluted into 40µl elution buffer whereas the other four were eluted into 80µl which should result in a two fold reduction in the DNA concentration. HT-STELA was performed using the eluted DNA (fig. 12c). A relatively consistent telomere length distribution profile was observed between the 40µl eluate replicates and between the 80µl eluate replicates, with a more intense banding pattern observed in the 40 µl elution compared to the 80µl elution. Although overall the DNA concentration given by the Nanodrop™ 3300 (Thermo Scientific®) was higher for the 40µl eluate replicates it was not double the concentration of the 80µl eluate; indicated that DNA elution is less efficient at 40µl compared to 80µl.

The relatively consistent telomere length distributions given using DNA extracted from the different eluate replicates from the DNA IQ™ system was encouraging, however

they consisted of a banding pattern as opposed to the desired telomere length distribution smear. In an attempt to achieve this smear an input DNA titration was performed using DNA from one of the replicates (80-4): 1µl, 3µl or 5µl was added to multiple HT-STELA PCR reactions (fig. 12d). When 1µl DNA is added per reaction the similar banding pattern to what was seen in fig. 12c was evident, however when the input DNA was increased to 3µl and 5µl a robust telomere length distribution smear was observed (fig. 12d). HT-STELA was then repeated for all the 4×10^5 replicates but with 3µl DNA added to PCR reactions as opposed to 1µl (fig. 12e). Robust telomere length smears were generated instead of banding patterns across all the PCR reactions for every replicate.

These experiments allowed us to optimise the DNA IQ™ system to provide robust HT-STELA smears.

Validation of HT-STELA in a CLL patient cohort

We tested the HT-STELA system in a cohort of 15 CLL samples. Blood samples were obtained and CD19+ cells purified using MACS® whole blood CD19 microbeads and an AutoMACS® Pro separator (Milenyi Biotech). Isolated cells were then counted using a VI-cell instrument (Beckman Coulter) and aliquoted into 4×10^5 cell pellets. The cell pellets were lysed for 1 hour and DNA was extracted from the lysates using the DNA IQ™ protocol Maxwell® 16 instrument (Promega®) and eluted into 40µl elution buffer. A portion of this eluted DNA was taken to be analysed by STELA (fig. 13b) and the rest was used for HT-STELA analysis. Briefly tel2 linker oligonucleotide was added to the eluted DNA to a final concentration of 250pM and 3µl DNA/tel2 mix was added to the 30µl HT-STELA PCR reactions, containing 0.2uM XpYpE5 and cycled 23 times. The PCR products were resolved and detected using the Fragment Analyzer™ (fig. 13a). Mean XpYp telomere length for each individual reaction was measured using the smear analysis function within the ProSize™ software and the resulting mean telomere lengths for the three replicates for each patient were combined and averaged giving a final XpYp mean telomere length. For the original STELA method band quantification using Phoretix software (Nonlinear Dynamics) was performed and mean XpYp telomere length was subsequently calculated. The mean telomere length measured by both techniques were then compared (fig. 13c). The output gel from the ProSize™ software shows that XpYp telomere length distribution smears were generated for 14 of the 15 samples tested. Importantly all the RFU values were greater

than the 25 RFU threshold which we had defined as the lower limit from which an accurate reproducible telomere length measurement could be made. The telomere length distributions generated with the HT-STELA and the original STELA methods were remarkably similar (fig. 13a&b) and the length estimates were significantly correlated ($R^2=0.97$; fig. 13c).

Patient stratification based on XpYp telomere length determined using STELA together with the fusogenic telomere length threshold, provides high resolution prognostic information for CLL patients (Lin et al., 2014). The entire HT-STELA process from a blood sample of a CLL patient, to the generation of a robust telomere length profile can be performed within 7.5 hours. The development of HT-STELA now facilitates the application of this technology for the routine prognostic assessment of CLL patients, as well as patients with other tumour types for which the prognostic thresholds are informative.

HT-STELA at additional chromosome ends

Once a reproducible HT-STELA assay was developed for the XpYp chromosome end, the technique was developed to allow the measurement of mean telomere length at additional chromosome ends. To achieve specificity for a particular chromosome end telomere-adjacent primers were designed incorporating telomere specific nucleotides at the 3' ends. It is this design parameter which gives specificity to one chromosome end as a mismatch will occur when the primer anneals to other subtelomeric sequences from other chromosome ends that exhibit high levels of sequence homology, resulting in failure of the Taq polymerase to extend from the primer in a PCR reaction and hence failure to produce a PCR product from other telomeres. Using this primer design approach a number of primers were designed for telomere adjacent regions at the 7q and 12q chromosome ends. Several primers specific to the 17p telomere adjacent region were already available as were additional XpYp specific primers. These primers were tested under the same conditions as XpYpE5 to determine whether they could generate telomere length distribution smears using HT1080 cl.2 DNA for their respective chromosome end (fig. 14).

At the 7q chromosome end primers designed around the 7q-specific nucleotides 1115T, 551A, 195G and, to a lesser extent, 29G produced robust telomere length

distribution smears (fig. 14a). The mean molecular weight of the fragments calculated using the smear analysis tool on the ProSize™ software showed the expected increase that was dependent on the distance from the start of the telomere repeat array. Calculation of the mean telomere length from these telomere length distribution smears by subtracting the distance from the primer to the start of the telomere repeat array resulted in a similar mean telomere length estimate irrespective of the distance from the start of the telomeres. 7q telomere length estimates with HT-STELA were comparable to those determined with the original STELA method (fig. 14a; fig. 16).

The HT-STELA development for the 12q chromosome end was also successful for some of the primers designed. Primers 350G and 550T (350 & 550 nucleotides from the TTAGGG repeats respectively) generated telomere length distribution smears with comparable RFU values. Like at 7q, the 12q mean telomere length calculated by HT-STELA was very similar to that calculated using the original STELA method (fig. 14b). The primer designed around the 1388G 12q specific nucleotide produced a very weak telomere distribution. The RFU values for this are too low for a mean telomere length measurement to be given with confidence and will therefore not be used in future experiments

Primers had previously been designed specific to the telomere-adjacent region at the 17p chromosome end and a further 8 primers were designed extending up to 6877bp from the telomere repeat array. All of the primers up to 3843bp from the telomere repeat tested in HT-STELA yielded smears consistent with telomeric amplification (fig. 14c). With the exception of 3843 bp (17p8) the HT-STELA smears could be quantified. The smear generated with 17p8 could not be quantified as it was too close to the 15 kb upper marker. Again there was a clear increase in the molecular weight of the amplified products as a function of the distance of the primer from the start of telomere repeat array, which following the subtraction of the telomere-adjacent sequences resulted in a consistent estimation of 17p telomere length from five different 17p primers with a mean of 6.5kb (fig. 14c). Interestingly this estimate was greater than that derived with the original STELA method, however as five separate primers yield the same length, we considered that the HT-STELA represented a more robust estimation of telomere length compared to the original STELA method using primer 17pseq1rev. We speculate that this may be related to the use of Southern

hybridisation to detect the telomere profiles with the original STELA method and the presence of telomere variant repeats that do not hybridise to the TTAGGG repeat sequence used or the hybridisation probe. These data also showed that robust HT-STELA profiles were generated using primers at least as far as 3.843 kb from the start of the telomere repeat array. All the 17p specific primers produced robust telomere length distributions with the exception of 17pseq1B located 1 nucleotide from the telomeric repeat tract. When this primer was used in HT-STELA a ladder-like distribution was observed that was not consistent with the robust amplification of a telomere (fig. 14c). Interestingly the performance of a telomere adjacent primer seems to be diminished if it located less 30 nucleotides from the telomeric repeat tract. Both 7q29G and 17pseq1B performed poorly compared to other primers designed further from the telomeric repeat tract at their respective chromosome ends.

The success of the XpYp telomere adjacent primers was more varied compared to that observed at the 17p telomere. XpYpM and XpYpO (1653 and 1625nt from TTAGGG respectively), or primers XpYpD, XpYpF, XpYpH and XpYpI all greater than 4.8 kb from the telomere, did not generate any PCR product whatsoever (fig. 14d). As expected XpYpE5 produced a telomere length distribution smear with a mean telomere length similar to what was generated using the original STELA method. XpYpB (2539 bp from the telomere) produce a smear of the expected size. XpYpC also produced a similar telomere length distribution, but with a higher RFU value than XpYpE5 suggesting that XpYpC is more efficient at generating XpYp telomere products and therefore XpYpC could be used instead of XpYpE5 for subsequent XpYp HT-STELA (fig. 14d). XpYpJ also produced a telomere length distribution smear however it was very weak. Like 7q29G and 17pseq1B, XpYpJ is located less than 30nt from the telomeric repeat tract and has failed to produce a robust telomere length distribution smear suggesting that there is a lower limit distance from the TTAGGG repeat tract below which primers cannot be designed which can sufficiently amplify telomeres.

These data demonstrate that HT-STELA can be applied to additional chromosome ends and that primers up to at least 3.843 kb from the start of the telomere repeat array yield robust telomere profiles. However it was also apparent that primers within 30bp of the telomere did not produce efficient or specific amplification.

Simplifying Target Amplification

The telomere lengths observed in samples derived from peripheral blood of normal individuals as detailed above can display considerable heterogeneity, with a diverse range of lengths ranging from close to zero to 25 kb or more. This can occasionally present a particular problem for PCR based amplification of telomeres, whereby the shorter telomeres are likely to amplify more efficiently than the longer telomeres, resulting in an underestimate of mean telomere length.

In such circumstances, we tested the concept of pre-digesting DNA with a restriction enzyme prior to HT-STELA, the idea being that it will reduce the complexity of the input DNA by digesting the non-telomeric DNA and provide a simpler target for amplification.

DNA samples were extracted and digested with the restriction enzyme as detailed in the Material and Methods. The choice of enzyme will depend upon the nature of the telomere repeat array to be amplified and it will be necessary to ensure that it does not cut between the 5' end of the oligonucleotide primer and chromosome telomere end. As proof of principle EcoR1 was chosen because it does not cut DNA between the 5' end of the XpYpC primer used for HT-STELA and the telomere repeat array, such that non-telomeric loci and other telomeres maybe cut, but the XpYp telomere will remain intact. Other enzymes could also conform to this specific requirement.

The DNA samples with, or without, EcoR1 digestion were subjected to HT-STELA and analysed with a Fragment Analyzer™ (AATI) (fig. 17). It was apparent that EcoR1 digestion prior to a HT-STELA successfully reduced the background signal, resulting the improved definition of the telomere smear.

Further experiments were undertaken to test the conditions for amplification, by titrating the numbers of PCR cycles and the amount of EcoR1 digested input DNA (fig. 18). It was clear from these experiments that an input DNA amount of 25 ng and 21-23 PCR cycle were optimal.

Adaptation of HT-STELA for pre-purified DNA samples

The above methodology was established for the analysis of telomere length in fresh/frozen cell and tissue samples obtained from either haematological or solid tumours. We next explored the ability to explore the technology using alternative DNA sample platforms, specifically previously extracted DNA samples. The ability to analyse previously extracted DNA samples expands the utility of HT-STELA, by for example, allowing the analysis of patient cohorts from clinical trials many of which will only have tumour DNA samples available, or indeed the analysis of any archived DNA sample.

The conditions under which the DNA IQ™ system is saturated by DNA was determined. To do this increasing amounts (200, 500, 1000 and 2000ng) of HT1080 cl2 DNA was added to DNA IQ™ lysis buffer and DNA extracted using the standard protocols on the Maxwell instrument (Promega®). This was performed twice: once using the DNA IQ™ casework pro kit (used when extracting DNA from CLL cell pellets) and also using the DNA IQ™ reference kit (fig. 19). The two kits differ in that a low elution volume (LEV) protocol is used with the casework pro kit whereas a standard elution volume (SEV) protocol is used with the reference sample kit. DNA is eluted into 80µl using the casework pro kit and into 300µl using the reference sample kit.

Differing amounts of eluted DNA was then tested in HT-STELA reactions. For the casework pro kit samples either 3, 6 or 9µl eluted DNA was added for each input DNA quantity added to the DNA IQ™ system. For the reference sample kit, due to the larger elution volume, 3, 10 and 20µl of eluted DNA was added to HT-STELA reactions. Again this was performed for each DNA quantity added to the DNA IQ™ system. The HT-STELA reactions were then subject to PCR under the same conditions as previously described and the resulting PCR products were resolved by capillary electrophoresis using the Fragment Analyzer™ (Advanced Analytical; fig. 19).

The quantity of DNA added to the DNA IQ™ casework pro kit cartridges had little impact on the resulting telomere length distribution smears. Whether adding 200, 500, 1000 or 2000ng to the system the resulting telomere length distribution smears appeared similar both in terms of MTL and normalised tel peak area % suggesting that the system is already at saturation point when 200ng is added to it (fig. 19a). A factor

which made more of an impression on the final telomere length distribution smear was the amount of eluted DNA added to HT-STELA reactions. The increase in DNA quantity added to HT-STELA reactions is reflected by the increase in signal intensity of the telomere length distribution smears for each DNA quantity set and for both kit types. For the DNA IQ™ casework pro kit, regardless of input DNA quantity, adding 6µl or less of the eluted DNA to the HT-STELA PCR reactions is not sufficient to generate a telomere length distribution smear with a normalised tel peak area % higher than the lower 25% threshold that we had previously defined as a quality threshold. Adding 9µl of eluted DNA to HT-STELA reactions produced a more robust telomere length distribution smears which, with exception of the 500ng samples, surpassed the lower normalised tel peak area % threshold. The MTL given by all of the 9µl replicates ranged from 6.084-6.357kb which is comparable to the HT1080 cl2 positive control MTL of 6.368kb.

Unlike the DNA IQ™ casework pro kit, the input DNA amount added to the DNA IQ™ reference sample cartridges affected the telomere length distribution smears observed on the HT-STELA output gel (fig. 19b). Adding DNA eluted from 200ng of input DNA into the HT-STELA reactions resulted in barely detectable telomere length distribution smears regardless of how much of the DNA was added to the reactions. Only when input DNA amount was increased to 500ng and above did the telomere length distribution smears become apparent. However this only occurred when 10µl or more eluted DNA was included in the HT-STELA reactions. This suggests that the DNA IQ™ system using the reference sample kit is not saturated using these DNA quantities. The only HT-STELA reaction that generated sufficient signal to surpass the 25% normalised tel peak area lower threshold, was when 20µl of the DNA eluted from the 2000ng DNA input was added to the HT-STELA reactions. Although this generated an HT-STELA signal of sufficient intensity and with an accurate MTL (6.224kb), however these conditions are not practical as too much DNA (2000 ngs) was required to generate a reliable signal. Thus DNA normalisation performed with the DNA IQ™ casework pro kit was preferred to the DNA IQ™ reference sample kit.

Our data showed that adding 9µl of eluted DNA from the DNA IQ™ casework pro kit with input DNA greater than 200 ngs resulted in telomere length distributions that yielded an accurate MTL and were above the 25% lower tel peak area % threshold.

However the tel peak area % was only marginally above the lower 25% threshold (mean 26%). For this reason and to test the reproducibility of the system a follow up experiment was performed in which either 200ng, 500ng or 1000ng DNA was added to the DNA IQ™ casework pro system (fig. 20a). Each DNA quantity had four replicates. In an attempt to increase the signal intensity and thereby the robustness of the HT-STELA assay 12µl of eluted DNA was added to HT-STELA reactions instead of the 9 µl used previously. The HT-STELA reactions were then subject to PCR and resulting PCR products were resolved by capillary electrophoresis. Figure 20a shows that the increase in DNA made the assay more robust with an increased tel peak area %. Although for two of the replicates (200-3 & -4) this value is still slightly below the lower 25% threshold, for the rest of the replicates it exceeds the threshold. The tel peak area % range increased with a range of 24.44 to 41.63% with a mean of 34% whereas when 9µl eluted DNA was added per reaction the range was 19.04-27.2% with a mean of 26%.

The XpYp mean telomere length measured from the PCR products generated from the normalised DNA samples was accurate ranging from 5.868kb-6.106kb which spans the 5.956kb measured MTL of the positive control HT1080 cl2 DNA. This MTL range gave an overall coefficient of variance of 1.65% (fig.20b). The %CV between the input DNA quantity replicates was similarly very low – 1.397% for 200ng, 2.152% for 500ng and 1.473% for 1000ng.

Together these experiments demonstrate that DNA samples containing 200ng DNA or more can be normalised using the DNA IQ™ casework extraction kit in conjunction with the Maxwell® 16 DNA extraction system thereby negating the need for time consuming DNA quantification. Furthermore adding 12µl of this eluted DNA to HT-STELA reactions can produce robust and reliable telomere length distribution smears from which highly accurate mean telomere length can be measured.

Adaptation of HT-STELA for DNA sample cards

We also explored the adaptation of HT-STELA to allow the analysis of blood samples stored on sample cards (such as Whatman® FTA/FTA Elute cards). FTA cards are a commonly used format for the convenient sampling and storage of blood samples for

subsequent downstream DNA analysis. It provides a quick method for patients that may require a telomere test, to provide a sample at home, without the requirement of a clinician or phlebotomist. A small blood sample is spotted onto an FTA card then returned to the testing facility by standard mail services. This will also have utility in the analysis of trial cohorts that have samples stored on FTA card.

To test the system, 125µl blood from three CLL patients was spotted onto FTA cards and allowed to air-dry overnight. The three patients had differing white blood cell (WBC) counts; patient 71 had a WBC count of 45×10^6 cells/ml whereas patient 74 had a count of 6×10^6 cells/ml and patient 75 had a WBC count of 101×10^6 cells/ml. The rationale behind attempting this procedure with patients with differing WBC counts was that it would give an indication as to the lower limit WBC count which would provide enough cells and hence enough DNA to be extracted for a reliable, robust telomere length distribution smear to be generated.

DNA was extracted from two 3mm punches of a dried blood spot with casework extraction buffer (Promega®) for 1 hour at 65°C with shaking at 1400rpm. The eluted blood cell lysates were then separated from the stripped punches and DNA was extracted with the Maxwell® automated DNA extraction system using the LEV blood kit. Negative control punches were also included (FTA card with no blood). As stated in the protocol (Promega®) LEV blood cartridges were used for the DNA extraction and DNA was eluted into 50µl elution buffer. 3µl of eluted DNA was added to each HT-STELA reaction in the same manner as previously mentioned. These reactions were then subject to 23 rounds of PCR amplification and the DNA fragments resolved with the Fragment Analyzer™ (Advanced Analytical; fig.21a). As expected the negative control did not produce any PCR products. However telomere length distribution smears were generated from DNA extracted from the dried blood of patients 71 and 75. The calculated mean telomere length (MTL) of these patients using this FTA-STELA method was very similar to MTL measured by STELA: for patient 71 the MTL for FTA-STELA was 3.889kb compared to 4.169kb measured by STELA. Similarly, for patient 75 the FTA-STELA derived MTL 2.036kb was very similar to the STELA MTL of 1.856kb. No telomere length distribution profile could be generated for patient 74. This doesn't appear to be due to the low WBC count (6×10^6 cells/ml) as the number of cells on two 3mm punches of this dried blood spot was estimated to contain 7.5×10^4

cells which, although less than the than the 4×10^5 cells required to produce robust telomere length profile, there should still have been sufficient DNA to yield a detectable profile (fig. 12b). Alternatively this negative result may be due to a technical issue that occurred within the experiment. The number of cells on two 3mm punches of the dried blood spots of patients 71 and 75 was estimated to be 5.63×10^5 and 1.575×10^6 . This difference in cell number is reflected not only in the difference in concentration of the eluted DNA (71 - $8.793 \text{ ng}/\mu\text{l}$, 75 – $70.113 \text{ ng}/\mu\text{l}$) but also in the tel peak area % of the telomere length distribution smears of the two patients (71 – 44.79%, 75 – 138.89%).

Large differences in both the concentration of the eluted DNA and the tel peak area % generated from the telomere length distribution smears was observed between the different samples analysed (fig.21a). We therefore tested DNA IQ™ Casework Pro kit in an attempt to normalise the DNA eluted from the FTA card extracts. We have previously shown that the number of cells known to saturate the DNA IQ™ system and therefore normalise DNA concentration is 4×10^5 . We estimated that this cell number would be present on two 3mm punches of a dried blood spot from a patient with a WBC count of 32×10^6 cells/ml. It was also likely however, that a telomere length distribution smear could still be generated from patients with a WBC count as low as 16×10^6 cells/ml a two 3mm FTA card punches from such a patient was estimated to contain 2×10^5 cells, a cell number that we have previously shown to produce a HT-TELA profile (fig. 12b). To test this an additional patient was included in the analysis: Patient 89, with a WBC count of 20×10^6 cells/ml. DNA was extracted from the dried blood spot of this patient as well as from the FTA sample of patient 91 (WBC count – 69×10^6 cells/ml), together with patients 71 and 75. Patient 74 was discounted as a telomere length profiles could not be achieved with either TELA, HT-TELA or FTA TELA.

The concentrations of the eluted DNAs indicate that the DNA IQ™ system successfully normalised the DNA eluted from each of the samples (fig. 21b). The DNA concentration ranged from 4.576 to $6.027 \text{ ng}/\mu\text{l}$, a lower range than observed when DNA was extracted using the LEV blood kit. Telomere length distribution smears were generated from all four eluted DNA samples (fig. 21b). Three of these four telomere length distribution smears were robust and exceeded the 25% lower threshold for tel peak area %. One of these robust telomere length distributions was generated from

DNA extracted from the dried blood of patient 89 (WBC count – 20) indicating that patients with a WBC count less than 32×10^6 can have XpYp MTL measured by FTA-STELA. The lower limit of WBC count for FTA-STELA is still unknown and will require elucidation in future assays. The MTL measured by FTA-STELA for patient 89 was 2.962kb, very similar to the MTL measured by STELA (2.75kb). The FTA-STELA MTL of patients 71 and 75 were similarly accurate: Patient 71 FTA-STELA MTL was 3.889kb compared to 4.169 as measured by STELA. Likewise patient 75 FTA-STELA MTL of 2.068kb was very similar to the STELA MTL of 1.856kb.

A less intense telomere length distribution smear was generated from the DNA extracted from the dried blood spot of patient 91 as reflected by the reduced tel peak area % (fig. 21b). A reduction in signal intensity has previously been shown to result in a decrease in accuracy in measuring mean XpYp telomere length and also increases variation between biological replicates (fig. 11). Indeed reduced accuracy in MTL measurement was seen in this sample as the FTA-STELA MTL of 6.293kb is a significant overestimation compared to the STELA measurement of 5.008kb.

Given the success of generating robust telomere length profiles from CLL patients blood samples stored on FTA cards, we undertook a larger study to test the ability of FTA-STELA to accurately measure mean XpYp telomere length in CLL patients. FTA STELA was performed in parallel with STELA on a cohort of CLL patients and mean XpYp telomere length measured by both techniques was compared. In total DNA was extracted from the dried blood of 26 CLL patients. FTA-STELA was performed and the resulting MTL measured was compared to the MTL generated with HT-STELA (fig. 21c). A clear correlation between the MTL determined with both techniques was observed ($R^2=0.9084$) suggesting that FTA-STELA can be used to measure mean XpYp telomere length in CLL patients accurately.

We have shown that the HT-STELA method can be adapted for the analysis of blood samples stored on FTA cards, this method will likely be applicable to other card based sample storage systems. This adaptation will provide considerable additional utility to the HT-STELA method by allowing patients to use standard self-sampling kits and send these direct to the testing facility for analysis. This will provide considerable cost savings in terms of blood sample acquisition and upstream processing.

Summary

Telomere length analysis, as representative of telomere dysfunction, provides a highly prognostic tool in human diseases, permitting considerable discrimination for clinical outcome following treatment. Moreover, prognostic power should enable clinicians to confidently predict the clinical course of these diseases and tailor treatments accordingly.

Therefore, a reliable method for measuring telomere length in a high throughput context represents a significant contribution to the ability of clinicians to determine, manage and treat telomere shortening illnesses.

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Table-1.

Chromosome end	Primer name	Genomic Coordinates (HG38)	Sequence
2p	2p2	chr2: 10755	GAGCTGCGTTTTGCTGAGCAC (SEQ ID No: 1)
4q	4qK1	chr4:190122098	ACGGTGGCATGCCTCTCTC (SEQ ID No: 2)
4p	4p4	chr4:10457	TCTGCGCCTGCGATGGCGCTATG (SEQ ID No: 3)
5p	5p3	chr5:12240	GCATTCTCTTCACCACAGATGTTG (SEQ ID No: 4)
7q	7q1533C 7q1115T 7q843T 7q551A 7q195G 7q29G	chr7:159334337 chr7:159334755 chr7:159335027 chr7:159335319 chr7:159335675 chr7:159335841	CCCACACAGTCATCTATTGTT (SEQ ID No: 5) GAGGTGCAGTAGTGGGGATCTAACT (SEQ ID No: 6) GGGACAGCATATTCTGGTTT (SEQ ID No: 7) GCACAGCCTTTGGGGTACCA (SEQ ID No: 8) AGTGGGAGATCCACACCGTAGCGTG (SEQ ID No: 9) CCaTGCAGTGCTAAGACAGCAATGAG (SEQ ID No: 10)
11q	11q13B	chr11:135076467	CAGACCTTGGAGGCACGGCCTTCG (SEQ ID No: 11)
12q	12q1511C 12q1387G 12q1036C 12q550T 12q350G 12q319G	chr12:133263363 chr12:133263487 chr12:133263838 chr12:133264324 chr12:133264524 chr12:133264555	CCTCTGGTCATTATGAATAGGGCTTC (SEQ ID No: 12) GGGGAAAAAATGCCCAAG (SEQ ID No: 13) CCTTCTCTTCTTGATGTC (SEQ ID No: 14) ACAGCCTTTTGGGGTACCGT (SEQ ID No: 15) GGCTTCATTGATGGTGAATACAATCG (SEQ ID No: 16) GCAGCGCTGAATATTCAGGGTG (SEQ ID No: 17)
16p	16prev1	chr16:10241	CACTTATTAGTTCAGTCTCTG (SEQ ID No: 18)
17p	17p8 17p7 17p2 17pseq4rev 17pseq2rev		AGAAGCAGCGAGGAGCTTCA (SEQ ID No: 19) CCTGGCATGGTATTGACATG (SEQ ID No: 20) GCTAGGAATGGAATCATTGACTC (SEQ ID No: 21) GATACTGGGAGGATCATATCTGGC (SEQ ID No: 22) CCATTAGCCTGTGGGGTCTGAT (SEQ ID No: 23)

	17pseq1rev 17pseq1B		GAATCCACGGATTGCTTTGTGTAC (SEQ ID No: 24) AAGCAGGTTGAGAGGCTGAGG (SEQ ID No: 25)
18q	18qrev4M	chr18:80262147	CACAGGGATGGTTAGGTATCTC (SEQ ID No: 26)
XpYp	XpYpB XpYpM XpYpO XpYpC XpYpP XpYpE2 XpYpE3 XpYpE4 XpYpE5 XpYpE6 XpYpE7 XpYpJ	chrX:12573 hg38 chrX:11688 chrX:11660 chrX:10896 chrX:10575 chrX:10427 chrX:10425 chrX:10429 chrX:10432 chrX:10437 chrX:10439 chrX:10064	CGAGCAAGCATCGGAACGTGACT (SEQ ID No: 27) ACCAGGTTTTCCAGTGTGTT (SEQ ID No: 28) CCTGTAACGCTGTTAGGTAC (SEQ ID No: 29) CAGGGACCGGGACAAATAGAC (SEQ ID No: 30) ACCAGGGGCTGATGTAACG (SEQ ID No: 31) TGTCTCAGGGTCCTAGTG (SEQ ID No: 32) TCTCAGGGTCCTAGTGTG (SEQ ID No: 33) GTTGTCTCAGGGTCCTAG (SEQ ID No: 34) GGGGTTGTCTCAGGGTCC (SEQ ID No: 35) TTCTAGGGGTTGTCTCAG (SEQ ID No: 36) TCTTCTAGGGGTTGTCTC (SEQ ID No: 37) CTAATCTGCTCCCWCCCAC (SEQ ID No: 38)

Table-2.

Lower allele										Upper allele									
Well	Sample ID	Range	gel 1	gel 2	gel 3	Average	SD	%CV		Well	Sample ID	Range	gel 1	gel 2	gel 3	Average	SD	%CV	
A1	SampA1	2000 bp to 4500 bp	2968	3040	3038	3015.333	41.00406	1.359852		A1	SampA1	6000 bp to 9000 bp	7507	7499	7458	7488	26.28688	0.351053	
A2	SampA2	2000 bp to 4500 bp	3020	3071	3020	3037	29.44486	0.969538		A2	SampA2	6000 bp to 9000 bp	7914	7747	7501	7720.667	207.7555	2.690901	
A3	SampA3	2000 bp to 4500 bp	2950	3035	2994	2993	42.50882	1.420275		A3	SampA3	6000 bp to 9000 bp	7487	7534	7335	7452	104.0144	1.395792	
A4	SampA4	2000 bp to 4500 bp	2948	3017	3022	2995.667	41.35618	1.380533		A4	SampA4	6000 bp to 9000 bp	7489	7512	7458	7486.333	27.09859	0.361974	
A5	SampA5	2000 bp to 4500 bp	2938	3047	2969	2984.667	56.16345	1.881733		A5	SampA5	6000 bp to 9000 bp	7662	7498	7402	7520.667	131.4737	1.748165	
A6	SampA6	2000 bp to 4500 bp	2964	2985	2982	2977	11.35782	0.381519		A6	SampA6	6000 bp to 9000 bp	7494	7465	7448	7469	23.25941	0.311413	
A7	SampA7	2000 bp to 4500 bp	2967	3025	3039	3010.667	38.371	1.274502		A7	SampA7	6000 bp to 9000 bp	7479	7439	7414	7444	32.78719	0.440451	
A8	SampA8	2000 bp to 4500 bp	3116	3039	3040	3065	44.17013	1.441113		A8	SampA8	6000 bp to 9000 bp	7711	7527	7402	7546.667	155.4359	2.059564	
A9	SampA9	2000 bp to 4500 bp	2962	3030	3007	2999.667	34.58805	1.153063		A9	SampA9	6000 bp to 9000 bp	7515	7503	7433	7483.667	45.28604	0.501781	
A10	SampA10	2000 bp to 4500 bp	2970	3048	2968	2995.333	45.62163	1.52309		A10	SampA10	6000 bp to 9000 bp	7574	7578	7366	7506	121.2601	1.615509	
A11	SampA11	2000 bp to 4500 bp	3132	3028	2952	3037.333	90.36223	2.975052		A11	SampA11	6000 bp to 9000 bp	7698	7500	7321	7506.333	188.5798	2.512276	
A12	SampA12	2000 bp to 4500 bp	2994	3045	2992	3010.333	30.03886	0.997858		A12	SampA12	6000 bp to 9000 bp	7466	7410	7349	7408.333	58.5178	0.789852	
B1	SampB1	2000 bp to 4500 bp	2950	3016	2979	2981.667	33.08071	1.10947		B1	SampB1	6000 bp to 9000 bp	7487	7497	7367	7450.333	72.34178	0.970987	
B2	SampB2	2000 bp to 4500 bp	2974	3059	2980	3004.333	47.43768	1.578975		B2	SampB2	6000 bp to 9000 bp	7600	7544	7421	7521.667	91.56601	1.213763	
B3	SampB3	2000 bp to 4500 bp	2982	3005	3036	3008	27.0555	0.899451		B3	SampB3	6000 bp to 9000 bp	7462	7464	7379	7435	48.50773	0.652424	
B4	SampB4	2000 bp to 4500 bp	2981	3019	3065	3020.667	42.194	1.396844		B4	SampB4	6000 bp to 9000 bp	7511	7539	7461	7503.667	39.53171	0.525952	
B5	SampB5	2000 bp to 4500 bp	3005	3074	3052	3043.667	35.24675	1.158036		B5	SampB5	6000 bp to 9000 bp	7524	7478	7264	7432	131.7046	1.637575	
B6	SampB6	2000 bp to 4500 bp	2966	2809	2966	2913.667	90.64399	3.110994		B6	SampB6	6000 bp to 9000 bp	7451	7852	7298	7533.667	286.102	3.797546	
B7	SampB7	2000 bp to 4500 bp	2962	3049	3002	3004.333	43.54991	1.449407		B7	SampB7	6000 bp to 9000 bp	7438	7466	7311	7405	82.60145	1.115482	
B8	SampB8	2000 bp to 4500 bp	2969	3068	2942	2993	66.34003	2.216506		B8	SampB8	6000 bp to 9000 bp	7486	7543	7238	7422.333	162.1614	2.184777	
B9	SampB9	2000 bp to 4500 bp	2978	3056	3014	3016	39.03844	1.294378		B9	SampB9	6000 bp to 9000 bp	7527	7478	7437	7480.667	45.05922	0.602342	
B10	SampB10	2000 bp to 4500 bp	2970	3075	2993	3012.667	55.1936	1.832051		B10	SampB10	6000 bp to 9000 bp	7403	7486	7341	7410	72.75301	0.981822	
B11	SampB11	2000 bp to 4500 bp	2942	3030	2971	2981	48.84412	1.504333		B11	SampB11	6000 bp to 9000 bp	7516	7484	7386	7461	61.02499	0.817367	
B12	SampB12	2000 bp to 4500 bp	2875	3069	3007	3017	47.79121	1.584064		B12	SampB12	6000 bp to 9000 bp	7495	7490	7399	7461.333	54.04011	0.724269	
C1	SampC1	2000 bp to 4500 bp	2885	3053	3022	3020	34.04409	1.127288		C1	SampC1	6000 bp to 9000 bp	7623	7581	7499	7554.667	67.98774	0.886754	
C2	SampC2	2000 bp to 4500 bp	2950	3003	3039	2997.333	44.76978	1.493654		C2	SampC2	6000 bp to 9000 bp	7556	7524	7404	7494.667	60.13322	1.069203	
C3	SampC3	2000 bp to 4500 bp	2923	3052	3007	2994	65.47519	2.18668		C3	SampC3	6000 bp to 9000 bp	7520	7415	7446	7460.333	53.94751	0.725125	
C4	SampC4	2000 bp to 4500 bp	2881	3002	3009	2964	71.96527	2.427978		C4	SampC4	6000 bp to 9000 bp	7480	7542	7444	7488.667	48.5715	0.661954	
C5	SampC5	2000 bp to 4500 bp	2994	3027	3006	3009	16.70329	0.555111		C5	SampC5	6000 bp to 9000 bp	7543	7508	7388	7479.667	61.29168	1.068935	
C6	SampC6	2000 bp to 4500 bp	2990	3083	3031	3034.667	46.6083	1.535982		C6	SampC6	6000 bp to 9000 bp	7541	7421	7429	7463.667	67.03198	0.893914	
C7	SampC7	2000 bp to 4500 bp	3000	3047	3014	3020.333	24.13158	0.798971		C7	SampC7	6000 bp to 9000 bp	7484	7475	7346	7435	77.20751	1.038433	
C8	SampC8	2000 bp to 4500 bp	3135	3018	3030	3061	46.36614	2.102781		C8	SampC8	6000 bp to 9000 bp	7734	7455	7465	7551.333	158.273	2.059361	
C9	SampC9	2000 bp to 4500 bp	2970	3057	2963	2996.667	52.36729	1.747538		C9	SampC9	6000 bp to 9000 bp	7538	7510	7317	7455	120.3287	1.614067	
C10	SampC10	2000 bp to 4500 bp	3062	3007	2924	2997.667	69.47382	2.31735		C10	SampC10	6000 bp to 9000 bp	7464	7462	7279	7468.333	192.5781	2.578595	
C11	SampC11	2000 bp to 4500 bp	2963	2687	3037	2895.667	184.4596	6.370194		C11	SampC11	6000 bp to 9000 bp	7429	7113	7317	7286.333	160.2165	2.198663	
C12	SampC12	2000 bp to 4500 bp	2937	3038	3051	3008.667	62.40459	2.074161		C12	SampC12	6000 bp to 9000 bp	7434	7434	7378	7415.333	82.33162	1.421831	
D1	SampD1	2000 bp to 4500 bp	2987	3027	3040	3018	27.62245	0.915257		D1	SampD1	6000 bp to 9000 bp	7537	7515	7399	7483.667	74.144	0.990744	
D2	SampD2	2000 bp to 4500 bp	2954	2936	2988	2959.333	26.40707	0.892332		D2	SampD2	6000 bp to 9000 bp	7553	7432	7428	7471	71.04224	0.950907	
D3	SampD3	2000 bp to 4500 bp	3002	3066	3050	3039.333	33.30666	1.058584		D3	SampD3	6000 bp to 9000 bp	7595	7457	7542	7551.333	69.61561	0.924346	
D4	SampD4	2000 bp to 4500 bp	2942	2979	3020	2981	38.03945	1.276064		D4	SampD4	6000 bp to 9000 bp	7552	7547	7511	7536.667	22.36813	0.296791	
D5	SampD5	2000 bp to 4500 bp	3018	3044	3073	3045	27.51363	0.903568		D5	SampD5	6000 bp to 9000 bp	7589	7479	7531	7533	55.02727	0.730843	
D6	SampD6	2000 bp to 4500 bp	2945	3038	3038	3007	53.69358	1.785619		D6	SampD6	6000 bp to 9000 bp	7587	7551	7565	7567.667	18.14754	0.239094	
D7	SampD7	2000 bp to 4500 bp	2923	2993	2992	2969.333	40.12896	1.351447		D7	SampD7	6000 bp to 9000 bp	7599	7569	7331	7566.333	34.07834	0.450394	
D8	SampD8	2000 bp to 4500 bp	2951	3058	2981	2996.667	55.1936	1.841833		D8	SampD8	6000 bp to 9000 bp	7459	7541	7331	7443.667	105.8364	1.421831	
D9	SampD9	2000 bp to 4500 bp	2981	3037	3032	3016.667	30.98825	1.027218		D9	SampD9	6000 bp to 9000 bp	7480	7439	7428	7449	27.40438	0.367893	
D10	SampD10	2000 bp to 4500 bp	3000	3023	2934	2985.667	46.19885	1.547354		D10	SampD10	6000 bp to 9000 bp	7473	7388	7297	7386	88.01704	1.191674	
D11	SampD11	2000 bp to 4500 bp	3027	2757	3037	2940.333	158.85	5.40245		D11	SampD11	6000 bp to 9000 bp	7537	7624	7461	7540.667	61.56184	1.081626	
D12	SampD12	2000 bp to 4500 bp	2988	3046	3027	3020.333	29.56913	0.979092		D12	SampD12	6000 bp to 9000 bp	7502	7470	7333	7435	89.77193	1.207423	
E1	SampE1	2000 bp to 4500 bp	2960	2998	3022	2993.333	31.26233	1.044399		E1	SampE1	6000 bp to 9000 bp	7570	7503	7490	7521	42.93018	0.570804	
E2	SampE2	2000 bp to 4500 bp	2967	2999	3047	3004.333	40.26578	1.340257		E2	SampE2	6000 bp to 9000 bp	7522	7507	7454	7494.333	35.72581	0.476704	
E3	SampE3	2000 bp to 4500 bp	2990	3007	3049	3015.333	30.36994	1.007184		E3	SampE3	6000 bp to 9000 bp	7601	7440	7474	7505	84.85871	1.130956	
E4	SampE4	2000 bp to 4500 bp	2992	2994	3000	2995.333	4.163332	0.138994		E4	SampE4	6000 bp to 9000 bp	7596	7437	7533	7522	80.06872	1.064461	
E5	SampE5	2000 bp to 4500 bp	2961	3017	3005	2994.333	29.48446	0.984675		E5	SampE5	6000 bp to 9000 bp	7465	7469	7428	7454	22.60531	0.303264	
E6	SampE6	2000 bp to 4500 bp	2976	2991	2976	2981	8.660254	0.250515		E6	SampE6	6000 bp to 9000 bp	7609	7522	7500	7543.667	57.63069	0.764608	
E7	SampE7	2000 bp to 4500 bp	3008	3061	3029	3032.667	26.68957	0.880069		E7	SampE7	6000 bp to 9000 bp	7452	7451	7325	7409.333	73.03652	0.985737	
E8	SampE8	2000 bp to 4500 bp	2961	2971	2969	2967	5.291503	0.17845		E8	SampE8	6000 bp to 9000 bp	7568	7454	7468	7461.667	102.7148	1.376367	
E9	SampE9	2000 bp to 4500 bp	3030	3036	3011	3025.667	13.05118	0.431349		E9	SampE9	6000 bp to 9000 bp	7576	7457	7479	7504	63.31666	0.845772	
E10	SampE10	2000 bp to 4500 bp	2979	3062	2952	2997.667	57.32655	1.912372		E10	SampE10	6000 bp to 9000 bp	7512	7482	7326	7440	98.8599	1.342203	
E11	SampE11	2000 bp to 4500 bp	2989	3038	3040	3025.667	2												

CLAIMS

1. A high throughput method for determining telomere length of mammalian chromosomal DNA comprising:
 - i) annealing a primer to a region adjacent a telomere repeat array of chromosomal DNA wherein said region is between 3843bp-30bp from said telomere repeat array;
 - ii) PCR amplifying 20ng-35ng of said chromosomal DNA using 21-23 cycles to generate an amplification product; and
 - iii) detecting the length of the amplification product.
2. The method according to claim 1 wherein part ii) comprises PCR amplifying 25ng-30ng of said chromosomal DNA.
3. The method according to claim 1 or 2 wherein part iii) involves attaching a fluorescent label to the PCR amplification product where said fluorescent label provides a signal of at least 25 RFU; or a signal that is at least 25% Telomere Peak area under a curve representative of generated product/ Marker peak area under a curve representative of generated marker product when 0.5ng/ μ l of marker is used at an injection rate of 1KV, 10 seconds.
4. The method according to claim 1, 2 or 3 wherein said chromosomal DNA was subjected to 23 PCR cycles or 30ng of chromosomal DNA was subjected to 23 PCR cycles.
5. The method according to any one of-claims 1-4 wherein said primer concentration is selected from the group consisting of: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and -0.8 μ M.
6. The method according to any one of claims 1-5 wherein said chromosomal DNA is extracted from at least one cell using an automated extraction method.
7. The method according to claim 6 wherein said cell is extracted from any biological sample, or processed derivative thereof.

8. The method according to claim 7 wherein said biological sample is a complex cell sample or a purified cell sample.
9. The method according to any one of claims 7 or 8 wherein said biological sample comprises whole blood.
10. The method according to any one of claims 6-9 wherein less than 6×10^5 cells are used for the extraction method.
11. The method according to claim 10 wherein between 2×10^5 and 6×10^5 cells are used for the extraction method.
12. The method according to claim 10 or claim 11 wherein 4×10^5 cells are used for the extraction method.
13. The method according to any one of claims 1-12 comprising digesting said chromosomal DNA with a restriction endonuclease that cleaves non-telomeric DNA prior to undertaking said method.
14. The method according to claim 13 wherein said restriction endonuclease does not cleave between the 5' end of said primer and the telomere repeat array or the 5' end of said primer and the chromosome telomere end.
15. The method according to any one of claims 1-14 wherein part iii) involves the use of a lower marker that is larger than 200bp but smaller than the smallest fragment size of a telomere generated with HT-STELA.
16. The method according to claim 15 wherein said lower marker is selected from the group consisting of: 882bp, 881bp, 880bp, 879bp, 878bp, 877bp, 876bp, 875bp, 874bp, 873bp, 872bp, 871bp, 870bp, 410bp, 409bp, 408bp, 407bp, 406bp, 405bp, 404bp, 403bp, 402bp, 401bp, 400bp, 399bp, 398bp, 397bp, 396bp, 395bp, 394bp, 393bp, 392bp, 391bp, and 390bp including all 0.1kb integers there between.
17. The method according to any one of claims 1-16 wherein part iii) involves the use of an upper marker selected from the group consisting of: 10kb, 11kb, 12kb, 13kb, 14kb, 15kb, 16kb, 17kb, 18kb, 19kb and 20kb, including all 0.1kb integers there between.

18. The method according to any one of claims 1-17 wherein said primer binds to telomere specific nucleotides at the 3' end.
19. The method according to any one of claims 1-18 wherein said primer binds to 7q chromosome.
20. The method according to claim 19 wherein said primer binds to at least one of the following 7q-specific nucleotides in chromosome 7q: chr7:159334755 hg38, or chr7: 159335319 hg38, or chr7: 159335675 hg38 or chr7: 159335841 hg38.
21. The method according to any one of claims 1-18 wherein said primer binds to 12q chromosome.
22. The method according to claim 21 wherein said primer binds to at least one of the following 12q-specific nucleotides in chromosome 12: chr12:133264524 hg38 or chr12: 133264324 hg38.
23. The method according to any one of claims 1-18 wherein said primer binds to XpYp chromosome.
24. The method according to claim 23 wherein said primer binds at least one of the following XpYp-specific nucleotides in chromosome XpYp: chrX:10432 hg38 or chrX:10896 hg38.
25. The method according to any one of claims 1-18 wherein said primer binds to 17p chromosome.
26. The method according to claim 25 wherein said primer binds to at least one of the following 17p-specific nucleotides in chromosome 17p: 292C with respect to the telomere repeat array beginning 17pseq1rev, 1018T with respect to the telomere repeat array beginning 17pseq2rev, 1837C with respect to the telomere repeat array beginning 17pseq4rev, 2559C with respect to the telomere array beginning 17p2, or 3018G with respect to the telomere array beginning 17p7.
27. The method according to any one of claims 1-18 wherein said primer binds to a chromosome selected from the group consisting of any one or more of: 2p, 4q, 4p, 5p, 11q, 16p, and 18q chromosome.

28. The method according to claim 27 wherein said primer binds to at least one of the following nucleotides: in chromosome 2p2 nucleotide chr2: 10755 hg38, in chromosome 4qK1 nucleotide chr4:190122098 hg38, in chromosome 4p4 nucleotide chr4:10457 hg38, in chromosome 5p3 nucleotide chr5:12240 hg38, in chromosome 11q13B nucleotide chr11:135076467 hg38, in chromosome 16prev1 nucleotide chr16: 10241 hg38 or in chromosome 18qrev4M nucleotide chr18:80262147 hg38.
29. The method according to any one of claims 1-28 wherein said method is undertaken to diagnose or prognose or to determine the risk of developing a condition selected from the group consisting of: cancer, ageing, neurological disorders including Alzheimer's disease, Parkinson's disease and other dementias, brain infarction, heart disease, chronic HIV infection, chronic hepatitis, skin diseases, chronic inflammatory bowel disease including ulcerative colitis, anaemia, atherosclerosis, Barrett's oesophagus and cancers including pre-cancerous conditions, infertility, telomere syndromes including dyskeratosis congenita, aplastic anaemia, idiopathic pulmonary fibrosis, familial myelodysplastic syndrome-acute myeloid leukaemia, Hoyeraal—Hreiderasson syndrome, Revesz syndrome, Coats plus syndrome, bone marrow failure, and cryptogenic liver cirrhosis.
30. The method according to any one of claims 1 - 29 wherein said method is undertaken to assess an individual's suitability to be a transplantation donor.
31. The method according to any one of claims 1-30 wherein said primer is selected from the group comprising:

GAGCTGCGTTTTGCTGAGCAC (SEQ ID No: 1);
ACGGTGGCATGCCTCTCTC (SEQ ID No: 2);
TCTGCGCCTGCGATGGCGCTATG (SEQ ID No: 3);
GCATTCTCTTCACCACAGATGTTG (SEQ ID No: 4);
CCCACACAGTCATCTATTGTT (SEQ ID No: 5);
GAGGTGCAGTAGTGGGGATCTAACT (SEQ ID No: 6);
GGGACAGCATATTCTGGTTT (SEQ ID No: 7);
GCACAGCCTTTTGGGGTACCA (SEQ ID No: 8);
AGTGGGAGATCCACACCGTAGCGTG (SEQ ID No: 9);

CCaTGcAGTGCTAAGACAGCAATGAG (SEQ ID No: 10);
CAGACCTTGGAGGCACGGCCTTCG (SEQ ID No: 11);
CCTCTGGTCATTATGAATAGGGCTTC (SEQ ID No: 12);
GGGGAAAAATGCCCAAG (SEQ ID No: 13);

CCTTCTCTTCTTGATGTC (SEQ ID No: 14);

ACAGCCTTTTGGGGTACCGT (SEQ ID No: 15);
GGCTTCATTGATGGTGAATACAATCG (SEQ ID No: 16);
GCAGCGCTGAATATTCAGGGTG (SEQ ID No: 17);
CACTTATTAGTTCCAGTCTCTG (SEQ ID No: 18);

AGAAGCAGCGAGGAGCTTCA (SEQ ID No: 19);
CCTGGCATGGTATTGACATG (SEQ ID No: 20);
GCTAGGAATGGAATCATTGACTC (SEQ ID No: 21);
GATACTGGGAGGATCATATCTGGC (SEQ ID No: 22);
CCATTAGCCTGTGGGGTCTGAT (SEQ ID No: 23);
GAATCCACGGATTGCTTTGTGTAC (SEQ ID No: 24);
AAGCAGGTTGAGAGGCTGAGG (SEQ ID No: 25);
CACAGGGATGGTTAGGTATCTC (SEQ ID No: 26);
CGAGCAAGCATCGGAACGTGACT (SEQ ID No: 27);
ACCAGGTTTTCCAGTGTGTT (SEQ ID No: 28);
CCTGTAACGCTGTTAGGTAC (SEQ ID No: 29);
CAGGGACCGGGACAAATAGAC (SEQ ID No: 30);
ACCAGGGGCTGATGTAACG (SEQ ID No: 31);
TGTCTCAGGGTCCTAGTG (SEQ ID No: 32);
TCTCAGGGTCCTAGTGTG (SEQ ID No: 33);
GTTGTCTCAGGGTCCTAG (SEQ ID No: 34);
GGGGTTGTCTCAGGGTCC (SEQ ID No: 35);
TTCTAGGGGTTGTCTCAG (SEQ ID No: 36);
TCTTCTAGGGGTTGTCTC (SEQ ID No: 37) and
CTAATCTGCTCCCWCCCAC (SEQ ID No: 38).

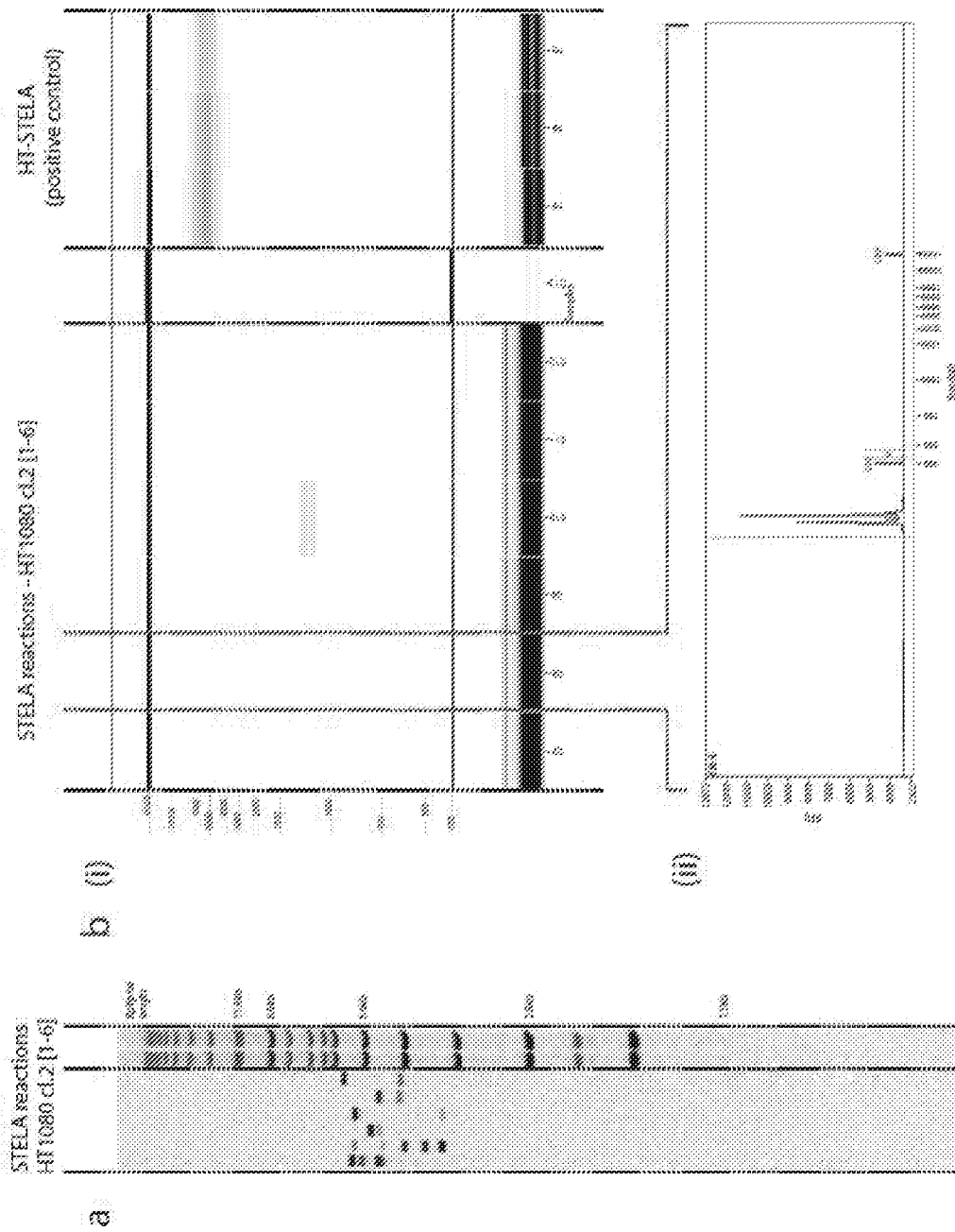


Figure-1.

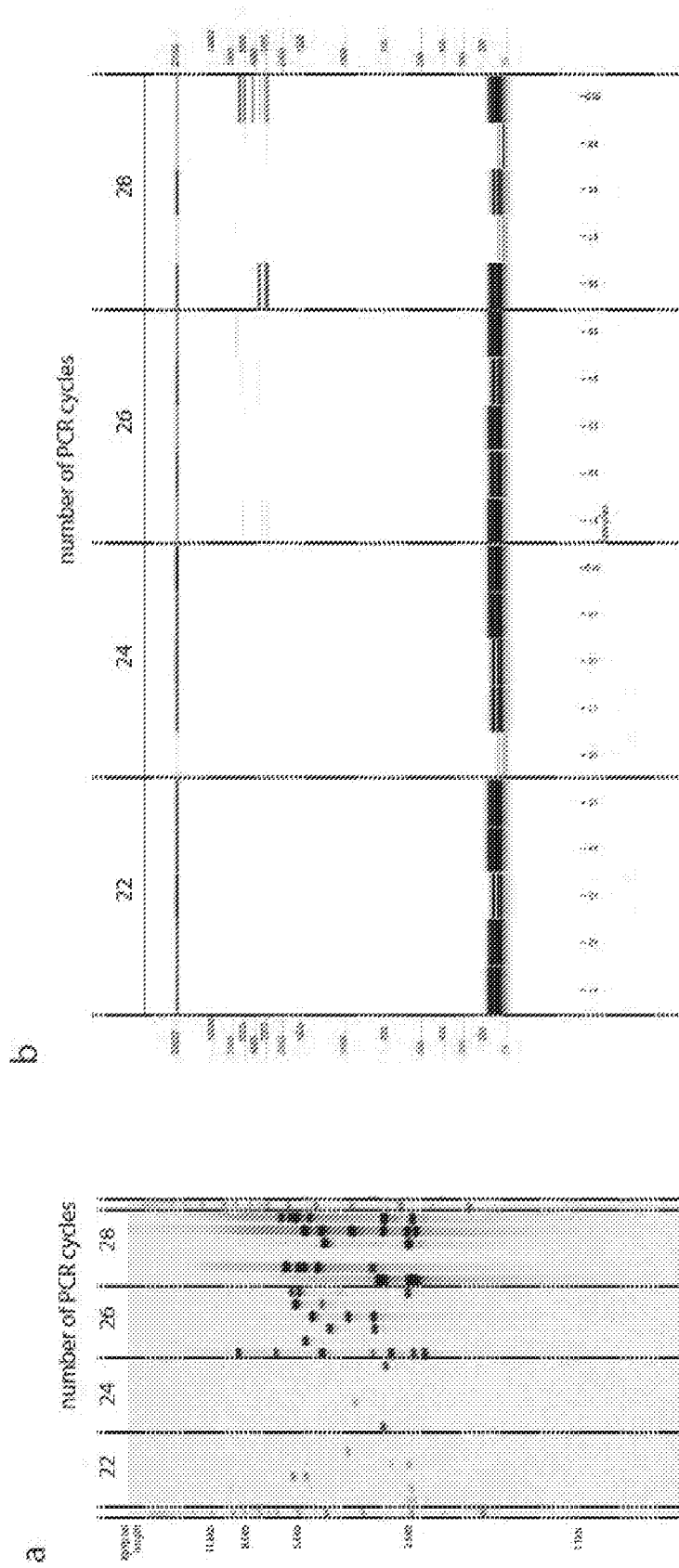


Figure-2.

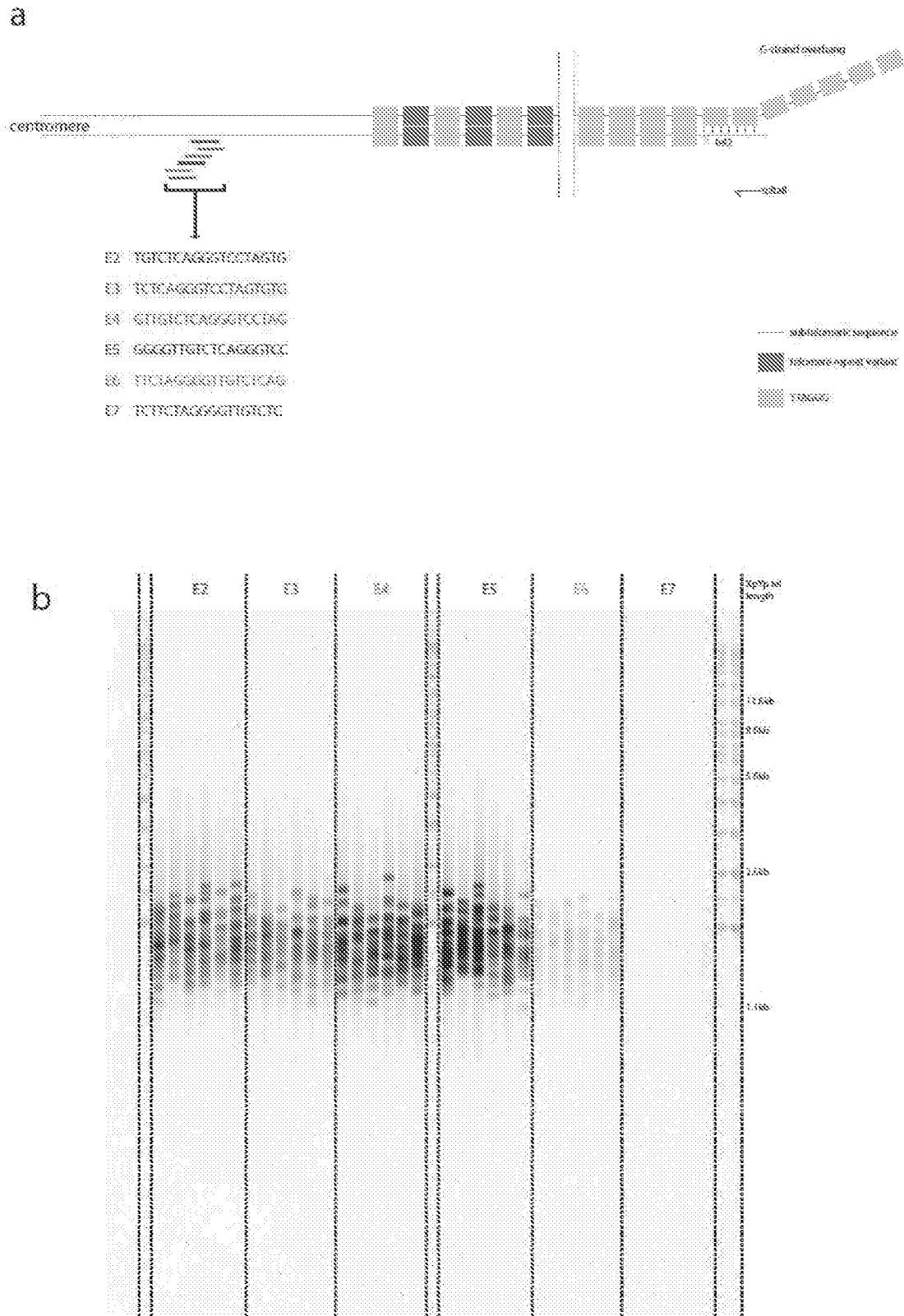
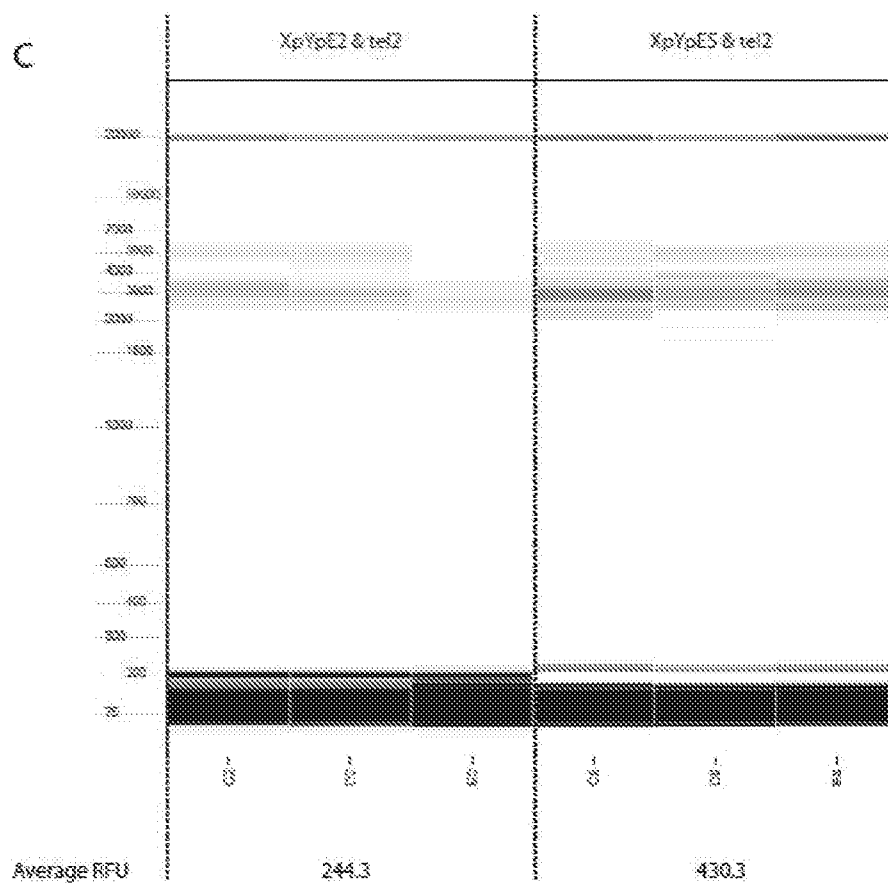


Figure-3A.

**Figure-3B.**

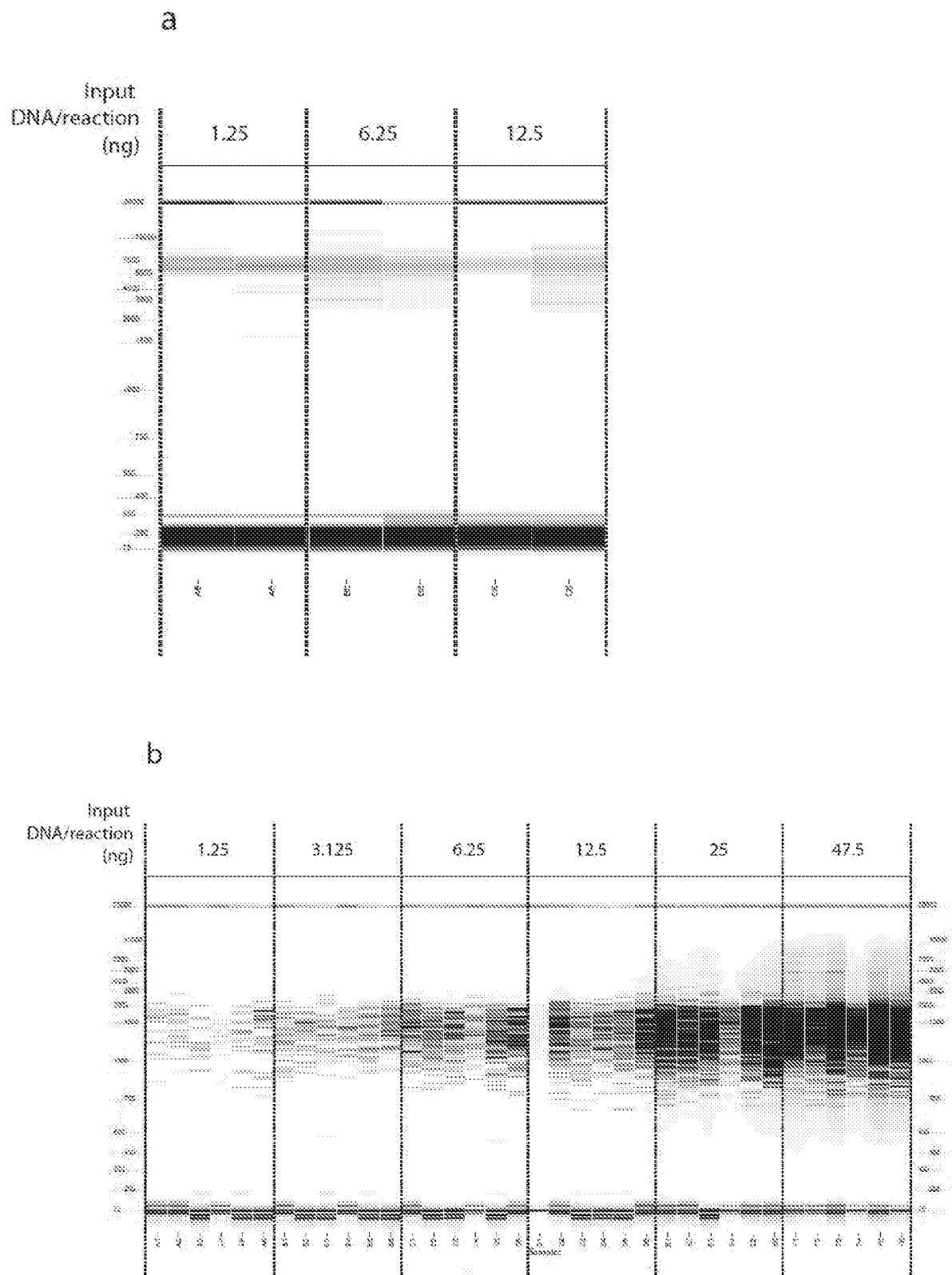


Figure-4.

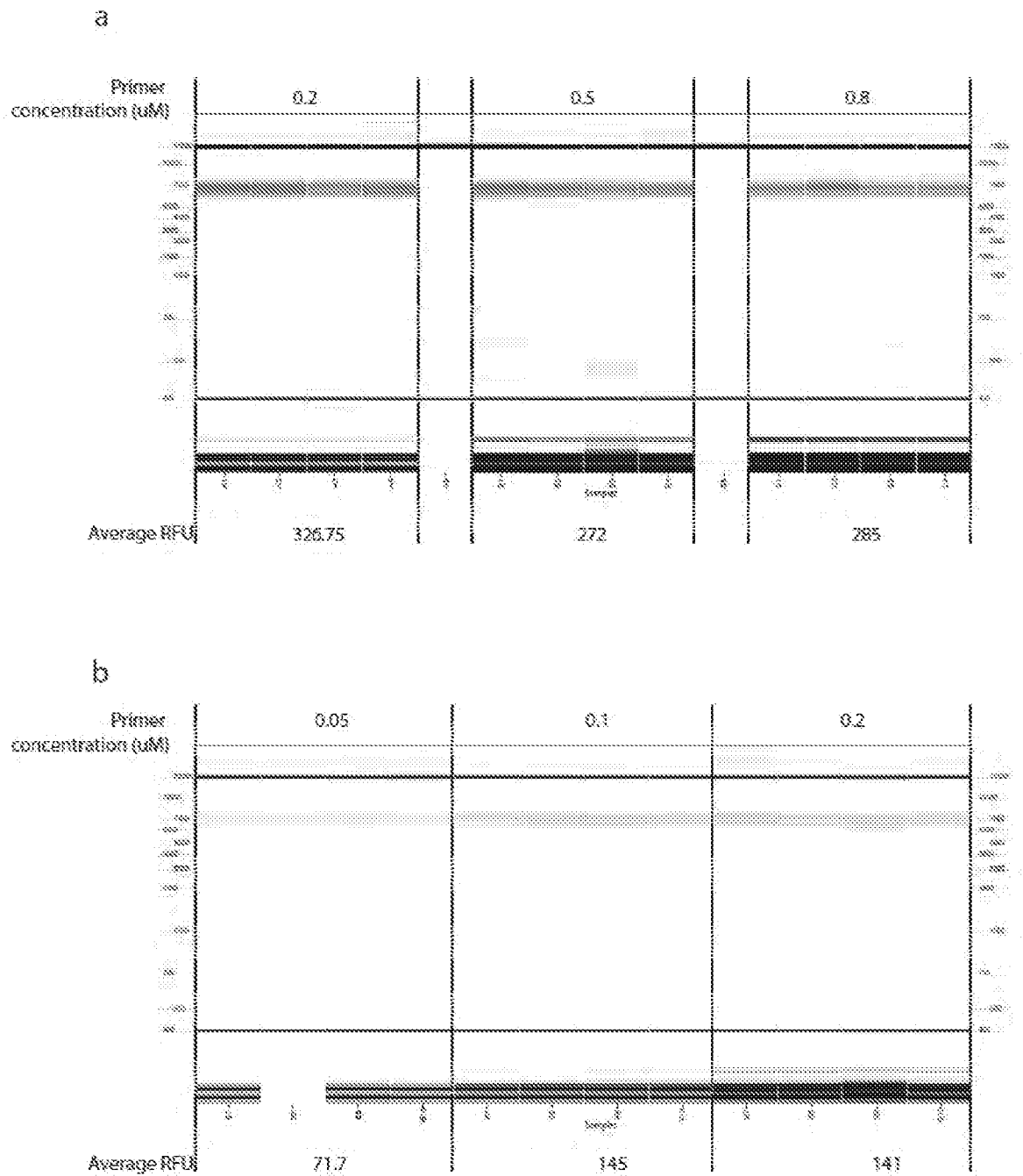


Figure-5.

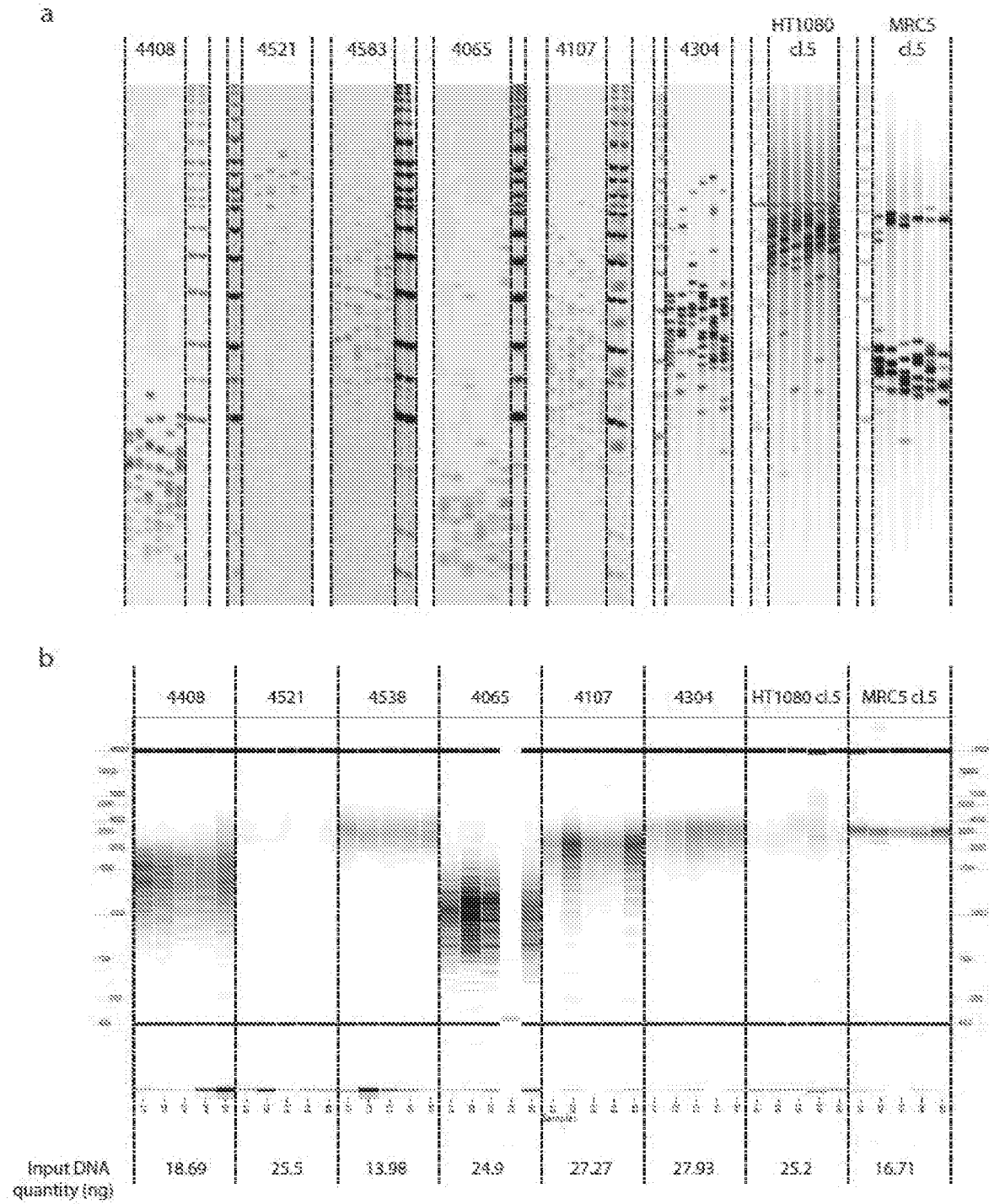


Figure-6A.

C

Sample	Mean telomere length (kb)	
	STELA	HT-STELA
4408	1.485	1.764
4521	8.598	4.591
4583	3.708	3.557
4065	1.29	1.317
4107	3.268	2.513
4304	4.268	3.368
HT3080 d.S	5.9	3.618
MRC5 d.S (LA)	3.048	3.702
MRC5 d.S (UA)	6.848	-

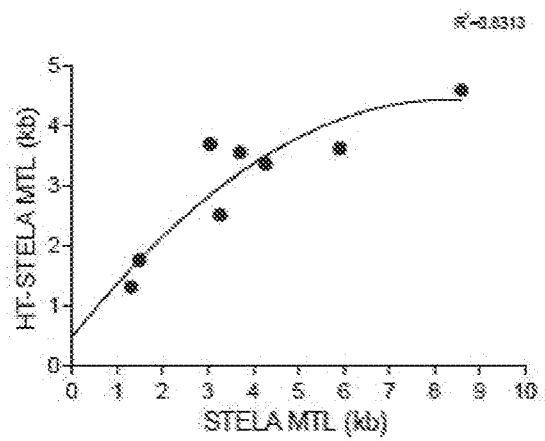


Figure-6B.

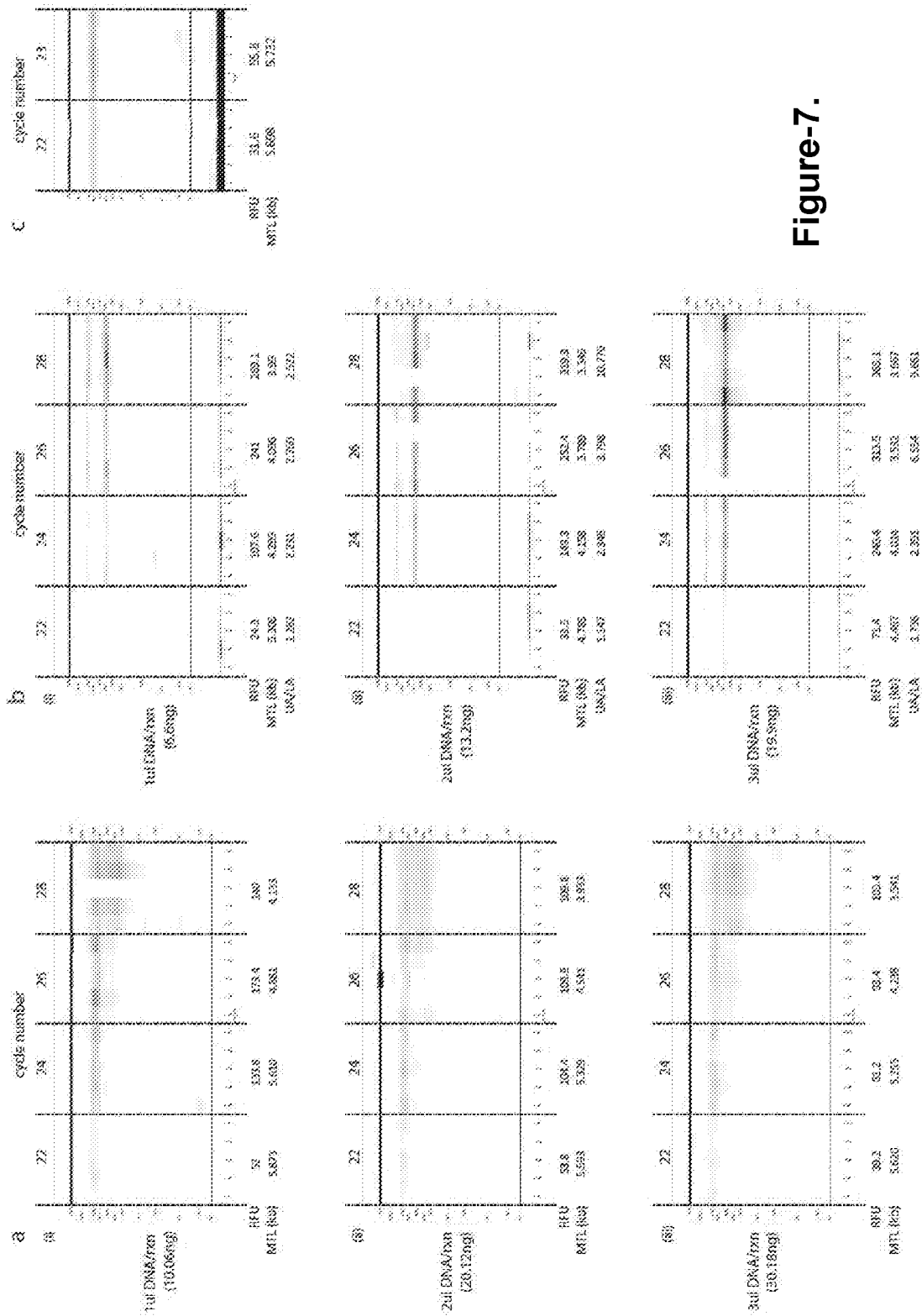
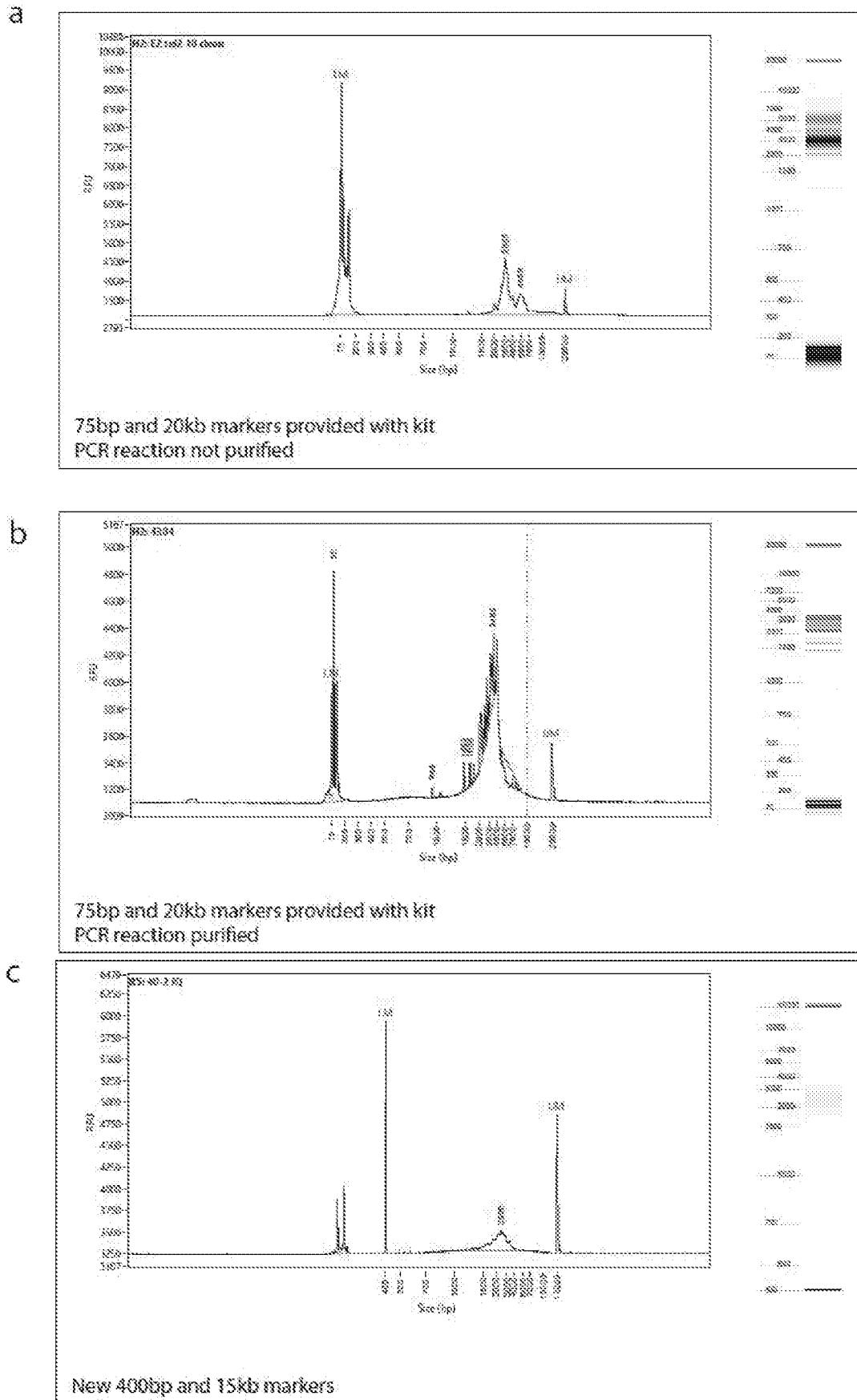


Figure-7.

**Figure-8.**

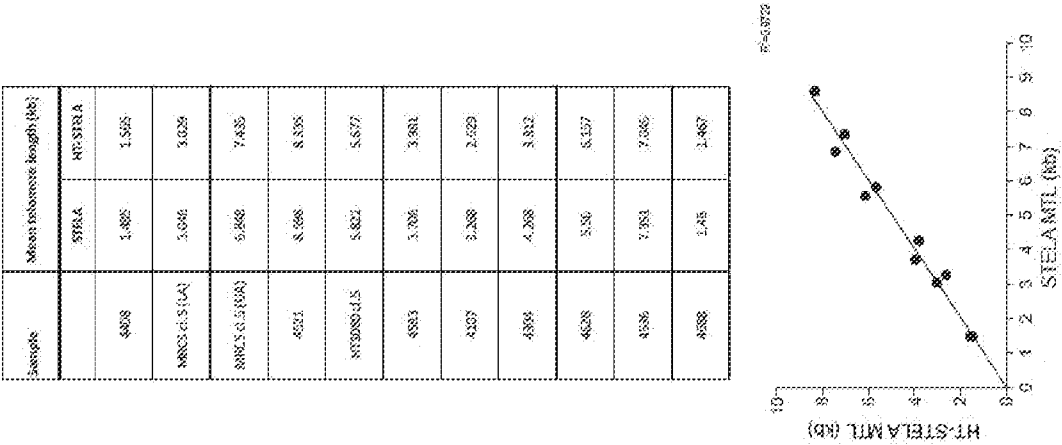


Figure-9.

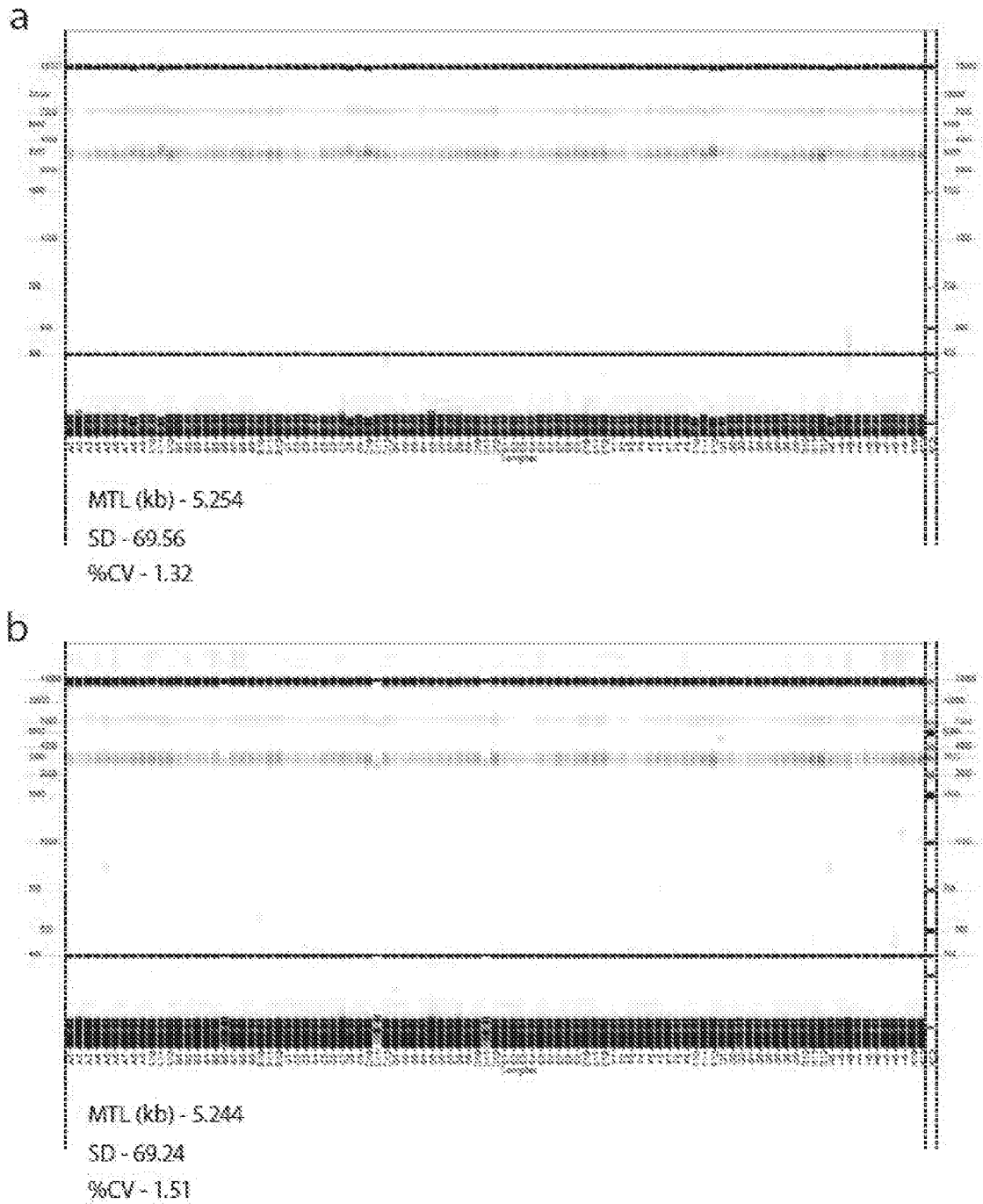
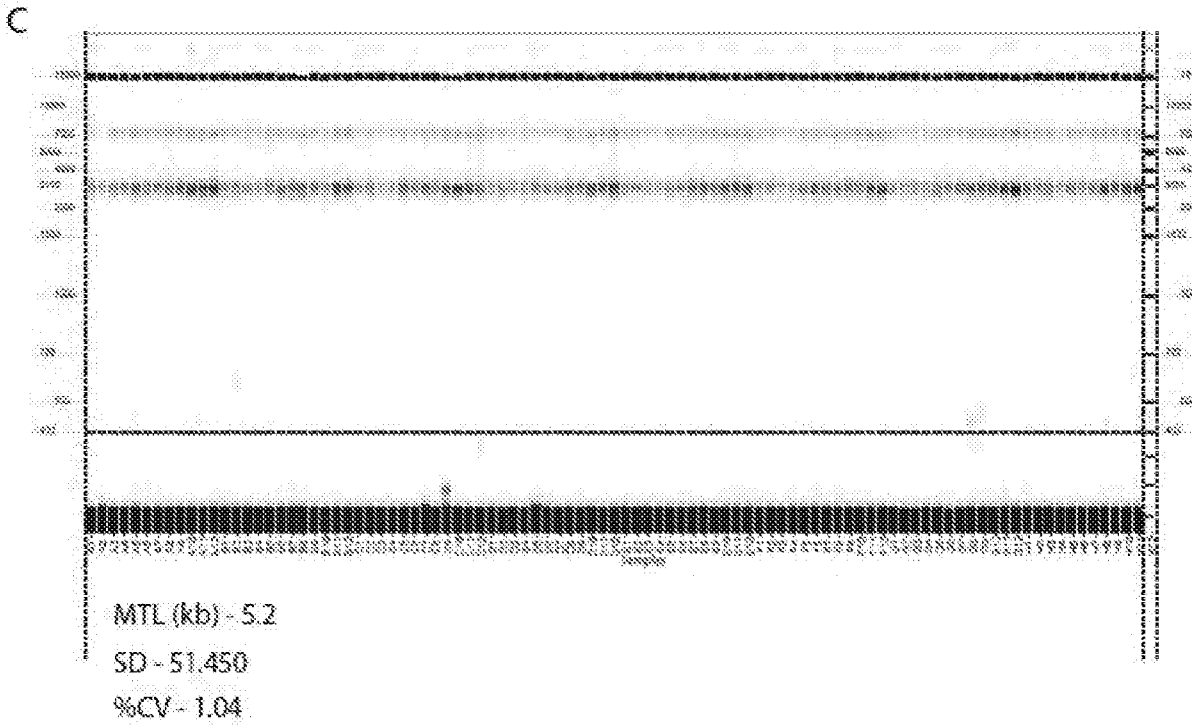


Figure-10A.

**Figure-10B.**

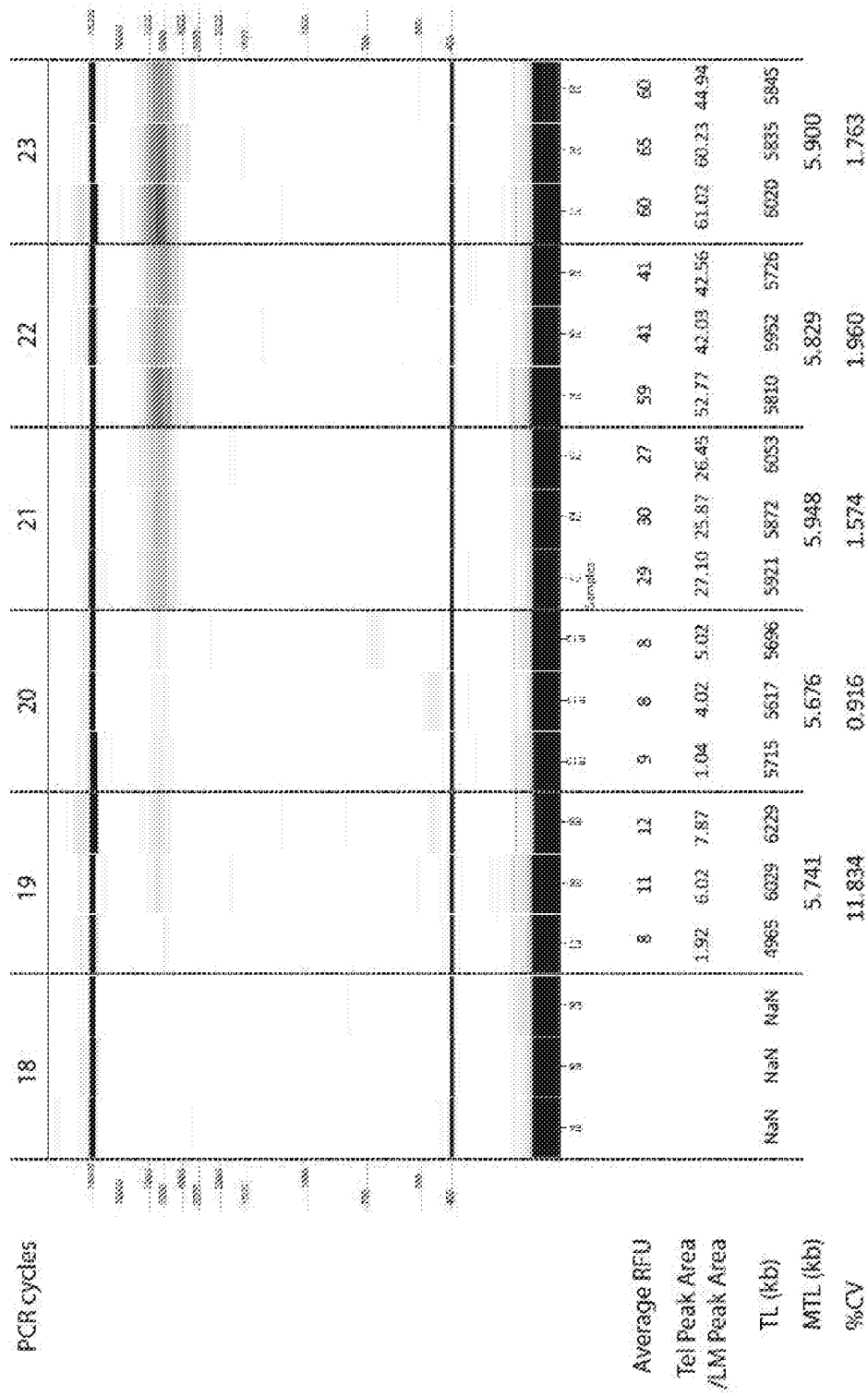
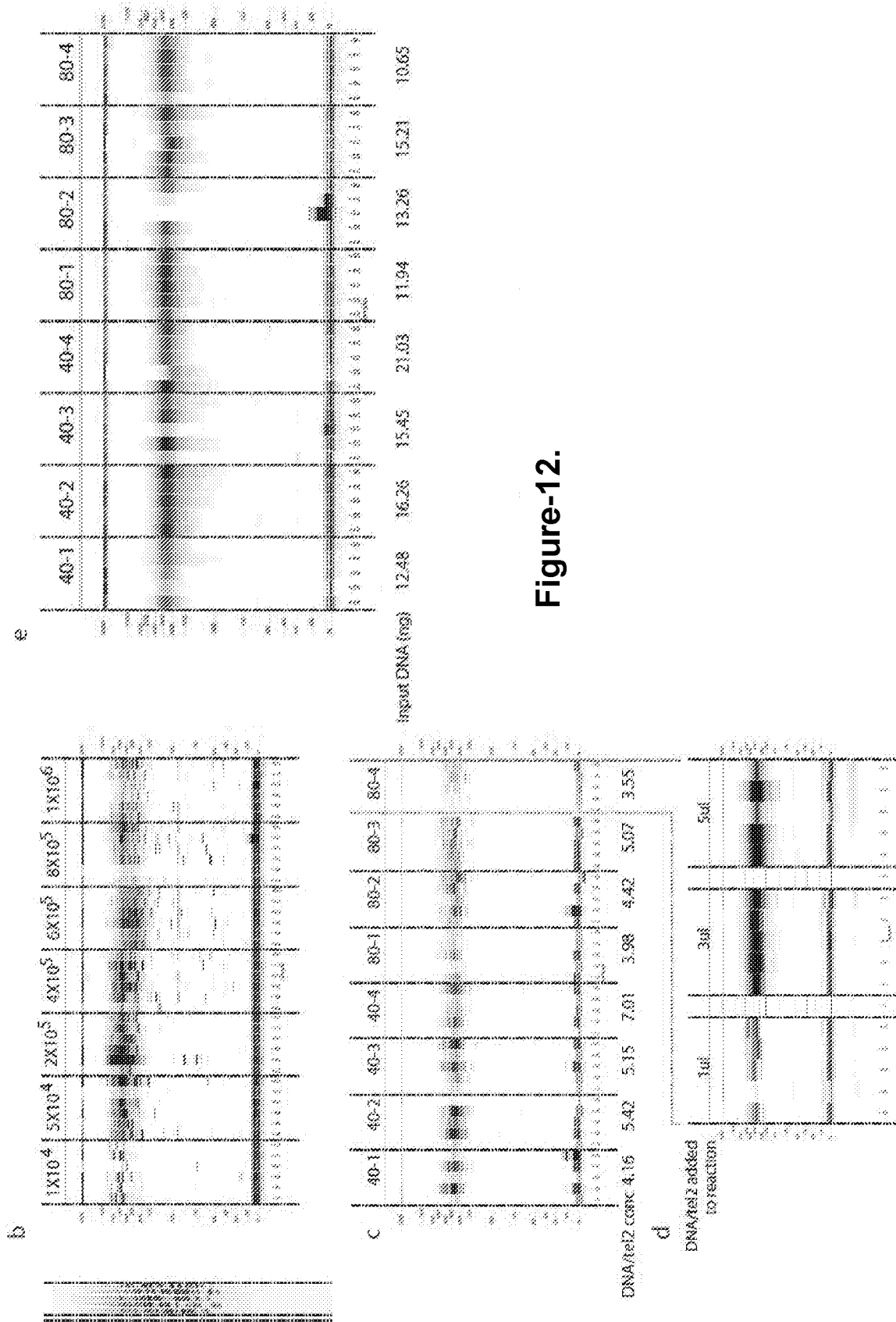
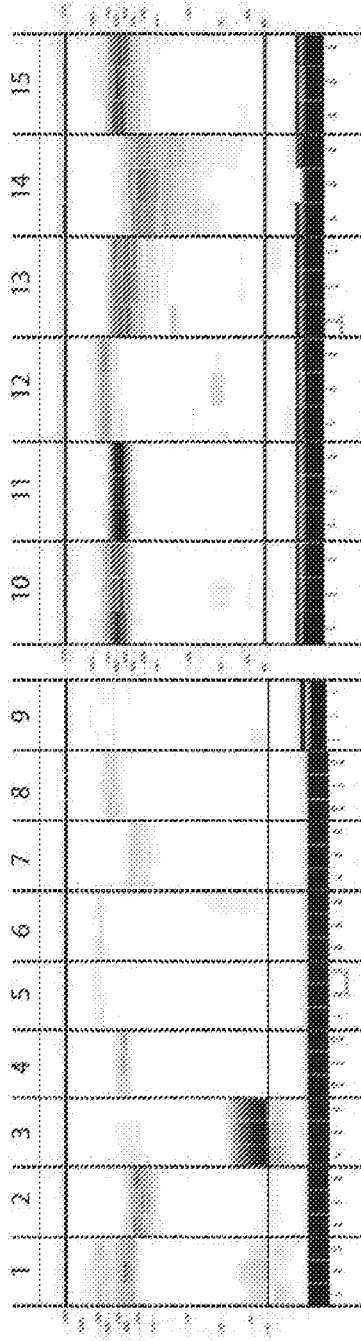
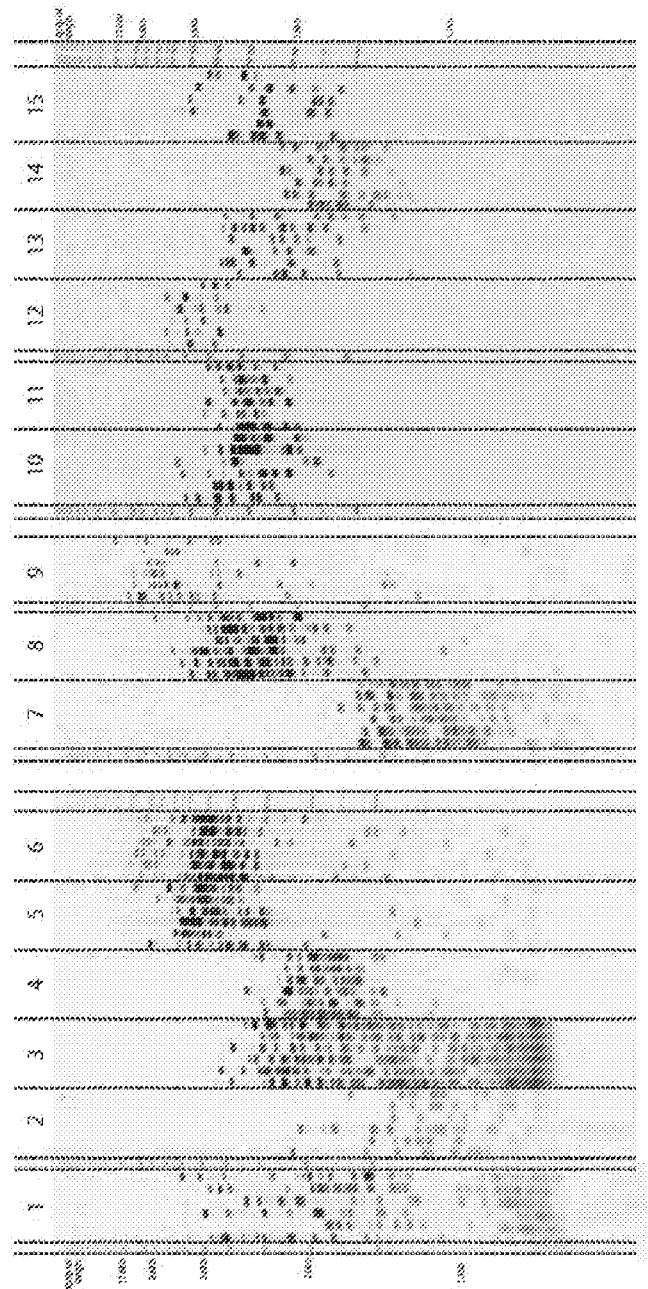


Figure-11.





a



b

Figure-13A.

C

Cell sample	Mean telomere length (kb)	
	STELA	HT-STELA
1.1A	2.235	2.595
1.1B	5.015	5.577
2	1.615	1.643
3	N/A	N/A
4	2.615	2.935
5	5.271	5.509
6	5.834	5.968
7	1.424	1.685
8	3.950	4.425
9	N/A	N/A
10	3.956	3.978
11	3.865	3.670
12	5.477	5.460
13	3.040	3.295
14	2.110	1.798
15	3.736	3.677

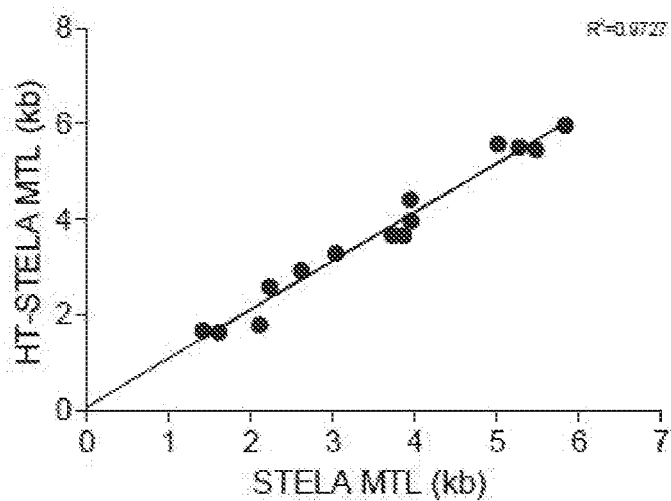


Figure-13B.

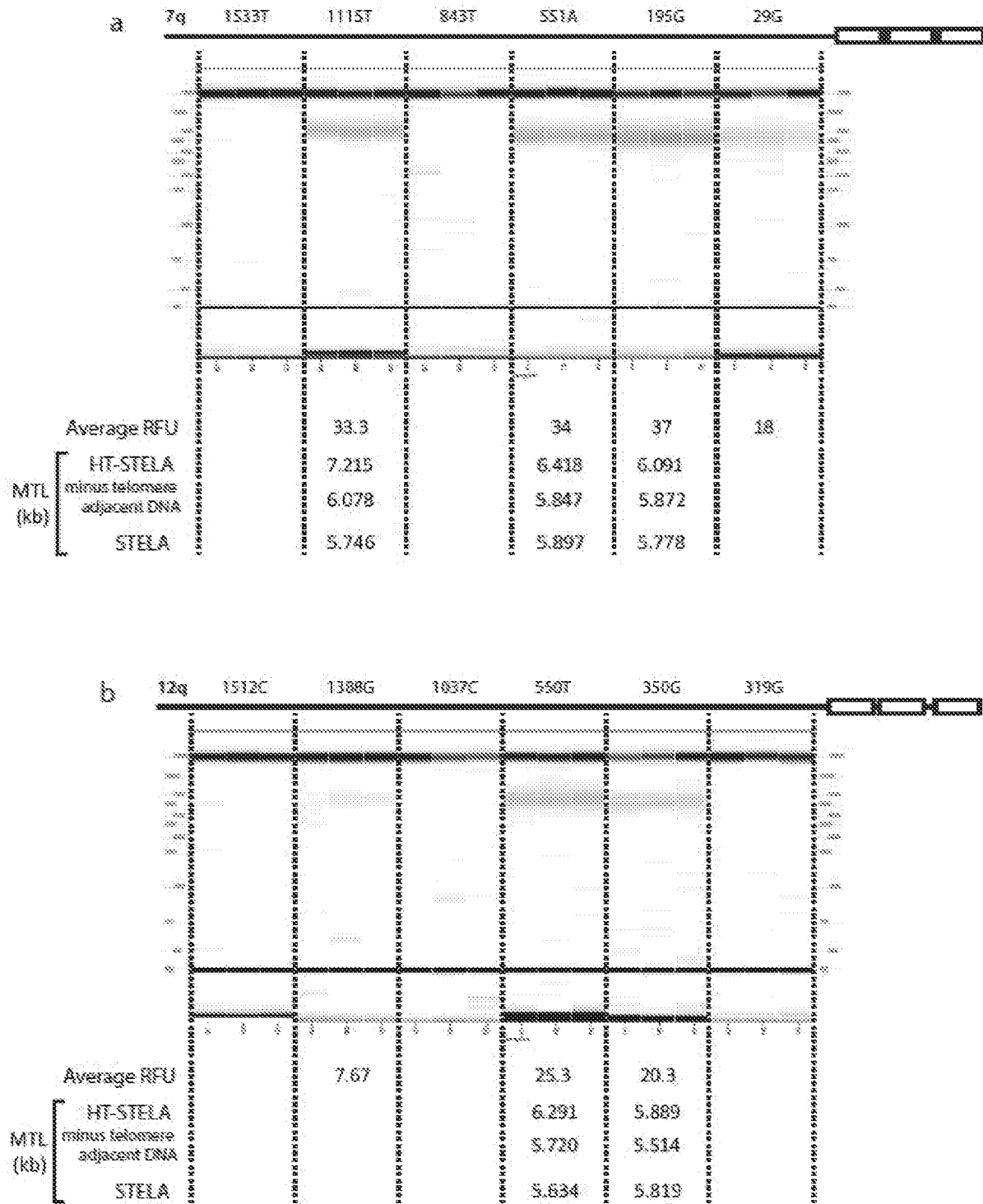


Figure-14A.

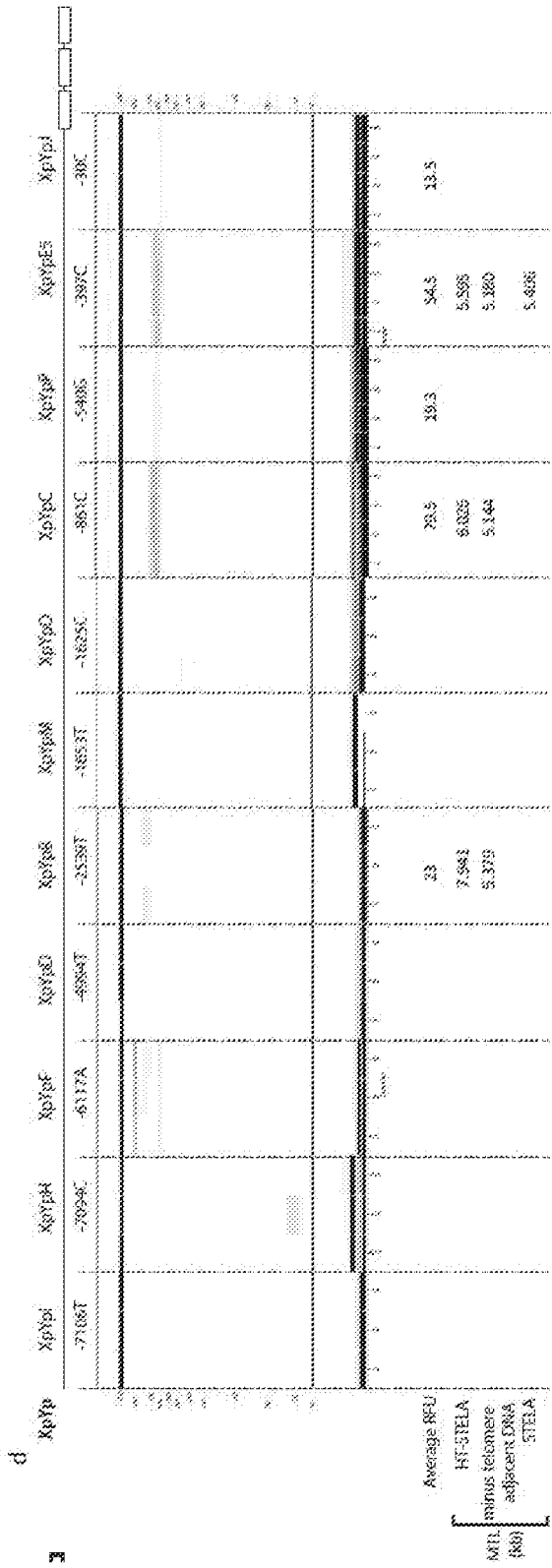
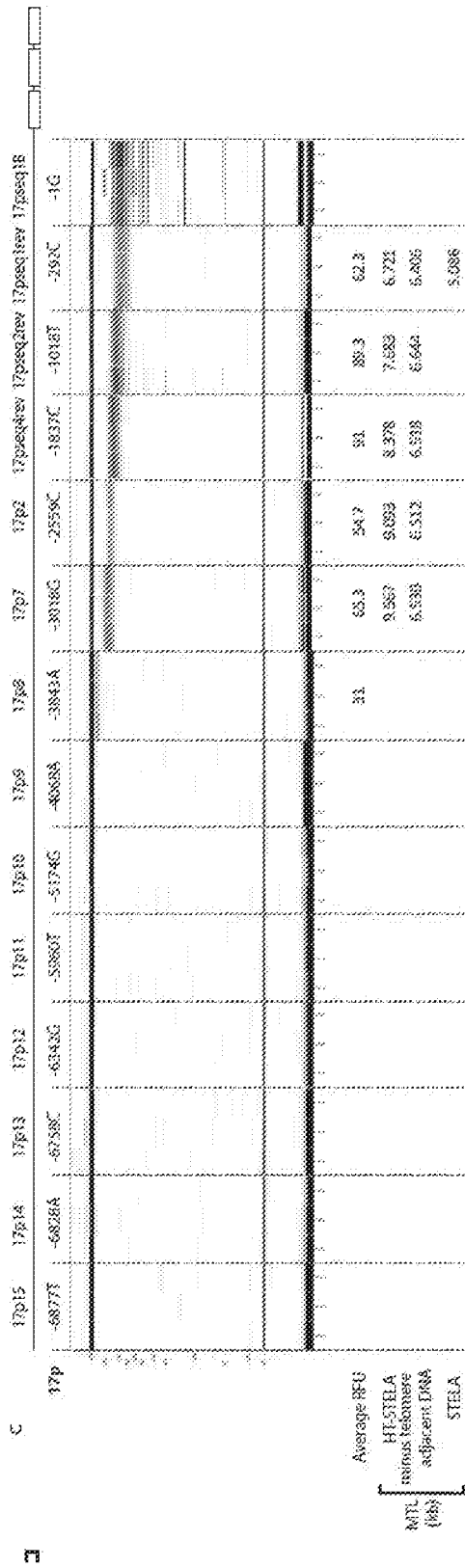


Figure-14B.

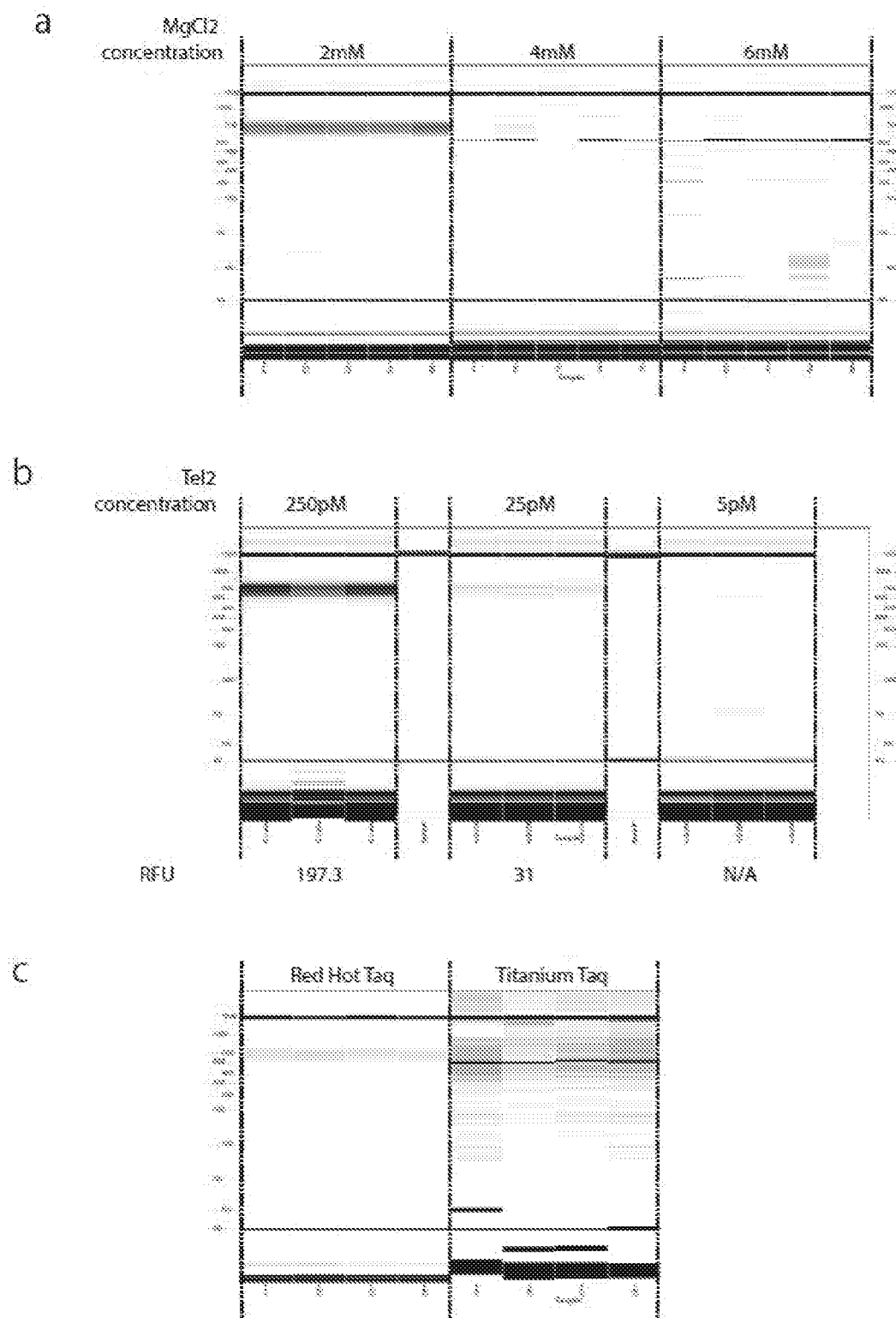


Figure-15A.

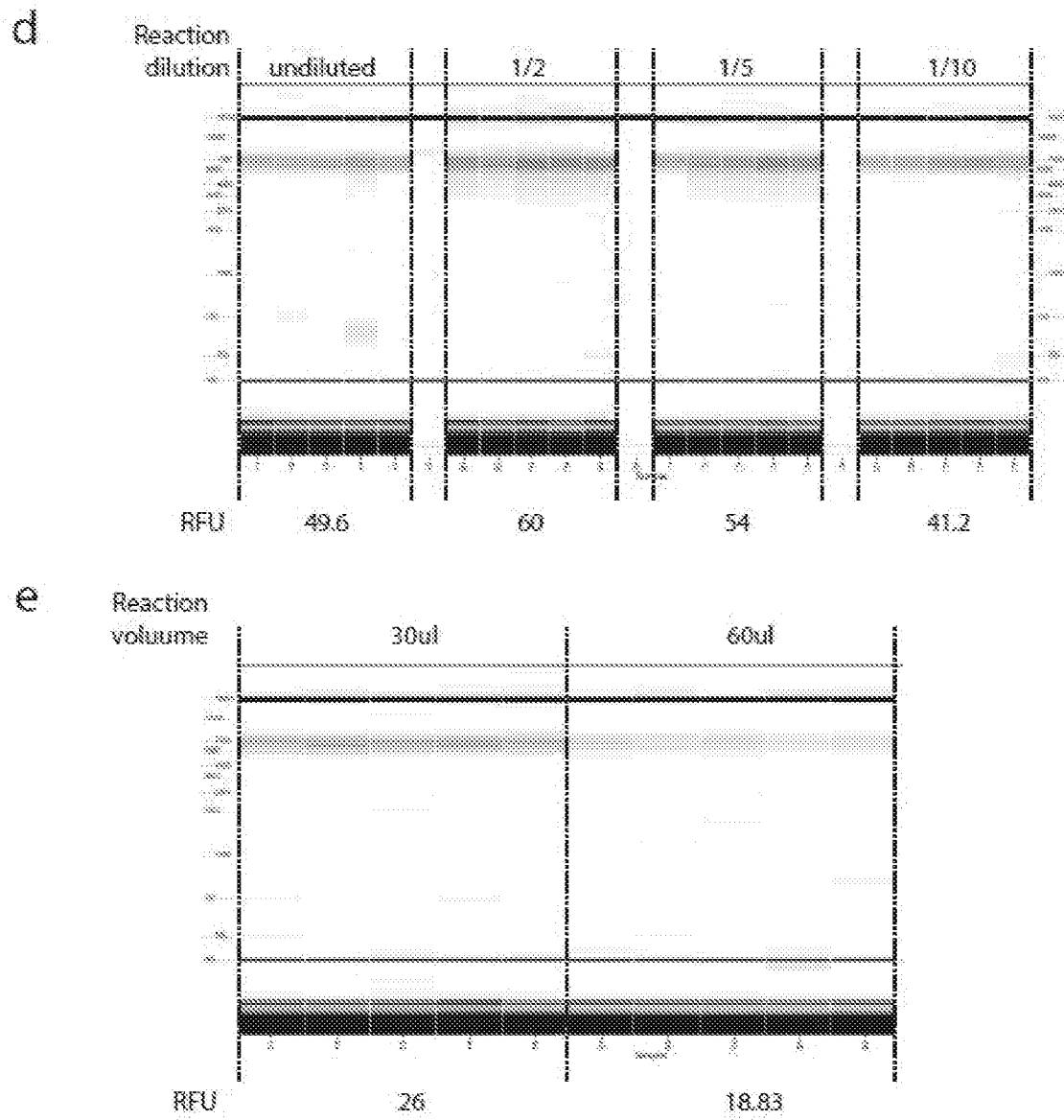


Figure-15B.

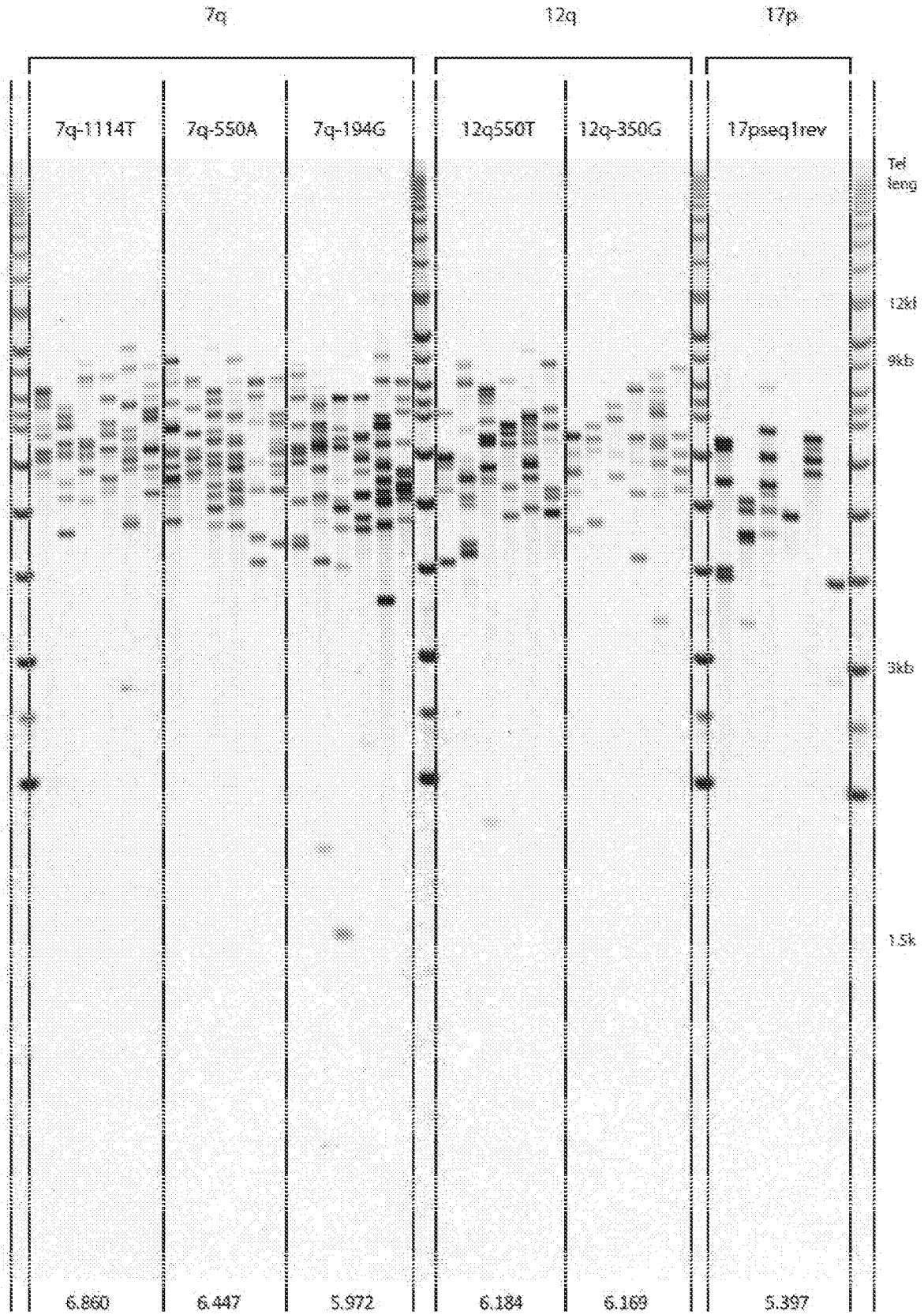


Figure-16.

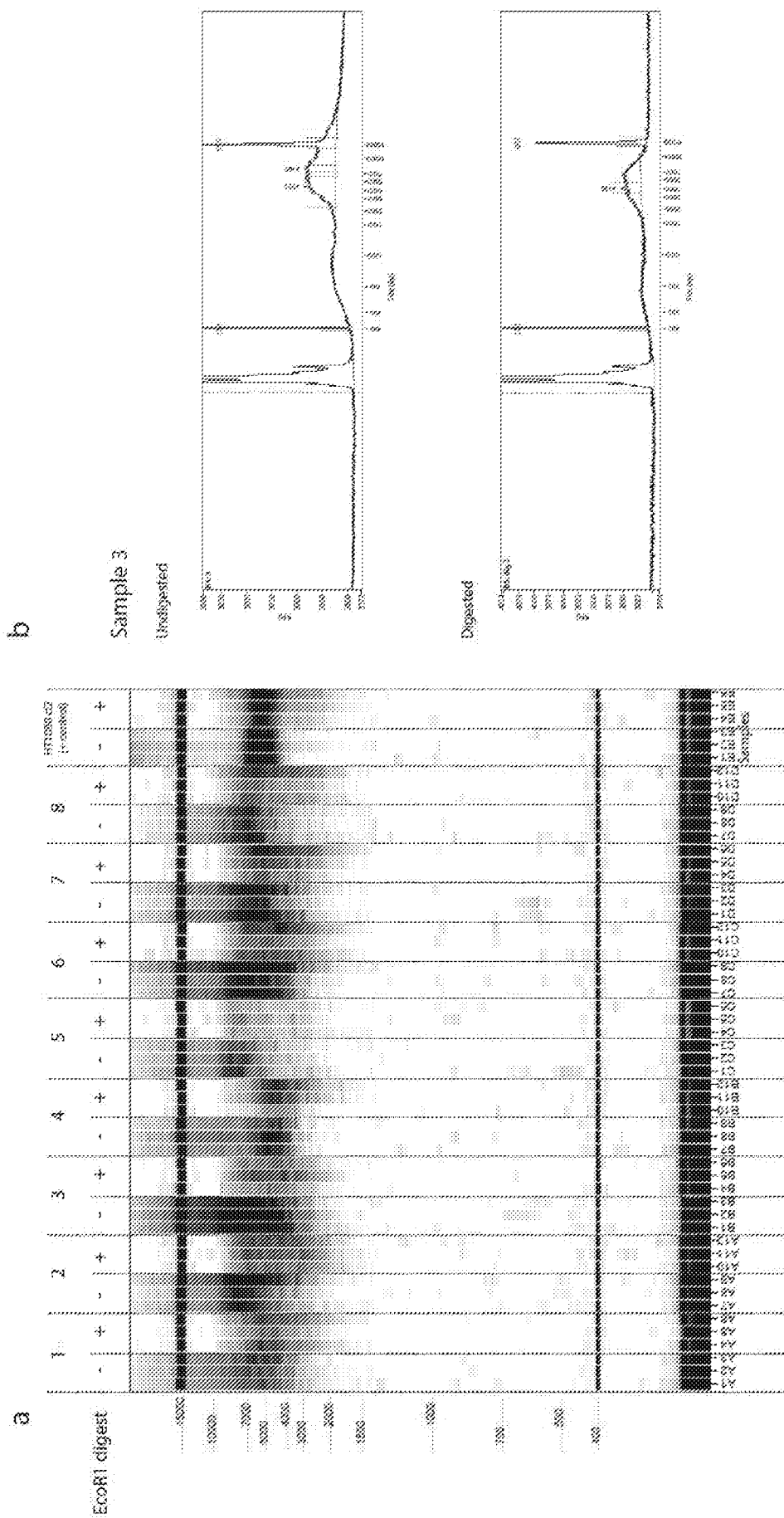


Figure-17.

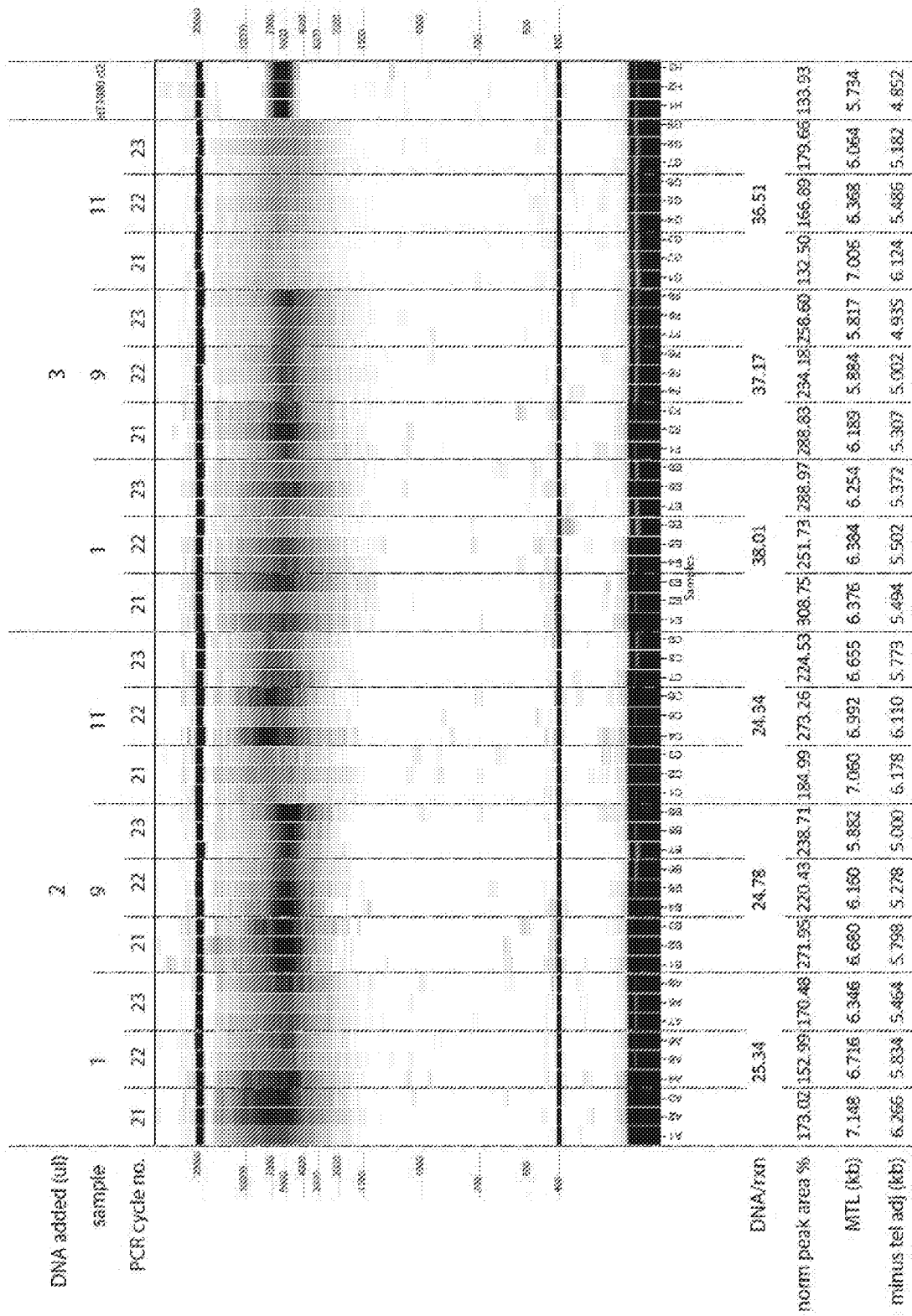
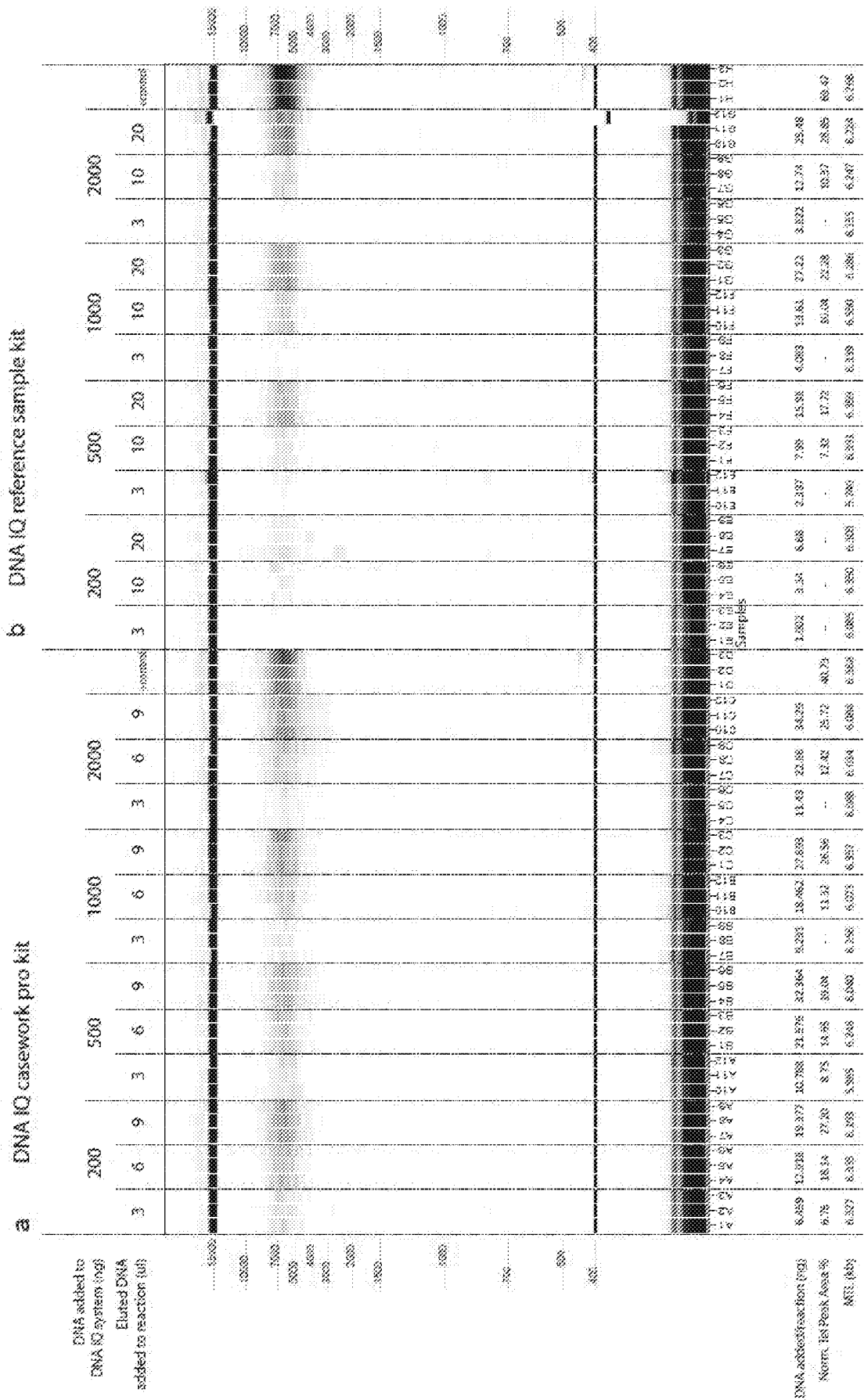


Figure-18



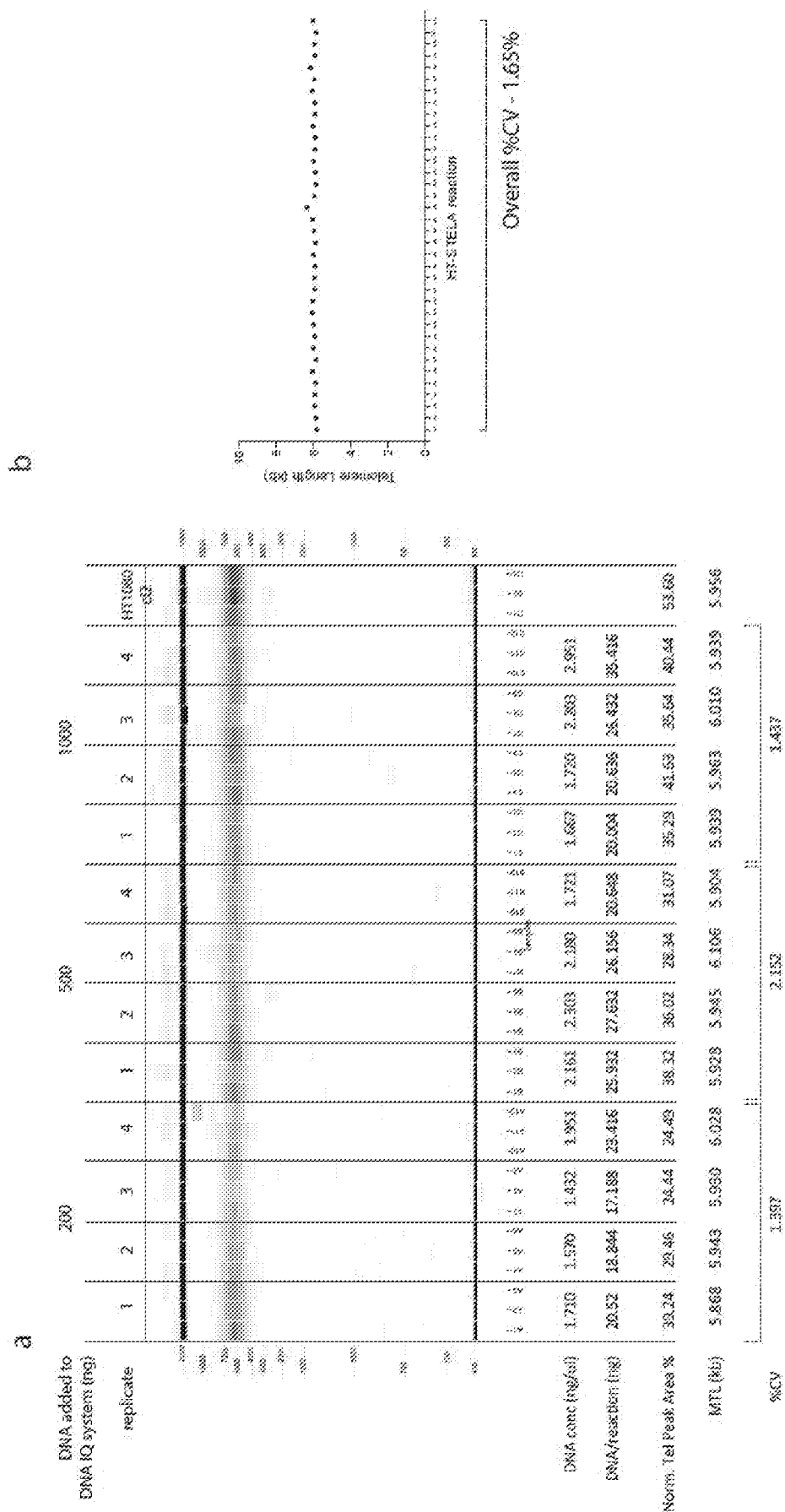


Figure-20.

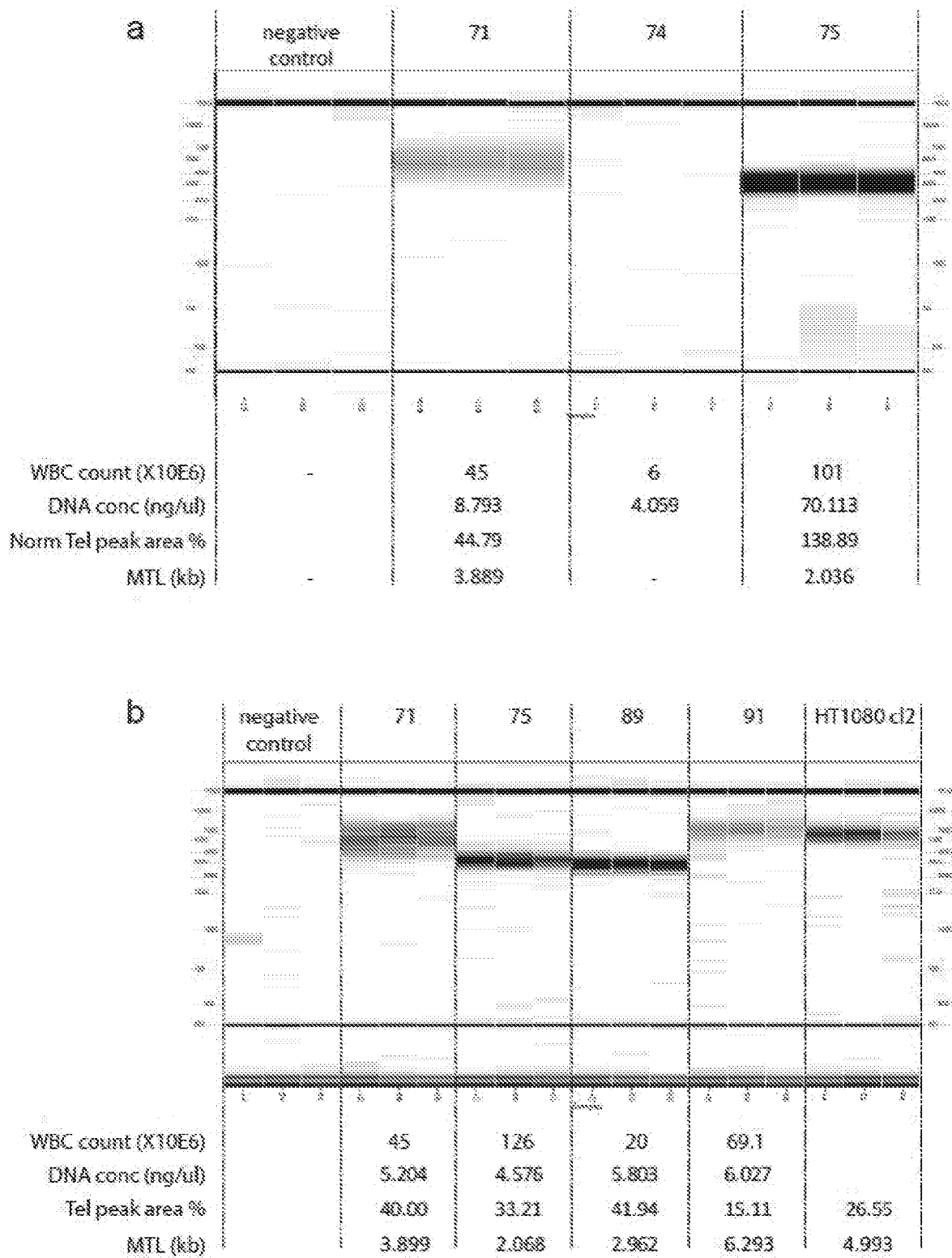
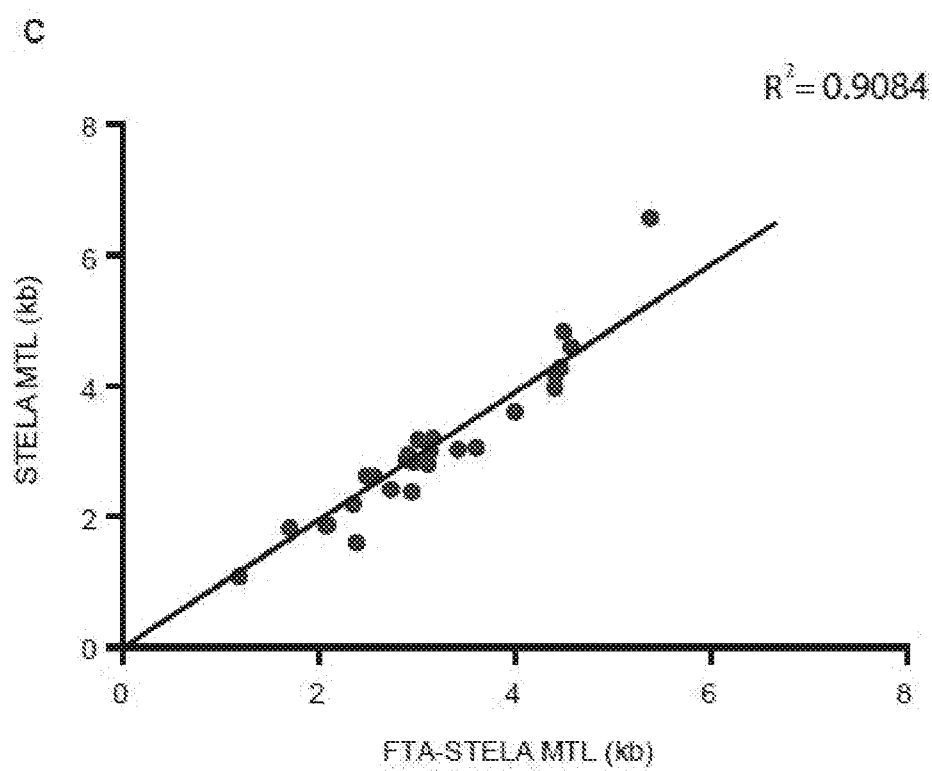
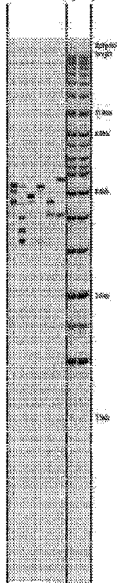


Figure-21A.

**Figure-21B.**

STELA reactions:
HT1080-cl.2 [1-6]

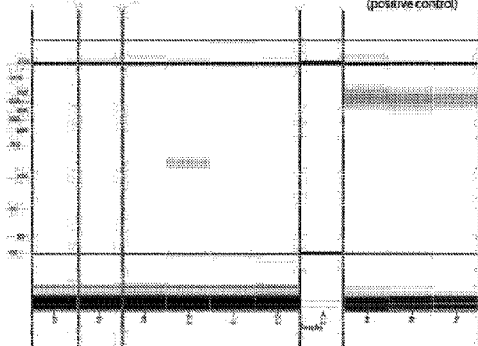
a.



STELA reactions - HT1080-cl.2 [1-6]

HT-STELA
(positive control)

b. (i)



(ii)

