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A Novel Pooled-Sample Multiplex Luminex Assay for High-Throughput Measurement of Relative Telomere Length

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Abstract

Objectives—Relative Telomere Length (RTL) is a potential biomarker of aging and risk for chronic disease. Previously, we developed a probe-based RTL assay on Luminex platform, where probes for Telomere (T) and reference gene (R) for a given DNA sample were tested in a single well. Here we describe a method of pooling multiple samples in one well to increase the throughput and cost-effectiveness.

Methods—We used four different microbeads for the same T-probe and four different microbeads for the same R-probe. Each pair of probe sets were hybridized to DNA in separate plates and then pooled in a single plate for all the subsequent steps. We used DNA samples from 60 independent individuals and repeated in multiple batches to test the precision.

Results—The precision was good to excellent with Intraclass correlation coefficient (ICC) of 0.908 (95% CI 0.856 – 0.942). More than 67% of the variation in the RTL could be explained by sample-to-sample variation; less than 0.1% variation was due to batch-to-batch variation and 0.3% variation was explained by bead-to-bead variation. We increased the throughput of RTL Luminex assay from 60 to 240 samples per run. The new assay was validated against the original Luminex assay without pooling ($r=0.79$, $p=1.44 \times 10^{-15}$). In an independent set of samples ($n=550$), the new assay showed a negative correlation of RTL with age ($r=-0.41$), a results providing external validation for the method.

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Competing interests: The authors declare they have no actual or potential competing financial interests.

Conclusion—We describe a novel high throughput pooled-sample multiplex Luminex assay for RTL with good to excellent precision suitable for large-scale studies.

Keywords

Relative Telomere length; Luminex; multiplex assay

Introduction

Telomeres are repeat sequences (TTAGGG)_n present at the end of the chromosomes that protect chromosomal integrity [Blackburn, 1991]. Telomere length (TL) is considered to be a biomarker of aging [Blackburn, 1991; Sanders and Newman, 2013]. The shortening of TL can cause genomic instability and may lead to carcinogenesis [Boardman et al., 2013; O'Sullivan and Karlseder, 2010; Zhang et al., 2007]. Telomeres are also shorter in age related diseases like chronic heart failure, atherosclerotic disease [van der Harst et al., 2007], myocardial infarction [Zee et al., 2009], and non-Hodgkin lymphoma [Widmann et al., 2007].

Aubert et al. (2012) recently reviewed the different methods of TL/content measurements in detail, including their advantages and disadvantages. Southern blot based on Terminal Restriction Fragment (TRF) analysis has been considered the gold standard of average TL measurement for many years [Harley et al., 1990; Kimura et al., 2010]. It requires large amounts of DNA (5-10ug) and is time consuming, which makes it poorly suited for large epidemiological studies. Measurement may also vary depending on the enzymes used and may include the sub-telomeric regions. STELA (single telomere length analysis) is a PCR-based technique for the analysis of individual telomeres which takes advantage of chromosome-specific sub-telomeric regions [Baird et al., 2003]. This method measures TL of individual chromosomes. It can detect very short telomeres but is less accurate for very long ones. This method is PCR-based, and primers depend on the G-rich overhang of the telomere. It tends to be very laborious and primer design is sometimes not possible. Quantitative fluorescence in situ hybridization (Q-FISH) uses fluorescently labeled probes to measure TL in fixed tissues and cells in metaphase, but it cannot give the actual length and is time consuming [Aubert et al., 2012]. Flow-FISH is a flow cytometry-based assay for cells in suspension [Baerlocher et al., 2006]. It, too, is labor intensive and cannot be done on fixed tissue.

Considering the high cost and low throughput of these methods, Cawthon (2002) developed a qPCR method for simple, rapid, and high-throughput measurement of TL. In 2009, the same group improved the assay by multiplexing to reduce well-to-well variation [Cawthon, 2009]. Mainly because of cost-efficiency and low DNA requirements, most of the large-scale studies use the qPCR method for telomere measurement [Eisenberg et al., 2015; Lapham et al., 2015]. However, a recent study across six labs reported substantial heterogeneity in per-sample intra-batch coefficient of variation (CV) (0-31%) and inter-batch CV (0.2-28%) [Martin-Ruiz et al., 2015].

In 2014, we described a novel multiplexed probe-based non-PCR method for TL measurement using extracted DNA [Kibriya et al., 2014]. We utilized (a) the ability of the

Luminex platform to “multiplex” both the probes for telomere and reference gene from the same DNA in one well, and (b) the signal amplification capability of Quantigene chemistry without PCR amplification of the DNA. The assay was validated against Southern blot of the TRF [Pierce et al., 2016]. The Luminex assay is also free of well-position effects [Kibriya et al., 2016]. Using the original Luminex assay, around 60 independent DNA samples can be tested for relative telomere length (RTL) in a single batch. Now, we describe a novel four-fold increase in the throughput of this assay (240 samples per batch). By using the same capture probe on four different Luminex beads, we can “pool” four different DNA samples in a single reaction well to increase the throughput. In this paper, we show the reproducibility of this new high throughput pooled-sample multiplex Luminex assay for RTL measurement using the same Luminex 200 platform. This approach may offer an alternative cost-efficient way to study RTL in a large number of extracted DNA samples.

Methods

Principle of Luminex-based Telomere assay

In the probe-based Luminex assay, fluorescent Luminex microbeads with capture probes (CP) are used to target DNA molecules. Only 50 ng DNA is required as starting material. For each DNA target, two target-specific probe sets are designed: (a) Capture extenders (CE) and (b) Label extenders (LE) and blocker (BL) probes (see Figure S1). A detailed design was shown in a previous publication [Kibriya et al., 2014].

CE has two parts - one part is complementary to the CP sequence on the bead and the other part is complementary to the target DNA sequence that is interrogated (in our case, the telomere repeats and the reference gene). LE have two parts - one is complementary to the target DNA sequence and the other is complimentary to the “pre-amplifier.” The target-specific regions of CE, LE and BL hybridize to contiguous sequences of the target DNA. The preamplifier binds with multiple biotinylated amplifiers. Each amplifier provides multiple hybridization sites for biotinylated label probes that bind Streptavidin R-Phycoerythrin (SAPE) producing fluorescent signals. The signal intensities from the Luminex bead and the conjugated SAPE are read on a Luminex 200 instrument. The signal is reported as median fluorescent intensity (MFI) from at least 60 beads read within 60 seconds. The sequence specific probes (both CE and LE) for the telomeric region (T) were designed to target the repeats “TTAGGG.” The 24-mer probe is targeted against four repeats – “TTAGGGTTAGGGTTAGGGTTAGGG.” For a reference single gene (R), we used ALK. A detail of the assay protocol was described in an earlier publication [Kibriya et al., 2014].

Increasing the throughput by pooling samples

In this pooled multiplex Luminex assay, we used four different Luminex micro-beads (T1, T2, T3 and T4) for the same T probe and four different Luminex micro-beads (R1, R2, R3 and R4) for the same R probe. Each pair of probe sets (set1: T1R1, set2: T2R2, set3: T3R3 and set4: T4R4) was used in four separate 96-well plates to hybridize to DNA (see Figure 1). In a single batch, we simultaneously processed four 96-well plates in the hybridization step, each plate containing 60 test DNA samples (the remaining wells are used for standard and controls), and the corresponding wells of four such plates were pooled in a single plate for

all the subsequent steps: signal amplification, attaching detection dye, wash and final detection of signal intensity (see Figure 1). Thus, in the final reaction plate for one batch, in each well, we had eight different micro-beads – T1-4 and R1-4 capturing Telomeric regions and Reference gene regions (multiplexing two different genomic regions) from four different DNA samples (pooling four samples in one reaction well). After reading the fluorescent intensity, we could de-multiplex the four different DNA samples in a single well by simply looking at the different bead sets.

All of the incubation steps were done in a VorTemp 56 shaking incubator. The assay plate was read in a Luminex 200 instrument. The median fluorescent intensity (MFI) generated by XPonent software was processed for quantification using Milliplex Analyst software. It should be noted that the MFI is proportional to (a) the number of repeats (which determines the TL) and (b) the quantity of DNA in the reaction well (see Figure 2). Quantification for Telomere (TEL) and ALK (reference gene) was done against the 8-point standard curve (one “no-template control blank” and seven serial dilutions of the “standard” DNA sample from 400 ng to 6.25 ng per well and each dilution was run in triplicate) generated by five parameter logistic (5 PL) algorithm [Gottschalk and Dunn, 2005; Ricketts and Head, 1999]. The standard curve statistics for TEL and ALK quantification have typically r^2 of 0.99 – 1.00. Figure 2 shows the concept for calculation of RTL using this assay. In the Luminex assay, the RTL is expressed as Telomere Quantity Index (TQI). The TQI is calculated as TEL/ALK. Therefore, TQI is a measure of RTL, which is normalized for quantity of DNA in a well, and it is relative to the “standard” DNA sample [Kibriya et al., 2016].

By using four different bead sets, we could process a total of 240 independent samples (four 96 well plates, each containing 60 test samples, and the rest of the wells were used for “standards” and quality control samples) at a time in one batch. This design increases the throughput of the assay by four fold. To assess the reproducibility of the new pooled-sample assay, we used the following two study designs.

Study design 1: many samples, with few replications

To test the precision of this novel high-throughput assay, we used DNA samples from 60 independent individuals and repeated them in three different batches (batch I, II and III). All DNA samples used in this work were extracted from whole blood samples using the FlexiGene DNA Kit (Qiagen). We used archived DNA samples with previously recorded DNA concentrations between 43 – 70 ng/μL using NanoDrop. The mean 260/280 ratio was 1.82 (95% CI = 1.80 – 1.85). We did not measure the sample concentration again before using the Luminex assay. Demographic data for these samples were not known to the laboratory. The replica plates of these DNA from 60 individuals were made at the same time and stored at -86C until use. The samples were placed in same well positions in the replica plates. Batch I and II were run one week apart, and Batch III was run after one month. Within each of the three batches, DNA from each individual was tested with two different bead-sets (set1: T1R1 and set3: T3R3). Thus, DNA from each of these 60 individuals was tested six times (i.e., twice within each batch and repeated in three batches).

Study design 2: Two control samples, with many replications—We used the same two control DNA samples from two donors in nine batches. In each batch, the control DNA samples were hybridized with four sets of beads (T1R1, T2R2, T3R3 and T4R4) in four different plates and then combined for subsequent steps. These control DNA samples were normalized to ~50 ng/μL concentration with TE buffer. From each normalized sample, multiple aliquots were made and kept at -86C. For each batch, one aliquot of each sample was used. Thus, each tube was frozen and thawed only once. We took the RTL measurements of these two samples from subsequent 9 batches of the assay for a given project. This also gave us the opportunity to test the reproducibility in all four bead sets used in this pooled multiplex assay.

For internal validation of the newly developed pooled-sample multiplex assay, we analyzed previously generated TQI of the same 60 samples (used in design 1) using the original lower throughput Luminex assay without pooling, and compared that with the TQI generated from the new assay using the same DNA as “standard” for generating the standard curve for quantification.

Statistical Analysis

We used a 2-way ANOVA model using method of moments [Eisenhart, 1947; Tamhane and Dunlop, 2000] for detection of effect of batch and bead-set.

$$\text{Model1: } Y_{ikl} = \mu + \text{Batch}_i + \text{Person_ID}_k + \varepsilon_{ikl}$$

Where Y_{ikl} represents the l -th observation on the i -th Batch, k -th Person_ID; μ is the common effect for the whole experiment, and ε_{ikl} represents the random error. The errors ε_{ikl} are assumed to be normally and independently distributed with mean 0 and standard deviation δ for all measurements. Person_ID is a random effect and Batch is a categorical variable with fixed effect.

$$\text{Model2: } Y_{jkl} = \mu + \text{BeadSet}_j + \text{Person_ID}_k + \varepsilon_{jkl}$$

Where Y_{jkl} represents the l -th observation on the j -th BeadSet, k -th Person_ID; μ is the common effect for the whole experiment and ε_{jkl} represents the random error. The errors ε_{jkl} are assumed to be normally and independently distributed with mean 0 and standard deviation δ for all measurements. Here BeadSet is a fixed effect and Person_ID is random effect.

The proportion explained by a variable (partial Eta squared) was calculated as the sum of squares for that variable divided by the total sum of squares for all the variables in the ANOVA model [IBM Corp. released 2016. IBM SPSS Statistics for Windows, Version 24.0, Armonk, NY: IBM Corp].

The intraclass correlation coefficients (ICC) estimates and their 95% confidence interval (CI) were calculated using IBM SPSS Statistics version 24 (Armonk, NY: IBM Corp.) based

on average-measures ($n=6$), absolute agreement, 2-way mixed-effects model. To select the correct way of calculating ICC, we determined the (a) Model (b) Type, and (c) Definition based on the standard guideline, as described in the literature [Koo and Li, 2016]. It has been suggested that ICC values less than 0.5 are indicative of poor reliability, values between 0.5 and 0.75 indicate moderate reliability, values between 0.75 and 0.9 indicate good reliability, and values greater than 0.9 indicate excellent reliability [Koo and Li, 2016; Shrout and Fleiss, 1979].

Results

Using the new assay as described above, we could successfully pool four samples from four different plates into one reaction well of the final plate (“pooling” samples) after hybridization to TEL and ALK probes (“multiplexing” two genomic regions of interest from a single sample). In each batch, we could process $4 \times 60 = 240$ test DNA samples for RTL measurement.

Design 1

We ran DNA samples from 60 unique individuals in three batches and in each batch the DNA was hybridized to two bead sets (T1R1 and T3R3). Thus, each sample was repeated six times. We tested whether the assay result differed by batch or by bead set. Table 1 shows the mean (95% CI) of quantification data from TEL, ALK and the TQI (measure of RTL) from all 360 samples. The data stratified by batch are presented in Table 1a and the data stratified by the bead set are presented in Table 1b. There was no statistical difference of RTL between the three batches (Table 1a) or between the measures using either of the two different bead-sets tested (see Table 1b). A complete data set is provided in the supplementary material as Table S1.

In the next step, in the ANOVA for TQI as the dependent variable, we included “Batch” as an independent variable in the model along with “Person.” The result is presented in Table 2. As expected, TQI was different from person-to person ($p=1.23 \times 10^{-45}$). In other words, significant variation in TQI could be explained by “person-to-person” variation (accounting for 68% variation, partial Eta squared = 0.676), whereas there was no significant “Batch” effect ($p=0.91$). Less than 0.1% of the variation in TQI could be explained by “Batch” (partial Eta squared = 0.0007, model 1 of Table 2). Similarly, only ~0.3% of the variation in TQI could be explained by the difference between bead sets (partial Eta squared = 0.0031, see model 2 of Table 2).

The precision of the assay was evaluated by ICC [Shrout and Fleiss, 1979]. To test the reproducibility of the assay, each of the same 60 samples was tested six times (3 batches $\times$ 2 bead types). Because these six tests may be considered as “rater” for a typical ICC measurement, we used a “two-way mixed-effects” model. To test the replicability, we used six measurements for each sample, i.e., an “average of 6-measurements” as type. Our objective was to see how close the measurements in the six tests were, and so we used “absolute agreement definition.” The ICC was 0.908 (95% CI 0.856 – 0.942) suggesting good to excellent replicability of this new high throughput Luminex assay. The TQI was also not dependent on the input DNA quantity ($r=0.01$, $p=0.94$, see Figure S2). Neither the TQI

nor the variation in TQI showed correlation with the 260/280 ratio of DNA (see Figure S3 and S4).

Design 2

We also checked the precision in a larger number of replicates from two QC samples, each of which were repeated more than 100 times in a subsequent nine batches for a given project. The reproducibility of TQI stratified by batch and bead sets is shown in Table 3. These two samples were tested with all four bead sets. Because of only two random samples in this design, ICC statistics may not be the most appropriate way to interpret the precision [Koo and Li, 2016]. There was no significant “Batch effect” or variation due to difference in bead sets.

Reproducibility in large-scale study

To test the reproducibility of the assay, RTL was measured for 3,123 tissue samples (up to 12 tissue types per donor) using this high throughput assay [Demanelis et al., 2017]. In that study, as a part of QC, a total of 233 samples were randomly duplicated and the laboratory was totally blinded to their identities. There was no difference between the two measurements ($1.11 \pm \text{SD } 0.348$ vs. $1.10 \pm \text{SD } 0.356$, paired t-test $p=0.614$). The strong correlation between the two measurements is shown in Figure 3. The ICC between these two measurements using an absolute agreement definition was 0.897 (95% CI 0.866 – 0.920).

The present study shows that, by using four different bead types, we have now increased the throughput of this RTL Luminex assay by four-fold (from 60 to 240 samples per run).

Internal validation of the assay

To validate this new assay, we compared two measurements of RLT (one from the old lower throughput assay and one from new the higher throughput assay with same bead set and the same DNA as “standard” to generate standard curves for quantification) obtained from the same samples. The original lower throughput multiplex Luminex assay was done almost one year ago. Pearson's correlation between these two measurements was strong ($r=0.79$, $p=1.44 \text{ E-}15$, see Figure 4). Although there was a significant difference in quantity of input DNA for these two assays ($74.10 \pm \text{SD } 24.82$ vs. $115.79 \pm \text{SD } 67.56$ ng/well for the new and old assay respectively, $p=2.58 \text{ E-}07$), the TQI measurements were very similar ($0.90 \pm \text{SD } 0.11$ vs. $0.89 \pm \text{SD } 0.10$ for new and old assay respectively, $p=0.18$). The average (95% CI) ICC between these two measurements using an absolute agreement definition was 0.882 (95% CI 0.807 – 0.927). In fact, the TQI values from old assay correlated well with corresponding values from all the six replications (separate scatter plots not shown). When we considered the mean of six replicates as TQI values from the new assay and compare those with the corresponding TQI values from the old lower throughput assay, the correlation was strong ($r=0.88$, $p=7.88 \text{ E-}19$, see Figure S5). Also, the ICC statistics between these two measurements – TQI values from the old assay and the mean TQI values of new assay - was consistent (ICC 0.93 (95% CI 0.891 – 0.963)). It may be noted that, previously we have validated the results from the original Luminex assay without pooling by conducting a blind comparison with TRF by Southern blot [Kibriya et al., 2014; B L Pierce et al., 2016]. The correlation coefficient (“r”) between the Southern blot and Luminex methods was between

0.65 and 0.75 in two different settings [B L Pierce et al., 2016]. The original Luminex assay also correlated well with conventional single-plex qPCR. We found the correlation coefficient (“r”) between 0.7 and 0.8 in different settings [Kibriya et al., 2014].

External validation of the assay

TL is known to have a negative correlation with age. Such a known negative correlate may be used as external validity of an assay [Eisenberg, 2016; Eisenberg et al., 2015]. In an independent set of DNA samples from 505 individuals (m=329, f=176), the leukocyte RTL was measured by this new high throughput assay [Demanelis et al., 2017]. The subjects were between 21 years and 70 years of age. Linear regression analysis suggested that, with an increase of age by 10 years, the TQI decreased by 0.08 (95% CI 0.09 – 0.06). The RTL negatively correlated with age ($r = -0.41$, $p < 0.0001$, see Figure 5). In other words, 17.5% of variation ($r^2 = 0.175$) in RTL was explained by variation in age. In our previous study using the original lower throughput Luminex assay in another set of samples (n=50), we found that 14.5% of variation ($r^2 = 0.14$) in RTL was explained by variation in age [B Pierce et al., 2016].

Discussion

In 2014, we developed an oligonucleotide probe-based Luminex RTL multiplex assay where we interrogated both the telomere region and the reference gene region of a DNA sample simultaneously from a single well reaction [Kibriya et al., 2014]. This approach allowed us to overcome the potential error arising from the difference in input DNA quantity for TEL and ALK quantification if those were measured from two different wells (traditional single-plex reaction). We processed 60 samples at a time in one batch. Considering the fact that the Luminex 200 platform can read up to 100 different bead types from a single well, and that for the RTL assay we use only two genomic regions of interest, we attempted to use the same DNA capture probes on different bead types to increase the assay throughput by processing a larger number of samples in one batch. We hybridized four sets of beads (each set consists of two bead types – one for TEL and one for ALK) in four different plates of DNA samples (each 96-well plate contains 60 different test DNA and the rest of the wells contain standards and QC). We then pooled them in one final plate and processed all 240 test samples for signal amplification in a single batch to measure RTL. We demonstrated the reproducibility of this novel high throughput RTL assay where we successfully increased the throughput from 60 to 240 samples in a single batch. In terms of the number of independent samples that can be tested for RTL in a single run, to our knowledge, we describe a novel pooled multiplex assay with highest throughput for RTL measurement.

This new high throughput pooled-sample Luminex assay was validated against the original Luminex assay without pooling. The ICC between the new and the original assay was 0.882 (95%CI 0.807 – 0.927) which is good to excellent. The reproducibility of this new assay measured by six replicates in 60 independent samples was also good to excellent with an ICC of 0.908 (95% CI 0.856 – 0.942). We did not find any significant “batch effects,” which is a potential advantage of this method for large-scale epidemiological studies. The

reproducibility was also checked in another independent set of duplicate samples (n=233), and good to excellent precision was reconfirmed with ICC 0.897 (95% CI 0.866 – 0.920).

Some recent papers show that the coefficient of variation (CV), while conventionally used in the TL measurement literature, may be confusing for “comparing the precision of qPCR and TRF” [Eisenberg, 2016; Verhulst et al., 2015; Verhulst et al., 2016]. The “comparison of CV” is invalid if measurement(s) from any of the assays do not have natural zero points [Eisenberg, 2016]. In theory, the zero point of RTL measurement by any assay represents the point of essentially no telomere repeat sequence in a sample. However, the TRF measurement includes the telomeric repeats and sub-telomeric sequences [Kimura et al., 2010]. Therefore, the CV reported from TRF may show a lower value [Eisenberg, 2016].

The Luminex assay is based on hybridization of telomeric repeat sequence-specific probes. For CV, the implicit assumption is that the variance is proportional to the mean. Some studies suggest that this holds true for TRF, but may not hold true for qPCR data [Verhulst et al., 2015]. Our data suggest that this holds true for our new high throughput assay, as well (see Figure S6). Verhulst and colleagues suggest using the ICC instead of CV as a measure of precision [Verhulst et al., 2015]. For accuracy, Eisenberg suggested that well-established correlates of RTL (for example, RTL decline with age) can be used as a marker of external validity [Eisenberg, 2016; Eisenberg et al., 2015]. Accordingly, we have used ICC for reporting the precision of this new assay. We have also provided data on negative correlation with age in an independent set of samples.

To increase the throughput, one of the largest studies addressing RTL using the qPCR method, utilized robots to process and handle 384 well plates [Lapham et al., 2015]. Because our method involves hybridization in 96-well plates, followed by pooling in a single plate, the assay can be done manually using a standard multi-channel pipette. Also, a traditional Luminex 100 or Luminex 200 scanner with a 96-well block is used to read the intensity. Thus, in terms of data point per batch, our assay is practically equivalent to a 384-well assay. It may be worth mentioning that the conventional qPCR (both single-plex and multi-plex qPCR) typically uses triplicate or quadruplicate [Ehrlenbach et al., 2009; Eisenberg et al., 2015; Lapham et al., 2015], whereas the Luminex assay does not require that. Our data provide proof of principle for effectively increasing the throughput of RTL Luminex assay using this pooled multiplex method. Theoretically, it may be possible to further increase the throughput to 1200 samples per run by pooling up to 20 bead sets.

Regarding the input DNA quantity, we recommend 50 – 100 ng per well. Since the TEL repeat regions are much more abundant than any single gene, it is possible to get good signal to noise from the TEL probe hybridized to an even lower quantity of DNA. However, it is difficult to get adequate signal to noise from the ALK probe hybridizing to a lower quantity of DNA. For this reason, this assay requires a little more DNA per well than a typical qPCR reaction per well. Even so, this is significantly lower than a TRF assay, and considering triplicates/quadruplicates in qPCR, the total requirement is almost the same as qPCR. Previously for the original Luminex assay without sample pooling, we demonstrated that the CV of TQI from 50 ng and 100 ng input DNA was below 5% [Kibriya et al., 2014]. The current study was not designed to show the effect of input DNA on TQI measurement, but

the data suggested that the TQI was not dependent on the input DNA quantity (see Figure S2).

The original Luminex assay for telomere correlates well with the traditional gold standard TRF [Kibriya et al., 2014; B L Pierce et al., 2016]. In a blinded comparison, the Luminex and Southern blot measurements for the same 50 DNA samples were taken in two independent laboratories (Ahsan Lab and Aviv Lab); each sample was measured twice, several months apart [B L Pierce et al., 2016]. The correlation (r) between Southern blot and Luminex was 0.65 in round-1 and 0.75 in round-2 [B L Pierce et al., 2016]. In addition, the lower throughput Luminex RTL assay did not show the well-effect that are often observed for the qPCR method of RTL assay [Kibriya et al., 2016].

In this study, we have used DNA extracted from whole blood. However, this assay can be used for any extracted DNA sample irrespective of source – e.g., T- and B-lymphocytes, different human tissues, or cell culture. It may be noted that neither the conventional qPCR nor the Luminex assay directly measures the actual TL, but instead measure the relative abundance of the telomere repeat sequence – compared to a particular standard sample. Therefore, this is a relative measure. However, it may be possible to generate absolute TL in kb only by calibrating against a DNA sample with known absolute TL. We acknowledge that calibration with TRF measurement may not be accurate because of the fact that TRF takes the subtelomeric region into account, and because, by design, the Luminex assay uses hybridization probes specific to the telomeric repeats only.

The Luminex assay is different from the qPCR (both single-plex and multiplex) with respect to the fact that the Luminex assay does not amplify the DNA and therefore does not introduce any variation in the RTL measure that reflects variation in DNA amplification efficiency – which is an important issue to consider for qPCR. Rather, this Luminex assay depends on hybridization of telomeric repeat sequence-specific probes to the telomeric DNA. In this respect, it has similarity to FISH or qFISH, although this assay can only be done in an extracted DNA sample, not in tissue or in-situ.

Like the TRF and qPCR assays, this Luminex assay also estimates only the average TL/content in the sample, and cannot address heterogeneity in TL/content across chromosomes. However, average TL measurement may have utility for different research questions. Different populations may have different average RTL in peripheral blood – e.g., people who have spent a significant portion of their lives in a polluted environment, drinking contaminated water, or eating food containing harmful additives. The average RTL may be associated with mortality [Brouillette et al., 2007; Dean et al., 2017; Fitzpatrick et al., 2011]. Chronic environmental exposure to a specific antigen may alter average RTL in T- and B-lymphocytes. In addition, different human tissue may have different average RTL [Dlouha et al., 2014].

Conclusion

We describe a novel high throughput pooled-sample multiplex Luminex assay for RTL measurement from a small quantity of DNA with good to excellent precision. The assay is suitable for large-scale studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation of the pooled multiplex assay for RTL. Upper pane shows the four 96-well plates, each containing 60 independent test DNA samples (marked by blue color) and the rest of the wells (marked by grey color) were used for “standard” and “control” samples. Samples in each of these four plates were hybridized with designated bead sets. Four different beads for TEL and four different beads for ALK are shown in different colors. The outer surface of all the four beads for TEL contain the same capture probe for TEL (shown in red) and the outer surface of all the four beads for ALK contain the same capture probe for ALK (shown in dark blue). Four different DNA samples in corresponding wells (e.g., well A12) of four plates are shown in different colors as horizontal lines attached to the beads. The lower panel shows the pooling of corresponding wells of all the four plates after hybridization. So in this final reaction plate, each well contains eight different beads. Four have captured TEL region from four different DNA samples and four other beads have captured ALK region from those four DNA samples. This final reaction plate was processed for signal amplification using pre-amplifiers, amplifiers and then StreptAvidin PhycoErythrin (SAPE) as described in method section and then read in Luminex 200 scanner for fluorescence.

Figure 2.

Cartoon to illustrate the concept of TQI measurement.

Four wells containing Standard and three samples are shown. Each line represents a DNA molecule. Difference in DNA input quantity is shown by the number of molecules in each well. TEL repeats are represented by star (red) and ALK region is represented by round (blue) marker. Median Fluorescent Intensity (MFI) is proportionate to the number of repeats (see standard and sample#1) as well as input quantity (see standard and sample#3).

Quantification of TEL and ALK is done in comparison to the standard. TQI is not affected by the input quantity of DNA (see standard and sample#3). Sample#2 shows an example of mixture of DNA molecule of varying number of TEL repeats.

Figure 3.

Reproducibility of the new high throughput assay for RTL measurement in a large-scale study. A total of 233 samples were run in duplicates as a quality control measure. The first measurement of TQI is shown on x-axis and the second measurement on y-axis.

Figure 4.

Validation of the new high throughput pooled-sample multiplexed assay against the original Luminex assay without sample-pooling. We compared the two measurements of TQI from the same samples (one measurement from the old lower throughput assay shown in x-axis and one from the new higher throughput assay with same bead set and the same DNA as “standard” to generate standard curves for quantification, shown on y-axis).

Figure 5.

External validation of the new high throughput pooled-sample multiplexed assay. The blood leucocytes RTL (TQI) shows significant negative correlation with age.

Table 1

Descriptive statistics: sixty independent samples, each repeated six times.

1a. Descriptive statistics by Batch									
Variable	Stratified by Batch	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	ANOVA p-value for Batch
TEL	Batch I	120	81.84	42.80	74.10	89.57	24.61	444.29	
	Batch II	120	85.78	40.65	78.44	93.13	31.15	363.80	
	Batch III	120	79.64	26.92	74.78	84.51	32.98	195.40	
ALK	Batch I	120	86.85	48.07	78.16	95.54	25.85	529.51	0.438
	Batch II	120	89.82	34.26	83.62	96.01	32.35	277.95	
	Batch III	120	84.10	24.76	79.62	88.57	38.64	221.98	
TQI	Batch I	120	0.95	0.12	0.93	0.97	0.69	1.29	0.488
	Batch II	120	0.95	0.14	0.92	0.97	0.72	1.36	
	Batch III	120	0.94	0.14	0.92	0.97	0.62	1.35	
0.963									

1b. Descriptive statistics by bead set									
Variable	Stratified by Bead set	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum	ANOVA p-value for bead set
TEL	Set_1	180	83.17	30.55	78.68	87.67	24.61	224.40	
	Set_3	180	81.67	43.32	75.30	88.04	30.00	444.29	
ALK	Set_1	180	87.28	28.94	83.02	91.53	25.85	225.18	0.704
	Set_3	180	86.57	43.56	80.16	92.97	29.52	529.51	
TQI	Set_1	180	0.95	0.12	0.93	0.97	0.69	1.35	0.856
	Set_3	180	0.94	0.14	0.92	0.96	0.62	1.36	
0.547									

Table 2

ANOVA models for “Batch” effect and “Bead set” effect on the TQI measurements.

	Variables in model	df	Mean Square	F	Sig.	Partial Eta Squared
a. Model 1:	BatchNo	2	0.001	0.098	0.91	0.0007
	Person_ID	59	0.071	10.551	1.23E-45	0.6763
b. Model 2:	BeadSet	1	0.006	0.939	0.33	0.0031
	Person_ID	59	0.071	10.613	6.09E-46	0.6768

Table 3

Reproducibility of the QC DNA samples

QC sample	Stratified by Batch	Mean	Std. Deviation	No. of times repeated	CV%	ANOVA p-value for Batch
F_2015_01	B	0.94	0.05	12	5.53	
	C	0.94	0.05	12	5.53	
	D	0.96	0.07	16	7.73	
	E	0.91	0.06	10	6.52	
	F	0.93	0.04	12	4.80	
	G	0.91	0.08	9	9.23	
	H	0.93	0.08	14	8.41	
	I	0.97	0.07	12	7.24	
	J	0.96	0.06	10	6.63	
						0.377
	Total	0.94	0.07	107	7.00	
K_2015_01	B	1.00	0.06	12	5.91	
	C	1.00	0.06	12	5.91	
	D	1.02	0.09	12	9.09	
	E	0.96	0.11	10	11.42	
	F	0.95	0.06	12	6.46	
	G	0.95	0.06	9	6.46	
	H	0.98	0.10	12	10.57	
	I	0.99	0.10	12	9.99	
	J	0.99	0.06	12	6.23	
						0.45
	Total	0.98	0.08	103	8.24	
QC sample	Stratified by Bead set	Mean	Std. Deviation	No. of times repeated	CV%	ANOVA p-value for Bead set
F_2015_01	Set_1	0.93	0.06	27	5.94	
	Set_2	0.94	0.08	24	8.00	
	Set_3	0.94	0.07	29	7.89	
	Set_4	0.94	0.06	27	6.30	

QC sample	Stratified by Batch	Mean	Std. Deviation	No. of times repeated	CV%	ANOVA p-value for Batch
K_2015_01	Set_1	0.99	0.08	26	8.54	0.941
	Set_2	0.98	0.09	23	8.73	
	Set_3	0.99	0.08	27	8.10	
	Set_4	0.97	0.08	27	7.97	
						0.8