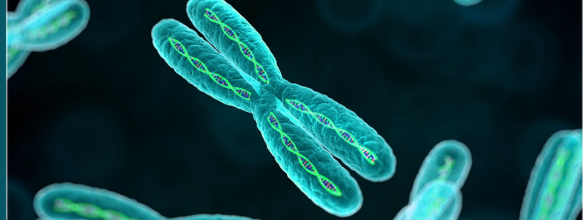


TRN protocols: Cross method precision study

Date submitted to TRN for TRN webpage upload:

1/18/2022

Date expected completion of study:

May 2022

PI (name, lab, university)

TRN network

Other laboratories contributing:

See Table 1

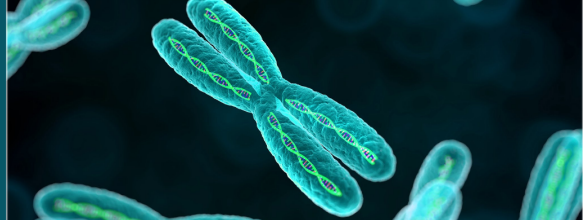
Brief summary

The goal of this study is to determine the precision of telomere length (TL) measurements by different techniques based on "blinded" analysis of the exact same DNA sample extracted in a single extraction and assayed on two different occasions. Before the formation of the TRN, and as part of the original RFA (https://grants.nih.gov/grants/guide/rfa-files/rfa-ag-19-022.html) a set of samples, specifically collected for cross-method comparisons were created by the National Institute of Aging. Following the completion of the first large scale cross methods multi-lab study related to DNA extraction, the TRN Steering Committee has identified a key protocol to further the understanding of the relation between different telomere length assays and to evaluate the varying degrees of precision in TL measurement by the respective assays in different laboratories.

Background and significance of study protocol

Except for ongoing TRN protocols, only two small studies performed "blind" and impartial comparisons of TL measurements by different methods. One study compared Southern blotting (SB), measured in one laboratory, and qPCR, measured in another laboratory in 50 samples of DNA extracted from leukocytes (1). Measurements were performed on two occasions. The inter-assay CV for was 6.45% for the qPCR and 1.74% for the SB. In the other study TL was measured in seven laboratories by qPCR, two laboratories by SB, and one laboratory by single telomere length analysis (STELA). Measurements on 10 samples (two from leukocytes) were performed on two occasions. The study's conclusion was that "Gel-based and PCR-based techniques were not different in accuracy." (2) Several challenges to the interpretation of these results exist including the reliance on CV for an estimation of precision and cross method comparisons resulting in significant scientific gaps in the understanding of the cross methodologic precision of different telomere length assays (3).

To date there is evidence to conclude that qPCR-based methodologies have decreased precision than SB approaches, however the sources of the decreased precision, and the extent of the differences in precision as well as the extent to which qPCR-based methods can be



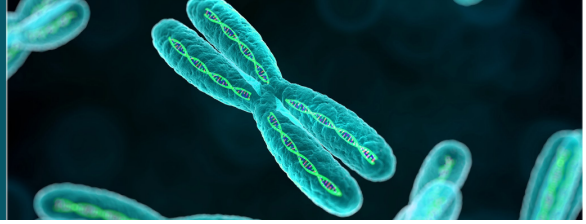
optimized remains unknown. Data presented by the TRN at the annual meeting in December of 2021 in New Orleans indicated both a substantial lab and a significant extraction method effect on precision for PCR-based measurement of TL. As the original TRN directed multi-lab study that has been completed was designed to assay the effect of DNA extraction and not to examine origins of between lab effects of PCR based or Southern Blot-based TL measurement, nor did it include MMqPCR and other regular utilized assays of TL measurement, this follow-up protocol is being performed.

In an effort to provide critical guidance to researchers about the precision of TL measurements conducted in different laboratories and utilizing different assays, the Steering Committee of the TRN designed this second large-scale cross laboratory and cross methodology study to evaluate TL measurement precision both across laboratories and, due to a substantially increased number of participating laboratories, also across methodology **utilizing a single DNA sample extracted at one time in a separate lab and measured on two distinct occasions by each of the other participating laboratories**. In this study samples are blinded at two stages and all analyses will be done by the TRN biostatistician. Acknowledging that protocol adaptations occur across laboratories and biological assays, and that even with the exact same protocol conducted in a different laboratory, differences arise, this study was designed to maximize the number of participating laboratories, including multiple labs conducting similar methods (e.g. monoplex qPCR, aTL, TRF, MMqPCR, etc.) to provide "real world" data related to the range of precision for the general assay categories (e.g. qPCR-based, Southern Blot, FISH etc.) and enhanced estimates of the between methodology precision differences. As DNA methylation (DNAm) has been proposed as a novel approach to generating and estimate of TL this was also included as a method acknowledging that the original estimates of DNAmTL were developed and trained on SB generated TL estimates (3). One U01 laboratory has been developing a high throughput DNA FISH based assay for TL measurement, as such a separate set of DNA samples are being created but stored for future measurement using this new method upon methodologic finalization. Data obtained from this study is expected to provide empirical data permitting the TRN to provide specific recommendations about assay selection, methodological precision, and ultimately feasibility and sample size requirements to researchers evaluating TL as a biomarker in population based studies.

Primary study comparison

Multiple-lab, multiple methodologic comparison of telomere length measurement and assay precision utilizing a single DNA sample. The data will enable an impartial assessment of the Intraclass correlation (ICC) for variation for replicates (repeatability) measured on the same occasion and on different occasions. For population-based telomere research, the relevant ICC is for replicates performed on different occasions on different extractions but for the purposes of this study and given the study results from the first TRN repeated extraction study, this protocol will use a single DNA sample extracted by one laboratory and sent to all participants.

Key words: Southern Blot, qPCR, MMqPCR, DNAm, aTL, FISH, telomere length, methodologic comparison, assay precision, repeatability, PBMCs



AIM 1:

Generate ICCs for TL measurements by different methods in different laboratories based on "blind" measurements on N=107 samples measured one month apart on the same DNA samples extracted from peripheral blood mononuclear cells (PBMCs) using the Qiagen Midi Prep.

Outcome 1:

Impartial ICC for duplicate samples measured by different TL measurement methods.

AIM 2:

Evaluate the relation between DNAm TL estimate and other TL estimations using SB, qPCR based, and FISH based estimates.

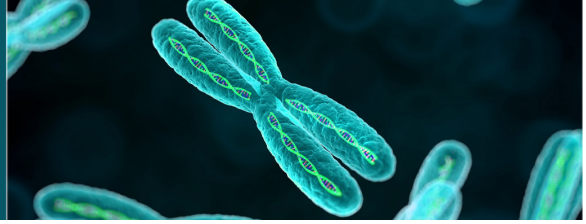
Outcome 2:

Correlation between TL measurements by assay type and by lab with DNAm estimation of TL

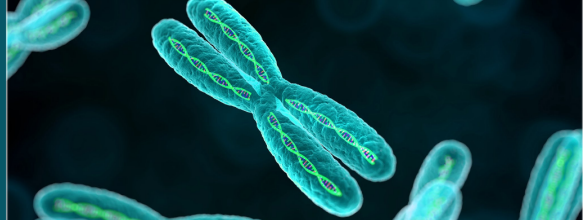
Methods:

Sample descriptions:

1. Prior to the first meeting of the TRN in December of 2019, NIA created 17 sets of between 3 and 5 million PBMCs from a total of 107 individuals. These samples have been stored at NIA since their creation and no demographic or identifying information has been shared with any member of the TRN or any of the participating laboratories.
2. NIA will send 4 matched sets of PBMCs (4 out of the original 17 sets created) from each of the 107 individuals, (a total of 428 tubes), to the u24 for DNA extraction. These samples were donated by healthy female and males (age range birth to 83 years, race and ethnicity are unknown) who were unrelated. The blinded codes will be sent to the U24 biostatistician.
3. The blinded set of 4 per individual will be sent on dry ice, overnight via fedex, with a temperature logger for tracking of any temperature deviation to the U24.
4. DNA will be extracted by U24 laboratory within one week of receipt from NIA and extracted DNA will be stored at -80 at temperature will be continuously monitored during storage.
5. PBMCs will be spun, washed and resuspended. The cells from all four tubes (expected between 15,000,000 and 20,000,000/individual) from each individual will be resuspended together in a final volume of 1ml following manufacturer's recommended protocol, with the following modification that the final volume will be due to two elutions of 500ul each for the Qiamp Midi kit resulting in an expected total yield of between 60-80ug of DNA based on test extractions in the U24 laboratory from each of the 107 individuals. The volume of the final stock DNA extraction will be 1000ul following two elutions of 500ul each to 1000ul/individual to ensure sufficient total volume for aliquoting across all sets for shipping to the participating laboratories with a minimum of 10ul/aliquot.



6. An 10ul aliquot of the stock sample will be obtained and utilized for nanodrop and QuBit estimation of 260/230; 260/280; and DNA concentration as well as DNA Tapestation for DIN generation. Samples will also be evaluated via agarose gel electrophoresis.
7. Based on the Qubit concentration 40 aliquots (20 pairs) from each of the 107 extractions (a total of 4280 individual aliquots) will be generated with 1 of the following total amounts of DNA and a minimum volume of 10ul
    a. 3 sets (pairs) will be generated 6ug/aliquot for TRF (36 ug total)
    b. 14 sets will be generated with 600ng/aliquot of DNA for PCR based assays (16.8ug total, minimum volume of 10ul/aliquot)
    c. 2 sets will be generated with 1.2ug
    d. I set of 1.2 ug is being sent to a U01 laboratory performing multiple different qPCR protocols related to different master mixes.
    e. 1 set will be generated with 600ng for DNAm analyses
    f. **NOTE**: 2/14 sets of 600ng and 1 set of 1.2ug will be stored and saved for future use, including use by the U01 laboratory optimizing DNA-FISH single telomere assay
8. Aliquot pairs will be blinded by the U24 between and within sets. Information regarding the relation between NIA blinded IDs and the U24 blinded IDs will be sent to TRN biostatistician. As such there will be two independent blinding steps such that only the U24 biostatistician has the ability to determine the paired individuals sent to each laboratory as well as the relation between specific individuals across laboratory
9. In February of 2022 one aliquot from each of the 107 individuals will be sent to all participating laboratories via fed ex on dry ice with a temperature logger, including the sample for DNAm analyses
10. All quality control data with matched IDs and DNA quantity will be sent to all participating laboratories with the samples on dry ice with a temperature logger and this data will also be sent to the U24 biostatistician
11. Participating laboratories will upload temperature tracking data and send this data to the U24 data architect.
12. Laboratories are expected to complete TL analyses within one to two months of sample receipt. All laboratories will utilize their own standardized protocols and machines and proceed with TL analyses as they routinely do in the laboratories. All laboratories will submit detailed protocols inclusive of all reagents, reaction parameters and provide sufficient level of detail to ensure reproducibility.
13. Laboratories will send TL measurement and associated sample ID to the U24 biostatistician
14. After the receipt of all TL data from a laboratory the duplicate set of 107 aliquots will blinded IDs will be sent to each laboratory for analysis with a target timeline of between 1 to 2 months.
15. After completion of TL analyses the TL measurement and associated sample ID will be sent to the U24 biostatistician
16. A set of PBMCs will be sent to one laboratory for HT-FISH



17. All data analyses will be done by the U24 biostatistician following the analyses protocol outlined below and will evaluate both between lab and between method variation.
18. The U24 biostatistician will provide individual laboratories their own ICCs
19. All other data reporting will be de-identified with respect to specific laboratories.
20. All participating laboratories are invited to participate in the peer reviewed reporting of the results of this study protocol with authorship determined based on standard guidelines

Analytic approach

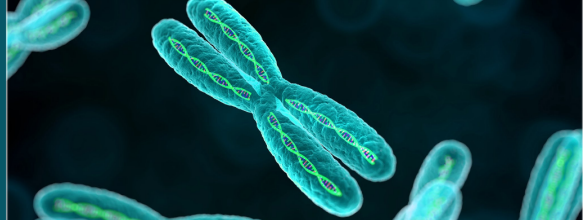
Prior to analyses, telomere length estimates on a relative scale (e.g. qPCR) will be transformed to a standard normal distribution (Z-score) for each single set for each laboratory. ICC estimates (±s.e. and 95% C.I.) will be estimated using the R-package rptR. Technique effects on ICC estimates will be evaluated using meta-analysis techniques using the R-package metafor, treating each estimate with associated uncertainty (s.e.) as a data point. Analyses will include analyses done separately without laboratories with commercial interest as well as the externally blinded MMqPCR done in the U24 (Drury) laboratory to minimize any potential for bias.

Primary and Secondary predictors

All TL analyses will be done blinded to individual and replicate. Blinded is expected to be at multiple levels with only the U24 biostatistician capable of unblinding. Primary analyses will be between methods where each laboratory provides one data point for the specific methods, this comparison will be done based on the ICCs between duplicate measurements. Secondary analyses will assess the aggregate comparisons of ICCs by general assay type (e.g., qPCR, MMqPCR, SB, etc.) Usefulness of the ICC as an indicator of precision will be verified, as was done previously in the cross method comparison study on the basis of associations with TRF. Secondary analyses will evaluate the correlation between TL measurement and age and gender acknowledging the limitation of this based on sample size.

Participating laboratories: Table 1

Lab	PI	Location	Method
1	Aviv	Rutgers	TRF
2	Shalev	Penn State	aTL
3	Lin (1.2 ug)	UCSF	qPCR
4	Zhang	Georgetown	qPCR
5	DeVivo	Harvard	qPCR
6	Notterman	Princeton	aTL
7	Cawthon	Utah	MMqPCR
9	Narwot	University Haslet	qPCR



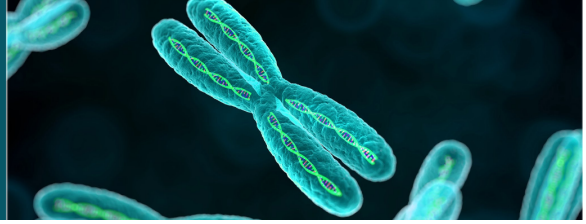
10	Bailey	Colorado State University	MMqPCR
12	Drury+	Tulane	MMqPCR
13	Benetos/Toupance	Université de Lorraine	TRF
14	Blasco **/*	CNIO	HT-FISH
15	Kobor	University of British Columbia	DNAm
16 #	Zhang (1.2ug)	Georgetown	HT-DNA qFISH
17	Pending*		TRF
18	Pending*		aTL

** lab will not receive DNA but will receive PBMCs given methodologic requirement; pending
# DNA will be stored until new methodology if optimized, expected Spring/Summer 2022
* Highlighted: indicate pending agreement. Target goal is a minimum of 3 labs per assay type (1/17/2022)
+ samples will be externally blinded by U24 biostatistician and relabeled by external team

Dissemination of results:

Dissemination of results will occur through several approaches. First the finalized protocol will be posted on the TRN website before any data analyses occurs and submitted as a preregistered study to Open Science. Second the data will be presented at the TRN annual meeting in December 2022 with the presentation record and stored on the TRN website as a freely accessible webinar. Third, the protocol and preliminary data, as well as the actual raw data, will be submitted as a white paper and located on the TRN website which will be accessible freely to the broader TRN and other invested groups and researchers. Finally, the TRN will work with the participating laboratories to prepare a peer reviewed manuscript describing the findings of this multi-laboratory multi-method study. Laboratories or investigators with commercial interest in telomeres or telomere length measurement or other interest that could be perceived as creating the potential for bias, who are involved in this study will declare those interests and full transparency will be ensured at each step in the development, implementation, reporting and dissemination of this protocol and subsequent results. All laboratories measuring telomere length agree to only perform the telomere length measurement by the proposed method outlined in table 1 and agree to not perform any other analyses. The incorporation of laboratories from multiple countries, as well as laboratories that provide fee for service analyses, was specifically done to ensure representative study design, maximize generalizability, and minimize potential for bias. Recognizing the importance of transparency and scientific rigor this study was designed to provide researchers, reviewers and agencies considering the funding of studies of telomere length in relation to population health and environmental and psychosocial exposures an unbiased estimate of assay precision and the relation between different methodological approaches to telomere length measurement.

References:



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