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Introduction

New to the field of telomere research? The Telomere Research Network has created this guide to assist researchers who are thinking about measuring telomere length (TL) in human population studies. This guide is intended to assist researchers in writing grants, designing studies, and conducting analyses involving telomere length measurements.

Although TL is a primary hallmark of aging and has been linked to various disease risks and exposures, inconsistencies across studies and procedures hamper our ability to test fundamental mechanisms and interactive models. Despite these limitations, the value of telomere research is significant, as meta-analytic findings have confirmed relationships between TL and all-cause mortality, depression, schizophrenia, cardiovascular disease, as well as presumed precursors including air pollution and early life stress.

In this guide, the TRN provides resources for investigators to maximize the scientific validity of telomere length measurement. A brief background on telomeres and telomere length is provided, along with a comparison of different methods for measuring telomere length.

Given field-wide concerns related to assay reproducibility and repeatability, for investigators who wish to measure telomeres in their own lab, we strongly recommend working with an established telomere lab and significant attention to reproducibility and repeatability of the assay (even if using commercial kits). We also strongly encourage assay validation with an external lab partner before initiating sample analysis, either with the same assay or another TL assay. For investigators working with an established lab, we also provide checklists for tracking important pre-analytic and analytic variables, as well as reporting recommendations of assay conditions for publications.

Additional resources for study design, analysis, and publication are available at the TRN website: https://trn.tulane.edu.

Updated March 4, 2021

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The little book of telomeres.

A primer for the ties between telomeres, health, and disease

A publication of the Telomere Research Network

December 2020

Authored by: Stacy S Drury, PI, Telomere Research Network
With special thanks to Celia Mayne, Waylon Hastings, Sue Rutherford Siegel, Jerry Shay

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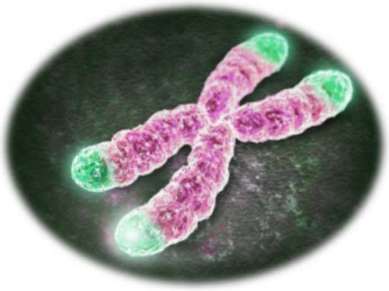
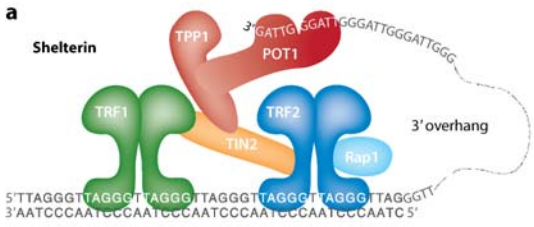
Telomere basics





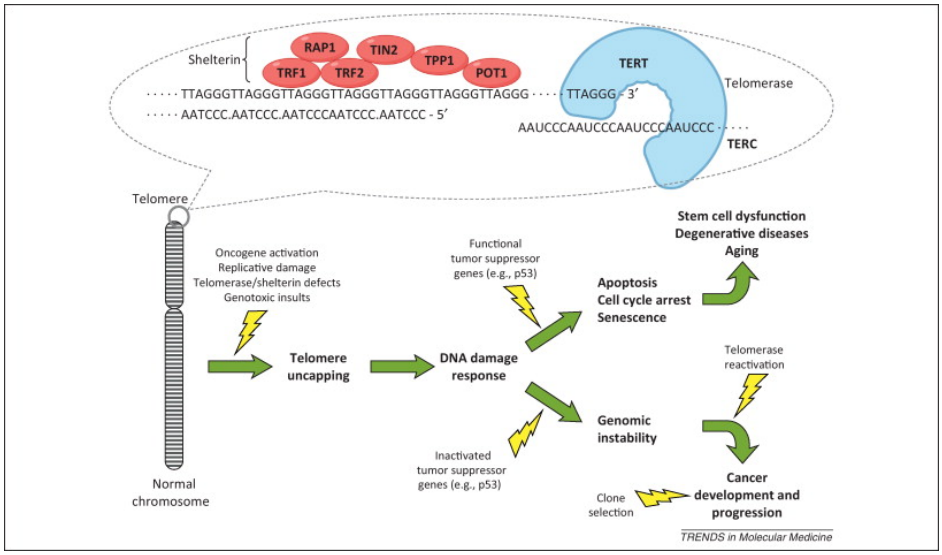
de Lange, 2018

Basics

1. DNA/protein/RNA complex at the end of all eukaryotic chromosomes
2. Repetitive, highly-conserved DNA sequence
3. Critical for chromosome integrity
4. Protects DNA sequence during replication due to incomplete lagging strand synthesis
5. Lengthened through telomerase OR alternative telomere lengthening (recombination)
6. Pathogenic role in certain cancers and telomere spectrum disorders
7. Linked to cellular senescence, apoptosis, cellular differentiation

2

The cancer vs degeneration balancing act- theory



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https://www.cell.com/trends/molecular-medicine/comments/S1471-4914(12)00226-2

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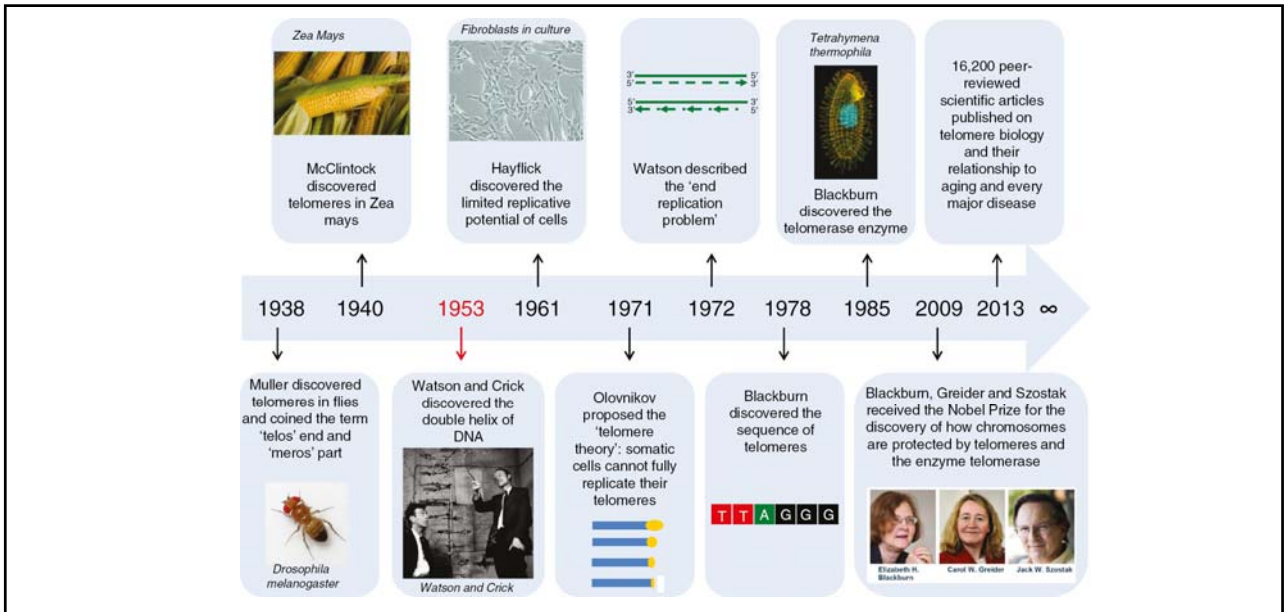

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Milestones in telomere biology discovery. Herman et al 2018

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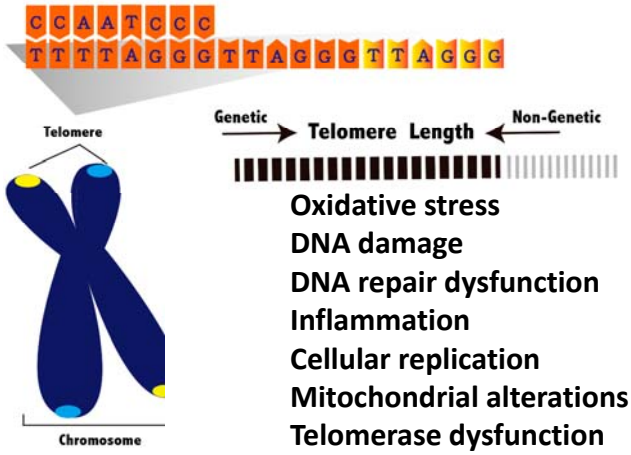
Biological Mechanisms?



Oxidative stress
DNA damage
DNA repair dysfunction
Inflammation
Cellular replication
Mitochondrial alterations
Telomerase dysfunction

CONSEQUENCES

- **Senescence**
- **Terminal Differentiation**
- **Apoptosis**
- **Altered chromosomal organization**
- **Altered gene regulation**
- **Altered meiosis, chromosome fusions**

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Adapted from Yeh et al 2016 https://www.mdpi.com/2073-4425/7/9/58/htm

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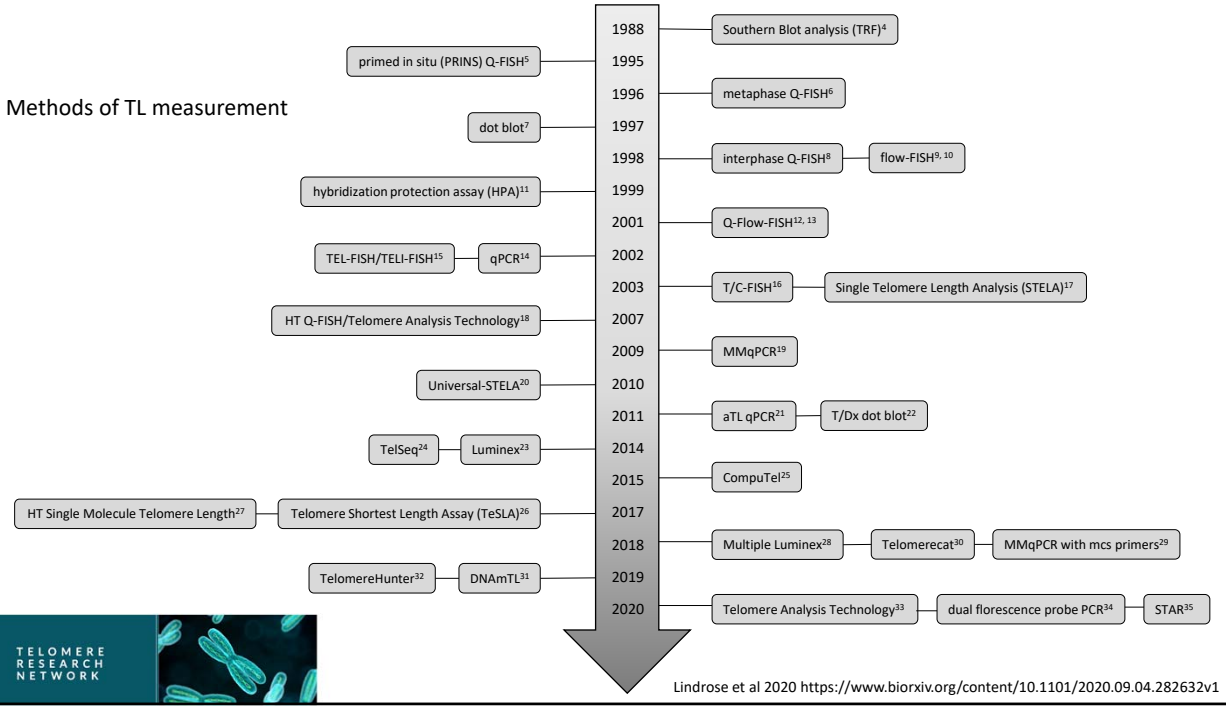
Methods of TL measurement



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Lindrose et al 2020 https://www.biorxiv.org/content/10.1101/2020.09.04.282632v1

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Metrics of TL measurement Overview (not comprehensive)

- Shortest TL
  - TeSLA, STELA, universal STELA
- Full distribution of telomere length (% short TL)
  - TeSLA, Telomere analyses technology
- Chromosome specific TL
  - FISH, STELA, universal STELA,
- Mean TL
  - TRF, qPCR, Flow FISH
- Estimated TL
  - DNAm, whole genome sequencing (Telohunter, Telocat, etc)

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Telomeres mental/physical health

> 50 outcomes with meta analyses and TL (not comprehensive)

MULTIPLE META ANALYTIC

- CARDIOVASCULAR DISEASE
- OBESITY
- EARLY LIFE ADVERSITY
- DEPRESSION
- SCHIZOPHRENIA
- PSYCHOSOCIAL STRESS
- CANCER (multiple types)

META ANALYSES

- ALL CAUSE MORTALITY
- AGING/FRAILTY
- ALZHEIMERS
- AIR POLLUTION
- ANXIETY
- PTSD
- ADVERSE BIRTH OUTCOMES
- SEX
- OPTIMISM
- LUPUS

INCREASING EVIDENCE

- GENERAL PSYCHOPATHOLOGY
- GESTATIONAL DIABETES
- MEDITATION
- IDIOPATHIC PULMONARY FIBROSIS
- PHYSICAL ACTIVITY
- PRENATAL STRESS
- PARITY
- INTRAUTERINE GROWTH RESTRICTION

NEGATIVE EVIDENCE OR CONFLICTING

- BIPOLAR
- SMOKING
- CURRENT HEALTH

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Key telomere length points

- High heritability (>60%) but strong environmental influence as well
- Newborn TL strongly associated with paternal and maternal TL, with somewhat greater association with maternal TL and a paternal age at conception (via sperm TL)
- Wide inter-individual variation in TL across the life span
- Telomerase activity varies across life span and across cell types
- Sex differences (female>male) present across the life course in humans
- Racial differences (self report- not geographic ancestry) present at birth and throughout life span, however significant need for increased diversity and consideration or race in most studies

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The importance of baseline TL

- Wide variation in newborn TL and across life span
  - Newborn TL range: 7.01kb to 11.6kb
  - Mother TL range (age 17-42): 6.19kb to 9.81kb
  - Father TL range (age 17-56): 5.83kb to 9.88kb
- Existing data suggests significantly greater TL attrition in first two decades of life compared to later ages (estimates range from 50 to 200bp annually, some evidence first three years greater attrition)
- The inter-individual variance at birth is substantial and less than the expected TL decline annually (e.g. 25-40bp /year)
- Multiple studies suggest that rank order of individuals relatively static with very small percentage of individuals changing more than one stratum over course of 5 to 15 years (all adult studies)

*Factor-Litvak et al 2016

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Long term questions for the TRN…..

- Do different metrics (e.g. shortest, mean, etc.) of TL differentially relate to psychosocial/environmental exposure?
- Do different metrics of TL differentially predict disease/health?
- What determines baseline TL (newborn)
- What is the importance of longer TL compared to shorter TL and does this change across the life span?
- For what conditions/exposures is TL and/or TL attrition relevant at the individual level
- For what conditions/exposures is TL and/or TL attrition at the population level
- Others……

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The end….. of the chromosome…
to be continued.

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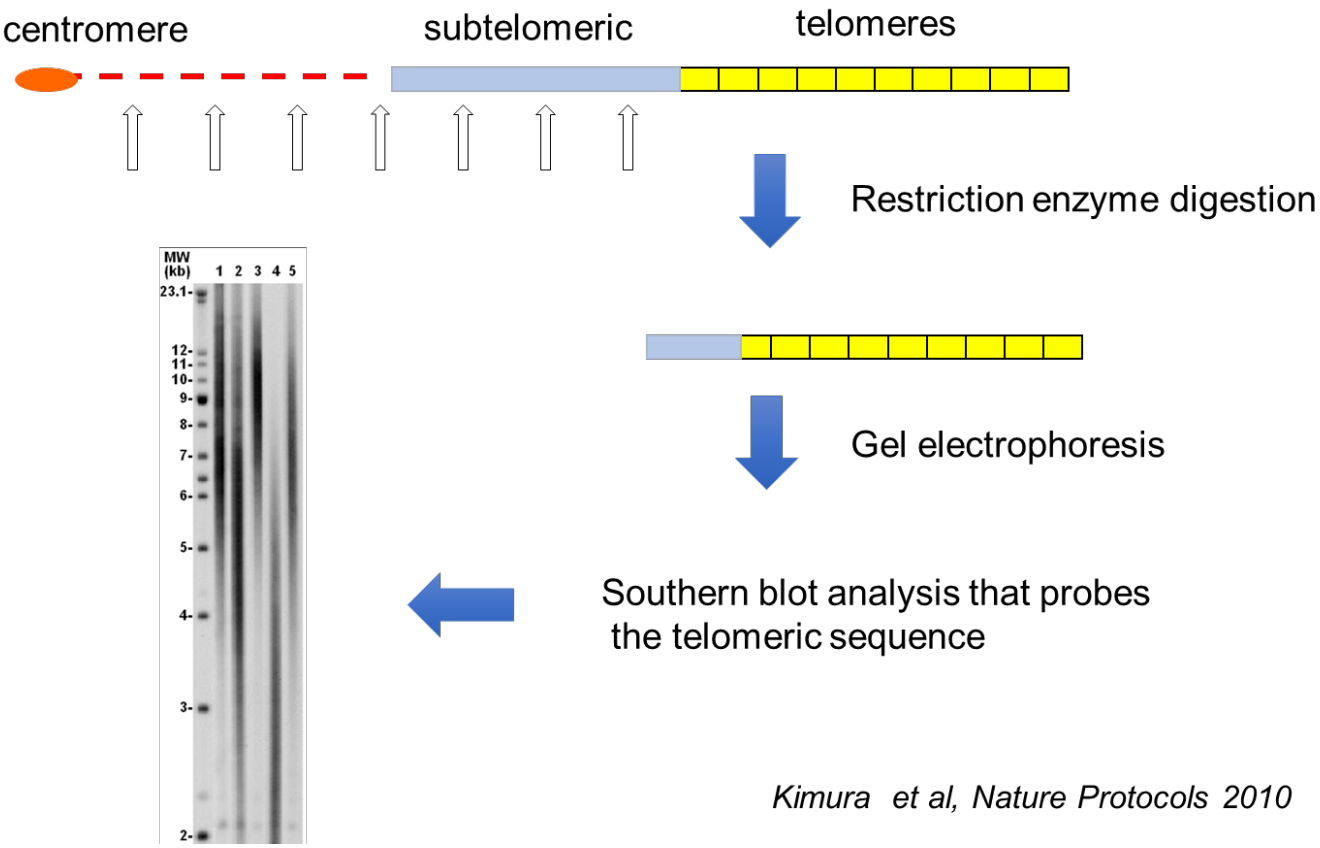
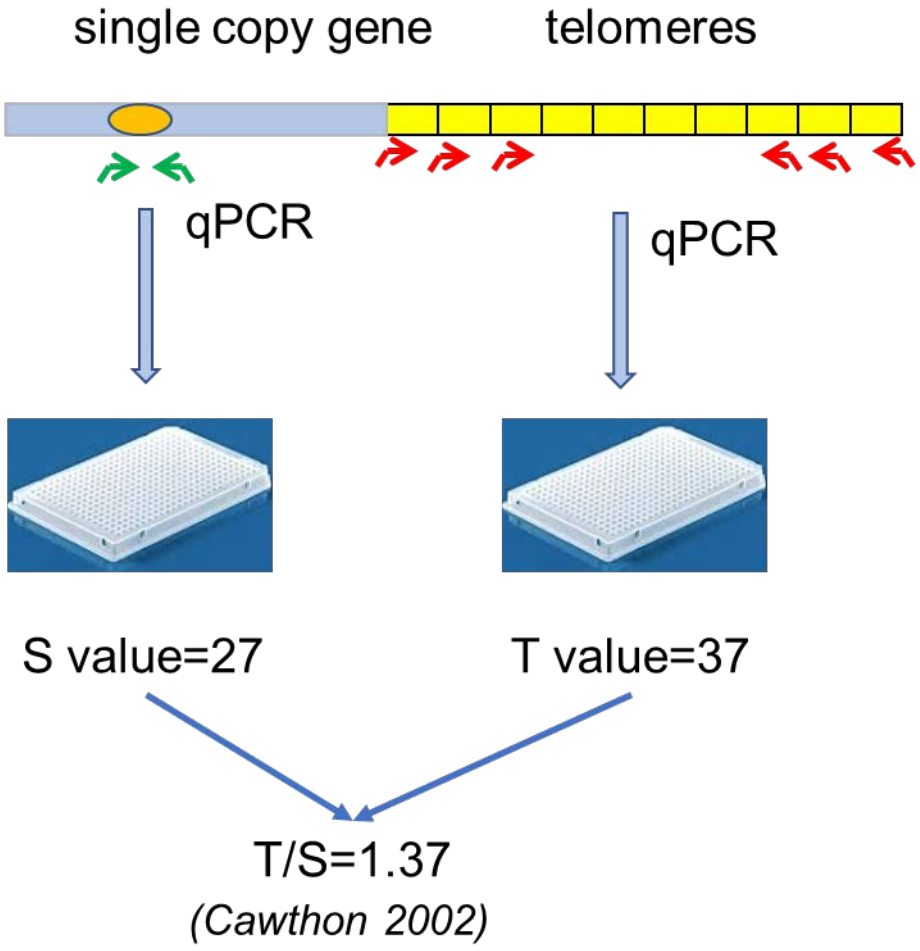
Telomere Restriction Fragment (TRF) Analysis with Southern Blot

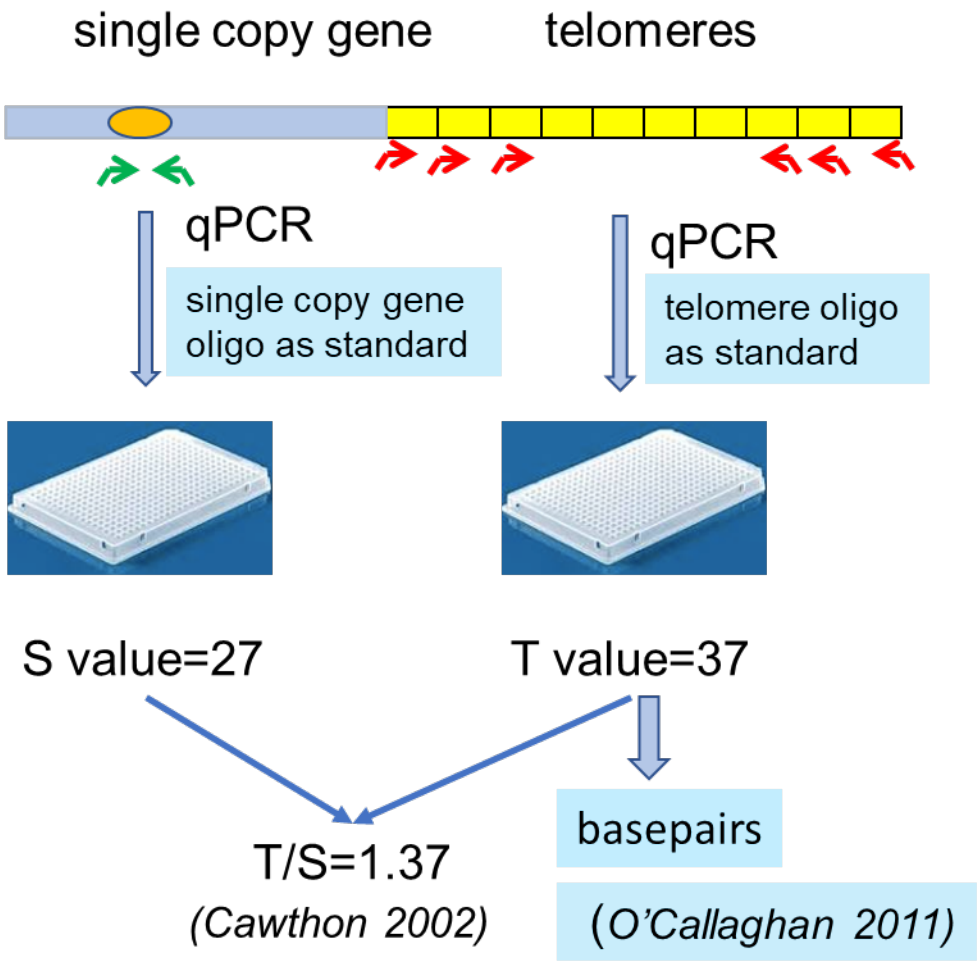


Kimura et al, Nature Protocols 2010

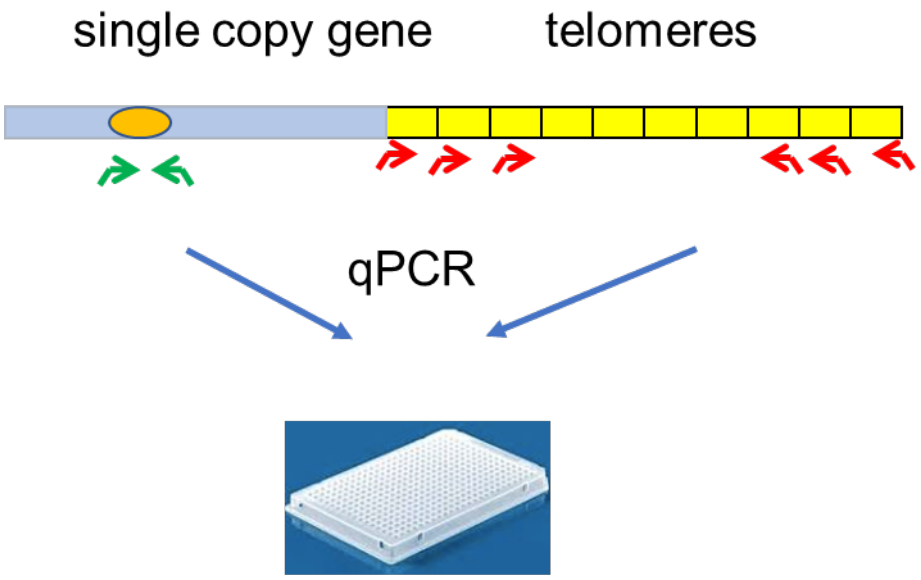
Telomere Length Measurement Using qPCR SinglePlex



Telomere Length Measurement Using qPCR Absolute TL Measurement (aTL)



Telomere Length Measurement Using qPCR Monochrome multiplex (MMqPCR)



S value=27, T value=37

T/S=1.37

(Cawthon 2009)

Key Methodologic Papers

- Shay JW, Wright WE. Telomeres and telomerase: three decades of progress. *Nat Rev Genet*. 2019;20(5):299-309. doi:10.1038/s41576-019-0099-1 https://www.nature.com/articles/s41576-019-0099-1

- Lai T, Wright WE, and Shay JW. Comparison of telomere length measurement methods. *Phil. Trans. R. Soc. B* 373:20160451. https://doi.org/10.1098/rstb.2016.0451

- Lindrose AR, McLester-Davis LWY, Tristano RI, et al. Method comparison studies of telomere length measurement using qPCR approaches: A critical appraisal of the literature. *PLoS One*. 2021;16(1):e0245582. Published 2021 Jan 20. doi:10.1371/journal.pone.0245582 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0245582

- Lin J, Smith DL, Esteves K, Drury S. Telomere length measurement by qPCR - Summary of critical factors and recommendations for assay design. *Psychoneuroendocrinology*. 2019;99:271-278. doi:10.1016/j.psyneuen.2018.10.005 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6363640/

- Kimura, M., Stone, R., Hunt, S. *et al.* Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. *Nat Protoc* **5,** 1596–1607 (2010). https://doi.org/10.1038/nprot.2010.124

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Telomere Length Measurement: Sample Collection & Storage Checklist

The Telomere Research Network (TRN) (trn.tulane.edu) was funded by the NIA/NIEHS to establish best practices for the measurement of telomere length in population-based studies. The TRN is currently investigating, in a systematic and rigorous set of experiments, the importance of multiple pre-analytic factors on telomere length measured using different assays. Over the next two years we expect to have specific guidance for these factors based on our data and comments from our colleagues and experts around the world. These recommendations are offered as initial guidelines for parameters that have the potential to impact the reproducibility, repeatability, and accuracy of telomere length measurement that should be recorded and evaluated for their impact on telomere length measurement. To make comments and/or request clarification please contact Stacy Drury, MD, PhD, Director of the TRN at telomerenetwork@gmail.com.

Sample:

- □ Specimen type[1,2]
- □ Collection procedure
- □ Lot number and expiration date of collection tubes or kit
- □ Sample storage temperature and buffer[3,4]
- □ Sample storage duration until processing and/or DNA extraction[4]
- □ Number of freeze-thaw cycles

Considerations for specific sample types:

- □ Blood: buffer/anticoagulant used in collection
- □ Buccal: method of stabilization (desiccant or stabilization buffer)
- □ Buffy coat, PBMCs, and other blood components: isolation procedure
- □ Saliva: volume of saliva collected and method of stabilization
- □ Organ tissues: fresh or frozen, stabilization matrix or storage media
- □ Cell lines: name of cell line, culture conditions (media and supplements), passage,

DNA:

- □ DNA extraction kit & reagent lot numbers, including mechanical vs manual extraction
- □ DNA extraction batches (e.g. lot number, batch)
- □ DNA storage temperature, duration, and concentration[5,6]
- □ DNA storage buffer/solution
- □ Method of measuring DNA concentration
- □ Number of freeze-thaw cycles[7]

Key Points

1. Sample storage and handling should be uniform within a study
2. Use same DNA extraction method/kit for all samples
3. If storage, handling, or DNA extraction differs within a study, assess this as an independent variable in all analyses and in relation to ICCs

Draft 1.0 2/23/2021

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1. Dlouha D, Maluskova J, Kralova Lesna I, Lanska V, Hubacek JA. Comparison of the relative telomere length measured in leukocytes and eleven different human tissues. *Physiol Res*. 2014;63 Suppl 3:S343-S350.
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7. Shao W, Khin S, Kopp WC. Characterization of effect of repeated freeze and thaw cycles on stability of genomic DNA using pulsed field gel electrophoresis. *Biopreserv Biobank*. 2012;10(1):4-11. doi:10.1089/bio.2011.0016

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In September 2019, NIA and NIEHS launched the Telomere Research Network (TRN) (trn.tulane.edu) to establish best practices for the measurement of telomere length in population-based studies. As a first step, this working document was drafted to reflect the current recommendations of the TRN. These recommendations are offered as initial guidelines for researchers, reviewers, and scientific research officers, and are considered *minimal* reporting guidelines for PCR-based measurement of telomere length. Over the next five years, the TRN expects to better define these parameters and integrate comments from our colleagues and experts around the world. To make comments and/or request clarification please contact Stacy Drury, M.D., PhD, Director of the TRN at telomerenetwork@gmail.com.

Minimum Reporting Recommendations for PCR-based Telomere Length Measurement

Sample type, storage, extraction and integrity:

- ☐ Sample type[1]
- ☐ Sample storage conditions, including temperature, duration, and buffer[2,3]
- ☐ DNA extraction method[4]
- ☐ DNA storage conditions, including freeze-thaw cycles[5,6,7]
- ☐ Method of documenting DNA quality and integrity[8]
- ☐ Percentage of samples specifically tested for DNA quality and integrity
- ☐ *For studies with repeated measures design, report the above for all time points*

qPCR assay:

- ☐ State whether qPCR, MMqPCR, aTL (absolute TL/PCR based) or other PCR based method
- ☐ PCR machine type[9]
- ☐ Source (manufacturer/home-made) of master mix and reagents, and final reaction volume[10]
- ☐ Telomere primer sequences and concentration[11]
- ☐ Single copy gene name, primer sequences, and concentration[11]
- ☐ Full PCR program description including temperature, times, and cycle numbers[11]
- ☐ PCR efficiency of single copy gene and telomere primers
- ☐ Source and concentration of control samples and standard curve[11]
- ☐ *For aTL PCR measurement only: sequence and concentration of oligo standards*

Data analysis:

- ☐ Mean and standard deviation or median and range of telomere lengths
- ☐ Number of sample replicates
- ☐ Level of independence of the replicates (plate vs day vs extraction)
- ☐ Analytic method, considering replicate measurements, to determine final telomere length[12]
- ☐ Method of accounting for variation between sample replicates
- ☐ Method for accounting for well position effects within plates[12]
- ☐ Method of accounting for between plate effects[12]
- ☐ % of samples repeated and % samples failing final QC and excluded from further analyses
- ☐ Acceptable range of PCR efficiency for the single copy gene and telomere primers
- ☐ ICCs of sample/study groups to address variability (not CV)[13,14]
- ☐ T/S ratio transformed to a z score prior before comparison across methods/studies[15]
- ☐ *For studies with family samples or repeated measure design: analytic method to account for this*[16,17]

Note: Currently, we do NOT recommend transformation of T/S measurement to base pairs for qPCR/MMPqPCR assays.

References:

1. Dlouha D, Maluskova J, Kralova Lesna I, Lanska V, Hubacek JA. Comparison of the relative telomere length measured in leukocytes and eleven different human tissues. *Physiol Res*. 2014;63 Suppl 3:S343-S350.
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ICC effect on statistical power – cross-sectional tests

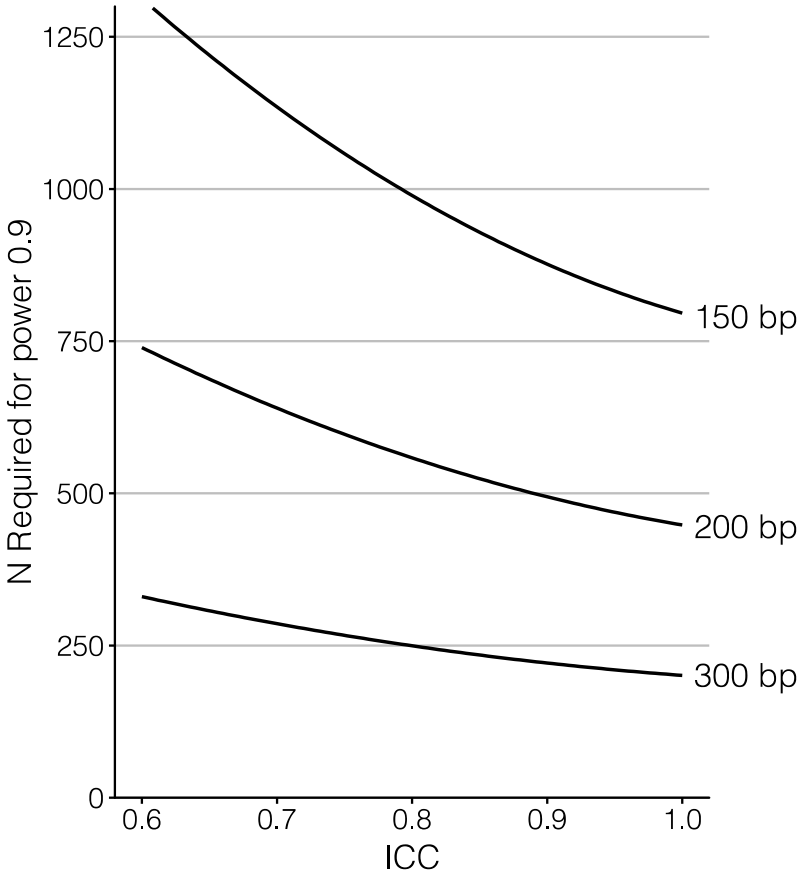

*Figure 1. The sample size required to test effect sizes of 150, 200 and 300 bp with a t-test with a power of 0.9, as a function of measurement error as expressed in the ICC (Intraclass Correlation Coefficient). To contextualize the differences: 150 bp is the approximate difference found between the sexes, and 300 bp is the approximate difference observed between individuals with and without atherosclerotic cardiovascular disease (e.g. Benetos et al 2018). Calculations assumed a realistic (true) standard deviation of 650 bp and power analysis was done using G*Power (Faul et al 2009). N is the combined n of the two groups to be compared and was assumed to be equally distributed among the two groups.*

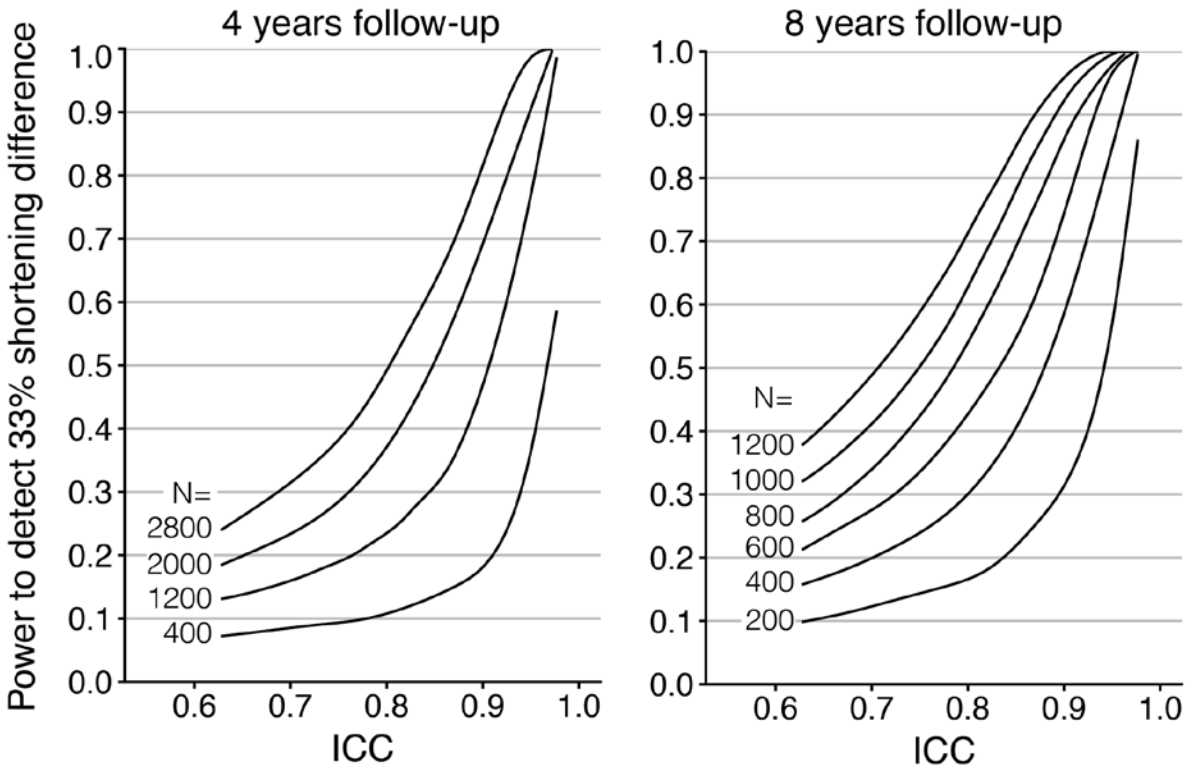

Figure 2. Statistical power to detect a significant difference in telomere shortening rate using longitudinal data as a function of measurement reliability expressed as the Intraclass Correlation Coefficient. Shown is power to detect a 33% change of telomere shortening rate, up or down, with p<0.05 relative to a baseline shortening rate of 25 bp/year. A. Four-year follow-up period. B. Eight-year follow-up period. Power was calculated for sample sizes as shown (200 – 2800), equally divided over the two levels of telomere shortening rate. Baseline telomere shortening was simulated assuming a Poisson distribution with mean / variance of 25, and population SD of telomere length was maintained at 0.65 kb at both time points.

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Faul F, Erdfelder E, Buchner A & Lang AG. Statistical power analyses using G*Power 3.1: Tests for correlation and regression analyses. *Behavior Research Methods* **41,** 1149–1160 (2009). https://doi.org/10.3758/BRM.41.4.1149

How to calculate the repeatability (ICC) of telomere length measures

Dan Eisenberg (dtae@uw.edu), Daniel Nettle (daniel.nettle@newcastle.ac.uk), and Simon Verhulst (s.verhulst@rug.nl)

Measurement repeatability is a critical component of assessing whether any measurement is reliable and for discerning statistical power to detect associations with TL (Nettle *et al*, 2019; Lindrose *et al*. 2021). In the past, TL measurement repeatability has typically been assessed using the coefficient of variation (standard deviation divided by the mean). However, for multiple reasons, the coefficient of variation (CV) is an invalid statistic for TL measurement and should not be used (Verhulst *et al*., 2015; Eisenberg, 2016; Verhulst *et al*., 2016). Instead, we urge the use of the repeatability, also known as intra-class correlation coefficient (ICC) for discerning assay repeatability. To this end, we provide detailed instructions how to calculate the ICC using R, which is freely available, and an example data set.

Note that measurement validity is determined by both precision (the closeness of measurements to each other) and accuracy (the closeness of measurements to a specific 'true' value). The ICC is informative on precision but provides no information on the accuracy.

Unlike the CV statistics, the ICC cannot be calculated for each biological sample individually, but instead is calculated for a set of biological samples. Thus, to be able to calculate the repeatability, (a subset of) samples should be re-measured one or more times in an identical fashion as TL is typically measured in one's laboratory. Two important considerations when selecting these samples are the following. Firstly, variation in TL measurement can arise at any point between sample collection and arrival at your TL estimate. Therefore, the ICC will be overestimated when, for example, the ICC is estimated over repeated measurements of extracted DNA relative to measurements where the DNA extraction is also repeated (but by how much is not known). Thus, the ICC you estimate from the data will be closer to the true ICC when more of the measurement process is repeated independently in the successive measurements. Secondly, the TL of the subset of samples needs to be representative of the complete set of samples that is analysed. This is true in particular for the range of TL in the sample – having a larger range in the subset than in the ultimate sample will overestimate the 'true' ICC, while having a smaller range in the subset than in the ultimate sample will result in underestimation of the ICC.

The ICC can be calculated in different ways, depending on how variation between batches is accounted for (i.e. not at all, as random effect, or as fixed effect), where 'batches' can be thought of as different plates, or gels, or measurement sessions that differ in any respect (e.g. day, person, equipment, laboratory). The best way to account for batch in the ICC calculation is to use the same approach as in the ultimate analyses in which hypotheses are tested.

The text below is an R script, mixing instructions with the actual script. When a line is precede by '#', this indicates it is a comment – and it will not be executed. It is left in the text here to make it possible to copy all text below (up to the References) to an R-script – see instructions below.


```
# R script to calculate the ICC (IntraClass Correlation), also known as 'repeatability'.
# The text below assumes you are new to R....
# '#' before text indicates it is a comment - will not be executed
# The other lines need to be 'run' and results will show in the console window.

# Before you start
# 1. it is advisable to do the analysis in Rstudio (freely downloadable and works on
# multiple platforms), which serves as a 'shell' to R, and copy this text to a new R-script.
# 2. A useful introduction by the authors of the package used in this script to calculate
# the ICC is recommended reading:
# https://cran.r-project.org/web/packages/rptR/vignettes/rptR.html
# The text below is only enough to get you started
# 3. Data format
# The data need to be in the 'long' format. This implies ALL the telomere estimates are
# in one column, with sample identity in another column. Data may have been collected in
# different 'batches' (plates, gels, days, labs) and batch identity is coded in a separate
# column.
# When your data is in wide format, this can easily be changed to a long format in Excel.
# So the data file usually has a minimum of three columns (with variable names in brackets):
# sample identity (id), telomere estimate (TL), batch (batch) [when you use different
# variable names, the names in the code below need to be replaced with those names].

# The packages listed below are needed and you will probably need to install them first.
# At the top of the bottom right panel in RStudio there is a tab 'packages' you can use.
library(readxl) #for when your data are in Excel format
library(MASS)
library(lme4)
library(rptR)

# To clear lists of objects – useful to run whenever you start an analysis:
rm(list=ls())
rm(list = ls(all = TRUE))

# Reading in the data.
# In the example below, the data were stored in Excel, but Rstudio reads many formats.
# Other formats may require loading another package - Rstudio will tell you this.

# Note that the first bit of the code below, 'd <-', you can read as 'd becomes'.
# We here arbitrarily name the data set 'd' (commands / names in R are case sensitive!).

# You can get the import code for your file location and correct command for your file format
```


```
# using the 'import dataset' tab in RStudio (above the top right frame on a mac).

# When you import a data set using RStudio, the dataset will have a name different from
# what is in the script below. I recommend copying the code you see after the "<-" to the
# script just before actually importing the data and copy this below after the text "d <-".
# Alternatively, you replace 'd' in the code with the name you have given your data set.

d <- read_excel("FILE LOCATION/example.xls") #importing the example data from Excel file
# The file 'example.xls' is available on the telomere network site.

# Calculating the ICC
# The ICC can be calculated in different ways, depending on how variation between
# batches is accounted for (i.e. not at all, as random effect, or as fixed effect).
# The best way to account for batch in the ICC calculation is to use the same approach
# as in the analysis for which the data were collected.
# Below is the code for different ways to include 'batch' in the analysis.

# When running the scripts below, depending on details,
# there may be 'Singularity' issues that are reported as errors.
# You can safely ignore these (see information on rptR package for details.)

# 1. No correction for batch
# Including 'id' only - i.e. batch is not in the model
# Note that the (1|something) codes for a random intercept for levels of 'something'
rpt(TL ~ (1|id), grname = "id", data=d, datatype = "Gaussian", nboot = 1000, npermut=0)

# 2. Including 'batch' as random effect
rpt(TL ~ (1|id) + (1|batch), grname = "id", data=d, datatype = "Gaussian", nboot = 1000,
npermut=0)

# 3. Including 'batch' as fixed effect
rpt(TL ~ batch + (1|id), grname = "id", data=d, datatype = "Gaussian", nboot = 1000,
npermut=0)

# Note that these models can be extended with other factors and covariates.
# See example below that includes age (as fixed effect)
rpt(TL ~ age + (1|id) + (1|batch), grname = "id", data=d, datatype = "Gaussian", nboot = 1000,
npermut=0)
# When running the model including age, the ICC is likely to become lower. The new (lower)
# ICC estimate is the more relevant estimate. This is so, because you will probably
# take age into account in your statistical analyses also. The remaining variation in the
# data will decrease as a consequence, and the ICC is calculated over the remaining variation.
```


```
# Adding other factors to the model, e.g. batch identity or procedure characteristics
# that you also include in the model with which you test hypotheses can also be added as
# factors to the model, and may increase the ICC.

# The script above assumes the TL measurements are normally distributed, but the
# rptR package can handle other distributions.

# Extrapolated repeatability – an important extension!
# Suppose your protocol includes rTL measurement in duplicate, using for example two plates
# with samples in triplicate on each plate, and you use the average of the two plates in
# the data analysis. You can then calculate the ICC over the two plates, but this will
# underestimate the ICC of the average of the two plates, which is based on more
measurements.
# However, the ICC of the mean of the two plates can be calculated with a simple equation
# once the ICC over the two plates is known:  when r is your repeatability (ICC) estimate,
# and n is the number of replicate measurements then the extrapolated repeatability (re):
# re = r / (r+1/n*(1-r))
# This is equation 37 in: Nakagawa, S. & Schielzeth 2010, Biological Reviews 85, 935-956.
```

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