Telomere Research Network Virtual Annual meeting December 3-4, 2020

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New Orleans La, 70118

TELOMERE RESEARCH NETWORK

NIH NIEHS

NIH NIA

U24 update and 2021 goals

TELOMERE RESEARCH NETWORK

TRN roadmap

Year 1:building the TRN network

- U01 method comparison study
- Analytic methodology
- qPCR reporting guidelines
- Telomeres in health and disease primer
- Pilot awards
- Development of subcommittees

Year 2-3: Methodologic rigor and innovation

- Empirically supported pre-analytic considerations
- Telomere study design check list
- Telomere methodology selection tool
- Pilot awards
- Telomere researcher database
- TRN quarterly newsletter
- Enhanced interface with basic telomere biology
- Relation between TL and other biomarkers
- Aging biomarkers and TL

Year 4-5: sustainability and impact

- Telomere length measurement workshops
- Larger research awards
- Methodological reporting recommendations
- Guidelines for new methodology validation
- From the bench to bedside- driving the clinical impact of TL
- Moving beyond telomere length to mechanism

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Year 1- building a Telomere Tool Kit

- Telomere basics for friends and family
- Study Design
- Study Analyses
- Seminal papers
- Cutting edge research
- RFAs, meeting announcements

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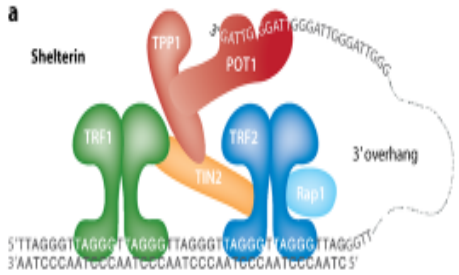
The little book of telomeres…

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

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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Tools you never thought you needed

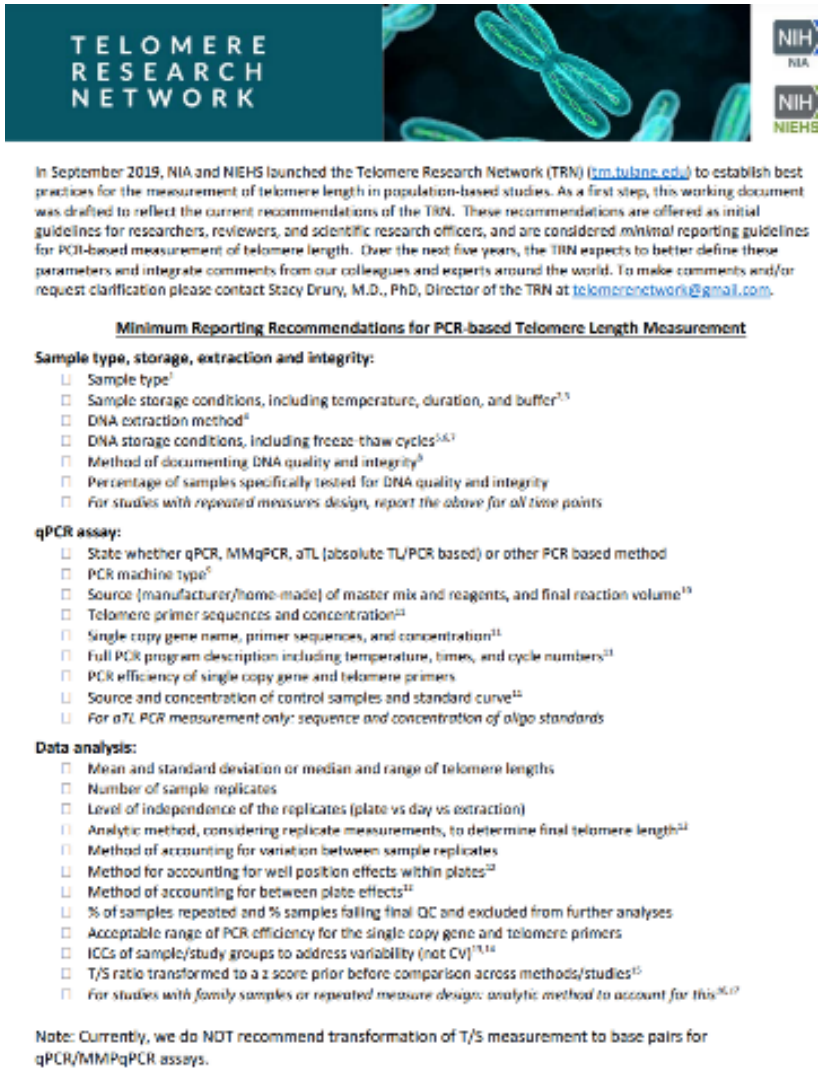
R-code to calculate ICC

```
rm(list=ls())

rm(list = ls(all = TRUE))

d <- read_excel("FILE LOCATION/example.xls")

rpt(TL ~ age + (1|id) + (1|batch), grname = "id",
data=d, datatype = "Gaussian", nboot = 1000,
npermut=0)
```



TELOMERE RESEARCH NETWORK

NIH NIA

NIH NIEHS

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[12]
- *For aTL PCR measurement only: sequence and concentration of oligo standards*

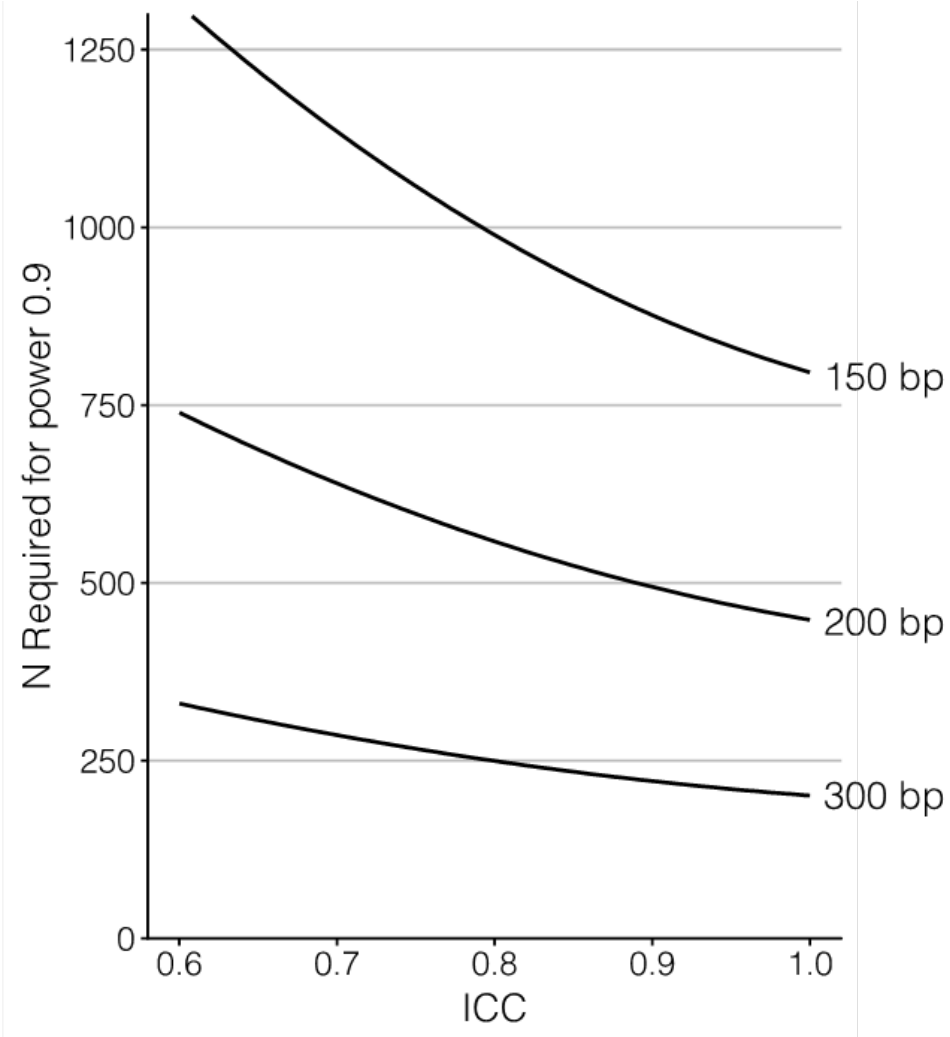
Data analysis:

- 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[13]
- 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)[3,14]
- 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.

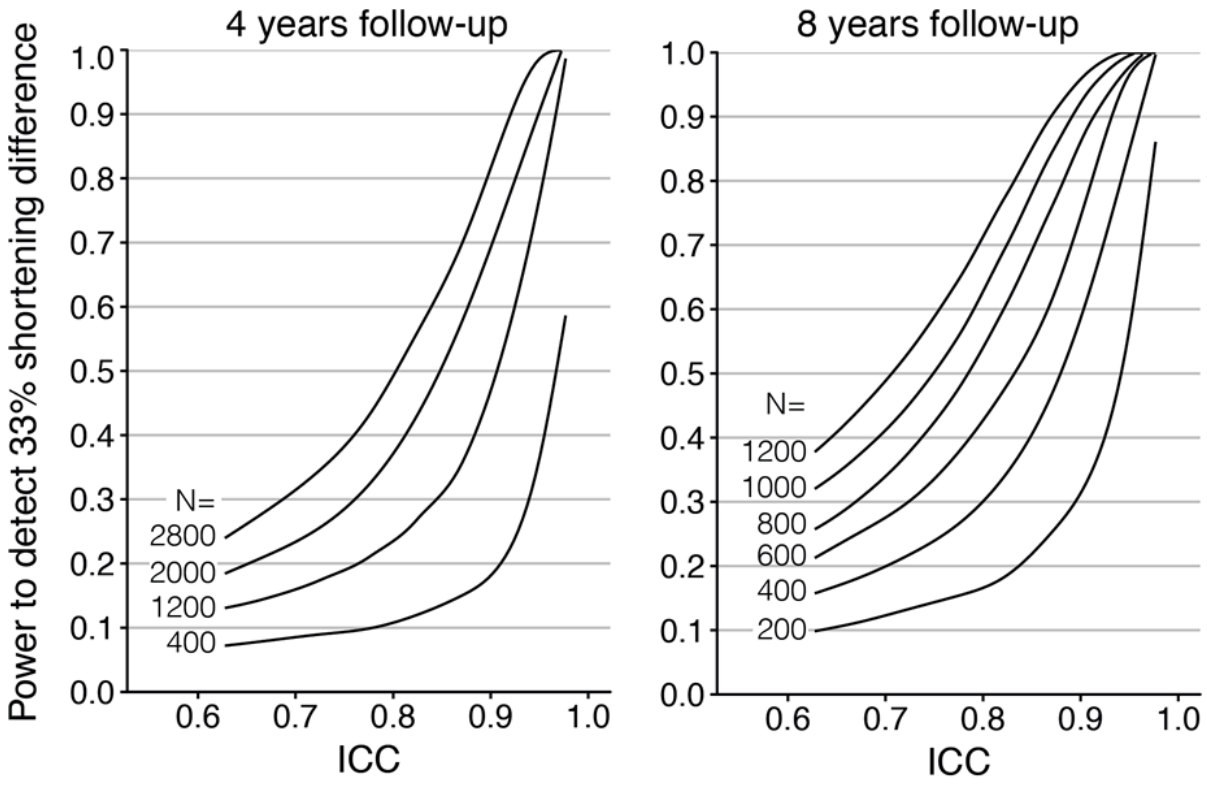
https://trn.tulane.edu/wp-content/uploads/sites/445/2020/10/How-to-calculate-repeatability.pdf

How big of a sample?



- *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.*

How long of a follow up?



- *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.*

Study design
- New study
- Archived
- Secondary data analyses

Sample type
- Fresh live cells
- Archived/nonviable

Cohort size
- Small < 300
- Moderate 300-600
- Large >600

DNA amount
- <1 ug
- >1ug

Cost
- >40/sample
- <40/sample

DNA integrity concerns
- Yes- major
- No- minor

Method selection tool:
Redcap link (planned) to guide researchers in deciding what/if TL measurement appropriate

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Pilot awards 2021

- Goals of pilot awards
  - Improve rigor and reproducibility of TL
  - Provide innovative date related to TL as a sentinel of environmental exposure, psychosocial stress and disease susceiptibilty
  - Determine the extent to which TL is responsive to changes in environment and how this is differs across development
  - Support new investigators in TL research
  - ** COVID related proposals are responsive to this RFA

- Logistics
  - March 1 deadline
  - Start date May 1, 2021
  - Presentation of final data: December 2022

- Review criteria
  - Large enough sample size (>200)
  - Innovative question that addresses existing gap
  - Appropriate consideration of age, sex, race/ethnicity

TRN topic webinars

- Available via TRN- email telomerenetwork@gmail.com
  - Introduction to the TRN- July 2020
  - Telomeres and COVID- August 2020

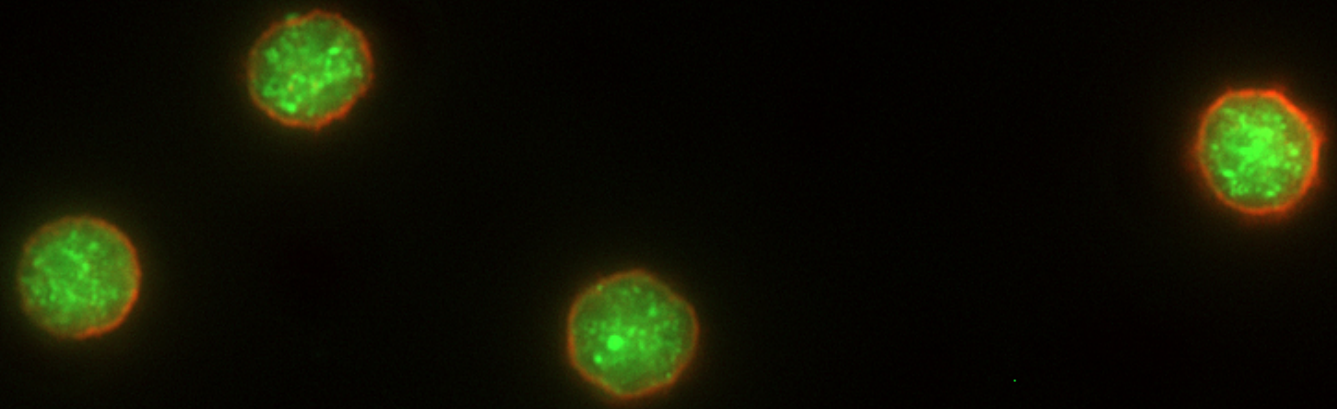
- UPCOMING
  - "The role of telomere length in understanding Health Disparities and the social determinants of health"
    - TBA- January 2021
  - "Moving telomere length into the clinical arena- current examples and future conditions"
    - transplantation, idiopathic pulmonary fibrosis, COVID disease severity/vaccine response
    - TBA – March 2021
  - Topics for May 2021?

TELOMERE RESEARCH NETWORK

Beyond Telomere Length:
Biological Consequences of Telomere Damage-Induced Cellular Responses

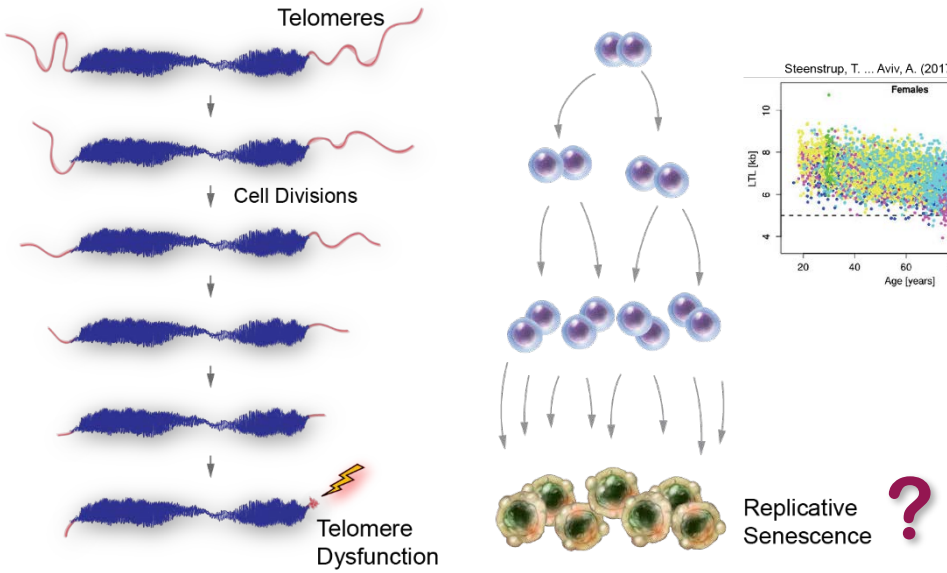
Utz Herbig, Ph.D.

Center for Cell Signaling, Department of Microbiology, Biochemistry, and Molecular Genetics.
New Jersey Medical School
Rutgers Biomedical and Health Sciences, Rutgers University, Newark, NJ, USA.

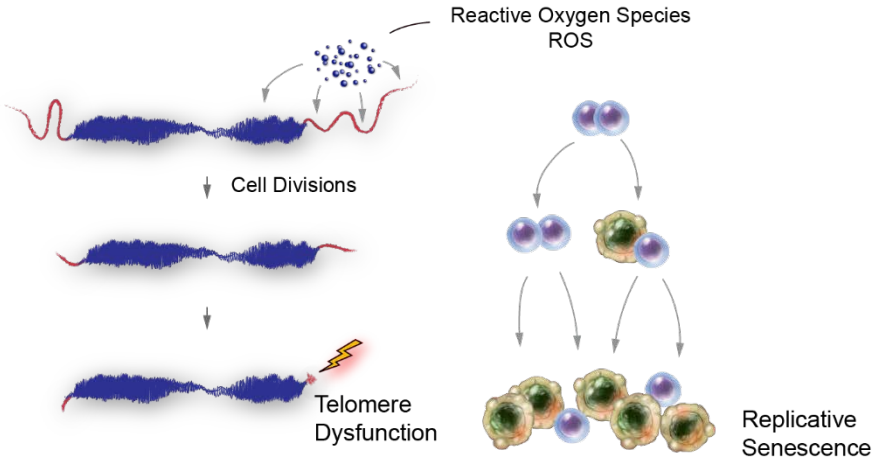


Replicative Senescence vs. Telomere Dysfunction-Induced Senescence

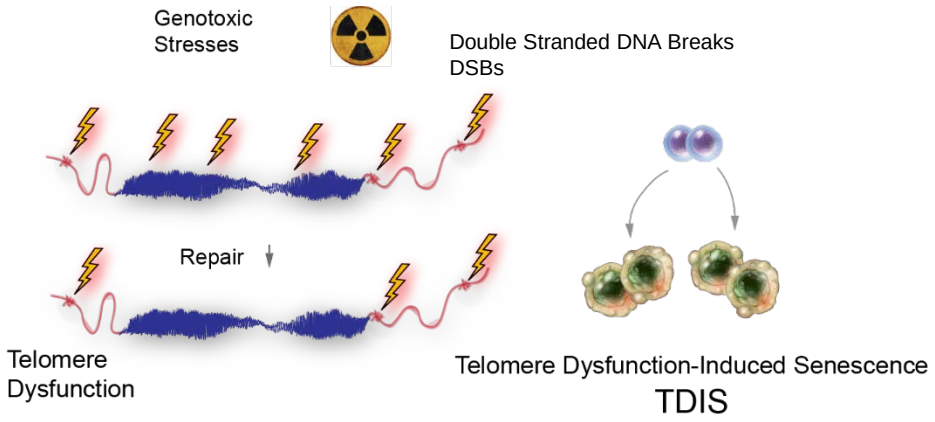
Normal

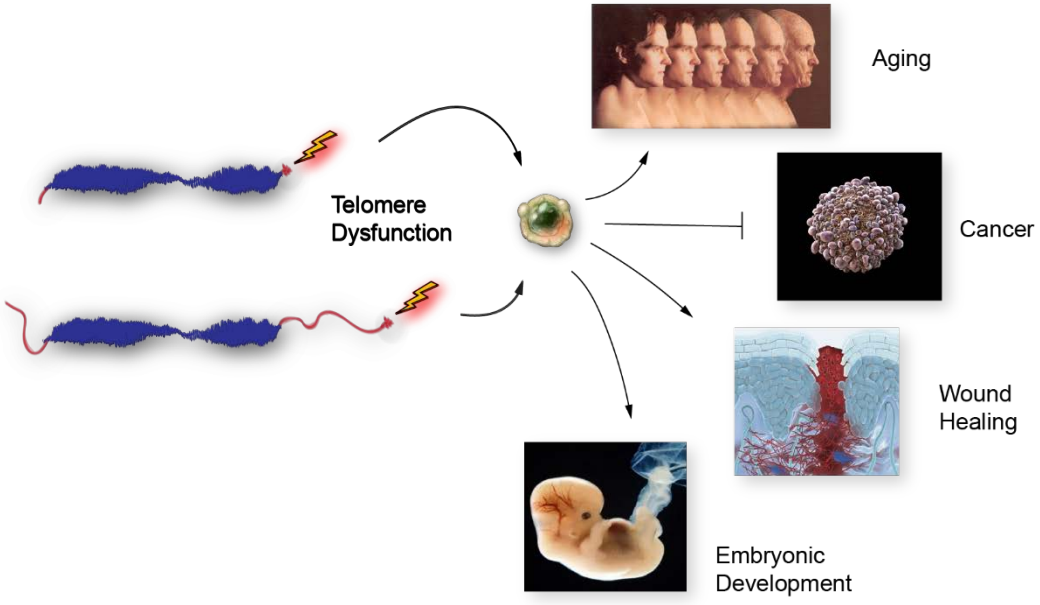


Accelerated

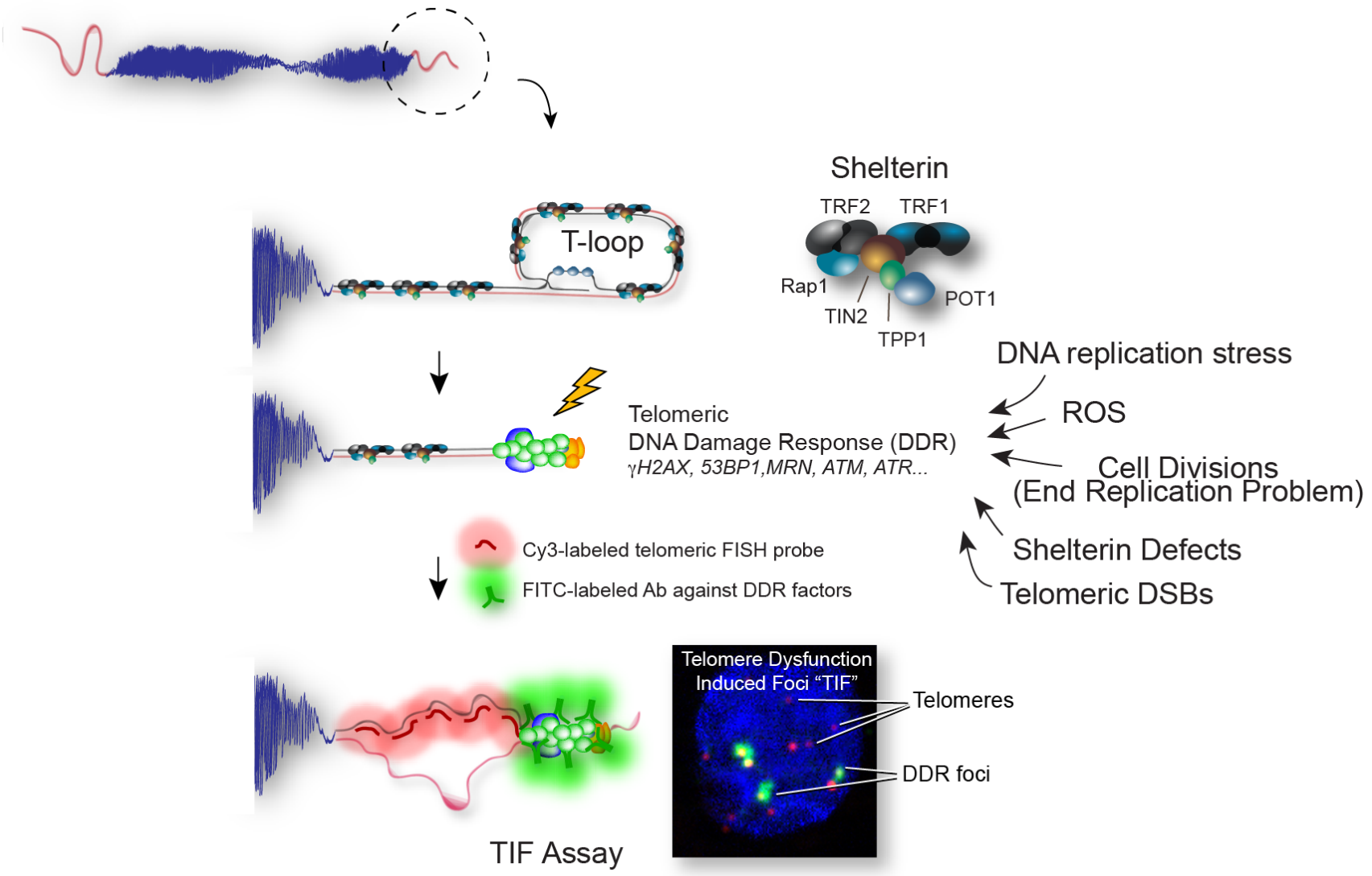


Rapid

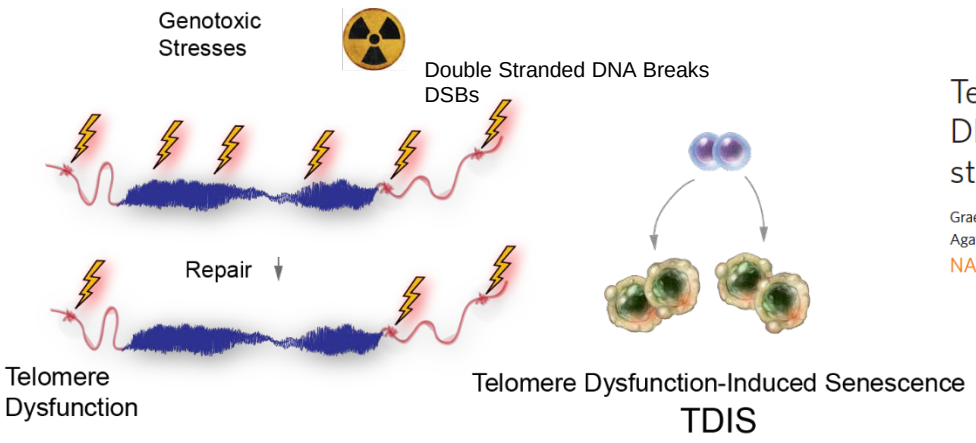




Telomere Dysfunction-Induced Senescence



DSBs in Telomeres Resist DNA Repair Regardless of Telomere Length



Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence

Graeme Hewitt[1,2,*], Diana Jurk[1,2,*], Francisco D.M. Marques[1,2,*], Clara Correia-Melo[1,2], Timothy Hardy[3], Agata Gackowska[3], Rhys Anderson[1,2], Morgan Taschuk[1,2], Jelena Mann[3] & João F. Passos[1,2]

NATURE COMMUNICATIONS | 3:708 | DOI: 10.1038/ncomms1708 |

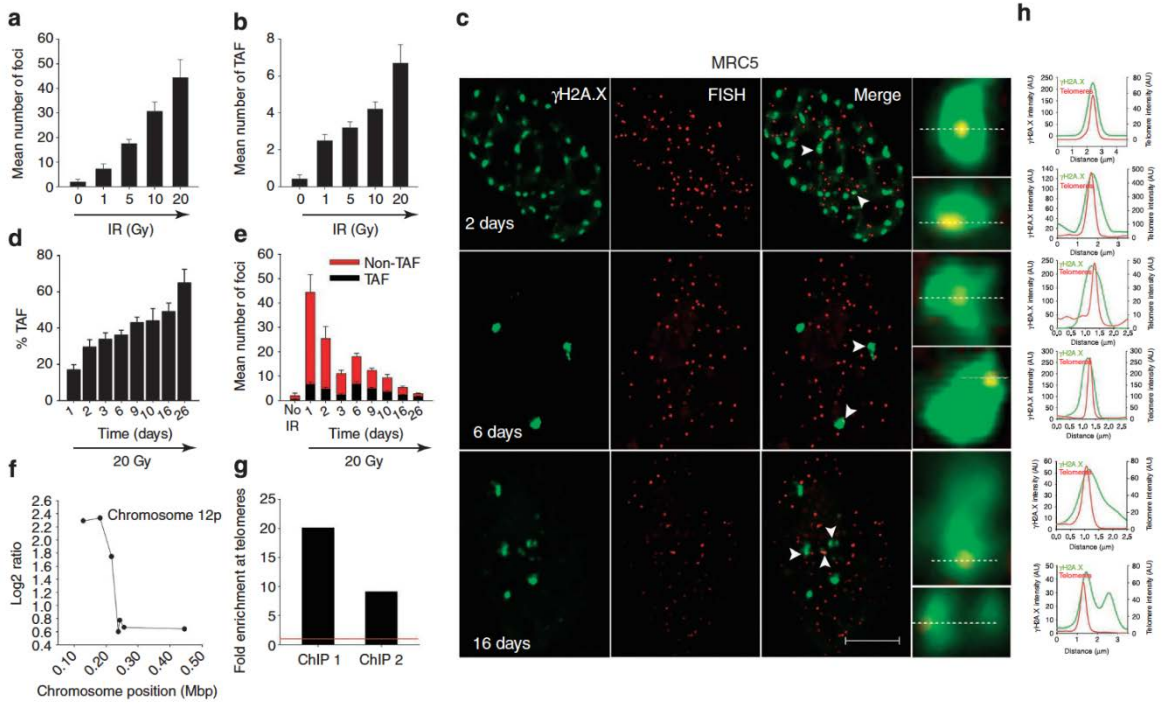
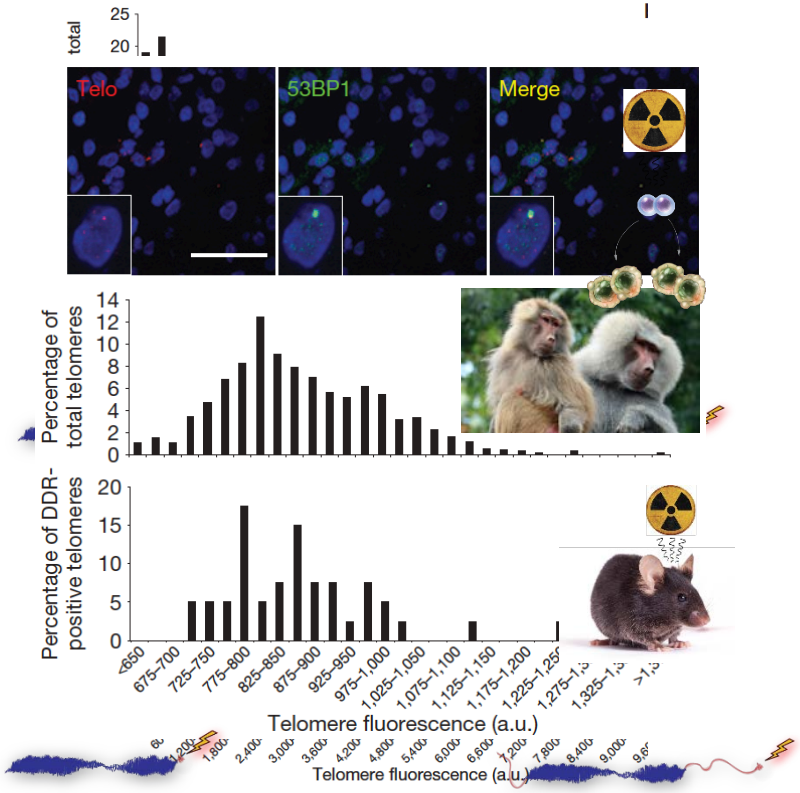


Figure 1 | TAF are persistent following X-ray-induced senescence in MRC5 fibroblasts with or without telomerase activity. (**a**) Total number of γH2A.

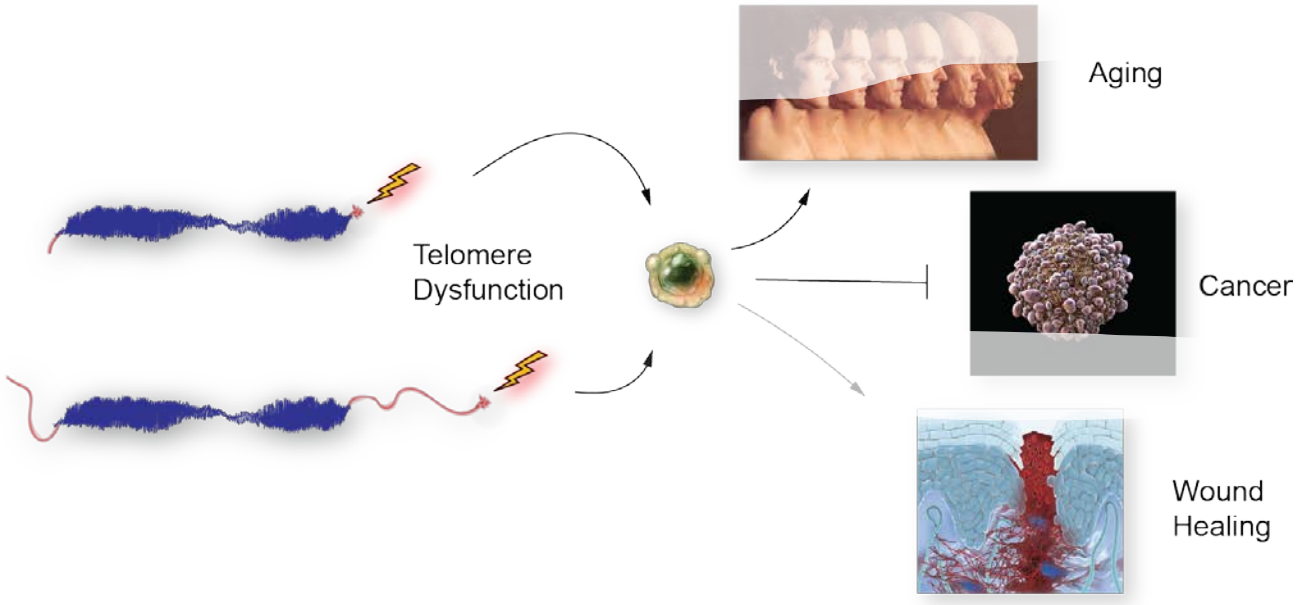
Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation

Marzia Fumagalli[1,9,10], Francesca Rossiello[1,10], Michela Clerici[2], Sara Barozzi[3], Davide Cittaro[4,5,9], Jessica M. Kaplunov[6], Gabriele Bucci[4,5], Miryana Dobreva[1,9], Valentina Matti[1], Christian M. Beausejour[7], Utz Herbig[6], Maria Pia Longhese[2] and Fabrizio d'Adda di Fagagna[1,8,11]

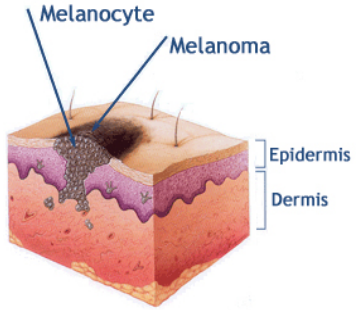
NATURE CELL BIOLOGY VOLUME 14 | NUMBER 4 | APRIL 2012

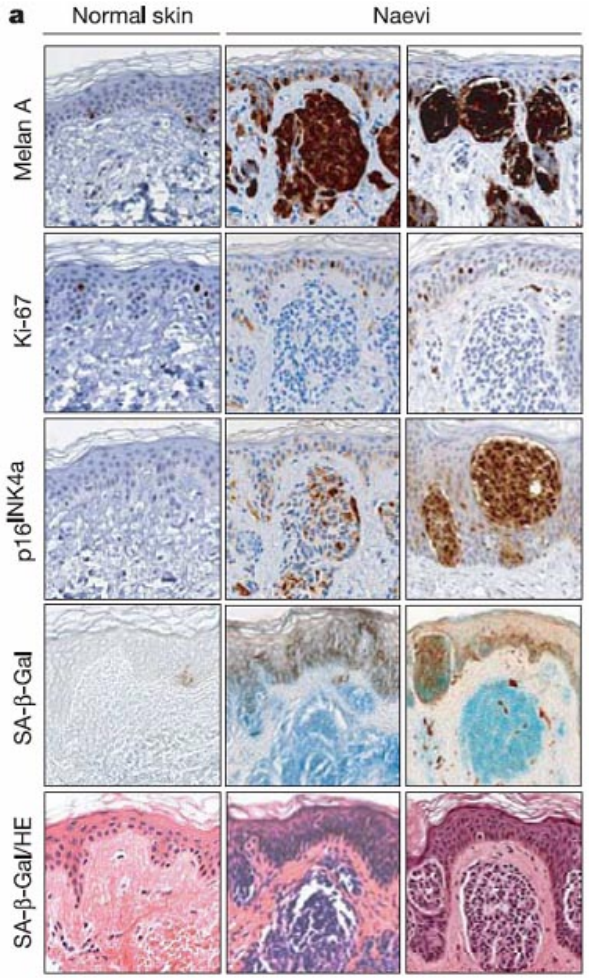


Dysfunctional Telomeres in Cancer



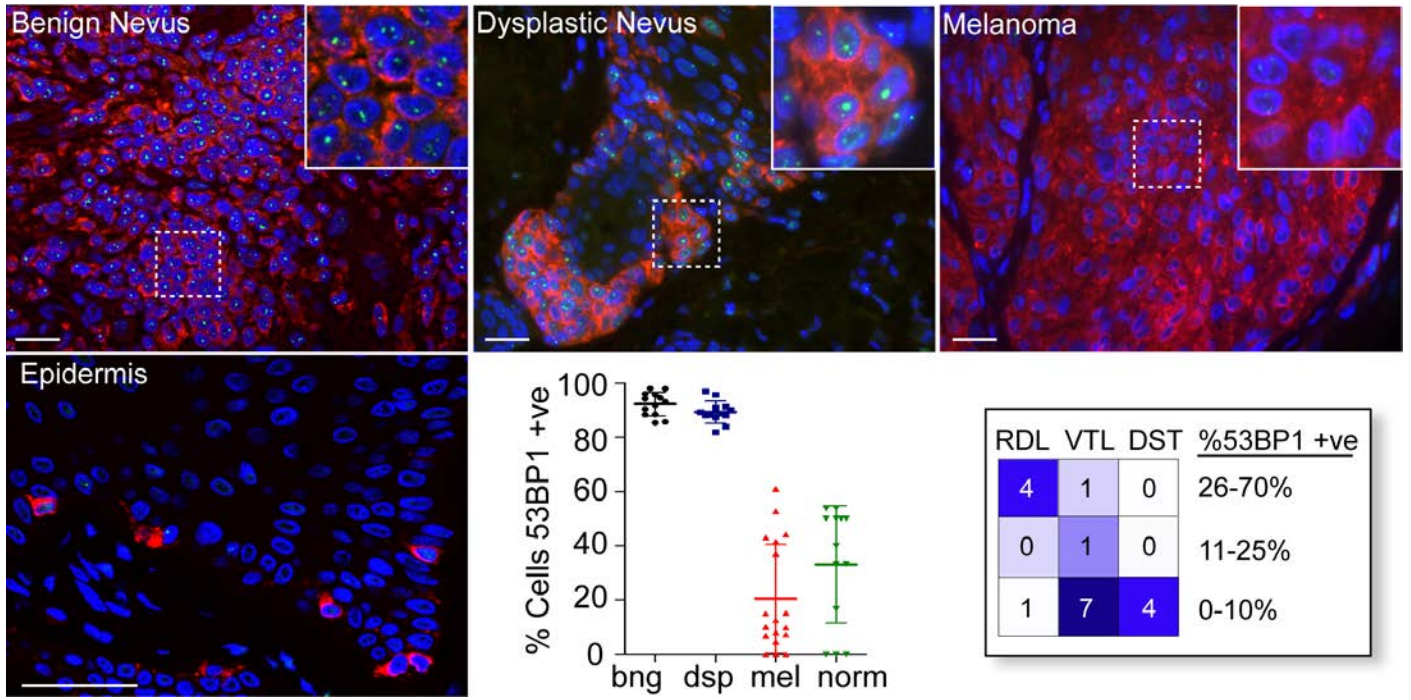
Dysfunctional Telomeres in Precancerous Human Neoplasms





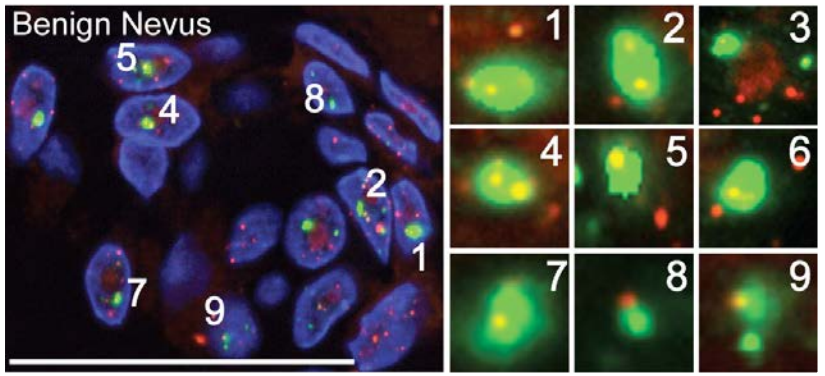
Michaloglou 2005 Nature **436** 720

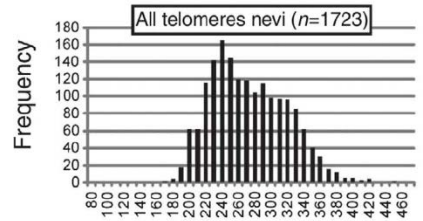
Red: Melan A
Green: 53BP1 (DNA damage)
Blue: Cell nucleus

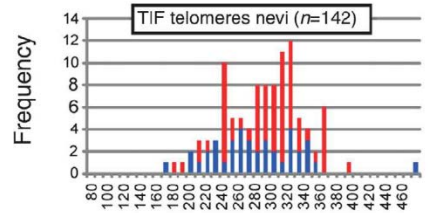


RDL	VTL	DST	%53BP1 +ve
4	1	0	26-70%
0	1	0	11-25%
1	7	4	0-10%

Red: telomere
Green: 53BP1 (DNA damage)
Blue: Cell nucleus

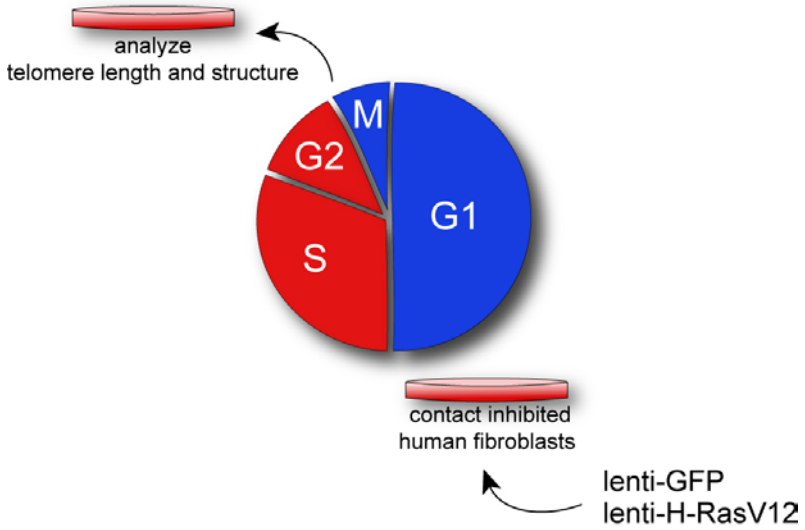


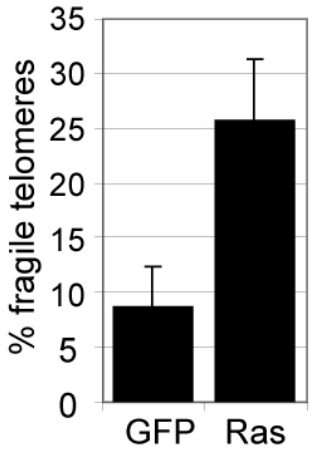


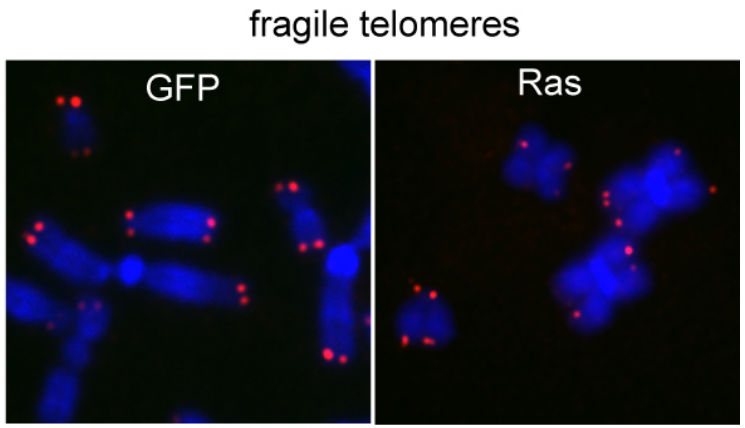


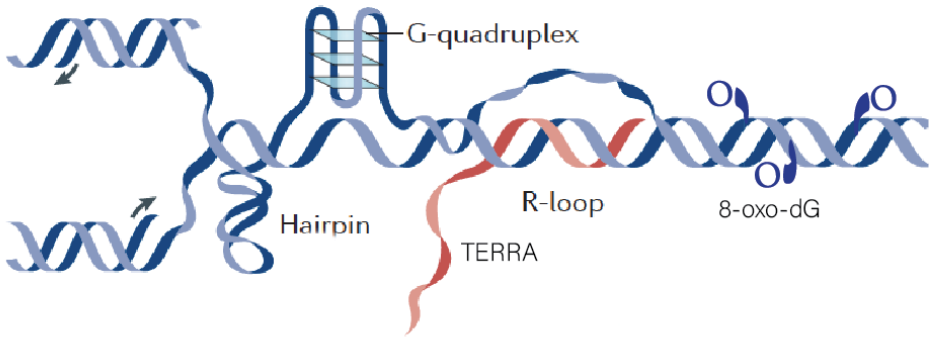
The EMBO Journal (2012) 31, 2839–2851 |

Oncogenes Cause Telomeric Double Stranded DNA Breaks and Fragile Telomeres





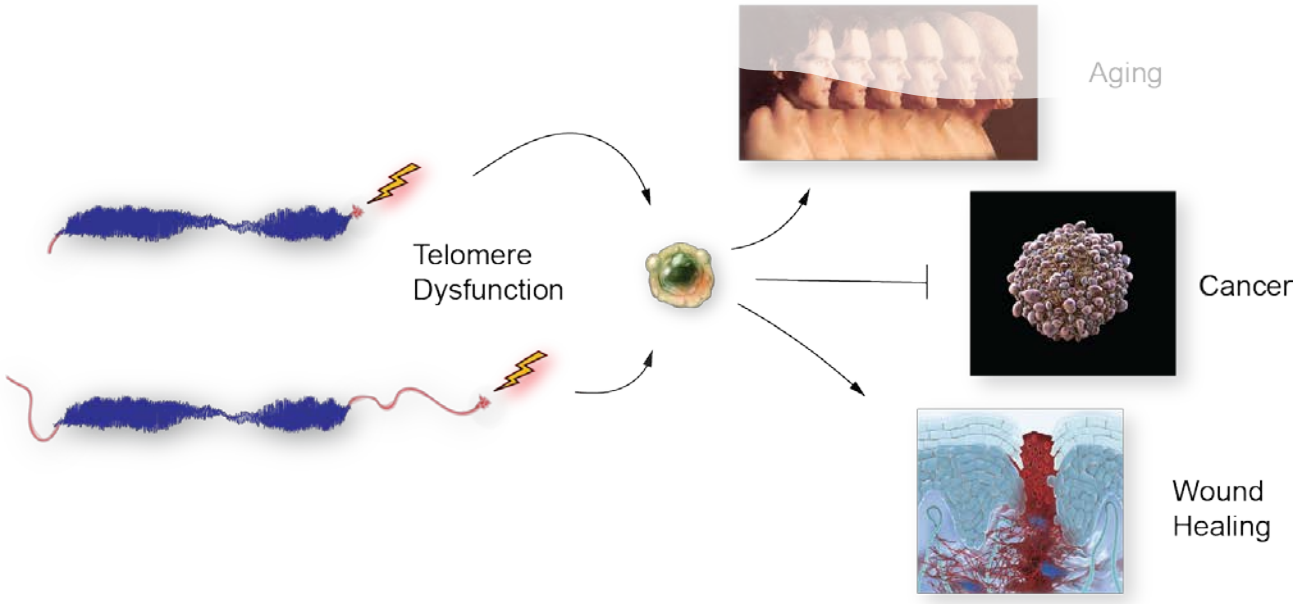




Telomeres are difficult to replicate!

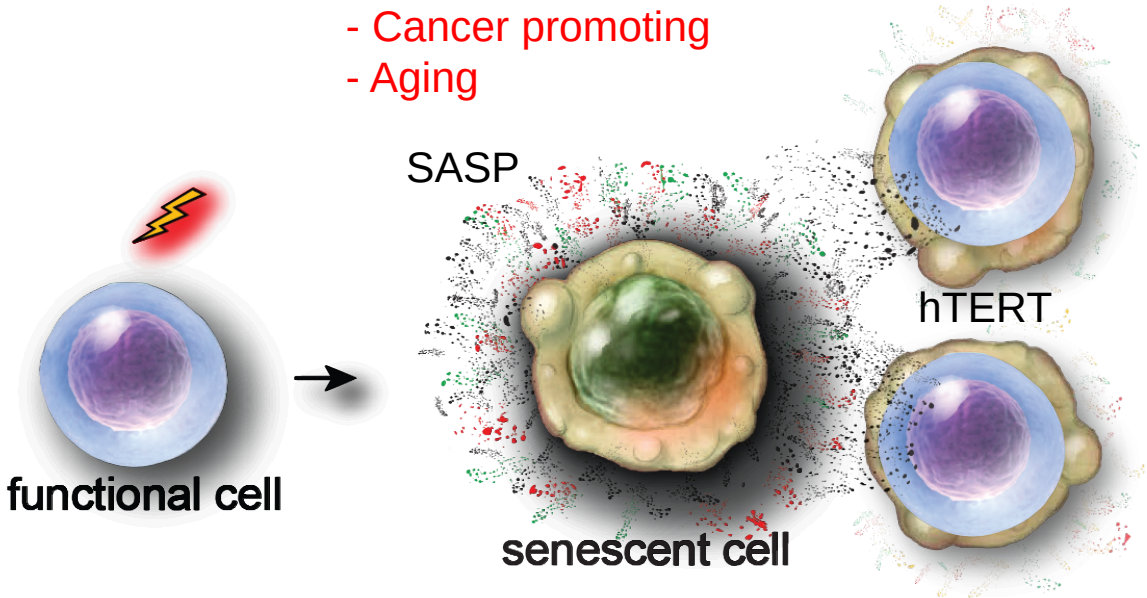
The EMBO Journal (2012) 31, 2839–2851 |

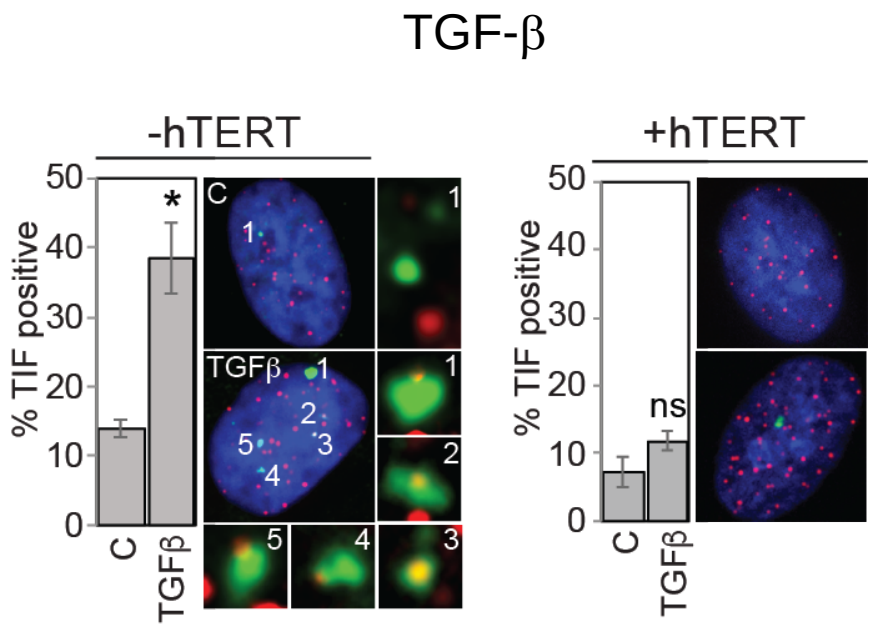
Dysfunctional Telomeres in Wound Healing

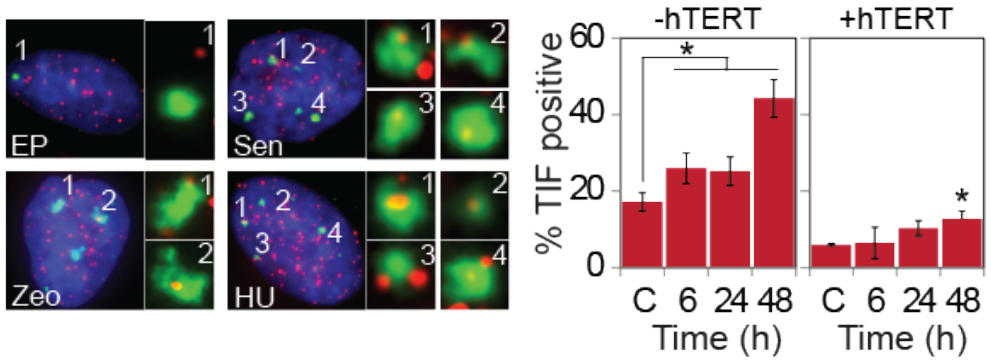


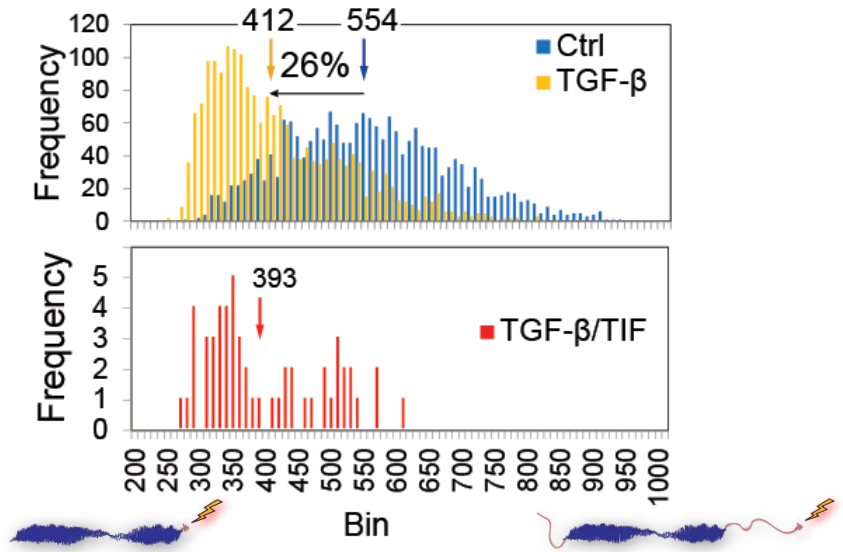
Senescence Associated Secretory Phenotype and Paracrine Senescence

- Inflammation
- Tissue Repair
- Cancer promoting
- Aging



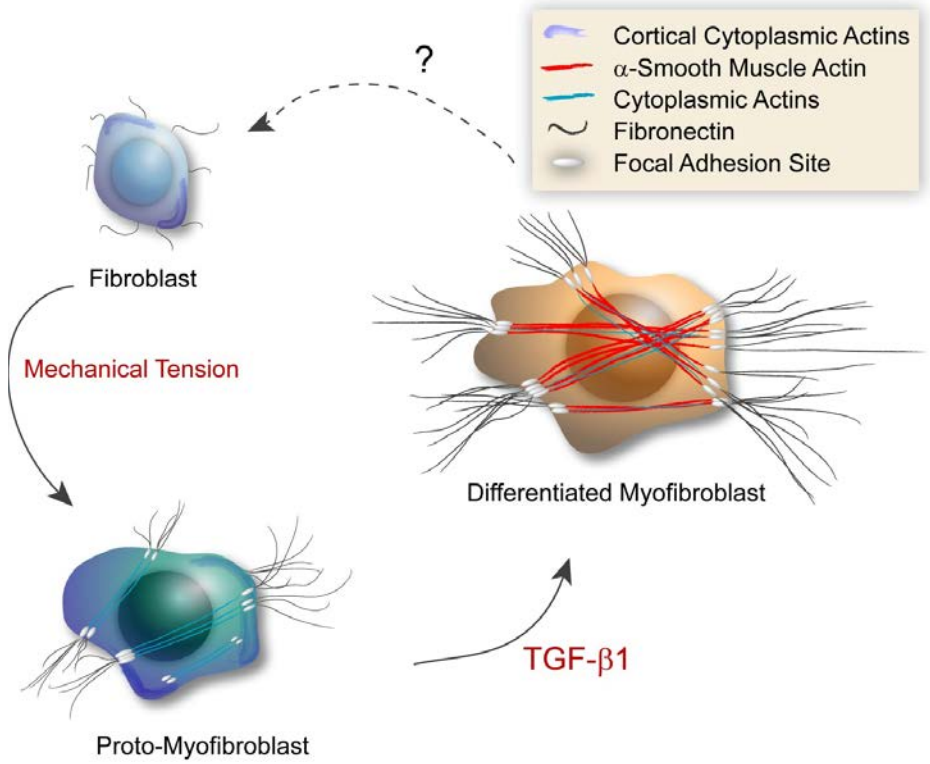


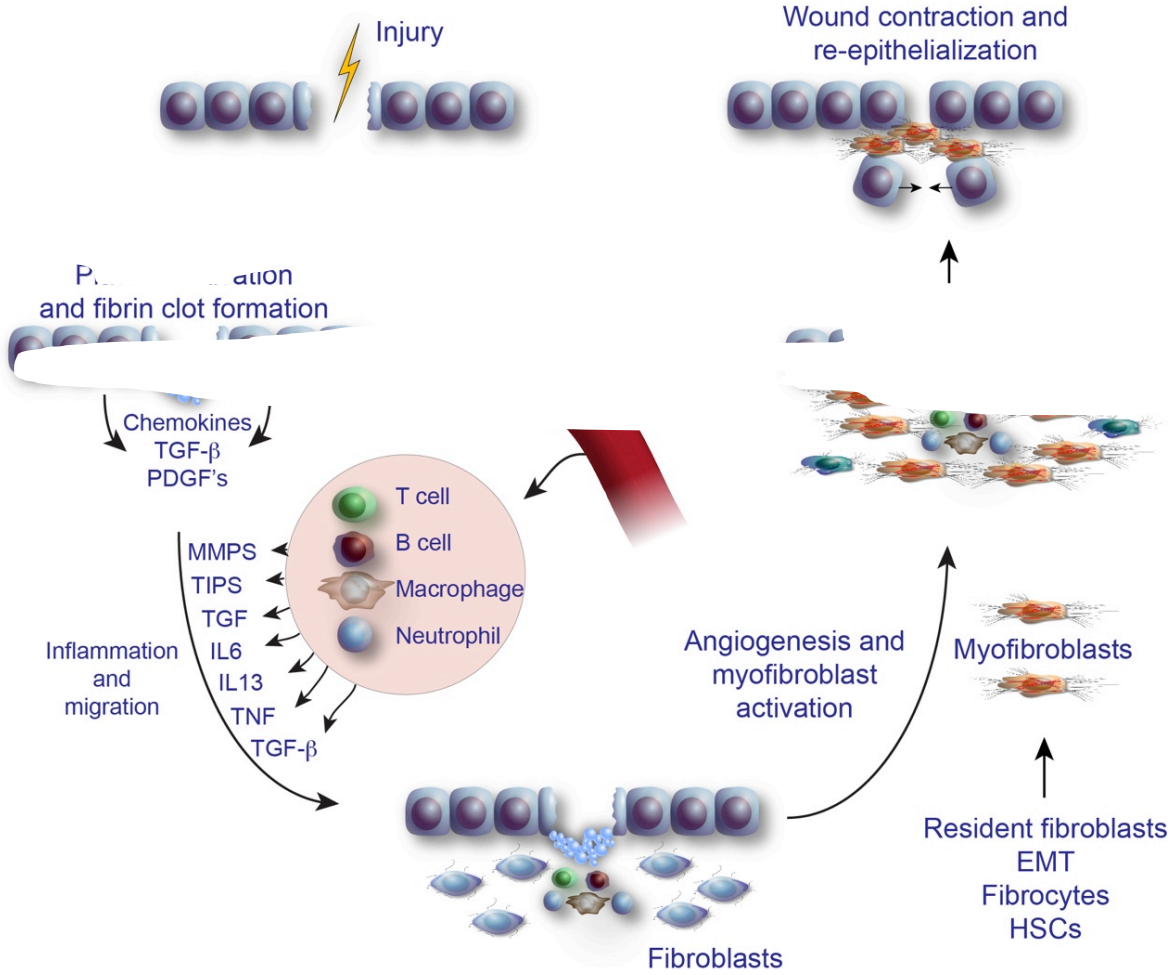




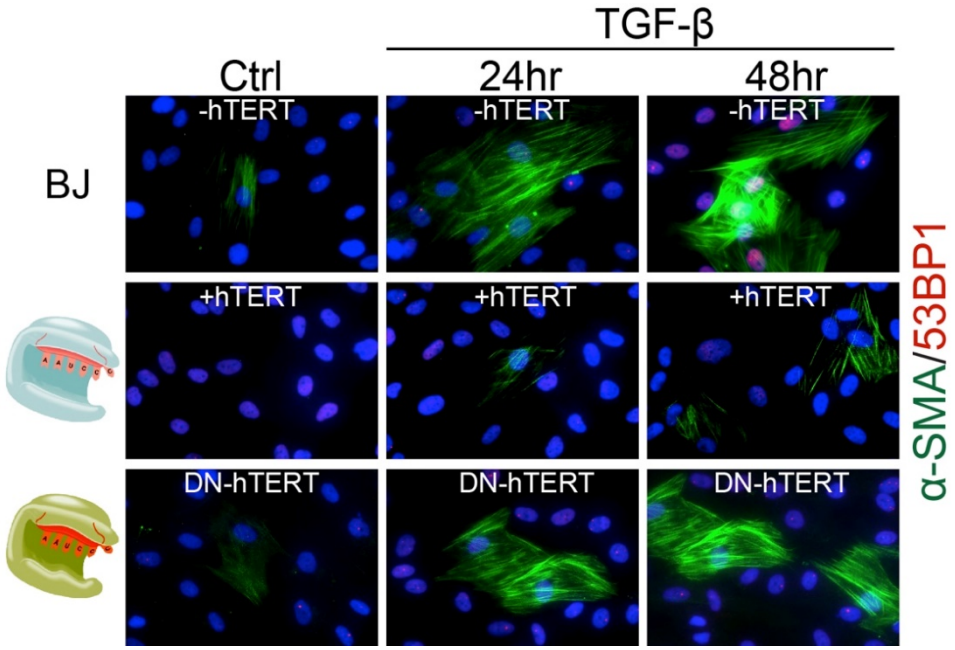
Razdan et al., 2018 Aging Cell

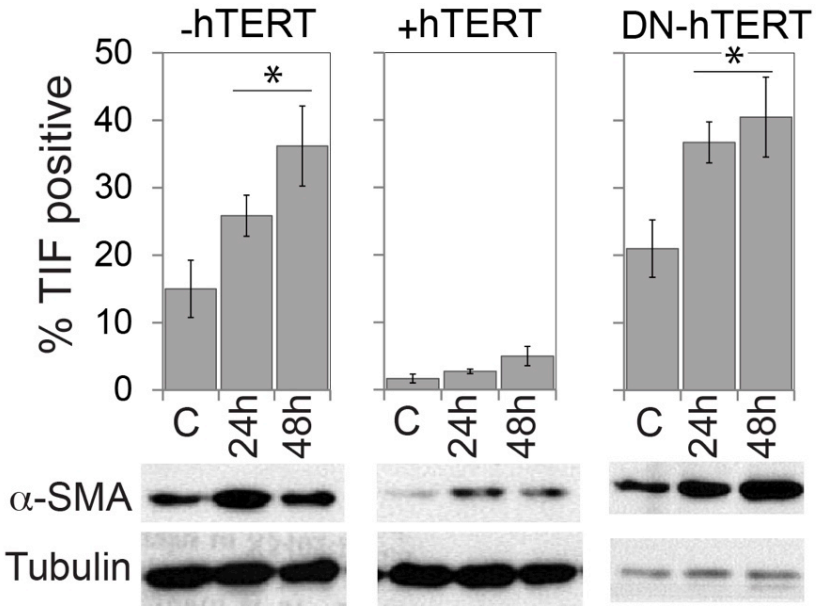
Wound Healing, TGFβ1, and Fibroblasts

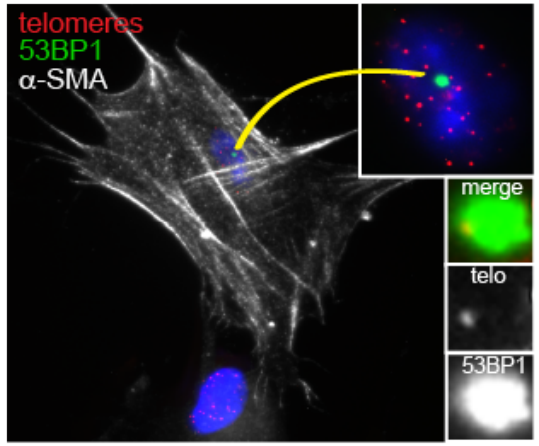


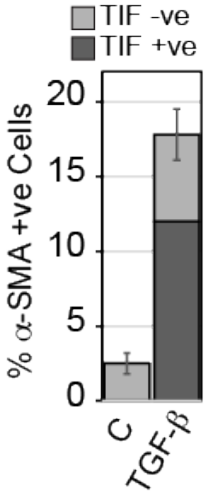


Cells Expressing Catalytically Active hTERT Resist Transdifferentiation



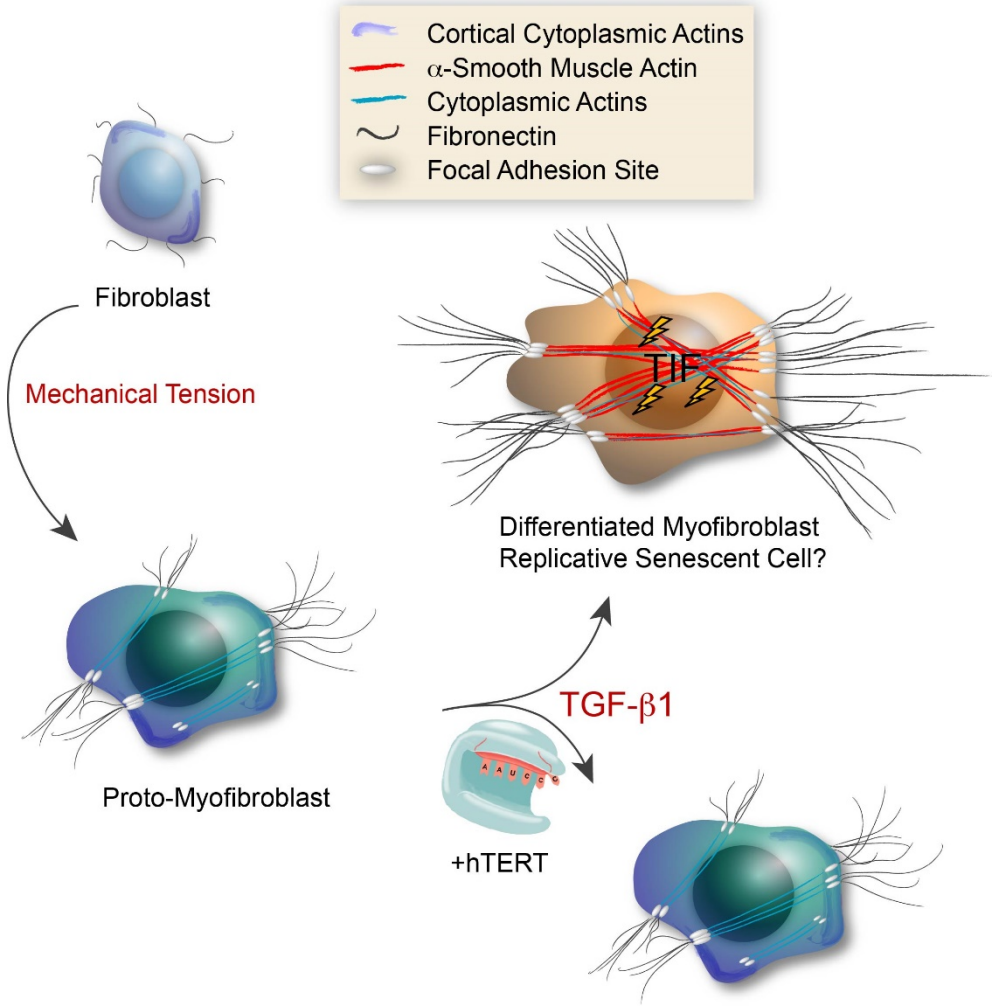




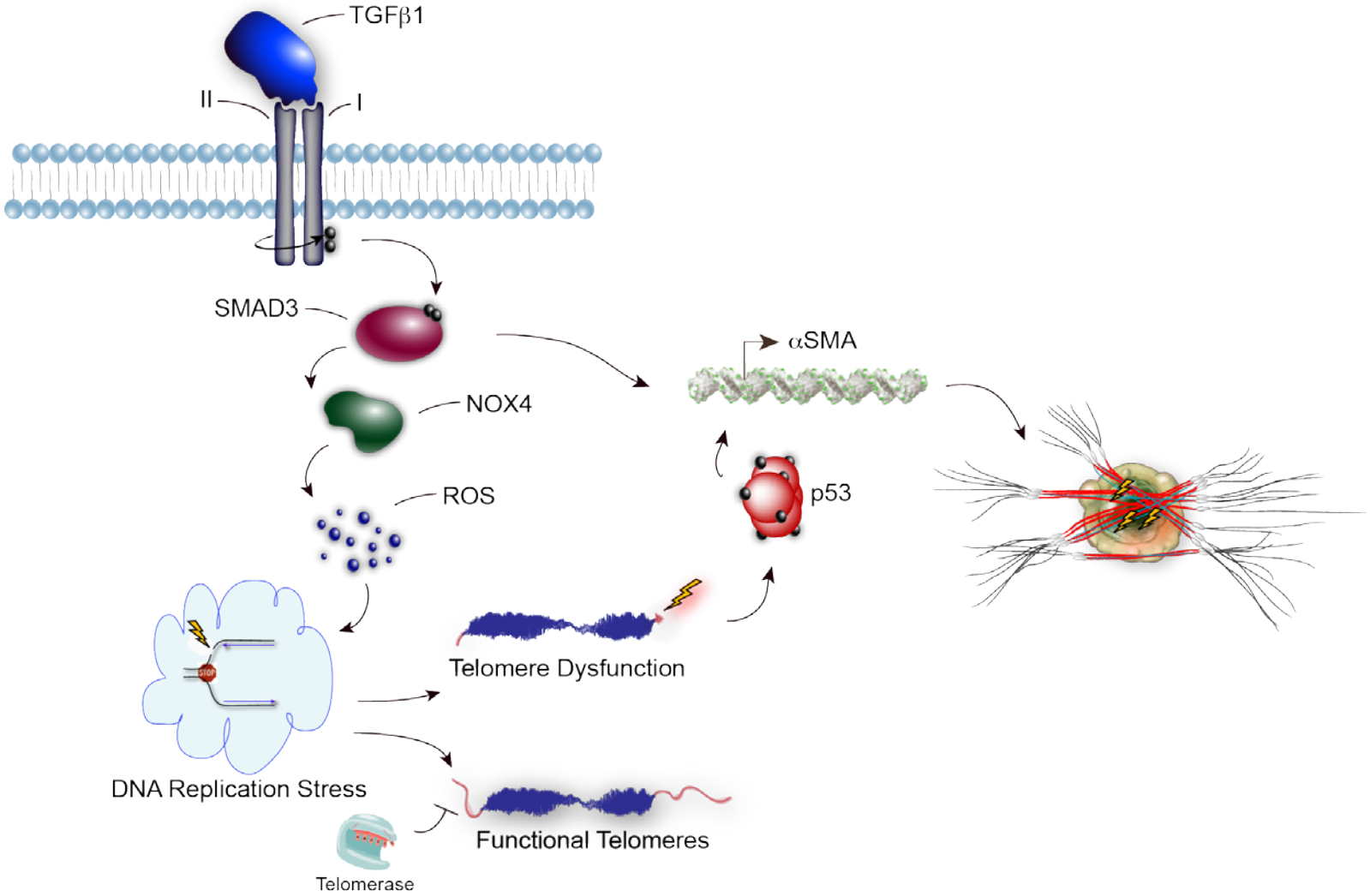


Razdan et al., 2018 Aging Cell

hTERT Suppresses Fibroblast to Myofibroblast Transdifferentiation



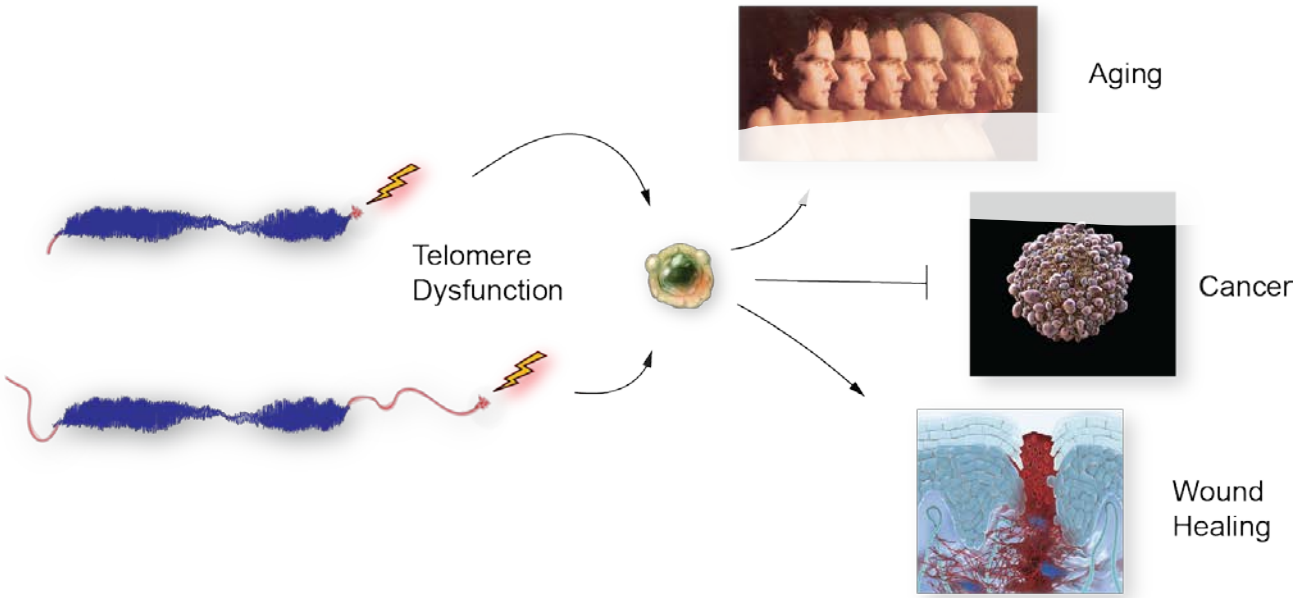
Telomere Dysfunction Promotes Myofibroblast Transdifferentiation

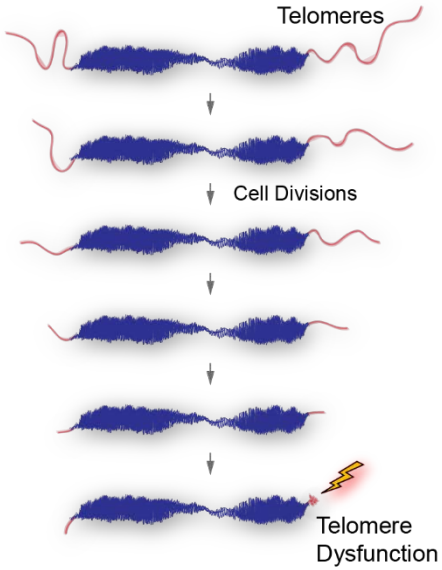


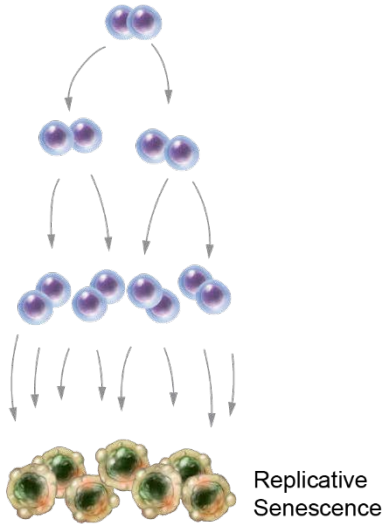
Summary I

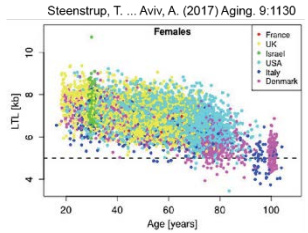
- Telomere length reflects both, the replicative history and the presence stresses that accelerate telomere erosion rates, such as oxidative stress and DNA replication stress
- Telomeres can activate cellular senescence regardless whether they are long or short
- Dysfunctional telomeres are both beneficial and damaging to the organism
  - Beneficial: tumor suppression, tissue repair
  - Damaging: (Potentially) aging and age associated diseases
- Telomere dysfunction can activate a transdifferentiation program without causing senescence (at first)

Dysfunctional Telomeres in Aging



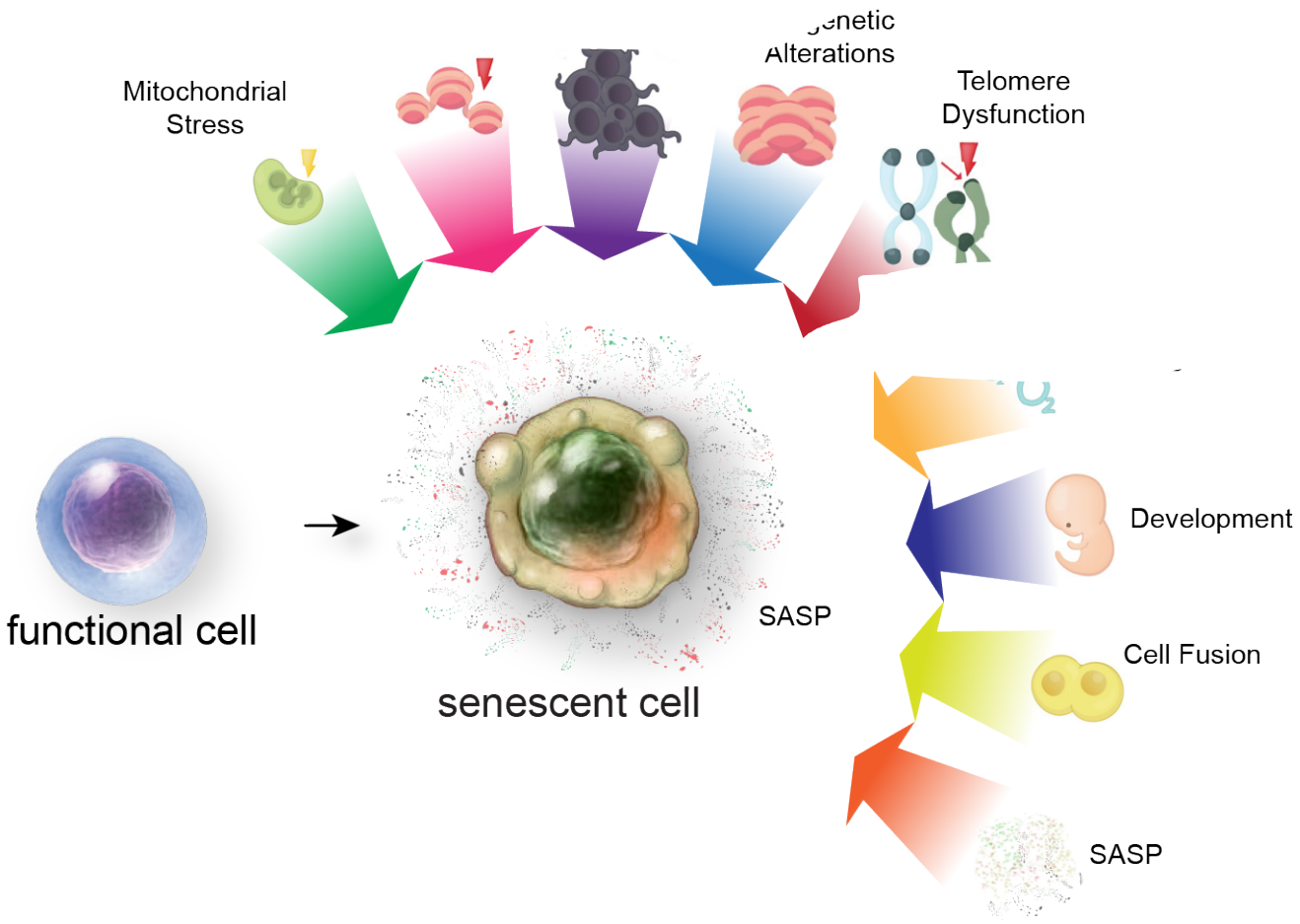




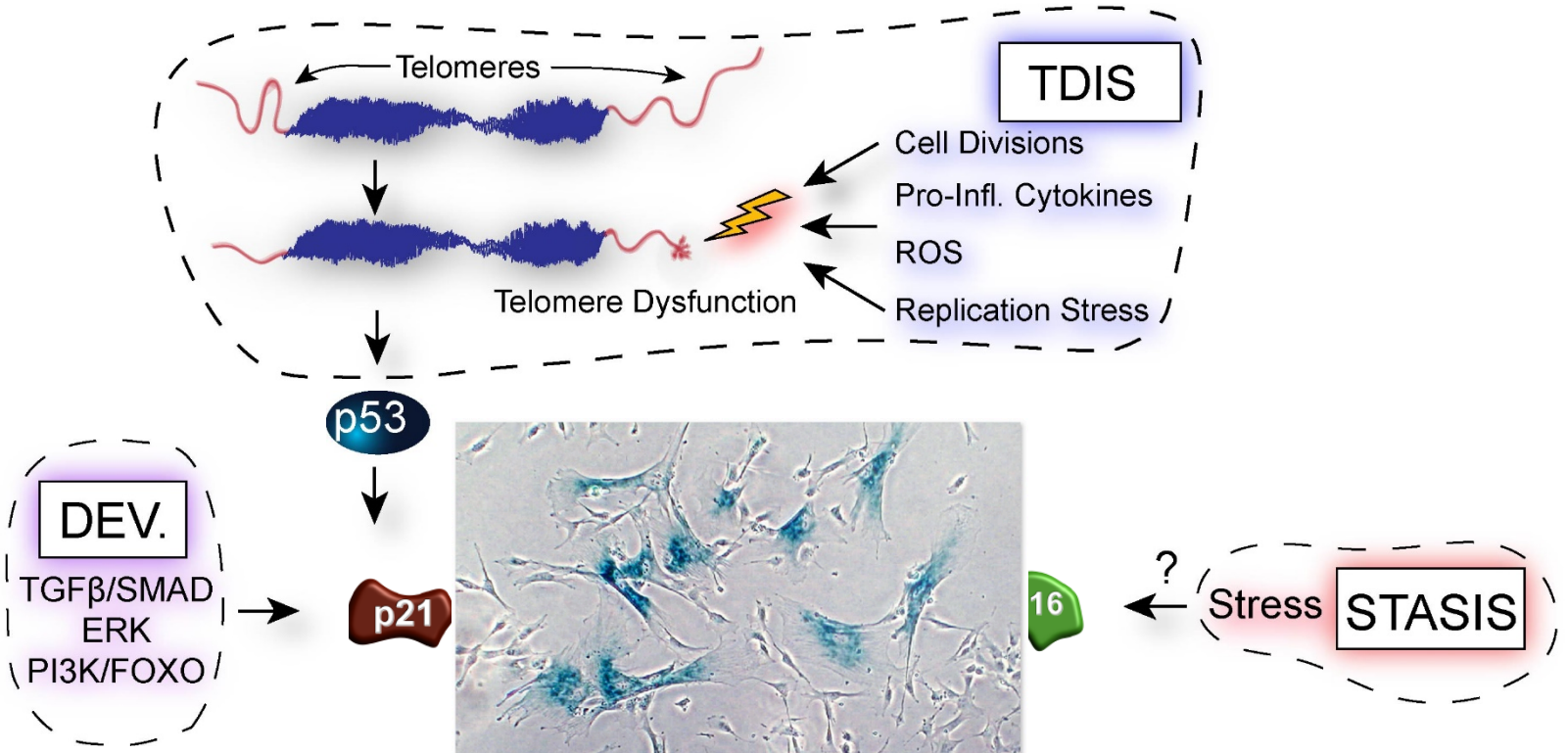


?

Senescence Inducers



At Least 3 Distinct Senescence Pathways

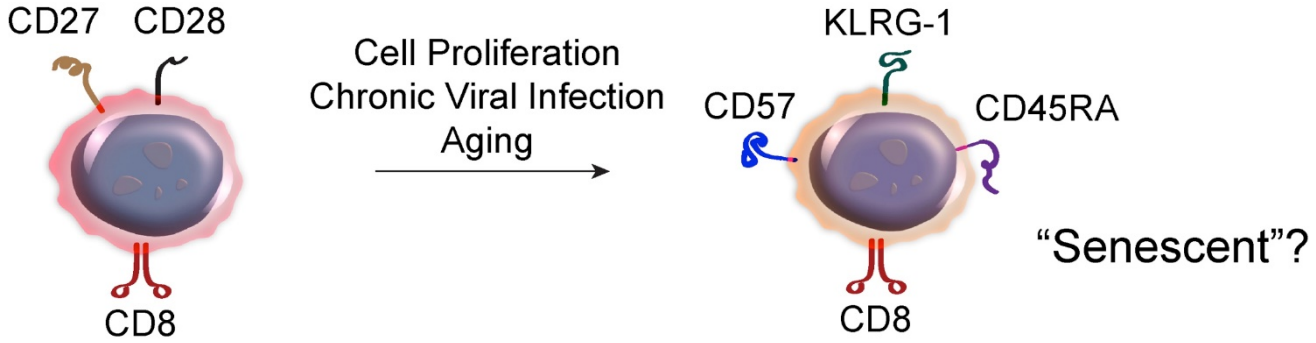


CDK Inhibitors
(p16^{INK4a}, p21)

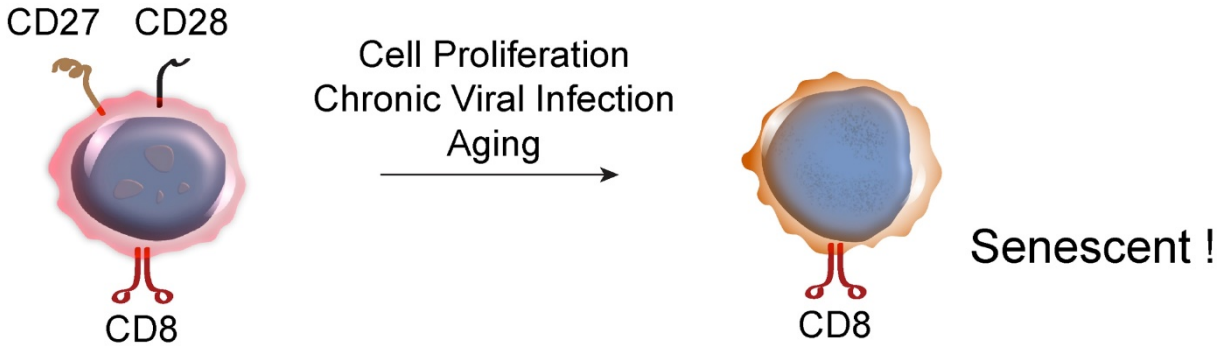
ochromatin Proteins
(macroH2A, HP1β, …)

Senescence Associated-β-Galactosidase
(SA-βGal)

What About Immune Cell - Senescence?

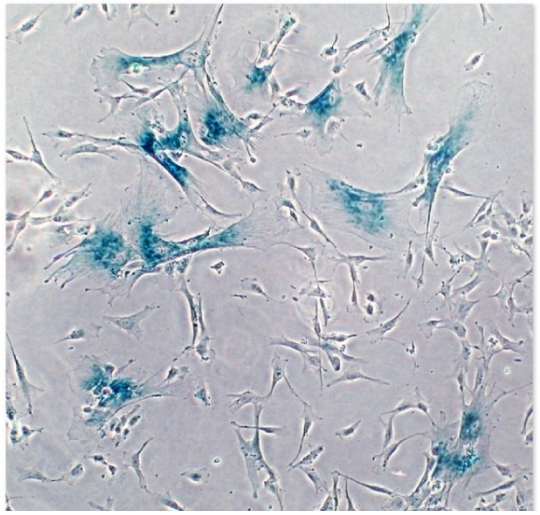


1. Increased Abundance in Older Humans
2. Lack of Proliferation, but reversible!
3. Shortened Telomeres
4. Low hTERT Activity
5. Elevated Levels of p16 (RT-qPCR)
6. Unique Cytokine production (Distinct From SASP)
7. "Exhaustion" Sometimes Interpreted as Senescence

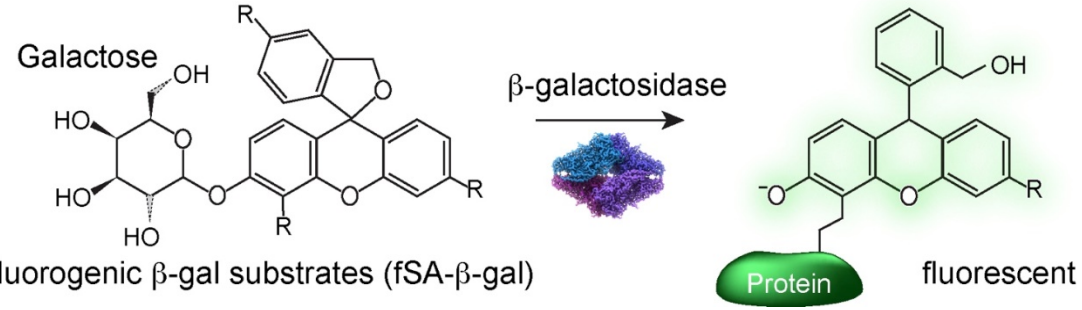


1. SA-β-Galactosidase Activity
2. Dysfunctional Telomeres
3. Elevated Protein Levels of p16
4. SASP
5. Gene Expression Profile
6. Etc.

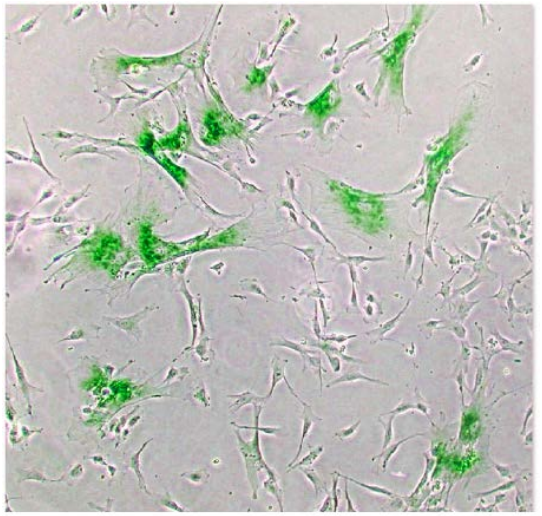
A Self-Immobilizing fSA-βGal Substrate to Detect and Isolate Senescent Mammalian Cells



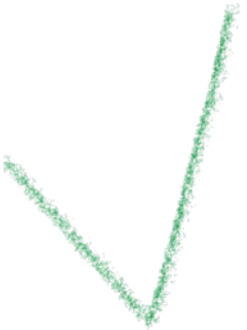
SA-βGal (X-gal)



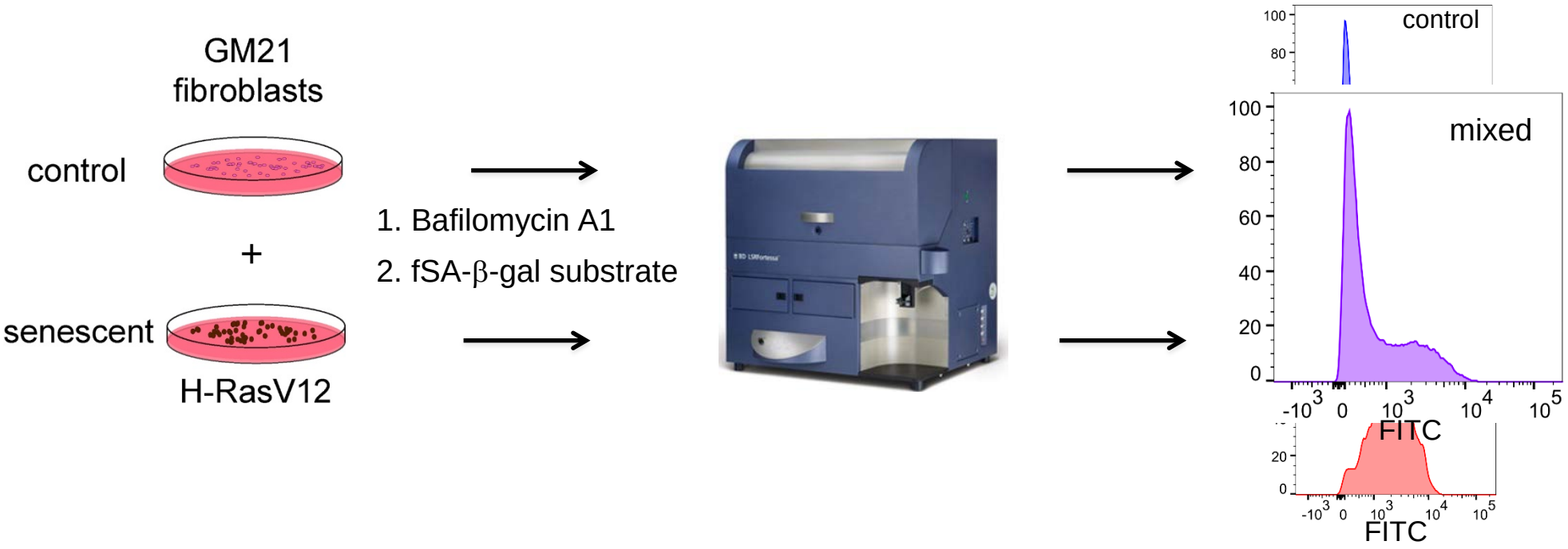
fluorogenic β-gal substrates (fSA-β-gal)



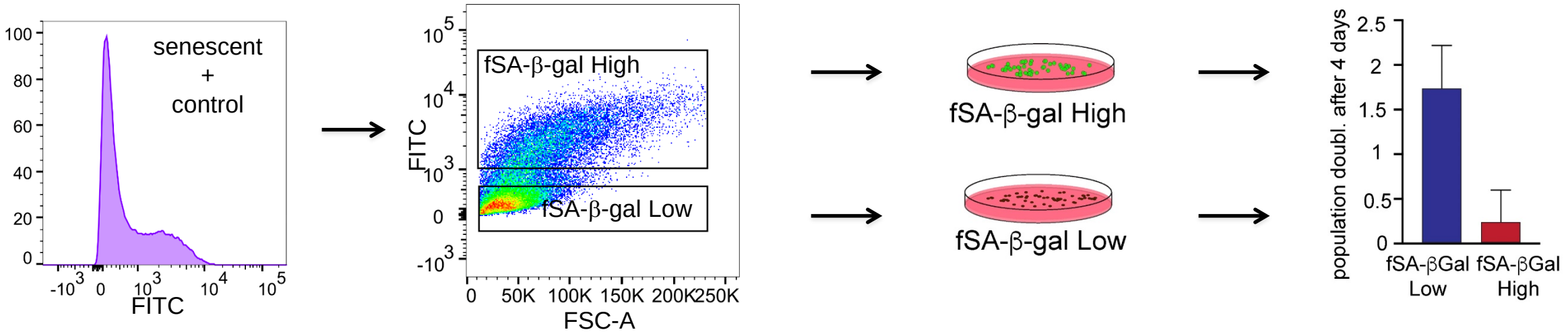
SA-βGal (fluorescent)

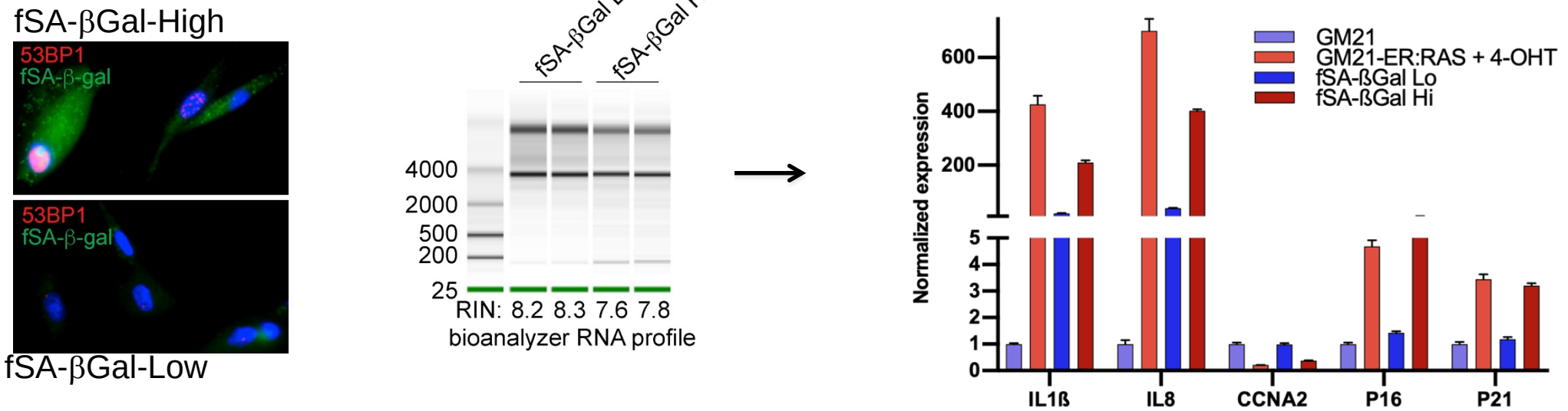


fSA-β-gal Substrates to Isolate Senescent Cells



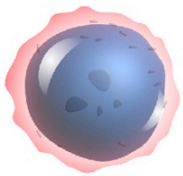
fSA-β-gal Substrates to Isolate Senescent Cells



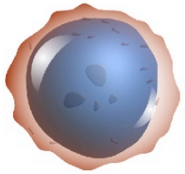


Subtypes of PBMCs

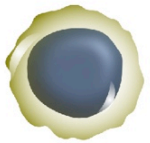
Collaborative Study Between Herbig and Fitzgerald-Bocarsly labs



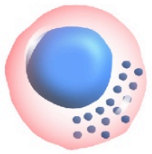
CD4+
T Cells
25-60%



CD8+
T Cells
5-30%

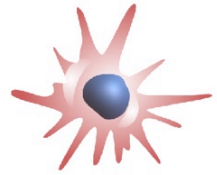


CD19+
B Cells
5-10%

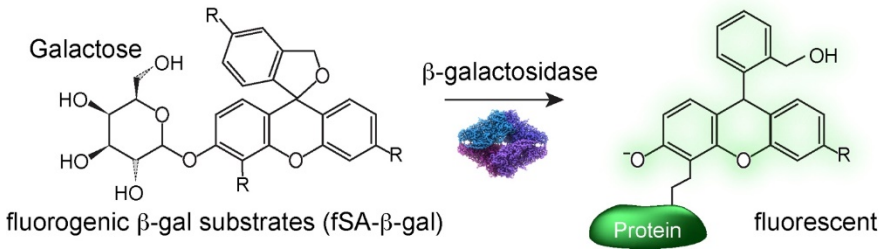


CD56+
NK Cells
10-30%

CD14+
monocytes
5-10%



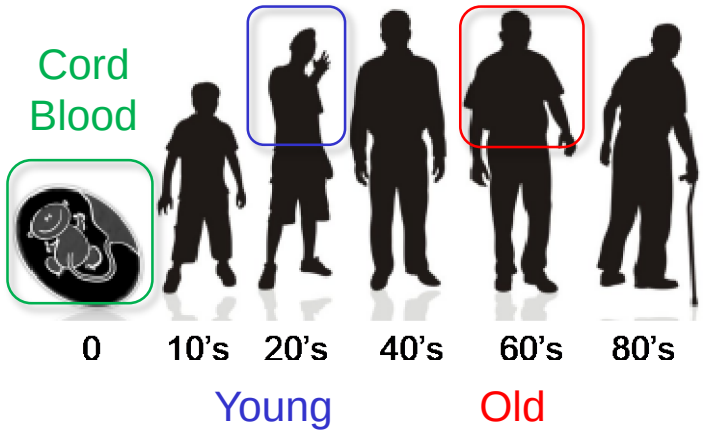
Dendritic
Cells
1-2%



fluorogenic β-gal substrates (fSA-β-gal)

fluorescent

PBMC Analysis Strategy



PBMCs

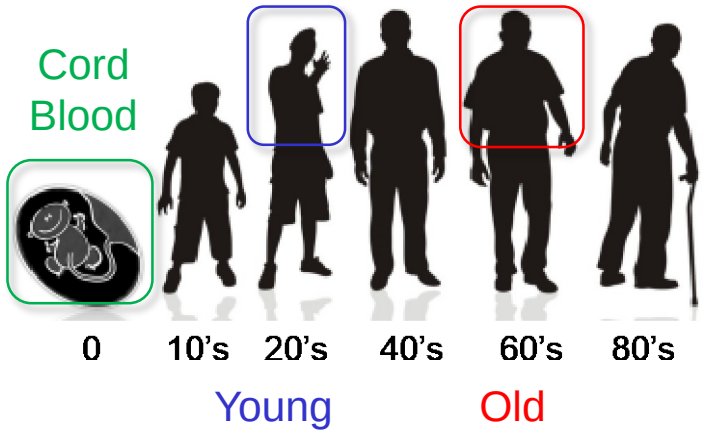
2) ISA-iSGal substrate

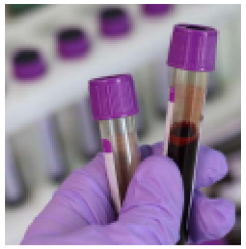
3) anti-CD45, CD3, CD8, CD4, CD123, BDCA2, CD14, CD19, CD56…

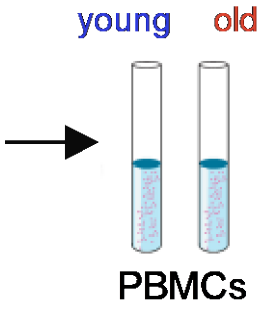
(SA-iSGal)

Cell Sorting
(Proliferation, RNA-seq, IF

PBMC Analysis Strategy







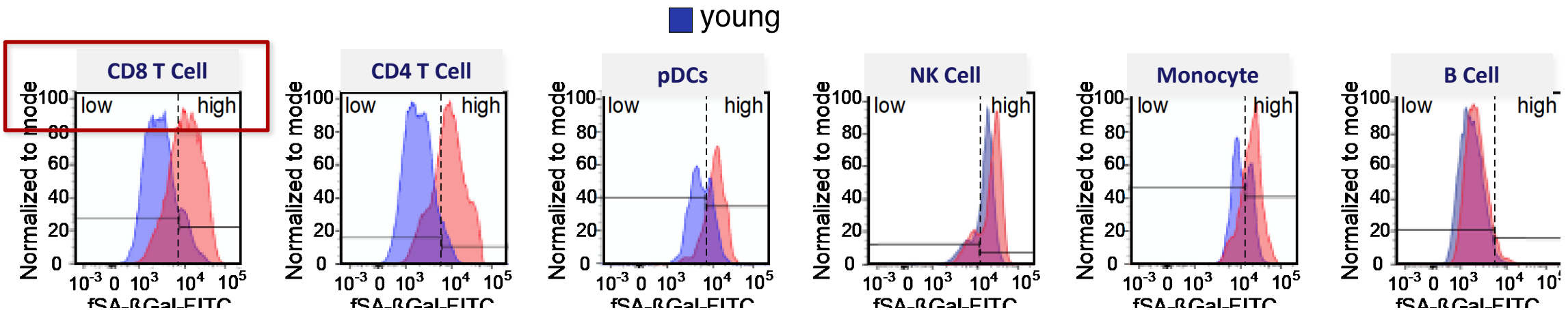
1) Bafilomycin A1
2) fSA-ßGal substrate

3) anti-CD45, CD3, CD8, CD4, CD123, BDCA2, CD14, CD19, CD56…

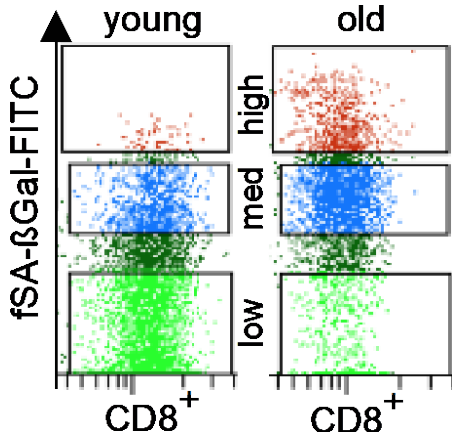
Flow Cytometry
(SA-ßGal)

Cell Sorting
(Proliferation, RNA-seq, IF

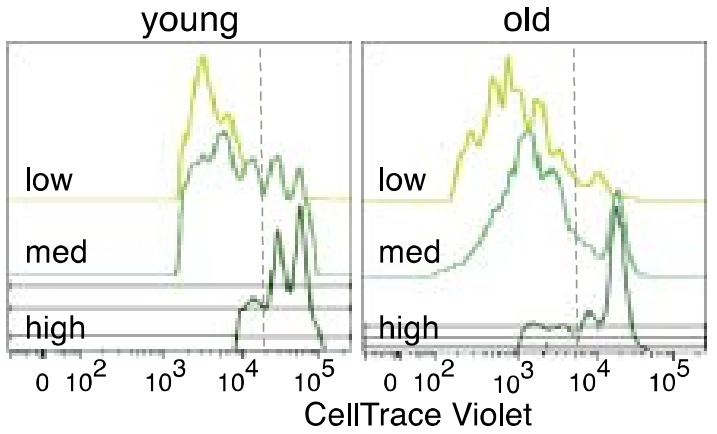
PBMC Analysis

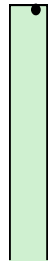


fSA-βGal Positive CD8 T Cells Display Hallmarks of Cellular Senescence

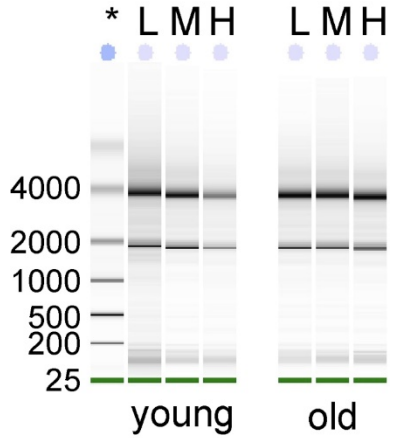


proliferation

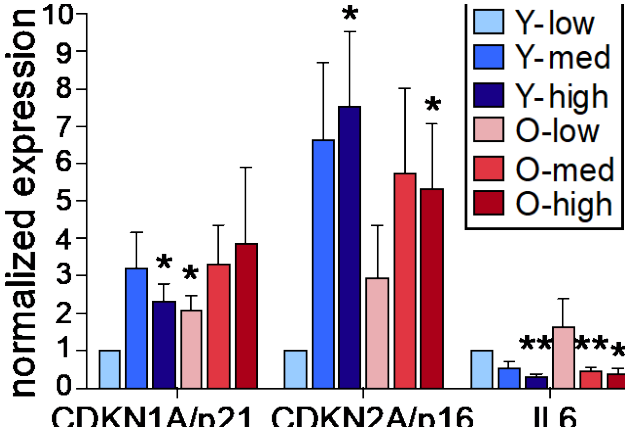




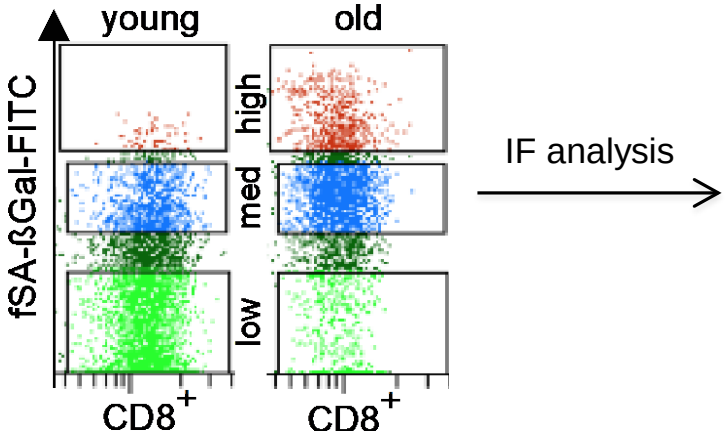
RNA



gene expression



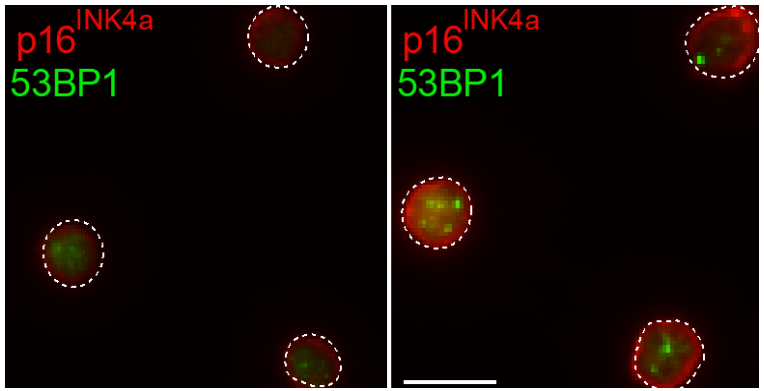
fSA-βGal Positive CD8 T Cells Display Hallmarks of Cellular Senescence



IF analysis

Young fSA-ßGal-low

Old fSA-ßGal-high



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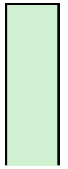
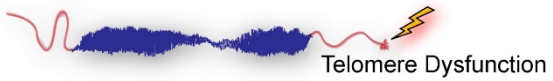
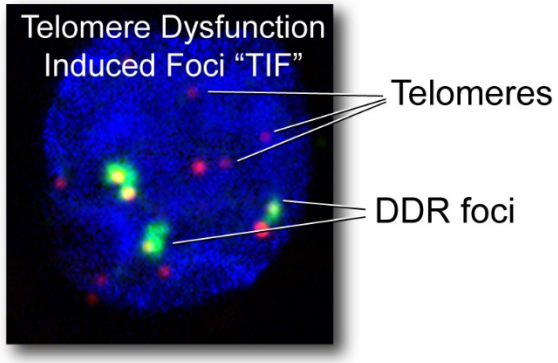
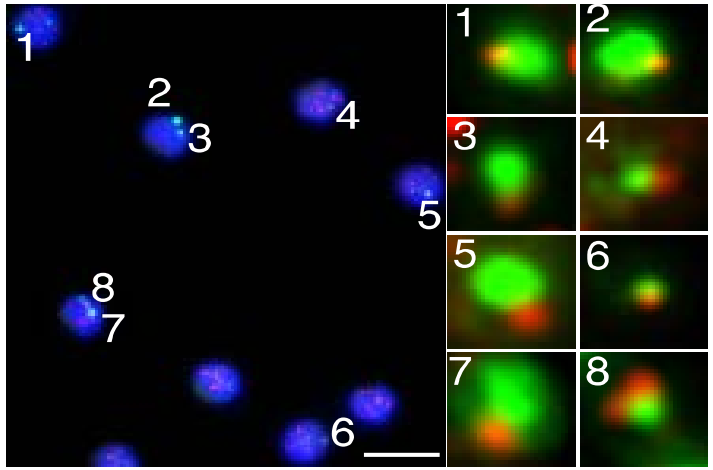


fSA-ßGal

Telomere dysfunction
Induced DNA damage
Foci (TIF) analysis





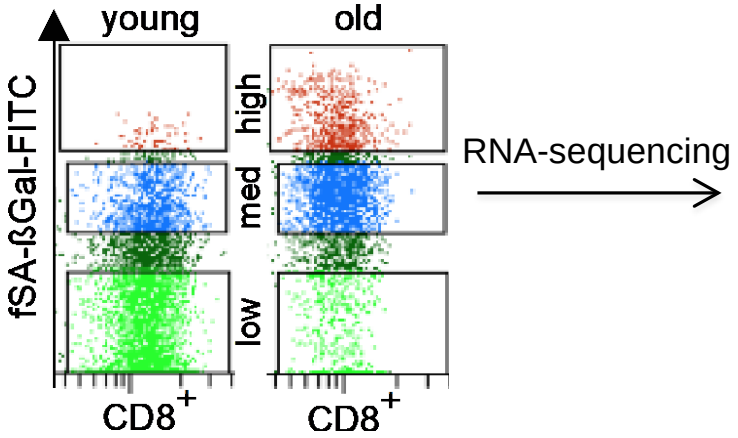


Telomere: red; 53BP1: green

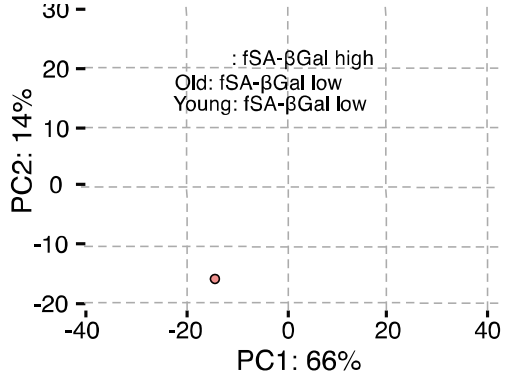
*

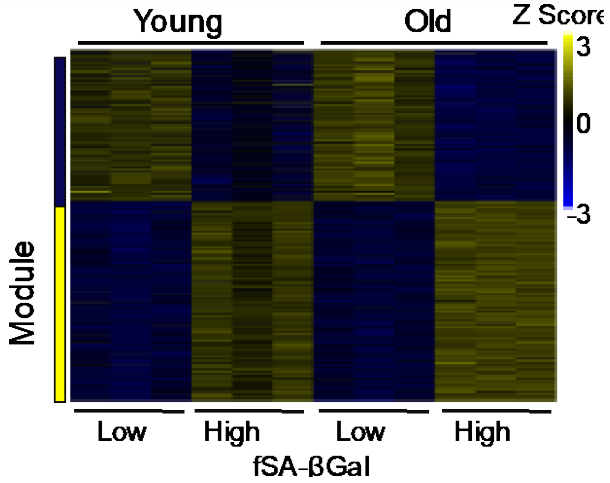
low med high
fSA-ßGal

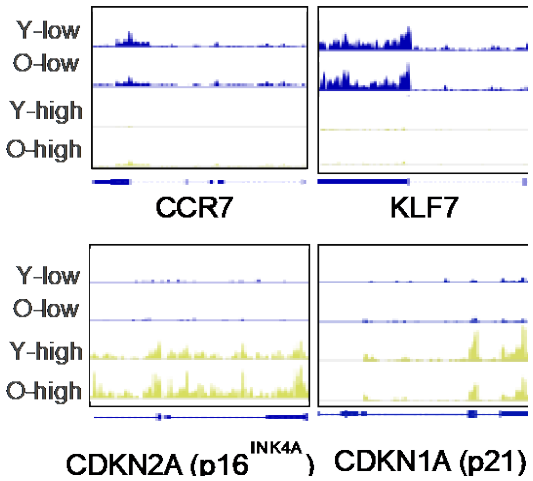
fSA-βGal Positive CD8 T Cells Display a Transcriptional Signature That Resembles a State of Deep Senescence

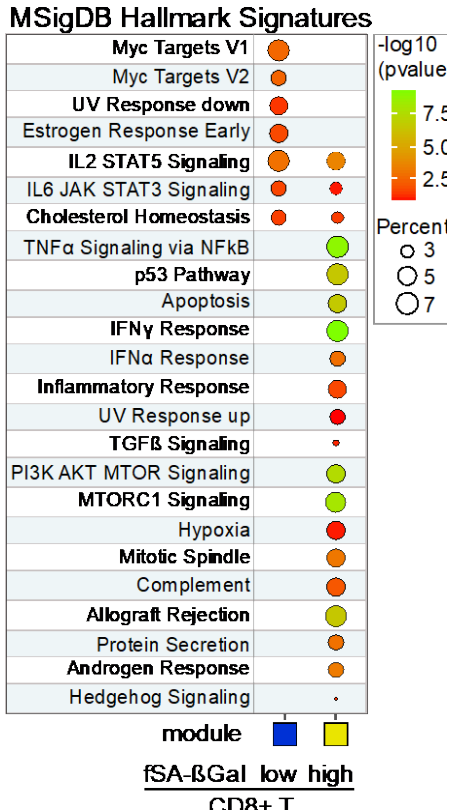


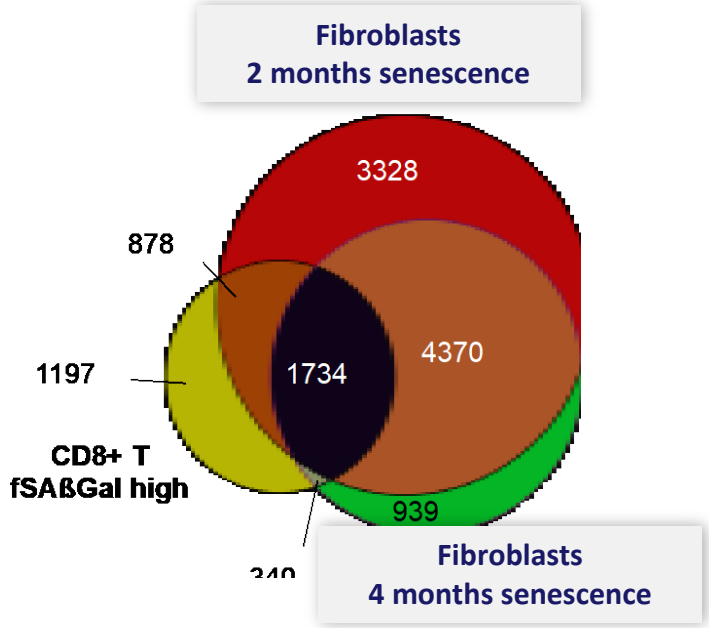
RNA-sequencing



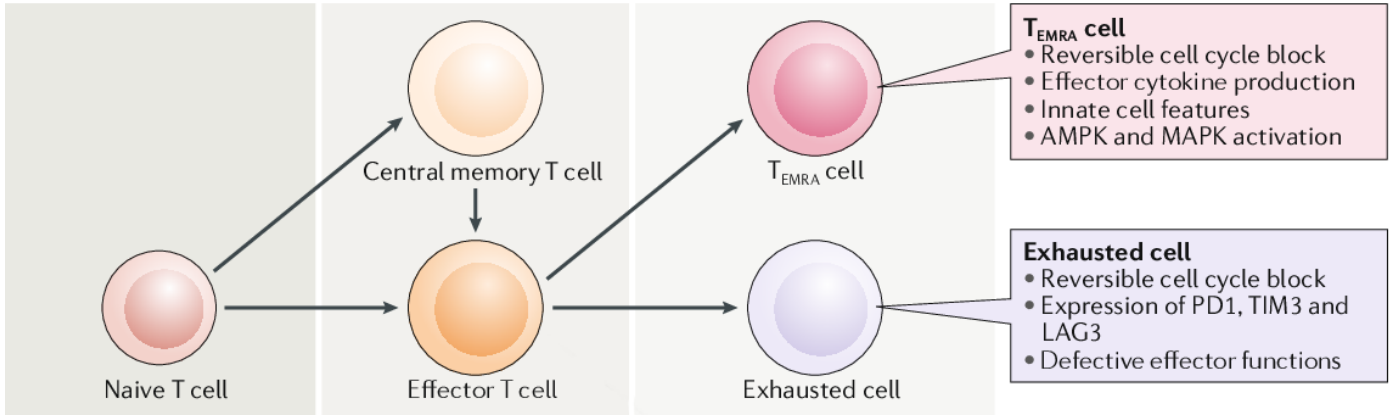


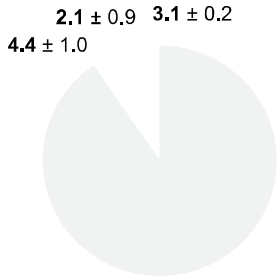




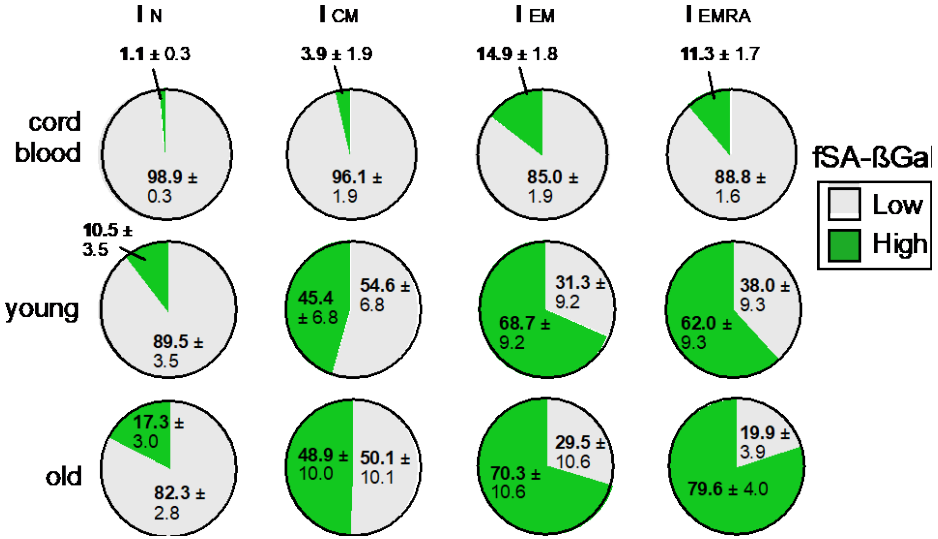


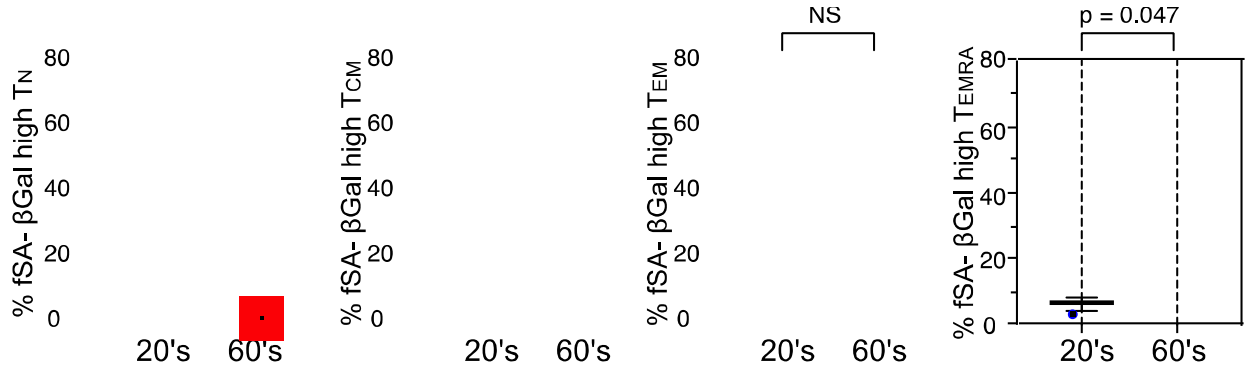
Senescent CD8 T Cells Develop in All Differentiation States



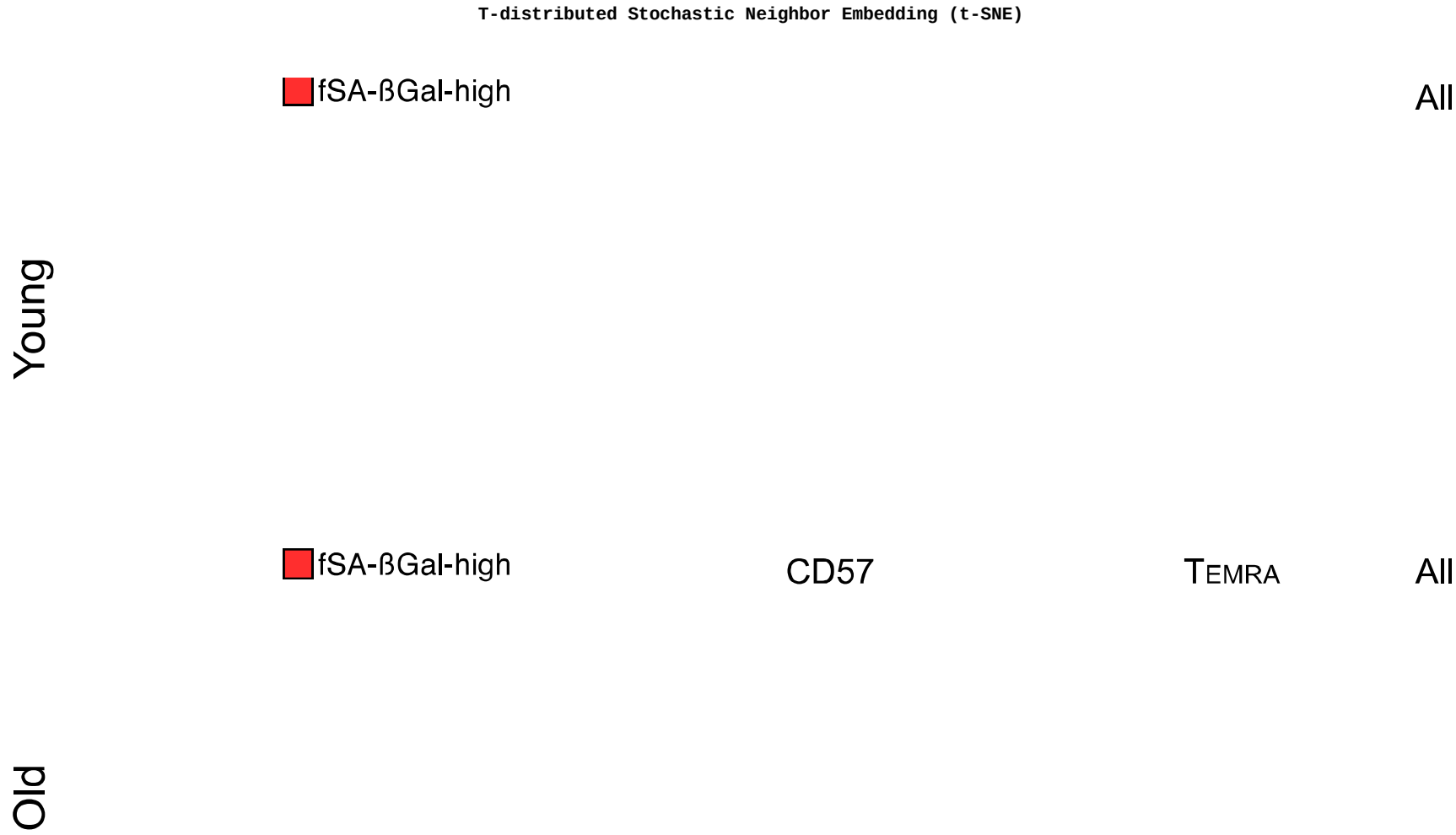


old

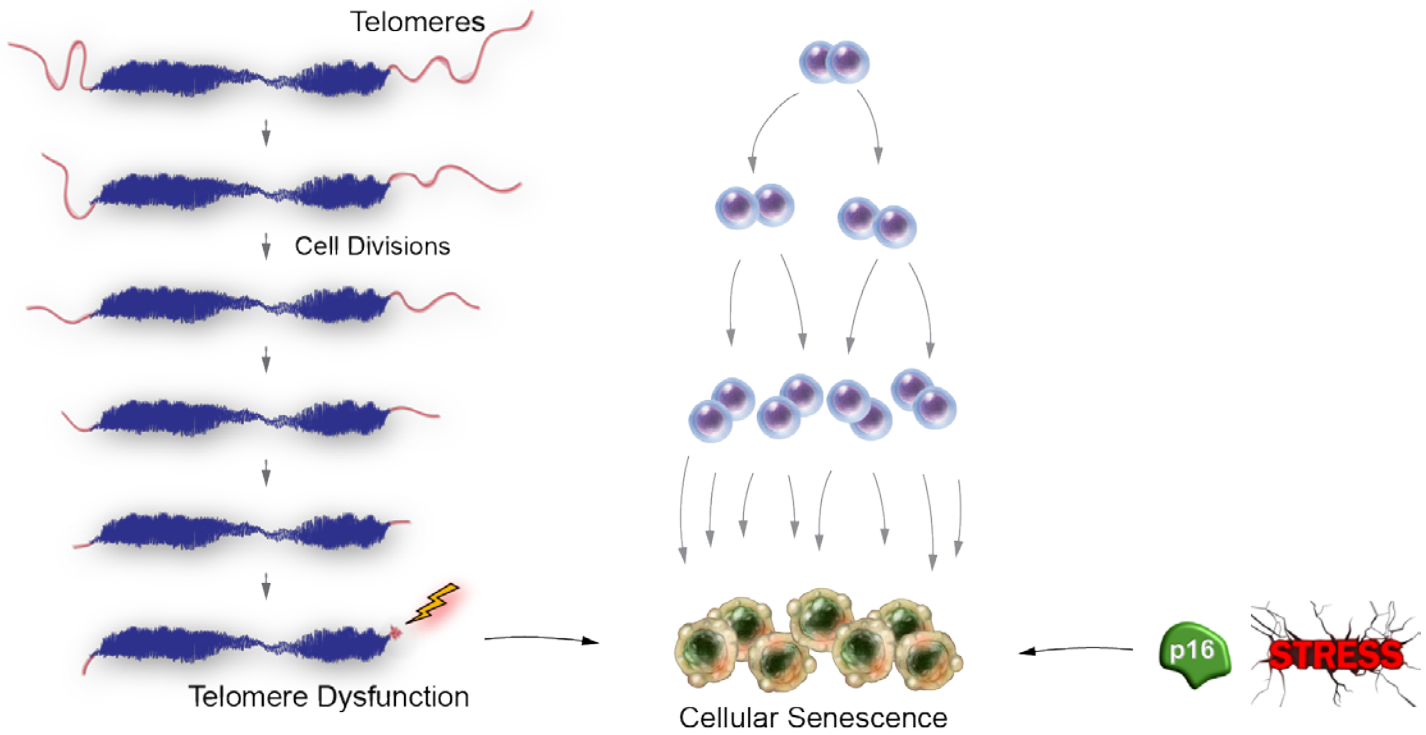


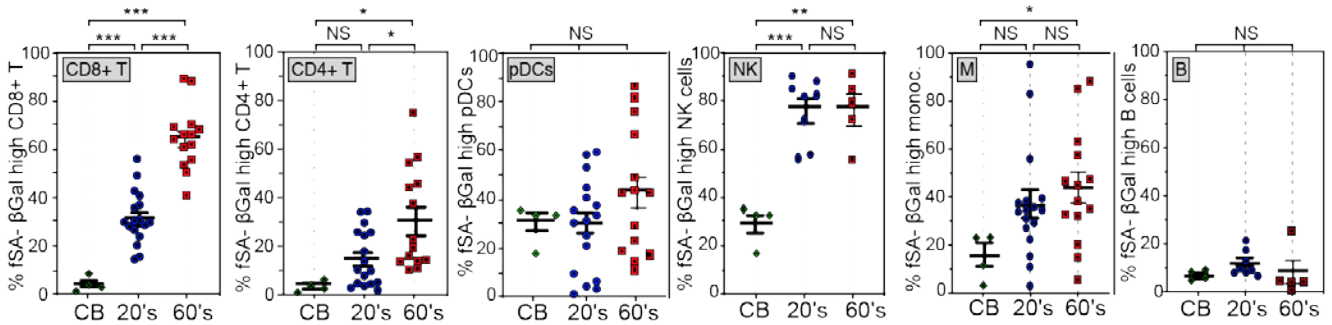


fSA-βGal Positive CD8 T Cells Are Distinct From Exhausted and TEMRA Cells



Summary II


Telomeres
Cell Divisions
Telomere Dysfunction
Cellular Senescence
p16
STRESS

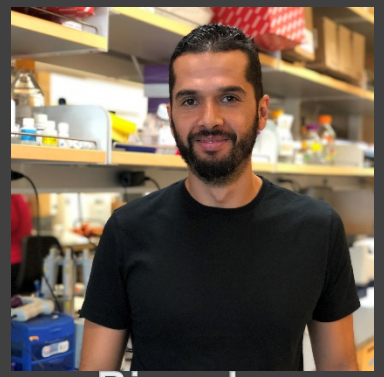

CD8+ T
CD4+ T
pDCs
NK
M
B
% fSA- βGal high CD8+ T
% fSA- βGal high CD4+ T
% fSA- βGal high pDCs
% fSA- βGal high NK cells
% fSA- βGal high monoc.
% fSA- βGal high B cells
CB
20's
60's
NS

Summary II

- Accurate method to detect, quantify, isolate, and characterize **senescent** PBMCs
  - Biomarker of biological age, acute disease, chronic disease
  - Prognostic marker for susceptibility to infection and disease outcome
- CD4 and CD8 T cells increasingly develop hallmarks of cellular senescence with advancing age
  - up to 89% of CD8 T cells are senescent in donors in their 60s; average 64%. CD4 T cells: up to 75% senescent; average 31%
- Senescent CD8 T cells display features of telomere dysfunction-induced senescence and of p16 mediated senescence, depending on donor
- Senescent CD8 T cells develop in all T cell differentiation states, including in naïve T cell populations
- CD8+ T cell senescence transcriptome resembles a state of prolonged or deep senescence
- Relevance for COVID-19 ?

Acknowledgements

Utz Herbig lab



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Priyanka Patel, PhD
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NIH NIA
NIH NIEHS
TELOMERE RESEARCH NETWORK

U01 Cross-Lab DNA Extraction Experiment: Preliminary Results

Jue Lin, Ph.D. UCSF
On behalf of the U01 and U24 labs

12/4/2020

Why We are Doing This Study

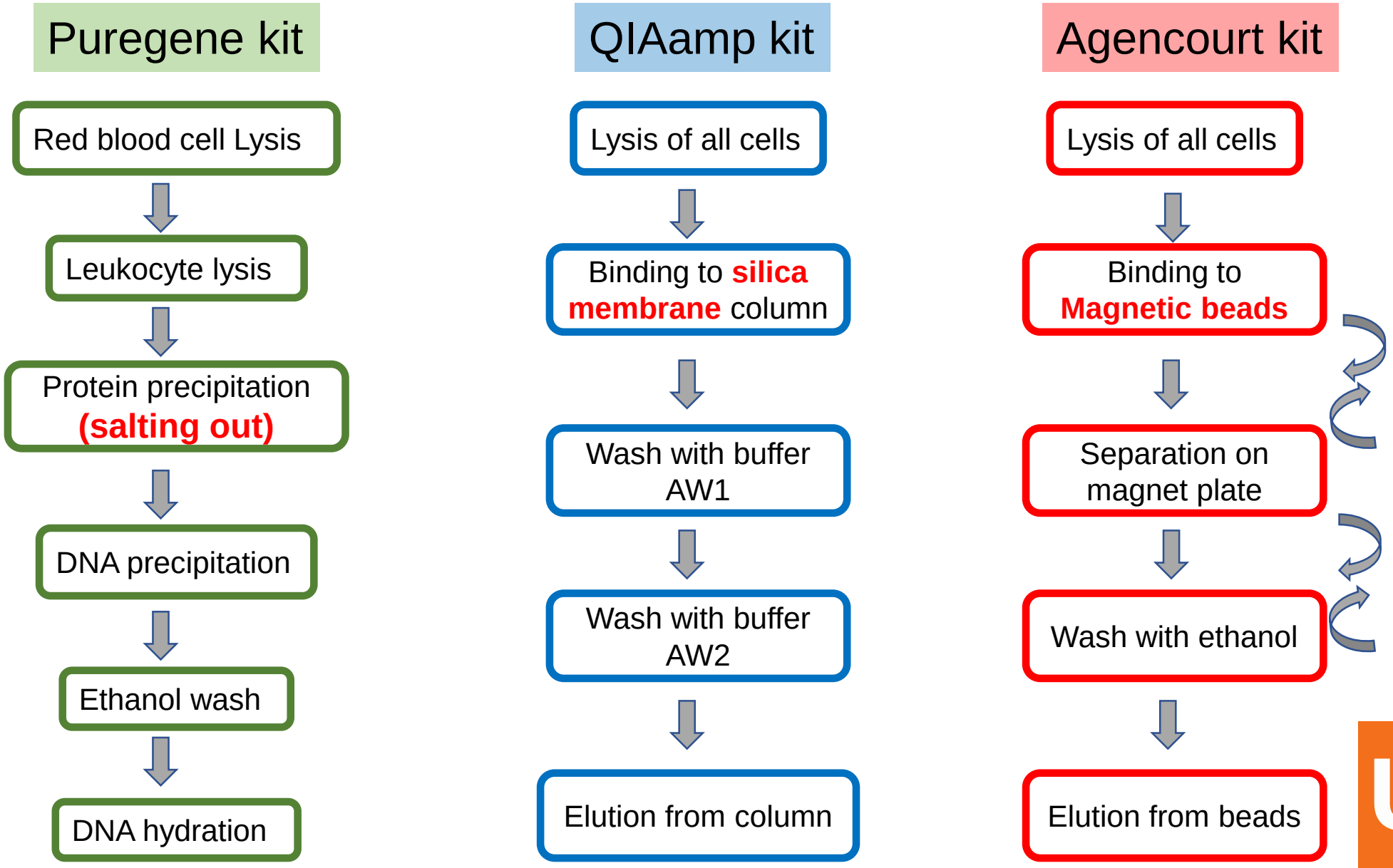
- One of the primary aims of the U24 and U01 grants are to develop recommendations for telomere research around biological sample collection, storage, and processing; laboratory methods; data and statistical analysis, and reporting requirements.

- Understanding the impact of DNA extraction methods on qPCR was identified as a critical step during the 2019 kickoff meeting.

Outline of the Talk

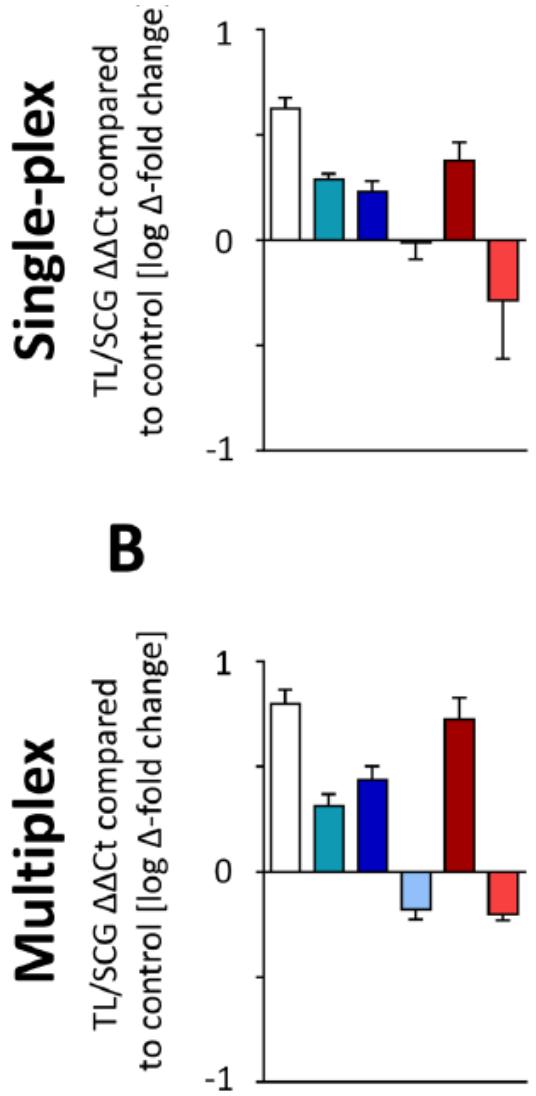
- Background
- Study design of cross-lab whole blood DNA extraction study
- Results of cross-lab whole blood DNA extraction study
- Results of saliva DNA extraction method study

Principles of DNA Extraction Methods Used



Summary of Prior Findings:

Significant difference between different DNA extraction methods



Preanalytical Conditions and DNA Isolation Methods Affect Telomere Length Quantification in Whole Blood

Alexander Tolios, Daniel Teupser, Lesca M. Holdt*

PLOS ONE, 2015

Magnetic Beads		Invitrogen GeneCatcher gDNA Kit
Spin Column		QIAGEN QIAamp DNA Blood Maxi Kit
		Marcherey-Nagel NucleoSpin Blood Kit
		5prime PerfectPure DNA Blood Kit
Precipitation		Stratec/Invisorb Blood Universal Kit
		DNA isolation protocol (IPP) according to [31]

Cunningham *Cancer Epidemiol Biomarkers Prev . 2013;*
Denham *BMC Research Notes 2014;*
Hofmann *Cancer Epidemiol Biomarkers Prev 2014;*
Boardman *Cancer Epidemiol Biomarkers Prev 2014*
Raschenberger *Scientifc Reports 2016;*
Dagnall *PLOS ONE 2017*

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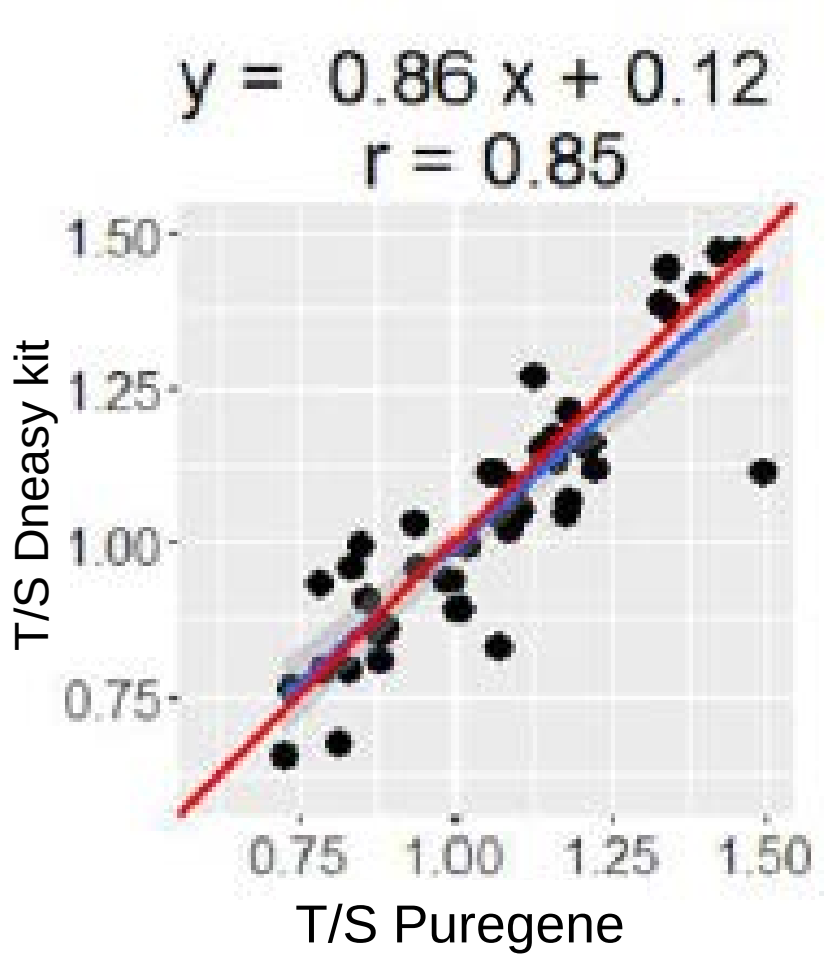
Summary of Prior Findings:

A systematic difference may allow for calibration



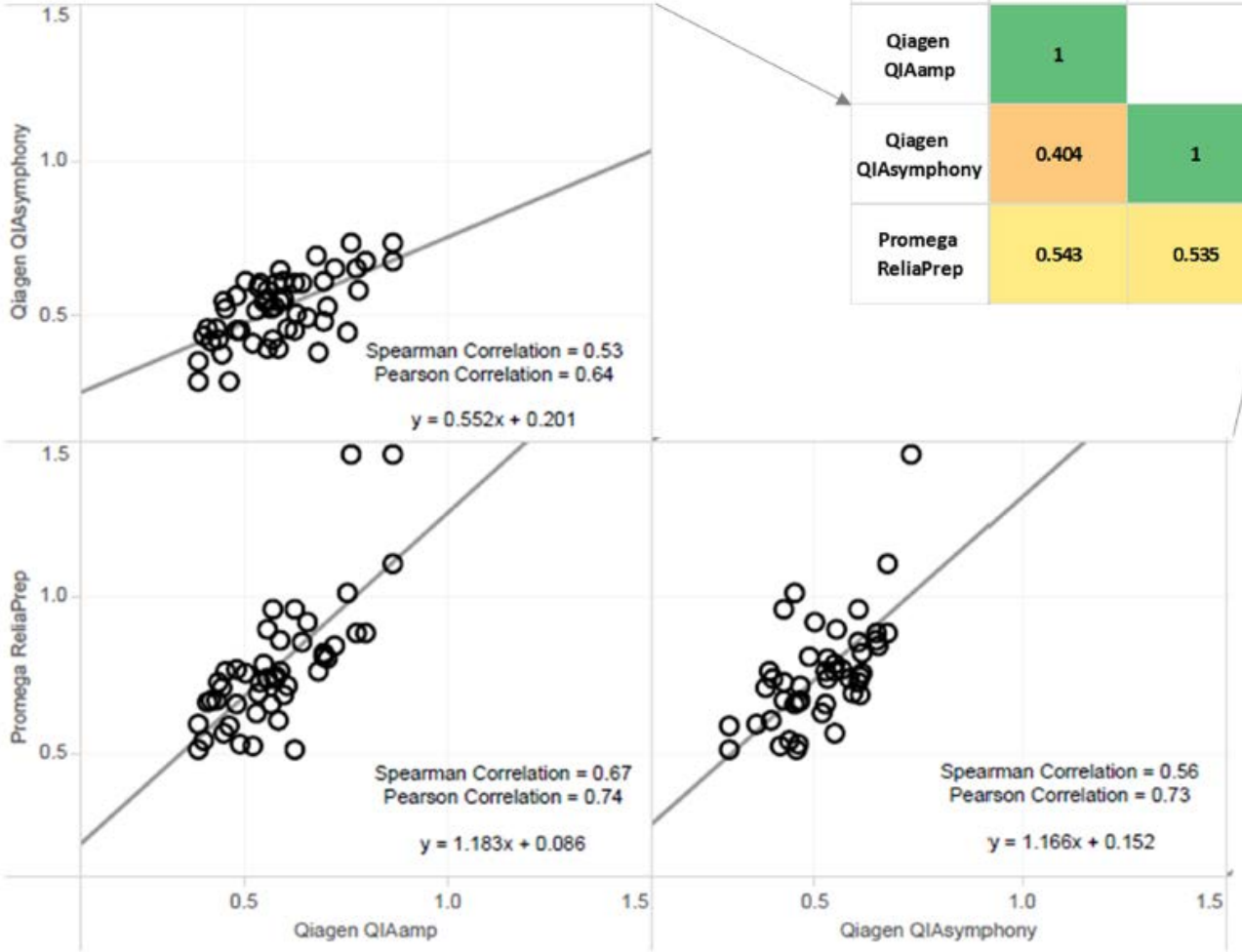
Method Specific Calibration Corrects for DNA Extraction Method Effects on Relative Telomere Length Measurements by Quantitative PCR

Luise A. Seeker[1,2]*, Rebecca Holland[3], Sarah Underwood[3], Jennifer Fairlie[3], Androniki Psifidi[2], Joanna J. Ilska[1], Ainsley Bagnall[4], Bruce Whitelaw[2], Mike Coffey[1], Georgios Banos[1,2], Daniel H. Nussey[3]

TL measurement in cattle

Seeker PLOS ONE 2016

Summary of Prior Findings



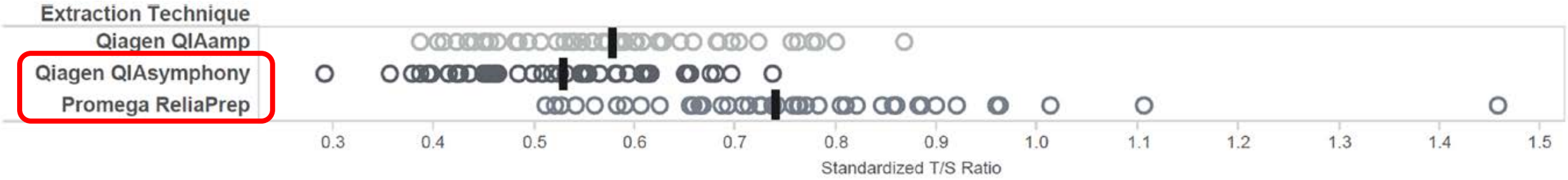
R^2	Qiagen QIAamp	Qiagen QIAsymphony	Promega ReliaPrep
Qiagen QIAamp	1		
Qiagen QIAsymphony	0.404	1	
Promega ReliaPrep	0.543	0.535	1

T/S ratios of the DNA samples from the same source material extracted by different methods are modestly correlated at best.

Dagnall PLOS ONE 2017

Summary of Prior Findings

T/S ratios of DNA extracted by different kits with the same principle are different

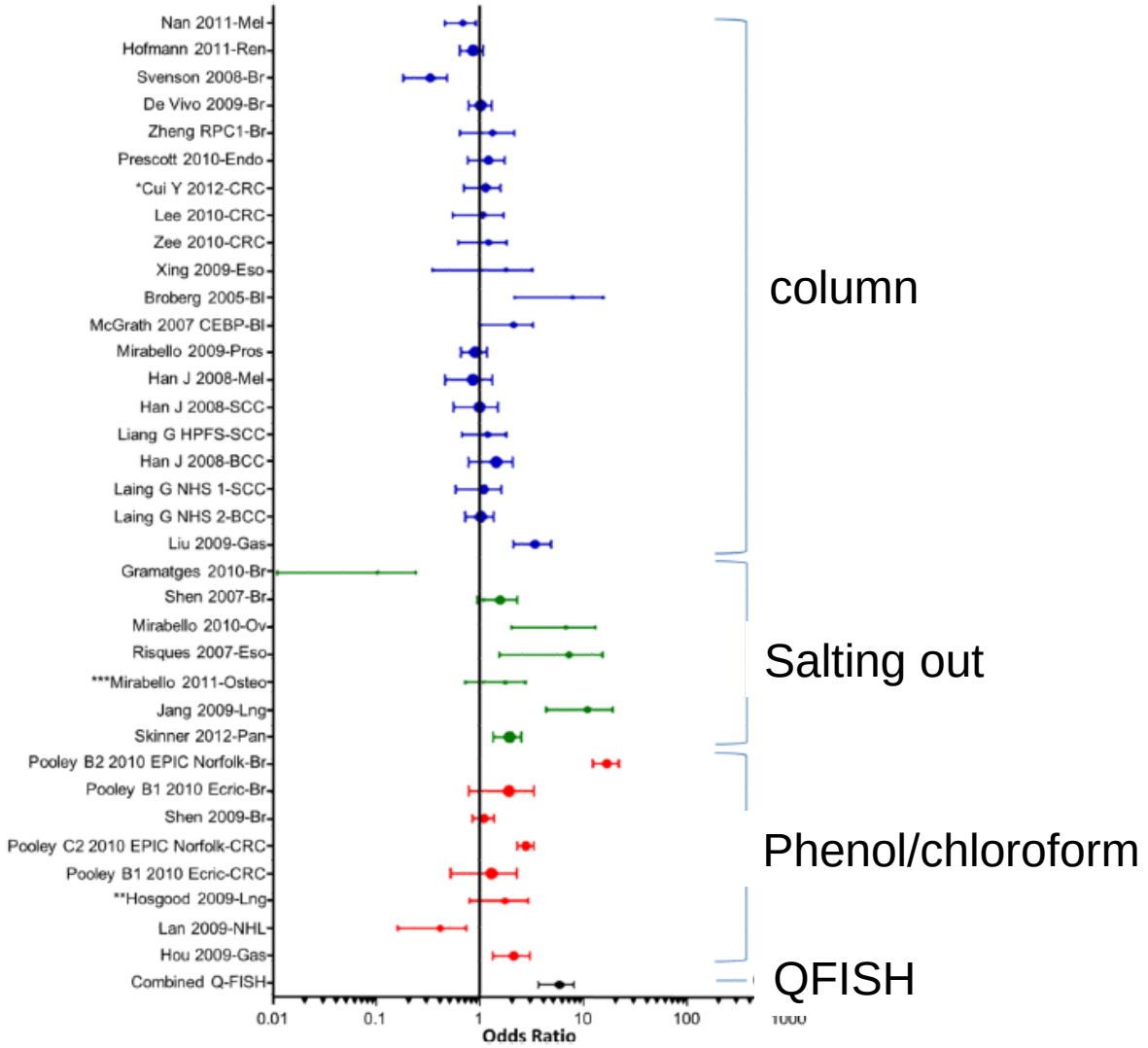


Extraction Technique	Number	Median	Range	P-Value*
Qiagen QIAamp	48	0.578	0.39 - 0.87	Reference
Qiagen QIAsymphony	48	0.529	0.29 - 0.74	0.00104
Promega ReliaPrep	44	0.74	0.51 - 1.46	< 0.000001

*Wilcoxon signed rank test for paired samples

Dagnall PLOS ONE 2017

Associations Between TL and Cancer Risk May be Impacted by TL and DNA Extraction Methods



Cunningham *Cancer Epidemiol Biomarkers Prev . 2013*

Associations Between TL and CVD May be Impacted by DNA Extraction Methods

DNA isolated by the EZ-1 kit (magnetic beads) results yielded no association of age-adjusted RTL with CVD.

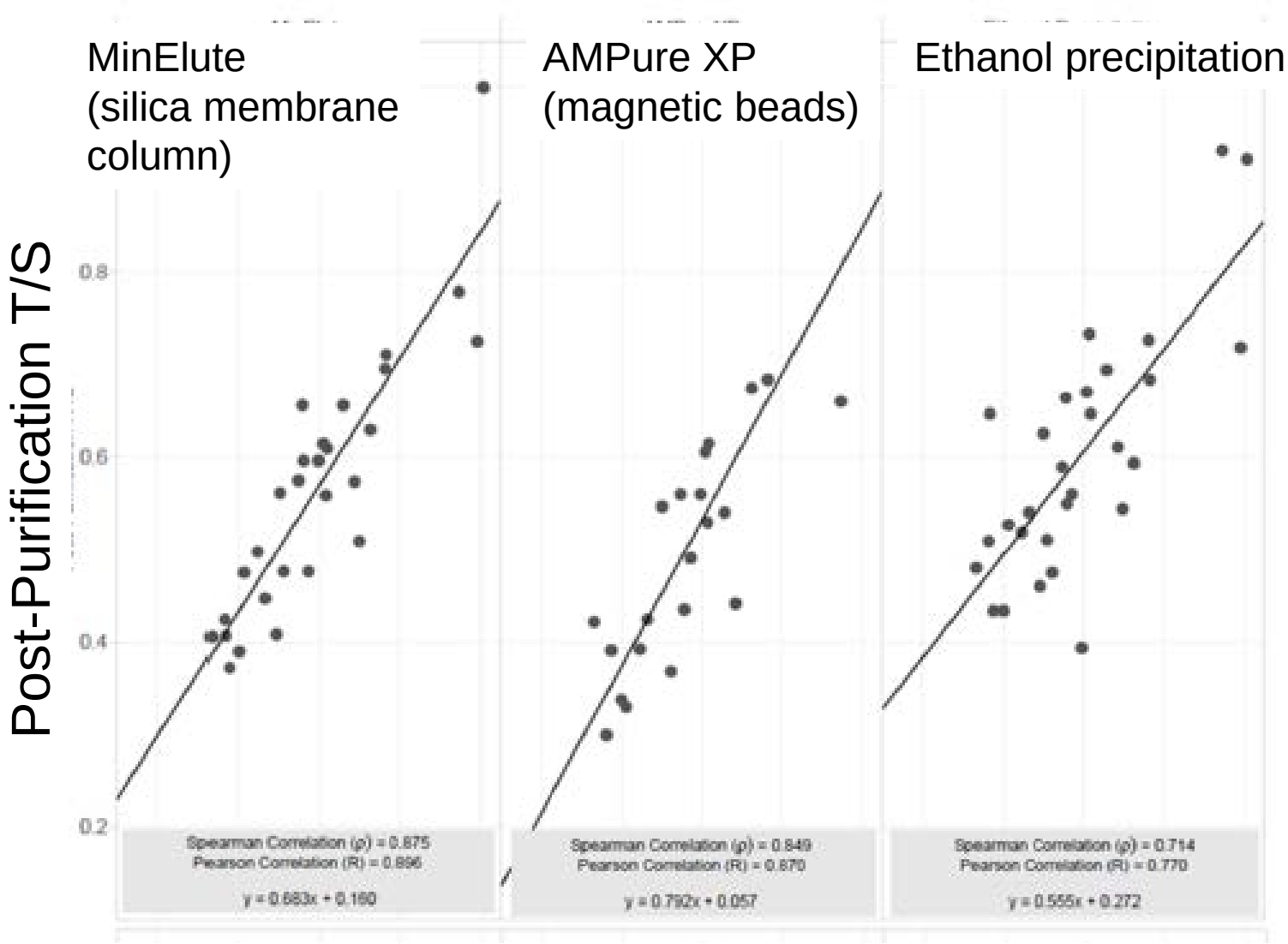
DNA isolated by INVISORB kit (salting out) resulted in highly significant odds ratios.

Raschenberger *Scientifc Reports 2016*

Why Do DNA Extraction Methods Impact qPCR TL Measurements?

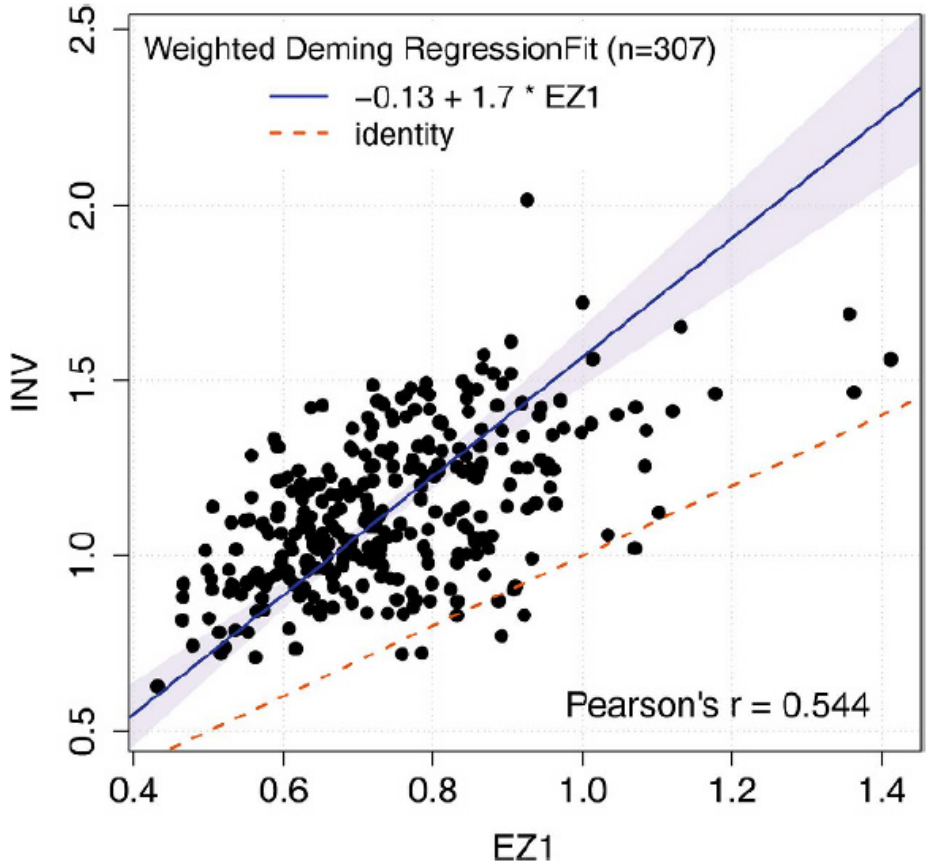
- Residual impurity (e.g. protein)
- Carry over chemical from the kit (e.g. salt, organic solvent?)
- DNA size and integrity (degradation)
- Different stability during storage

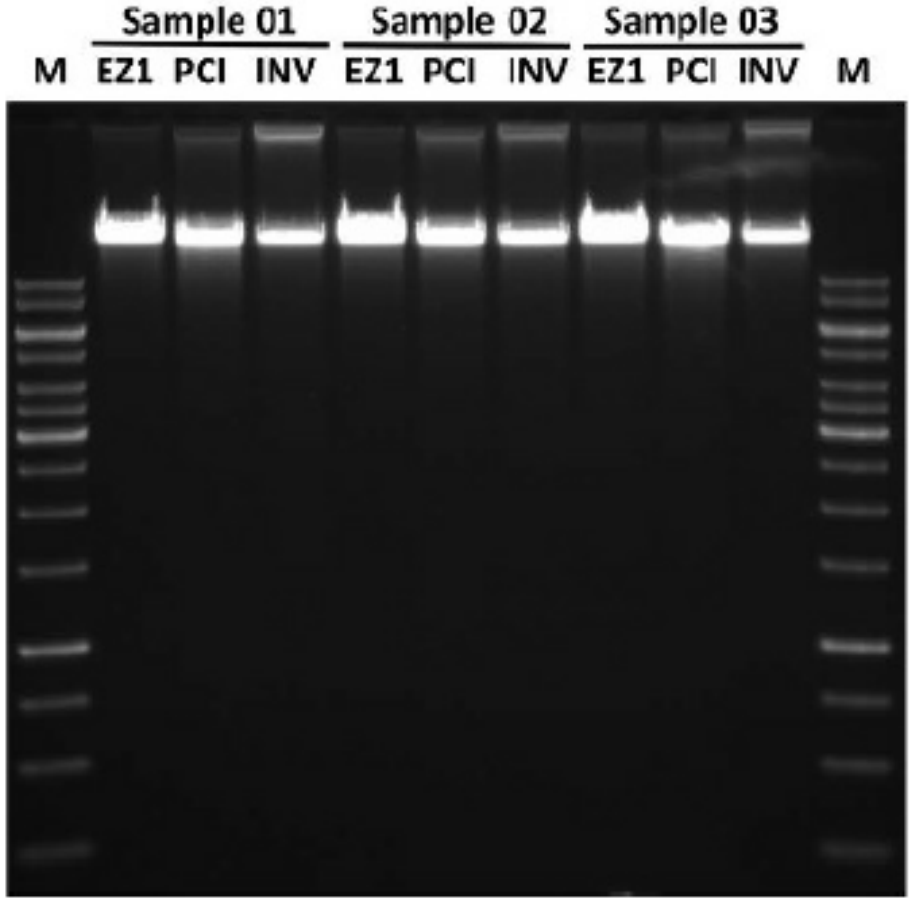
Post-Extraction Purification Introduces Variability



Dagnall PLOS ONE 2017

DNA Integrity Alone Does Not Explain the Discrepancy Between Different DNA Extraction Methods





Raschenberger *Scientifc Reports 2016*

OD260/OD280 and OD260/OD230 Ratios May Have an Impact on T/S Ratios

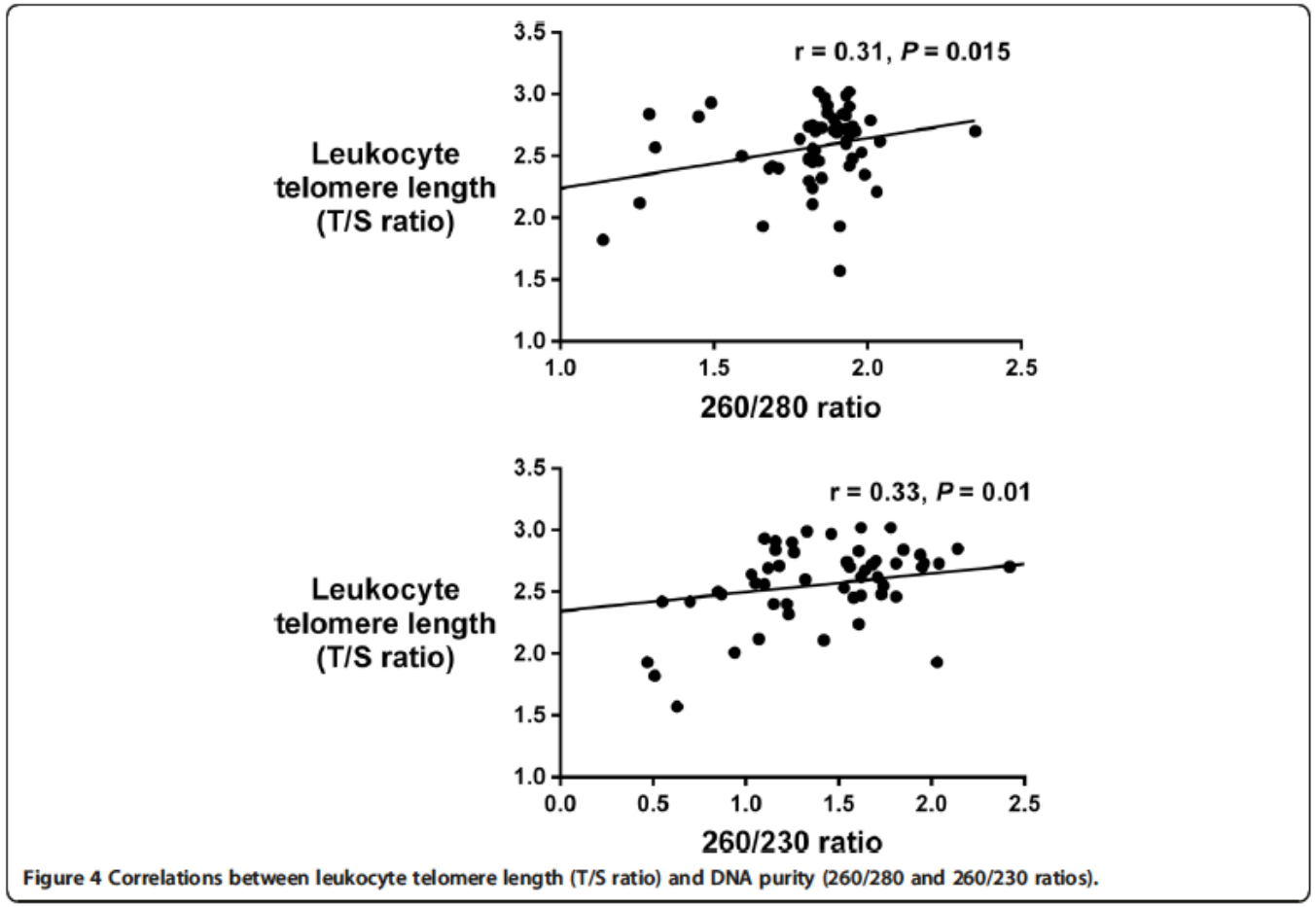


Figure 4 Correlations between leukocyte telomere length (T/S ratio) and DNA purity (260/280 and 260/230 ratios).

***Denham** BMC Research Notes 2014*

But, this is a correlational observation, not a systematic experimental approach of comparing DNA from the same source material

DNA Extraction Methods Also Impact mtDNA Copy Number Assay

Table 1. Differences in leukocyte TL and mtDNA copy number by DNA extraction method in paired samples from the same subjects

		Distributions of measurements								Spearman ρ^b
	N	Min	10th	25th	50th	75th	90th	Max	P^a	(95% CI)
TL										
QIAamp	40	0.77	0.96	0.99	1.13	1.27	1.42	1.72	<0.001	0.71 (0.51–0.84)
ReliaPrep	40	1.08	1.22	1.34	1.48	1.65	1.84	2.15		
mtDNA copy number										
QIAamp	48	82	149	179	212	265	341	372	0.005	0.46 (0.21–0.66)
ReliaPrep	48	94	137	157	184	230	271	462		

Abbreviations: CI, confidence interval; TL, telomere length; mtDNA, mitochondrial DNA.

[a]Wilcoxon signed-rank test.

[b]Spearman rank correlation coefficients evaluating agreement between measurements of the same analyte in paired samples of DNA extracted from the same source material using different methods.

Hofmann *Cancer Epidemiol Biomarkers Prev 2014*

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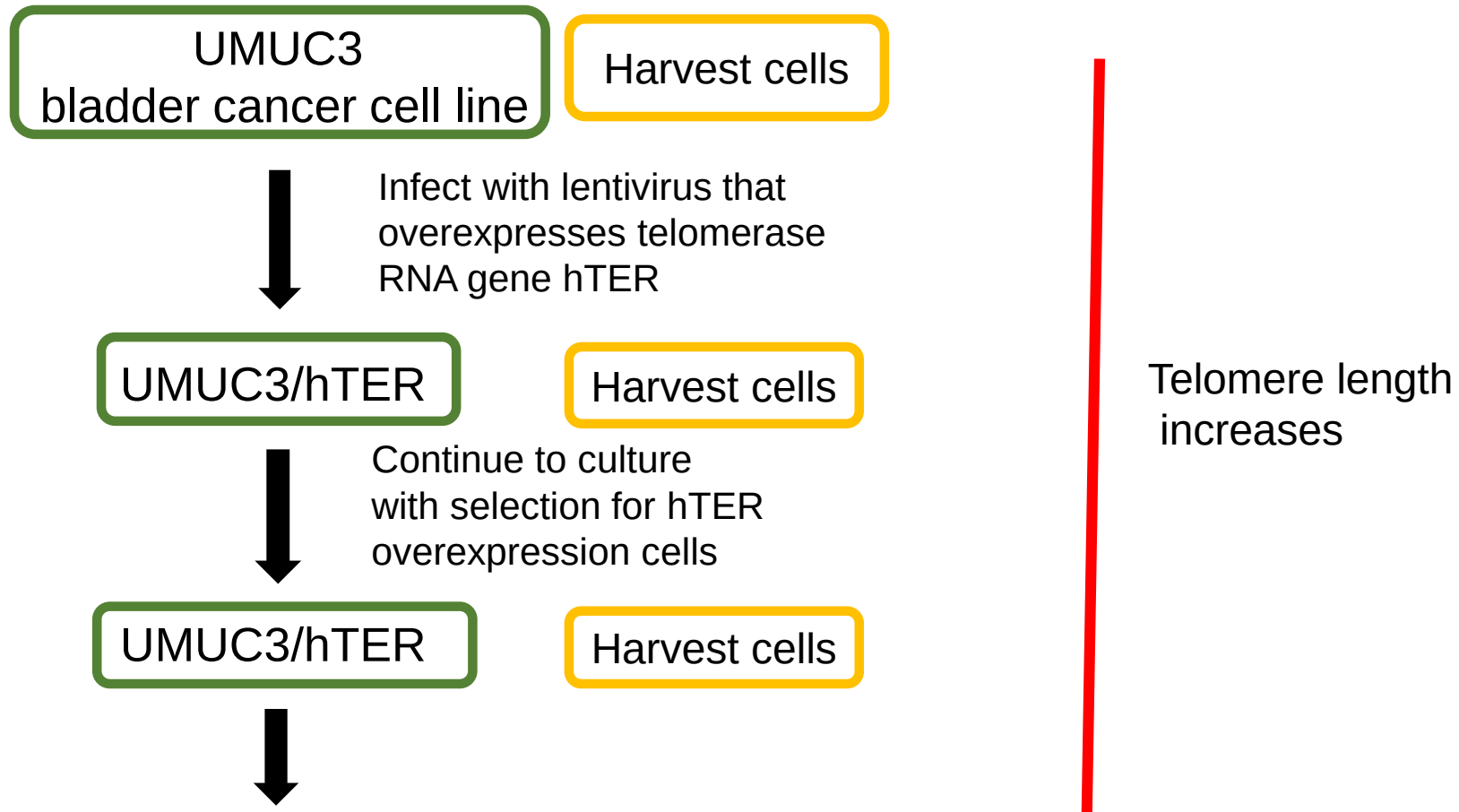
Experimental Setup for DNA Extraction Method Comparison in Cultured Human Cells



Results From All Three DNA Extraction Methods Are Highly Correlated Using DNA from UMUC3 cells

qPCR/UMUC3 cells

Pearson Correlation	QIAamp	PureGene	Agencourt
QIAamp		0.946	0.977
PureGene			0.988

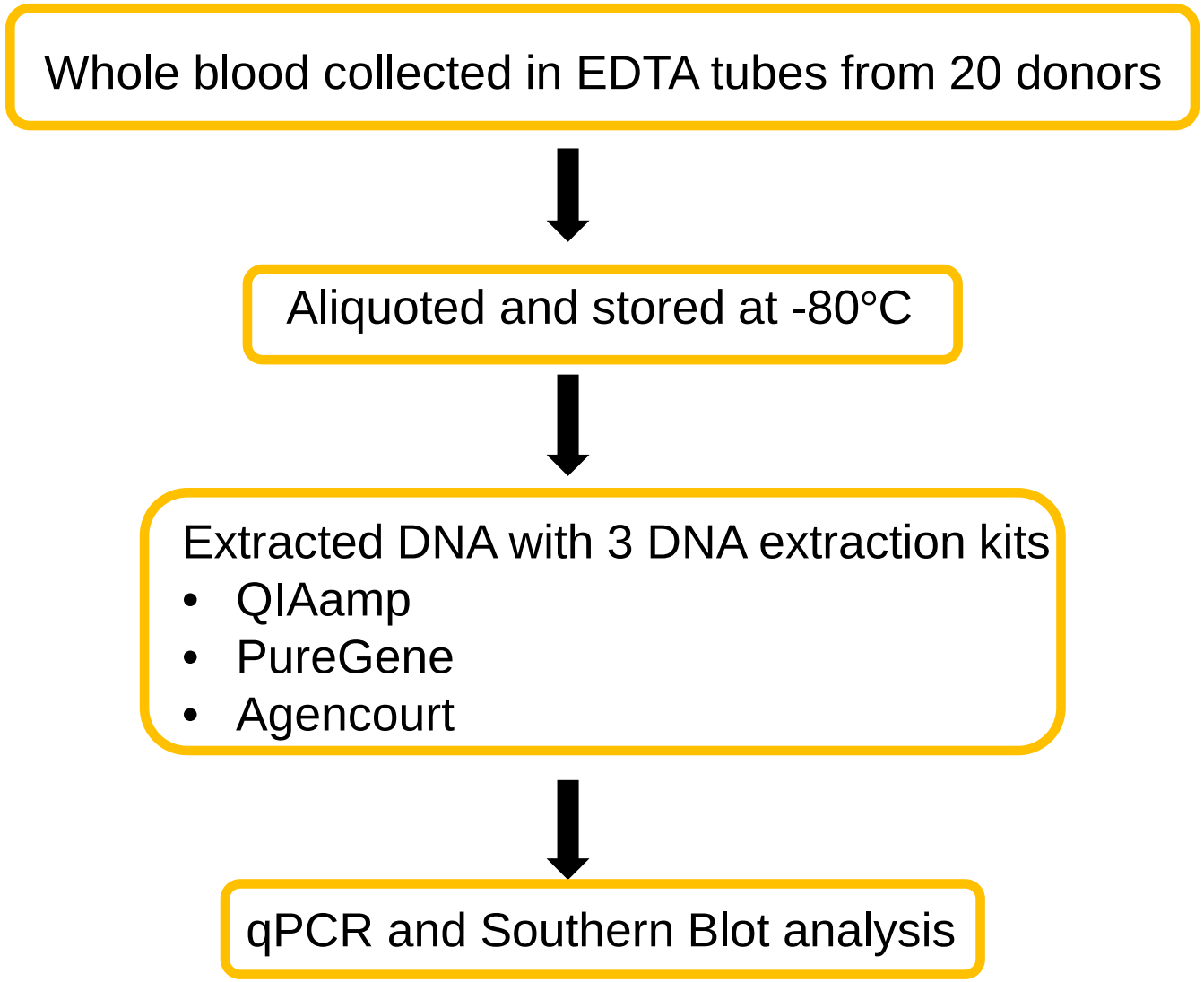
Southern Blot/UMUC3 cells

Pearson Correlation	QIAamp	PureGene	Agencourt
QIAamp		0.992	0.992
PureGene			0.995

qPCR vs. Southern Blot/UMUC3 cells

	Pearson Correlation
QIAamp	0.93
PureGene	0.96
Agencourt	0.979

Experimental Setup for DNA Extraction Method Comparison From Whole Blood: pilot study



Results From PureGene Extracted DNA Show Lower Correlations with Those by QIAamp and Agencourt

qPCR/whole blood

Pearson Correlation	QIAamp mini	PureGene	Agencourt
QIAamp midi	0.96	**0.649**	0.949
QIAamp mini		**0.669**	0.921
PureGene			**0.73**

Southern Blot/whole blood

Pearson Correlation	QIAamp midi	PureGene	Agencourt
QIAamp midi		0.88	0.9024
PureGene			0.904

qPCR vs. Southern Blot/whole blood

	Pearson Correlation
QIAamp	0.754
PureGene	**0.212**
Agencourt	0.879

Unresolved Issues with Previous Studies

- Although differences in DNA quality (OD260/OD280, OD260/OD230) have been described, the impact of DNA quality has not been examined systematically.
- Some studies used blood and DNA samples were stored for a long period of time, therefore the confounding impacts of sample storage can not be parsed out.
- Relationship between DNA integrity and T/S has not been carefully examined.
- Impact of DNA extraction maybe different for different qPCR assay platforms and different specimen types.

Unresolved Issues with Previous Studies

- The TL data from qPCR methods were not compared with another TL method, e. g. Southern Blot.
- Recommendation from previous studies: consistently use one method for all samples within a study.
- When low to modest correlations were found, it is not clear which method can be recommended.

Purpose of the Current Cross-lab DNA extraction Study

Determine the effect of DNA extraction methods for whole blood, both within and between labs, on the intra-class correlation (ICC) of qPCR measurement in relation to telomere restriction fragment (TRF) telomere length determination.

Intraclass Correlations (ICC), not Coefficient of Variations (CV)

International Journal of Epidemiology, 2016, 1295–1298
doi: 10.1093/ije/dyw191
Advance Access Publication Date: 30 August 2016
Letters to the Editor

Letters to the Editor

Telomere length measurement validity: the coefficient of variation is invalid and cannot be used to compare quantitative polymerase chain reaction and Southern blot telomere length measurement techniques

From Dan TA Eisenberg

Intraclass Correlations (ICC), not Coefficient of Variations (CV)

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Telomere length measurement validity: the coefficient of variation is invalid and cannot be used to compare quantitative polymerase chain reaction and Southern blot telomere length measurement techniques

From Dan TA Eisenberg

- ICC allows assessment of consistency or reproducibility of quantitative measurements made by different observers measuring the same quantity.
- ICC compares data structured as groups, rather than paired observations.

Study Overview

EDTA whole blood from 50 anonymous donors aliquoted and shipped to from Lin lab to U24 lab

U24 blinds and ships samples to U01 labs

- Aviv Lab
- Gadalla Lab
- Lin Lab
- Shalev Lab

- Two randomized sets of 50 samples extracted with each DNA extraction method
- Extraction of paired duplicates performed on different days
- Duplicate TL runs performed on different days

Donor demographics sent to U24 statistician

Data transfer to U24 data scientist

Unblind and data analysis by to U24 statistician

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Primary Analysis

- Intra class correlation (ICC) of duplicate qPCR runs of the same DNA sample
- ICC of duplicate DNA extractions of the same extraction method within each lab for both qPCR and Southern Blot
- ICC of the same DNA extraction method between labs
- ICC of qPCR and SB for each DNA extraction method

Primary Analysis

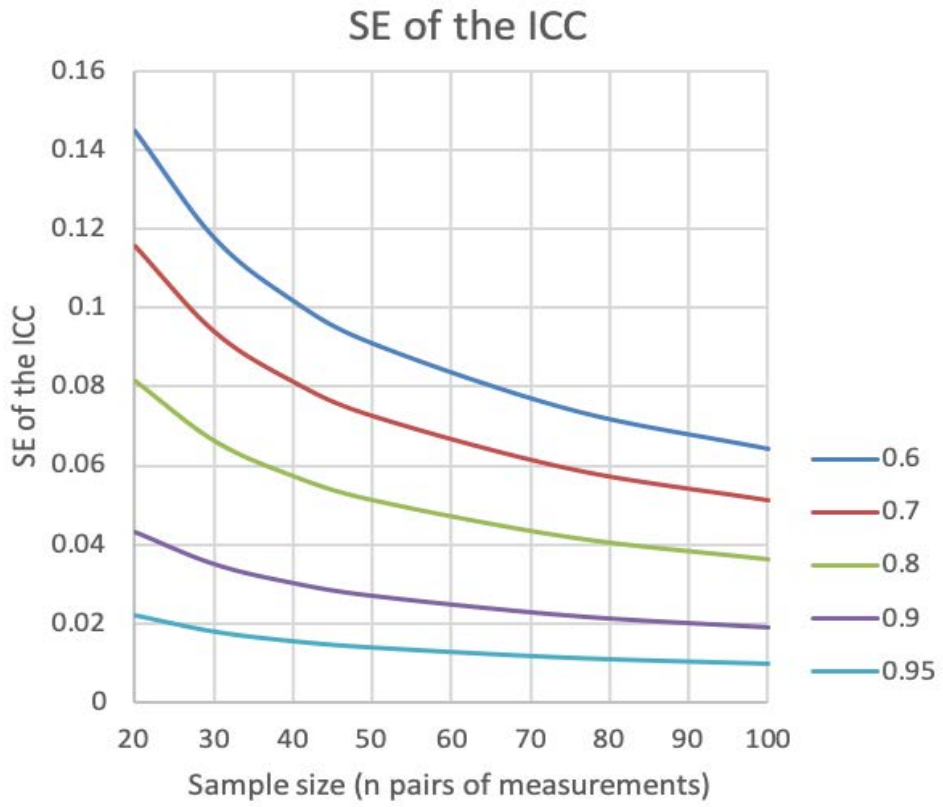
- ***Intra class correlation (ICC) of duplicate qPCR runs of the same DNA sample***
- ***ICC of duplicate DNA extractions of the same extraction method within each lab for both qPCR and Southern Blot***

- ICC of the same DNA extraction method between labs
- ICC of qPCR and SB for each DNA extraction method

Data Quality Assurance Measurements

- Written approved protocols
- Centralized sample and data management
- Temperature monitoring and documentation of shipments
- Each tube is barcoded
- Randomization and blinding of samples
- Detailed documentation of all relevant data
- Adherence to TRN TL reporting guidelines

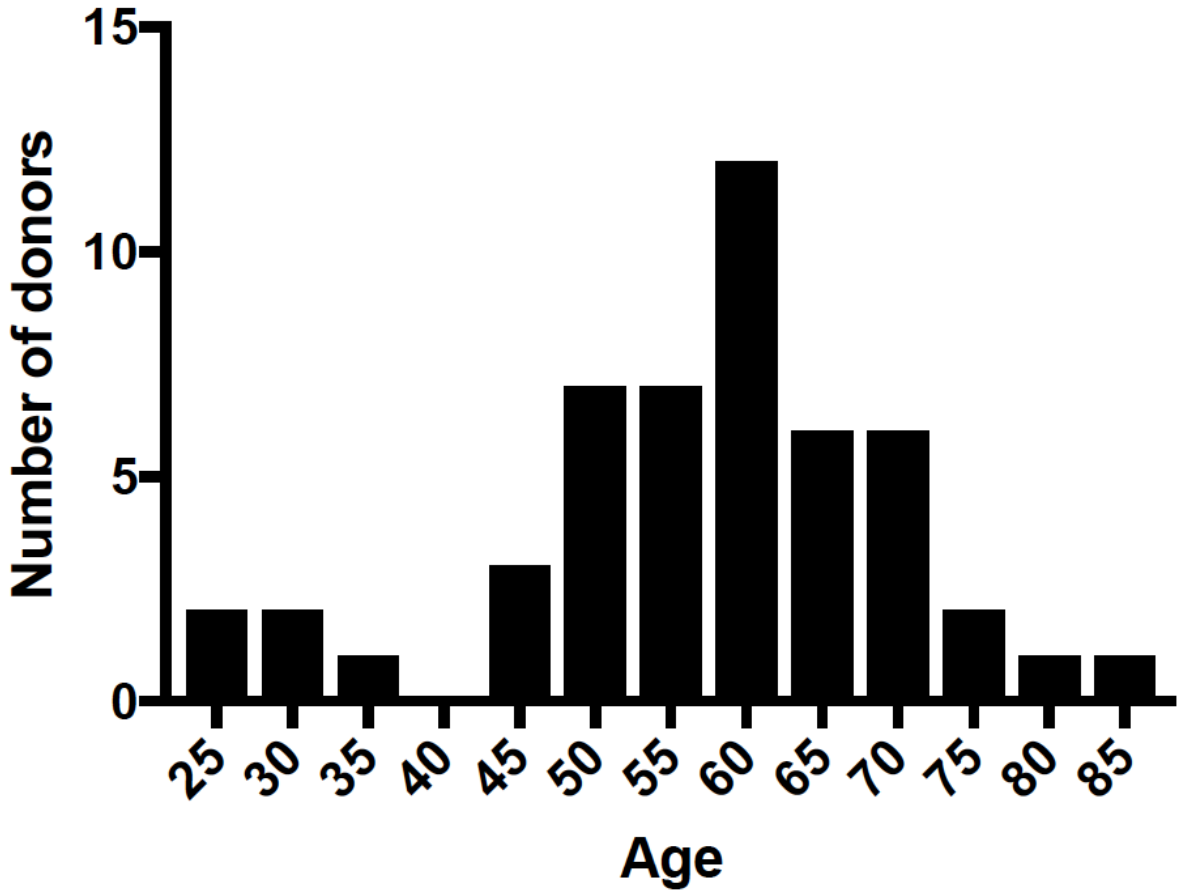
Power Calculations



With 50 pairs of measurement (2 extractions per sample for each DNA extraction method), and an estimated ICC of 0.8, the 95% confidence of the ICC will approximately be 0.69 - 0.91.

Age Distribution of the 50 Donors

Whole blood in EDTA tubes purchased from Stanford Blood Center as research products



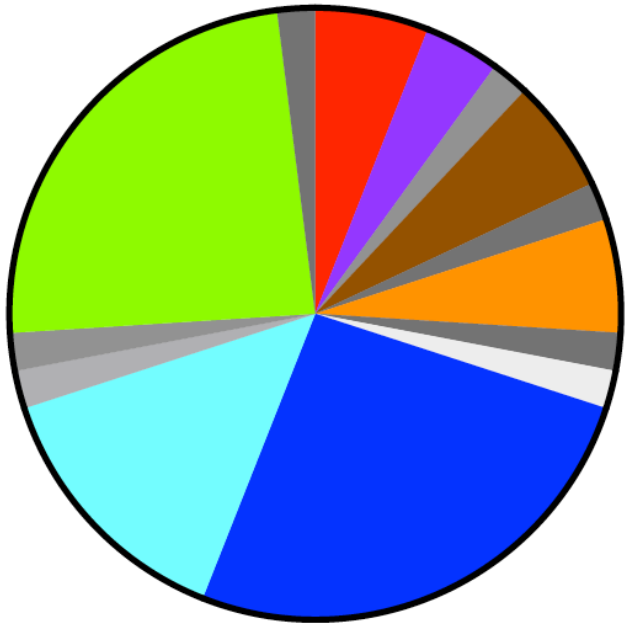
Min	27 yr
Median	59 yr
Max	84 yr
Mean	57.7 yr

Gender distribution

18% Female

82% Male

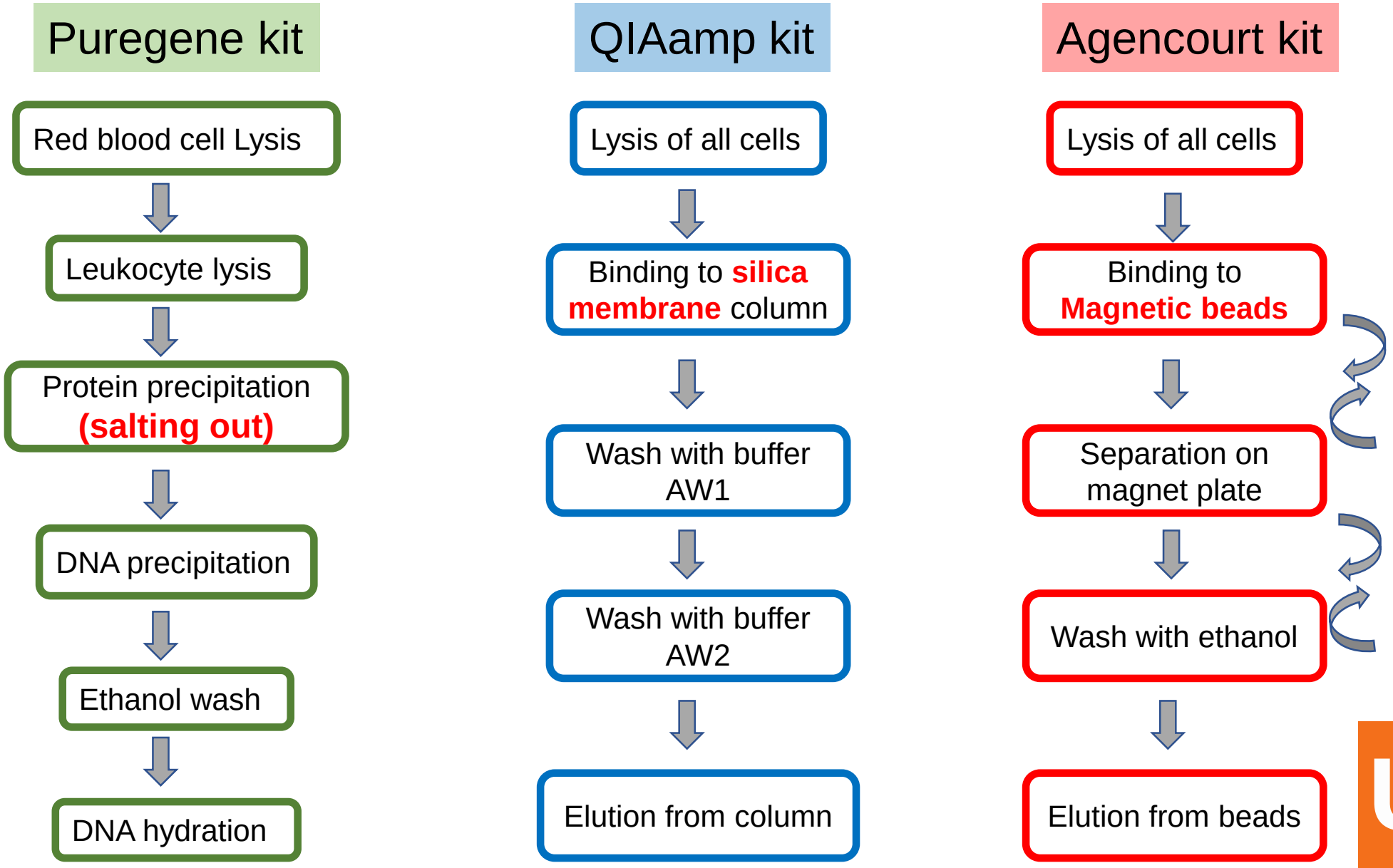
Ethnicity Distribution of the 50 Donors



Total=50

Chinese	6 %
Eastern European	4%
Filipino	2%
Hispanic or LatinX	6%
Indian	2%
Japanese	6%
Mediterranean	2%
Mexican	2%
North American	**26%**
North European	**14%**
other	2%
other white	2%
Western European	**24%**
White/Asian	2%

Principles of DNA Extraction Methods Used



List of DNA Extraction Kits Used

Lab	DNA extraction principle	kit	size	TL assay
A	Salting out	Puregene	midi	SB
B	Salting out	Puregene	mini	qPCR
D	Salting out	Puregene	mini	qPCR
B	Silica membrane column	QIAamp	mini	qPCR
D	Silica membrane column	QIAamp	mini	qPCR
D	Silica membrane column	QIAamp	midi	qPCR
C	Magnetic bead	King Fisher	mini	qPCR
D	Magnetic bead	Agencourt GenFind	mini	qPCR

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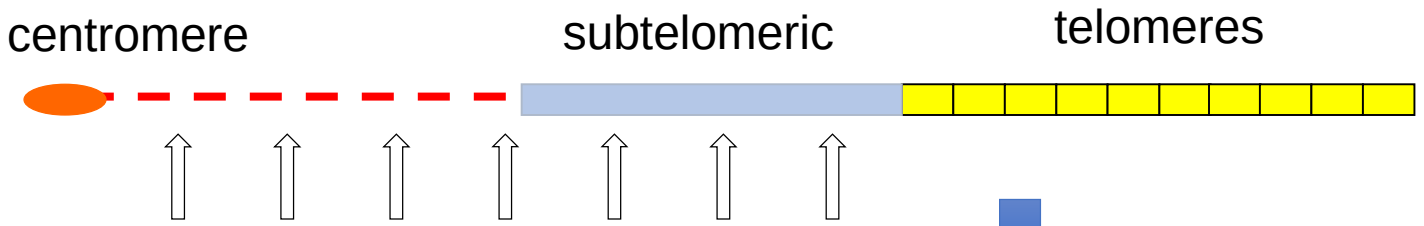
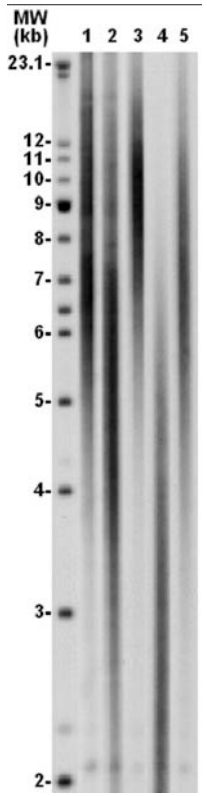
Terminal Restriction Fragment (TRF) Analysis with Southern Blots



Restriction enzyme digestion

Gel electrophoresis

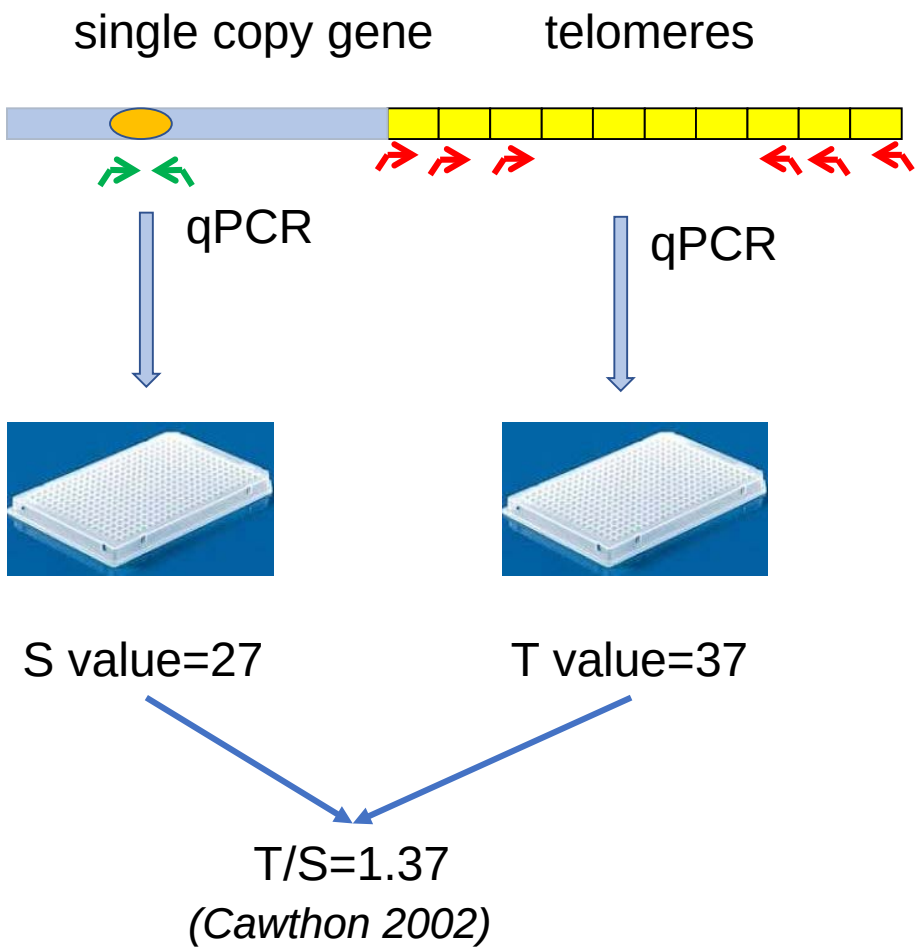


Southern blot analysis that probes the telomeric sequence

Kimura et al, Nature Protocols 2010

Telomere Length Measurement Using qPCR

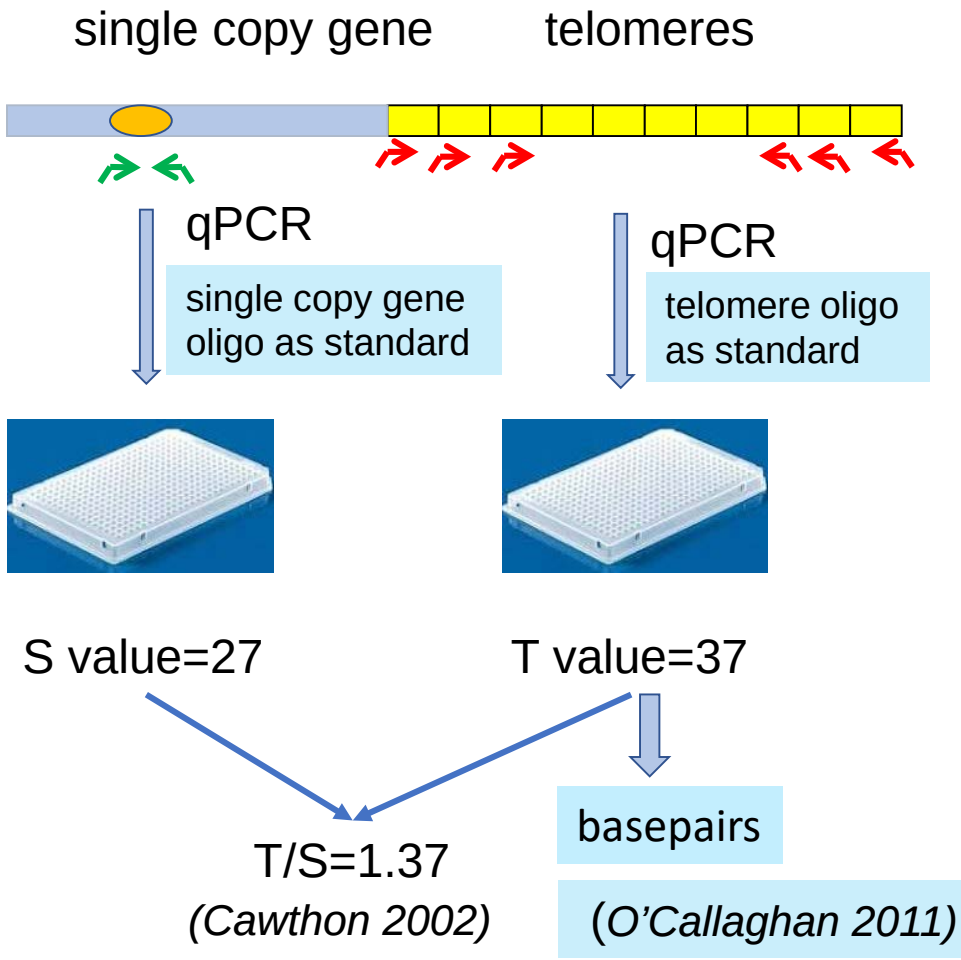
SinglePlex



Telomere Length Measurement Using qPCR

SinglePlex

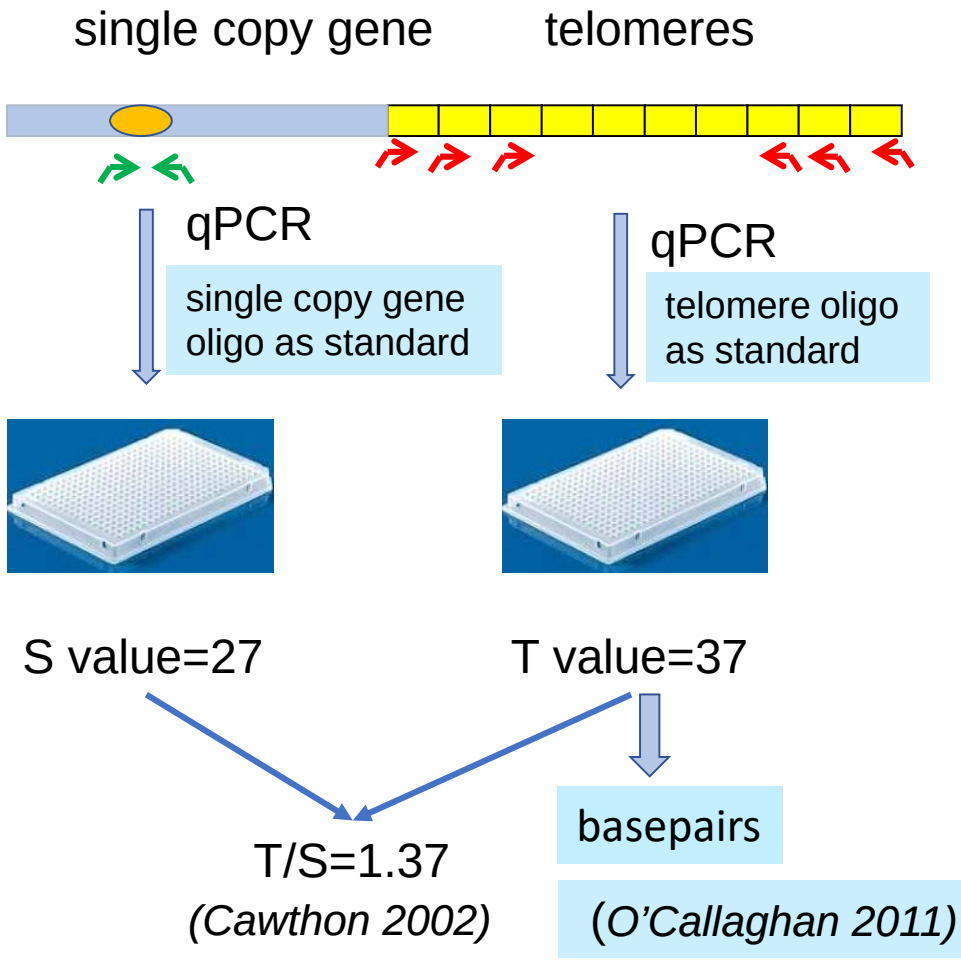
Absolute TL measurement (aTL)



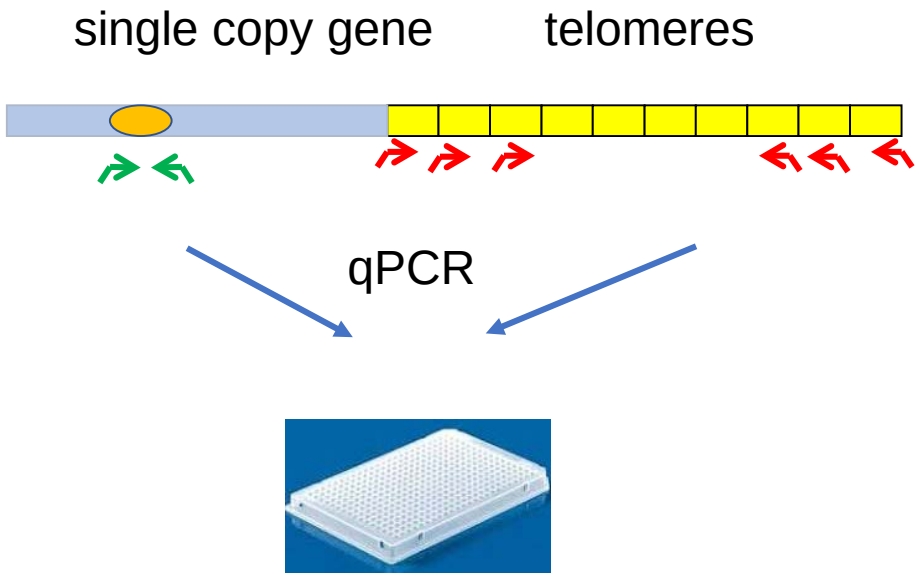
Telomere Length Measurement Using qPCR

SinglePlex

Absolute TL measurement (aTL)



Monochrome multiplex (MMqPCR)



S value=27, T value=37

T/S=1.37

(Cawthon 2009)

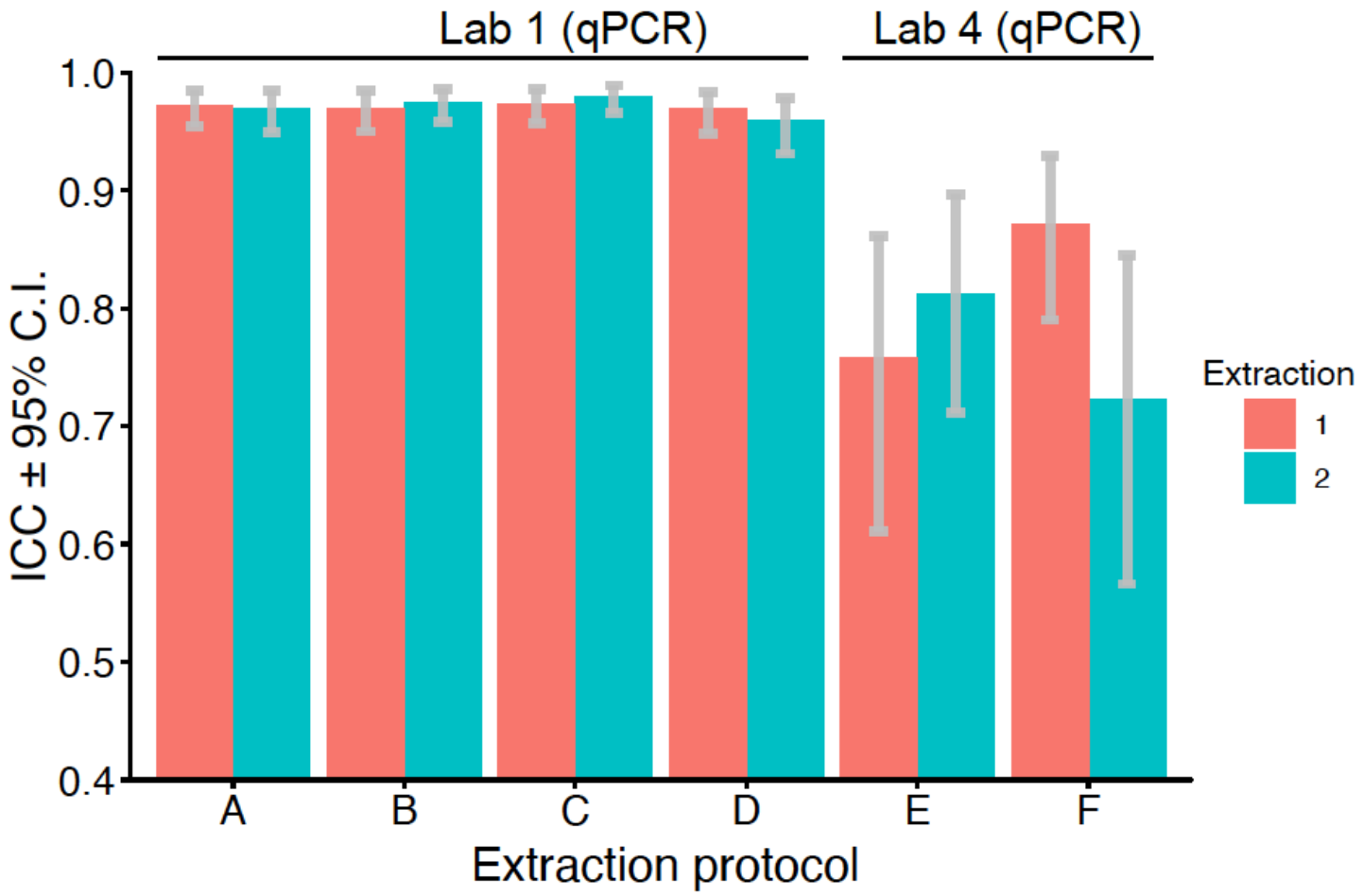
qPCR Methods Used in This Study

Lab	Overall format	Master mix	Single copy gene	Reference standard	Instrument
4	Singleplex	homemade	Beta-globin	Commercial human genomic DNA	Roche LightCycler 480
3	Singleplex	2X Rotor-Gene SYBR Green PCR Master Mix-QIAGEN	36B4	Pooled reference samples	Roche LightCycler 480
2	Absolute qPCR	QuantiTect SYBR Green PCR Kit - QIAGEN	interferon beta 1	84-bp duplex telomere oligo (T) and 82-bp duplex interferon beta 1 oligo (S)	QIAGEN RotorGene Q real-time PCR cycler

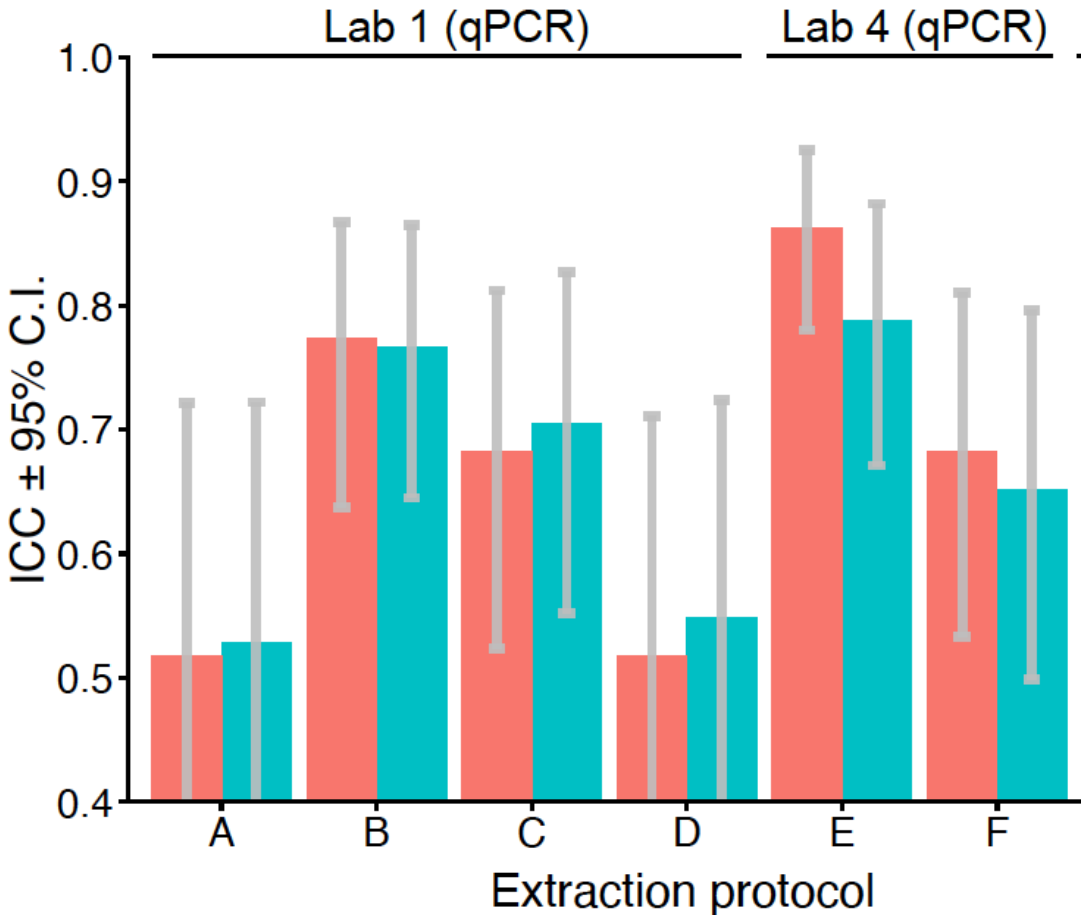
Preliminary Data Analysis

- 2 sets of randomized blood were extracted by the same method
  3 different DNA principles, with 7 different protocols based on 3 labs for this preliminary data
- For qPCR, each DNA sample was run twice on 2 different days
- For SB, the same DNA sample was only run once
- For lab 1, DNA extraction and qPCR TL assay were performed by 2 operators

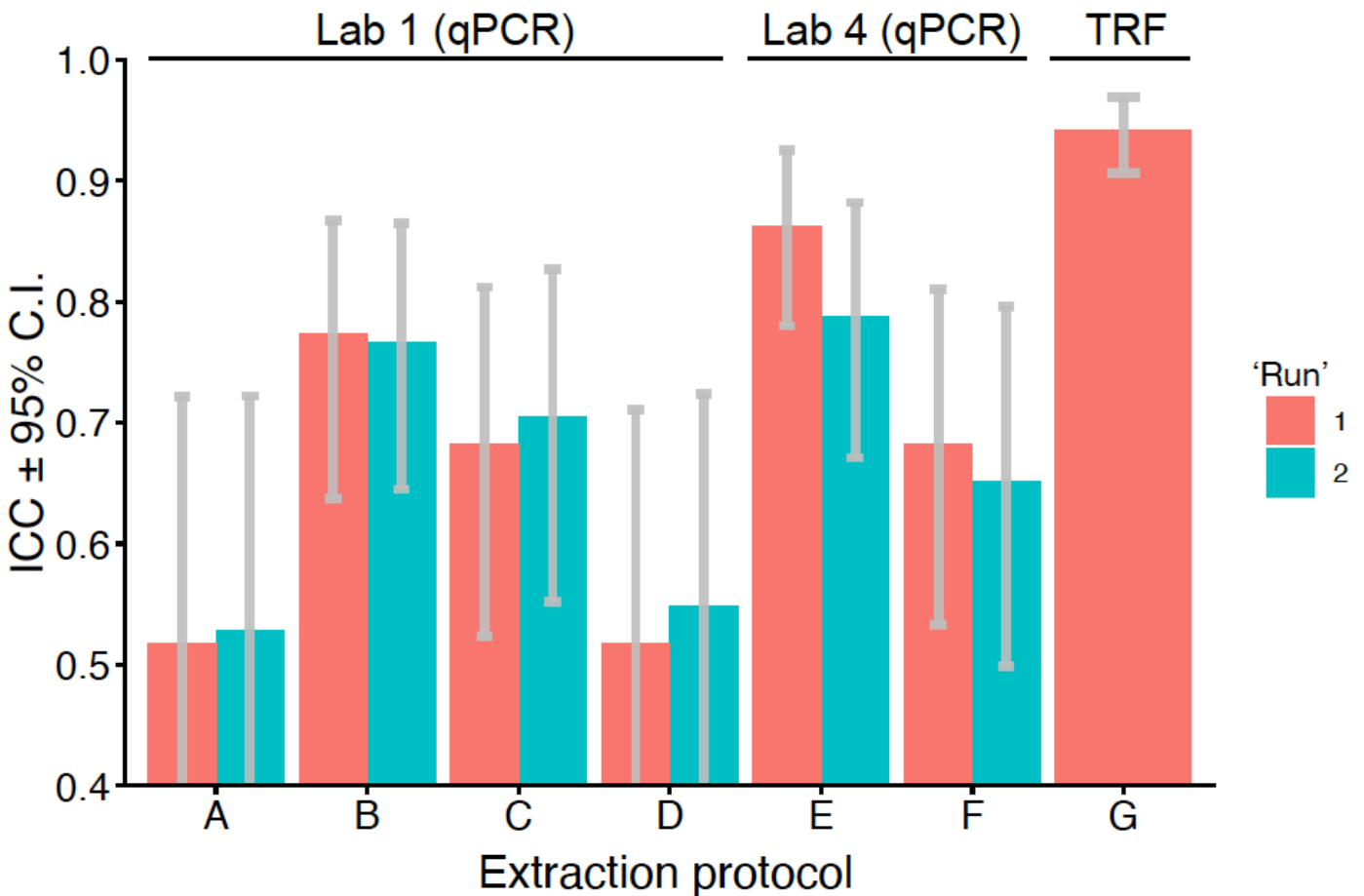
Intra Class Correlations (ICC) of the Same DNA Sample with Duplicate qPCR Runs Are High



Intra Class Correlations (ICC) of Duplicate DNA Extractions with the Same Extraction Protocol for qPCR Are Much Lower



Intra Class Correlation (ICC) of Duplicate DNA Extractions with the Same Extraction Protocol for Southern Blot Is High



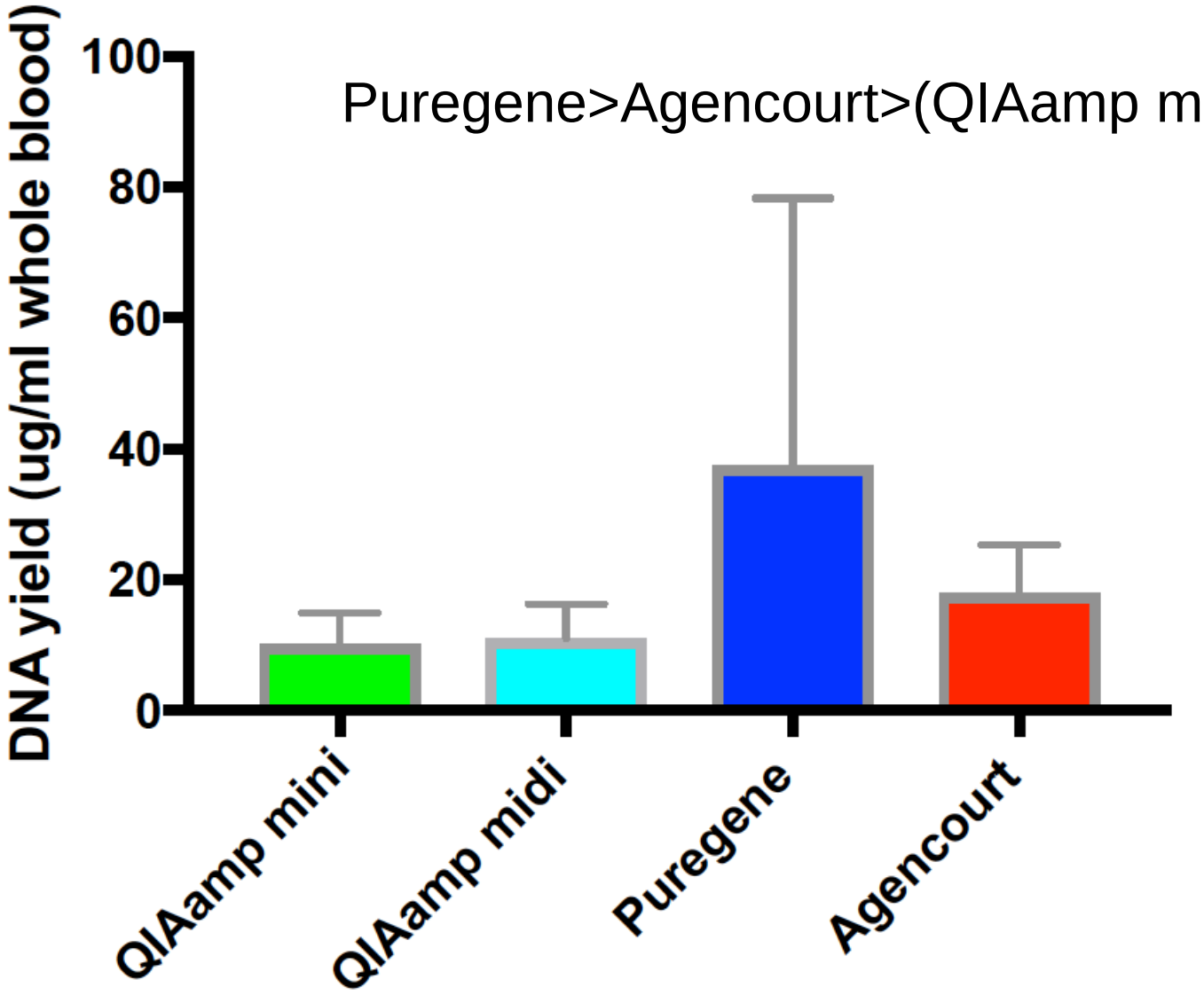
Secondary Analysis

Preliminary data on DNA yields and quality assessment

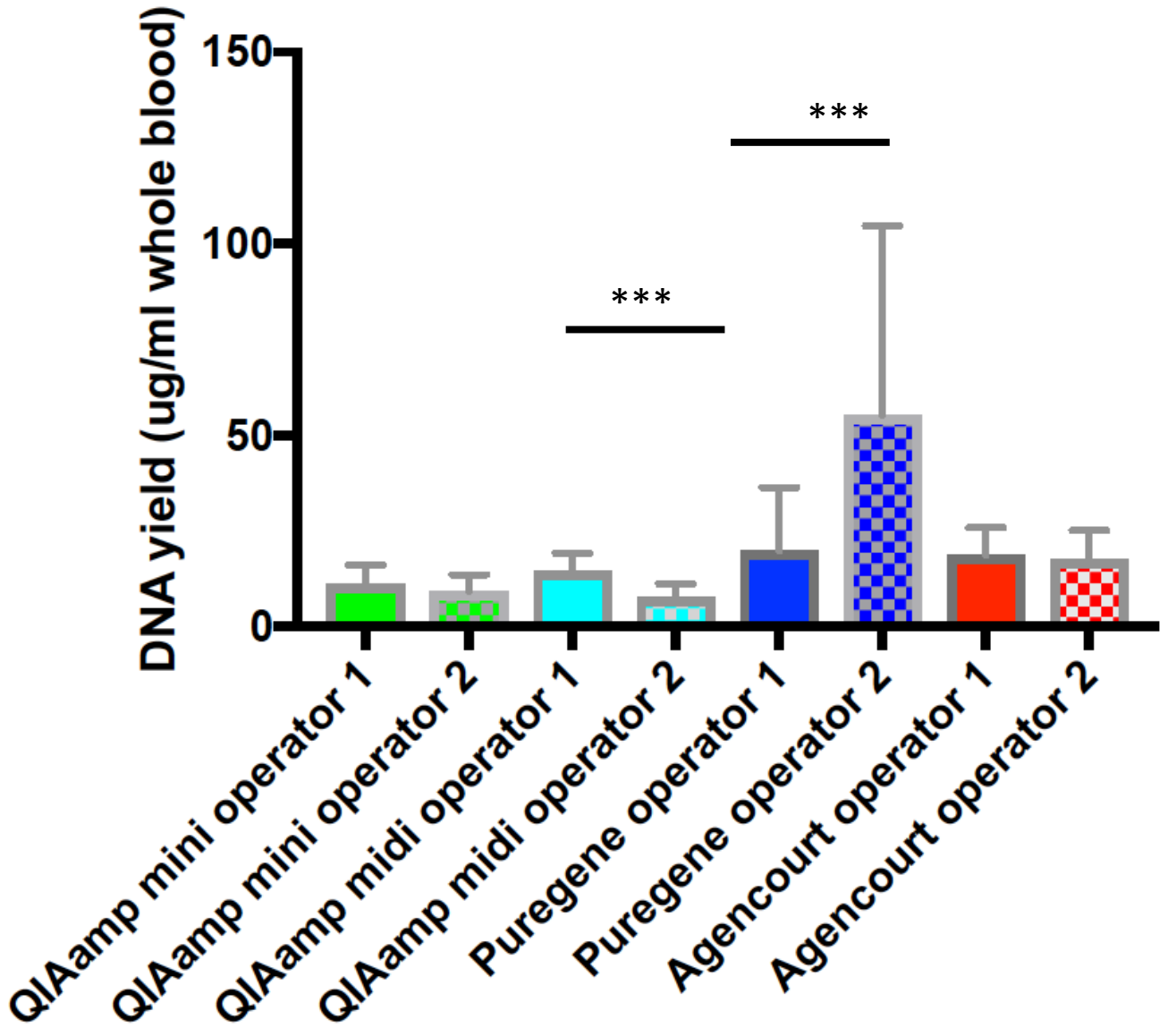
Blinded samples

Different DNA Yields with Different Extraction Methods

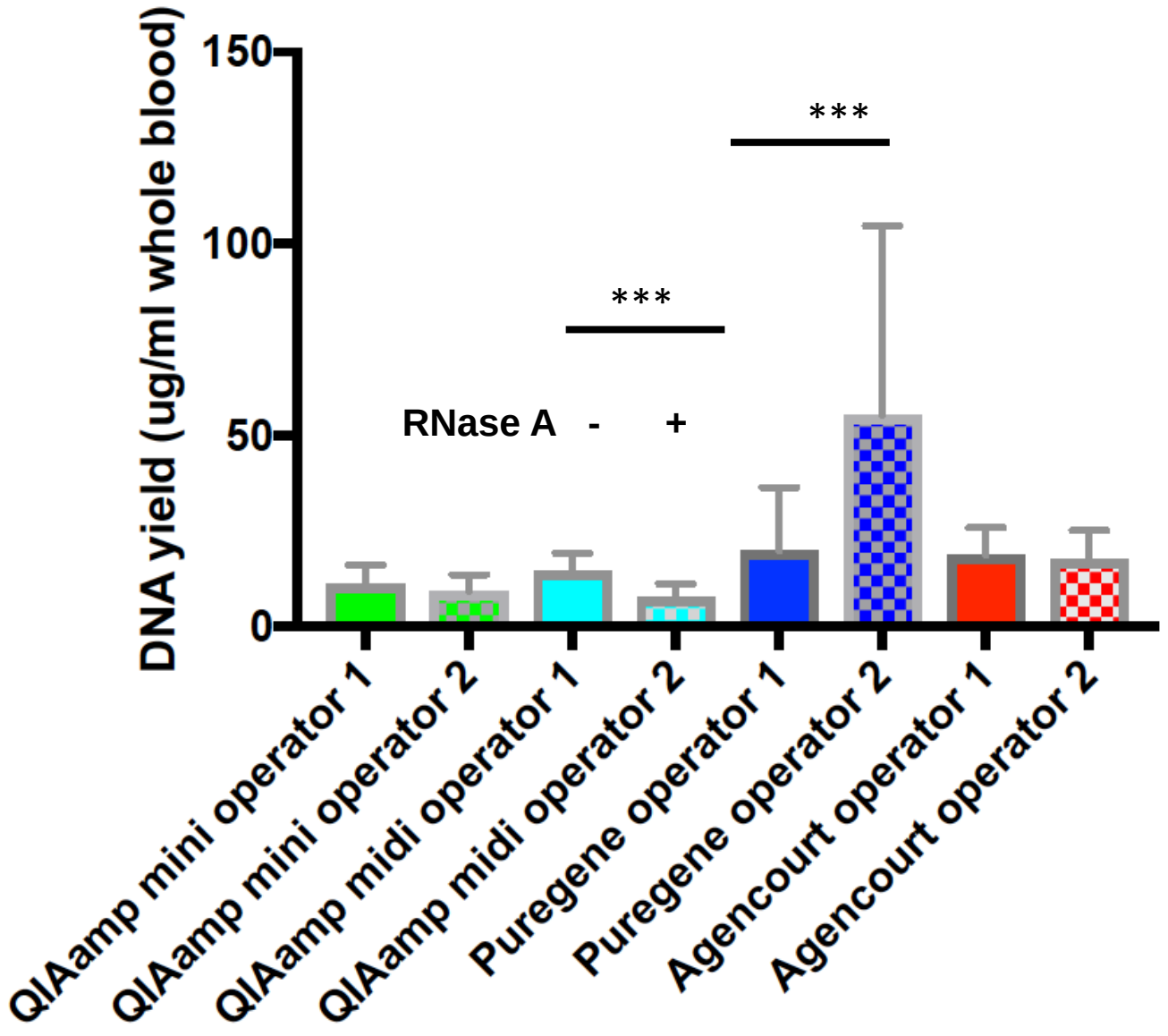
Puregene>Agencourt>(QIAamp midi=QIAamp mini)



DNA yields: Puregene Yields are More Variable Between 2 Operators

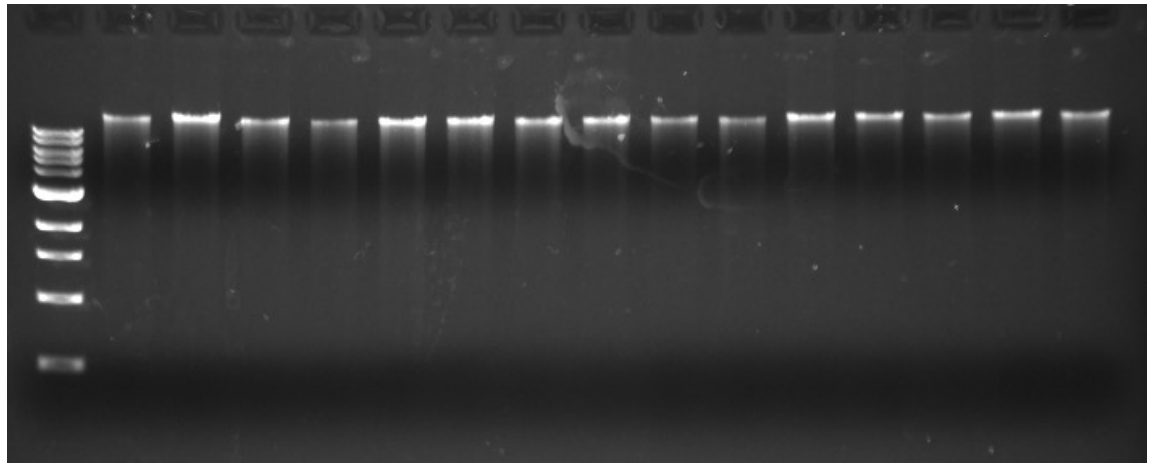


DNA yields: Puregene Yields are More Variable Between 2 Operators

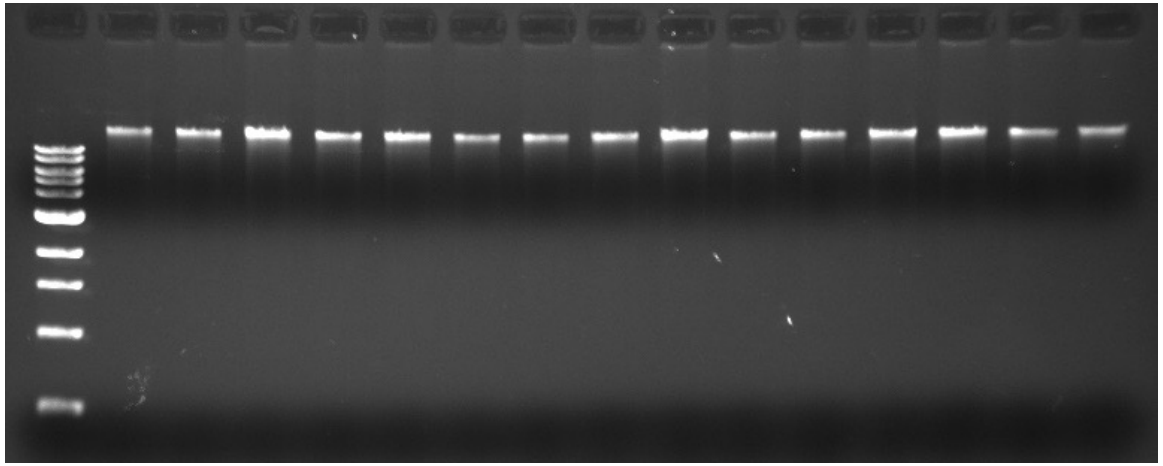


QIAamp Mini DNAs Are Partially Degraded

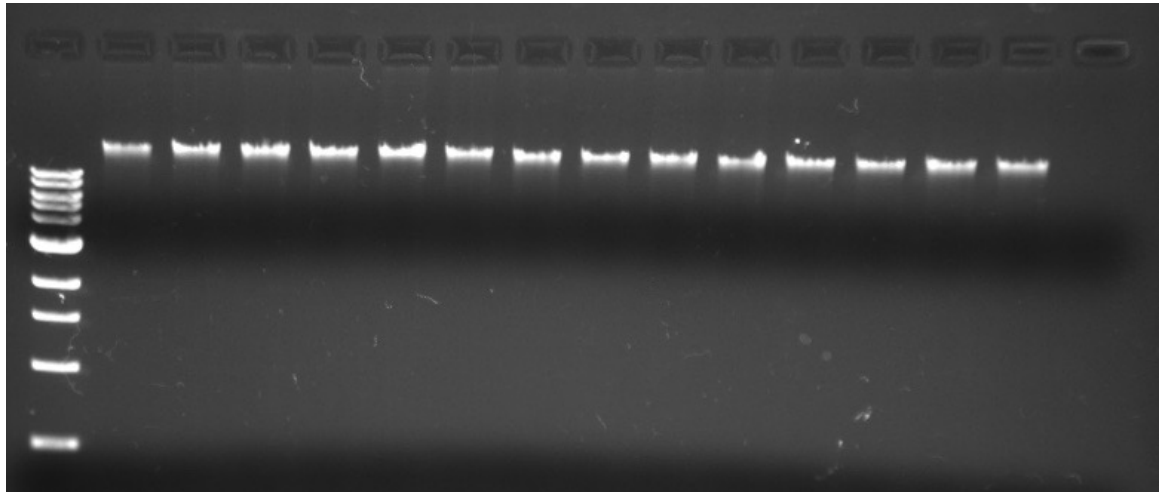
QIAamp Mini



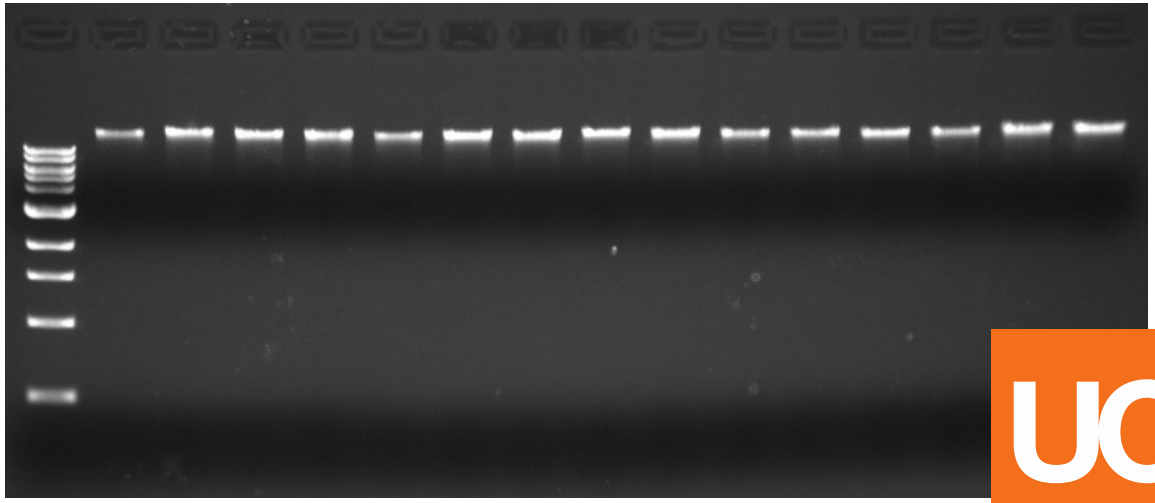
QIAamp Midi



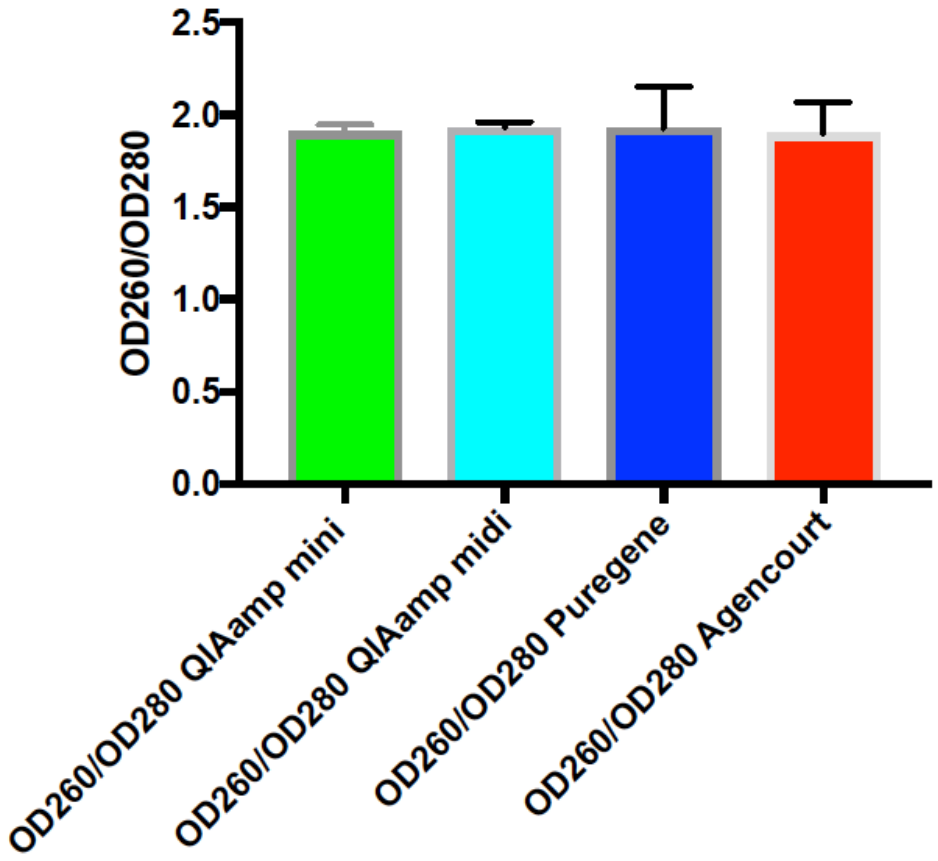
Puregene

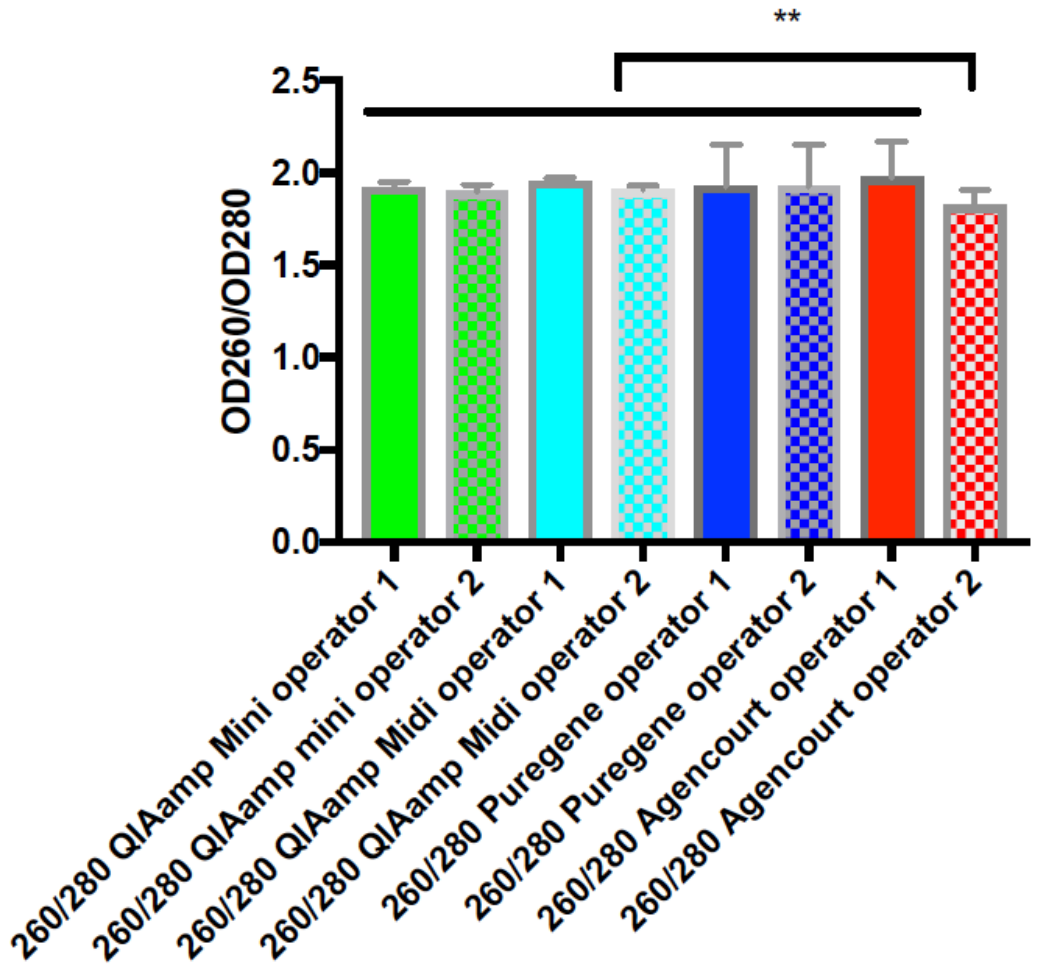


Agencourt

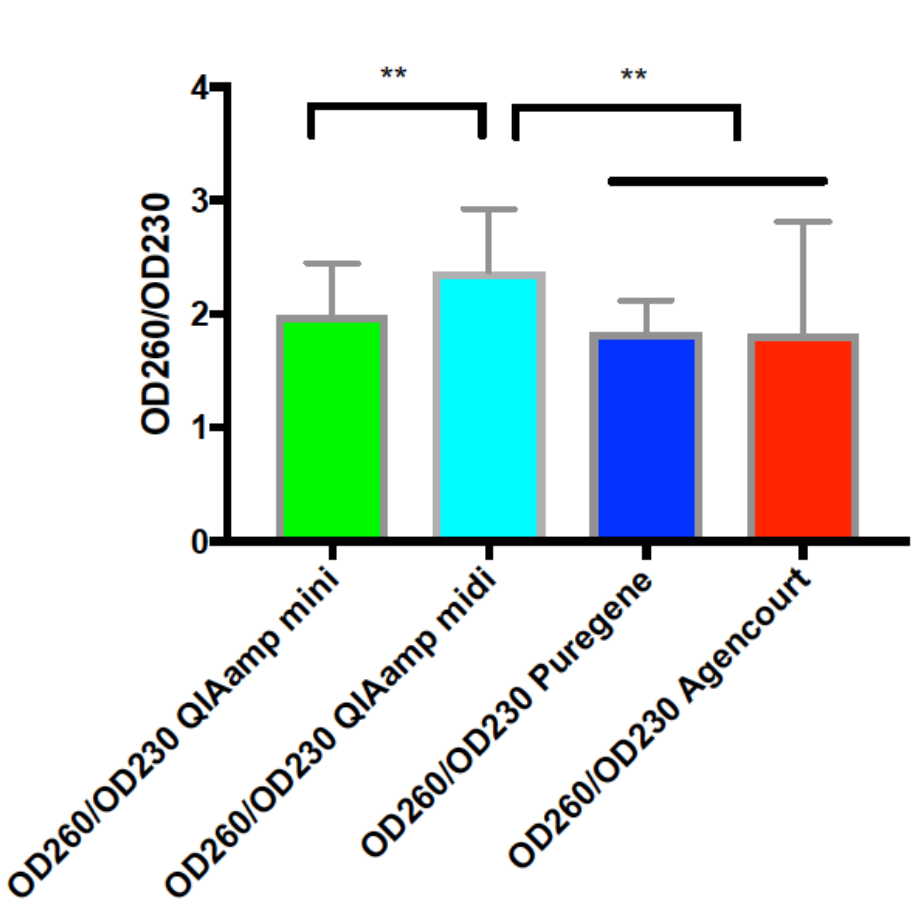


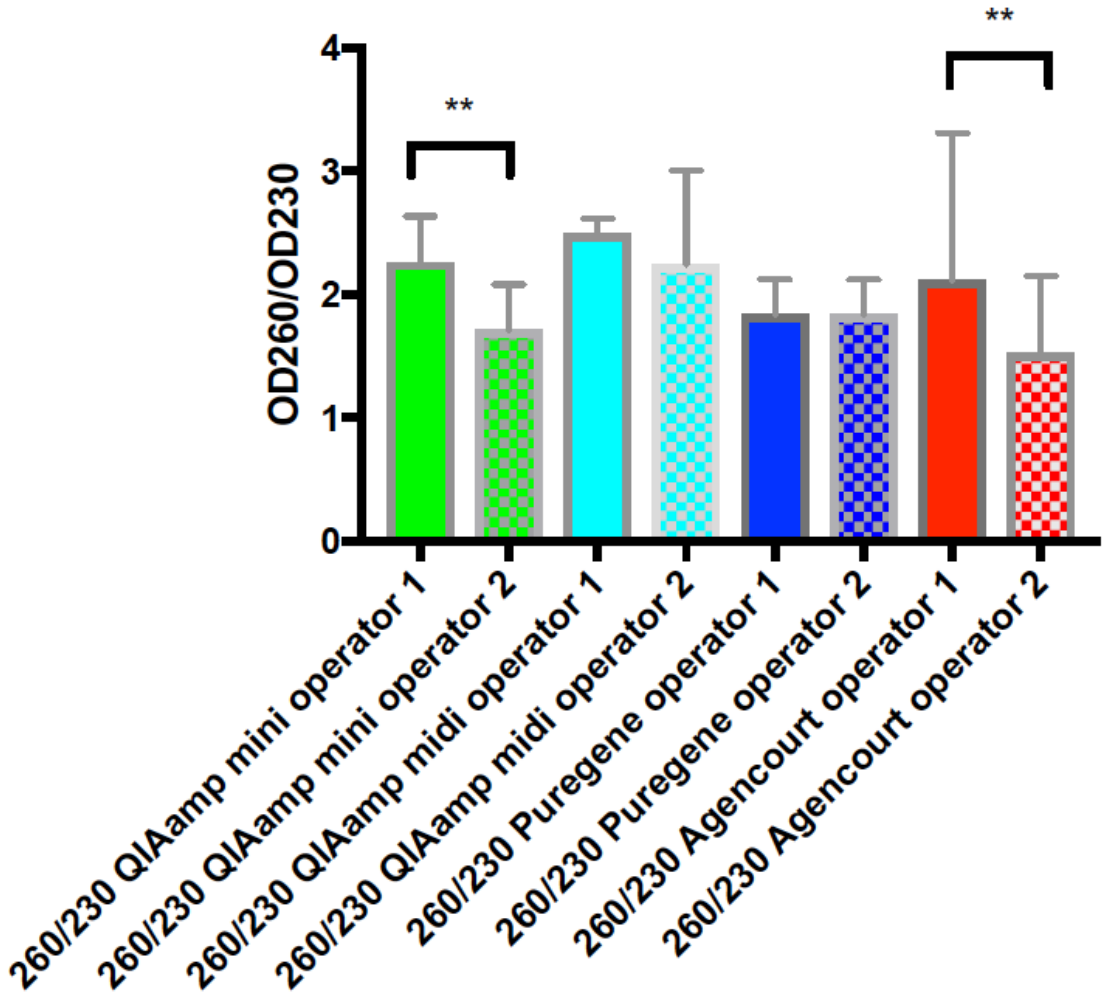
OD260/OD280 Ratios Vary by Operators



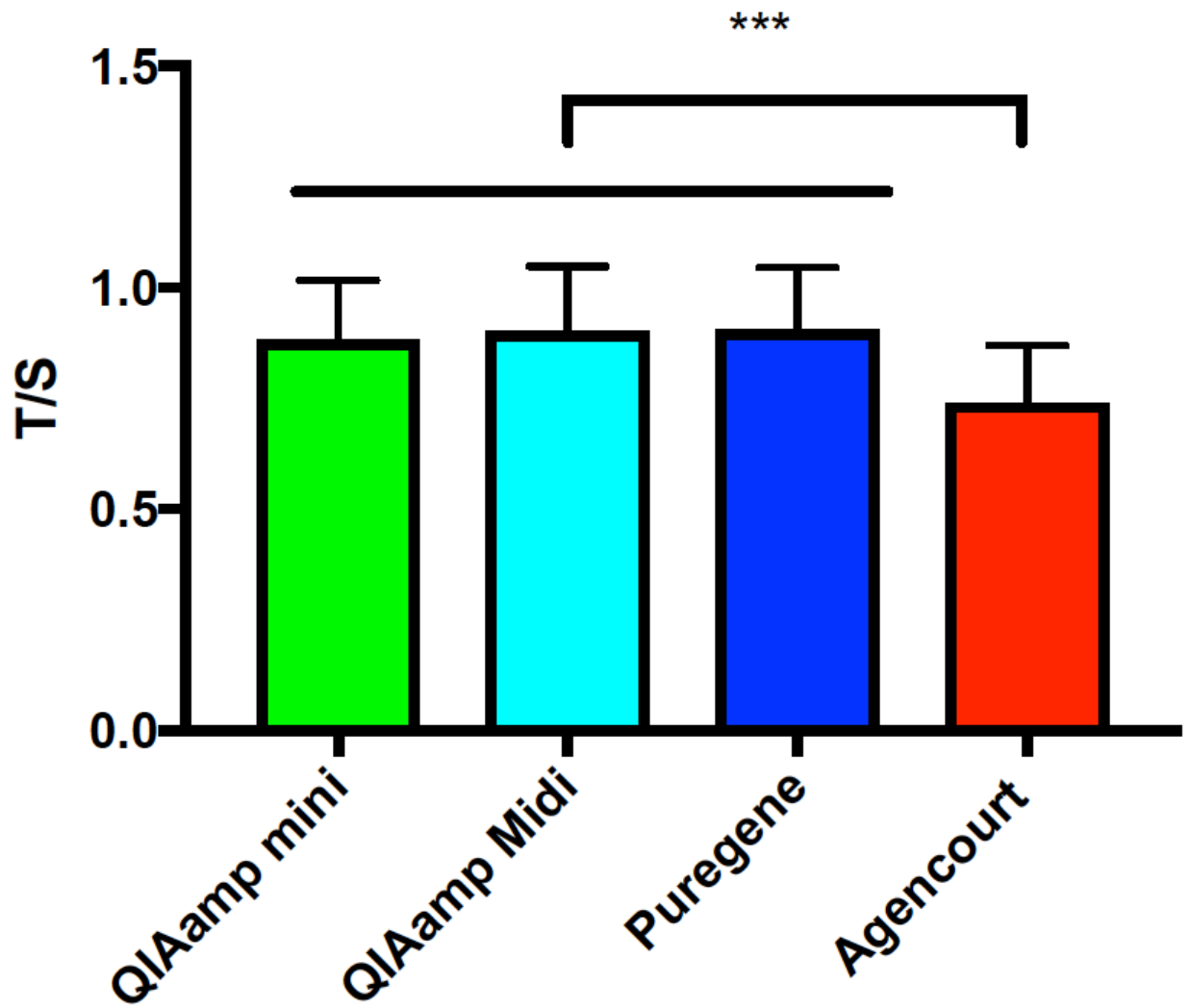


OD260/OD230 Ratios Vary by Operators





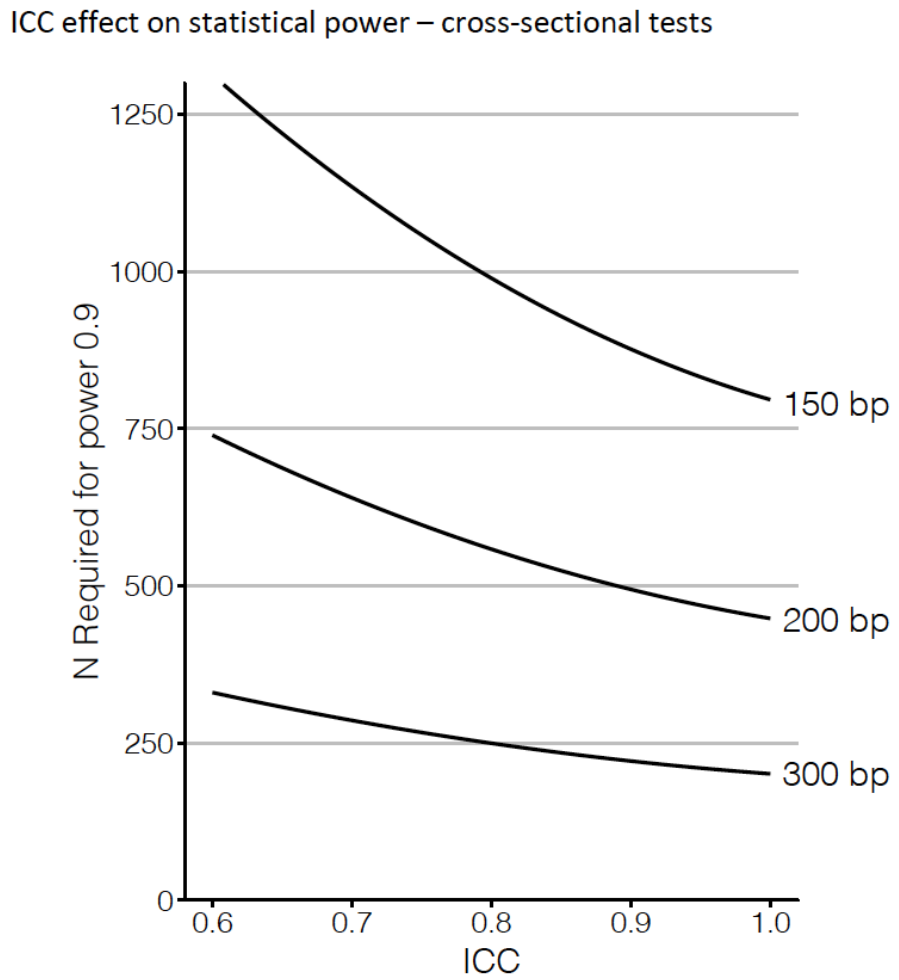
Systematic Differences of qPCR TL: Agencourt Has lower T/S



Summary of the Cross-lab Whole Blood DNA Extraction Method Study

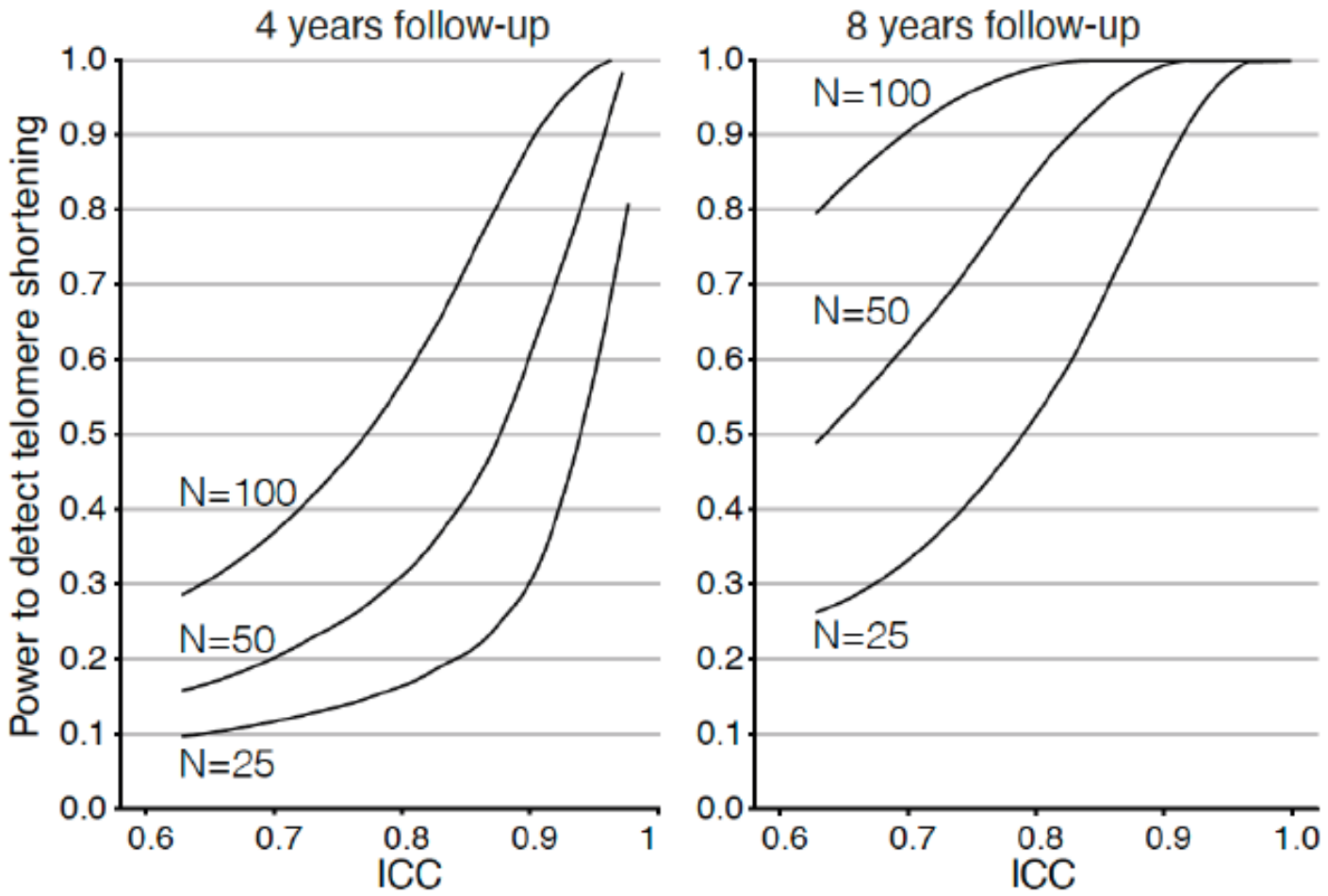
- The ICC of independent DNA extractions using the same extraction protocol for qPCR is lower than that of SB. ICCs of qPCR runs are not informative.
- Some DNA extraction protocols have higher ICC than others.
- Lab differences exist for ICC of independent DNA extractions.
- Operator differences exist for some DNA extraction protocols.
- The relationships between sample shipping condition, DNA quality need further investigation.

Impact of ICC on Power Calculations



Benetos et al, Cir Res, 2018; Faul et al, Behavior Research Methods, 2009

Impact of ICC on Power Calculations



Benetos et al, Cir Res, 2018; Faul et al, Behavior Research Methods, 2009

Next Steps

- Continued analysis of ICCs and other factors
- Impact of DNA integrity on qPCR TL (Shalev, Drury ad Aviv labs)
- DNA storage condition and time (Aviv, Lin and Shalev labs)
- Dried blood spots (DBS) DNA extraction methods (Lin and Drury labs)

Impact of DNA Extraction Methods on qPCR TL from Saliva Collected in DNA Genotek' Oragene Kits

Study Overview

48 saliva samples collected between Nov 30, 2015 to April 5, 2016 In the NGHS study (Barbara Laraia, Elissa Epel, PIs)

Extracted by 2 operators using 3 DNA extraction kits

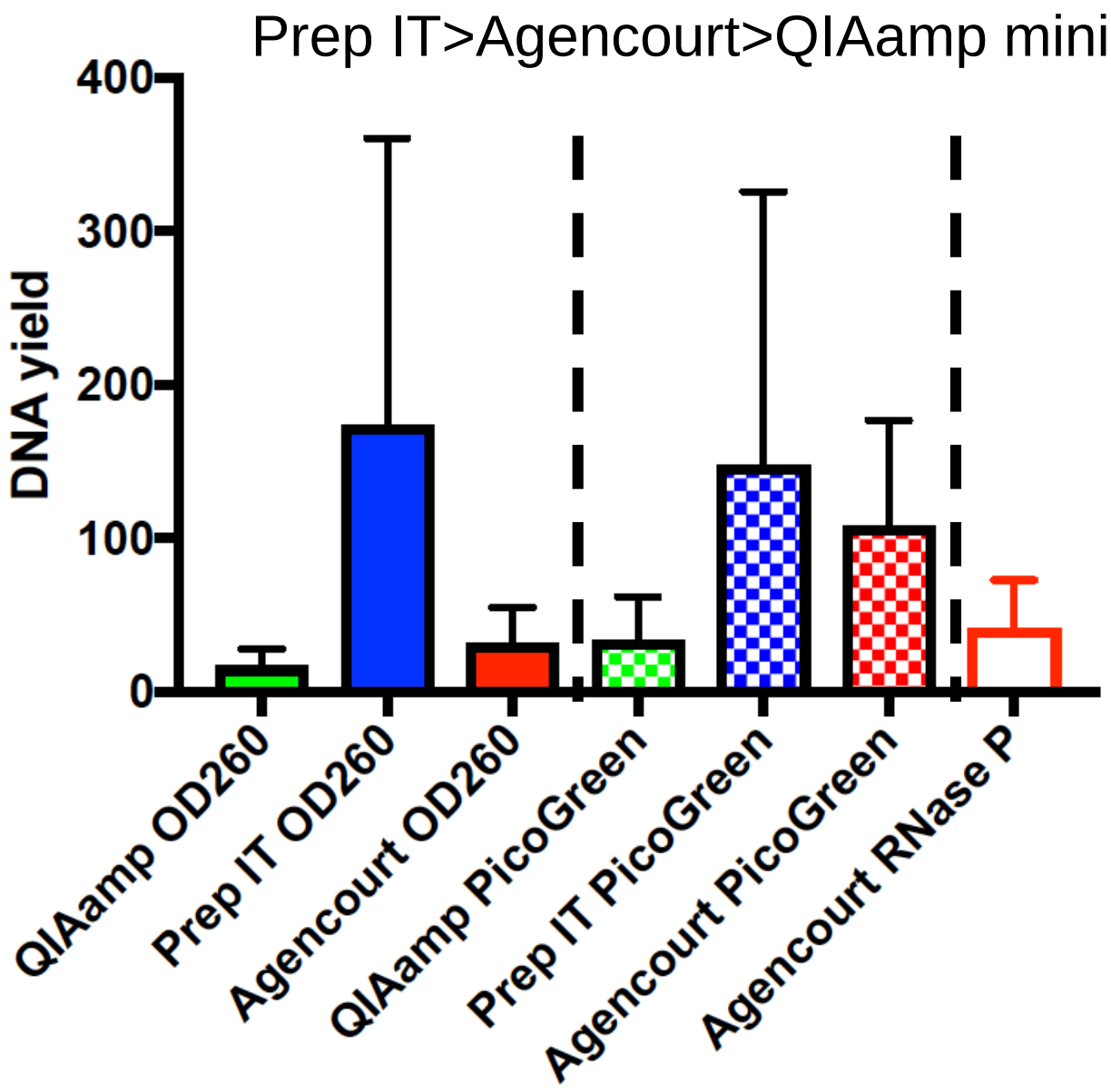
- QIAamp mini
- Prep IT (salting out)
- Agencourt

DNA quantification and quality assessment

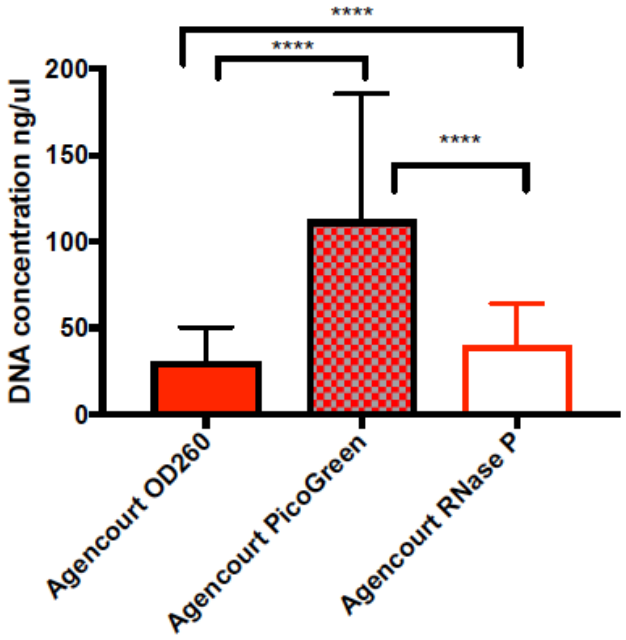
- OD260/OD280&OD260/OD230
- Picogreen
- Quantification with RNase P
- gels

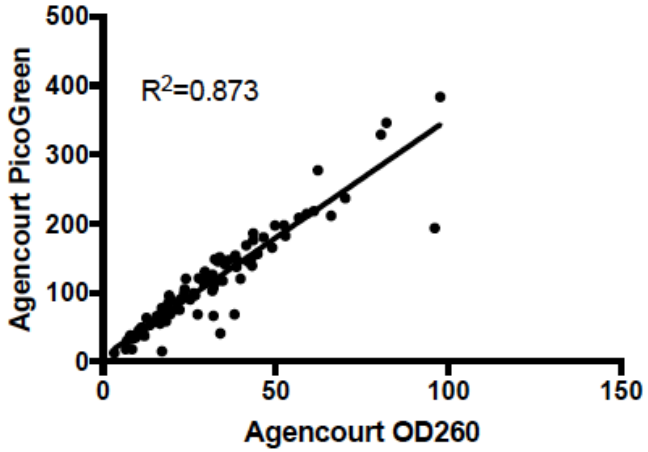
TL by qPCR
Data analysis

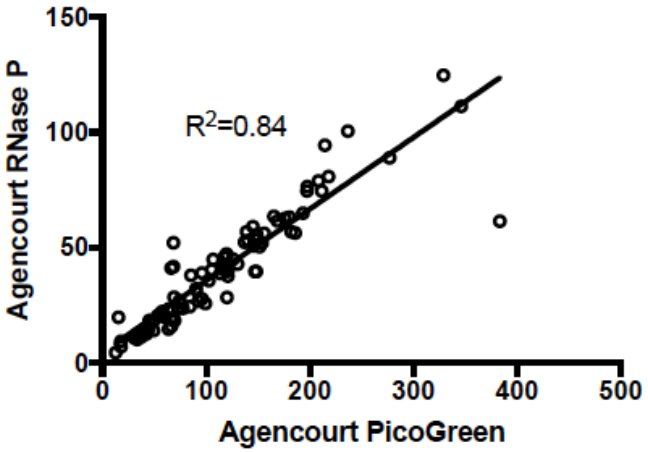
DNA Yield Measured by OD260, PicoGreen and RNase P

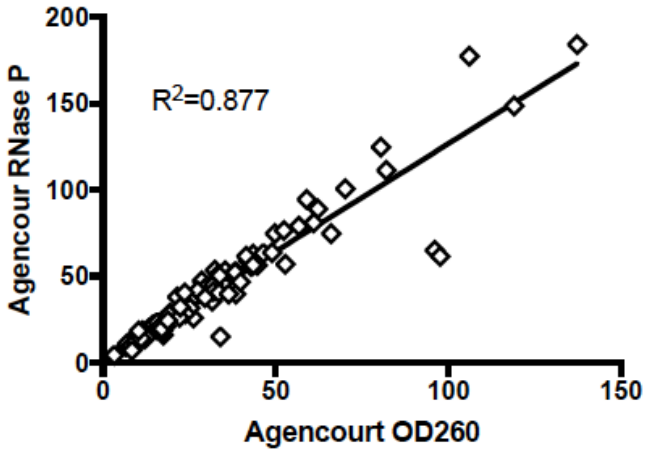


DNA Yield Measured by OD260, PicoGreen and RNase P Are Highly Correlated



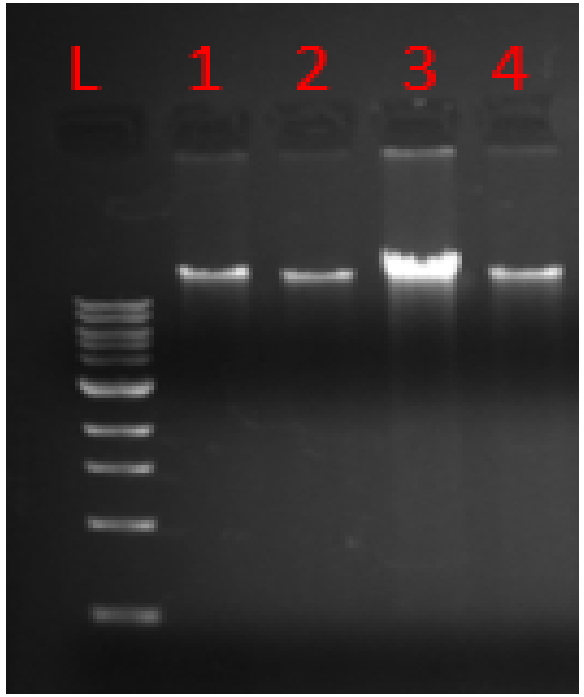




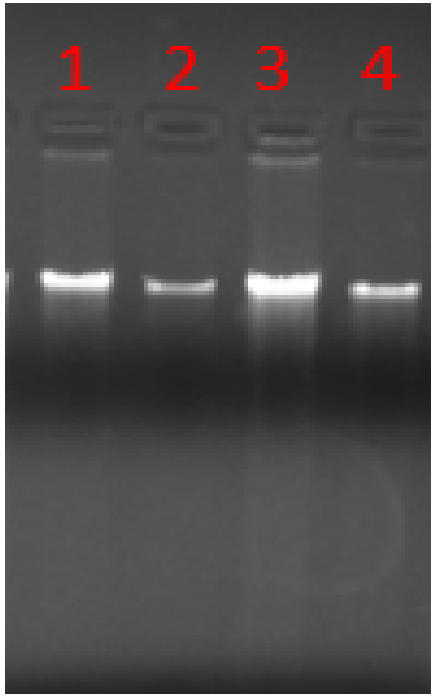


Saliva DNA Extracted by QIAamp Mini Kit is Partially Degraded

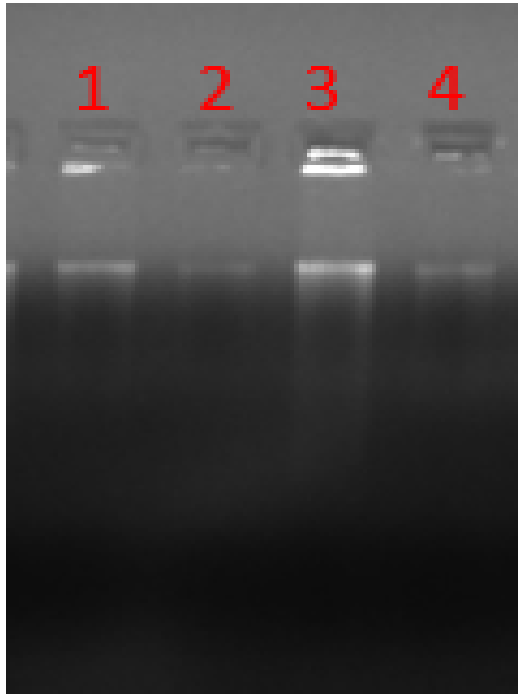
Agencourt



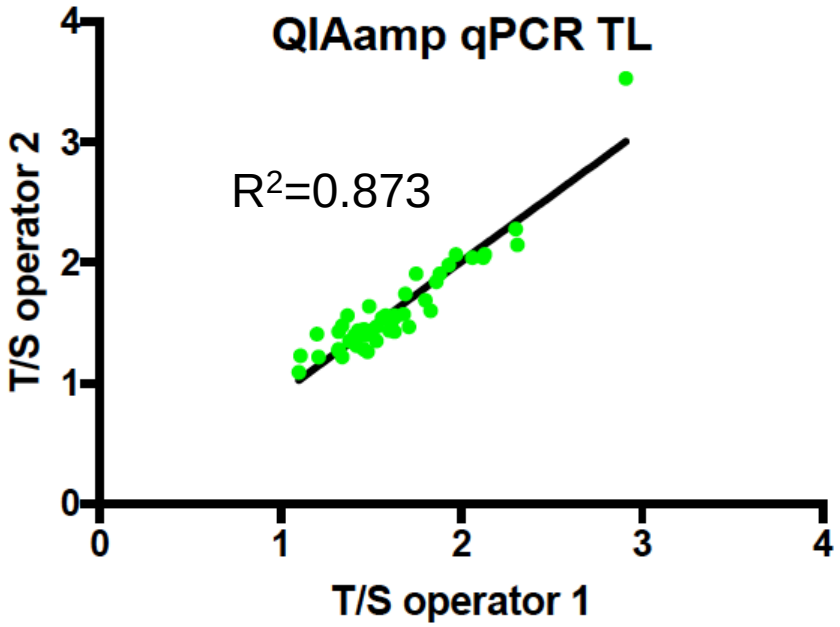
Prep IT

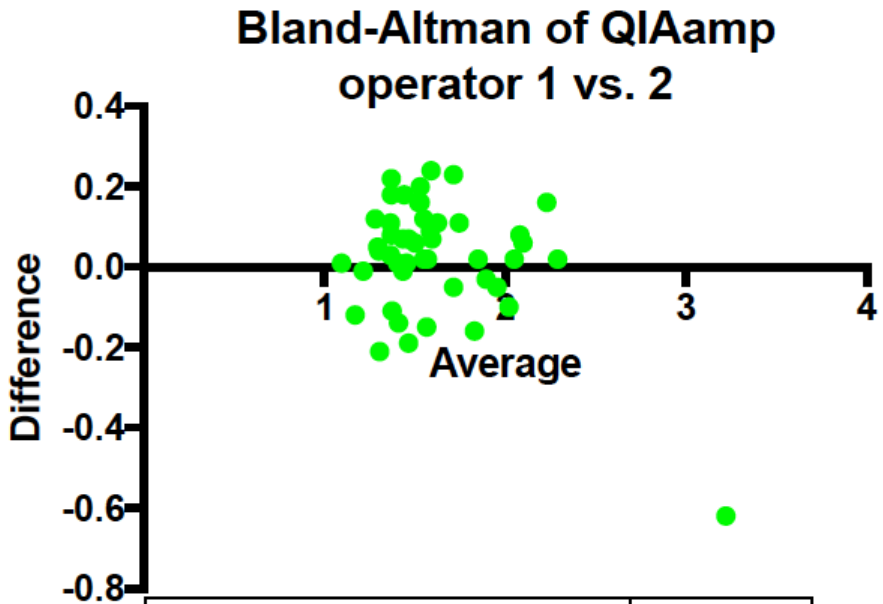


QIAamp



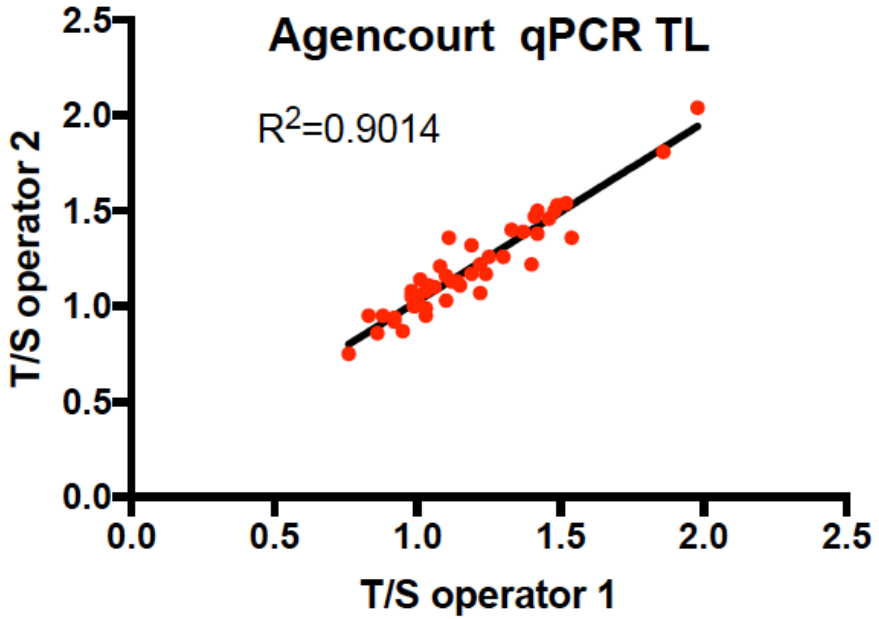
High Correlation of T/S ratios Between the Two Operators for QIAamp Mini DNAs

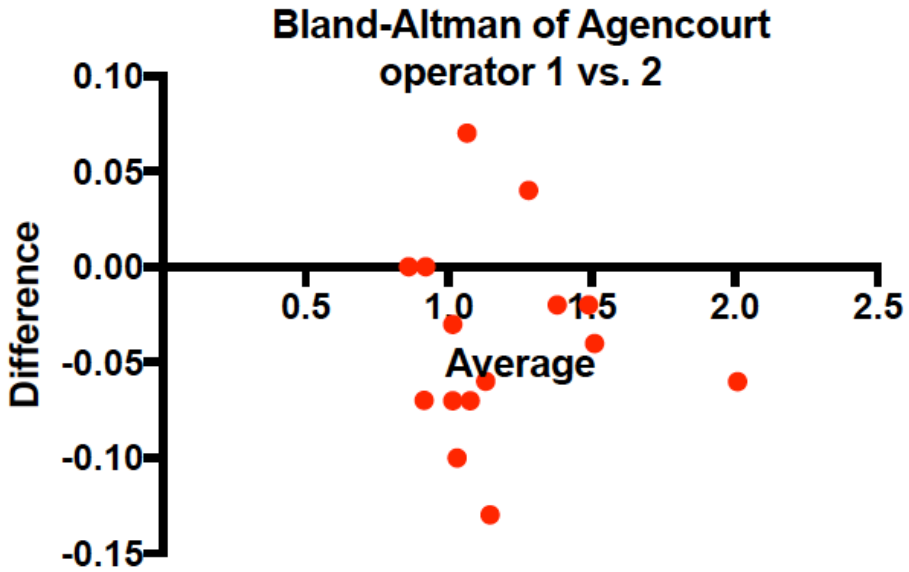




Bias	0.02511
SD of bias	0.1475
95% Limits of Agreement	
From	-0.264
To	0.3142

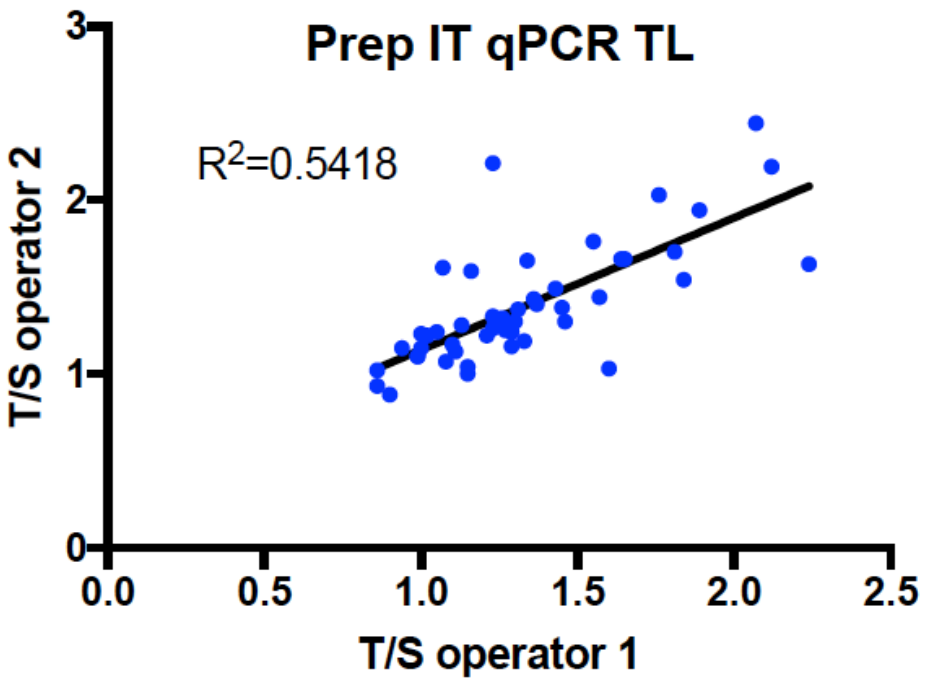
High Correlation of T/S ratios Between the Two Operators For Agencourt DNAs

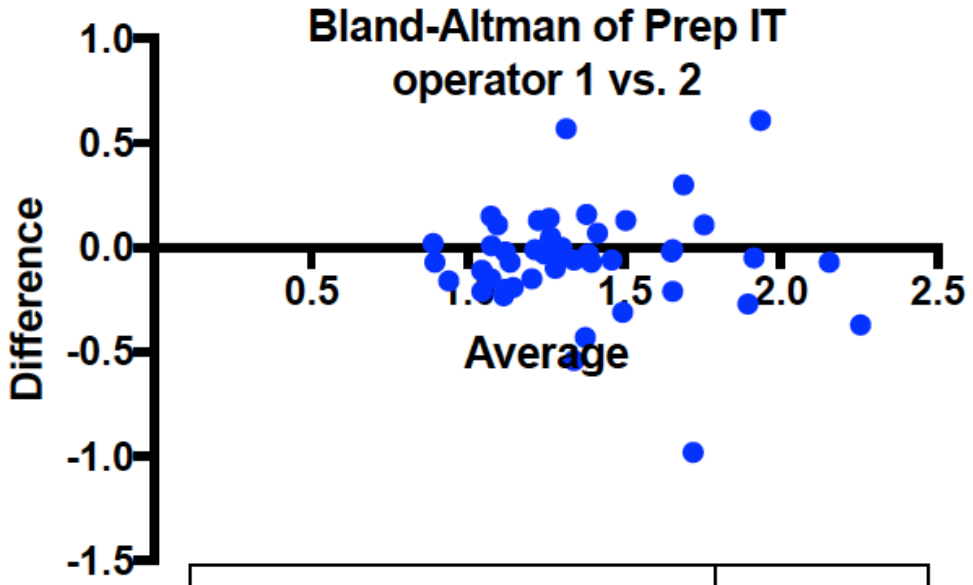




Bias	-0.03733
SD of bias	0.05189
95% Limits of Agreement	
From	-0.139
To	0.06437

Modest Correlation of T/S ratios Between the Two Operators For Prep IT DNAs





Bias	-0.05833
SD of bias	0.2466
95% Limits of Agreement	
From	-0.5417
To	0.4251

Correlation Matrix between Two Operators and Three Extraction Methods: Prep IT is More Variable

R^2	Agencourt 1	Agencourt 2	Prep IT 1	Prep IT 2	QIAamp 1	QIAamp 2
Agencourt 1	1					
Agencourt 2	0.901	1				
Prep IT 1	0.807	0.750	1			
Prep IT 2	0.589	0.586	0.542	1		
QIAamp 1	0.768	0.715	0.755	0.411	1	
QIAamp 2	0.726	0.690	0.638	0.454	0.873	1

Correlation Matrix between Two Operators and Three Extraction Methods: Prep IT is More Variable

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QIAamp 1	0.768	0.715	0.755	0.411	1	
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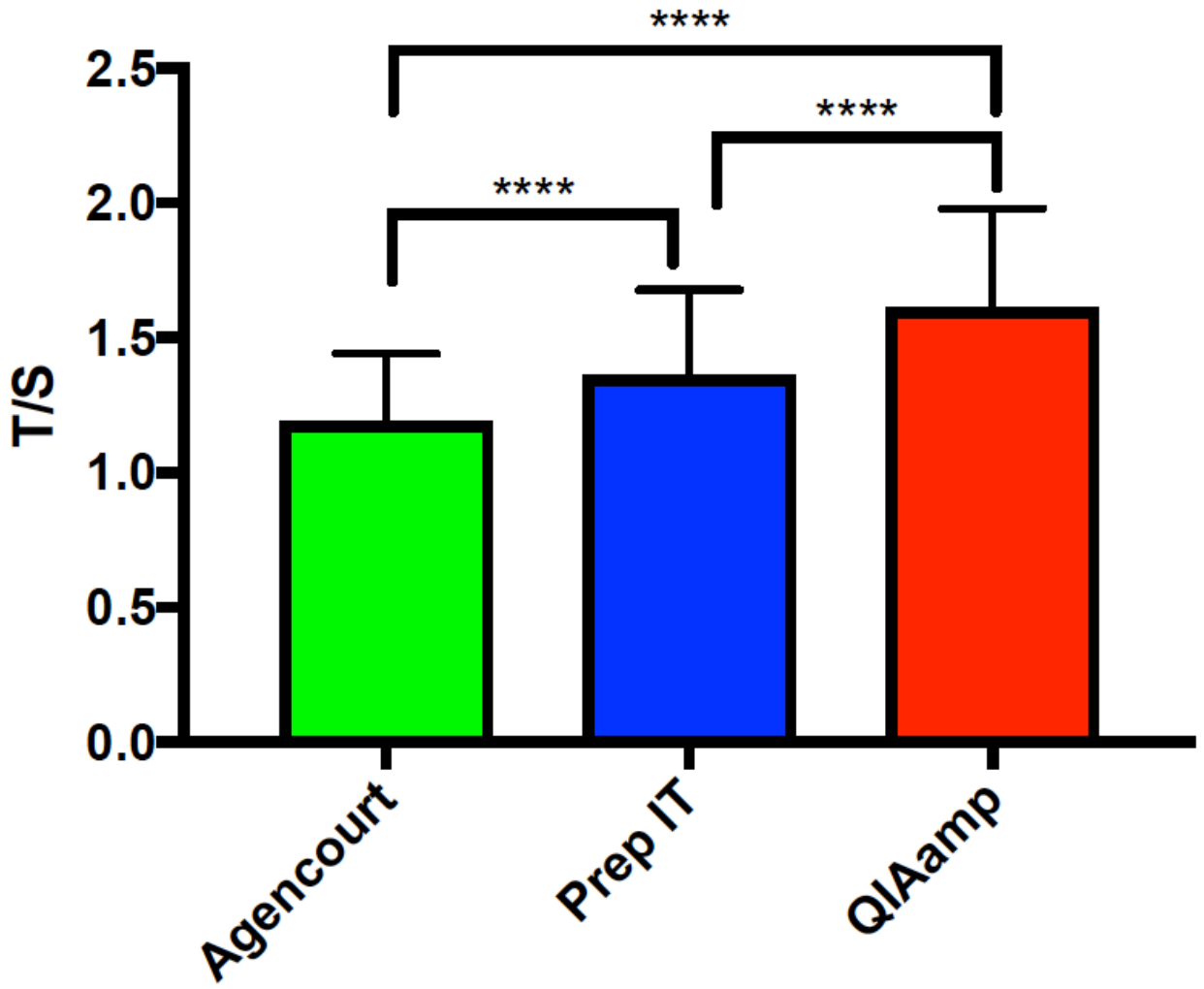
Systematic Differences Between Three DNA Extraction Methods



Summaries and Next Steps for Saliva Samples

- Differences in DNA yields and integrity and T/S ratios for different extraction kits
- Both Agencourt and QIAamp kits provide high correlations of T/S ratios between 2 extractions done by 2 operators, while T/S ratios of Puregene extracted DNA by 2 operators are more variable.
- T/S ratios are highly correlated between Agencourt and QIAamp kits.
- Southern Blot analysis will be performed to determine the correlation of qPCR and TRF

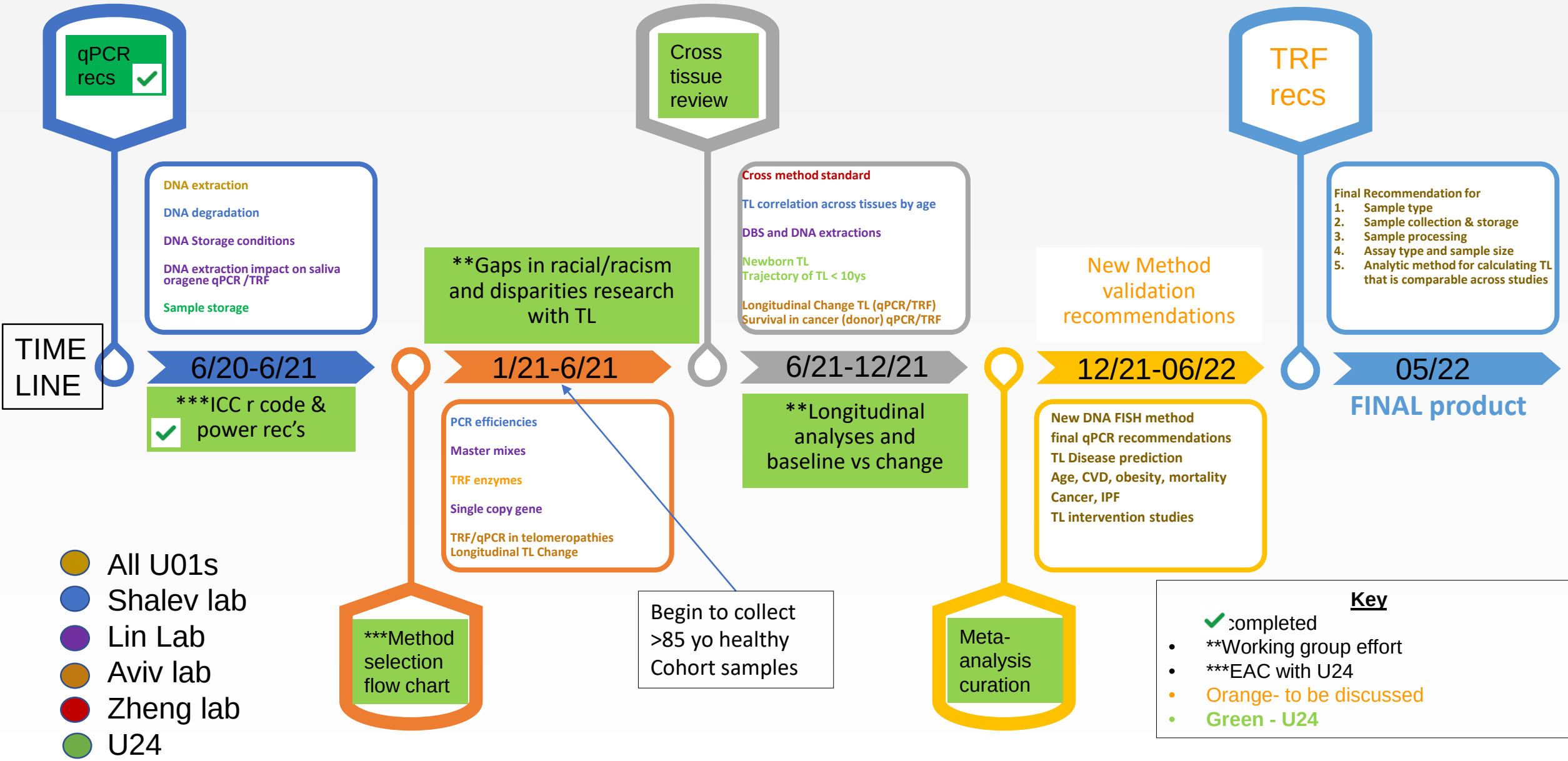
Collaborating Groups for the Whole Blood Cross-lab DNA Extraction Study

- Aviv lab - Rutgers
  Tsung-Po Lai
- Gadalla lab – NCI
  Casey Dagnall
- Lin lab – UCSF
  Dana Smith
  Calvin Wu
- Drury lab – Tulane University
  Alyssa Lindrose
  Camilo Fernandez Alonso
- Shalev lab - University of Pennsylvania
  Thomas Heller
  Christopher Chiaro
  Waylon Hastings
- Zheng lab- Georgetown University
  Ying Wang
- Simon Verhulst - University of Groningen

Thank you!

Funding source: U01AG064785

YEAR 1 to 3:cross method timeline



- TRF/ qPCR/TESLA/Luminex/qFISH prediction of disease…

1. Age, CVD, obesity, mortality
2. Cancer, **PF, Telomere syndrome disorders
3. TL intervention studies
4. Infection risk
5. Environmental exposures
6. Psychosocial stress
7. COVID

Use of NIA samples

1. Unlikely to be useful for end point (e.g. BMI, sex) outcome
2. Option 1- ICC between labs/cross methods comparisons
3. Option 2- involve larger group of labs that are not the “premiere” labs; potential end point could be age
4. Option 3 (live cells) examine if qPCR and TRF can detect cell subtype differences- what is the next step? How would this impact the field? (can it drive increased clinical utility?)
5. Cells that can be used for something deeper in terms of understanding telomere function- RNA, expression, associations with other markers of cell senecense/apoptosis

FINAL goals by year 3 for U01s

- Final Recommendation for

1. Sample type
2. Sample collection & storage
3. Sample processing
4. Assay type and sample size
5. Analytic method for calculating TL that is comparable across studies

Closing thoughts and 2021 time line and goals

TELOMERE
RESEARCH
NETWORK

The importance of the methodology laboratory and best practice for the field….

TELOMERE
RESEARCH
NETWORK

Publication analysis of telomere length studies in pediatrics

Tom Qian

Chao Zhang

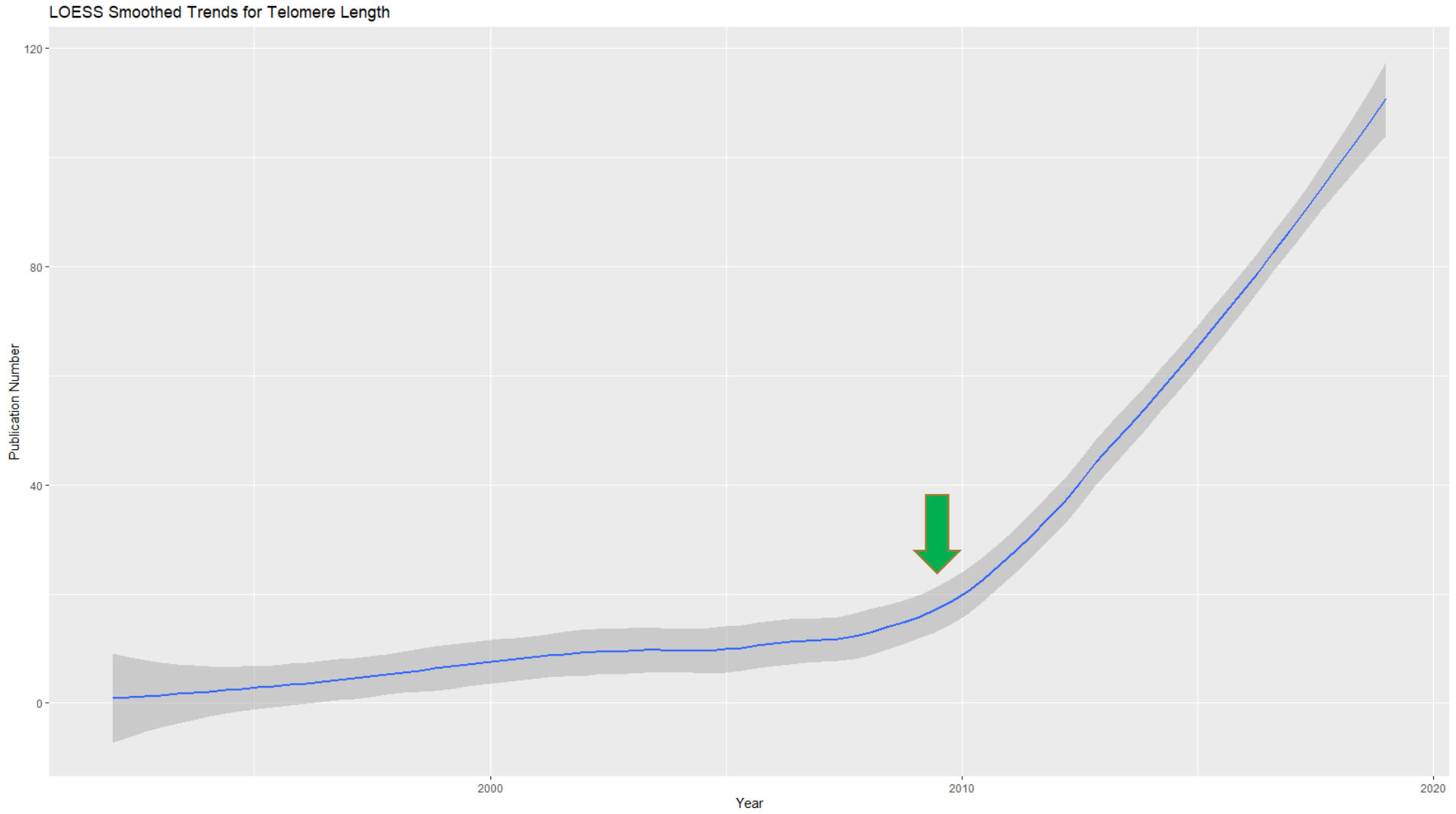
Hannah Piersiak

Kathryn L. Humphreys

Colter Mitchell

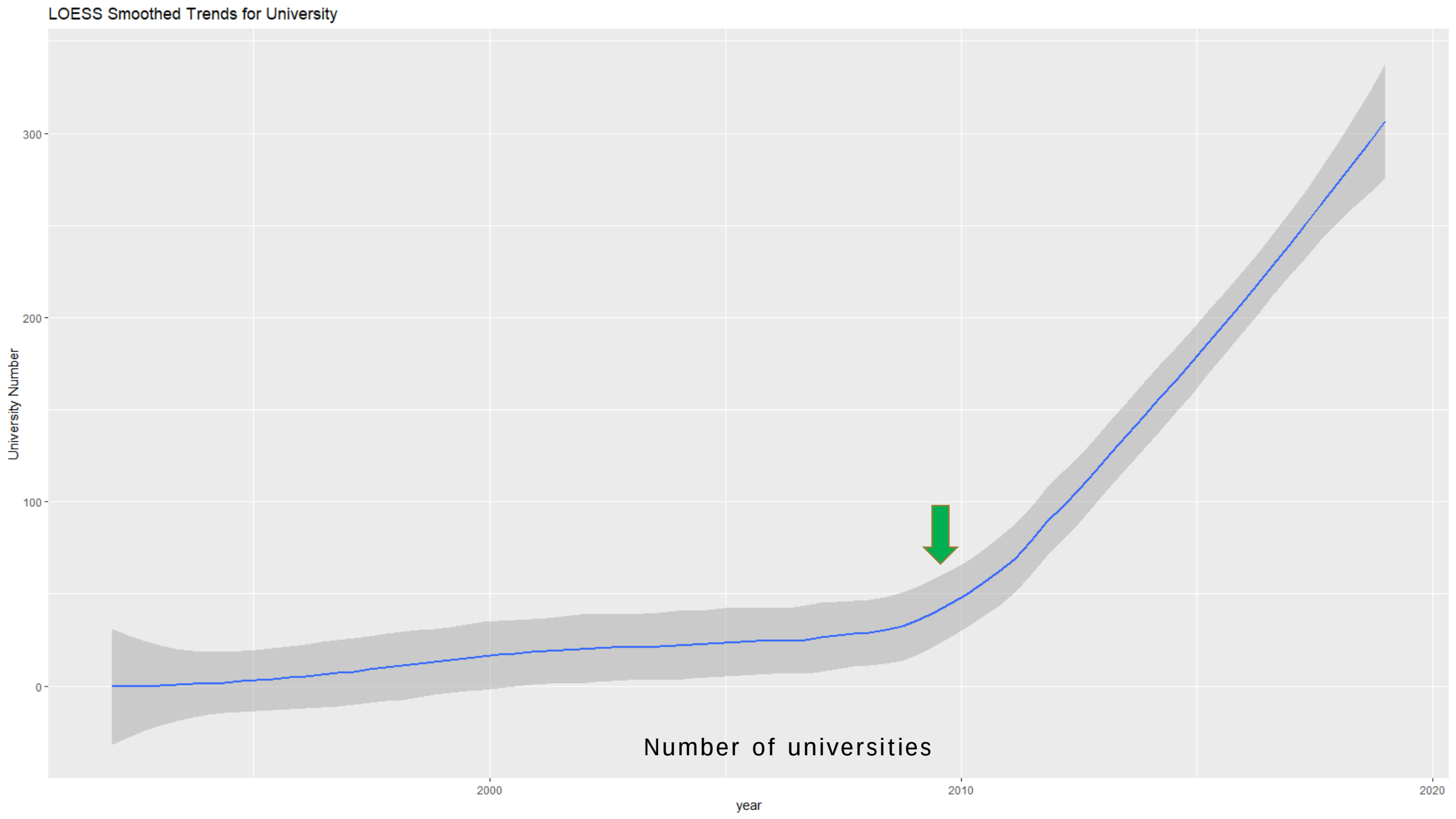
VANDERBILT UNIVERSITY

SEA LAB

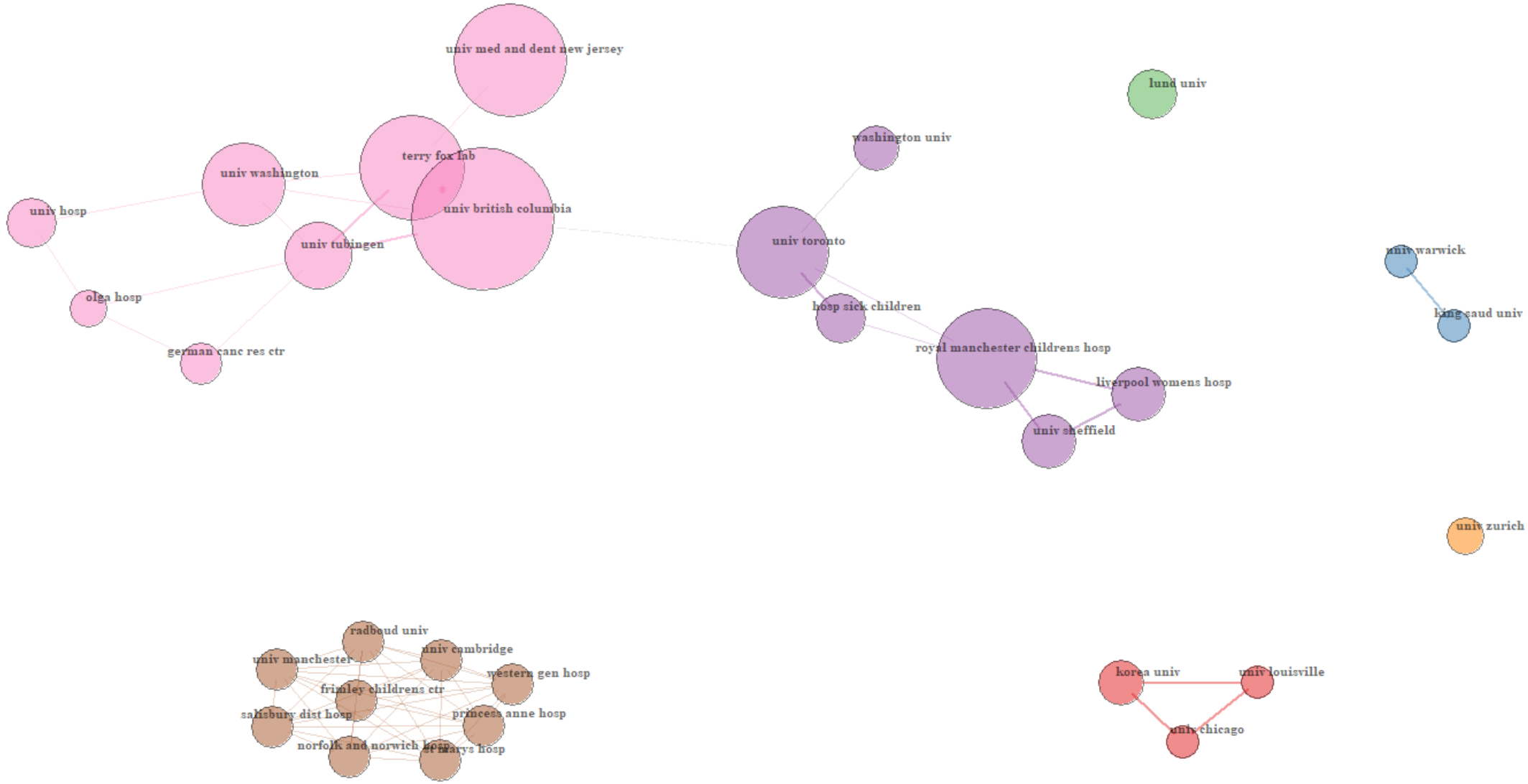


Raw publication number

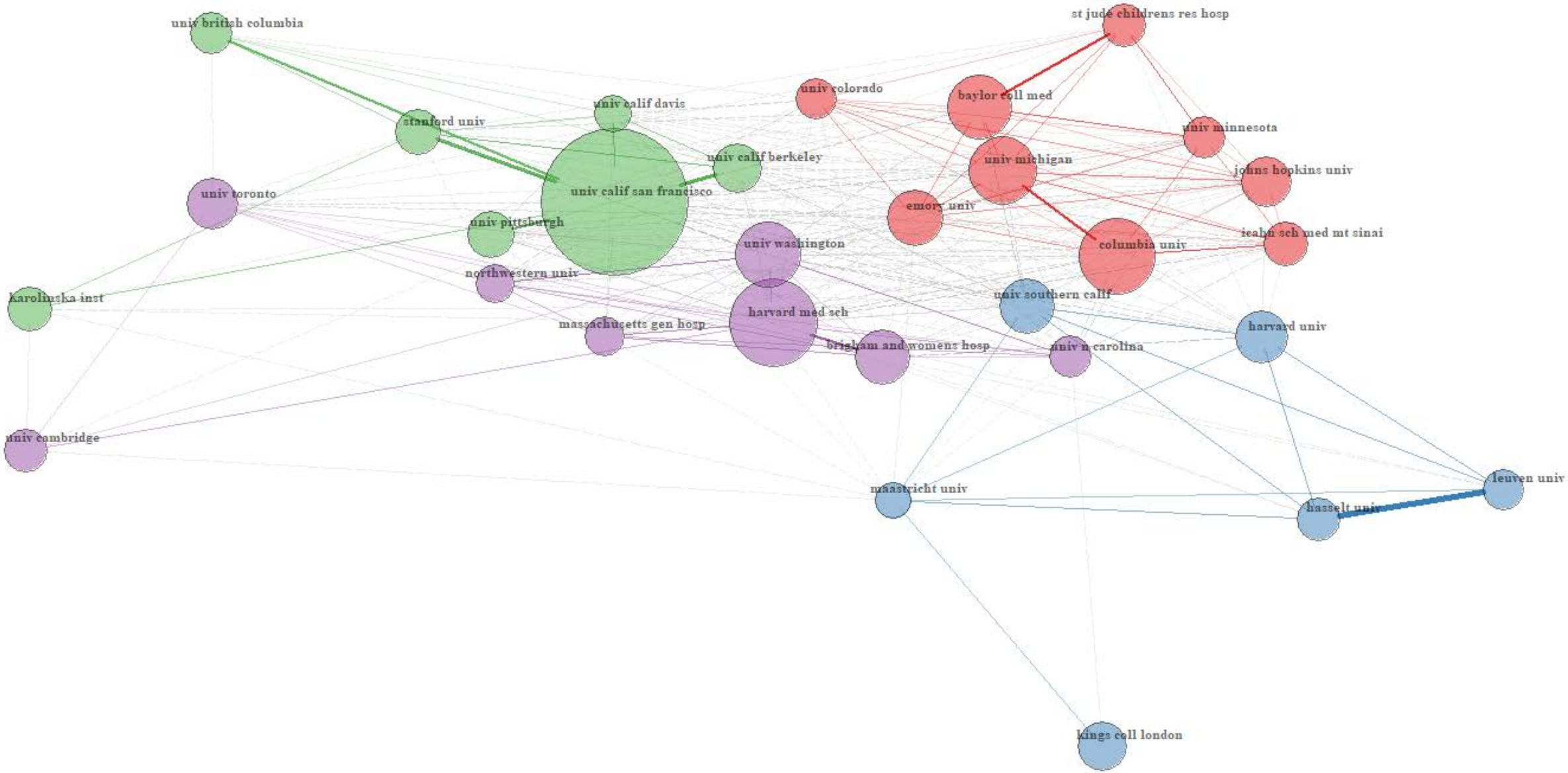
VANDERBILT UNIVERSITY


LOESS Smoothed Trends for University
300
200
100
0
University Number
2000
2010
2020
year
Number of universities

University Collaboration Network (1992-2010)



University Collaboration Network (2011-2020)



Next steps

- Refine network analyses
- Ensure that TRN reaches out to networks
- Utilize data to drive development of subcommittee/research networks

U24 NEXT STEPS

- Quarterly news letter
- Sample collection and storage conditions check list
- How to pick a telomere collaborator?
- Telomere methods selection on-line survey
- MARCH 1st Pilot award RFA
- Further methods comparisons

TELOMERE RESEARCH NETWORK

TRN dissemination of results

- Web page
- TRN quarterly news letter
- Include link to qPCR reporting guidelines when reviewing or writing peer reviewed manuscripts

- Other ideas for ways to disseminate data driven best practices?

- Consultation- email telomerenetwork@gmail.com

TELOMERE RESEARCH NETWORK

THANK YOU!