Telomere Considerations in Health ABC

The following sets of questions and responses and comments are included with the telomere assays to clarify issues in analysis of these specimens and to address questions frequently asked about the specimens. If there are any questions, please email Dr. Cawthon (rcawthon@genetics.utah.edu) and copy Emily Kenyon (EKenyon@psg.ucsf.edu) and Wen-Chi Hsueh (wen-chi.hseuh@ucsf.edu) on the correspondence.

1. How were specimens sent to Dr. Cawthon?

The samples sent from Vermont were in 96-well plates, with one microliter of concentrated DNA per well (about 100 nanograms of DNA in one microliter). Dr. Cawthon only received 45 plates from Vermont, each with at most 64 samples, although the last plate probably had fewer than 64. So at most, he received 2880 samples. Because some samples simply failed to PCR amplify, there are TL data for 2,750 individuals.

2. What method was used to prepare the specimens for analysis?

To process a plate of Health ABC DNA samples, a dilution buffer was freshly prepared and used to dilute the DNA in the plate. Any left over dilution buffer was discarded before processing a new plate.

Plate number is a proxy for each separate preparation of dilution buffer.

3. How were the specimens processed?

"Whenever I was ready to do a run of the assay, I added a buffer to each well, greatly diluting the DNA, so that I could then use an electronic multi-tip pipetor to pull up all I needed so that I could distribute each separate DNA sample into 6 separate reaction tubes, consisting of 3 tubes for the "t" reactions, and 3 tubes for 's' reaction. The 3 't' tubes then went into 3 separate PCR machines which were then run simultaneously for about 1 hour; the 3 's' tubes sat for the duration of the 't' runs. At the end of the "t" runs, the "t" tubes were discarded, and replaced with the "s" tubes, and another PCR run of all 3 machines commenced, also for about one hour. So from the time the samples were diluted to the time the "t" and "s" runs were completed it was usually about 2 and a half hours. Samples were diluted only once, on a plate-by-plate basis, with the PCR runs following immediately in most cases."

Each of the 3 't' runs and 3 's' runs of a plate's samples included a serial dilution series of the same reference DNA, allowing standard curves to be generated for all 6 runs. The same reference DNA was used for all 45 Health ABC plates. Every Health ABC sample's 't' and 's' values were determined by the standard curve method, relative to this one reference DNA.

The batch number variable refers to the calendar date on which a given plate's PCR reaction tubes were all set up and sealed (even if only a subset of those tubes were subjected to a quantitative PCR run on the day of setup).

4. Is there any other potential source of variability besides plate (dilution solution) and batch (calendar date on which a given plate's PCR reaction tubes were all set up and sealed)?

Technically, there is a third variable which would be "day run" which in most cases is the same as the batch number, except for a few plates whose runs were split across two or three days.

Note from Emily Kenyon: Variation in the telomere results associated with this "day run" variable, if any, has yet to be assessed but this is likely to be small since this affected only a few plates.

5. How were the t/s ratios calculated?

"For the full set of Health ABC runs, I used 3 new quantitative PCR machines that are not block-based. They use a circular carousel to hold the reaction tubes, and the carousel centrifuges at about 500 rpm throughout the run. With this system, there is no evidence of any well position effects. Therefore, it seemed reasonable to switch to taking the 'ratio of the averages'. Also, since I had 3 machines, I thought it best to distribute the 3 't' measurements over the 3 machines, and also to distribute the 3 's' measurements over the 3 machines. One advantage of taking the 'ratio of the averages' over taking the '"average of the ratios' is that with the 'average of the ratios' approach, one has to throw out both the 't' and 's' measurements from a given well if either reaction fails; however, with the 'ratio of the averages' approach, one only has to throw out the reactions that fail."

6. Why are there no true replicates?

Because Dr. Cawthon needed more concentrated DNA than the center at the University of Vermont usually made available to investigators, the routine replicate samples were not included in the specimens sent to him. He suggested a substitute plan but it turned out not to be feasible in part because DNA samples that are diluted as much as all the Health ABC samples were as he measured them do not give reproducible QPCR results, after more than a few hours of storage at the diluted concentration. (In contrast, concentrated DNA samples re-assayed across days or months or years give reproducible QPCR results.).

<u>Further observations from Wen-Chi Hsueh -- very helpful summary:</u>

Both (PCR and Southern blot) methods have different sources of measurement error (e.g. the Southern blot results are smears, so the TL measurement cannot be exact and more importantly, it includes subtelomeric segment, estimated to range in length from 2500bp

to 6000 bp) which we have no good idea whether it matters or not). For Q-PCR, in essence, the result is a ratio of the copy number of the 6-bp repeat divided by the copy number of a single copy gene. So it is not a direct measurement but it does measure only the telomeres. As several labs have also learned, both methods require highly trained individuals and DNA of good quality to obtain data of high quality.

Other practical considerations include the amount and quality of DNA needed (Southern blot requires several ug of DNA vs. < 100ng for q-PCR; the q-PCR method can tolerate DNA of lesser quality), which is often an issue for archived DNA samples from many years ago, and the costs in time (q-PCR is > 10x more efficient) and money (Southern blot is more expensive).

Measurement

Both assays have obvious batch/plate effects. We know it exists in Health ABC, but it also exists for Southern blot (personal communication with people who used the Southern blot method), it is just not reported in papers. Furthermore, the quality of Southern blot results probably vary greatly depending on which lab did the assay (some examples shown below), but more real "data points" (studies) will be needed to evaluate this. We probably all had dealt with data with similar issues, such as observer effect, interviewer effect etc.). Thus, this is not desirable but it is by no means a unique example. Therefore, the question is, whether we can use statistical means to reduce the impact from these factors.

According to Dr. Cawthon's paper, the correlation between the Southern blot and the Q-PCR method (based on 95 samples) was 0.7. [reference: PMID: 12000852].

As for reproducibility, we were able to identify a few papers that specifically mention the inter-assay CV for the TL assay (no info on sample sizes for calculating CV was available). This is not a comprehensive list but hopefully informative.

- Southern Blot done by Dr. Aviv's group at different time, with different versions of the assay protocol done on the same group of samples:
  - 12% (published in 2006)
  - 5%, in 2007
  - 0.95% in 2008

- Southern blot done by a different group
  - 5.1%, 2005

- Q-PCR performed in the Cawthon Lab
  - 5.8%, published in 2002
  - 4-8% in the Amish, 2007, unpublished

- Q-PCR done in different labs
  - 2.2%, published in 2007 (PMID: PMID: 17416776)

As the q-PCR method is a relatively new invention, studies using this method have only been published in recent years.

Heritability of telomere data based on PCR measurements vs. Southern blot
- we have family data to show that the heritability of TL is 50%, which is comparable to what had been published using the Southern blot. Family data are more susceptible to measurement errors, so if the Q-PCR data are not informative, we wouldn't have been able to get such an estimate.

Plates vs. batch in Health ABC
There are 45 plates done in 20 different occasions (batches) for the Health ABC samples. The number of plates per batch ranges from 1-5. The correlation between plate and batch is 0.99. After adjusting for covariates (age, sex, site, race), there was a strong association ($p<0.001$) between TL and plates/batches. Batch alone, plate alone and plate+batch together account for 6%, 9% and 14% of total TL variation, respectively. In fact, even the interaction between plate and batch was significant.

We further evaluated the effect of including plate alone, batch alone, and both variables in the models when we examined the association between TL and phenotypes (e.g. survival) and did not observe any significant impact on the results among the three different models.

Note from Tammy: Although plate and batch are correlated (likely due to temporal associations) they each represent different sources of variation. While the method of testing that Dr. Hsueh suggests is reasonable, at least one of the TL variation measures should be included in the final model.