The Epigenetic Clock Applies to Human Urine

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Abstract

Several recent articles describe epigenetic biomarkers of aging based on methylation levels. For example, the epigenetic clock method (Horvath 2013, PMID: 24138928) is based on forming the weighted average of 353 epigenetic markers (CpGs) which leads to an estimate of age (in units of years), which is referred to as "DNA methylation age" or as "epigenetic age". The epigenetic clock method for estimating age has been found to apply to sorted cell types (e.g. neurons, glial cells, monocytes, helper T cells, cytotoxic T cells) and complex tissues (e.g. blood, brain, lung, kidney, bone).

However, it is not yet known whether the epigenetic clock method applies to DNA extracted from human urine. Urine is an attractive (I think this word is not the right word) fluid because a) it can be easily collected and b) it probably reflects the epigenetic age of cells shed from the urinary tract system, kidney and/or bladder. Thus, urine-based test of epigenetic age might obviate the need for collecting biopsies from inaccessible organs.

To extract DNA from urine samples, we used the *Quick*-DNA™ Urine Kit from Zymo Research (Irvine, CA, USA). This kit features uniquely formulated urine conditioning buffer (UCB) that allows stabilization of DNA in urine for up to 1 month at ambient temperature. Cellular and cell-free DNA are easily and reliably purified from up to 40 ml of urine. On average, 1 ml urine yielded 6-1000 ng and 2-20 ng of DNA for healthy female and male individuals, respectively.

We evaluated the accuracy of the epigenetic clock method in human urine samples (n=34) from subjects with an age range from 2 to 84 years. DNA methylation age was highly correlated with chronological age (r=0.96, p=3e-19).

Overall, our study demonstrates that the epigenetic clock applies to human urine samples. This study is a first step for future studies that will evaluate whether epigenetic age of urine is predictive of health outcomes including cancer outcomes.

The Epigenetic Clock

Figure 1. The weighted methylation average of the 353 clock CpGs versus chronological age in the training set. The rate of change of the red curve can be interpreted as tick rate. Organismal growth (and concomitant cell division) leads to a high ticking rate that slows down to a constant ticking rate (linear dependence) after adulthood. Points are colored and labeled by data set. (S. Horvath. *Genome Biology*, 14:R115)

Methods

Urine Conditioning Buffer (Zymo Research; Cat. No. D3061-1-140) was immediately added to voided urine samples and stored at 4 °C prior to DNA extraction. Urine DNA was extracted using *Quick*-DNA™ Urine Kit (Zymo Research; Cat. No. D3061) and stored at -20 °C.

Methylation analysis was performed using Infinium HumanMethylation450 BeadChip Kits (Illumina). 500 ng of DNA was bisulfite converted using EZ DNA Methylation Kits (Zymo Research) and subsequently processed for HumanMethylation450 BeadChips following manufacturer's instructions. Following hybridization, BeadChips were scanned using the Illumina HiScan System.

The 353 clock CpGs were previously selected by a penalized regression model regressing a calibrated version of chronological age on Illumina DNA methylation array datasets of 8,000 samples from 51 healthy tissues and cell types. DNA methylation age (DNAmAge) was defined as predicted age.

A linear regression model's coefficients b_0, b_1, …, b_{353} relate to transformed age as follows

$$F(\text{chronological age}) = b_0 + b_1 CpG_1 + \cdots + b_{353} CpG_{353} + \text{error}$$

DNAmAge is estimated as follows

$$DNAmAge = inverse.F(b_0 + b_1 CpG_1 + \cdots + b_{353} CpG_{353})$$

Quick-DNA™ Urine Kit

Isolate Cellular and/or cell-free DNA from up to 40 ml of Urine

Figure 2. Simplified protocol for *Quick*-DNA™ Urine Kit.

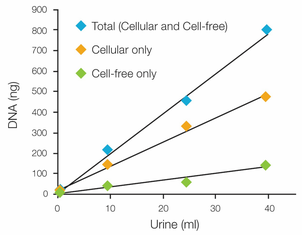


Figure 3. DNA yields increase linearly with increasing volumes of urine from healthy subjects extracted using the *Quick*-DNA™ Urine Kit. DNA was isolated from 1 ml, 10 ml, 25 ml, and 40 ml urine. DNA concentration was quantified using the **Femto™ Human DNA Quantification Kit** (Zymo Research, E2005).

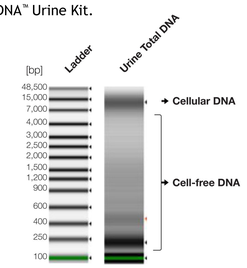
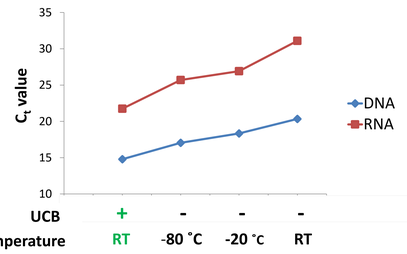


Figure 4. Both cellular and cell-free DNA can be effectively purified from urine using the *Quick*-DNA™ Urine Kit. 5 ml of urine from a healthy female donor was processed and DNA was eluted in 20 µl final volume. 1 µl of the sample was analyzed using a 2200 TapeStation.

UCB Provides Superior Preservation vs. Conventional Methods



I am going to send you a modified graph, because this was wrong.

Figure 5. DNA: 10 ml urine (with or without UCB) from a healthy female was stored at different storage conditions: Room Temperature (RT), -20˚C, and -80˚C. After two weeks of storage, total DNA (blue) was purified using the ***Quick*-DNA™ Urine Kit** (Zymo Research). The C_t values were determined by qPCR using the **Femto™ Human DNA Quantification Kit** (Zymo Research, E2005). Experiment was done in duplicates and averaged. **RNA**: 10 ml urine (with or without UCB) from a healthy male was spiked in with HeLa cells and stored at different storage conditions: Room Temperature (RT), -20˚C, and -80˚C. After two weeks of storage, total RNA (red) was purified using a custom urine RNA isolation protocol from Zymo Research. Corresponding C_t values were obtained from RT-qPCR. Experiment was done in duplicates and averaged.

UCB Prevents Microbial Growth at Ambient Temperature

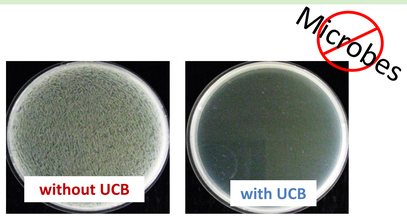
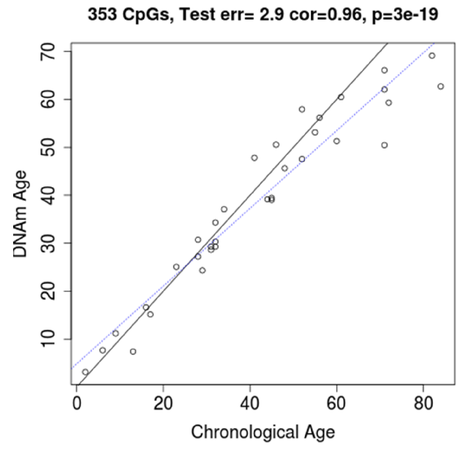


Figure 6. Urine was stored with and without UCB for 6 days. On day 6, microbial colony formation was determined by spreading 500 µl urine on LB plates and incubating at 37 ˚C overnight.

Result

Methylation Age Highly Correlates with Chronological Age



n = 34

Figure 7. DNA methylation age was highly correlated with chronological age. Human urine DNA samples from 34 subjects with an age range of 2 to 84 years were bisulfite converted, and methylation levels were measured by Infinium HM450 array. DNAmAge were then predicted by the Horvath epigenetic clock and plotted against chronological age. The average age difference was only 2.9 years with r=0.96 and p=3e-19. The dotted line was the regression line.

Accelerated DNAmAge of Blood DNA of Patients

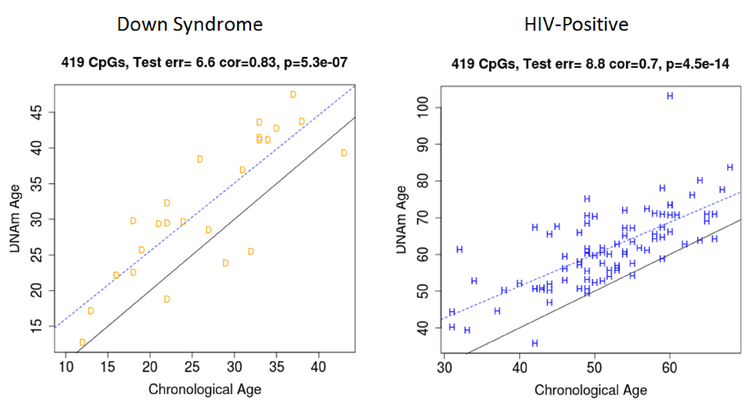


Figure 8. Accelerated epigenetic aging was observed in blood DNA of Down syndrome and HIV-positive patients. Zymo scientists identified a group of age-associated CpGs using HM450 methylation array datasets of blood DNA of healthy subjects. Using these markers for age prediction, DNAmAge of blood DNA of Down Syndrome (D) and HIV-positive (H) patients was 6.6 years and 8.8 years older than chronological age with p=5.3e-07 and p=4.5e-14 respectively. The dotted line is the regression line.

Conclusion

Our study demonstrates that the epigenetic clock applies to human urine samples. We developed a simple, reliable total solution for urine collection, stabilization, purification and detection. This study is a first step for future studies that will evaluate whether epigenetic age of urine is predictive of health outcomes including cancer outcomes.