G-tail telomere HPA: simple measurement of human single-stranded telomeric overhangs

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Accurate measurement of telomeric 3′-overhang (G-tail) lengths is essential for investigation of the biological effects of telomere dysfunction. G-tail telomere hybridization protection assay (Gt-telomere HPA) has the advantages of being simple to perform, accurate and highly sensitive for G tails as short as 20 nucleotides. Furthermore, Gt-telomere HPA is specific and quantitative for human G tails, and can be used to assay cell lysates as well as genomic DNA.

Telomeric DNA is composed of many 5′-TTAGGG-3′ repeats. The terminus of each telomeric DNA has a single-stranded, 3′ overhang of between 75 and 300 bases in the G-rich strand, the so-called G tail[1,2]. Telomeric G tails are essential for proper telomere function[3,4].

Several methods are available for measuring the lengths of telomeric G tails (**Table 1**)[5–7]. Although the telomeric-oligonucleotide ligation assay (T-OLA)[6], the primer extension–nick translation (PENT) and the 3′ overhang protection assay[5] are all suitable for measuring G tails, they are complicated assays that require at least two days to complete. A further drawback is that they are difficult to apply in large-scale and high-throughput screening of samples. To overcome these problems, we have developed a new method for measuring telomeric G tails using a hybridization protection assay (Gt-telomere HPA). HPA has been used previously for detection of total telomere length and telomerase activity[8,9]. Our new method is based on an HPA format that uses oligonucleotide probes labeled with a highly chemiluminescent acridinium ester. Gt-telomere HPA is a single tube–based assay that can be completed in less than 40 min (**Table 1** and **Fig. 1a**). Whereas Gt-telomere HPA cannot be used to measure either variation in G-tail sizes within cells or the lengths of the G tails of individual chromosomes, the method has several key advantages: sensitivity for detection of short G tails, direct applicability to cell lysates, and the potential for large-scale screening using a 96-well format luminometer.

To determine the sensitivity and specificity of Gt-telomere HPA, we incubated serial dilutions of 84-mer telomere oligonucleotides, 5′-$(TTAGGG)_{14}$-3′, with 3×10^7 relative light units of luminescence (rlu) of 29-mer telomere HPA probes (**Supplementary Methods** online). We observed a linear increase in signal intensity with increasing oligonucleotide amount across the range 0.05 fmoles to 10 fmoles (**Fig. 1b**). The HPA probes can therefore be used to detect target mammalian telomere DNA sequences (**Supplementary Fig. 1** online). In a similar experiment, we obtained a linear response over a range of 1 μg to 20 μg of nondenatured genomic DNA (**Fig. 1c**). Thus, we now typically use 5 μg of nondenatured genomic DNA in this assay. In parallel experiments, we treated DNA samples with exonuclease I (ExoI) to remove single-stranded nucleotides in the 3′ to 5′ direction, thus deleting the telomere G tails. All the samples were ExoI-sensitive (**Fig. 1c**), confirming that the luminescent signals detected were specifically telomeric, single-stranded G tails. In contrast, treatment with T7 exonuclease, which removes the telomeric C strand in a 5′ to 3′ direction, thereby increasing 3′ telomeric G tails, increased the signals in a time-dependent manner (**Supplementary Fig. 2** online). At 90 s, the observed rlu value indicated that G tails with a mean length of approximately 1,600 nucleotides (nt) had been generated (**Supplementary Fig. 3** online). Taken together, the results of these enzyme-treatment experiments showed that Gt-telomere HPA can be used to specifically and quantitatively detect telomeric G tails.

Table 1 | Comparison of Gt-telomere HPA with known techniques to determine G-tail length

Method	Detection range (nt)	Detection time	Radioisotope	Assay in crude extracts	Electrophoresis	Determination of G-tail size distribution	High-throughput analysis
T-OLA	24–650	2 d	Required	Not possible	Required	Possible	Not possible
PENT	130–210	2 d	Required	Not possible	Required	Possible	Not possible
Electron microscopy	225–650	2 d	Required	Not possible	Required	Possible	Not possible
3′ overhang protection assay	45–384	2 d	Required	Not possible	Required	Not possible	Not possible
Gt-telomere HPA	20–1,600	40 min	Not required	Possible	Not required	Not possible	Possible[a]

[a]96-well plate screening is available.

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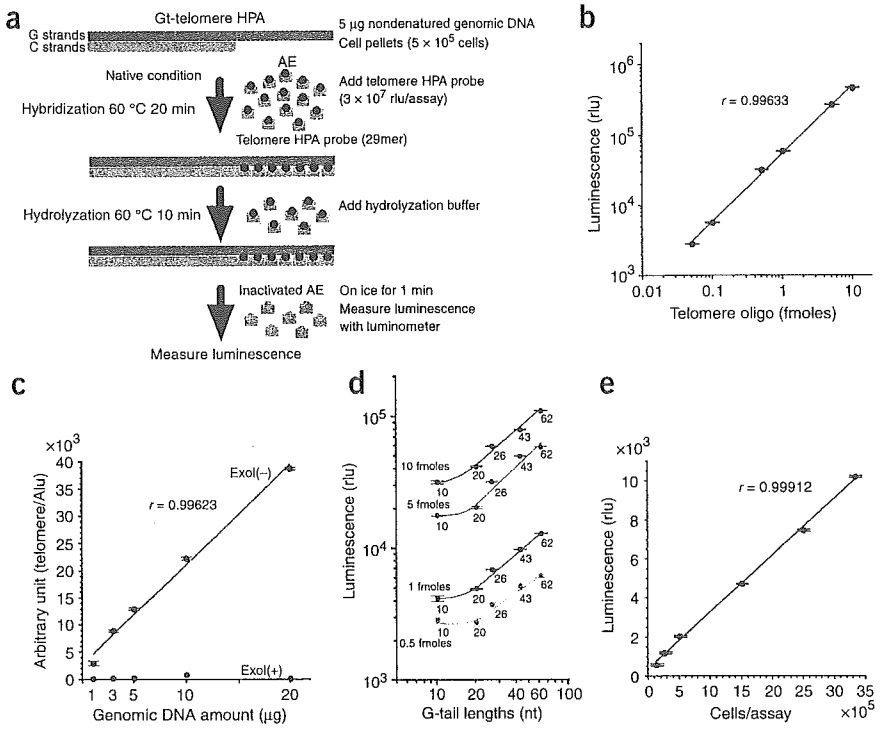

Figure 1 | Strategy, linearity, specificity and sensitivity of Gt-telomere HPA. (**a**) The Gt-telomere HPA strategy (see **Supplementary Methods** online). (**b**) Linearity of Gt-telomere HPA. Hybridization of acridinium ester–labeled telomere HPA probes (29-mer) with the indicated amounts of wild-type telomere 84-mer, 5′-$(TTAGGG)_{14}$-3′. Error bars, s.e.m.; $n = 3$. (**c**) Specific detection of mammalian telomere sequence by Gt-telomere HPA. Genomic DNAs were pretreated with ExoI or without ExoI before G tails were assayed. Data are mean ± range, $n = 2$. (**d**) Quantitative detection of 20-nt G tails by Gt-telomere HPA. In each experiment indicated amounts and indicated sizes of G-tail DNA were used. Error bars, s.e.m.; $n = 3$. (**e**) Detection of G tails in SiHa cell lysates. Data are mean ± range, $n = 2$.

We evaluated the sensitivity of Gt-telomere HPA, especially in terms of the minimal G-tail length that can be detected, using synthetic telomeric DNA constructs with G-tail lengths of 10–62 nt (**Supplementary Fig. 4** online). Despite the fact that the HPA probe is a 29-mer, we obtained HPA signals when we used G-tail DNAs shorter than 29-mer G-tail DNA probe (**Fig. 1d**). We predicted that this was due to the partial hybridization of telomere HPA probe to G-tail DNA as shown in **Supplementary Fig. 4**. Although 10-nt G tails were detectable and ExoI sensitive (data not shown), linearity was obtained only between 20 nt and 62 nt, indicating that Gt-telomere HPA can be used to quantitatively detect 20-nt G tails (**Fig. 1d**). To estimate how many perfect nucleotide pairs in hybrids between telomere repeats and the probe were required for resistance of acridinium ester to alkaline treatment, we hybridized mutant oligonucleotide and mutant HPA probes. We found that mismatches six bases away from the acridinium ester–labeled position did not affect HPA signal (**Supplementary Fig. 5** online).

To measure G-tail lengths in cell lysates, we lysed cell pellets from the SiHa cancer cell line in a high-salt concentration hybridization buffer that contained the detergent, lithium lauryl sulfate. Gt-telomere HPA displayed a good linearity in the range 1×10^5 to 3.5×10^6 cells (**Fig. 1e**). Thus, a sample of 5×10^5 cells is sufficient for measurement of telomeric G tails.

Next, we carried out experiments to evaluate the utility of our new method. First, we measured mean telomeric G-tail lengths in genomic DNA (**Fig. 2a**) and cell pellets (**Fig. 2b**) of normal and hTERT-infected TIG-3 human fibroblasts and of SV40-transformed TIG-3 cells at crisis. Our results (**Fig. 2**) are consistent with the previous reports that human telomeric G tails are reduced during cellular senescence[10] and at crisis in SV40-transformed cells[5]. We observed no reduction of G-tail lengths in human telomerase revere transcriptase, hTERT-expressing TIG-3 cells. Second, we examined the effect of *TERF2*$^{\Delta B \Delta M}$ (that encodes TERF2 lacking both N-terminal basic domain and C-terminal Myb domain), the dominant negative allele of telomere repeat binding protein 2 (*TERF2*, also known as *TRF2*) that is known to reduce the lengths of G tails[11] in SiHa cells. We found that these cells had the expected reduction in their G-tail lengths (**Fig. 2**). These findings are consistent with those of previous reports[1,2,12]. In both of these experiments, the data from cell pellets were consistent with those from purified genomic DNA (**Fig. 2**). Thus, direct measurement using cell pellets is feasible with Gt-telomere HPA. *Alu* elements are a class of short interspersed elements (SINEs) that have expanded to a copy number of more than one million elements in primate genomes. The *Alu*-HPA probe can be used to normalize the amount of total genomic

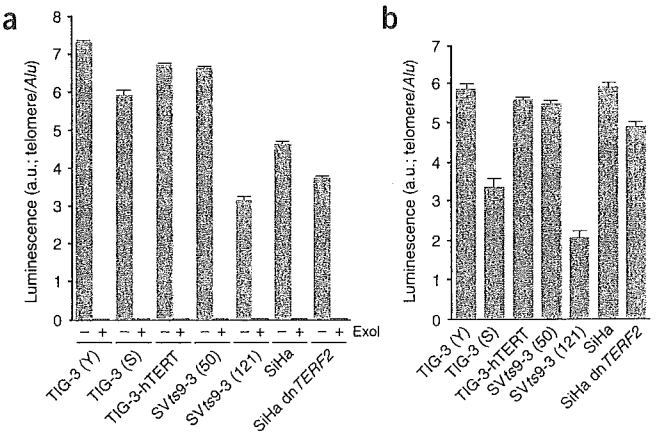

Figure 2 | Mean G-tail lengths in cultured human cells. (**a**) G-tail length measured in purified genomic DNA from human cells either with or without ExoI pretreatment expressed in arbitrary units (a.u.): ((rlu by telomere probe ÷ rlu by *Alu* probe) × 100). TIG-3(Y), normal young at 28 population doubling levels (PDLs); TIG-3 (S), senescent cells at 81 PDLs; TIG-3-hTERT, hTERT-introduced cells; SV*ts*9-3 (50), young SV40-transformed cells at 50 PDLs; SV*ts*9-3 (121), crisis stage cells at 121 PDLs; SiHa, control vector-infected; SiHa dn*TERF2*, dominant-negative allele *TERF2*$^{\Delta B \Delta M}$-infected. Error bars, s.e.m.; $n = 3$. (**b**) Gt-telomere HPA was performed using lysates derived from 5×10^5 cells. Data are mean ± range, $n = 2$.

DNA, even when cell numbers have been adjusted before performing the assay (**Supplementary Fig. 6** online). As the number of *Alu* sequences may vary between individuals, some variation in normalization is possible with our method. Direct measurement from lysates of 5×10^5 or fewer cells may eliminate genomic DNA loss during sample preparation. This may be an advantage when limited numbers of cells are available as occurs, for example, in clinical samples from blood inspection, fine needle aspiration or cancer cells in urine.

Although the rlu values produced by Gt-telomere HPA give only the average luminescence produced by G tails in a cell population, mean G-tail lengths can be estimated from those rlu values using an analytical curve (**Fig. 1b**) and chromosome karyotype information (**Supplementary Fig. 7** online). The estimated average size of the G tails produced by Gt-telomere HPA are comparable to those reported using other methods. Although our method can be used to estimate the difference in mean G-tail lengths between cell populations, if specific G-tail shortening in a specific chromosome was the trigger for a biological effect, then Gt-telomere HPA would not be suitable for identifying this change. Likewise, Gt-telomere HPA could not detect the known differences in G-tail lengths between leading strand and lagging strand ends[2,13]. Despite these limitations, however, Gt-telomere HPA offers other advantages such as large-scale screening. In addition, Gt-telomere HPA will have practical benefits for the basic research in cancer, aging, chromosome and telomere biology.

Note: Supplement ·ry information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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