Generation of digoxigenin-incorporated probes to enhance DNA detection sensitivity

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Telomere length in humans has been correlated with cancer and age-related diseases. The standard method to measure telomere length relies on Southern blot analysis with radioactively or non-radioactively labeled probes containing several telomeric DNA repeats. However, this approach requires relatively large amounts of genomic DNA, making it difficult to measure telomere length when a limited amount of sample is available. Here, we describe a non-radioactive labeling method that uses 3´ fill-in combined with lambda exonuclease digestion to incorporate one or more digoxigenin (DIG) molecules into bridged nucleic acid (BNA)–containing oligonucleotides (ONTs). Using our method, we were able to generate probes to detect both C- and G-rich telomeric DNA strands. Compared with commercially available DIG-labeled telomere probes, probes generated using this new approach significantly enhance the sensitivity of telomere length measurements.

Telomeres, the ends of chromosomes, are composed of tandem 5´-TTAGGG-3´ repeats bound by shelterin proteins to prevent chromosome degradation and end-to-end fusions (1). In humans, telomeres gradually shorten in almost all dividing cells (2), triggering DNA damage responses and cellular senescence that, in combination with additional oncogenic changes, can lead to genomic instability and cancer progression (2–4). Terminal restriction fragment (TRF) analysis directly detects different sizes of telomeres using telomere-specific probes for Southern blotting (5). Traditionally, ^{32}P-labeled telomere-specific oligonucleotides (ONTs) have been used for TRF analyses. However, radioactive probes for filter hybridizations not only have less stability but require relatively long exposure times. Moreover, there are mounting concerns when it comes to hazardous waste disposal and safety issues associated with radioactivity.

Digoxigenin (DIG)-labeled probes are widely used as an alternative to radioactive probes for detecting targeted DNA or RNA. These probes are stable and can be used multiple times without losing activity (6). Moreover, DIG-labeled probes can be more sensitive than ^{32}P-labeled probes when detecting repetitive genomic DNA sequences (7). Therefore, DIG-labeled probes are ideal hybridization probes for telomere detection.

The most common approach to labeling ONTs with DIG is attaching a single DIG molecule to the 3´ or 5´ end of an ONT using either a enzymatic or chemical reaction (2000. DIG Application Manual for filter hybridization. Roche Diagnostics GmbH, Mannheim, Germany). However, the sensitivity of end-labeled probes is lower than that of probes labeled by other methods (8). Although incorporation of DIG molecules can be increased by 3´-end tailing, probe specificity decreases due to addition of nonspecific nucleotides at the 3´ end (8). Here, we have developed a method to incorporate multiple DIGs into telomere-specific ONTs, which increases both the sensitivity and stability of telomere detection.

A schematic and details of our methodology are shown in the Supplementary Material and in Supplementary Figure S1. Initially, we prepared template DNA for DIG-labeled telomere C-rich (TC) or G-rich (TG) probe synthesis by annealing the G-rich or C-rich template ONT to a universal priming ONT. The 5´-phosphorylated template ONT begins with seven C-rich or G-rich telomeric repeats followed by a short non-telomeric sequence (Supplementary Table S1). The universal priming ONT is also 5´-phosphorylated and contains a sequence complimentary to the non-telomeric sequence in the template ONT with additional thymine and adenine bridged nucleic acids (BNAs) at the 3´ end (Supplementary Table S1) such that it anneals to the template ONT in the correct spot. We decided to use BNA-containing ONTs since they have a greater resistance to

METHOD SUMMARY

We present a non-radioactive labeling method for incorporating multiple digoxigenin (DIG) molecules into bridged nucleic acid (BNA)–containing oligonucleotides to intensify detection sensitivity in Southern blot analysis.

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nuclease digestion and a higher affinity for the target DNA or RNA as compared with locked nucleic acids (9,10).

Exo⁻ Klenow Fragment (exo⁻ KF) (New England Biolabs, Ipswich, MA), along with a dNTP mix containing DIG-11-dUTP (Roche Applied Sciences, Mannheim, Germany), was used for 3´ fill-in reactions. After the 3´ fill-in reactions, we applied T4 DNA polymerase (New England Biolabs) for DNA blunting in order to remove the additional nucleotide at the 3´ end from the template DNA generated by 3´ fill-in reactions. This reaction greatly improved the specificity of DIG-labeled telomere probes. To obtain single-stranded telomere probes, lambda exonuclease (New England Biolabs) was used to digest the 5´ phosphorylated template ONT and non-telomeric sequence in the priming ONT (5´→3´ direction). Lambda exonuclease is unable to degrade BNA-containing telomere-specific single-stranded DNA.

To quantitatively compare the sensitivity of the DIG-labeled TC and TG probes to a commercially purchased ONT containing three C-rich telomeric repeats labeled with a single DIG (SD) at the 3´ end (Supplementary Table S1) (Sigma-Aldrich, St. Louis, MO), dot blot assays were performed. One microliter of a serial dilution of each probe (0.78–100 fM) was spotted on the positively charged nylon membrane (Roche Applied Sciences), and labeled probes were fixed on the membrane in a UV crosslinker set at "autocrosslink" (Stratagene, La Jolla, CA). Fixed probes were detected using standard DIG detection procedures (2000. DIG Application Manual for filter hybridization. Roche Diagnostics GmbH, Mannheim, Germany) with an anti-DIG antibody (Roche Applied Sciences) and a chemiluminescence substrate, CDP-Star (Roche Applied Sciences). The chemiluminescence signals of each probe were then visualized using a G:BOX (Syngene, Frederick, MD). Our quantifications showed that 100 fM of the commercial SD probe generated a similar signal intensity to 3.13 fM of our TC and TG probes (Figure 1, B and C), demonstrating the increased sensitivity of our labeling method with multiple DIG molecules incorporated into the primer. We next performed Southern blot analysis (Supplementary Material) with different probes using a 1.6 kb telomeric repeats isolated from pSX-Neo-1.6T2AG3 (11) with digested lambda DNA to test TC and TG probe specificity. The results showed that TC, TG, and SD probes all hybridized to the 1.6 kb telomeric DNA, and no hybridization was detected with digested lambda DNA.

The TeloTAGGG telomere length assay (Roche Applied Sciences) is a commercially available assay that uses a DIG-labeled telomere probe (Telo) for TRF analysis. To further test the performance of SD, TC, TG, and Telo probes in telomere detection, we used varying amounts of genomic DNA extracted from HeLa cells for TRF analysis (see Supplementary Material) with different probes (Figure 2, A and B). After normalizing the signal intensity of each sample to a 4.3 Kb band from the DIG-labeled DNA Molecular Weight Marker II (Roche Applied Sciences), the sensitivities of the TC and TG probes for telomere detection were significantly higher than for the SD and Telo probes (Figure 2C). We should note that the TG probe has 2- to 3-fold higher sensitivity when compared with the TC probe. This finding is, however, consistent with a previous study that found radioactively labeled G-rich probes have ~2-fold better signal compared with C-rich probes for detecting telomeres. One potential explanation for this differential sensitivity may be due to the formation of G-quartets by telomeric G-rich strands, which may reduce hybridization by the TC probe (12).

Here we present a method for generating highly sensitive DIG-labeled telomere specific probes. Although we have only examined the use of this labeling method for TRF analysis, we expect probes generated using this approach will be useful for other telomere applications, such as telomere overhang analysis (13) and

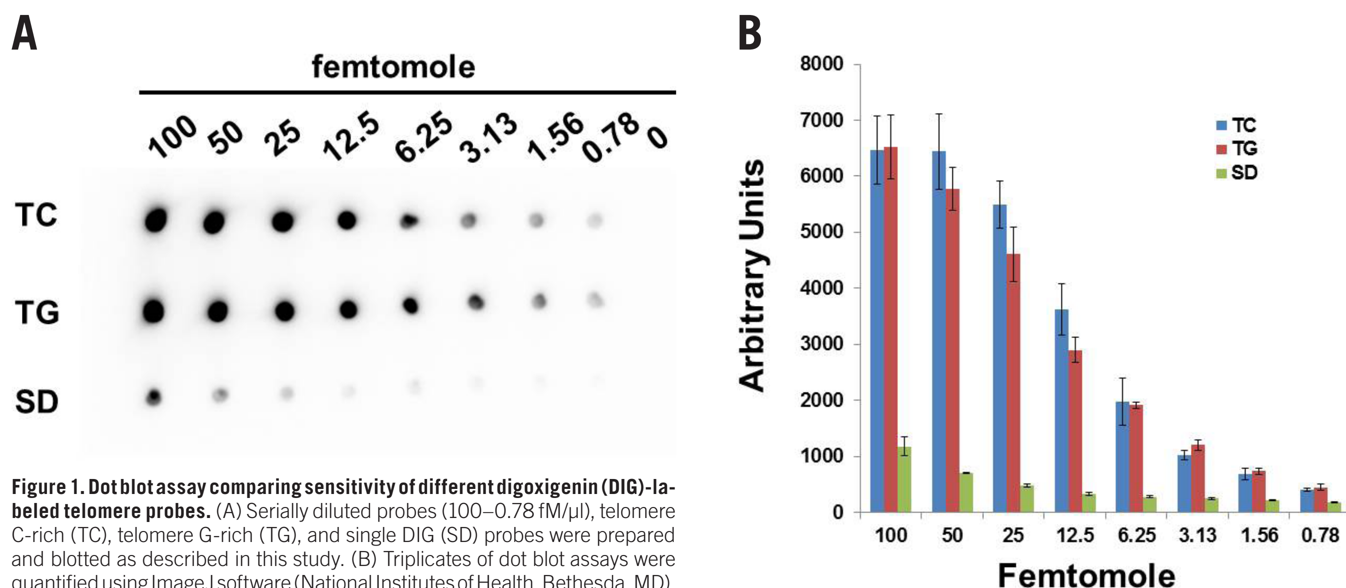

Figure 1. Dot blot assay comparing sensitivity of different digoxigenin (DIG)-labeled telomere probes. (A) Serially diluted probes (100–0.78 fM/µl), telomere C-rich (TC), telomere G-rich (TG), and single DIG (SD) probes were prepared and blotted as described in this study. (B) Triplicates of dot blot assays were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

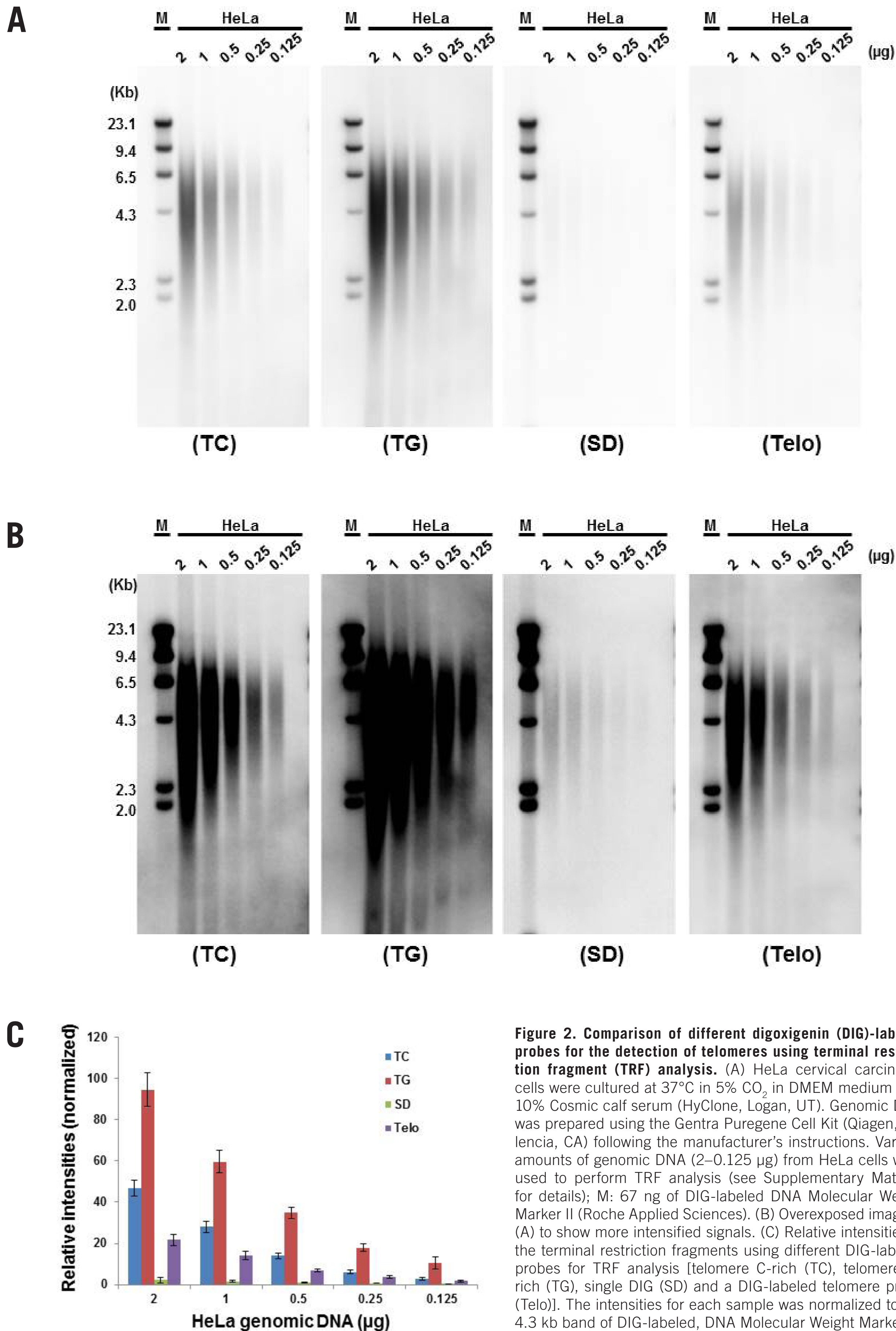

Figure 2. Comparison of different digoxigenin (DIG)-labeled probes for the detection of telomeres using terminal restriction fragment (TRF) analysis. (A) HeLa cervical carcinoma cells were cultured at 37°C in 5% CO_2 in DMEM medium with 10% Cosmic calf serum (HyClone, Logan, UT). Genomic DNA was prepared using the Gentra Puregene Cell Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Varying amounts of genomic DNA (2–0.125 µg) from HeLa cells were used to perform TRF analysis (see Supplementary Material for details); M: 67 ng of DIG-labeled DNA Molecular Weight Marker II (Roche Applied Sciences). (B) Overexposed image of (A) to show more intensified signals. (C) Relative intensities of the terminal restriction fragments using different DIG-labeled probes for TRF analysis [telomere C-rich (TC), telomere G-rich (TG), single DIG (SD) and a DIG-labeled telomere probe (Telo)]. The intensities for each sample was normalized to the 4.3 kb band of DIG-labeled, DNA Molecular Weight Marker II.

single telomere length analysis (14). Finally, our new DIG labeling strategy should also be immediately utilizable in labs outside the field of telomere biology that are interested in increasing the sensitivity of Southern blot applications.

Author contributions

T.P.L. conducted all experiments. T.P.L., W.E.W., and J.W.S. designed the study, analyzed the data, and wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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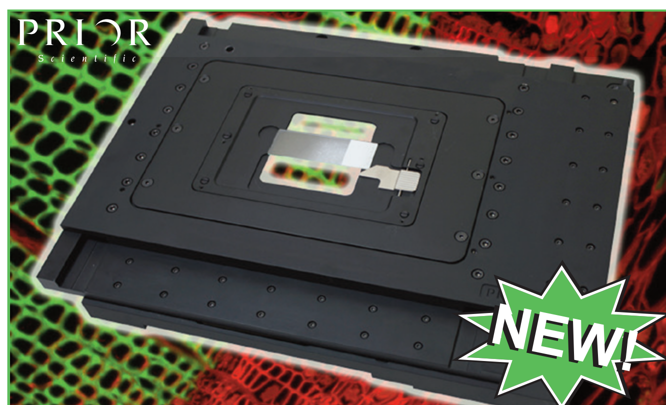

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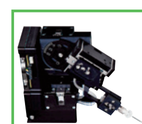
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