Generation of digoxigenin-incorporated probes to enhance DNA detection sensitivity

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BioTechniques 60:306-309 (June 2016) doi 10.2144/000114427
Keywords: oligonucleotide; telomere; bridged nucleic acid (BNA); terminal restriction fragments (TRF)

Supplementary material for this article is available at www.BioTechniques.com/article/114427.

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.

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.
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 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% CO2 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.

Acknowledgments
We acknowledge Henrietta Lacks and her family members for the important contributions “HeLa” cells have made to this work. This work was performed in laboratories constructed with support from NIH grant C06 RR30414. This work was supported by AG01228 from the National Institute on Aging (WEW and JWS) and by a CPRIT Postdoctoral Cancer Intervention and Prevention Discoveries Training Program grant RP140110 (T.P.L.). We also acknowledge the Harold Simmons NCI Designated Comprehensive Cancer Center Support Grant (CA142543), and the Southland Financial Corporation Distinguished Chair in Geriatric Research (J.W.S. and W.E.W.). This paper is subject to the NIH Public Access Policy.

Competing interests
The authors declare no competing interests.

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Received 17 November 2015; accepted 01 March 2016.

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