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3′ cycle-labeled oligonucleotides with predictable length for primer extension and transgene analysis

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ABSTRACT

Efficient labeling of short oligos at their 3′-ends was achieved through polymerase chain reaction. The length of cycled-labeled oligos can be accurately predicted by omitting one or more dNTPs in the labeling step. Thus, labeled oligos can be simply column-purified, eliminating the need for tedious gel purification. We demonstrated the effectiveness of this technique in determining the transcription start site of a given gene and in transgene analysis to differentiate the transcript of an endogenous gene from that of an introduced homologous gene. This technique could be widely extended to other molecular biology applications in which labeled oligos are employed.

Efficient labeling of short oligos with α-^{32}P-labeled radionucleotides at their 3′-ends was achieved through polymerase chain reaction (PCR) based on the working principle of cycle sequencing (1). By omitting one or more dNTPs during the cycle labeling step, 3′-ends of labeled oligos could be accurately defined which obviates the need for tedious gel purification. The use of α-^{32}P radionucleotides in the labeling step also eliminates the exclusive requirement of γ-^{32}P radionucleotide used in the 5′-end-labeling reactions. Moreover, radiolabeled primers can be synthesized directly from plasmid templates with this method. Using this technique, the labeled oligos can be used to hybridize to target RNA for primer extension through reverse transcription after a simple column purification to remove unincorporated nucleotides. Sequencing ladders can also be prepared using the same reagent/enzyme system with the same labeled oligos without purification. This further avoids the employment of additional enzymes, such as T7 DNA polymerase or sequenase. Using this protocol, the transcription start site (TSS) of aspen (*Populus tremuloides* Michx.) caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (OMT) transcript was successfully mapped.

A minus strand primer (primer I), 5′-GGAGTCATCTGAGTTTCACCTGTTG located 5 bp downstream from ATG of the aspen OMT cDNA (2) was synthesized (Integrated DNA Technology) and subsequently incorporated with [α-^{32}P]dATP, [α-^{32}P]dCTP and dTTP, but not dGTP in the labeling reaction. Plasmid DNA containing the 5′-flanking region of the aspen OMT gene (3,4) was used as the template. The labeling was carried out using the Δ*Taq* cycle sequencing kit (USB) with 10 pmol of primer I, 0.05 pmol of template DNA, 1 µl of dTTP mix, 5 µCi of [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), and the following PCR conditions: 80 cycles of 95°C/15 s and 60°C/30 s in a thermal cycler (Perkin-Elmer). After the labeling cycles, 3 µl of the reaction mixture was diluted to 17.5 µl with template, Δ*Taq* polymerase and water for subsequent preparation of sequencing ladders according to the manufacturer's protocol. The rest of the cycle-labeled oligos were column-purified using Chroma spin-10 column (Clontech). Three microliters of purified radiolabeled primer I (1.9×10^6 c.p.m.) were hybridized with total RNA isolated from developing xylem of aspen (5). The hybridization was carried out at 65°C for 1 h in a final volume of 20 µl containing 4 µl of 5× first strand buffer (GIBCO BRL). After brief centrifugation, the resulting primer–RNA hybrids were added to 20 µl extension solution containing 4 µl of 5× first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 2.5 µg/ml actinomycin D (GIBCO BRL) and 100 U of SuperScript^{TM}II (GIBCO BRL) and subjected to reverse transcription at 42°C for 1 h. The extension products were precipitated, vacuum-dried, and resuspended in 5 µl of 0.1 mM NaOH + 1 mM EDTA (6) with 5 µl of loading dye (sequencing stop solution + 10 mM NaOH). After denaturation, 2–4 µl of the products were electrophoresed on a 6% Long Ranger (FMC) sequencing gel in parallel with the sequencing ladders.

The cycle-labeling step incorporated 10 nt into the primer I, of which 7 nt were radioactive, resulting in a primer 35 bases long. Using this cycle-labeled oligo for primer extension, 5′ terminus of aspen OMT transcript could be effectively detected using as little as 5 µg of total RNA in only 3 h autoradiography (Fig. 1A). As seen in this figure, good resolution of sequencing ladders with a long readable length indicates the applicability of this technique to resolve even longer primer extension products. Another primer was thus employed to test the sensitivity of this technique further. This primer (primer II), 5′-CTGGCTAGTTGCATGGCAAAG) is located ~50 bp downstream from primer I and allows only 1 radionucleotide to be incorporated in the labeling step. The labeling reaction was carried out as described above, with the exception that only [α-^{32}P]dATP along with dTTP and dGTP mix were added to the reaction, and 5 µl of purified labeled primer (3×10^5 c.p.m.) was used for primer extension. As seen in Figure 1B, these internally single-base labeled primers could also detect the OMT 5′ terminus after 3 h of autoradiography using total RNA of ≥20 µg. In this case, mapping of the 5′ terminus using a limited amount of RNA should also be achievable with longer exposure times. The 5′ end of the aspen OMT gene transcript was mapped at 124 and 175 nt away from primer I and primer II, respectively,

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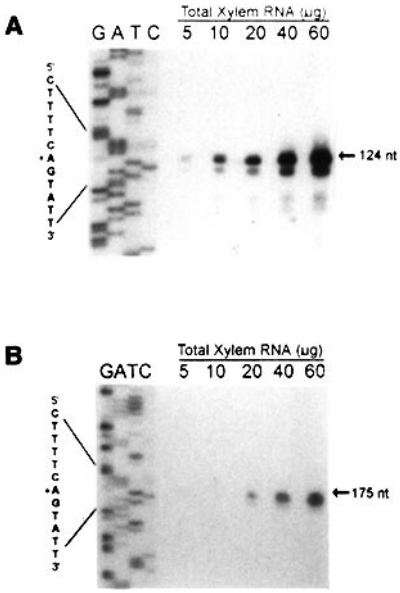

Figure 1. Mapping of the TSS of aspen OMT gene using cycle-labeled oligos of different specific activity with various amounts of total RNA extracted from aspen xylem. (**A)** Primer extension using cycle-labeled primer I. (**B)** Primer extension using cycle-labeled primer II. G, A, T, C indicate the four sequencing reactions. The plus-strand DNA sequences are shown and the TSS (A*) is indicated as 124 and 175 nt, representing the length from the TSS to the end of the primers I and II, respectively.

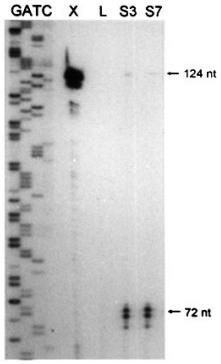

Figure 2. Transgene analysis by primer extension using cycle-labeled primer I. Lanes G, A, T, C indicate the sequencing reactions; lanes X and L, primer extension reactions of total RNA extracted from xylem and leaf tissue of wild-type aspen, respectively; lanes S3 and S7, primer extension reactions of total RNA extracted from leaves of two transgenic aspen over-expressing OMT. The transcription start sites are indicated by arrows. The numbers represent the length from the transcription start site to the end of primer I.

giving a consistent result of adenine as the TSS according to the OMT gene sequence (4). A putative TATA box was found 31 bp upstream from the TSS.

The same technique was also successfully applied in the analysis of transgenic aspen to differentiate the expression of endogenous OMT gene from that of an introduced homologous OMT cDNA (7). The endogenous OMT is preferentially expressed in developing xylem but poorly expressed in leaves (2). When the cycle-labeled primer I was used in the primer extension analysis, 5′ terminus of the transgene was mapped in leaves of two transgenic plants (S3 and S7) at 72 nt upstream from primer I, but not in leaves of wild-type (L) aspen, as shown in Figure 2. Whereas, the TSS of the native OMT gene was mapped at 124 nt upstream from the same primer in xylem (X) of wild-type aspen. A very low level of signal at 124 nt could also be seen in wild-type and transgenic leaves (L, S3 and S7) due to the expression of endogenous OMT gene in midveins of leaves. A 40 bp 5′-untranslated leader was identified for the introduced OMT gene as expected according to the organization of OMT fusion gene in the binary vector (7,8). It is therefore conceivable that the expression of introduced OMT gene in xylem of transgenic plants could not be explicitly distinguished from the signal of native OMT transcripts in a Northern blot (data not shown), because of the strong expression level of the endogenous OMT gene, which has a similar size to the introduced OMT gene. Thus, primer extension analysis would be the best, if not the only, alternative to Northern analysis for verifying the expression of homologous transgenes in tissue where the corresponding native transcripts are abundant. Under this circumstance and in the context of rapidly increasing research efforts on engineering transgenic plants, the simplicity and the effectiveness of our method for oligo labeling should be a timely addition to the general molecular biotechniques.

The labeling technique presented here offers a straightforward and easy-to-perform alternative to the existing ones. In our method, oligos can be efficiently labeled with predictable length and desired level of specificity. Since 3′-ends of radiolabeled oligos can be accurately defined, this protocol facilitates the use of plasmid DNA rather than the preparation of a specific PCR fragment as the template in the labeling reaction. The irritating problems in generating labeled oligos of varied lengths usually associated with other PCR-based techniques are also overcome, which further eliminates the need for gel purification. Although this study demonstrated the effectiveness of applying [α-^{32}P]dNTP for oligo labelling, radionucleotides with lower emission energy isotopes, or non-radioactive nucleotides should also be adequate in this technique. The protocol could be further modified to use any thermostable DNA polymerase of choice in the primer labeling reaction, if cycle sequencing is not regularly performed in the lab. In this case, the preparation of sequencing ladders using the labeled oligos needs to be done according to any routine methods employed in the lab. This oligo labeling technique could also be applicable to other molecular biology studies, such as oligo hybridization and *in vitro* transcription.

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