is usually not affected if the extraction is done within a few minutes. We simultaneously obtained good yields of high quality DNA and RNA in a very short time period.

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Reducing "Double Sequences" in Automated DNA Sequencing with T7 DNA Polymerase and Internal Labeling

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We describe a modification of the standard automated DNA sequencing protocol with T7 DNA polymerase, unlabeled primers and internal labeling (3) that helps to largely overcome ambiguous sequences that are occasionally observed. The ambiguous traces result from annealing of primers to secondary sites on the template and the subsequent generation of two or more different sequencing products. Such traces usually cannot be interpreted. These so-called "double sequences" appear mainly when unknown template DNAs are sequenced where secondary binding sites cannot be excluded upon design of the primers. They become increasingly prominent when sequencing large templates. The modification of the standard protocol described here is based on improved primer design (4), which is adapted to the labeled dNTP used in the reaction.

The sequencing of reactions with unlabeled primers and internal labeling with T7 DNA Polymerase (Autoread™ kit [Pharmacia Biotech, Uppsala, Sweden] and Sequenase® [United States Biochemical, Cleveland, OH, USA]) are usually carried out in two steps. (i) In the labeling step, primers are extended by the addition of a labeled dNTP (e.g., fluorescein-15-dATP). In standard reactions, the other, unlabeled, dNTPs (dC, G and TTP) are present in low concentrations (60 nmol/L). This is to achieve labeling, even when the first position, where the labeled dNTP can be incorporated, is located two or more bases downstream of the primer binding site. Alternatively, fluorescein-labeled dCTP (Du Pont NEN, Boston, MA, USA) can be used in sequencing reactions. (ii) In the extension/termination step, the products are extended in the presence of all unlabeled dNTPs in high concentration until a dideoxynucleotide is incorporated.

We have previously shown that the

Benchmarks

quality of the sequence signals increases when the labeled dNTP is incorporated as close to the primer as possible (4). This has led us to design primers where the labeled dNTP (dATP or dCTP) is incorporated directly downstream of the primer. One advantage of this practice is the consistently strong sequences that are generated. Increased specificity of labeling is another improvement, achievable by eliminating all unlabeled dNTPs in the labeling reaction.

Despite careful primer design that takes into account, for example, the binding profile of primers (1) and possible hairpin loop formation (Gene-Skipper [EMBL, Heidelberg, Germany] and Oligo 4.1 program [Medprobe, Oslo, Norway]), we occasionally observe the occurrence of "double sequences". These are due to secondary binding of primers to their templates (Figure 1A) when sequencing reactions are carried out with the standard labeling mixture (Pharmacia Biotech). This mixture not only contains fluorescein-labeled dATP (10 $\mu\text{mol/L}$) but also other, unlabeled, dNTPs (1 $\mu\text{mol/L}$). However, when the standard labeling mixture is replaced with 10 $\mu\text{mol/L}$ of either fluorescein dATP (Pharmacia Biotech; Boehringer Mannheim

[Mannheim, Germany]) or fluorescein dCTP (Du Pont NEN), in most cases the resulting sequence is unambiguous as shown in Figure 1B with the same primer/template combinations.

Presuming a GC content of 50%, in three out of four cases the next base 3' of the primer will not be an "A" but rather "C", "G" or "T" at a secondary binding site. In these instances, no label will be incorporated when only labeled dATP is present in the labeling step. Binding of the primer to these sites will be silent and the sequence resulting from the correct binding site will be unambiguous. We have tested this presumption with 17 primers that had led to ambiguous sequences with the standard protocol. The primers were used again in sequencing reactions with only fluorescein dATP in the labeling reaction. Fourteen of these sequences were unambiguous when reactions were repeated with the modified protocol.

This was, however, only true when sequencing reactions were carried out with primers that do not carry an "A" as 3'-terminal base or when native T7 DNA polymerase was replaced with Sequenase. Native T7 DNA polymerase carries 3'→5' exonuclease activity which would replace the 3'-terminal unlabeled nucleotide (e.g., "A") of the

primer with a labeled nucleotide (4). This would also be the case in the sequence shown in Figure 1. Sequenase (Version 2.0), in contrast to native T7 DNA polymerase, does not have 3'→5' exonuclease activity (2) and sequencing reactions carried out with Sequenase lead to clear sequences (Figure 1B).

We conclude that sequencing reactions are best carried out (i) when the labeled dNTP is incorporated immediately downstream of the primer, (ii) with only the fluorescently labeled dNTP being present in the labeling step and (iii) with Sequenase instead of native T7 DNA polymerase if the primer terminates with the same nucleotide that is employed for labeling.

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Figure 1. Sequencing reaction carried out with Sequenase and labeling mixture (A) or fluorescein-15-dATP (B) present in the labeling step. Sequencing products were analyzed on an A.L.F.TM DNA Sequencer (Pharmacia Biotech). The bases were called automatically and were extended in the 5' direction to also include the primer sequence (underlined).