Comparison of DNA Polymerases for Quantification of Single Nucleotide Differences by Primer Extension Assays


Differential termination of primer extension can be used to detect and quantify previously characterized single nucleotide differences in DNA (3) or RNA (1,2). The method relies on the extension of a labeled primer with its 3′ end situated just 5′ to the site of the divergent nucleotides. Differential termination is effected by selectively limiting the complement of dNTPs (3), possibly by the incorporation of a dideoxy analog at the site of the sequence variation (1,2). Using a standard method (2) on PCR products from genomic DNA, we observed an imperfect termination of the extension products, a phenomenon deleterious to any quanti-
tative analysis. To choose the optimal enzyme among a variety of polymerases that have been used for the analysis (1–3), in this study we compare the activities of the Klenow fragment of DNA polymerase I, AMV reverse transcriptase and modified T7 DNA polymerase for quantitative assays of primer extension on plasmid templates.

For comparative studies of different DNA polymerases in quantitative primer extension analysis, we applied the assay to a plasmid DNA template to distinguish between a wild-type G and a mutated A at a single site in the sequence (Figure 1). The 18-mer extension primer anneals with the 3' end, four residues away from the variable site. Extension in the presence of ddATP, dGTP, dCTP and dTTP results in either of two radiolabeled products, a 24-mer from a wild-type template or a 22-mer from a mutated template, because the reaction is expected to terminate at the site of incorporation of the dideoxy analog. The 18-mer extension primer (Figure 1) was end-labeled using γ-32P labeled ATP and T4 polynucleotide kinase, purified on a 15% polyacrylamide gel and annealed to a heat-denatured plasmid template by incubation for 15 min at 37°C in 10 µL of the respective extension buffers [Klenow large fragment of DNA polymerase I: 10 mM Tris, pH 8.0, 5 mM MgCl2, 50 mM KCl; AMV reverse transcriptase and modified T7 DNA polymerase (Sequenase™ version 2.0) as supplied with the enzymes. All enzymes were purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden]. Extension was carried out for 10 min at 37°C in the presence of 5 µL of extension mix [1 mM dideoxyATP (Amersham Pharmacia Biotech), 0.1 mM each of deoxyCTP, deoxyGTP and deoxyTTP, and either 1 U of Klenow enzyme, 6 U of reverse transcriptase or 3 U of modified T7 DNA polymerase], terminated by the addition of 10 µL of formamide and heated to 95°C for 2 min. The samples were analyzed on 18% polyacrylamide gels, and the radioactivity was measured in a PhosphorImager® SF (Amersham Pharmacia Biotech).

Extension with the Klenow fragment on wild-type template results in the 24-mer (Figure 2, lane 7), but also, readthrough products of 30 and 32 nucleotides appear that correspond to termination at the subsequent adenosines. Incomplete termination is apparent also on the mutated template (lane 8), with the major 22-mer constituting approximately 95%. The same bands appear with the Klenow fragment from a different batch (lane 9). Lowering the concentration of the Klenow enzyme by a factor of 10 does not eliminate the readthrough product (data not shown). On the contrary, reverse transcriptase and modified T7 DNA polymerase extend no longer than to the first adenosine (lane 10 and 11), given a detection limit of 0.2% of the most intensive...
band. However, reverse transcriptase appears to terminate prematurely, as evidenced by the extra band between the primer and the 24-mer. Since reverse transcriptase does not discriminate strongly between deoxy and dideoxynucleotides (4) and lacks 3′–5′ exonuclease activity, this termination may be caused by misincorporation of a ddATP, but it could also result from structural impediments on the template.

In other cases with reverse transcriptase, this intermediate band was absent or less pronounced (data not shown), but it was never seen with the modified T7 DNA polymerase. While the 3′–5′ exonuclease activity of the Klenow fragment results in digestion of residual primer (lanes 6–9), it may also play a role in the readthrough activity of the enzyme, if a ddATP is removed and replaced by a misincorporated nucleotide, which allows for further extension. Also, misincorporation in the first place could be the cause of readthrough, as the Klenow fragment discriminates 700-fold against dideoxyATP relative to dATP (5). Notably, a test of the Klenow fragment, without the addition of the dideoxy analog, still showed a minor readthrough product (data not shown).

The modified T7 DNA polymerase has been chemically or genetically modified to improve its properties for use in dideoxy sequencing reactions. These properties include high processivity, low 3′–5′ exonuclease activity and efficient incorporation of nucleotide analogs, with the enzyme preferring deoxy to dideoxynucleotides by a factor of two (4). Evidently, these properties are also valuable in primer extension analysis; modified T7 DNA polymerase is the only enzyme tested here, which results in a single, properly terminated extension product. This is true also for enzyme concentrations that are three to six times as high (data not shown). Low or missing proofreading activity of an enzyme may be a concern when they are used in quantitative assays, but when combined with a high ability to incorporate the dideoxy analog as in the modified T7 DNA polymerase, it appears to be optimal. Batch-to-batch variation might occur. With a single batch, we have seen weak, nonspecific bands and retention of label in the slots, problems that might be eliminated by phenol extraction of the samples.

In summary, we have compared the activities of the Klenow fragment of DNA polymerase I, AMV reverse transcriptase and modified T7 DNA polymerase in differential termination of primer extension. We found the modified T7 DNA polymerase to be superior because it results in a single, properly terminated extension product.

REFERENCES


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