Benchmarks

Protocol

1. The project is best started with a base by cutting an approximately 8 × 26 cm wood board. Place two right-angled shelving braces at one end of the base, with the brace ends facing each other and touching. The braces will serve as supports and the rotisserie motor will be mounted on the braces (Figure 1). Fasten the braces to the base with 3/16 × 1" bolts.
2. From the rotisserie kit, attach the rotisserie brackets to the shelving braces. Adapter pieces supplied with the kit are not needed. Fasten the rotisserie mounting brackets to the shelving braces with the supplied bolts. Ensure the bracket into which the motor inserts is on the side of the base left vacant by the fastened braces (Figure 1). It may be necessary to drill additional holes in the braces to fix and secure the rotisserie mounting brackets in place.
3. Cut the rotisserie rod to a length of 24 cm. Cut a 4 cm length from the 4 × 4 cm wood beam. With a router, bore a square slot the same width as the rotisserie rod in the wood block. Alternatively, a round hole can be bored with a drill. Fasten the grip clips to the remaining four sides of the wood block.
4. Place the wood block in the center of the rod and secure in place. Silicone or epoxy will serve well to fix the block in position. Attach rubber feet to the bottom of the base.
5. Mount the motor and insert the rod. Insert test 50 mL conical bottom centrifuge tubes on the initial use to ensure the apparatus has a level operation.

cal rpm range is between three and seven, depending on the rotisserie motor kit you choose. Widely available power bars can serve as an on/off switch for motors not equipped with their own.

Membranes are inserted into the 50 mL tubes. The surface carrying the nu-

cleic acids or proteins should face inwards. Membranes should be moist to prevent breakage and air bubble formation from surface tension. To start the operation, reagent solution is added to the tube, which is then capped and inserted into a grip clip for incubation.

Regular procedures for western, northern, northwestern or Southern blotting, including washing, can be used with this apparatus. This apparatus saves time, mess, expensive reagents and can substitute for relatively expensive equipment at room temperature or 65°C inside a dry oven. Results are comparable to those obtained with a commercial rotisserie apparatus.

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

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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-

Figure 1. Rotisserie hybridizer. (1) Rotisserie motor; (2) wood block; (3) grip clip; and (4) shelving braces.

Benchmarks

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 - ^{32}P 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 MgCl_2 , 50 mM KCl; AMV reverse transcriptase and modified T7 DNA polymerase (SequenaseTM 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 nu-

cleotides 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 con-

centration 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

Figure 1. Schematic representation of the primer extension assay. In the presence of ddATP, the end-labeled primer will be extended into a 24-mer if the plasmid template contains the wild-type (upper bold C) nucleotide, or into a 22-mer if it has the mutated (lower bold T) nucleotide at the position indicated by the shorter arrow.

Figure 2. Primer extension with different DNA polymerases. Lanes 1–4 show a dideoxy sequencing ladder of the relevant area in a mutated template; the primer is shown as an arrow. Sequencing was done with the modified T7 DNA polymerase as recommended by the supplier except that annealing of the end-labeled primer was as described for the extension assay. Lanes 5–11 show the primer extension analysis in the presence of the template and enzyme as indicated on top of each lane. At the top, the (-) indicates a no enzyme/template; K = the Klenow fragment of DNA polymerase I; K* = the Klenow enzyme from a different enzyme batch; RT = reverse transcriptase; T7 = modified T7 DNA polymerase; Wt is wild-type plasmid template; and Mut = the template with a point mutation. The straight lines (—) on the right point to residual primer, 22-mer from mutated template, 24-mer from wild-type template, and 30- and 32-mer readthrough products, respectively.

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

1. **Dabiri, G.A., L. Fang, R.A. Drakas and K. Nishikura.** 1996. Editing of the GluR-B ion channel RNA in vitro by recombinant double-stranded RNA adenosine deaminase. *EMBO J.* 15:34-45.
2. **Egebjerg, J., V. Kukekov and S.F. Heinemann.** 1994. Intron sequence directs RNA editing of the glutamate receptor subunit GluR2 coding sequence. *Biochemistry* 91:10270-10274.
3. **Picketts, D.J., C. Cameron, S.A.M. Taylor, K.V. Deugau and D.P. Lillicrap.** 1992. Differential termination of primer extension: a novel, quantifiable method for detection of point mutations. *Hum. Genet.* 89:155-157.
4. **Tabor, S. and C.C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* 84:4767-4771.
5. **Tabor, S. and C.C. Richardson.** 1995. A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc. Natl. Acad. Sci. USA* 92:6339-6343.

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