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An improved sequencing method using Sequenase™ that is independant of template concentration

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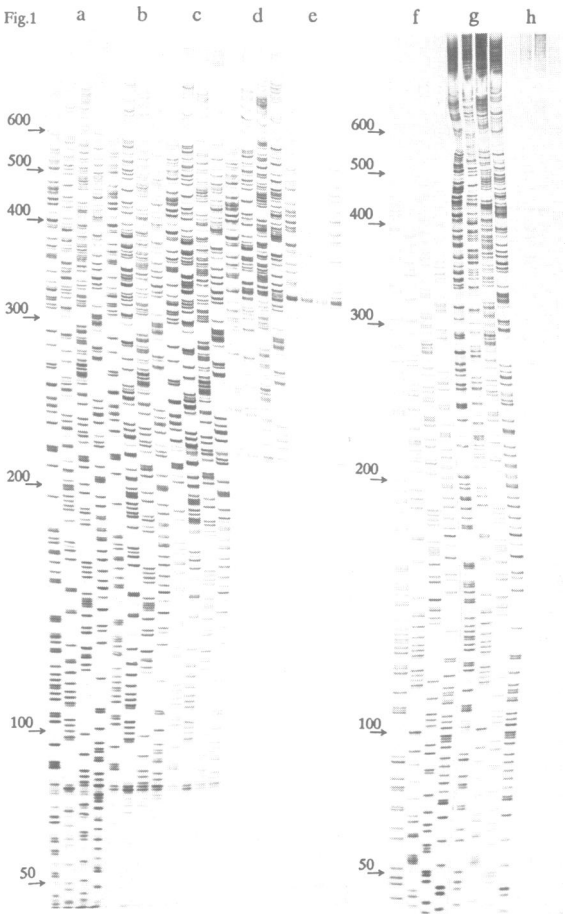
The recommended protocol for Sequenase suffers from severe dependence on relatively large (1-2μg) and constant amounts of template. At lower template concentrations, band intensity close to the primer becomes weak and sequence becomes unreadable (Fig.1, a-e: 1 μg, 500, 250, 100, 60ng template respectively). This arises because the method uses limiting concentrations (0.2μM) of dNTPs to provide a set of extended fragments of appropriate length. Lower template concentrations increase the ratio of dNTP:template and thus increase the mean length of the extended products. This is a major disadvantage in any large sequencing project as it is frequently difficult to obtain high yields of some clones, particularly those with large inserts. We have used an improved protocol that generates a more uniformly labelled sequence ladder in the range 1 to 600 nucleotides using 60-600ng template.



-nplate DNA was annealed to 0.2pmol primer (Biolabs #1224) and a dCTP-minus extension was carried out using 2.5μCi ^{35}S-dATP; dGTP plus dTTP both at 0.2μM; 6 mM dithiothreitol and 2 units of Sequenase (5 mins, 37°C). 2 μl of this reaction were added to 2 μl of each termination mix (80 μM dNTPs, 8 μM ddNTPs; USB kit) and the reaction incubated for a further 5-10 mins at 37°C. This method results in addition of 9 nucleotides, including four ^{35}S-dATP residues in every extended molecule (1st stage), followed by base-specific termination to provide products of which the length is determined purely by the dNTP:ddNTP ratio (2nd stage). Using the USB termination mixes we obtain readable sequence from 1 to >500 nucleotides with 200ng template (Fig.1f). Altering the ratio from 10:1 to 100:1 (dNTP:ddNTP) gives a more evenly distributed sequence ladder (1 to >600) using 600ng or 60ng of template (Fig.1g & h respectively). This protocol works well with other universal and specific primers, although it may be necessary to omit an alternative nucleotide in the "minus" reaction.

Reference: Tabor, S. and Richardson, C.C. (1987) *Proc. Natl. Acad. Sci., USA*, **84**, 4767-4771. Sequenase™ is a trademark of United States Biochemicals.