A simple method for sequencing the complementary strand of ssDNA from M13 clones

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The genomes of bacteriophage M13 and related filamentous phages can be purified from the virion as (+) strand ssDNA which is highly suitable for sequencing by the chain termination method (1, 2). However, existing methods for obtaining the (−) strand are difficult to apply routinely to large numbers of clones (3). We describe a method based on primed synthesis of the minus strand which has three main features: firstly, the forward primer extension step is carried out in the presence of a limiting amount of dNTPs, so that most of the nucleotides are incorporated into the newly synthesized (−) strand. Secondly, the concentration of the sequencing nucleotide mix is high enough to prevent any residual dNTPs from the previous step from unacceptably altering the dNTP/ddNTP ratio. Thirdly, the use of labelled primers rather than nucleotides excludes detection of any residual forward primer extended during the sequencing reaction. These features obviate the need to purify the template prior to sequencing. Instead, the sequencing nucleotide mix consisting of deoxy- and dideoxynucleotides is added directly following the extension step, together with reverse primer.

The method as implemented is based on linear amplification sequencing using Taq polymerase (4), performed on templates prepared in 96 well microtitre plates (5). The amount of DNA used was estimated to be 0.03 to 0.05 pmol per base-specific reaction. The amount of dNTPs added for the extension step (200 pmols) was calculated to allow approximately 4000 nucleotides per template to be incorporated. Sequencing reactions were fractionated and read using an Applied Biosystems 373A DNA sequencer (software version 1.0.2).

Step 1 (extension). 2 μl template DNA was added to individual wells of a heat resistant microtitre plate (Techne FMW11). An extension mix was prepared on ice containing (per clone): 8 μl 10×Taq buffer (100 mM Tris−HCl pH 8.5, 500 mM KCl, 15 mM $MgCl_2$), 4 μl 50 μM dNTP mix, 0.8 pmol unlabelled forward primer, 2 U Taq DNA polymerase (Cetus), and water to 55 μl; 13 μl was added to each well. The reactions were overlaid with a drop of paraffin oil, the plate was covered with a lid, centrifuged briefly, and placed on a pre-heated thermal cycler (Techne MW-1 or PHC-3). The reactions were cycled once at 92°C 1 min, 55°C 2min, 72°C 3 min. *Step 2 (cycle sequencing).* The plate was removed from the cycler and 4μl of the appropriate 5×termination mix (A mix, 0.8 mM ddATP; C mix, 0.8 mM ddCTP; G mix, 0.16 mM ddGTP; T mix, 1.25 mM ddTTP; each mix also contains 25 μM of each dNTP) and 2 μl of 5′-labelled reverse primer (0.125 pmol/μl) were added the sides of the wells, above the oil. The plate was centrifuged briefly and returned to the heat block for 30 cycles of 92°C 1 min, 55°C 2 min and 72°C 2.5 min. The four reactions for each clone were then pooled and ethanol precipitated prior to loading on a sequencing gel.

A total of 53 recombinant M13 clones were sequenced using this procedure, of which 50 yielded accurate sequence data. While shorter reading lengths were observed in some cases, the average reading length was nonetheless close to that observed for the forward reaction (397 versus 416 nucleotides for the same fifty clones). The average insert length was 1000 bp. The wide range of insert sizes in the sample, 317 to 3780 bp, further demonstrates the robustness of the method. We have also used ^{32}P-labelled primers, and the approach should prove suitable for multiplex DNA sequencing (6, 7).

There are two primary advantages of this procedure in relation to large scale sequencing projects using M13 cloning vectors. Firstly, the number of recombinant clones required to complete a project can be reduced substantially, producing significant savings in labour. Secondly, the boundaries of the insert DNA within the vector are accurately defined, which should benefit directed sequencing strategies in the later stages of a project.

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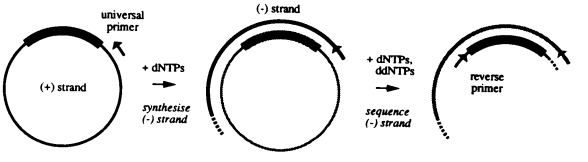

Figure 1. Principle of reverse primer sequencing (3). Universal primer is annealed to M13 (+) strand ssDNA and extended to synthesise the (−) strand, which is then sequenced using reverse primer. The insert to be sequenced is shown as a shaded box.