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# Large-scale production of DNA sequencing templates by microtitre format PCR

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Large scale DNA sequencing projects require gel readers with high throughput and good base-calling accuracy with as little manual intervention as possible. The automatic sequencing machines now available meet these requirements quite well, indeed, a single ABI 373A machine can read at least around 500 samples/200 000 nt per week. Thus, a full-time sequencing laboratory will need to produce thousands of sequencing templates per week, in order to make full use of several ABI machines. Efforts to address the problems of template production have focussed on automating the processing of miniprep cultures (1–5).

PCR methods (6–9) obviating the processing of miniprep cultures would offer a great time saving but as yet, reliable large scale methods are not available. One problem is to remove excess primers and nucleotides as well as larger amounts of truncated amplification products from the PCR reaction prior to sequencing. So far complicated clean-up procedures have been suggested which are expensive and not compatible with large numbers of templates. Another problem is the set-up of PCR reactions in a convenient format with as little manual interference as possible.

We present a fast and reliable method for sequencing plasmid and M13 phage libraries based on PCR production of seqencing templates in microtitre dishes, from cultures stored and grown in the same format. This method consists of four steps: (1) Pick colonies or plaques into microtitre dishes and grow overnight; (2) Aliquot PCR mix with multi-channel pipette into PCR

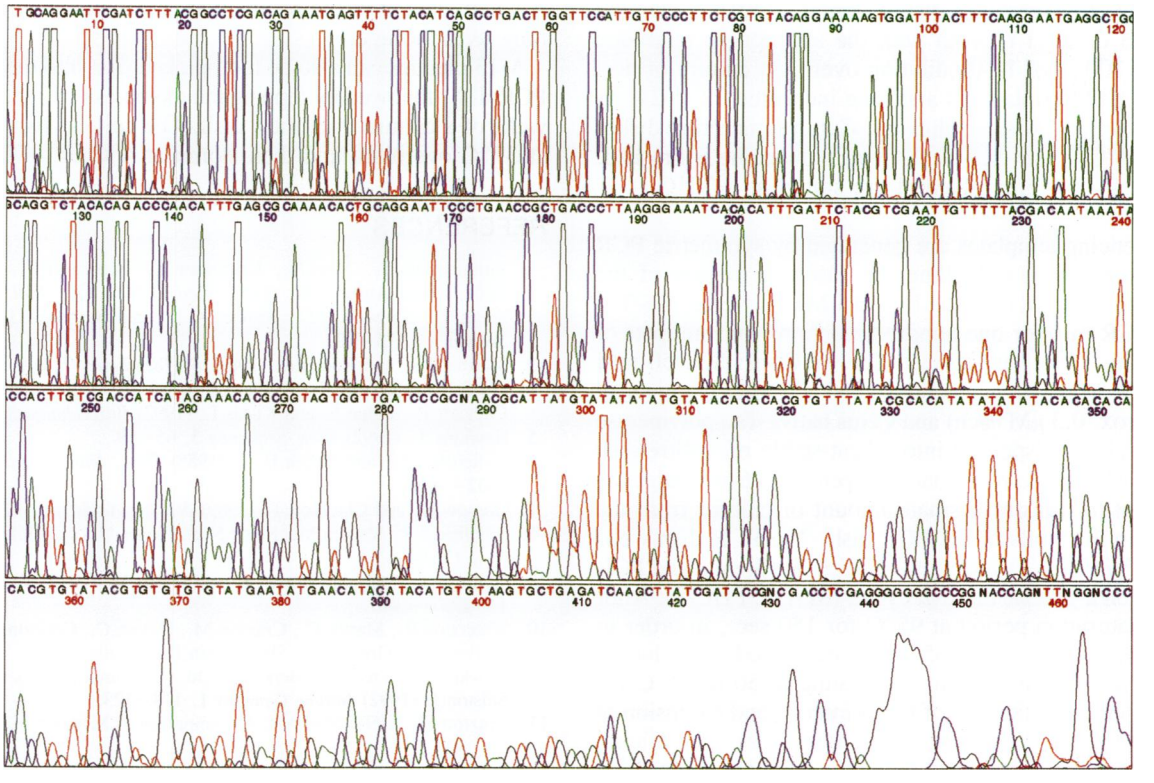

**Figure 1.** Typical fluorescent plot of a PCR template sequenced with dye terminators. The template V-H12 is one of a collection of 576 recombinant clones obtained by cloning of *Alu*I-digested genomic DNA from the Japanese pufferfish *Fugu rupripes rupripes* into the *Eco*RV site of Bluescript II KS vector. Template DNA was generated by PCR with M13 (−21) forward and reverse primers as described in the protocol. It has a 396 bp insert (nt 15 to nt 410) and was sequenced with SK primer.

microtitre dish, load DNA using a 96-pin hedgehog device, and run PCR; (3) Remove excess primer, nucleotides and low molecular weight truncated PCR fragments by one step precipitation of templates with polyethylene glycol (PEG); (4) Cycle sequence PCR products using e.g. dye-terminator or dye primer chemistry on the ABI 373A sequencer.

Lambda and M13 phage libraries may also be handled by a modification of steps 1 and 2.

We have used the method for sequencing around two thousand PCR templates obtained from both plasmid and phage libraries. The success rate of the method (number of PCR templates with inserts greater than 250 bp/number of high quality sequence reads) was greater than 90%. The method has the following advantages. PCR amplification is only limited by the number of available PCR machines and a single worker can easily produce between 300 to 900 templates per day. No conventional miniprep of M13, plasmid or lambda DNA is required. PEG precipitated templates can be sequenced from both ends, each read yielding up to 400 nt of highly accurate sequence (Figure 1). Bacterial stocks and PCR templates for sequencing are conveniently stored in microtitre dishes. The PCR templates can also be used for directed sequencing using custom primers. Thus, our method is suitable for large scale sequencing projects. Most of the protocol (steps 1, 2, and 4) can be further automated by robotic handling of colony/plaque picking and reagent dispensing. Step 3, involving high speed centrifugation in microtitre dishes will be more difficult to automate.

## PROTOCOL

*Step 1.* Recombinant colonies or plaques are toothpicked into separate wells of a microtitre dish (Corning 25850) containing 100 $\mu$l of TB or 2×TY broth with the appropriate antibiotic (colonies) or 100 $\mu$l of 1/100 dilution overnight culture of host cells in 2×TY (M13 plaques) and then incubated 12 to 24 hrs with lids on, at 37°C without shaking. Culture microtitre dishes are stored at 4°C for several weeks until sequencing is finished. Replica dishes containing glycerol are stored at −70°C.

*Step 2.* Sequencing templates are generated by symmetric PCR using universal primers flanking the insertion region of the plasmid/phage e.g.: M13 −21 forward/M13 reverse, T3/T7, KS/SK. A PCR mix for one hundred reactions (or multiples of this) containing 1×buffer (10 mM Tris−HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), nucleotides (250 $\mu$M each), universal primers (approx. 0.3 $\mu$M each) and Cetus native Taq polymerase (0.5 U per well) is dispensed into a heat-stable microtitre dish (Techne FMW1) using a multichannel pipette. A 96-pin hedgehog device is used to transfer a small amount of culture from the culture dish to the wells of the PCR dish. The PCR dish wells are then overlaid with 20 $\mu$l of light mineral oil (Sigma) and PCR is performed on a Techne thermal cycler (MW-1, PHC-3). After an initial denaturation period at 95°C for 150 sec., in order to free some template DNA, 30−35 cycles are carried out including denaturation at 95°C for 1 min., annealing at 50 to 55°C for 1 min. (depending on the Tm of the primers), and extension at 72°C for 1−3 min. (depending on the insert size). An aliquot of the PCR product is examined by agarose gel electrophoresis to estimate insert size and check the specificity of the PCR.

*Step 3.* A large portion of the aqueous phase of the PCR products is transferred to 0.5 ml snap-cap tubes containing an equal volume of PEG mix (26.2% PEG 8000, 6.6 mM $MgCl_2$, 0.6 M NaOAc pH 5.2) (10), and then mixed thoroughly. The PEG mix is dispensed in advance using a multiple pipettor (eg. Eppendorf 4780). The PEG mixture selectively precipitates DNA of more than 100 bp to 150 bp, leaving residual primers, nucleotides and truncated PCR products in the supernatant. After 5 min at room temperature, samples are spun at 13000 rpm for 5 min. and the supernatant is carefully removed with a yellow tip, avoiding the usually invisible DNA pellet. Pellets are washed once with ethanol and air dried, then dissolved in water. The final volume of the template depends on the volume of the PCR reaction e.g. PCR products from a 30 $\mu$l reaction are dissolved in 10 $\mu$l $H_2O$ and PCR products from a 50 $\mu$l reaction are dissolved in 30−50 $\mu$l $H_2O$.

*Step 4.* Cycle sequencing is performed in microtitre dishes using dye terminators (Taq DyeDeoxy™ Terminator Cycle Sequencing Kit from Applied Biosystems) or dye primers (11) (Applied Biosystems or home-made). With dye terminators, 96 templates can be sequenced in a single microtitre dish and excess dye terminators are removed 24 samples at a time by gravity chromatography using a perspex block of microcolumns scaled down to microtitre format (12). With dye primers, 24 templates can be sequenced in a single microtitre dish. Radioactive methods for cycle sequencing may also be used.

*Modification for lambda/M13 libraries.* Because of the difficulty of obtaining reliably good phage titres in liquid culture, we recommend picking lambda plaques directly into the PCR microtitre dish. 1−2 $\mu$l of top agar containing phage is transferred by yellow tip to the PCR dish and mixed with the PCR mix. A small amount of the resultant PCR product may be saved for long term storage.

M13 plaques can also be toothpicked first into the wells of the PCR dish, followed by dipping the toothpicks into the appropriate wells of a culture microtitre dish. The culture dish is incubated at 37°C overnight and then stored at 4°C or −70°C.

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