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Direct sequencing of double-stranded PCR products without intermediate fragment purification; digestion with mung bean nuclease

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Initial problems with the direct sequencing of double-stranded PCR products have been largely overcome by carrying out the sequencing reactions in a PCR-like manner (1), i.e. using one of the PCR primers (or an internal primer), *Taq* DNA polymerase and thermal cycling. The only PCR reactants that are incompatible with the subsequent PCR-like sequencing reactions are the PCR primers. Conventionally, the PCR product is purified to remove these primers prior to sequencing. The recent report that S1 nuclease is active at neutral pH in the presence of Mg^{2+} (3) prompted us to assess the usefulness of such nucleases for the removal of PCR primers from PCR reactions. This treatment would have the added advantage of removing misprimed, single-stranded amplification products from the PCR reaction.

A portion of the 16S mitochondrial RNA gene was amplified from three species of Hymenoptera; *Apis mellifera, Venturia canescens* and *Myrmecia forficata* (F.). Genomic DNA was extracted from frozen insect thoraces as described in (6). PCR reactions were then performed essentially as described in (4). Reactions contained 67 mM Tris–HCl buffer [pH 8.8, containing 16.6 mM $(NH_4)_2SO_4$, 0.2 $mg.ml^{-1}$ gelatin, 0.45% Triton X-100], 2 mM $MgCl_2$, 25 μM dNTPs, 0.13 μM primer 16Sa [CGTCGATTTGAACTCAAATC; edited from (5)], 0.13 μM primer 16Sb [CACCTGTTTATCAAAAACAT; edited from (5)] and 0.5 units of *Taq* DNA polymerase in a total volume of 20 μl. Reactions were overlaid with 20 μl of mineral oil, and heated to 75°C for 5 min before the addition of DNA. The 16S fragment was then amplified under the following conditions; denature for 1 min at 93°C, anneal for 1 min at 50°C and extend for 1 min at 72°C. Thirty cycles were performed followed by an extension for 20 min at 72°C. Reactions were then stored at 4°C. PCR reaction products were analyzed by electrophoresis through 1% agarose and visualized with ethidium bromide (6). This indicated the amplification of a single, 500 base-pair fragment.

PCR reaction product was then (a) purified by the Spinbind procedure (FMC Bioproducts), (b) digested with mung bean nuclease, or (c) untreated. For the mung bean nuclease digestion, aliquots of the PCR reaction (6 μl) were made 10 mM with respect to $MgCl_2$ and 0.1 mM with respect to EDTA in a final volume of 12 μl. EDTA was added to complex the Zn^{2+} present in the supplied mung bean nuclease preparation (New England Biolabs). This is possible because of the greater affinity of EDTA for Zn^{2+} compared with Mg^{2+} (2). Mung bean nuclease (2 units) was then added, the reactions overlaid with 20 μl of mineral oil, and the PCR reactions digested at 25°C for 30 min. The reaction was stopped by heating at 100°C for 5 min, and the digests stored at 4°C. Preliminary experiments indicated that (a) this treatment with mung bean nuclease was sufficient to remove all PCR primers from the PCR reaction, and (b) heating at 100°C for 5 min was necessary to completely denature the mung bean nuclease (data not shown). Aliquots of the variously treated PCR product were then used as the template for *Taq* cycle sequencing reactions, as outlined in the protocol for the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit from Applied Biosystems.

The accuracy of the sequence data produced from the variously treated PCR products was assessed by comparing the sequence generated during a single sequencing run with the consensus sequence (obtained after Spinbind purification of the PCR product and sequencing both strands from two individuals). This comparison was then repeated in three different hymenopteran species. When Spinbind-purified PCR product was used, the sequence generated during a single sequencing run was 98. ± 1.7% accurate (300 bases). The quality of the sequencing data was high (Figure 1A) — the baseline was low with few ambiguities. When mung bean nuclease-digested PCR product was used, the sequence generated during a single sequencing run was 98.7 ± 0.7% accurate. The baseline was higher (Figure 1B) compared to Spinbind purified PCR product (e.g. nucleotides 70 to 80). However, ambiguities were resolved as accurately as they were when Spinbind purified PCR product was used as template. When unpurified PCR product was used, the sequence generated during a single sequencing run was only 88.0 ± 2.8% accurate. The baseline was high, and many more ambiguities were evident (Figure 1C). Presumably, the non-sequencing primer (at approximately 7 nM, sequencing primer at 160 nM) inititiated a competing sequencing reaction that contributed to the errors in base calling.

The present report demonstrates that mung bean nuclease can be used to generate sequencing templates directly from PCR reaction products. The entire procedure is carried out in a thermal cycler and could easily be adapted to both amplify and sequence DNA fragments in a single tube.

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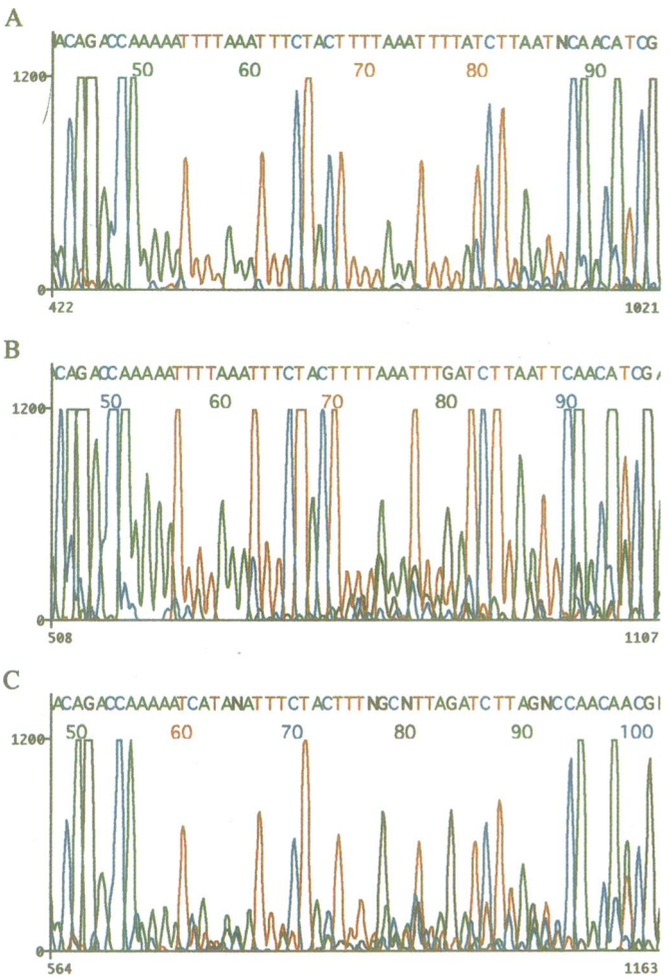

Figure 1. Comparison of the fluorescent sequencing plots generated from the variously treated PCR product. A portion of the 16S mitochondrial RNA gene from the wasp *Venturia canescens* was amplified by PCR and the PCR product (A) purified by the Spinbind procedure (FMC Bioproducts), (B) digested with mung bean nuclease, or (C) untreated. An aliquot from each treatment was then sequenced with one of the PCR primers (16Sa) using the *Taq* DyeDeoXy™ Terminator Cycle Sequencing Kit from Applied Biosystems.

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