Preparation of DNA from Numerous Individual Microscopic Organisms for PCR-Based Assays of Environmental Samples

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Polymerase chain reaction (PCR)-based assays have been developed to identify microscopic organisms that cause disease (8) or have an environmental impact (1). In both cases, DNA is extracted from pooled individuals. The pooled sample has typically been created for bacteria by culturing and for disease vectors by taking advantage of their reproduction in the host. PCR-based assays are being developed for a slightly larger class (50–500 µm) of microorganisms for which it is important to identify individuals rather than pooled samples (2,4). The usual extraction methods require many steps of centrifugation, vortex mixing, incubation and precipitation (4). Microorganisms with impervious exteriors (shells or chitinous exoskeletons) are especially resistant to lysis methods of DNA extraction. Rapid and easy methods for DNA extraction are needed so that large numbers of individuals can be screened in PCR-based assays of environmental samples.

We describe a technique called fast resin extraction (FRE)-PCR to amplify the DNA from large numbers of individual microscopic organisms rapidly. To test the technique, we used early larval stages of marine bivalves (Phylum Mollusca) and naupliar stages of copepods (Phylum Arthropoda) from marine plankton samples. In both cases, the small size and lack of morphological differentiation at the magnifications available by light microscopy make them difficult to identify to species. Higher magnifications, available with scanning and transmission electron microscopy, are time-consuming and costly, limiting the number of individuals that can be examined.

Larvae of the surfclam, Spisula solidissima, were cultured in the laboratory for use in testing the extraction methods. Plankton tows were taken with a plankton net on the continental shelf near Tuckerton, NJ. Individual bivalve larvae and copepod nauplii were sorted live with the aid of a dissecting microscope, transferred with <8 µL of seawater into individual tubes (200 or 500 µL or into wells in a 96-well V-bottom microplate) and frozen at -80°C until used.

Plastic pipet tips were used to make pestles that fit snugly in the bottom of the microcentrifuge tubes, leaving no sample unground. The insufficient grinding of commercial pestles was discovered by examining the results with the aid of a compound microscope. Snugly fitting pestles were made by softening the pointed end of a pipet tip (200 µL) in a flame for a few seconds and pushing it to the bottom of a microcentrifuge tube. Tubes used to mold pestles were the same size as the sample tubes. Pestles for the 96-well plates were molded in 200-µL tubes because the 96-well V-bottom plates melted when exposed to hot pipet tips. Pestles were reused after soaking overnight in 3 N HCl and thorough rinsing in deionized water before autoclaving.

Tubes and plates with individual larvae and nauplii were transferred directly from the freezer to a heating block and heated for 4 min at 100°C to denature DNases. The individual larva in each tube was ground with a plastic pestle. Twenty microliters of a 5% solution of Chelex®-100 resin (Bio-Rad, Hercules, CA, USA) in sterile water were added to each tube before heating for 10 min at 100°C. After heating, the Chelex resin settled quickly to the bottom of the tube without centrifugation.

Figure 1. Agarose gel containing PCR amplification mixture (10 µL) of an individual S. solidissima larva. Lane 1: an 1100-bp piece of the 18S rRNA gene from a day-1 veliger; lane 2: negative control (no DNA); lane 3: 1-kb molecular weight ladder.
and the supernatant was transferred to a 96-well V-bottom microplate and stored at -80°C.

The 600-bp fragment of the 18S rRNA gene was amplified by PCR in a second 96-well microplate. The primers used were forward (1F): 5’-CTGGTGTGATCGTCCAGT and reverse (536R): 5’-G ATCCCGCGGC G/T GCTG. A PCR cocktail was prepared and distributed into each well with a 12-channel pipettor. Each 50-µL reaction mixture contained 5 µL 10x PCR buffer (Boehringer Mannheim, Indianapolis, IN, USA), 0.2 mM dNTP, 0.2 µM of each oligonucleotide primer, 20 µg bovine serum albumin (BSA) and 0.5 U Taq DNA Polymerase (Boehringer Mannheim). The template DNA was 5 µL of the Chelex supernatant from an individual bivalve larva or copepod nauplius. A RoboCycler® Gradient 96 Temperature Cycler (Stratagene, La Jolla, CA, USA) was used for the first 5 cycles with the following parameters: 95°C for 1 min, 40°C for 1.5 min, 72°C for 2 min; and the next 35 cycles at 95°C for 1 min, 50°C for 1.5 min, 72°C for 2 min. This was followed by one final elongation cycle at 72°C for 7 min. After amplification, the presence of a PCR product was verified by electrophoresis of 10 µL of reaction mixture in a 2% TBE agarose gel containing ethidium bromide. The use of a 12-channel pipettor accelerated the process of sample transfer from extraction to detection if the Chelex supernatant and the PCR amplifications were in 96-well plates, and the electrophoresis gels were poured with microtiter format combs (Shelton Scientific, Shelton, CT, USA). Alternative detection by dot-blot hybridization was accelerated by transferring samples directly from the 96-well plate to the nylon membrane with the 12-channel pipettor.

FRE-PCR was tested first on D-stage veligers of a commercial bivalve species (S. solidissima) cultured in the laboratory. For this PCR, the primer 926R (5’-CCGCTAATCATTGAGTTTT) was substituted for the 536R primer to demonstrate that FRE-PCR could be used to amplify a large fragment of DNA from the smallest larvae. DNA from the smallest veligers (day 1: shell length = 80 µm) was sufficient to perform the PCR on an 1100-bp fragment (Figure 1).

Since DNA from the smallest larva was successfully amplified, the potential existed to amplify unknown larvae from plankton samples. The FRE-PCR was performed on 42 larvae sorted from a plankton sample collected August 14, 1995 (Figure 2A). With this method, amplification was highly successful with 33 of 42 lanes showing successful PCR bands. The few empty lanes can be explained by an unsuccessful PCR or lack of a template. During the process of sorting swimming larvae from dishes into tubes, a larva might not get transferred from the pipet into the tube because of human error. The species’ identities of the larval PCR products were later determined by species-specific and universal bivalve 18S probes in dot-blot hybridizations (3). Since probe detection is more sensitive than ethidium bromide detection (8), many of the samples that presented empty lanes contained enough PCR product for probing. With probe detection, 39 of 42 samples (93%) demonstrated a successful PCR. The negative control (Figure 2A, slot 48) showed neither a PCR band nor a positive hybridization.

In a second experiment, the same 600-bp fragment was amplified from 4 nauplii and 2 adult copepods sorted from a plankton sample collected in July 1996 (Figure 2B). In other experiments, the mt16S rRNA gene was amplified from bivalve larvae. Thus, we have demonstrated that FRE-PCR can be used on a variety of microorganisms.
with impervious exteriors, such as shells or chitinous exoskeletons. Advantages to this method are high throughput, high success rate and enough template DNA for several different assays (FRE-PCR used only 5 µL of the 20-µL supernatant). Other extraction methods that were tried and discarded for low success rates included boiling in water or PCR buffer, grinding before boiling, grinding with commercial pestles and grinding without Chelex.

Until now, PCR-based assays of small marine eukaryotes using probes and restriction enzymes have been applied to a pooled sample of 10 individual larvae (2) or to large, soft-bodied, individual larvae (6). Córte-Real et al. (5) amplified single bivalve larvae with a 67% success rate, but larvae were cultured, and all were selected for extraction before the shell had developed. Now FRE-PCR can be applied to numerous microscopic individuals from marine environmental samples as well as to microscopic organisms in the same size range (e.g., nematodes, insects, polychaetes) from other environments.

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Site-Directed Mutagenesis: A Two-Step Method Using PCR and DpnI

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In recent years, a two-step mutagenesis protocol has been developed that requires the polymerase chain reaction (PCR) (2,3). In the first step, a specific mutation is introduced by using a mutagenic primer and flanking primer (Figure 1). The PCR product is then used as a primer along with an opposite end-flanking primer for a second PCR step. The final PCR product is cut at unique restriction sites and subcloned. The advantages of this two-step method are: (i) it permits mutagenesis at virtually any site; (ii) it is inexpensive because only one specific primer is required for a given mutagenesis reaction; and (iii) it allows the mutated PCR product to be easily introduced into different vectors without further subcloning steps.

We found that a significant problem with this two-step method was the generation of a high frequency of unmutagenized recombinant clones as a result of contamination with the parental plasmid used as a template for PCR. Gel purification was insufficient to correct this problem because we found that contaminating nonmutagenized supercoiled plasmid can migrate similar to PCR products. Reducing template concentration was also an inadequate solution because low-input template concentrations typically led to low or no detectable PCR product.

We provide an easy modification that simplifies the procedure and vastly improves the frequency of mutagenized products available for subcloning. The DpnI restriction enzyme is used to cleave contaminating parental plasmid before gel purification and final subcloning. DpnI recognizes a methylated A residue in its target sequence GA\(^\beta\)TC, and thus it will cleave the bacterially generated parental plasmid but not the PCR products. Although DpnI is used in some commercially available mutagenesis kits (e.g., from Stratagene, La Jolla, CA, USA), it should be stressed that other than the use of DpnI, the method described in this report differs fundamentally from these methods. In addition, our method is considerably less expensive than commercial kits and involves the use of only a single nonmodified specific oligonucleotide. Our method works efficiently in most laboratory bacterial strains. In situations in which Dam\(^\beta\) bacteria are required, a restriction enzyme that cuts at a unique site in the parental plasmid must be substituted for DpnI.

To test our modified method, we mutagenized a mouse T-cell receptor \(\beta\), 1.8-kb Sall/BamHI genomic fragment [containing the \(V_{\beta8.1}\) leader exon, a rearranged \(V_{\beta8.1}D_{\beta8.1}J_{\beta8.2.3}\) exon and a third exon composed of the \(C_{\beta2.1}\) and \(C_{\beta2.4}\) exons fused together (1)] inserted in the pBluescript\textsuperscript{\textregistered} KS(+)- vector (Stratagene). As shown in Figure 1, the