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An alternative hot start technique for PCR in small volumes using beads of wax-embedded reaction components dried in trehalose

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Extra bands, caused by mispriming during the first screening cycle of the PCR, may become a problem in certain kinds of applications. For instance in forensic medicine, where PCR has facilitated the identification of minute and even degraded stains of blood or sperm, this amplification problem may hamper the interpretation of the results. 'Hot start' is one of the means to overcome this problem. In a hot start, a reaction component which is essential for polymerization, is excluded from the reaction mixture. After heating the reaction mix above the temperature at which mispriming can occur, the missing component is added into the reaction mixture, in a small volume. This method is laborious and prone to cross-contamination. Recently, a hot start has been developed in which the first part of the reaction mixture is covered with melted wax, which hardens at room temperature and separates the components from the second part of the reaction mixture (1). It is therefore possible to assemble the second part of the reaction set-up outside the thermal block. In this method the ready-made portions of the first part of the reaction mixture must be stored in a freezer prior to use. In addition the wax barrier raises the total volume of the material in the tube, prolonging the temperature transition times. We describe an alternative method in which one of the reaction components is dried in trehalose and embedded in a wax bead, which can be stored at room temperature. The small, 2 μl volume of the bead enables; i) faster cycling in conical microtubes especially in thermal cyclers which require no oil overlay, ii) the wax barrier method to be applied in capillary pipettes of i.d. less than 1 mm, iii) more convenient pipetting after cycling because of the thinner layer of hardened wax on top of the reaction mixture.

The reaction mixture can be pipetted directly into the well of the electrophoresis gel if Ficoll 400® is included into the reaction.

According to Colaco *et al.* (2), modifying enzymes and other reaction components can be dried in the presence of trehalose and stored at room temperature without severe loss of activity. We dried the primers or the nucleotides mixed with trehalose, pipetted the mix on polyethylene threads, and embedded the crystallized drops and the thread with melted wax (Figure 1A). The pieces of threads with the beads carrying the missing component of the reactions were placed on the bottom of microtubes, or on top of the rest of the reaction mixture in a capillary pipet. The wax starts melting at 57°C and floats up, exposing the trehalose drop, which dissolves within a few seconds.

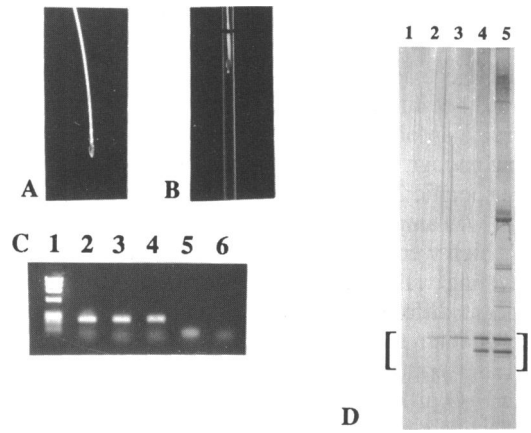

Figure 1. A. The polyethylene thread carrying the wax bead on the tip. **B.** The bead in the PCR mix in a capillary pipette at room temperature. **C.** 1% ethidium bromide stained agarose gel. Each lane contains 7 μl of the 15 μl PCR mixture. The reaction vessels were 25 μl capillary pipettes of i.d. less than 1 mm. 15 mm total program time with 30 fast cycles was carried out manually in water baths. The reaction mixture was 50 mM Tris, pH 8.8, 1.5 mM $MgCl_2$, 20 mM KCl, 0.5 mg/ml BSA, 200 μM each dNTPs, 2% (wt/vol) Ficoll® 400, 0.75 units of Taq polymerase per 15 μl. 15 pmol of each of the primers; 5′ CGAGGCCGGG-ATGATTTGCTATGAG 3′ and 5′ CCTTTCGCCTCGGTTCGGCTTC 3′ was included in the bead or in H_2O. The template DNA was 10 ng of denatured genomic DNA template of *R.galegae*. The program was; 30× [(95°C. 8s), (60°C, 7s), (75°C, 15s)]. 0.25s ramp time can be reduced from each step time. Lane 1. 500 ng of φ174 DNA digested with *Hae*III (Promega). Lane 2. *R.galegae* DNA, primers in H_2O. Lane 3. *R. galegae* DNA, primers in the bead without bromphenol blue. Lane 4. *R.galegae* DNA, primers in the bead containing bromophenol blue. Lane 5. Primers in H_2O, no template DNA. Lane 6. Primers in the bead, no template DNA. **D.** Silverstained 10% PAGE gel (4). Each lane contains 18 μl samples of the 50 μl PCRs detecting the human tyrosine kinase gene intron 1 microsatellite locus. As templates 10 ng of homozygous K562 human DNA as a control or whole blood quick lysate DNA (5), corresponding to 5 μl of patient blood sample. The reaction mixture was 1 μM of primer-1: 5′-GTGGGC-TGAAAAGCTCCCGATTAT-3′ and primer-2: 5′-GTGATTCCCATTGGCC-TGTTCCTC-3′, 50 mM Tris–HCl, pH 8.8, 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 200 μM dNTPs and 1.25 units Taq-polymerase (Promega). PCR was carried out in a Perkin-Elmer Cetus 9600 thermal cycler. Following the initial denaturation at 94°C for 4 minutes, the conditions were: 30× [(95°C, 30s), (60°C, 30s), (72°C, 60s)]. Lane 1. As a negative control, H_2O instead of DNA. Lane 2. 10 ng of homozygous control DNA K562 primers in the bead. Lane 3. 10 ng of K562, primers in liquid added to the reaction mix at room temperature. Lane 4. Quick lysate whole blood DNA sample. Primers in the bead. Lane 5. Quick lysate sample, primers in liquid added to the reaction mix at room temperature. [ ] indicates the area in which the bands representing the correct alleles should appear.

The primer mix for 100 15 μl reactions contained 2–5 μl trehalose solution (SIGMA, T 9531) (1 g in 1 ml H_2O filtered through 0.22 μm filter), 1.5 mnol of each of the primers, 0.5 μl H_2O saturated with bromphenol blue at room temperature, 2 μl 1 M Tris–HCl pH 8.8 and H_2O to 300 μl. Two-sided adhesive tapes were attached on top of a supporting frame 2–5 cm from each other. 20 pieces of 0.3 mm thick polyethylene threads were attached perpendicularly to the tapes 5–10 mm from each other. This set-up was rinsed with sterile distilled water and treated with short wavelength UV irradiation for 15–30 min. Then 3 μl drops of the mixes were pipetted onto the threads. The drops were dried at 65°C for 1 h. The threads were cut 0.5 mm from the dried drop and attached around an aluminium cap via the tape. The tips of the threads, which were hanging over the edge, were dipped three to five times in melted wax. The hardened wax covered 3–5 mm of the tips of the threads carrying the crystalized trehalose mixture. The volume of the bead produced this way is approximately 2 μl if the thread is cut 3–5 mm from the end. The beads can be stored at room temperature. The purpose of the bromphenol blue is to indicate that the drops are not dissolved before the tubes are put into the incubator due to leaky wax cover, and to serve as a marker during the subsequent electrophoresis. The bromphenol blue and the trehalose did not inhibit the reactions. If used in thermal cyclers which require an oil overlay, the thread above the wax should be left long enough to prevent the bead from floating in the oil.

A strain specific 261 bp fragment of *Rhizobium galegae* (ATCC 43677) was amplified in 15 μl reaction volumes in capillary pipettes, which were sealed with a Bunsen burner (Figure 1C). 15 min cycling was done manually in water baths. We found no significant difference in yield or specificity of the 261 bp fragment whether the primers were in the bead or in water. However, the control reaction without template DNA using primers in H_2O produced more primer-dimer. To show that the method effectively prevents extra band formation, a microsatellite locus of the human tyrosine hydrolase gene intron1 (HUMTHO1) serving as a marker in forensic DNA-typing (3) was amplified using wax-primers in the beads or in H_2O (Figure 1D).

Our method saves time and effort when large numbers of samples should be analyzed in minimal time.

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