Wax-embedded PCR Reagents

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PCR has revolutionized nucleic acid analysis in many scientific disciplines, including molecular biology, medical diagnostics, population genetics, and forensic analysis.⁽¹⁾ PCR is a highly sensitive technique that can selectively enrich for a specific target from a background of nonrelated sequences. However, if all of the reaction components (nucleotides, buffer containing magnesium, primers, a thermostable DNA polymerase, and sample containing the target to be amplified) are mixed at room temperature prior to thermocycling, premature mispriming events such as non-specific annealing of primers to nontarget nucleic acid sequences and the production of primer oligomerization (primer dimer) artifacts may occur.⁽²⁾ In these cases, the undesired nonspecific products that are generated can interfere with amplification and detection of the specific target DNA sequences, especially when low-copy-number amplifications are performed.

Hot start is a recent variation of PCR that discourages these pre-PCR mispriming events prior to initial denaturation.^(3,4) In hot start PCR, one of the essential reaction components is deliberately excluded until the temperature of the reaction exceeds the temperature at which the primers anneal to the target template. The result is typically enhanced sensitivity and specificity in the PCR reaction.⁽⁵⁾ In one version of hot start PCR, the missing component is added to the tubes in the thermal cycler block at high temperature. Although this method is simple, it involves manually opening the tubes once they have reached the appropriate temperature. This is not only cumbersome and time-consuming when many samples are processed simultaneously but is also prone to cross-contamination.

Recent variations of hot start PCR involve the use of paraffin wax to segregate the reaction components in solution and require step-wise assembly of the reaction mixture. All reagents are present in the tubes prior to thermocycling, thereby eliminating the need for opening the tubes manually. One method includes an AmpliWax vapor barrier whereby a layer of solid wax is used to separate the retained reagent(s) from the bulk reaction mix until the initial denaturation step melts the wax.⁽⁶⁾ The upper and lower aqueous layers are mixed by convection, and the wax then forms a

vapor barrier on the surface of the aqueous mixture. With this approach, the wax must first be melted and solidified on top of the lower aqueous layer prior to addition of the missing component(s). In a similar approach, wax is melted and solidified to cover the lower aqueous layer completely.⁽⁷⁾ The missing reagent is then layered onto the wax cap, followed by two drops of mineral oil. When the temperature of the tubes in the thermocycler exceeds the melting temperature of the wax, the wax rises to the top of the tube and the upper and lower aqueous layers mix by convection.

Another variation of the hot start technique involves drying one of the reaction components in trehalose and embedding the dried reactant in a wax bead.⁽⁸⁾ The wax bead containing the missing reactant (in this example, the primers) is then added to the PCR reaction at room temperature, and the reactant is released when the temperature in the thermocycler melts the wax. With this approach, there is no need to melt the wax prior to assembling the reaction.

Our approach to the problem of pre-PCR mispriming is simple and convenient. We have cosolidified PCR reagents with wax, omitting either the template or the DNA polymerase. With our approach, the reagents can be stored conveniently at room temperature and delivered to a reaction by simply adding water and the template or enzyme to the cosolidified reagents. Thermocycling is performed directly with no need to melt the cosolidified reagents prior to assembling the reaction. The reagents are released from the wax when the temperature reaches the melting point of the wax. All aqueous reagents mix, and the wax then forms a vapor barrier on the surface. We anticipate that this method will be useful in other amplification systems with similar considerations.

We have expanded this approach to cosolidify nonthermostable reagents for first-strand cDNA synthesis by reverse transcriptase-PCR (RT-PCR). RT-PCR is a simple modification of PCR that allows for the selective amplification of extremely small amounts of target RNA.^(9,10) Cosolidification is particularly useful for preparing, storing, and delivering first-strand cDNA for eventual PCR amplification when samples are available in isolated areas. Samples can be prepared easily, first-strand cDNA synthesis performed using cosolidified re-

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agents, and the first-strand cDNA product cosolidified in the wax for convenient storage when PCR amplification is not performed immediately. This method also allows for simple delivery of the stored product to a subsequent PCR reaction.

MATERIALS AND METHODS

Preparation of Cosolidified Reagents

Cosolidified PCR reagents were prepared as follows: For each sample, selected PCR reagents (in a volume of 6 or 7 μ l) were mixed in a 0.5 ml Eppendorf tube just prior to cosolidification. A single AmpliWax PCR Gem 100 (Perkin-Elmer Cetus) was melted in a separate 0.5 ml microcentrifuge tube by incubating the tube at 65°C for ~2 min. The mixed PCR reagents were then added to the side of the tube containing the melted wax, and the tube was tapped until the reagents were in contact with the wax. The tube was then placed at 65°C just until the wax melted (~1 min). The PCR reagents and wax cosolidified following vortexing. Alternatively, PCR reagents could be pre-dispensed into tubes containing solid wax and then cosolidified by melting and vortexing.

Cosolidified first-strand cDNA synthesis reagents were prepared as described above, except 11 μ l of first-strand cDNA synthesis reagents containing oligo(dT)12–18 primer (cDNA Synthesis Kit, Pharmacia) were cosolidified with wax.

PCR Amplification

The following reagents were used for PCR amplification in the experiments involving cosolidified PCR reagents: pBR322, linearized with *Pvu*II (1 ng/ μ l), 1 μ l; SF-4 primer, 5'-GATAAGCTTTAAT-GCGGTAGTTTATCACAG-3', (50 pmol/ μ l, 1 μ l); SF-5 primer, 5'-AGAGGATCCA-CAGGACGGGTGTGGTCGCCA-3', (50 pmol/ μ l), 1 μ l; 50 $\times$ buffer, 500 mM Tris-HCl (pH 8.3), 2.5 M KCl, 75 mM MgCl₂, 2 μ l; DNA polymerization mix (Pharmacia), 20 mM each of dATP, dCTP, dGTP, and dTTP, 1 μ l; and AmpliTaq DNA polymerase (5 U/ μ l), 1 μ l.

Prior to PCR amplification, all PCR reagents, except enzyme or template, were either cosolidified in wax or left in solution. Triplicate samples of each were

stored at either room temperature or on ice for 2 hrs. For each sample, the missing component and 93 μ l of sterile distilled water were added to the other reagents in solution or to the cosolidified wax preparation. An AmpliWax PCR Gem 100 was added to those tubes that did not contain cosolidified reagents. Tubes were placed into a thermocycler (Perkin-Elmer, DNA Thermal Cycler 480), and PCR was performed using the following program: 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min.

Preparation of Whole Blood for First-strand cDNA Synthesis

Whole human blood (EDTA-treated) was prepared for first-strand cDNA synthesis as described by Shi and Liu.⁽¹¹⁾ For each of five samples, 90 μ l of RNase-free water (treated with diethylpyrocarbonate) was added to 10 μ l of whole human blood in a 0.5 ml microcentrifuge tube. Samples were incubated on ice for 5 min to lyse the erythrocytes and centrifuged at 6000 rpm in a microcentrifuge for 10 min. The supernatants were discarded. Each pellet was resuspended in 20 μ l of RNase-free water containing 24 units of RNA-guard ribonuclease inhibitor (Pharmacia). Samples were incubated on ice for 20 min to release total RNA from the leukocytes. Immediately prior to first-strand cDNA synthesis, samples were incubated at 65°C for 10 min to denature the template RNA.

RT-PCR

The composition of the first-strand mixture is as follows (from cDNA synthesis kit, Pharmacia): 136 mM Tris-HCl (pH 8.3), 204 mM KCl, 27 mM MgCl₂, 27 mM DTT, 0.2 mg/ml of BSA, 5.4 mM each of dATP, dCTP, dGTP, and dTTP, 60 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (cloned), 30 units of RNAGuard, and 5 μ g of oligo(dT)12–18.

The following reagents were used for amplification of first-strand cDNA: PCO4 primer (Perkin-Elmer), 5'-CAACTTCATC-CACGTTACC-3'; GH20 primer (Perkin-Elmer), 5'-GAAGAGCCAAGGACAGGTAC-3'; AmpliTaq DNA polymerase.

For each sample, the entire volume (20 μ l) of total RNA was used in a first-strand cDNA synthesis reaction. For one sample, total RNA was added to a first-strand reaction and incubated at 37°C for

1 hr. For two samples, total RNA was added to a first-strand reaction and tubes were incubated at 65°C for ~2 min prior to the 1 hr incubation at 37°C. For the two tubes containing cosolidified first-strand reagents, 20 μ l of total RNA was added to each tube and the tubes were heated at 65°C for 1 min to melt the wax. The tubes were then vortexed to mix the sample with the reagents and incubated at 65°C for an additional minute to remelt the wax. The tubes were then placed at 37°C for 1 hr. The wax formed a solid layer over the top of the aqueous reactants. Fifty pmol of each primer specific for human β -globin (PCO4 and GH20), 64 μ l of sterile distilled water, and 1 μ l (5 units) of AmpliTaq were added to each sample. An AmpliWax PCR Gem 100 was added to the samples that did not contain cosolidified reagents. Tubes were placed into a thermocycler (Perkin-Elmer, DNA Thermal Cycler 480), and PCR was performed using the following program: 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min.

RESULTS AND DISCUSSION

To demonstrate that cosolidified PCR reagents are useful for decreasing nonspecific, higher-molecular weight products in PCR reactions, all PCR reagents, except enzyme, were either cosolidified with wax or left in solution and stored at room temperature or on ice. For each sample, 5 units of AmpliTaq and 93 μ l of sterile distilled water were added to the other reagents in solution or on top of the cosolidified wax preparation. Negative (containing no template) and positive control reactions were also prepared using reagents stored at -20°C. Tubes were placed into a thermocycler, and PCR amplification was performed as described. Figure 1 shows the results of this experiment. Using this model system, a specific product of ~350 bp is expected. No visible PCR product is present in the negative control lane (lane 1), indicating that cross-contamination of samples was not a problem. All other lanes show a specific PCR product with the expected size and intensity equivalent to that of the positive control lane (lane 2). Samples in which PCR reagents were stored in solution, however (lanes 9–14), also indicate the presence of undesirable higher-molecular-weight products. These nonspecific products are absent in

FIGURE 1 PCR amplification using reagents (excluding enzyme) in wax or in solution. Reactions were prepared in triplicate (except for negative and positive control). Ten microliters of each PCR reaction was electrophoresed on a 1% agarose gel containing ethidium bromide to visualize the ~350-bp fragment. (Lane 1) Fresh reagents excluding template (negative control); (lane 2) fresh reagents including template (positive control); (lanes 3–5) reagents in wax, stored on ice; (lanes 6–8) reagents in wax, stored at room temperature; (lanes 9–11) reagents in solution, stored on ice; (lanes 12–14) reagents in solution, stored at room temperature; (lane 15) 100 Base-Pair Ladder (Pharmacia).

FIGURE 2 PCR amplification using reagents (excluding template) in wax or in solution. Reactions were prepared in triplicate. Ten microliters of each PCR reaction was electrophoresed on a 1% agarose gel containing ethidium bromide to visualize the ~350-bp fragment. (Lanes 1–3) Reagents in wax, stored on ice; (lanes 4–6) reagents in wax, stored at room temperature; (lanes 7–9) reagents in solution, stored on ice; (lanes 10–12) reagents in solution, stored at room temperature; (lane 13) 100 Base-Pair Ladder.

samples prepared using reagents cosolidified with wax (lanes 3–8).

If all PCR components except the template are cosolidified, specificity of the reaction can be enhanced. An experiment similar to the one described above was performed, except the template, rather than the enzyme, was excluded. Triplicate samples were incubated at room temperature or on ice for 2 hr. Prior to PCR amplification, 1 ng of pBR322-*Pvu*II and 93 μ l of sterile distilled water were added to the other reagents. Thermocycling was as described. It is evident from the results shown in Figure 2 that nonspecific, lower-molecular-weight products (primer-dimers) were generated when reactants were stored in solution (lanes 7–12). This problem was avoided, however, by using reactants cosolidified with wax (lanes 1–6). Also, the intensity of the specific PCR product is somewhat reduced in those lanes where primer-dimers are present, suggesting that formation of artifacts may deplete the reaction.

In both of the experiments described above using a model system, cosolidification of PCR reagents appeared to significantly reduce or eliminate nonspecific products that arise from premature interaction of reagents. From these data, it is reasonable to assume that if reagents are cosolidified with wax, their diffusion is greatly impeded and thus the probability of their interaction is reduced.

To show that cosolidification of nonthermostable reactants with wax is also feasible, first-strand cDNA reagents were cosolidified with wax as described in Materials and Methods. Five samples of total RNA were prepared from whole human blood and RT-PCR was performed as described. Figure 3, lanes 4 and 5, shows a specific PCR product (268 bp) equivalent in intensity to the control reaction where first-strand reagents were not subjected to an initial incubation at 65°C (lane 1). The reagents comprising the first-strand mixture can thus tolerate brief incubation at 65°C, a prerequisite to cosolidification with wax. Lanes 2 and 3 show that a significant amount of specific PCR product can be generated from reactions performed with cosolidified first-strand reagents, despite the fact that the reaction components may be considered thermolabile.

In summary, we have developed an extremely simple and convenient method for preparing, storing, and deliv-

FIGURE 3 RT-PCR using first-strand reagents in wax or in solution. RNA isolated from 10 μ l of whole human blood was used as a template for first-strand cDNA synthesis. PCR amplification was performed following first-strand synthesis. Primers specific for human β -globin were used for amplification. Ten microliters of each PCR reaction was electrophoresed on a 1% agarose gel containing ethidium bromide to visualize the 268-bp fragment. (Lane 1) Fresh first-strand mix; (lanes 2–3) first-strand mix in wax; (lanes 4–5) first-strand mix in solution, heated for 2 minutes at 65°C; (lane 6) 100 Base-Pair Ladder.

ering PCR reagents. Using a model system, the utilization of reaction components cosolidified with wax resulted in significant reduction of nonspecific products, of both high and low molecular weight, that can interfere with the generation and/or detection of specific PCR products. Samples can be prepared in minutes with no special equipment required. Delivery of reagents to a PCR reaction is direct with no requirement for the melting of the wax prior to PCR amplification.

We have extended this method of cosolidification to other molecular biology reagents, including restriction enzymes, DNA polymerases, modifying enzymes, and enzyme-conjugated antibodies (data not shown). These reagents exhibited significant activity following delivery

from the cosolidified mixture, and several preparations were more stable upon storage than similarly stored samples in solution (data not shown).

We have also successfully demonstrated the principle that cosolidification can be used for more complex mixtures such as first-strand reaction mixes. The use of such cosolidified reagents alone or in combination with reagents dried in the presence of a carbohydrate polymer⁽¹²⁾ should be amenable to automation and should facilitate the application of all aspects of PCR in routine practice.

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