Single-Tube Nested PCR Using Immobilized Internal Primers

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The detection of specific DNA sequences by PCR has proved extremely valuable for the analysis of genetic disorders and the diagnosis of a variety of infectious disease pathogens (7,8,10). However, not unusually, high sensitivity and specificity are required for diagnostic purposes. In these situations, nested PCR has been devised to improve the PCR assay (10,11). Nested PCR is a two-step procedure in which the products of a first PCR using outer primers are re-amplified using a second set of inner primers located within the previously amplified sequence. Although nested PCR is more sensitive than conventional PCR, an inherent drawback is the need to open tubes after the first round of amplification to transfer products to a second PCR amplification reaction that utilizes a different primer pair or to introduce new reagents and/or primers (9). This process increases dramatically the risk of cross-contamination of negative samples with PCR products derived from positive specimens during the first round of amplification. Aiming at minimizing the risk of cross-contamination, some proposals for single-tube nested PCR were devised (4,5,12,15). Nevertheless, all of them are operationally complex or require the design of special primers. Thus, this paper describes the design and development of a novel single-tube nested PCR in which the internal primers are separated from the components of the first round of amplification by immobilization onto the inside of the microtube cap (patent pending). In addition, comparative data on the relative levels of sensitivity of the single-tube nested PCR in which the internal primers are separated from the components of the first round of amplification by immobilization onto the inside of the microtube cap (patent pending) were used in the first PCR, and 50 pmol internal primers (Schfo17 and Schre19) were used in the second PCR. Two microliters of the products of the first PCR were used as templates for the second PCR. Single-tube nested PCR was optimized for concentrations of dNTPs, Mg$_{2+}$, and outer and inner primers. Ten microliters containing 50 pmol inner primers (Schfo17 and Schre19) with traces of bromophenol blue were previously immobilized onto the inside of the microtube cap by incubating the tubes at 37°C until the solution had dried. The first amplification round consisted of 15 cycles (92°C for 30 s, 50°C for 30 s, and 72°C for 1 min), whereas the second round of amplification consisted of 45 cycles (the annealing temperature was increased to 55°C). The first amplification round of the single-tube nested PCR was performed in a 50-µL volume containing 10 mM Tris-HCl, 50 mM KCl, 0.1 mM MgCl$_2$, 0.2 mM each dNTP, 50 pmol each primer (Schfo17 and Schre19), 2.5 U Taq DNA polymerase, and 2 µL of the DNA solution was added into the PCR mixture. Thirty microliters of mineral oil were added to overlay the reaction mixture. After the first-round PCR, the thermal cycler was turned to 92°C, and the closed tubes were inverted several times to dissolve the inner primers inside the cap, briefly centrifuged, and returned to the machine for the second round of amplification. Products (10 µL) were separated by electrophoresis in 1% agarose gels, and ethidium bromide-stained gels were visualized and photographed over a UV light using the MP4+ Polaroid System (Sigma, St. Louis, MA, USA).

For the optimization of primer-pair studies, single-tube nested PCR assays were set up using 1 and 0.1 ng purified S. mansoni DNA as template and various primer-pairs ratios. The relative amounts of long, short, and intermediate amplification products were gauged by agarose gel electrophoresis so that the best yield of the desired 721-bp Schistosoma product could be achieved (Figure 1). When 1 ng was used as template, the strongest band appeared with the outer primer set;internal primer set ratio (OP:IP) of 1:10, whereas the OP:IP 1:100 produced the most intense band when 0.1 ng DNA template was used, suggesting that the optimal ratio would be in this range. Thus, we decided to use the ratio of 1:10 (5:50 pmol) in all subsequent experiments. The novel single-tube nested PCR developed was specific for the detection of...
Schistosoma DNA, not amplifying as expected, DNA from vertebrate or invertebrate hosts of the parasite (data not shown). The detection limit of the STNPCR was 1 fg S. mansoni genomic DNA (Figure 2). The detection limit obtained by 30-cycle conventional PCR was 1 pg (this is barely visible in the figure, although it was clear in the original gel). In addition, a conventional PCR with 45 cycles was performed resulting in a detection limit of 0.1 pg, although it was accompanied by intense primer dimer formation (data not shown). Thus, single-tube nested PCR is potentially 100–1000 times more sensitive than conventional PCR and, theoretically, can detect DNA corresponding to less than a single cell of the multicellular parasite S. mansoni. In comparison to two-step nested PCR (detection limit of 0.1 fg; data not shown) single-tube nested PCR was just 10 times less sensitive.

The major disadvantage of two-step nested PCR is the increased probability of carryover contamination of negative specimens with products derived from positive amplifications (9). To avoid this additional risk, several methods have been developed to perform the first and second rounds of PCR in a single tube, which does not have to be opened until the product analysis stage when the amplification reaction itself is complete. One possible advantage of conventional two-step nested PCR over single-tube nested PCR is the dilution of potential Taq DNA polymerase inhibitors during transfer of amplification products from the first step of amplification to new reactions in the second stage of amplification (9). To achieve maximal efficiency in single-tube nested PCR, inhibitors must be removed by an appropriate sample preparation procedure. However, the advantage associated with significantly decreasing the possibility of carryover contamination outweighs this disadvantage. Most of the methods devised to perform single-tube nested PCR are based on the use of pairs of primers with different G+C contents, resulting in primers sets with vastly different annealing temperatures (5,12,15). Nonetheless, this approach restricts the selection of primers pairs, as pairs of primers with significantly different annealing temperatures are required. Another approach involves the physical separation of the components for the first and second round of amplification, by means of a thin layer of mineral oil (1). Although conceptually simple, this is not efficient, as the components of the second-round PCR usually cannot be kept properly separated during the time necessary to finish the first amplification process. Other systems are based on the sequestration of reaction mixture (including thermostable DNA polymerase) embedded into agarose resin inside a central chamber of the reaction tube screw-top (14). Although interesting, this method is cumbersome, requiring the use of specially designed reaction tubes and upper cooling plates to protect the gel matrix. In addition, the presence of agarose, extraneous to the PCR mixture, may interfere with the PCR. The same comments can be applied to methods using trehalose matrix instead of agarose (13).

To overcome the shortcomings of the approaches for single-tube nested PCR currently available, we developed a very simple, reproducible, and consistent system that allows for single-tube nested PCR. We have noticed that only the internal oligonucleotides needed to be sequestered, and this could be efficiently done by a simple procedure in which the internal primers were immobilized by drying onto the internal face of the microtube cap and easily dissolved into the reaction mixture to participate in the second round of amplification by inverting the PCR tube. In addition, conveniently, standard PCR

**Figure 1.** Agarose gel electrophoresis for the optimization of the proportion of external and internal primers of single-tube nested PCR. (A) 1 ng or (B) 0.1 ng S. mansoni DNA were used under different ratios of external to internal primers. Lane 1, 1:1; lane 2, 1:10; lane 3, 1:100; lane 4, 1:1000; lane 5, 1:10000; lane 6, 0:50; lane 7, 50:0. M, molecular weight markers (λ HindIII) (Sigma). The arrows indicate the sizes of the PCR products.

**Figure 2.** Agarose gel electrophoresis showing the detection limit of PCR (A) and single-tube nested PCR (B). The amounts of S. mansoni genomic DNA in panel A are: lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng; lane 4, 10 pg; lane 5, 1 pg; lane 6, 0.1 pg; lane 7, 10 fg; lane 8, 1 fg; and in panel B are lane 1, 0.1 ng; lane 2, 10 pg; lane 3, 1 pg; lane 4, 0.1 pg; lane 5, 10 fg; lane 6, 1 fg; lane 7, 0.1 fg. M, molecular weight markers (λ HindIII). The arrow indicates the size of the PCR product.
Benchmarks

and cycling conditions can be used in the single-tube nested PCR devised. The first round of amplification includes limiting the concentrations of primers so that they are exhausted at the completion of the first PCR. Since the concentration of the outer primers is only 0.1 μM, the larger product synthesized by them does not contribute significantly to the overall yield of products; the larger product serves only to boost the second stage amplification reaction. When the first PCR was performed, the primers for the second PCR were not available to anneal to the template. When the second PCR was running, the outer primers were not practically available because they were almost completely used up during the first amplification.

In schistosomiasis, several applications for PCR can be envisaged: detection of parasites in infected water collections, as well as diagnosis of infection in vertebrate and invertebrate hosts. Indeed, a few approaches based on the detection of Schistosoma DNA were published for the detection of the parasite in snails (3,6) and cercaria contaminated water collections (2). The diagnostic system proposed here can be easily adapted to be used for PCR detection of other infectious pathogens. In this direction, we have also adapted the single-tube nested PCR for the diagnosis of malaria (unpublished results).

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PCR-Based Method for Identification of Integration Events in the Pichia pastoris Genome

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The yeast Pichia pastoris is a frequently used microorganism for the cloning and expression of eukaryotic genes. These yeast cells have many of the advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and posttranslational modification. P. pastoris is a methylotrophic yeast that is capable of metabolizing methanol as its sole carbon source. The promoter regulating the production of alcohol oxidase is used to drive heterologous protein expression in yeast. Stability of recombinant molecules is ensured by homologous recombination between transforming DNA and regions of homology within the genome. Such integrants show extreme stability in the absence of selective pressure.

The presence of a recombinant gene in the appropriate site of the yeast genome is usually confirmed by PCR on isolated genomic DNA. Isolation of genomic DNA is a laborious and time-consuming method, which moreover requests expensive lytic enzymes for the digestion of yeast cell walls. Alternatively, glass-bead-based extraction methods are used for the rapid extraction of DNA for PCR. For routine applications in our laboratory, we have developed a method that is based on the

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