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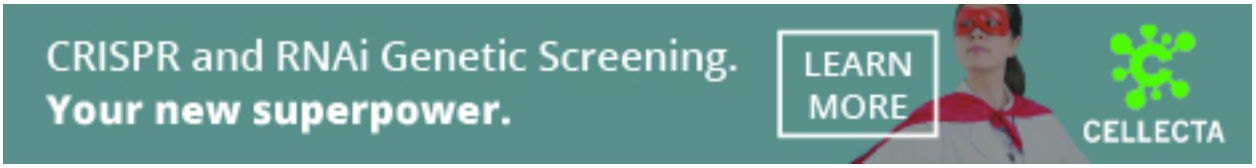

Optimized Conditions for Cycle Sequencing of PCR Products

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The recent development of the cycle (or linear amplification) sequencing technique has greatly improved the ability to sequence directly double-stranded PCR products,[1–3] which would otherwise require elaborate preparation before sequencing such as asymmetric PCR [4] and single-stranded DNA purification.[5–7] However, conventional cycle sequencing is dependent on additional primers for sequencing–primers that bind to sites internal to those used for PCR amplification.[1–3,8] This is a major drawback when one considers the cost and effort of making multiple sets of primers for each template, especially for a large-scale application. Therefore, in our current project to develop PCR-based polymorphic cDNA markers for the mouse genome,[9] a protocol for obtaining highly accurate sequence from double-stranded PCR products using the same primers for PCR and sequencing was needed. With such a protocol, cycle sequencing can be directly applied to the sequencing of PCR-amplified genomic DNA, useful in finding sequence polymorphisms and in the analysis of unclonable DNA fragments. To date, we have successfully obtained sequences from 108 templates (PCR products) derived from the genomic DNAs of two mouse strains. On the basis of our observations, we present optimized cycle sequencing conditions.

The primers used were designed from the 3′-untranslated region of cDNAs using the Primer Detective program,[10] prepared with a model 391 synthesizer (Applied Biosystems), and purified by OPC column. Samples of mouse DNA (25 ng) were amplified by standard PCR conditions using a Thermal Cycler model 9600 (Perkin-Elmer Cetus). The PCR products (average length, 200 bp) were purified after agarose gel electrophoresis by phenol–chloroform extraction to eliminate nonspecific PCR products. Estimation of DNA concentration was done by comparison of band intensities between a standard and samples on EtBr-stained agarose gels. The cycle sequencing reactions were carried out using the *Taq* DyeDeoxy Terminators (Applied Biosystems) according to the supplier's guide, and the samples were run on a model 373A DNA Sequencing System (Applied Biosystems). Sequencing accuracy was defined as the percent homology between the original cDNA sequences and our sequence results.

In our preliminary sequencing experiments we obtained sequence information while varying DNA concentration over a range of 5–25 ng per reaction at standard cycle sequencing conditions. In contrast to previously reported procedures,[3] we found that our cycle sequencing results had a narrow range for optimal template concentration, from 5 to 10 ng per reaction. It was found that 7.5 ng gave the best signal intensity, as well as the most accurate sequence.

Using the supplier's recommended conditions (three-temperature PCR), we sequenced the first 72 templates with primers from both ends (Table 1). A significant portion of them did not produce successful results (successful defined here as homology ≥90%) and overall success rate was only 65%. We assumed that the actual melting temperature (T_m) value of the primers was one of the parameters having an influence on the sequencing quality. When the success rate was evaluated according to the T_m value of the primer, it was noted that primers with T_m values <60°C were less successful (Table 1). With this idea in mind, we tried a variety of PCR conditions and found, rather empirically, that primers with T_m values <60°C can be successfully sequenced by two-temperature PCR (96°C–55°C) and primers with T_m values ≥60°C can be successfully sequenced by two-temperature PCR (either 96–55°C, 96–60°C, or 96–65°C). Of the unsuccessful primers using the standard three-temperature PCR conditions, 58% (7/12) with T_m < 60°C and 39% (15/38) with T_m ≥60°C were rescued according to these empirically obtained conditions.

TABLE 1 Success Rate by the Standard Condition

Primer T_m value[a]	Percent fraction of successful primers (number of sequenced templates)
<60°C	50 (24)
≥60°C	68 (120)

Standard cycle sequencing conditions for the first 72 primers were 96°C for 1 min, 25 cycles of 96°C for 15 sec, 50°C for 1 sec, 60°C for 4 min.

[a]A melting temperature value for each primer was calculated by the nearest neighbor method[11] using the computer program OLIGO (National Biosciences) at conditions of 1 M salt and 0.6 pM of primer.

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TABLE 2 Success Rate by the Adapted Conditions

Primer T_m value	Percent fraction of successful primers (number of sequenced templates) 96–55°C	96–60°C	96–65°C
<60°C	**100** (5)	25 (8)	N.D.[a]
≥60°C	73 (22)	**88** (60)	83 (12)

Cycle sequencing conditions are 96°C for 1 min, 25 cycles of 96°C for 15 sec, and 55°C, 60°C or 65°C for 4 min. The recommended conditions for optimal cycle sequencing of PCR products are shown in boldface type.
[a]Not determined.

After establishing these conditions, we applied this new strategy to an additional group of 36 templates. As shown in Table 2, we were able to obtain quality sequencing results starting 5 bp downstream of the primer to the end of the PCR product from the first trial for most primers. Compared with the 65% success rate by the supplier's recommended conditions (Table 1), the 89% success rate by our recommended conditions was a significant improvement. In contrast to using the direct coupling of PCR amplification and cycle sequencing (CAS) technique,[12] our recommended protocol offers a recourse for primers that give poor quality sequence. Although the accuracy of the sequencing here is not 100% (average 95%), the accuracy obtained by this protocol is sufficient to reconstitute the exact sequence by comparison of overlapped sequences obtained from a pair of primers.

In summary, we recommend trying the following cycle sequencing conditions first for double-stranded PCR products: (1) ~7.5 ng of template per reaction, (2) two-temperature PCR (96°C for 1 min, 25 cycles of 96°C for 15 sec, and 55°C for 4 min) for primers with T_m <60°C and 2-temperature PCR (96°C for 1 min, 25 cycles of 96°C for 15 sec, and 60°C for 4 min) for primers with T_m≥60°C.

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