Shortened Protocol for Terminal Deoxynucleotidyl Transferase-Dependent PCR

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Several methods have been developed for determining the sequence specificity of DNA damaging agents in single-copy genes in mammalian cells (10). The ligation-mediated PCR (LMPCR) technique can be used for those compounds that cleave DNA and produce a 5'-phosphate (9, 14). For those compounds that form stable adducts with DNA (e.g., cisplatin), there are compounds that cleave DNA before gel electrophoresis. These steps were repeated on a 6% (w/v) polyacrylamide DNA sequencing gel and scanned using a PhosphorImager® (Amersham Pharmacia Biotech).

The main aim of this research was to establish a shortened protocol for TDPCR. The original TDPCR protocol (7) had the following order of 12 steps: linear amplification with a gene-specific oligonucleotide, ethanol precipitation 1, terminal transferase ribo-G tailing, ethanol precipitation 2, ligation of linker oligonucleotides, PCR with a nested gene-specific oligonucleotide, phenol extraction, ethanol precipitation 3, electrophoresis, electroblotting, hybridization with a third gene-specific oligonucleotide, and autoradiography.

We first wished to replace the labor-intensive final steps of phenol extraction, ethanol precipitation 3, electroblotting, hybridization with a third gene-specific oligonucleotide, and autoradiography. These steps were replaced by PCR with a 32P-labeled nested gene-specific oligonucleotide, electrophoresis, and autoradiography. This latter modification is commonly used for LMPCR (2, 16).

Using this modified protocol, cisplatin damage could be detected (Figure 1, lanes 7 and 8), and the procedure was shown to work efficiently. The modified protocol for TDPCR was used for LMPCR sequence. We have compared the modified
fied protocol to a three-oligonucleotide TDP PCR method (13) in which a second PCR step is used to directly label the DNA (Figure 1, lanes 9–12). It can be seen that the three-oligonucleotide method (despite poor resolution) gave rise to similar results to our modified protocol in terms of sites of damage and relative damage intensity.

After establishing the viability of the procedure, we attempted to shorten the procedure further by eliminating the first ethanol precipitation step. This gave a final shortened protocol containing seven steps: linear amplification with a gene-specific oligonucleotide, terminal transferase ribo-G tailing, ethanol precipitation, ligation of linker oligonucleotides, PCR with a 32P-labeled nested gene-specific oligonucleotide, electrophoresis, and autoradiography. The results from this shortened protocol can be seen in Figure 1, lanes 1–4 and 13–16. These results show that the shortened procedure works as effectively as the longer procedure.

To eliminate the first ethanol precipitation step, products from the linear amplification reaction were directly added to the terminal transferase reaction. As shown in Figure 1, lanes 13–16, various volumes were added. Above 2 µL no increase in signal was observed, and hence 2 µL was subsequently used routinely.

In contrast to Komura and Riggs (7), who found that 10 linear amplification cycles gave the best signal-to-noise ratio, we found that 20 and 30 cycles gave a better signal-to-noise ratio (using densitometric analysis of the phosphorimage) (data not shown). This could be due to a number of factors because the procedures were significantly distinct. The different gene sequences analyzed are probably the main cause of this difference.

It is important that dithiothreitol is not used in the [32P]-end-labeling of the nested primer because the dithiothreitol reacts with the cobalt chloride in the TdT buffer. This results in a brown precipitate forming on addition of the PCR mixture to the ligation reaction (containing residual TdT buffer), which was found to inhibit the reaction.

In summary, with this shortened protocol, we have enabled this important technique to be carried out in two days rather than four to five days without significantly diminishing the efficiency or accuracy of the method. The removal of several tedious steps (e.g., phenol extraction and electroblotting) should also make this technique more widely available. Chromatin structure footprinting analysis in intact human cells is one potential application of this technique (4).

REFERENCES

Figure 1. Phosphor image of a DNA sequencing gel showing cisplatin damage in human genomic DNA in the epsilon-globin gene promoter using the TDP PCR procedure. Lanes 1, 2, 5, 6, 9, and 10 are control samples derived from undamaged DNA. Lanes 3, 7, 11 and 13–16 are from human DNA samples treated with 0.7 µM cisplatin, and lanes 4, 8 and 12 are from samples treated with 1 µM cisplatin. Lanes 1–4 and 13–16 have been subjected to the shortened TDP PCR procedure; lane 13 represents 1 µL directly added from the linear amplification reaction; lanes 1–4 and 14, 2 µL; lane 15, 3 µL; and lane 16, 5 µL. Lanes 5–8 were from samples that were ethanol precipitated after the linear amplification step. Lanes 9–12 were derived using the three-oligonucleotide procedure (13), with a primer of sequence 5'-TCACGT-CACTGCACCACCTTAAAG-3' being used for first-strand synthesis. See text for further details.
PCR has been widely adapted for the introduction of site-specific mutations into target DNA (8,12). In most cases, mutagenic oligomers are incorporated using a two-step DNA amplification strategy (e.g., Reference 4). Approaches such as the “megaprimer” method (10) are often very effective, but DNA sequences containing repeating elements or encoding extensively paired RNA molecules can be highly recalcitrant to homogeneous amplification, resulting in very heterogeneous populations of products. In our own studies on the role of spacer sequences in rRNA maturation, such heterogeneity often has been observed in the form of broad streaks (e.g., Reference 6). Standard modifications to increase the specificity of the reaction by alterations in annealing temperature, amount of primer, amount of template, magnesium concentration, or the cycle number have provided little improvement. Even specialized approaches such as hot-start PCR (2), touchdown PCR (3), and overlap extension (5,11) have not provided a satisfactory solution. Eventually, a single-strand overlap extension (SSOE) technique was applied successfully (6), but this approach necessitated the separation and fractionation of overlapping single strands, clearly a labor-intensive approach.

In the case of these past trials, it became apparent that alternate pairing between complementary sequence elements within the megaprimer results in heterogeneous intermediates during DNA replication, which gave rise to the highly recombinant populations of replicated molecules. When the SSOE technique is used, the complementary strands are removed during strand purification. In the present report, the recombinations were avoided or minimized by a simplified alternative approach in which the need for strand purification was eliminated entirely. In some respects, the new strategy is a variation of the PCR-ligation-PCR approach.