Evaluation of Different Amplification Protocols for Use in Primer-Extension Preamplification

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ABSTRACT

Different amplification protocols were evaluated for use with primer-extension preamplification (PEP). We hypothesized that a protocol known to improve amplification of long DNA fragments would improve efficacy of PEP. Eight DNA samples were preamplified by PCR using different protocols. Treatments consisted of the use of Taq DNA polymerase (T), Taq plus a second polymerase obtained from Pyrococcus furiosus (E) or Stoffel fragment (S) in PEP. After preamplification, six genetic markers were genotyped, and the number of scorable genotypes was recorded. A control reaction (C) consisted of amplification using genomic DNA as template. A second experiment was performed to evaluate preamplification efficiency using Taq DNA polymerase (5 units) and exponential dilutions of Pfu DNA polymerase. After preamplification, the same procedure was used to obtain a number of scorable genotypes. In the first experiment, treatment E was the most reliable approach for amplifying genomic DNA in PEP. Treatments T and S produced fewer scorable genotypes than treatments E or C. In the second experiment, low concentrations of Pfu DNA polymerase produced a similar percentage of scorable genotypes as higher concentrations. Low concentrations of Pfu DNA polymerase combined with Taq DNA polymerase is the most cost-effective procedure to maximize amplification of limited DNA samples in PEP.

INTRODUCTION

Primer-extension preamplification (PEP) is an in vitro procedure developed to duplicate a large fraction of the genome from limited amounts of DNA, such as that derived from a single haploid cell (16,17). PEP involves repeated primer extensions using a mixture of 15-base random oligonucleotides. The diversity of oligonucleotide sequence helps ensure amplification of segments throughout the genome. It is estimated that at least 78% of the genomic sequence in a single human haploid cell can be copied no less than 30 times (17). Through PEP it is possible to perform multiple genotyping experiments on DNA from single sperm (11,15,17), oocyte (3) or blastomere (5,12,16) cells. PEP may also be an alternative method to increase the number of possible genotypings from limited DNA samples used in genetic mapping experiments.

Several modifications of the original polymerase chain reaction (PCR) protocol have been developed to enhance its utility. One modification is substitution of AmpliTaq® DNA polymerase, Stoffel fragment for Taq DNA polymerase. The Stoffel fragment is a 61-kDa, highly thermostable, recombinant DNA polymerase with no associated exonuclease activity. This polymerase lacks the N-terminal 289-amino acid portion of the full-length polymerase, thus causing increased thermal stability. The Stoffel fragment has been very useful for genetic mapping using primers with random-amplified polymorphic DNA (RAPD). It possesses low processivity, making it most useful for short DNA fragments (6,13).

Another modification of the original procedure is the addition of a second DNA polymerase, obtained from Pyrococcus furiosus, known as Pfu DNA polymerase (2). This second polymerase is used as an additive to improve the reliability and yield of PCR amplifications. Regardless of the concentration in which it is present in the reaction, it enhances the efficiency of DNA extension by Taq DNA polymerase (1). Because of an increased processivity, it increases the proportion of extension reactions that produce long fragments of DNA in each PCR cycle (8).

Although these modifications have been designed to enhance PCR protocols, no evaluation has been made of their use with PEP. We hypothesized that the efficacy of PEP for subsequent genotyping is related to processivity of the polymerase. The objective of this paper is to test this hypothesis by examining genotyping success after PEP with polymerases varying widely in processivity.
MATERIALS AND METHODS

Primer-Extension Preamplification

Template DNA for the study was obtained from eight pigs, picked at random within a large half-sib family (pigs have a common sire). DNA was extracted from skin biopsies using a modification of the salt extraction method (7).

Three different variations of PEP were used in this study to evaluate their ability to produce scorable genotypes from PCR. The PEP reaction was performed alternatively with Taq DNA polymerase (T), with Taq plus Pfu DNA polymerase (E) and with Stoffel fragment (S).

For PEP with Taq DNA polymerase, the reaction consisted of a 33 µM solution of random primers (Operon Technologies, Alameda, CA, USA), 1× Taq DNA polymerase buffer (50 mM Tris-HCl, pH 9.0), 2.5 mM MgCl₂, 750 µM of each dNTP, 5 ng of template genomic DNA and 5 U of Taq DNA Polymerase (Promega, Madison, WI, USA) in a volume of 60 µL. DNA concentration in the PEP PCR is tenfold less than that normally used in our PCR-based genotyping.

The PEP reaction with Taq and Pfu DNA polymerase differed from the above by substitution of 1× Taq Extender™ buffer [20 mM Tris-HCL pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton® X-100, 0.1 mg/mL nuclease-free bovine serum albumin] (Stratagene, La Jolla, CA, USA) for Taq DNA polymerase buffer and addition of 5 U of Pfu DNA polymerase (Stratagene).

The Stoffel fragment reaction substituted 1× Stoffel fragment buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl) and 5 U of AmpliTaq DNA Polymerase, Stoffel fragment (PerkinElmer, Norwalk, CT, USA) for Taq DNA polymerase and Taq buffer.

Fifty primer-extension cycles were performed in an MJ Research Model PTC-100 thermal cycler (Watertown, MA, USA). Each cycle consisted of a 1-min denaturation step at 92°C, a 2-min annealing step at 37°C, a programmed ramping step of 10 s per degree to 55°C and a 4-min polymerase extension step at 55°C (17).

A second PEP experiment was performed to evaluate amplification of long DNA fragments with constant amounts of Taq DNA polymerase and exponential dilutions of Pfu DNA polymerase. Taq DNA polymerase was used in the amount of 5 U per 60 µL reaction. Pfu DNA polymerase was used in the amounts of 5, 2.5, 1.25, 0.625 and 0.3125 U per 60 µL reaction, corresponding to dilutions of 1:1, 1:2, 1:4, 1:8 and 1:16, respectively, of the Taq DNA polymerase used. The remaining materials and conditions were kept the same as before.
Marker Genotyping

Six different microsatellite markers (IGF-1, S0088, Sw72, Sw122, Sw129 and Sw957) were chosen (4,10) to be amplified from PEP product or genomic DNA. The size of the fragments amplified by all markers used in the study ranged from 101–157 bp. PCR was performed in 15-µL reactions with a final concentration of 200 µM of each dNTP, 1× *Taq* DNA polymerase buffer, 1.5 mM MgCl₂, 0.025 U of *Taq* DNA polymerase/µL, 0.2 µM of each primer and 1 µL of PEP product. A control (C) reaction was produced by amplifying each marker from genomic DNA (0.83 ng/µL).

Thirty-three PCR cycles were performed with the first 5 cycles consisting of a 1-min denaturation step at 95°C, a 50-s annealing step at 58°C and a 50-s extension at 72°C. In the remaining 28 cycles, denaturation temperature was lowered to 90°C. Samples were electrophoresed in 8%, 19:1 acrylamide:bis-acrylamide gels for 1800 volt-hours. Gels were stained with ethidium bromide and results recorded photographically.

Presence or absence of scorable microsatellite genotypes was independently recorded by two individuals blinded to the treatment categories. Proportions of average scorable genotypes from the eight samples were obtained. Data were transformed to normalize the distribution using the arc-sine of the square root of the proportion (9). In both experiments, an analysis of variance was performed that included the effects of the individual scoring the sample, genetic marker and treatment.

RESULTS

A significant difference (*P* <0.001) due to the type of PEP treatment method was observed for the percentage of scorable genotypes in the first experiment (Table 1). Different percentages (*P* <0.05) of scorable genotypes were obtained for the control and treatment E (high processivity). Treatments T and S (low processivity) yielded significantly fewer (*P* <0.005) scorable genotypes when compared with treatment E or control. Genotyping success was similar (*P* = 0.16) for treatments T and S (low processivity).

In the second experiment, no differences (*P* = 0.1) in percentage of scorable genotypes were observed with different concentrations of *Pfu* DNA polymerase (Table 2). The percentage of samples amplified varied from 95% (5 U) to 100%. Regardless of the concentration of *Pfu* DNA polymerase used, similar efficiency in the percentage of scorable genotypes was found. Only in the first experiment was a significant effect (*P* <0.01) of marker observed. Marker Sw129 amplified 61% of scorable genotypes, while the remaining markers, while the remaining markers had an average success of 83% (Table 1). In the second experiment, a consistent amplification of
scorable genotypes was obtained. The range of numbers of scorable genotypes was from 95%–100% with an average of 98% of the samples (Table 2).

In both experiments, the effect of the individual scoring the samples was not significant ($P > 0.20$). The proportion of samples that were scored similarly by the two individuals in the first experiment was 92%, while the proportion was 98% in the second experiment. The quality of scorable genotypes produced from preamplified samples was similar for all treatments. In some cases, the PEP treatment resulted in fainter bands when compared with the control. For those experiments that yielded a scorable genotype, the banding pattern was clear and distinguishable with no artifacts or background amplification that could lead to misinterpretation. For experiments that did not yield a scorable genotype, the lane was free of any background or banding pattern.
DISCUSSION

Although use of PEP was originally developed to amplify whole genomes from single cells (5,11,12,16,17), it can also be used to extend the number of possible genotypings of limited DNA stock samples for genetic analysis. The use of Pfu DNA polymerase in the PEP reaction produced a high number of scorable genotypes with microsatellite markers amplified by PCR, relative to Taq or Stoffel alone. Pfu DNA polymerase, used as an additive, has the ability to increase the processivity of Taq DNA polymerase (8). This makes PEP a feasible alternative to direct use of genomic DNA when the original samples are limited.

The results obtained with the two treatments with low processivity (T and S) are considerably lower (Table 1) when compared with the control and treatment E (combined polymerases). The lower genotyping success rate of the low processivity treatments may reflect less complete genome coverage in the preamplification.

Samples treated with Taq DNA polymerase (T) and with the Stoffel fragment (S) gave a similarly low percentage of scorable genotypes. It is known that Taq DNA polymerase has low processivity (14), so long templates will not be effectively amplified by the enzyme. There was no evidence of difference in number of scorable genotypes due to the use of Stoffel fragment when compared with Taq DNA polymerase. Given the low processivity of Stoffel fragment relative to Taq DNA polymerase, a lower percentage of scorable genotypes was anticipated. This result may indicate that processivity affects PEP efficacy in a nonlinear fashion or that factors in addition to processivity have important effects.

Dilutions of Pfu DNA polymerase had no effect (P > 0.05) in the percentage of scorable genotypes observed in the study (Table 2). The lowest percentage of scorable genotypes observed was 95% from the treatment with equal amounts of Taq and Pfu DNA polymerase (5 U). This was not different from other treatments, which yielded up to 100% scorable genotypes. In other studies, high yields of long DNA fragments were amplified from λ bacteriophage template using different ratios of Taq and Pfu DNA polymerases (1). When Taq DNA polymerase was kept constant and a range of concentrations similar to that employed here of Pfu DNA polymerase were used as an additive, long DNA fragments were equally and efficiently amplified. This allows the use of about 99% of the regular and 1% of the second polymerase (1). These results indicate that the presence of Pfu DNA polymerase is important to improve amplification efficiency of long DNA fragments; however, there was no effect within the range of concentrations used in this study.

A significant effect (P < 0.05) of genetic markers was observed in the first experiment. Although marker Sw129 produced 61% scorable genotypes, which is a significantly lower percentage when compared with the remaining markers (Table 1), amplification treatment differences followed the same pattern observed for other markers. In contrast, Sw129 produced scorable genotypes with equal efficacy compared to all the other markers in experiment 2. Further study with Sw129 and other markers under treatments E and S similarly revealed no effect of marker (data not shown). Consequently, these results suggest that statistical differences observed between markers in experiment 1 can be attributed to sampling.

Use of sample preamplification can greatly increase the number of scorable genotypes obtainable from a limited DNA sample. Based on our usual microsatellite genotyping protocol and PEP as outlined here, roughly a 150-fold increase in the number of trials could be performed. Each PEP reaction utilized 5 ng of DNA, which is normally 40% of the amount of DNA used in one genotype reaction. This 5 ng of genomic DNA was used to produce 60 µL of PEP reaction with 1 µL of the PEP-produced material used subsequently in a microsatellite typing assay. Thus a 150-fold increase in the potential number of reactions was achieved before accounting for the genotyping success rate. This approach could probably be extended further through the use of less DNA in the initial PEP reaction.

Increasing extension time in the PEP reaction beyond that used here, to increase product length, is probably unnecessary. Previous reports show that annealing and extension time of at least 10 min but no more than 22 min successfully amplifies targets longer than 20 kb (2). The annealing and extension time used in this study was 9 min (2 min of annealing time, a ramping time of 10 s per degree and 4 min of extension time). This time was in the lower limit suggested to successfully amplify long targets of genomic DNA. Given that the percentage of scorable genotypes obtained from template DNA resulting from PEP with a combination of Taq and Pfu DNA polymerases was similar to the percentage obtained with genomic DNA, there appears to be little need to increase this time further.
Cases in which limited amounts of DNA are available are not unusual. When genetic mapping studies employ markers throughout the genome, many genotyping assays and much DNA may be required. As a consequence, the intensive use of samples may result in their depletion. Cost of obtaining biological specimens or availability may limit the initial amount of DNA attainable. In some cases the nature of the biological organism may limit the amounts of DNA available, as would be the case with Drosophila. In still other situations, laboratory accidents may result in the loss of DNA samples. PEP is a procedure that can be useful in increasing the number of scorable genotypes obtained from DNA samples in these situations.

REFERENCES


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