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Investigating the Effects of DMSO on PCR Fidelity Using a Restriction Digest-Based Method

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Dimethyl sulfoxide (DMSO) is commonly used in PCR to relieve secondary structures when amplifying high GC templates, but we have little understanding of how it affects PCR fidelity based on its interaction with DNA molecules. To evaluate the possible mutational effects, we developed a restriction digestion-based method to rapidly detect erroneous PCR products and compared our result to normal as well as error-prone PCR conditions. We successfully detected PCR mutations generated by the error-prone condition using three different restriction enzymes. The presence of 2% DMSO in PCR did not appear to affect the yield or the proportion of mutations significantly. Overall, our method is a rapid way to assess the occurrence of mutations in a pool of PCR amplicons and can easily be extended to more sensitive studies.

When performing the polymerase chain reaction (PCR), three criteria are critical to its efficacy: high specificity, i.e. the amplification of only the intended target sequence; high efficiency, i.e. a sufficiently large product yield; and high fidelity, i.e. the lack of sequence errors in the final product. In studies of heterogeneous populations in which PCR is employed, such as studies of allelic polymorphisms, allelic stages in single sperm cells, single DNA molecules, or rare mutations in tissues or a population of cells, PCR fidelity is important as every polymerase-induced error introduced in a cycle will be amplified in subsequent cycles. Taq polymerase (Taq), which is commonly used in PCR, possesses no proof-reading ability and has an error rate of 10^{-4} base pairs, making PCR fidelity a concern when performing studies where sequence accuracy of the product is crucial (3, 10).

Dimethyl sulfoxide (DMSO) is an organosulfur compound with a high polarity and high dielectric constant, that is used in PCR to disrupt secondary structure formation in the DNA template. DMSO is believed to hydrogen bond to the major and minor grooves of template DNA, and as a result destabilizes the double helix structure (9). This is particularly useful in templates with high GC content because the increased hydrogen bond strength increases the difficulty of denaturing the template and causes intermolecular secondary structures to form more readily, which can compete with primer annealing (4). Thus the addition of DMSO can greatly improve yields and specificities of PCR priming reactions. However, the effect of disrupted base-pairing imposed by DMSO introduces the worthwhile consideration that mismatched base-pairing could result during the primer annealing steps of PCR, leading to increased mutation rates to the priming region. Another consideration is the effect DMSO may have on the fidelity of Taq, in which proper base-pairing is dependent on the formation of correct hydrogen bonds. Again, mutation rate could be increased as a result of this decreased fidelity.

To detect mutations arising from PCR amplification, a restriction enzyme-based method is employed. The purpose of our study is to investigate whether the presence of DMSO has an effect on PCR fidelity.

MATERIALS AND METHODS

Amplification of *lacZ* gene under different PCR conditions. Based on the sequence of the pUC19 plasmid (Invitrogen, CA), the region from 126 to 595 of the *lacZ* gene open reading frame was targeted to give a 470 base pair (bp) product. Primers: forward 5'-GTGAGCGCAACGCAATTAAT-3', reverse 5'-GGGTGTCGGGGCTGGCTTAA-3', were designed and analyzed using Oligoanalyzer 3.1 (IDT, CA). For all reactions, 1x PCR buffer (Qiagen, CA), 0.25 mM dNTPs, 50 µM of each primer, 50 pg of pUC19 template DNA (Invitrogen, CA) and 1U of Taq polymerase (Qiagen, CA) were added. Appropriate amounts of 100% DMSO and/or deionized water were added to give a 50 µl final volume for standard and 2% DMSO conditions. Error-prone PCR conditions were applied as reported previously (1) by including an additional 5.5 mM of $MgCl_2$, 0.5 mM of $MnCl_2$ and 0.75 mM each of dCTP and dTTP to make the final 50 µL reactions. Amplifications were performed in Biometra T-gradient thermocycler (Biometra, Germany) using the following cycling protocol: denaturation at 94°C for 5 min, 40 cycles of incubation at 94°C for 30 s, 45°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 5 min. For accurate downstream analysis, 9 PCR reactions were performed for each of the conditions (standard, 2% DMSO and error-prone), and 3 reactions were pooled per replicate.

Identification of PCR mutants. Restriction digests were performed on the PCR products using EcoRI-HF (New England Biolabs, MA), BamHI (Gibco, CA), RsaI (Gibco, CA), HindIII

TABLE 1. Restriction enzymes used in this study. (*) denotes the cut site.

Restriction Enzyme	Recognition Sequence/Cut Site	Expected Band Sizes (bp)
EcoRI	5'---G*AATTC---3' 3'---CTTAA*G---5'	296, 174
BamHI	5'---G*GATCC---3' 3'---CCTAG*G---5'	275, 195
RsaI	5'---GT*AC---3' 3'---CA*TG---5'	241, 184, 45
HindIII	5'---A*AGCTT---3' 3'---TTCGA*A---5'	326, 144

(Fermentas, ON) and their corresponding buffers at 1X concentration. These enzymes vary in the sequence and number of their recognition sites (Table 1). Restriction digests were performed by adding either 35 U (EcoRI, RsaI, HindIII), or 20 U (BamHI) of enzyme to 30 µL of pooled PCR product, as well as the appropriate buffer to 1X concentration. A manganese digest that included 0.5 mM $MnCl_2$ was performed to assess whether the presence of Mn^{2+} had an effect on restriction digestion, such as star activity (11). The digests were then incubated overnight at 37ºC. To visualize the digested products, gel electrophoresis was carried out using 16 µL of product loaded on 1% agarose gels in the presence of 0.25 µg/mL ethidium bromide. Gel visualization was performed with Alpha Imager 2000 and AlphaEase FC image software v4.1.0 (Alpha Innotech Corporation, CA).

Quantification of gel bands. The gels were analyzed for relative intensities using the ImageJ program (Research Service Branch, National Institute of Health, MD). For each lane, the closest 500 bp band was used as the reference intensity for all samples. Two areas were used for measurement. Measurement of undigested bands was done using an area sufficient to encompass the largest visible undigested (470 bp) band in the entire gel as well as the 500 bp standard. Measurement of total DNA was done using an area of identical width but with the height extended to include all visible bands.

RESULTS

Effects of DMSO and error-prone conditions on PCR yield. The full gene encoding the *lacZα* was successfully amplified from the pUC19 plasmid in all PCR conditions. In the error-prone PCR, the yield was significantly lower compared to the standard PCR method. Under this condition, the relative intensity observed was only 0.21±0.01 ($p<0.05$), compared to the standard PCR conditions, which had a relative intensity of 0.24 ± 0.01. In contrast, the addition of DMSO did not seem to affect PCR yield significantly as the relative intensity from its band was 0.23 ± 0.01.

Changes in the relative amount of undigested PCR product of *lacZα* amplified in error-prone PCR conditions. Standard PCR samples digested in the presence of 0.5 mM $MnCl_2$ showed no star activity or change in efficacy, demonstrating that the presence of Mn^{2+} in the error-prone PCR conditions does not affect restriction digestion (data not shown). A significant increase in the percentage of undigested PCR products was observed in EcoRI, RsaI and HindIII samples (Fig. 1). In EcoRI-digested samples a 1.45-fold increase was observed in the undigested PCR product of the error-prone PCR over the standard PCR (8.76% to 12.60%) (Fig. 1). RsaI-digested samples showed a 1.62-fold increase (10.14% to 16.44%) in the proportion of undigested product from the standard PCR condition to error-prone PCR condition (Fig. 2). In the case of HindIII, the difference was most prominent as there was a 1.85-fold increase from 6.64% to 14.40% undigested PCR products from the standard PCR to the error-prone PCR respectively (Fig. 2).

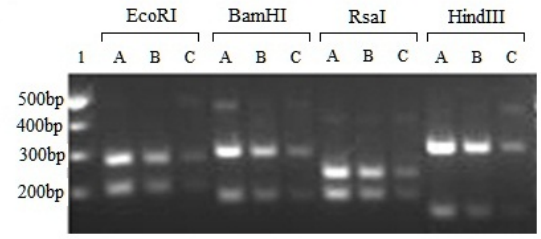

FIG. 1. Effect of DMSO in the PCR on the pattern of restriction fragments generated by digesting the PCR product of *lacZ* with various restriction enzymes. The experiment was performed in triplicates; one representative sample is shown here. Lane Key: 1- 1 kb plus ladder (Fermentas), A- Standard PCR, B- 2% DMSO, C- Error-prone PCR.

Although the BamHI-digested samples did not have a significant increase in the proportion of undigested PCR products (Fig. 2), they did demonstrate a similar trend. A 1.2-fold increase (11.98% to 14.38%) from standard to error-prone PCR was observed (Fig. 2). Therefore, amplification of the template under error-prone PCR conditions increased the proportion of undigested PCR products in all restriction enzymes tested. This is logical, since an increase in mutational frequency should result in a higher proportion of undigested PCR products.

The addition of Mn2+ into the digest of standard PCR showed that the Mn2+, in the error-prone PCR product, did not affect the restriction analysis(data not shown).

Changes in relative amount of undigested PCR product amplified in the presence of 2% DMSO. The addition of 2% DMSO did not affect the percentage of undigested PCR product significantly ($p<0.05$) (Fig. 1B). The trend was not consistent over all four enzymes. RsaI and HindIII digested samples had higher mean proportions of undigested PCR products in 2% DMSO than the standard conditions (Fig. 2). The other two digests, EcoRI and BamHI, had higher mean proportions of undigested PCR products under standard conditions (Fig. 2). This suggests that no significant

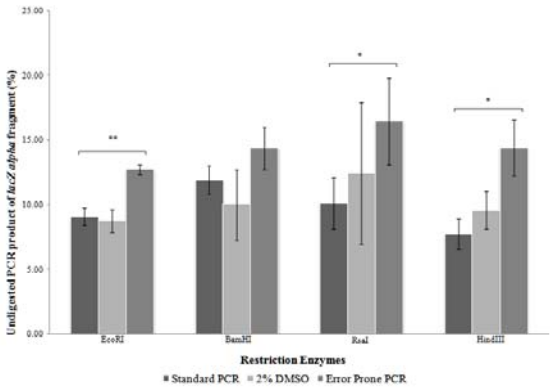

FIG. 2. Comparison of the proportion of residual undigested PCR product of *lacZ alpha* relative to total amplified PCR product following restriction enzyme digest. The relative quantities of the digests were visualized via gel electrophoresis and analyzed with ImageJ Software. Statistical significance at $p<0.05$ (*) or $p<0.005$ (**) was determined by Student's T-test. Error bars represent a standard deviation of n=3 replicates

change in mutational frequency between standard and 2% DMSO was detected using our assay system.

DISCUSSION

The standard PCR conditions used are expected to show an error frequency of ~0.01% per base (i.e., 1 error in 10,000 bases) (10). According to the formula derived by Cadwell and Joyce (1), this should result in proportions of 95% wild type, 4.5% products with 1 error, and <0.5% products with 2 errors or more. Compounded with the probability of a single error being located in a restriction site (1.2% for a 6-bp site), approximately 0.5% of the final product should be unaffected by restriction digestion. However, calculated proportions of undigested PCR products were over 6% even in standard conditions. This may be a consequence of poor resolution in the gel quantification software or incomplete digestion by the restriction enzyme even after overnight incubation. Since the conditions used for error-prone PCR had no effect on restriction digestion, it can be assumed that the proportion of incompletely digested PCR product in error-prone samples is approximately equivalent to that in the standard samples(data not shown). The primary discrepancy is that the amount of total DNA in the error-prone PCR samples is lower. The presumed effect, if any, of the lower DNA amount would be an increase in digestion (since the same amount of enzyme is present in both cases) and therefore an underestimation of the proportion of undigested PCR products. Consequently, the observed increase in undigested PCR products in error-prone PCR remains valid. The assumption is also made that the proportion of incompletely digested DNA is not significantly different between the standard and 2% DMSO samples, although it would have been optimal to perform a similar control for the effect of 2% DMSO on restriction digestion.

An exception is found in the BamHI-digested samples. Unusually high amounts of undigested product can be seen for all conditions, including the standard PCR (Fig. 1). This is most likely a result of either enzyme degradation from age or the lower amount of enzyme used, due to limitations in the amount and quality of enzyme available. Although the trend remains apparent, the increase in background as well as the poor resolution of this quantification method resulted in a lack of statistically significant data. Therefore, although the increase in mutant product expected from PCR performed under error-prone conditions was clearly observed by the use of this assay, the sensitivity of quantification is suboptimal. For instance, though the proportion of mutations in error-prone PCR is more than 20-fold greater than that found from standard conditions (1), < 2-fold increases were observed in our experiment. Although the analysis method used was ostensibly quantitative, interference from background noise and incomplete digestion limits the resolution such that small increases in mutation frequency are likely to go unnoticed. Accordingly, although no significant difference was observed between the standard and 2% DMSO conditions, the possibility nevertheless remains that DMSO increases mutation frequency but at a level that is undetectable using this assay.

In this experiment, the length of recognition sites varied from 4 bp (RsaI) to 6 bp (BamHI, HindIII) (Table 1); RsaI is equivalent to an 8 bp long site, however, since two 4 bp sites are present in our product. The longer the recognition sequence, the more likely it is that a given single base pair substitution will result in a defective cut site - for our 470bp product, there is a probability of 0.85% for a 4-cutter versus a probability of 1.28% for a 6-cutter - and therefore a greater proportion of the mutants will be insensitive to restriction digestion and detectable by this assay. On the other hand, enzymes with a shorter recognition site possess a higher potential for the formation of new digest sites by a single base pair substitution. The probability of a given 4 bp potential site (i.e., varying from the restriction sequence by one nucleotide) occurring in a sequence is considerably higher than for a 6 bp potential site, since the latter needs two more bases in the correct position than the former to be a potential restriction site. Digestion of a mutant product with one or more new recognition sites would result in

unexpected bands. However, in this study, only predicted bands were observed in RsaI-digested samples, even under error-prone PCR conditions (Fig. 1). One possible explanation is that the undigested band represents 24 possible mutations (4 bp x 2 sites x 3 alternate nucleotides) while the creation of a new cut site would only represent one specific mutation. The bands arising from new cut sites would therefore have much lower intensities and might not be detected in gel imaging. This would suggest that detecting the elimination of a digest site is more suitable for this method.

Despite the fact that Cadwell and Joyce reported no significant difference in yield between the standard and mutagenic PCR protocols (2), the yield of PCR product in the undigested error-prone PCR sample was considerably lower than that from the standard conditions. The most likely source for this discrepancy is the 5-fold decreased amount of Taq used in this protocol due to limited availability of supplies. An elevated amount of Taq promotes the extension of the product beyond a mismatch (1), so it is possible that using only 1 unit resulted in early termination of product elongation and a subsequent decrease in the amount of full-length product seen after gel electrophoresis. The most important consequence of this effect is that this may cause PCR products in the error-prone reaction to be biased towards wild-type since it can be extended without difficulty. This would lower the apparent proportion of mutants.

We have shown the ability of this restriction digestion-based assay to assess an increase in mutant PCR products in a simple, efficient and high-throughput fashion. Relative to commonly-used phenotype-based assays (6, 7), this assay boasts considerable flexibility because any product that contains one or more restriction sites or possible restriction sites can be used. Using this assay, mutation frequencies were not found to be significantly elevated upon the addition of 2% DMSO to the PCR conditions. However, since the sensitivity of this assay has not yet been determined, it is possible that the presence of 2% DMSO has a significant adverse effect on PCR fidelity that falls below the threshold for detection in our experiment.

FUTURE DIRECTIONS

Our current approach is limited by its resolution, but it is easily modified. To detect undigested mutations better, one could clone the restriction digest products in a Δ*(lacZ)*M15 background. Colonies that still exhibit alpha-complementation of the *lacZ* deletion, indicated by a Lac+ phenotype, would arise from the cloning of a gene that is intact and therefore insensitive to restriction digestion. For even higher resolution, a selection-based method could be exploited using an antibiotic or auxotrophic marker. In that case, only the colonies that still have the intact gene could survive on the selective growth condition. Also, a number of other gel-based methods are available such as denaturing gradient gel electrophoresis (DGGE) (5) or carbodiimide treatment (8) to sample PCR amplicons directly, but there is a trade-off between sensitivity and simplicity. Furthermore, to look at mutations caused by Taq polymerase more directly, one could sample PCR amplicons for specific mutations such as opal codon reversion assay for base pair mutations and 3'->5' exonuclease activity for exonucleolytic removal activity (10).

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