Reports

Improving sequencing quality from PCR products containing long mononucleotide repeats

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Stutter products are a common artifact in the PCR amplification of frequently used genetic markers that contain mononucleotide simple sequence repeats. Despite the importance of accurate determination of nucleotide sequence and allele size, there has been little progress toward decreasing the formation of stutter products during PCR. In this study, we tested the effects of lowered extension temperatures, inclusion of co-solutes in PCR, PCR cycle number, and the use of different polymerases on sequence quality for a set of sequences containing mononucleotide A/T repeats of 10–17 bp. Our analyses showed that sequence quality of mononucleotide repeats ≤15 bp is greatly improved with the use of proofreading DNA polymerases fused to nonspecific dsDNA binding domains. Our findings also suggest that the number of nucleotides with which the DNA polymerase interacts may be the most important factor in the reduction of slipped-strand mispairings in vitro.

Introduction

Simple sequence repeats (SSRs), or microsatellites, are repetitive nucleotide sequences composed of 1–6 bases found in both organellar and nuclear genomes. These highly repetitive motifs make microsatellites particularly prone to mutation via slipped-strand mispairing (1,2). The relatively rapid rate of mutation, high number of alleles, and their high frequency in genomes have made SSRs popular markers for population genetics, linkage mapping, genetic fingerprinting, and taxonomic study (3,4).

The natural process of slipped-strand mispairing that results in SSR mutation in vivo also occurs in vitro during PCR-mediated DNA replication. Mutations at SSR sites during in vitro enzymatic replication of SSRs are usually the result of insertion or deletion of repeats in the extending, or nascent, DNA strand sequence (5). In order for slipped-strand mispairing to occur, the DNA polymerase enzyme first stalls and dissociates from the dsDNA complex during replication of the repeated motif. If base pairing is disrupted after polymerase dissociation, then a loop of one or more repeat units may form in either the nascent or the template strand prior to re-association and cause the insertion or deletion of one or more units, respectively, in the newly formed DNA strand (1,2). Deletion mutations are believed to be more common as they require fewer nucleotides of the dsDNA to dissociate and therefore are more energetically favorable than insertion mutations (5–7).

In PCR, these artifacts of the process have a greater mutation frequency with greater repeat numbers, and smaller sizes of repeat motifs (8–10). They occur at the greatest rate in mononucleotide repeats (also known as homopolymer runs) composed of eight or more nucleotides (9), which is the estimated number of bases that fill the active site of *Taq* DNA polymerase and many other DNA polymerases (11–13). It is assumed that when the active site of a polymerase is full of identical nucleotides, it is more likely to dissociate (9) and allow the DNA strands an opportunity to misalign.

These mutations can result in stutter products in microsatellite images or in sequence chromatograms, and may confound the delimitation of the true repeat number, as stutter products can be generated in similar or even greater proportions than the true product (9,14). Additionally, the quality of sequence data after a mononucleotide repeat of 10 or more bases is often greatly reduced, often to the point of being unreadable.

This issue is regularly encountered by those who sequence PCR products derived from genomic DNA. In particular, A/T rich regions such as intergenic spacers of the plastid genome—widely used by investigators of plant phylogenetics (15)—often contain mononucleotide repeats. While contigs can usually be generated by sequencing both strands, this necessitates doubling the amount of sequencing effort required in order to obtain a minimum 2-fold coverage. Confounding the problem further are samples that have two or more regions of mononucleotide repeats within targets of interest. Determining the nucleotide sequence between these regions is usually impossible to do with any confidence without the added step of designing internal sequencing primers (16). Slipped-strand mispairing does seem to be primarily (although not wholly) a function of the PCR process. Kieleczawa (16) reported that sequences containing A/T repeats up to 50 bp can be readily sequenced from plasmids, indicating that slippage during the sequencing reaction is not the main source of reduced sequence quality.

Here we report our efforts designed to improve the quality of sequence data generated from PCR-amplified genomic regions containing mononucleotide repeats. We first attempted to reduce the degree of stutter generated with *Taq* DNA polymerase–mediated PCR by increasing the affinity of the polymerase by varying the PCR conditions. We then tested new-gener-

ation DNA polymerases that are designed to have increased performance relative to *Taq* DNA polymerase.

Materials and methods

For our experiments, we focused on a particular genomic region and a set of 25 plant samples (Supplementary Table S1) that we knew contained mononucleotide repeats that negatively impacted sequence quality. Total genomic DNA was extracted from dried leaf material of each sample using the Plant II DNA extraction kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's specifications, yielding DNA concentrations of 40–150 ng/µL per sample. As a starting point for PCR amplification, we used the following standard conditions, from which we subsequently altered various parameters: 20-µL reaction volumes containing 1 U AmpliTaq Gold Polymerase with 1× GeneAmp PCR Buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) (Applied Biosystems, Foster City, CA, USA), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0. 2 µM each primer and 20 ng genomic DNA. Thermal cycling was performed on a Veriti PCR thermal cycler (ABI) as follows: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 1 min, 58°C for 30 s, and 72°C for 1 min; hold at 72°C for 5 min; and an indefinite hold at 4°C. We targeted the plastid *psbA-trnH* intergenic spacer using the primer psbAF (17) (5′-GTTATGCATGAACGTAATGCTC-3′) and a modified version (2 base pairs shorter) of the primer trnH2 (18) (5′-CGCATGGTGGATTCACAATCC-3′).

Amplification products were sequenced directly using the same primers used for PCR, under the following conditions: 10.5-µL reaction volumes containing 0.5 µL BigDye terminator mix v3.1, 1.88 µL 5× sequencing buffer (Applied Biosystems), 1 µM primer and 0.5 µL PCR product. Sequencing thermal cycling parameters were 96°C for 2 min; 30 cycles of 96°C for 30 s, 55°C for 30 s, and 60°C for 4 min; and a 4°C hold. We cleaned cycle sequencing products from each reaction on Sephadex columns (Cat. no. S5897; Sigma-Aldrich, St. Louis, MO, USA) and ran the samples on an ABI 3730 sequencer (Applied Biosciences). Sequences generated under these conditions served as the baseline for quality comparisons. For the various trials below, these conditions were held constant while only the indicated parameters of interest were changed.

Lowering PCR extension temperature

Reducing the extension temperature has previously been shown to be useful for amplification of A/T-rich regions of DNA (19). We hypothesized that if stutter products are caused by dissociation of the polymerase from the template strand, then the generation of slipped-strand products may be mitigated by decreasing extension temperatures in attempts to reduce DNA melting and increase binding affinity of *Taq* DNA polymerase. Since the affinity of *Taq* is not at its optimum at the normal extension temperature of 72°C (20) and DNA melting of A/T-rich regions may occur at 72°C (19), our first experiment was to determine whether sequence quality could be improved by lowering the extension temperature to 60°C. To accommodate the reduced extension speed of the polymerase at this temperature, we also increased the extension time to 3 min.

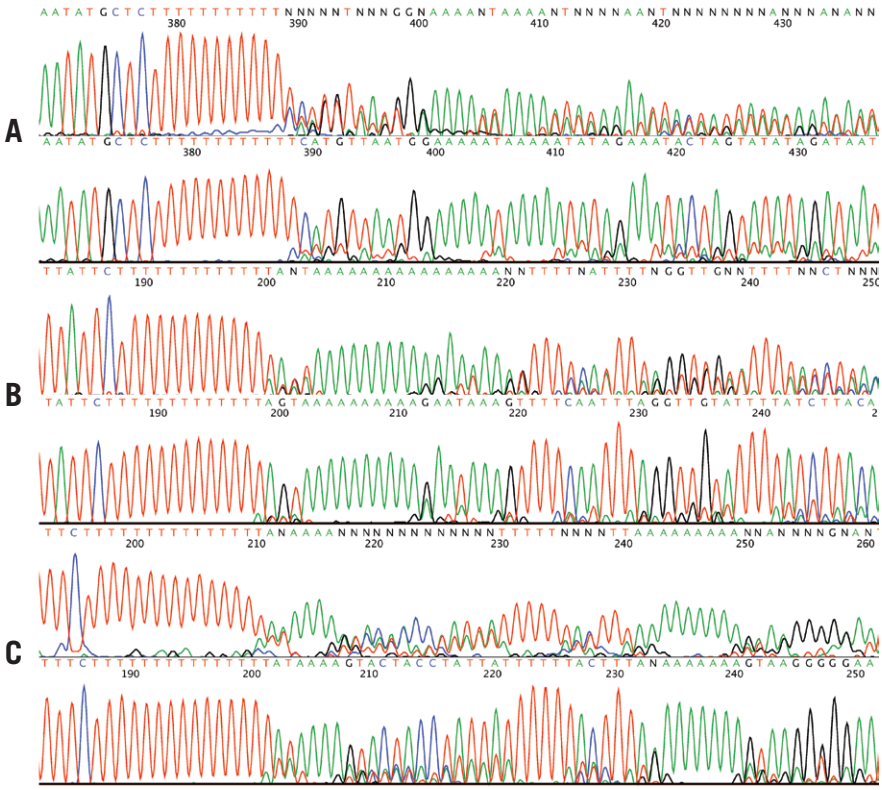

Figure 1. Comparisons of sequence chromatograms derived from the *trnH-psbA* intergenic spacer of three different species. Chromatograms on the top were generated with Ampli*Taq* Gold, and chromatograms on the bottom were generated with Phusion. (A) mononucleotide run of 12 A's (*Hieracium pilosella*). (B) mononucleotide runs of 13T's and 10A's (*Malus pumila*). (C) mononucleotide run of 15 T's (*Lepidium campestre*).

Reducing the number of cycles in PCR

Some reports indicate that PCR-generated errors during amplification increase toward the end of the cycling phase of PCR (21,22). In order to determine whether the main cause of stutter product formation was a consequence of excessive cycle numbers, we performed a series of reactions using the same standard conditions, but with reduced total cycles: 22, 24, 26, 28, and 30 cycles instead of 35.

Inclusion of co-solutes in PCR

With a priori knowledge of the target sequences of our samples, we were aware that most samples likely possess significant secondary structure due to the presence of two mononucleotide repeats of complementary bases. In attempts to minimize the amount of polymerase dissociation caused by template secondary structure, we tested the inclusion of three co-solutes—DMSO, betaine, and trehalose—that have been shown to improve PCR efficiency by lowering the DNA melting temperature (23,24) and reducing secondary structure formation (25). Therefore, we tested the inclusion of (*i*) a 3% (v/v) final concentration of DMSO, (*ii*) a 1 M final concentration of betaine, and (*iii*) a 5% (w/v) final concentration of trehalose in both the PCR and sequencing reactions.

New generation polymerases

Following these experiments we progressed to the evaluation of three new-generation enzymes that are reported as having levels of processivity more than two times greater than *Taq* DNA polymerase, and lower error rates: Phusion (Finnzymes, Espoo, Finland), Herculase II Fusion (Agilent, Santa Clara, CA, USA), and KAPAHiFi (Kapabio-

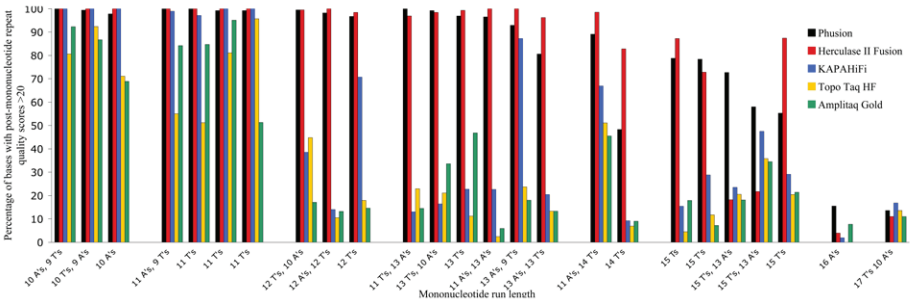

Figure 2. Quantitative assessment of post-mononucleotide repeat sequence quality. Bar graphs indicate the percentage of nucleotides between the 3′ end of the first mononucleotide run and the reverse primer with a quality score greater than 20 for various repeat lengths using five different polymerases.

systems, Boston, MA, USA) (see technical data sheets at www.finnzymes.com/pdf/phusion_brochure_fph6_low.pdf, www.kapabiosystems.com/public/pdfs/kapa-hifi-pcr-kits/KAPA_HiFi_Brochure.pdf, and www.stratagene.com/lit_items/PCR Brochure 6-21-07.pdf).

Phusion is a *Pyrococcus*-like DNA polymerase that has been attached to the nonspecific dsDNA binding protein Sso7d, resulting in increased catalytic activity and processivity (26). Herculase II Fusion is also a *Pfu*-based DNA polymerase with an added double-stranded DNA binding domain, and is provided with a reaction buffer that eliminates the inhibition of proofreading enzymes by PCR byproducts. KAPAHiFi is a high-fidelity engineered DNA polymerase that has been modified for increased affinity to DNA resulting in high processivity without fusion based proteins.

We also evaluated a fourth polymerase, Topo Taq HF (Fidelity Systems, Gaithersburg, MD, USA) which is a hybrid enzyme that is linked to multiple nonspecific DNA binding HhH domains (27) and is blended with a topoisomerase, conferring strand displacement activity.

Reaction and cycling protocols generally followed manufacturers' recommendations. For Phusion, we used 20-µL reaction volumes containing 0.2 U polymerase with 1× Phusion HF Buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 µM each primer, and 20 ng genomic DNA. For KAPAHiFi, we used 20-µL reaction volumes containing 0.4 U polymerase with 1× KAPAHiFi Fidelity Buffer (containing 2.0 mM $MgCl_2$), 0.2 mM dNTPs, 0.2 µM each primer, and 20 ng genomic DNA. For Herculase II Fusion, we used 20-µL reaction volumes containing 0.4 U polymerase with 1× Herculase II reaction buffer (containing 2.0 mM $MgCl_2$), 0.25 mM dNTPs, 0.2 µM each primer, and 20 ng genomic DNA. For Topo Taq HF, we used 20-µL reaction volumes containing 1 U polymerase with 1× amplification buffer (containing 3.0 mM $MgCl_2$), 0.5 mM dNTPs, 0.3 µM each primer, and 20 ng genomic DNA.

Since the Phusion and KAPAHiFi polymerases have been designed for reduced cycling times, we used increased denaturation and annealing temperatures and shortened thermal cycling times (per the manufacturer's recommendations) as follows: an initial denaturation at 98°C for 1 min; 30 cycles of 98°C for 20 s, 64°C for 15 s, and 72°C for 20 s; hold at 72°C for 5 min; and an indefinite hold at 4°C. To ensure that any improvements in sequence quality were not a result of changing the thermal cycling parameters alone, we also performed PCR with AmpliTaq Gold polymerase using the adjusted thermal conditions.

Reactions containing Herculase II Fusion, or Topo Taq HF were amplified with conditions similar to those for AmpliTaq Gold, as follows: an initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 30 s; hold at 72°C for 5 min; and an indefinite hold at 4°C. Amplification products were sequenced using the methods outlined for generating the baseline quality scores..

To ensure that any improvements in sequence quality were not a result of changing the thermal cycling parameters alone, we also performed PCR with *Taq* DNA polymerase using the adjusted thermal conditions used for the other enzymes.

Data analysis

Sequence trace files were inspected and edited using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Most sequence chromatograms were qualitatively inspected by visual means to assess whether there was any improvement over standard reaction conditions. For most trials, this was sufficient to determine that there was no significant improvement in quality. PCR products that were amplified more than once under the same PCR conditions showed little variation in sequence quality (data not shown).

In order to obtain a quantitative assessment of quality improvement for improved chromatograms, Sequencher was used to generate quality scores for sequences generated under standard PCR conditions with AmpliTaq Gold, and compared with sequences using the other four enzymes. Poor sequence quality as a result of mononucleotide repeats are typically restricted to nucleotides that are after the 3′ end of the repeat. In order to provide an estimate of quality unbiased by the relative position of the mononucleotide repeat in the sequence, we restricted the quality assessment to the portion of the sequence that was between the 3′ end of the mononucleotide repeat (the first repeat in cases where there were two) and the reverse priming site. For each sequence, we determined the percentage of bases within this region with quality scores >20.

Results and discussion

Despite the importance of accurate sequencing and genotyping of microsatellites to various biological and forensic applications, limited progress has been made toward the reduction of in vitro frameshift mutations. To date, manufacturers have focused primarily on improving polymerase error rate, performance with difficult GC-rich templates, and processivity. While significant improvements have been made in these particular areas, the options for sequencing through long mononucleotide repeats in PCR products are few.

The most effective method of alleviating frameshift error is still the use of thermolabile polymerases at low extension temperatures (3). Hite et al. (3) hypothesized that the low extension temperature (37°C) used with thermolabile polymerases decreases the likelihood of dissociation of the 3′ end of the nascent strand, thereby reducing the occurrence of slipped-strand mispairings. Although the use of thermolabile DNA polymerases may be effective in this regard, the application to PCR and cycle sequencing reactions is not generally practical due to the need to add enzyme after each denaturation step. Computational methods have been developed to model and interpret stutter patterns for automated genotyping applications (4,28) but the optimal solution would be to reduce the degree of stutter product formation in the first place.

All of our attempts to improve the quality of sequence data from AmpliTaq Gold–generated PCR products by altering various parameters of the PCR constituents or the thermal profile (including the same thermal conditions as used for the other polymerases) failed to yield any improvements in quality.

The use of different polymerases yielded varying results. The sequence quality of samples amplified using Topo Taq HF was similar to that generated using AmpliTaq Gold. Improvement in sequence quality scores was demonstrated with the use of KAPAHiFi relative to AmpliTaq Gold for sequences containing runs of 10 or 11 bp, but sequences from samples with longer runs showed no consistent improvement.

In contrast however, we found that use of fusion enzymes (Phusion, Herculase II Fusion) resulted in a marked improvement in reducing stutter product formation. Quality scores >20 were determined for nearly 100% of the bases derived from samples that had mononucleotide repeats ≤13 bases in length (Figures 1 and 2). Considerable improvement in quality scores for repeats of 14 and 15 bases was also observed. Two sequences we tested with 16- and 17-base repeats were little improved with fusion enzymes over reactions that used *Taq* DNA polymerase. This improvement could be due to a number of mutually nonexclusive phenomenon.

Since the formation of stutter products necessitates the dissociation of the DNA polymerase, it is possible that the increased processivity of the fusion-based enzymes decreases the likelihood of dissociation during replication of a mononucleotide repeat and therefore reduces stutter product formation. If this was the mechanism, one would expect to see similar results from other enzymes with high processivity. Our quality results from KAPAHiFi, however, showed no consistent improvement over AmpliTaq Gold polymerase for samples with mononucleotide repeats greater than 12 bp, which is consistent with previous work (3) that failed to find a link between processivity and frameshift error.

A separate possible mechanism for reducing stutter product formation that we considered is proofreading ability. A study of T7, T4, and *Pfu* DNA polymerases found that exonuclease-deficient mutants of the enzymes produced more mutations than their proofreading native forms, indicating that proofreading ability may enable the enzymes to correct frameshift mutations. However, the ability of proofreading polymerases to correct frameshift mutations was greatly reduced as the repeat size reached 8 nucleotides (10). Kroutil et al. (10) found that proofreading T7 DNA polymerase decreased deletion frameshift errors over a non-proofreading–deficient mutant by 160× for repeats 3 nucleotides in length, but this advantage decreased to only 7× for runs of 8 nucleotides in length, indicating an upper limit to the ability of proofreading polymerases to reduce stuttering. Our data also indicates that proofreading ability has little effect in reducing frameshift errors associated with mononucleotide repeats. Our trials using KAPAHiFi, a proofreading polymerase with the lowest error rate currently available (2.8×10^{-7} errors per nucleotide), yielded little to no improvement in sequence quality versus the non-proofreading AmpliTaq Gold enzyme. This result fits well with the hypothesized process of slipped-strand mispairing. Despite formation of a loop in one strand during replication of a long mononucleotide repeat, the 3′ end of the nascent strand could be paired properly with the template strand at any point along the repeat, leaving the 3′-to-5′ proofreading ability nothing to operate on.

An additional possibility is that some property of polymerases makes them susceptible to dissociation, at least in vitro, when their active sites are entirely occupied with repetitive sequences (9). One explanation of fusion enzymes' ability to decrease stutter is that the additional DNA binding domain effectively increases the contact surface with the DNA, enabling accurate replication of larger repeat regions. The maximum rate of mutation in homopolymer runs has been found to occur in vitro at runs 8 bp in length (9), which corresponds to the estimated number of nucleotides that fill the active site of *Taq* and many other DNA polymerases (12–14). It is interesting to note that in this study the quality of sequences generated with Phusion (as well as Herculase II Fusion) declined rapidly after 13 mononucleotides (Figure 2). This represents a 5-bp improvement over previous studies that found maximal mutation rates in runs ≥8 bp (9,10) and corresponds to the 4–5 bp estimated to interact with the Sso7d protein (29–31). This is suggestive that the mechanism of decreased stutter product formation observed with the Phusion enzyme in this study is a property of the increased contact between enzyme and DNA afforded by the fusion of the Sso7d protein to the polymerase.

Another potential benefit of the Sso7d protein is the ability to increase the melting temperature of dsDNA by up to 39°C (32). This attribute may be generally beneficial in the amplification of A/T-rich amplicons. Some DNA melting may occur at 72°C during the elongation phase that would cause the termination of elongation (19) and result in potential stutter product formation.

Although the use of fusion enzymes improved sequence quality for a number of our samples, the improvement appears to reach a limit at mononucleotide repeats of 15 bp, with no improvement in quality of sequences with longer runs. Further optimization of the Phusion-based PCR reactions and/or cycle sequencing reaction may yield further improvements in sequence quality; however, the initial trials we have performed have not yielded significantly positive results.

Additional improvements in quality for sequences with repeats >15 bp may require development of other accessory proteins, or novel polymerases. We note the recent report of SsoDPo1 (33), which can form trimeric complexes with DNA resulting in a large contact surface and extreme processivity (900 bp).

Here we have reported an unexplored attribute of fusion polymerases, and a resulting simple and cost-effective way to reduce genotyping errors in PCR-based sequencing. These findings will be of broad utility to investigators interested in optimizing sequence quality and allele detection in simple sequence repeats. Our findings also indicate that neither processivity nor proofreading ability alone can account for the mitigation of slipped-strand intermediates and suggest that the reduction of stutter product formation may be a result of increasing the polymerase contact surface.

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Competing interests

The authors declare no competing interests.

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