Mutational Analysis of the *ATM* Gene in Familial Breast Cancer Using iProof™ High-Fidelity DNA Polymerase

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Introduction

Breast cancer is the most common cancer among women in the developed world. In 5–10% of the cases it is thought to be heritable. Two highly penetrant breast cancer genes have been identified (BRCA1 and BRCA2). However, a large number of cases with a familial predisposition to breast cancer cannot be explained by mutations in these two genes. Therefore, other genes predisposing to breast cancer can be assumed to exist. One candidate is the *ATM* gene, which is mutated in the recessive neurodegenerative disorder ataxia telangiectasia (AT). AT is characterized by progressive neuronal degeneration, oculocutaneous telangiectasia, immunodeficiency, hypersensitivity to ionizing radiation, and an increased risk of lymphoma and leukemia, as well as breast cancer. More than 80% of the mutations found in patients with AT are nonsense mutations.

The *ATM* gene is located on human chromosome 11q22.3. It consists of 66 exons, of which four are noncoding. The *ATM* gene expresses a 370 kD serine protein kinase that consists of 3,056 amino acid residues and is located primarily in the nucleus. It is activated by ionizing radiation and DNA damage. The activated ATM protein phosphorylates and regulates proteins involved in DNA repair and cell cycle control.

Several epidemiological studies have reported an increased risk of breast cancer in female relatives of patients with AT who are obligate heterozygous carriers of *ATM* mutations. However, several subsequent case-control studies have failed to detect a higher frequency of *ATM* mutation carriers in patients with breast cancer. These conflicting results may have been caused by the inclusion in the studies of cases without a familial history of breast cancer. Moreover, most of the case-control studies used methods that detected only protein truncating mutations. Therefore, a possible association of missense mutations with an increased risk of breast cancer could have been missed.

In order to clarify the role of the *ATM* gene in familial breast cancer, an international NIH joint project with the Queensland Institute of Medical Research in Australia was initiated. The aim was to perform mutational analysis of the *ATM* gene in a large number of familial breast cancer cases using a combination of bidirectional dideoxysequencing and the multiplex ligation-dependent probe amplification (MLPA) assay (Schouten et al. 2002). To that end, all exons and adjacent intronic sequences of the *ATM* gene were amplified in a total of 65 fragments for sequence analysis. Concurrently, the performance of iProof high-fidelity DNA polymerase from Bio-Rad Laboratories, Inc. was compared to that of a widely used hot-start non-proofreading Taq polymerase from a different vendor.

Methods

For comparison of the iProof polymerase with the alternative Taq polymerase, seven exons were amplified with both polymerases and sequenced. Primers with similar melting temperatures were designed, which enabled the use of the same cycling protocol for all fragments (Table 1).

Table 1. Primer sequences and amplicon sizes of the *ATM* gene fragments used in the comparative analysis of the iProof polymerase from Bio-Rad and a hot-start Taq polymerase obtained from a different vendor.

	Amplicon Length, bp	Forward Primer, 5'–3'	Reverse Primer, 5'–3'
Exon promoter	595	TCAACTCGTAAGCTGGGAGGCA	CGCATCCAGTATCACGCGGT
Exon 1a	600	GAAATGAAACCCGCCTCCGT	GAGGGAGGAGTCAAGGGCCA
Exon 35	581	AAGGAAGTTCAGATTCATTCCCTA	TGAATACTACAGGCAACAGAAAACA
Exon 53	600	CACACTCAGATCACATTTGTCTTCC	AAAGGCAGAGGCCTATGAGGAAT
Exon 60	554	CCCAATGCTGTGATGCCACC	CCTGCCAAACAACAAAGTGCTCA
Exon MLPA 30*	1,987	GAGCTGTCTTGACGTTC	GCAGTCTTTCTATCCTGTTCTT

* The primers for exon MLPA 30 have been designed with Primer3.

PCR amplification with 0.3 U iProof polymerase (Bio-Rad) was carried out in a 15 µl volume containing 25 ng of genomic DNA, iProof HF master mix (Bio-Rad), 400 µM of each dNTP, and 0.48 µM of each forward and reverse primer. Thermocycling was performed in a 96-well GeneAmp PCR system 9700 thermocycler (Applied Biosystems). Initial denaturation of DNA at 98°C for 30 sec was followed by 35 cycles of denaturation at 98°C for 10 sec, primer annealing at 60°C for 10 sec, and primer extension at 72°C for 10 sec. A final elongation step was performed at 72°C for 10 min. Different times for annealing and elongation had been tested, but the best results were achieved with the indicated times.

For the competing Taq polymerase, PCR was also carried out in a 15 µl volume containing 25 ng of genomic DNA. Reactions contained 0.38 U Taq polymerase, 30 mM Tris-HCl, 100 mM KCl, 400 µM of each dNTP, 5 mM $MgCl_2$, and 0.16 µM of each forward and reverse primer. Thermocycling was performed in a 96-well GeneAmp PCR system 9700 thermocycler (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 5 min was followed by 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 45 sec, and primer extension at 72°C for 45 sec. The final elongation was performed at 72°C for 10 min.

The quality of the PCR amplicons was evaluated by electrophoresis on a 2% agarose gel in 1x Tris-boric acid-EDTA buffer (TBE). Amplicons generated by either polymerase were then cleaned up with Agencourt AMPure kit (Agencourt Bioscience Corporation) on a Biomek NX laboratory automation workstation (Beckman Coulter, Inc.). The purified DNA was eluted in 20 µl of HPLC water (Merck KGaA).

Sequence reactions were carried out with a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). Reactions were performed in a total volume of 10 µl, containing 2 µl PCR product, 2 µl 5x sequencing buffer, 0.32 µM M13 forward or M13 reverse primer, and 0.25 µl BigDye. Cycle sequencing was conducted in a 96-well GeneAmp PCR system 9700 thermocycler (Applied Biosystems) with the following program: initial denaturation at 96°C for 1 min was followed by 25 cycles of denaturation at 96°C for 10 sec, primer annealing at 50°C for 5 sec, and primer extension at 60°C for 90 sec. Unincorporated ddNTPs, dNTPs, and primers were removed with a CleanSEQ kit (Agencourt Bioscience Corporation). The purified products were eluted in 30 µl 0.1 mM EDTA.

The DNA cycle sequencing reaction products were analyzed with a 3730 DNA analyzer and SeqScape software version 2.5 (Applied Biosystems).

Results and Discussion

The hot-start non-proofreading Taq polymerase commonly used in our laboratory repeatedly failed to yield sufficient products for some of the *ATM* gene fragments (Figure 1). As a consequence, sequencing either failed or yielded sequence traces of a quality too low for unambiguous base calling. In contrast, the iProof polymerase yielded amplicons of high quality for the same sequences. There was no or little background in the sequence traces, and the base signals were of sufficient quality for unambiguous base calling (Figure 2).

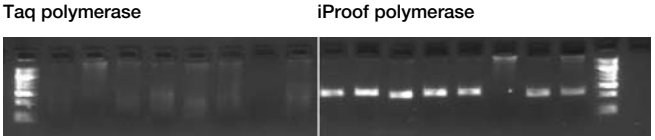

Fig. 1. Analysis of PCR fragments. Comparison of PCR yield for eight different samples of exon 35 of the *ATM* gene amplified with either hot-start non-proofreading Taq polymerase (left) or the iProof polymerase (right).

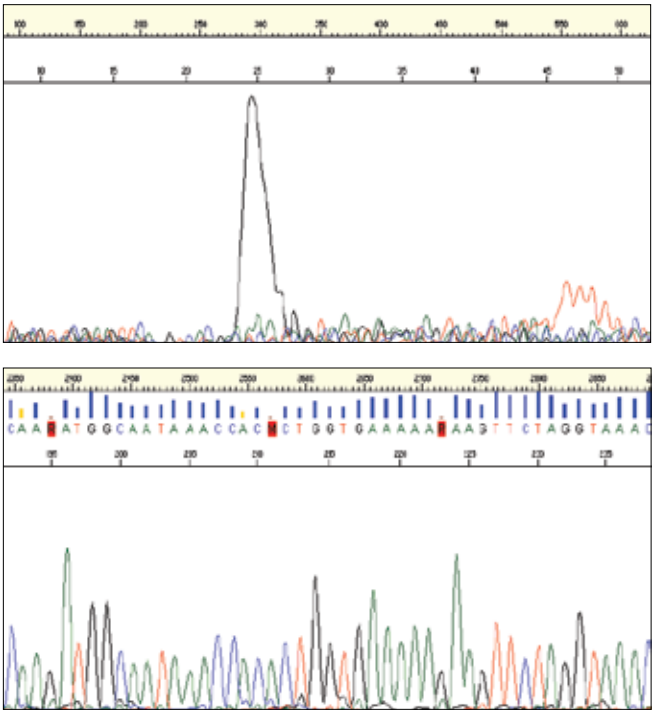

Fig. 2. Sequencing of PCR-amplified DNA. Comparison of analyzed DNA sequence trace records of a sample of exon 35 PCR amplified with either hot-start non-proofreading Taq polymerase (top) or iProof polymerase (bottom).

A further advantage of the iProof polymerase was the reduced PCR cycling time of 28 min compared to 95 min for the Taq polymerase. The short elongation time of 10 sec worked well for amplicons of different length without a loss of quantity or quality. Consequently, all *ATM* gene fragments that had failed to amplify with the Taq polymerase were subjected to PCR amplification with the iProof polymerase. Consistently excellent amplification (Figure 3) and sequencing results were obtained. The iProof polymerase was also tested for the high-throughput PCR amplification and sequencing of *ATM* exons 35 and 53 in a 384-well format. Again, the iProof polymerase consistently yielded amplicons of high quality and quantity and thereby maximized productivity (not shown).

The iProof polymerase also worked well for the amplification of DNA fragments larger than 1 kilobase in length that were required for the analysis of break points of exons that were found to be duplicated or deleted using the MLPA assay (Figure 4). Successful PCR amplification of these fragments took only 53 min.

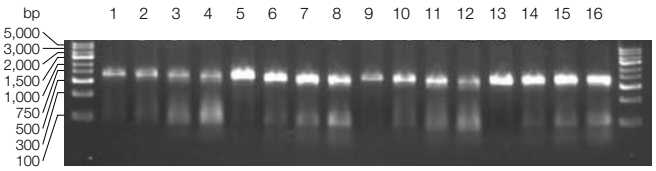

Fig. 3. Effective amplification of *ATM* gene sequences 600–750 bp in length that are difficult to amplify. PCR yields, with the iProof polymerase, of different samples and exons that had proven difficult or impossible to amplify with hot-start non-proofreading Taq polymerase. Lanes 1–4, exon 53; lanes 5–8, exon 60; lanes 9–12, promoter region; and lanes 13–16, exon 1a.

Fig. 4. Amplification of DNA fragments ~1.2 kb in length. Lanes 1 and 2, negative control; lanes 3 and 4, with hot-start non-proofreading Taq polymerase; lanes 5 and 6, with iProof polymerase.

Conclusions

In summary, the iProof polymerase has significantly reduced PCR cycling time and successfully and reliably amplified sequencing quality DNA fragments that had failed repeatedly with the Taq polymerase from another vendor.

Reference

Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30, e57.

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