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Polymerase chain reaction fidelity and denaturing high-performance liquid chromatography

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Abstract

Incorporation of non-complementary nucleotides during polymerase chain reaction can result in ambiguous denaturing high-performance liquid chromatography profiles that reduce both sensitivity and specificity of mutation analysis. The use of proofreading DNA polymerases increases the fidelity of polymerase chain reaction and, consequently, reduces background noise in the chromatograms. This is demonstrated for several *BRCA1* and *BRCA2* mutations hat had yielded previously chromatograms of poor quality using non-proofreading enzyme for amplification. Interestingly, despite the reduced level of background heteroduplices, the ability of denaturing high-performance liquid chromatography to detect mutant alleles at a frequency <10% in pools of chromosomes did not improve significantly.

Keywords: Polymerase chain reaction fidelity; Denaturing HPLC; Mutation analysis; DNA polymerase

1. Introduction

Partially denaturing high-performance liquid chromatography (DHPLC) has emerged as the most sensitive physical mutation screening method in a recent comparison with single strand conformational polymorphism analysis, conformation sensitive gel electrophoresis, and two-dimensional gene scanning [1]. The comparative study was based on a set of blinded samples containing 58 distinct mutations in *BRCA1.* Only the laboratory employing DHPLC identified correctly each of the mutations. The other laboratories, in contrast, failed to report correctly 9–40% of the mutations. This study confirmed other less rigorously designed studies that had attested DHPLC a mutation detection rate consistently in excess of at least 96% [2,3]. Most studies have also reported few if any false positives [2]. One exception has been a recent evaluation of DHPLC for mutation detection in the fibrillin 1 gene with an overall specificity of only 48% [3]. However, all false-positive calls were associated with amplicons of poor or medium quality. Generally, it has been recommended that the quality of amplified fragments should be similar to that suitable for direct conventional sequencing or subcloning [2]. This is achieved by careful primer design, elimination of pre-PCR artifacts using ''hot-start'' approaches, attention to optimal Mg^{2+} concentration, avoidance of excessive cycle number, and conditions that maximize standard *Taq* polymerase fidelity, such as adequate template concentration (0.5–1 ng/μl of reaction volume) and the use of stoichiometrically balanced deoxynucleotide triphosphate concentrations to prevent ex-

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cessive misincorporation of nucleotide bases [4]. Specialty low error rate thermostable DNA polymerases are not considered generally necessary, though they have been reported to reduce the amount of background heteroduplices significantly [5]. Recently, Transgenomic (Omaha, NE, USA) and Stratagene (La Jolla, CA, USA) have introduced proofreading DNA polymerases under the brand names Optimase™ and AccuType™, respectively, that do not only seem to amplify genomic targets with high fidelity and yield but are also compatible with the poly-(styrene–divinylbenzene) separation matrix whose performance can deteriorate rapidly in the presence of metal cations such as manganese [2,5].

In the present study, we evaluated the quality of chromatograms and the detection of heterozygous carriers of mutations in *BRCA1* and *BRCA2* following amplification with AmpliTaq™ Gold (Applied Biosystems, Foster City, CA, USA) and Optimase™, respectively. We also investigated whether the use of a proofreading DNA polymerase would improve the ability of DHPLC to detect mutations present at low frequency.

2. Materials and methods

2.1. DNA samples

Ten of the 11 sequence variants in *BRCA1* and *BRCA2*, which are listed in Table 1, had been discovered previously during the mutational analysis of hereditary breast and/or ovarian cancer families referred to the Division of Senology at the University of Vienna. The mutation 478 C>T in exon 3 of *BRCA2* had been identified at the Peter MacCallum Cancer Institute in Melbourne, Australia. Written consent had been obtained from all individuals prior to analysis.

Table 1
PCR and DHPLC conditions for the mutational analysis of selected *BRCA1* and *BRCA2* mutations

Mutation[a]	Gene	Exon/intron	Primers	
			Forward (5′–3′)	Reverse (5′–3′)
300 T>G	*BRCA1*	5	GTTGTGAGATTATCTTTTCATGGC	CTTCCAACCTAGCATCATTACCA
1630 G>A	*BRCA1*	11	GGTTCTGATGACTCACATGATGGG	TCATCACTTGACCATTCTGCTCC
1806 C>T	*BRCA1*	11	GAGCCACAGATAATACAAGAGCGTC	GCAGATTCTTTTTCGAGTGATTCTATTGGG
2201 C>T+2430 T>C	*BRCA1*	11	AGCAACTGGAGCCAAGAAGAGTAAC	TCTGTGTCATTTCTATTATCTTTGGA
2382 G>T	*BRCA1*	11	GCAACTGGAGCCAAGAAGAGTAAC	TCTGTGTCATTTCTATTATCTTTGGA
3232 A>G	*BRCA1*	11	TCAATGTCACCTGAAAGAGAAATGG	CAGGATGCTTACAATTACTTCCAGG
5272+66 G>A	*BRCA1*	18	GGCTCTTTAGCTTCTTAGGAC	GAGACCATTTTCCCAGCATC
460 C>T or 478 C>T	*BRCA2*	3	TTCCTTATGATCTTTAACTGTTCT	GCTAAGATTTTAACACAGGTTTGC
7772 C>T	*BRCA2*	15	GGCCAGGGGTTGTGCTTTTT	ATTTCATTCATCCATTCCTGC

	Amplicon size (bp)	AmpliTaq™ Gold		Optimase™		DHPLC conditions	
		Annealing temp. (°C)	$MgCl_2$ (n*M*)	Annealing temp. (°C)	$MgCl_2$ (n*M*)	Gradient[b]	Column temp. (°C)
300 T>G	208	63–56	3.5	63–56	1.5	50–54% B in 0.5 min, 54–61% B in 3.5 min	53
1630 G>A	460	65–58	3.0	65–58	1.5	50–59% B in 0.5 min, 59–66% B in 3.5 min	56
1806 C>T	273	60	2.0	67–60	1.5	50–55% B in 0.5 min, 55–62% B in 3.5 min	56
2201 C>T+2430 T>C	458	63–56	3.0	63–56	1.5	50–58% B in 0.5 min, 58–65% B in 3.5 min	57
2382 G>T	458	63–56	3.0	63–56	1.5	50–58% B in 0.5 min, 58–65% B in 3.5 min	57
3232 A>G	301	55	3.5	63–56	1.5	50–57% B in 0.5 min, 57–64% B in 3.5 min	56
5272+66 G>A	352	60	1.5	63–56	1.5	50–57% B in 0.5 min, 57–64% B in 3.5 min	56
460 C>T or 478 C>T	406	65–58	2.5	61–54	1.5	50–55% B in 0.5 min, 55–62% B in 3.5 min	56
7772 C>T	370	55	1.5	61–54	1.5	55–52% B in 0.5 min, 52–59% B in 3.5 min	59

[a] Genomic position in relation to Genbank Accession numbers U14680 and U43746 for *BRCA1* and *BRCA2*, respectively.
[b] Eluant A: 0.1 *M* triethylammonium acetate, pH 7.0; eluant B: 0.1 *M* triethylammonium acetate, pH 7.0, 25% (v/v) acetonitrile.

2.2. Polymerase chain reaction

Polymerase chain reactions with AmpliTaq™ Gold were carried out in a 50-μl volume containing approximately 100 ng of genomic DNA, 200 μ*M* of each dNTP, 12.5 pmol of each forward and reverse primer (Table 1), 1× PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 1.5–3.5 m*M* $MgCl_2$ (see Table 1), and 1.0 Unit of AmpliTaq™ Gold DNA polymerase (Applied Biosystems). Reactions carried out with Optimase™ contained 1× Optimase™ buffer (Transgenomic), 1.5 m*M* of $MgSO_4$, and 2.5 Units of Optimase. All other reagent concentrations were identical to those used with AmpliTaq Gold. Thermocycling was performed in an Applied Biosystems 9700 thermocycler. In case of AmpliTaq Gold, initial denaturation (of DNA)/activation (of enzyme) at 95 °C for 4 min was followed by 14 cycles of denaturation at 95 °C for 20 s, primer annealing under touchdown conditions starting at either 65 or 63 °C (see Table 1) for 1 min and decreasing by 0.5 °C in each consecutive cycle, and primer extension at 72 °C for 20 s. We then performed 31 additional cycles at 94 °C for 20 s, 56–58 °C for 20, and 72 °C for 20 s. A final elongation was performed at 72 °C for 7 min, after which the reaction was held at 4 °C. In four instances, no touchdown protocol was used. Instead, 40 cycles were performed at 94 °C for 45 s, 55–60 °C (Table 1) for 30 s, and 72 °C for 30 s. Amplifications performed in the presence of Optimase were started with an initial denaturation step at 95 °C for 5 min, followed by 14 cycles of denaturation at 95 °C for 30 s, primer annealing under touchdown conditions starting at either 61–67 °C (see Table 1) for 30s and decreasing by 0.5 °C in each consecutive cycle, and primer extension at 72 °C for 1 min. Subsequently, 20 cycles were performed at 95 °C for 30 s, 54–60 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min.

2.3. Denaturing HPLC

Denaturing HPLC compares two or more chromosomes as a mixture of denatured and renatured amplicons [2]. The presence of single or multiple mismatches is revealed by the appearance of one or more early eluting peaks or shoulders in the chromatographic profile representing heteroduplex species. To ensure the proper formation of homo- and heteroduplices, all PCR products were denatured once more at 95 °C for 3 min and allowed to renature over 30 min by decreasing the temperature from 95 to 65 °C. Amplicons were then stored at 4 °C until analyzed on a WAVE® System 2100A equipped with a DNASep™ column, both of which were purchased from Transgenomic. The mobile phase was 0.1 *M* triethylammonium acetate (Transgenomic), pH 7.0. DNA fragments were eluted at a flow-rate of 0.9 ml/min with a linear acetonitrile (Merck, Darmstadt, Germany) gradient that varied as a function of amplicon size and column temperature (Table 1). Chromatographic conditions for detection of mutations of interest had been optimized previously [6].

3. Results and discussion

Fig. 1 shows the chromatograms obtained for homozygous controls carrying the wild type allele and heterozygous carriers of eleven different *BRCA1* and *BRCA2* mutations, one of which was a double heterozygote (2201 C>T and 2430 T>C), following amplification with either AmpliTaq™ Gold or Optimase™. *BRCA1* and *BRCA2* are among the genes investigated most extensively by DHPLC [1,6–15]. The mutations had been picked because they had proven either difficult to detect (e.g. 300 T>G in exon 5 of *BRCA1*) or had yielded chromatograms of poor quality characterized by the presence of additional peaks and shoulders preceding the main PCR product peak (e.g. 1630 G>A in exon 11 of *BRCA1*) upon amplification with a DNA polymerase lacking proofreading function. It is obvious that the use of Optimase DNA polymerase, in comparison to AmpliTaq Gold, led to a significant reduction of background heteroduplices, thus facilitating interpretation of elution profiles. Nevertheless, the detection of 300 T>G in exon 5 of *BRCA1* remained a challenge.

Direct sequence analysis of PCR products is still regarded as the benchmark against which alternative strategies for mutation detection should be measured.

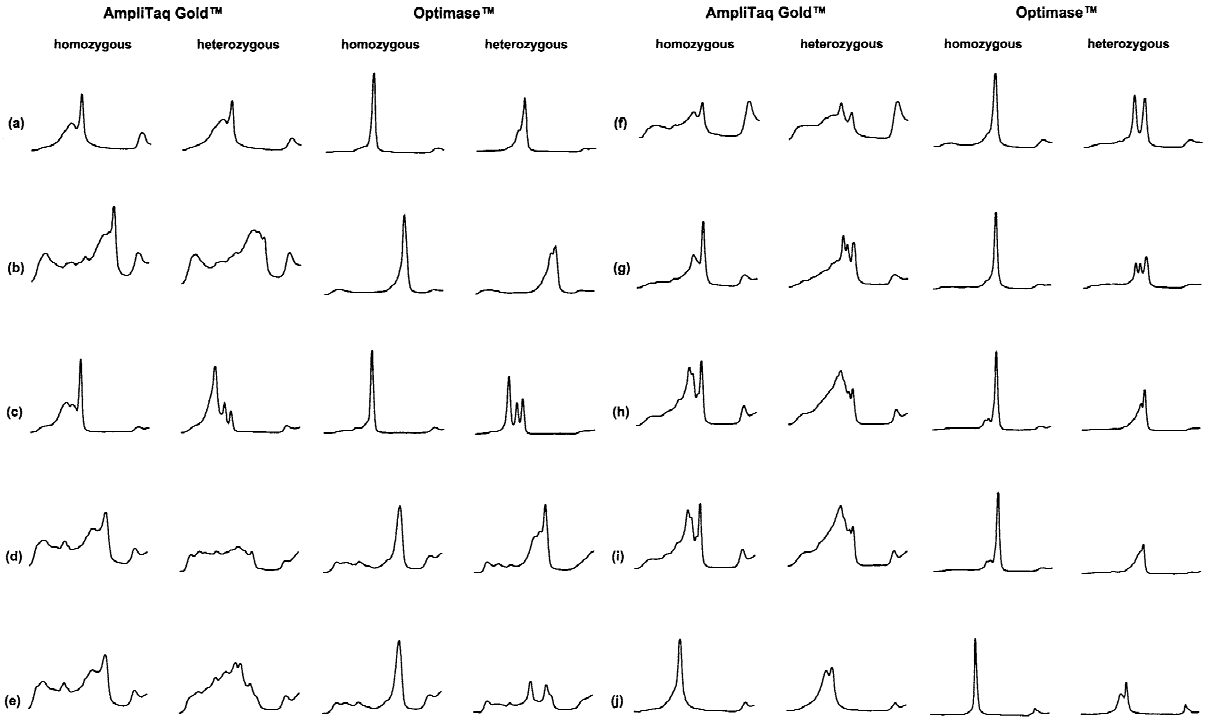

Fig. 1. Comparison of denaturing high-performance liquid chromatography elution profiles obtained for homozygous and heterozygous amplicons generated by non-proofreading AmpliTaq™ Gold and high-fidelity Optimase™ DNA polymerase, respectively. Sample identification: (a) 300 T>G, (b) 1630 G>A, (c) 1806 C>T, (d) 2382 G>T, (e) 2201 C>T+2430 T>C, (f) 3232 A>G, (g) 5272+66 G>A, (h), 460 C>T (i), 478 C>T, and (j) 7772 C>T. For experimental conditions, see Table 1.

However, the success rate of dye-terminator sequencing in detecting mutations decreases increasingly the lower the relative amount of mutant or derived allele present in a DNA sample. At a frequency of 20%, only about 80% of mutant alleles will be detected successfully by sequencing [16]. Denaturing HPLC, in contrast, has succeeded repeatedly in detecting mutations present at a frequency of ≥10% [17–20], and in one reported instance as low as 0.5% [18]. This is of significant practical utility in the detection of somatic mosaicism, such as in mitochondriopathies [18] and tuberous sclerosis [19,20]. Pooling of chromosomes also constitutes an effective mean for increasing the throughput in mutational screening of point mutations induced in the genomes of model organisms by chemical mutagenesis using either ethylmethanesulfonate (EMS) or ethylnitrosurea (ENU) [21–23]. Preliminary studies using DHPLC to discover induced lesions in a few selected genes in *Arabidopsis thaliana* [21], *Drosophila* [22], and mouse [23], showed that one might expect, on average, a functional change for every 0.2–2.4 Mb of coding sequence screened. If one were to restrict amplicon size to approximately 600 base pairs to ensure maximum sensitivity of DHPLC [24], one had to screen about 330–4000 fragments to discover a lesion. Aside from the use of capillary high-performance liquid chromatography arrays in combination with multi-color fluorescence detection [25], chromosome pooling constitutes the most effective way of accelerating mutation discovery. Therefore, it was investigated whether the use of high-fidelity DNA polymerase would allow more chromosomes to be pooled while maintaining the same level of mutation detection sensitivity. Surprisingly, despite

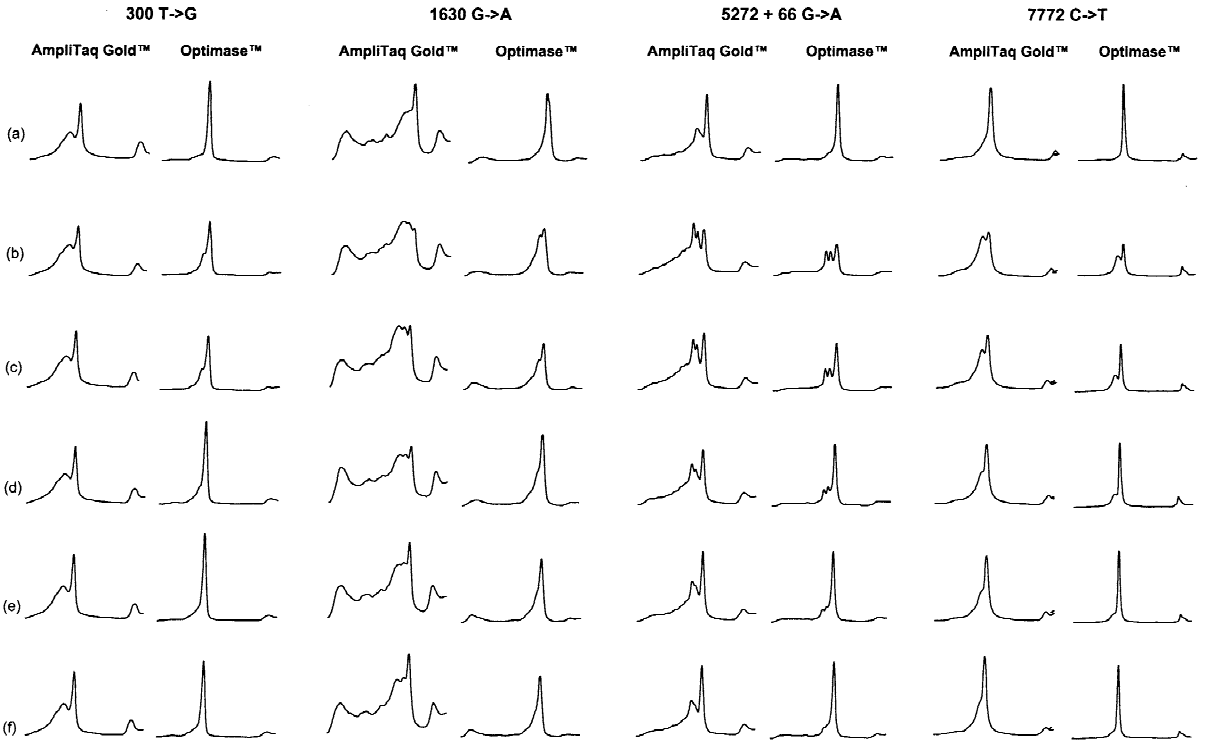

Fig. 2. Comparison of denaturing high-performance liquid chromatography elution profiles obtained for various mixtures of mutant to wild type allele following their amplification by non-proofreading AmpliTaq™ Gold and high-fidelity Optimase™ DNA polymerase. The four mutations investigated were 300 T>G, 1630 G>A, 5272+66 G>A, and 7772 C>T (see also Table 1). Ratios of mutant to wild type chromosomes were (a) 0:2, (b) 1:1, (c) 1:3, (d) 1:7, (e) 1:15, and (f) 1:23. Experimental conditions were identical to those in Fig. 1.

the significant reduction in background noise, amplification with Optimase™ improved only marginally the percentage threshold at which a mutant allele could be detected (Fig. 2). Consequently, it is not recommended to pool more than about 12 chromosomes.

In conclusion, the replacement of AmpliTaq™ Gold with Optimase™ led to a significant reduction in background heteroduplices, which in turn should facilitate automated calling of aberrant elution profiles. Although the price per unit is about the same for the two enzyme preparations, it proved necessary in our hands to use two and a half times more units of Optimase per PCR than AmpliTaq Gold to obtain the same yield of amplicon. Hence, one may opt to restrict the use of Optimase to DNA fragments that yield consistently poor-quality chromatograms upon amplification with non-proofreading DNA polymerase.

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