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Quantitative Ratio of Primer Pairs and Annealing Temperature Affecting PCR Products in Duplex Amplification

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ABSTRACT

The quantity of PCR products that are simultaneously amplified from two different loci in a duplex amplification (DA) are significantly lower for one of the loci, as compared to identical PCR amplification in separate single-band amplifications (SBA). This difference in amplification probably occurs already after the second cycle of amplification. To further analyze this phenomenon, we tested different reaction conditions, including annealing times, a wide range of temperatures, various quantities of the template, several nucleotide concentrations, different amounts of TaqI DNA Polymerase, number of amplification cycles and various amounts of primers and primers ratio. Changing the ratio between the sets of primers in DA had the most significant effect on the relative levels of amplification of the loci with an optimal ratio of 4:1 in favor of the set of primers used to amplify the underrepresented fragment. The optimal annealing temperatures for the tested sets of primers were identical in SBA and different in DA. Possible reasons for this phenomenon are discussed.

INTRODUCTION

The efforts invested in studying single-band amplification (SBA) by polymerase chain reaction (PCR) have led to defining the components and the reaction conditions, including the annealing temperature, the number and duration of the cycles and the quantity of the template (10,11,22). In some cases, such as screening for several markers in restricted amounts of sample, there is a need to perform a duplex PCR amplification (DA). For example, during in vitro fertilization, one or two blastomeres can be removed from an embryo and be used for genetic analy-

sis before the transfer of this embryo to the mother (6,19). In addition, prenatal sex determination of embryos that are at risk for X-linked disorders can be detected by qualitative DA (11). Thus, DA allows the detection of more than one mutation from just one or two cells in a relatively short time and has the advantage of requiring less equipment and components (14,19). Previous studies used either ethidium bromide staining (1,7) or radioactive-labeling (15) to estimate the yield of the products. These studies, however, did not discuss the possibility that amplification of one fragment may interfere with the amplification of the other.

Quantitative PCR has the potential to become a useful tool in diagnostics and in research applications that require quantitative results (2,3,8,10,13,22). For example, aneuploidities, in chromosomes 21 and 18, can be detected by the quantitative PCR approach (16,17,22). This technique determines the quantity of the template by exploring the quantity of the amplified products. Although some efforts have been made to perform quantitative PCR in the plateau phase (12), it is mostly performed in the exponential range of amplification, which usually ranges between 25–30 cycles, in which there is an optimal linear proportion between the quantity of the template and the PCR products (1).

There are different ways to estimate the quantity of products in a quantification assay. One way is to use external controls, in which known quantities of DNA are amplified under the same reaction conditions as the experimental sample and used to build a standard curve from which the concentration of the sample DNA is calculated (9,18). Because the quantity of the PCR product may be inconsistent in independent amplifications, internal standards, in which the sample and the internal control are amplified in the same tube, are preferred. The internal standard approach eliminates the differences in the reaction conditions and ensures reliable internal controls. The internal control can be either a competitive co-amplification, using labeled primers of the same sequence as the primers of the target to be quantified, within the PCR (5), or a competitive reaction that uses

different primers, specific to a genomic template of known frequency (4). In both cases, amplification levels are compared between the experimental and the control fragments.

The quantification of DA is based on the assumption that the components of the reaction are not limited, while the two or more amplified sites are different and are usually located on separate chromosomes. Therefore, there is no obvious reason for any kind of interference between the primer pairs. This means that amplification of one primer pair will have no effect on the amplification of the others in the same tube and production in SBA of these pairs will be the same as in DA. Any difference in the quantity of products of two different fragments in DA can be explained as poor-quality bands that are the result of a technical rather than intrinsic problem (16).

While performing quantitative PCR amplifications on human loci, we noticed that there were significant differences in the amounts and ratios of the amplification products in DA when compared to SBA. We found that the difference started after the second cycle of amplification and looked for the parameters that affected it. Changing the relative amounts of primer pairs had a major effect and changing the annealing temperature had a minor effect on the relative amounts of the amplification products in DA. In contrast, changing other parameters in the reaction, such as the amounts of enzyme, the nucleotide concentrations, the number of cycles, the amount of the template and the annealing time had little or no effect on the relative amounts of the amplification products.

MATERIALS AND METHODS

For DNA preparation, DNA was extracted by the phenol-chloroform method from peripheral blood and collected in an EDTA tube as described (20). The PCR primers used in this study were designed from different chromosomes. They all have a similar annealing temperature, and the amplified products give distinguishable lengths: for chromosome 21: (p.11.2., amplified fragment of 490 bp), 21p2A-

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5'-CTCGAGGATCCCATCCACACT-3'. 21p2B-5'-GAGCCTCAGTTTTCTCCTCTG3'. For chromosome 1: (q.31-32, amplified fragment of 301 bp); 1p5A-5'-CCTAACTCCTGTCCCGTAACT-3'; 1p5B-5'-CTGCTCCTGGAAGGTGACAAT-3'. For chromosome 11: (p.15.5, amplified fragment of 177 bp); 11p7A-5'-CCTAGACATTGCCCTCCAGA-3'; 11p7B-5'-ATCCAGCAGCGTGTAGTG-3'. For chromosome Y: (SRY gene, amplified fragment of 230 bp); SRYA-5'-CATGAACGCATTTCATCGTGTGGTC-3'; SRYB-5'-CTGCGGGAAGCAAAGT-GCAATTCTT-3'.

Hot start PCR was performed in a total volume of 25 µL for single and duplex PCR. Each experiment was performed in 5–10 replicates. Because only one of the components was tested each time, the others were as follows (standard conditions): DNA 50 ng; 1× Reaction Buffer (50 mM KCl, 10 mM

Tris-HCl, pH 8.0, 15 mM MgCl₂); 0.2 µM dNTP for single-band PCR and 0.3 µM for duplex PCR; 100 ng of each primer, 1 U for single-band and 1.5 U for duplex of *TaqI* DNA Polymerase (Promega, Madison, WI, USA). Amplification conditions were: 5 min at 94°C, 30 cycles of 60 s at 94°C; 60 s at 58°C; 30 s at 72°C and final extension of 7 min at 72°C. The PCR was conducted using Robocycler™ (Stratagene, La Jolla, CA, USA) with a “golden gradient” annealing block. Nine microliters of PCR product were loaded together with 5 µL of loading-buffer on a 2% agarose gel and subjected to gel electrophoresis at 120 mA for 1 h. The gel was post-run stained with 1 µg/mL ethidium bromide gently shaken for 30 min and washed twice, each 10 min, with distilled water. Intensity of the bands was determined by Eagle Eye® II Image analysis (Stratagene). Quantitative analysis was performed using the

RFLP SCAN software (Scanalytics, Fairfax, VA, USA).

RESULTS

Differences in the amounts of PCR products between SBA and DA. The quantities of PCR products were compared in SBA and DA, using specific primers for human loci 1p5 (chromosome 1), 11p7 (chromosome 11), 21p2 (chromosome 21) and SRYA (chromosome Y). The optimal annealing temperatures in SBA was always 58°C for the above loci (data not shown). However, at 58°C, DA of two of the loci always yielded significantly lower amounts of one locus as compared to the other locus. There was no correlation between the size of the amplified fragment, its origin and its final amount. For example, DA reactions of 11p7 and 1p5 loci always yielded significantly

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lower amounts of 11p7 locus as compared to 1p5 locus, or to SBA of 11p7, while the amplification of 1p5 was left relatively unchanged (Figure 1).

The annealing temperature has an effect in DA. One possible reason for the difference in the quantity between the two amplified fragments could be a requirement for different annealing temperatures for SBA as compared to DA. Duplex amplifications were performed at 12 different annealing temperatures starting with 54°C and rising in steps of one degree. Both 11p7 and 1p5 fragments in SBA had an optimal annealing temperature of 58°C, where as in DA, the optimal temperature was 62°C for fragment 1p5 locus and 55°C for fragment 11p7 locus (Figure 2). Similar results were obtained in DA of all other pairs of loci (data not shown).

The relative amounts of the primers in DA have a major effect on amplification. The effect of different amounts of pairs of primers on PCR amplification was tested in DA on 5 different sets of primer pairs. In these experiments, the quantity of one pair of primers was 100 ng, while the quantity of the second pair of primers was 25–400 ng (1:4–4:1 ratio). The results of the experiment with 21p2 and SRY loci are shown in Figure 3. Increasing the amounts of the SRY primers resulted in an increased amplification of the SRY fragment, while hardly affecting the amplification of the 21p2 fragment. At 400 ng of the SRY primers (4:1 ratio), amplification was roughly equal

for both 21p2 and SRY loci (Figure 3). Changing the amount of SRY primers to 800 ng (8:1 ratio) did not affect the production of either fragments (data not shown). In addition, changing the primer quantity to 400 ng had almost no effect on amplification in SBA (data not shown). Similar results were obtained in DA of all other sets of loci (data not shown).

The amount of the template DNA within a certain range does not affect the ratio between the two amplified fragments in DA. The effect of the quantity of the template DNA on DA was analyzed for 1p5 and 11p7 loci, using 1 ng, 10 ng, 20 ng, 30 ng or 50 ng of template DNA. In the tested concentration range of the template, the ratio between the two amplified loci remained roughly the same (Figure 4). In the range of 1–30 ng of template DNA, the level of amplification was in rough correlation with the quantity of template DNA (Figure 4).

Different annealing times, nucleotide concentrations or amounts of *TaqI* DNA Polymerase do not affect the ratio between the products in DA.

The possibility that the difference in amplification between the two loci in DA is due to different annealing times of the two pairs of primers was investigated for 21p2 and SRY loci. Extending the annealing periods from 1–2 min did not affect the ratio of amplification of the two loci (Table 1). The effect of different nucleotide concentrations (0.1 μ M, 0.3 μ M, 0.6 μ M and 1.2 μ M) was analyzed. The results showed that nucleotide concentration had no effect on the product ratio in DA (Table 1). As expected, increasing the concentration of nucleotides, without increasing the amount of MgCl_2 in the PCR buffer, inhibited the reaction. The effect of different quantities of *TaqI* DNA Polymerase was also examined. Within the tested range of enzyme concentrations (1–6 U), the quantity of PCR product did not change (data not shown). In DA, 0.5 U of *TaqI* DNA Polymerase was not enough to produce DNA amplification (Table 1).

Differences in the amounts of PCR products between SBA and DA appear from the second cycle of amplification. To investigate the PCR

Figure 1. Comparison of PCR products of loci 1p5 and 11p7 between SBA and DA. PCR amplification was performed under standard conditions, and the amplified products were subjected to 2% agarose gel electrophoresis. Two independent SBA reactions of 1p5 locus (1,2) and 11p7 locus (6,7) and three independent DA reactions of 1p5 and 11p7 loci (3–5). Lane M represents a 100-bp DNA ladder.

Figure 2. The effect of the annealing temperature on DA amplification of 1p5 and 11p7 loci. (A) Lane 1 represents DA performed at 54°C and each subsequent lane represents an increase of 1°C in the annealing temperature. Lane M represents a 100-bp ladder of DNA. (B) The relative amounts of DA products at the different temperatures. The units of the Y-axis are arbitrary and represent the relative amounts of the PCR product as calculated from the ethidium bromide staining by the RFLP SCAN software.

stage in which the quantitative differences between the fragments takes place in the DA assay, we took 1- μ L samples from the DA reaction of 1p5 and 11p7 loci following 1–6, 10, 15, 20 or 27 cycles and used it as a template for two separated SBA reactions of 1p5 and 11p7. Six repeats of this experiment all revealed that following the first cycle of amplification the amounts of PCR products in DA and SBA were roughly similar, while the difference in the amounts of PCR products between DA and SBA was created after the second cycle of amplification (Figure 5).

DISCUSSION

PCR amplification depends on the denaturing of the template, the annealing of the primers to the template and the successful elongation by the *TaqI* DNA Polymerase. It is difficult to estimate the efficiency of a PCR when a single pair of primers always yields the same product. In contrast, the relative efficiencies of primer pairs and amounts of products can be monitored during duplex amplification. DA results in a difference in the amounts and the relative ratio of amplification products

Figure 3. The effect of the relative amounts of pairs of primers on PCR amplification in DA. (A) An agarose gel staining of DA of 21p2 and SRY loci. The amount of primers for 21p2 locus was kept at 100 ng, while the amount of primers for SRY locus was 100 ng (1,2), 200 ng (3,4), 400 ng (5,6), 50 ng (7,8) or 25 ng (9,10). Lane M, a 100-bp ladder of DNA. (B) Quantification of the results shown in A. The amount of the primers of the SRY locus are shown below the bars. The units of the Y-axis are arbitrary and represent the relative amounts of the PCR product as calculated from the ethidium bromide staining by the RFLP SCAN software.

Figure 4. The effect of DNA template on PCR amplification. PCR was performed in duplicates under the standard conditions, using 1, 10, 20, 30 or 50 ng of human DNA template in: SBA of the 11p7 locus (a); DA of the 11p7 and 1p5 loci (b); SBA of the 1p5 locus (c). M = a 100 bp ladder of DNA; T = an amplification in the absence of template; D = amplification in the absence of *TaqI* DNA Polymerase.

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Table 1. The effect of annealing time, nucleotide concentration and amounts of *TaqI* DNA Polymerase on the ratio of the PCR products obtained in SBA and DA of 21p2 and SRY loci.

	SBA-Primer 21p2 SBA-Primer SRY	DA-Primer 21p2 DA-Primer SRY
Annealing Time (n = 6) (100-ng primers)		
1 min	ND	2.43 ± 0.225
2 min	ND	2.60 ± 0.145
Nucleotides concentration (n=5)		
0.1 μM		2.20 ± 0.82
0.2 μM	1.01 ± 0.01	
0.3 μM		3.50 ± 0.36
0.4 μM	2.01 ± 0.12	
Units of <i>Taq</i> Polymerase (n=5)		
1 U	1.30 ± 0.005	
1.5 U		2.56 ± 0.35
2 U	1.48 ± 0.07	
3 U		2.29 ± 0.23
6 U		1.82 ± 0.08
The results represent an average of 5 (nucleotide concentration and <i>TaqI</i> DNA Polymerase) or 6 (annealing time) independent experiments. The units are arbitrary and represent the relative amounts of the PCR product as calculated from the ethidium bromide staining by the RFLP SCAN software. ND = not determined.		

of the two loci. A priori, this phenomenon is not expected because the loci are amplified from different loci in the human genome and, with nothing limiting, should theoretically be amplified independently. The question that is raised is why one primer pair has an ad-

vantage over the other primer pair, while the conditions used in DA are identical to those that give high yield for both pairs in SBA. It is possible that small differences in the ability of the primers to find the target sequences and to properly anneal to them will eventu-

ally result in different efficiency of amplification where the more efficient primer set prevails. The ratio between the primer pairs had the most significant effect in DA and by changing this ratio, it was possible to overcome the differential amplification of the two loci. Thus, increasing the quantity of primers to the fragment that is apparently less available in advance can increase the potency of hybridization and amplification of that fragment.

The annealing temperature had a milder effect on the loci amplification in DA. One possible explanation for the difference in the optimal annealing temperature in DA, as compared to SBA, is that the annealing efficiency of one pair of the primers is lower in DA, as compared to SBA. Thus, lowering the annealing temperature provides an advantage to the pair of primers with the lower efficiency in DA. An indication for this explanation is that the minimal difference in the amount of products of the two loci in DA was always observed at a lower annealing temperature.

Interestingly, the difference in the yield between the two loci was not the result of interference to the annealing of one pair of primers by the other pair of primers since, under standard conditions, increasing the annealing time from 1 to 2 min had no apparent effect on the ratio between the amplified fragments. Also, changing the quantities of the nucleotides in SBA and DA affected neither the yield nor the relative amounts of the two amplified fragments. As expected, a high concentration of nucleotides without an increase in the amount of $MgCl_2$ in the buffer inhibited PCR amplification (12).

The difference in the quantity of the products in DA, as compared to SBA, was not affected by the amount of *TaqI* DNA Polymerase, since changing the quantity of the enzyme did not affect the quantity of PCR product in either SBA or DA. Again, as expected, when the quantity of the *TaqI* DNA Polymerase was too small, PCR product could not be detected. These findings and the observation that the difference in amplification occurs from the second cycle of amplification all indicate that, in spite of the fact that the DNA is a large molecule in comparison to the specific fragment that is amplified,

Figure 5. Differences between SBA and DA appear after the second cycle of amplification. SBA and DA reactions were performed 6x on the 1p5 and 11p7 loci. The ratio was calculated from the ethidium bromide staining of the PCR products by the RFLP SCAN software. The cycle number refers to the number of cycles in the DA and SBA reactions from which 1 μL samples were removed and used as templates for separate SBA reactions.

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fragment amplification along the DNA molecule and the presence of fragments from the first round of amplification in the reaction mixture can make other loci less available to amplification.

Understanding the mechanism beyond the interference in amplification in DA should lead to better performance in quantitative PCR analysis. However, this study suggests several ways to overcome the difference in amplification of two loci in DA, including increasing the amount of the pair of primers of the underrepresented locus or lowering the annealing temperature of the reaction.

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