JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 96, No. 4, 317–323. 2003

REVIEW

Bias and Artifacts in Multitemplate Polymerase Chain Reactions (PCR)

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Received 1 July 2003/Accepted 2 July 2003

Polymerase chain reaction (PCR) is often used for the amplification of a mixture of homologous genes. PCR bias and artifact formation can occur in multitemplate PCR, and provide incorrect information on the abundance and diversity of genes. PCR bias and artifact formation occur at a higher rate during the last few cycles of the reaction, and therefore can be avoided by stopping the PCR earlier.

[**Key words:** PCR bias, chimera, heteroduplex, microbial community analysis, 16S rRNA gene]

PCR has become widely used in molecular biology, applied microbiology, and microbial ecology. In many cases, the template is a mixture of homologous genes. In multitemplate PCR, some factors give large bias and the relative abundance of homologs in the final amplification products does not necessarily reflect the gene ratio in the starting mixture (1–4). Moreover, artifacts are sometimes produced during the amplification and give the sequence data of nonexistent genes (5–15). However, many researchers seem to ignore these problems, and there have been only a few reports on PCR bias and artifacts (16).

In microbial ecology, to reveal microbial community structures in environmental samples, the 16S rRNA gene (16S rDNA) is often amplified by PCR with universal primers which have a sequence common to all microorganisms. The PCR product is a mixture of 16S rDNA from various microorganisms, and is analyzed by cloning and sequencing (17), denaturing gradient gel electrophoresis (DGGE) (18, 19), terminal restriction fragment length polymorphism (T-RFLP) (20, 21), and other methods (22). In these analyses, PCR bias results in incorrect population data on microorganisms, and artifacts show nonexistent varieties of microorganisms. However, these problems are not fully understood, and in many cases, PCR has been performed with little attention to these issues. To obtain reliable data from such PCR-based analyses, the elimination of PCR bias and artifacts is essential.

The present review focuses on the mechanism of PCR bias and artifact formation in multitemplate PCR, and discusses suitable methods for the elimination of these problems to increase the reliability of the data from PCR-based analyses.

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I. PCR BIAS

PCR bias due to differences in primer binding energy

The primer set used in multitemplate PCR should have a sequence common to the targets. However, often there are no common sequences in the targets. If a primer has one mismatch with some targets, the amplification efficiency is usually very low, and therefore, a large bias in the amplification will occur. To avoid this bias, a degenerate primer which is a mixture of primers with a nucleotide sequence corresponding to the variation among homologs is often used. Polz and Cavanaugh (3) examined the extent of bias in PCR amplification with degenerate primers. They amplified bacterial 16S rDNA with the very popular degenerate primers 27F and 1492R, each of which contains a single degenerate position as shown in Table 1. First, they prepared the mutagenized *Escherichia coli* 16S rDNA fragments Eco(GC), Eco(AT), and Eco(AT)m as templates. Eco(GC) and Eco(AT) differed at a single degenerate position in each of the priming sites for primers 27F and 1492R. Eco(AT)m was made from Eco(AT) by changing six nucleotides in the middle of the molecules for the quantification of Eco(AT)m in the mixture of Eco(AT) plus Eco(AT)m or Eco(GC) plus Eco(AT)m. When the mixture of Eco(AT) and Eco(AT)m at ratios of 1:1, 1:5, 1:10, and 1:20 was amplified by PCR with primers 27F and 1492R, the ratio was maintained in the final PCR products after 25 cycles. This result shows that the six nucleotide difference in the middle of the template did not influence the product ratio. When an equal amount of Eco(GC) and Eco(AT)m was amplified with primers 27F and 1492R, Eco(GC) was amplified better than Eco(AT)m at the product ratios of 1.4, 1.7, and 2.2 after 15, 25, and 35 cycles, respectively. This result shows the gene fragments containing G or C in the degenerate position were preferably amplified probably because of a higher binding energy between G and C than A and T. Next, a mixture of equal

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TABLE 1. Degenerate primers used by Polz and Cavanaugh (3)

Templates	Primers of perfect match	
	Forward primer (27F)[a]	Reverse primer (1492R)[a]
Eco(GC), *B. subtilis* 16S rDNA	AGAGTTTGATCCTGGCTCAG	TACGGCTACCTTGTTACGACTT
Eco(AT), Eco(AT)m, *V. fischeri* 16S rDNA	AGAGTTTGATCATGGCTCAG	TACGGTTACCTTGTTACGACTT

[a] The underlined nucleotides are at the degenerate positions.

amounts of total genomic DNA of *Bacillus subtilis* and *Vibrio fischeri* was amplified with primers 27F and 1492R, and as a result, *B. subtilis* rDNA containing G at the degenerate positions was amplified with higher efficiency than *V. fischeri* rDNA containing A and T at the degenerate positions. The product from the *B. subtilis* genome was 2.3 times more abundant than that from the *V. fischeri* genome after 25 PCR cycles. Considering the *rrn* operon number and the genome size of both bacteria, this means that 1.7 times more product was obtained from one *B. subtilis rrn* operon than from one *V. fischeri rrn* operon. Thus, the template with the GC-rich permutation in the priming site was amplified better than that with the AT-rich permutation. The extent of the bias in PCR amplification would depend on the PCR conditions. In general, stricter annealing conditions cause a larger bias (23). Therefore, the extent of the bias should be examined in each case when degenerate primers are used. Whenever possible, degeneracy should be avoided.

PCR bias to a 1:1 ratio Suzuki and Giovannoni (1) have reported a strong bias towards 1:1 mixtures of genes in final PCR products, regardless of the initial ratio of the templates. They prepared mixtures of two 16S rDNA fragments (27F-1492R fragment) at 1:4, 2:3, 3:2, and 4:1, and amplified them by PCR with primers 27F and 338R. The PCR product ratio after 35 cycles was nearly 1:1 in all cases. Mathieu-Daudé *et al.* (2) has also found this bias in the amplification of cDNA. The bias was strongly dependent on the cycle number. The original differences in concentration decreased as the number of PCR cycles increased. This bias is explained by rehybridization of the PCR products. When the amplification reaction proceeds, the concentration of PCR products becomes high enough to allow the rehybridization of the products to some extent while the temperature is lower than the DNA melting point. If multiple PCR products are amplified in the same tube, rehybridization occurs faster for the more abundant PCR product and interferes with primer binding or extension. Consequently, the amplification rate for abundant PCR products declines faster than that for less abundant products in the later PCR cycles, and the difference in starting template concentrations decreases. This bias can be eliminated by limiting the number of PCR cycles.

II. HETERODUPLEXES

Formation of a heteroduplex At the denaturing temperature in the PCR cycle, all DNA will be single stranded. When the temperature decreases for annealing, three kinds of duplexes can be formed, a homoduplex between complementary strands, a heteroduplex caused by the cross-hybridization of heterologous sequences, and a duplex between primers and templates. When the primer concentration is much higher than the template concentration, the duplex between primers and templates is most abundant at the annealing step, and then, the homoduplex is produced by primer extension. Therefore, after the extension step, the homoduplex is dominant. On the other hand, in later PCR cycles, most primers are used up, and the amplified product concentrations are very high. In these conditions, a significant amount of heteroduplex would be formed.

Problems caused by the heteroduplex Heteroduplexes cause several problems in PCR-based analyses such as cloning and sequencing, DGGE, and T-RFLP (24). When heteroduplexes are cloned, the mismatch repair system in the host (*E. coli*) can convert a heteroduplex into a homoduplex (25–27). Since there is no way for the repair enzymes to identify the parent strand of the heteroduplex, the enzymes independently choose either strand as a template for resynthesis of the complementary base. This random choice can occur at each repairing position in one heteroduplex. As a result, the repaired sequence is a mixture of the two parent heterologous sequences (28). This result gives an artifactual sequence and increases the sequence diversity. Speksnijder *et al.* (13) mixed seven closely related 16S rDNA fragments in equal quantities, amplified the mixture by PCR, and analyzed the sequence by cloning and sequencing. Two of 66 clones showed an artifactual sequence resulting from the mismatch repair of the heteroduplex.

In the electrophoretic analysis of PCR products, a heteroduplex can give extra bands or peaks. Qiu *et al.* (14) have found an extra band on polyacrylamide gel. They equally mixed cloned 16S rDNAs from two strains, amplified nearly the entire 16S rDNA by PCR, and analyzed the PCR products by PAGE. The migration of heteroduplexes was retarded and showed an extra band on the gel. The decrease in the mobility of the heteroduplex was inversely proportional to the sequence similarity of the two parental molecules. This decrease would be due to the bulky form of the mismatch sites. Thompson *et al.* (28) have found extra peaks in capillary electrophoresis. They mixed genomic DNA from three bacterial species, *Vibrio cholera*, *V. parahaemolyticus*, and *V. vulnificus*, amplified a 114-bp region of the 16S rDNA by PCR, and separated the PCR products by constant denaturant capillary electrophoresis (CDCE). As a result, six peaks appeared. Three peaks corresponded with homoduplexes of the genes from the three species, while the three extra peaks corresponded with heteroduplexes among the genes. Thus, heteroduplexes showed a nonexistent diversity of genes. When heteroduplexes are applied to DGGE, they will surely give extra bands on the gel, and may lead to wrong conclusions.

Judo *et al.* (12) experienced difficulty in restriction fragment length polymorphism (RFLP) analysis because of a significant amount of heteroduplex. They used a 590-bp

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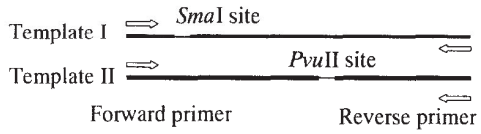

FIG. 1. Schematic representation of PCR templates and primers used by Judo *et al.* (12). Templates I and II differed in only two nucleotide pairs shown by thin lines.

DNA fragment obtained from a pUC-based plasmid. They separately mutagenized this fragment to introduce a *Sma*I site and a *Pvu*II site (Fig. 1). The two 590-bp fragments produced differed in only two nucleotide pairs, to encode a *Sma*I site at position 63 in one fragment, and a *Pvu*II site at position 345 in the other. They amplified the mixture of these two fragments by PCR, and analyzed the products by RFLP. If heteroduplexes form between these two fragments, they will have no restriction sites, and show an uncleaved 590-bp band in RFLP analysis. As expected, a clear 590-bp band appeared, and this showed that a significant amount of heteroduplex was produced. In T-RFLP, the same problem will occur.

Elimination of the heteroduplex Thompson *et al.* (28) have succeeded in the elimination of heteroduplexes by 'reconditioning PCR'. They diluted the PCR products tenfold with a fresh reaction mixture for PCR, and applied the mixture to PCR for three cycles. This procedure resulted in the absence of heteroduplex peaks in CDCE.

Qiu *et al.* (14) have shown that PAGE can separate heteroduplexes, but the separation may be difficult when highly similar fragments are amplified. They have tried the elimination of heteroduplexes with T7 endonuclease I which can cut the single-stranded DNA bubbles in a heteroduplex molecule and chop them into small DNA fragments. This attempt worked well, but the experimental conditions for this treatment were critical. Low concentrations of enzyme or a short incubation time did not remove all heteroduplexes, whereas high concentrations of enzyme or a long incubation time could digest the homoduplex molecules.

Since heteroduplexes are formed in significant amounts in the later PCR cycles when the concentration of PCR product is high enough to compete with the primers for annealing, the formation of heteroduplexes is avoidable by limiting the number of cycles.

III. CHIMERA

Two kinds of mechanism for chimera formation have been reported, annealing of an incompletely extended primer, and template switching during DNA synthesis (15).

Formation of a chimera from an incompletely extended primer An incompletely extended primer can act as a primer in the subsequent PCR cycles. If the incomplete fragment anneals to a different template having a partially homologous sequence, a chimeric product results (29).

Pääbo *et al.* (6) have reported the formation of chimeras from partially homologous templates. They mixed two templates I and II which had partially homologous sequences at the ends (Fig. 2A). The forward primer was specific for template I, and the reverse primer was specific for template II.

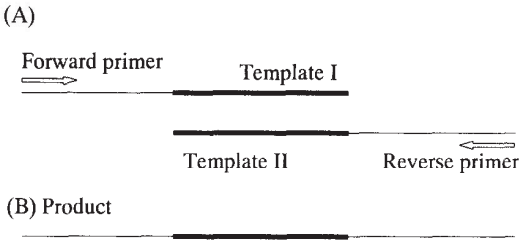

FIG. 2. Schematic representation of PCR templates and primers (A) used by Pääbo *et al.* (6) for the formation of a chimera, and the PCR product (B). Templates I and II had the same sequences at the ends indicated by thick lines.

By 40 cycles of PCR, chimeric products (Fig. 2B) were generated which had the expected size and sequence. This shows that PCR products having a partially homologous sequence at the 3′ end can act as primers in the subsequent PCR cycles to produce chimeric molecules.

Judo *et al.* (12) utilized this mechanism to produce a large amount of chimeric molecules. They used two 590-bp fragments differing in only two nucleotide pairs (Fig. 1). They mixed these two fragments and amplified them by PCR in cycling conditions altered to enhance chimera production (94°C for 45 s, 50°C for 10 s, 94°C for 45 s, 72°C for 10 s, 50°C for 45 s, and 72°C for 45 s). First, the reaction mixtures were cooled from 94°C to 50°C for annealing, and soon the temperature was raised to 94°C to discourage complete extension. The formation of incompletely extended primers was enhanced by this step. After the second denaturation step for 45 s, the mixtures were cooled to 72°C to allow incompletely extended primers to anneal to the templates under conditions where unextended primers cannot compete to bind the templates. Next, the temperature was decreased to 50°C for annealing, raised to 72°C for extension, and raised to 94°C for denaturation as a standard PCR cycle. This cycling resulted in many more chimeras than the standard PCR cycle (94°C for 45 s, 50°C for 45 s, and 72°C for 45 s). This experiment has clearly shown that the chimeras form from incompletely extended primers.

The formation of chimeras by this mechanism occurs in the later cycles of PCR when the concentration of the incompletely extended primers is high enough to compete with the original primer for annealing. Therefore, chimera can be avoided by limiting the number of PCR cycles.

Formation of a chimera by template-switching During primer extension, the extending strand switches templates from the original to one which annealed to the original downstream of the priming site, and a chimeric molecule is produced.

Odelberg *et al.* (8) showed that chimeras formed during a single round of primer extension. First, they prepared a reaction mixture (Fig. 3A) containing one template and one primer pair (a ^{32}P-labeled forward primer, pr2A, and a nonlabeled reverse primer, pr2B), and performed a single thermal cycle consisting of (i) denaturing at 94°C for 5 min and (ii) primer-annealing and extension at 60°C for 2 min. As a result (Fig. 3B), autoradiography showed the existence of the expected 426-nt fragment (the product by the ^{32}P-labeled forward primer extension on the template) and an additional

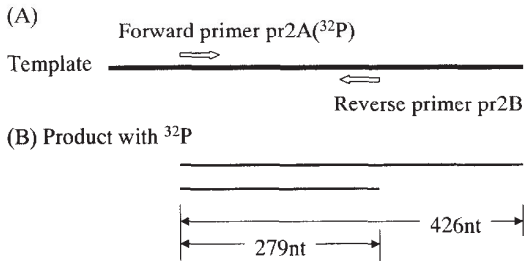

FIG. 3. Schematic representation of the PCR template and primers (A) used by Odelberg *et al.* (8) for the formation of chimeras, and products (B) after a single thermal cycle.

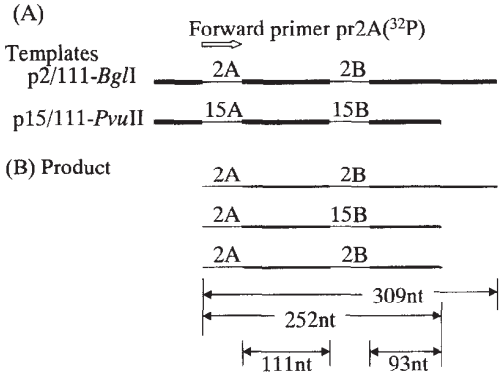

FIG. 4. Schematic representation of the PCR templates and primer (A) used by Odelberg *et al.* (8) for the formation of chimeras, and products (B) after a single thermal cycle. The two templates had the same sequence except for the two 20-bp sites shown by thin lines.

279-nt fragment in a small amount. This result suggested that some of the extending strand from pr2A switched templates from the original to the extending strand from pr2B. Next, they prepared two templates, p2/111-*Bgl*I and p15/111-*Pvu*II, differing in size and in the two unique 20-bp sites, 2A, 2B, 15A, and 15B (Fig. 4A), and performed a single thermal cycle with these two templates and one primer, pr2A. The 252-nt fragment (Fig. 4B), apparently generated by a switch of template, was detected. In this experiment, two types of template-switches are possible, a switch that occurs between sites 2A and 2B, and a switch that occurs after site 2B. The first type will generate a chimera with the 15B sequence. The second type of switch will generate a non-chimeric molecule. Quantification of the products in this experiment showed that nearly the same number of chimeric and non-chimeric molecules existed in the 252-nt fragment. Since the number of nucleotides between sites 2A and 2B was approximately equal to the number of nucleotides downstream of site 15B (111 nt versus 93 nt), the result suggests that the template-switching does not exhibit a sequence preference. From these and some additional experiments, Odelberg *et al.* (8) have proposed models for the formation of chimeras. Two of the models (Fig. 5A, B) are for the template-switching process, and one model (Fig. 5C) is for the incomplete extension process: (A-1) Partial hybridization occurs between partially complementary strands and the primers anneal to their respective targets. (A-2) Primer extension begins through polymerase-catalyzed strand

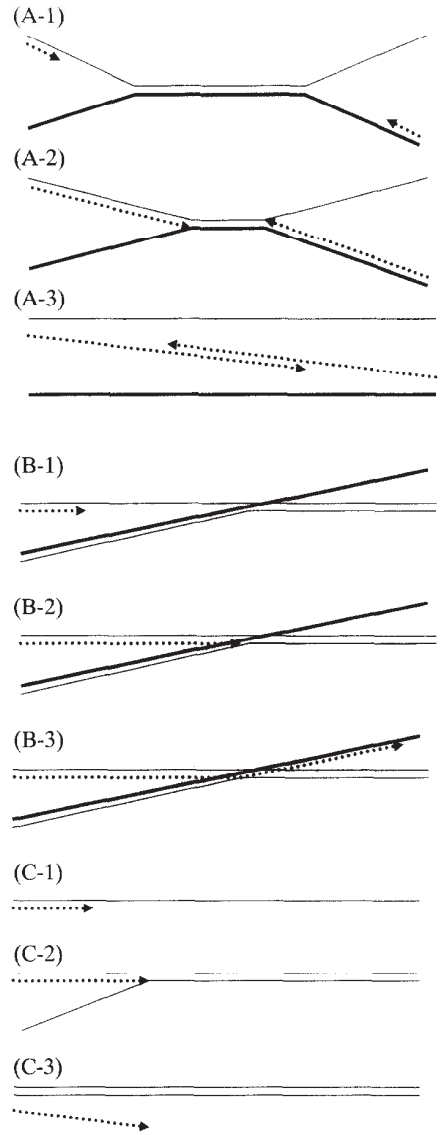

FIG. 5. Models of how chimeras form (8).

displacement (or, more likely, by progressive hydrolysis of the interfering strand). (A-3) As the two extending strands meet, a template-switch occurs, and the continued synthesis generates chimeric molecules. (B-1) A single template strand partially hybridizes two different template strands, and the primer anneals to its target. (B-2) Primer extension begins, and the extending strand encounters the branch. (B-3) The extending strand switches to the other template, and generates a chimera. (C-1) A primer anneals to its target, and primer extension begins. (C-2) Before the extension is completed, a complementary strand begins to reanneal to the template strand and meet the extending strand. (C-3) Continued reannealing of the complementary strand displaces the extending strand, and thus, the incompletely extended single strand is produced. Then, this strand anneals to the secondary template or another incompletely extended strand containing the complementary region, and continues DNA synthesis to generate a chimeric molecule. These models

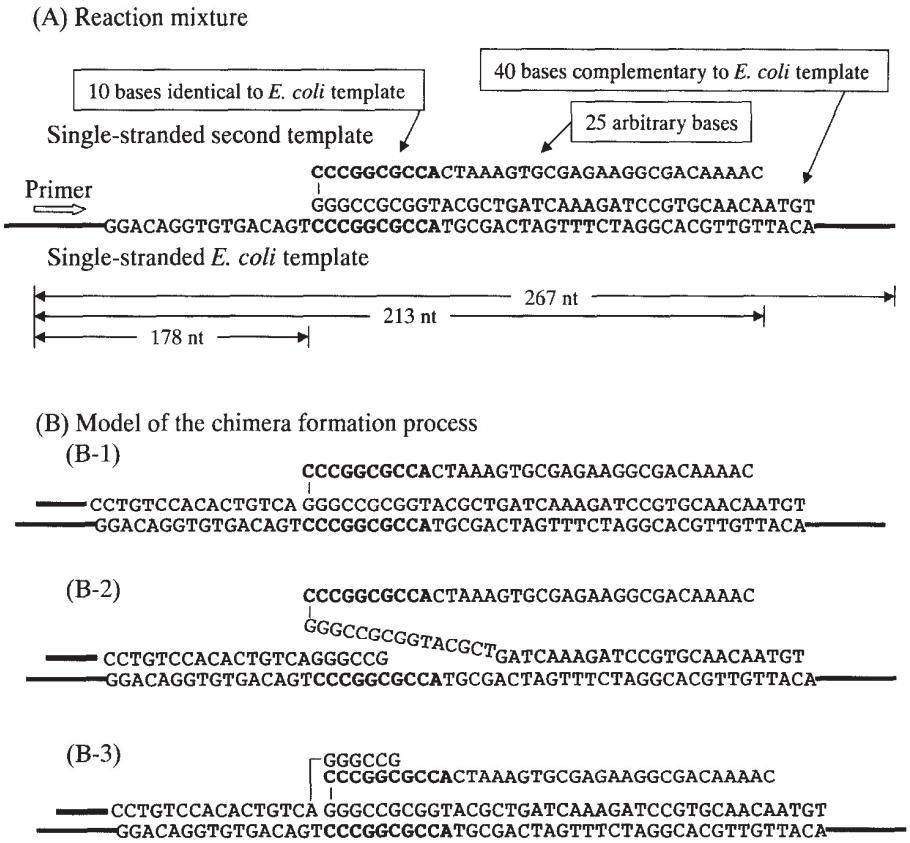

FIG. 6. Schematic representation of the PCR templates and primer used by Patel *et al.* (9) for the formation of chimeras (A), and a model of the process (B). The sequence in boldface type in the second template was the same as that in the *E. coli* template.

would be expected to occur at a higher rate during the last few cycles of PCR when the template concentration is high enough to allow the reannealing of the templates before or during primer extension.

Patel *et al.* (9) have tried to explain the mechanism by which chimeras form, using unique templates. They prepared two single-stranded templates for template switching (Fig. 6A). One was a 640-base *E. coli* template having a primer binding site. The other (second template) was a 75-base template, part of which bound to the *E. coli* template at a position downstream from the primer binding site. The second template had a 40-base sequence at its 3′ end which was complementary to the *E. coli* template. The 10-base sequence that followed was identical to the *E. coli* template at the 5′ end of the site binding to the second template. The 25 bases at its 5′ end were arbitrarily designed. The primer extension reaction was performed only once. The reaction mixture containing the *E. coli* template, the second template, the primer, and dNTP was heated to 95°C for 4 min and *Pfu* polymerase was added. The mixture was then incubated for 5 min at 75°C at which temperature the second template would bind to the *E. coli* template, but the primer would not. Next, the mixture was cooled and kept at 66°C for 30 min. As a result, three products were obtained, a 178-base product which would be formed by blocking the extension at the binding site of the second template, a 267-base product which would be formed by full-length extension on the *E. coli* template, and an intermediate length product which would be a chimera formed by template switching. Patel *et al.* (9) explained the mechanism of this template switch as follows (Fig. 6B): The primer extension reaches the binding site of the second template (Fig. 6B-1). Then the extension becomes less efficient in the double-stranded section (Fig. 6B-2). When the 3′ end of the extended strand becomes free by chance, it can bind to the second template because the appropriate sequences are present (Fig. 6B-3). When further extension is performed on the second template, a chimeric molecule is produced. Patel *et al.* (9) carried out several experiments changing the extension temperature and the length of the repeated sequences in the second template. They found that a higher extension temperature and longer repeated sequence favored the formation of chimeras. At higher temperatures, the binding of base pairs becomes less stable, and the primer extension in the double-stranded section becomes easier. Moreover, the 3′ end of the extended strand would more easily become free at a higher temperature, and this would increase the amount of chimeric products. This type of process would also depend on the template concentration, and would be expected to occur only during the last few cycles in PCR.

IV. RESULTS OF RANDOM EVENTS

Misannealing of primers and misincorporation of bases

by DNA polymerases can produce artifacts (30). When these events happen in the early stages of PCR, a large amount of artifact may be produced, especially with a low initial amount of template, and can lead to the wrong conclusions. Some other mechanisms may also exist to produce artifacts or cause amplification bias by accident. In any case, these events will happen randomly, and so, the results will not be reproducible. Therefore, the influence of random events is avoidable by performing the several replicate PCR amplifications, and mixing them before further analysis.

V. ELIMINATION OF PCR BIAS AND ARTIFACTS

PCR bias and artifacts which occur randomly can be avoided by mixing the replicate PCR amplifications. Reproducible events are considered to occur in the late stages of PCR, and can be avoided by stopping the PCR earlier. However, a stoppage too early will result in not enough PCR product for subsequent analyses. One needs to know when to stop the PCR to obtain enough product while avoiding PCR bias and artifacts. For this purpose, real-time monitoring of the PCR product would be useful. There are three methods for monitoring the PCR product in real time (31), the use of (i) double stranded DNA-specific dyes (32), (ii) sequence-specific fluorescent probes (33, 34), and (iii) sequence-specific fluorescent primers (35).

Real-time monitoring of PCR products

Double-stranded DNA-specific dyes The DNA-binding fluorogenic molecules, SYBR® Green I and YO-PRO-1, are often used for monitoring the amount of PCR product. These molecules emit fluorescence when associated with double-stranded DNA, and so the fluorescence intensity indicates the amount of PCR product. When used in PCR, the fluorescence intensity increases as the amount of PCR product increases, and reaches a maximum value (32). The maximum fluorescence intensity is obtained when all the fluorogenic molecules in the reaction mixture are associated with the PCR product, or when there is no further increase in PCR product. Therefore, careful consideration of the data is needed to decide when to stop the PCR.

Sequence-specific fluorescent probes TaqMan® probes (33) and HybProbes (34) are very commonly used for the real-time monitoring of PCR products. TaqMan® probes (33) hybridize to the targeted PCR products, and are degraded by *Taq* polymerase with its exonuclease activity at the extension step. By this degradation, the fluorophores on the probes are let free and emit fluorescence. HybProbes (34) consist of two probes, and emit fluorescence when both hybridize to the targeted PCR products. To monitor multitemplate PCR products, one must find a sequence common to these products to design the probes. However, in many cases, the design is difficult because of differences in the sequence among targets even in conserved regions. When there are suitable sequences in the products, one can design probes and use these methods for real-time monitoring. With these methods, no increase in the fluorescence intensity may indicate the total consumption of the probes, and careful consideration is needed to decide when the PCR should be stopped.

Sequence-specific fluorescent primers Real-time monitoring with a fluorescent primer has recently been developed (35). When a primer containing a BODIPY® FL-modified cytosine at its 5′ end is used for PCR, its fluorescence is quenched by the guanine in the target, complementary to the modified cytosine, and the quench rate is proportional to the amount of PCR product. With this method, the quench rate shows the rate of consumption of the modified primer, and therefore, this method is suitable for monitoring the late stages of PCR. In PCR with this primer, a slight but significant decrease in the fluorescence intensity means that the PCR is approaching the end, and one should stop the reaction at this point. The fluorescence of some other dyes, 5-FAM, TAMRA and so on, is also quenched by their interaction with a guanine (36), and these dyes also can be used for this method.

CONCLUSION

In multitemplate PCR, PCR bias to a 1:1 ratio, the formation of heteroduplexes, and the formation of chimeras have been reported to occur at a higher rate during the last few cycles of PCR, and provide incorrect information on the abundance of specific genes and diversity of gene sequences. These problems can be avoided by stopping the PCR earlier. By monitoring the PCR product in real time, one can judge when best to stop the reaction.

PCR bias and artifact can also occur by accident. Neither of the two is reproducible, however, and therefore, the problem can be prevented by mixing PCR products from independent amplifications.

In conclusion, multitemplate PCR should be carried out using several replicates and stopped at an early stage. The PCR products in the replicates should be mixed before further analysis.

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