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BRIEF COMMUNICATION

Reduction of heteroduplex formation in PCR amplification

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Abstract

Heteroduplex formation is known to occur during mixed-template polymerase chain reaction (PCR) using universal primers, and may represent a serious problem in several PCR-based analyses. A common way to eliminate heteroduplex formation is to use reconditioning PCR. Because we detected that reconditioning PCR was not always sufficient to prevent heteroduplex formation, we focused on developing methods for the elimination of heteroduplexes during PCR. We detected that the heteroduplex to homoduplex ratio can be decreased by the addition of *Taq* polymerase and by a decrease in the number of PCR cycles. An appropriate combination of both of these approaches can be a method generally applicable to decrease the formation of heteroduplexes.

Additional key words: polymerase chain reaction, reconditioning PCR, mixed-template PCR.

PCR amplification of a mixture of homologous sequences is often used in medical diagnostics, forensic science (Ruano and Kidd 1992), molecular biology, applied microbiology, microbial ecology (reviewed in Kanawaga 2003), and analysis of genetic diversity (Acinas *et al.* 2005). However, during the amplification of mixed templates using universal primers, severe PCR artifacts can occur (reviewed in Speksnijder *et al.* 2001, Kanawaga 2003). Formation of heteroduplex DNA molecules is problematic because it can give data for either nonexistent genes (Kurata *et al.* 2004) or non-existent varieties of microorganisms (Kanawaga 2003). The elimination of PCR artifacts is thus particularly essential in many different disciplines using PCR (Hayward-Lester *et al.* 1995, Qiu *et al.* 2001, Thompson *et al.* 2002, Kurata *et al.* 2004, Gu *et al.* 2008).

Although several methods were proposed to eliminate heteroduplex formation (Qiu *et al.* 2001, Thompson *et al.* 2002), they are not generally applicable (*e.g.* Qiu *et al.* 2001, Erhart *et al.* 2006). The best simplified system to study a dynamics of heteroduplex formation during PCR seems to be a system where the ratio of templates is exactly defined. To avoid an artifact imbalance of templates as a consequence of pipetting errors, the best are "natural systems" as genes possessing two alleles or homologous gene copies present on the sex chromosomes. We have studied the formation of heteroduplex DNA using dioecious *Silene* species (*Caryophyllaceae*) with homogametic (XX) females and heterogametic (XY) males (Negrutiu *et al.* 2001, Mrackova *et al.* 2008) where the ratio of sex-linked genes with copies on both the X and the Y chromosomes is 1:1 in male individuals.

As a model system we used PCR amplification of the gene fragments of spermidine synthase from genomic DNA of *Silene diclinis*, with both X- and Y-linked copies that are amplified using the same pair of primers, and using the same PCR conditions (Filatov 2005, Marais *et al.* 2008). The sequence alignment of *S. diclinis* X- and Y-linked copies (1001 and 1003 bp long; accession numbers EU122955 and EU122956, respectively) enabled us to find a *Sau*96I restriction site distinguishing the two copies (Fig. 1*A*). The restriction analysis of PCR products [digestion of 0.01 cm^3 of PCR products with 1.5 U of *Sau*96I (*New England Biolabs*, Ipswich, USA)

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Abbreviation: PCR - polymerase chain reaction.
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overnight to ensure complete digestion] enabled us to distinguish the X-X and the Y-Y homoduplexes, which were cut (Fig. 1*A*), from the X-Y or Y-X heteroduplexes, which remained uncut (Fig. 1*B*). The X-linked copy was digested into two fragments 568 bp and 433 bp long, whereas the Y-linked copy was digested into two fragments 365 bp and 638 bp long. The restriction endonuclease *Sau*96I is an enzyme that cuts double stranded DNA in the recognition site. The double stranded X-X and Y-Y homoduplex molecules had the recognition sites at the same positions on both strands; thus it enables to cut the homoduplex molecules. Each strand of the double stranded X-Y and Y-X heteroduplex molecules had the recognition site in different positions, which did not enable digestion of the heteroduplex molecules. We confirmed the presence of the heteroduplex molecules in the uncut fraction using gel extraction of this uncut fraction, its cloning, and sequencing of 13 random clones. All of the sequenced clones coming from the uncut fraction contained either solely X-derived (6 clones) or Y-derived (7 clones) copies of spermidine synthase, thus the heteroduplex was probably converted by the mismatch repair system of the host microorganism used for cloning.

To study the accumulation of heteroduplex molecules during individual PCR cycles, we measured the amount of homoduplex and heteroduplex DNA every 2 cycles from cycle 21 onward. The 0.02 cm^3 volume PCR mixture contained 0.4 U of *Taq* DNA polymerase (*Top-Bio*, Prague, Czech Republic), 1× *Taq* polymerase buffer (PCR *Blue Buffer* complete, *Top-Bio*), 8 pmol of each primer (c2B12+1 and c2B12-2; Filatov 2005), and 4 nmol of each dNTP (*Fermentas*, Burlington, Canada). The PCR amplification was performed on a *T3000* thermocycler (*Biometra*, Göttingen, Germany), and consisted of a 180 s denaturation step at 94 °C followed by a different number of cycles (21, 23, 25, 27, 29, 31, 33, or 35) for 30 s at 94 °C, 60 s at 60 °C and 120 s at 72 °C. To estimate the proportion of heteroduplex molecules, PCR products were digested with *Sau*96I. The size and quantity of resulting PCR products was analyzed by electrophoresis using a 2 % agarose gel. DNA was stained after electrophoresis using 10 000-fold diluted *SYBR® Green I* (*Molecular Probes*, Eugene, OR, USA). The images were scanned using *Storm*, and the quantity of PCR products was measured using *ImageQuant v. 5.0* (both *Molecular Dynamics*, Sunnyvale, CA, USA). The experiments were carried out two times with similar results. We found that heteroduplexes are detected at the 25th cycle afterward, and that the fraction of heteroduplex molecules increases with the increasing number of PCR cycles (supplementary Fig. 1, http://www.ibp.cz/labs/LPDG/EMBP). The ratio of heteroduplexes to homoduplexes increases rapidly in the later PCR cycles. The

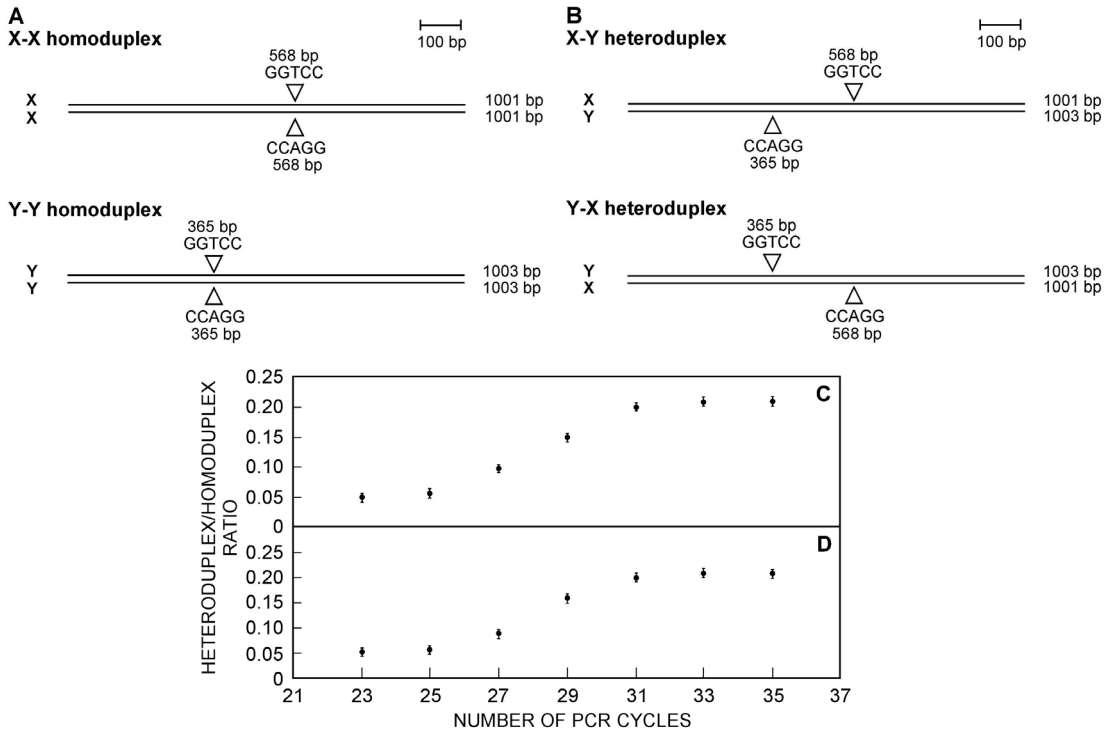

Fig. 1. Restriction patterns of the X- and Y-linked copies of the *S. diclinis* spermidine synthase gene digested with *Sau*96I (*A, B*) and analysis of heteroduplex formation under standard PCR conditions (*C*) and after the supplementary addition of primers (*D*). *A* - The X-linked copy is digested into two fragments 568 bp and 433 bp long. The Y-linked copy is digested into two fragments 365 bp and 638 bp long. *B* - The double stranded X-Y and Y-X heteroduplex molecules are not cut by the restriction enzyme. *C* - The heteroduplex to homoduplex ratio increases with the increasing number of PCR cycles. *D* - The heteroduplex to homoduplex ratios after the extra addition of primers. Means ± SE, $n = 2$.

most rapid increase is between the cycles 26 - 31 (Fig. 1*C*). As Thompson *et al.* (2002) hypothesized, heteroduplex formation could be caused by a low concentration of primers in the later PCR cycles. We attempted to eliminate heteroduplex formation by adding extra 8 pmol of primers (to the standard PCR mixture already containing 8 pmol of each primer) at the 18th cycle to avoid primer depletion. This procedure did not lead to the decrease of the heteroduplex formation as the most rapid increase in the heteroduplex to homoduplex ratio is between the cycles 26 and 31 even after the extra addition of primers. The increase in the heteroduplex to homoduplex ratio and the maximum heteroduplex content occurred at approximately the same cycle of PCR as in the case without extra addition of primers. Thus, the heteroduplex to homoduplex ratio did not differ due to primer addition (Fig. 1*D*).

Thompson *et al.* (2002) proposed that a 10-fold dilution of the amplification product, followed by a three-cycle re-amplification ("reconditioning") could effectively remove heteroduplexes from template amplifications. We tried to decrease the fraction of heteroduplex DNA molecules using this reconditioning PCR (35 cycles + reconditioning). However, the reconditioning PCR was in our case not sufficient to prevent the formation of heteroduplexes. As an increasing number of PCR cycles is connected with an increasing amount of PCR products, the most likely explanation of our results is a low ratio of active *Taq* polymerase molecules to PCR products in the later PCR cycles, because we have shown that the limiting factor was not the primer depletion. Also the concentration of free dNTPs cannot be the limiting factor, because the difference between the dNTP concentration at the beginning and/or the end of PCR is negligible (Ruano *et al.* 1991). Different annealing times also did not change the heteroduplex to homoduplex ratio (data not shown).

Theoretically, heteroduplex formation could be caused by the reduced activity of DNA polymerase in the later cycles of PCR. To investigate the efficiency of DNA amplification, we terminated PCR after 21, 23, 25, 27, 29, 31, 33, and 35 cycles, and measured the intensities of all bands. The experiments were carried out twice with similar results. The results demonstrated that the amount of PCR products was not significantly increased after cycle 33 (supplementary Fig. 2*A*, http://www.ibp.cz/labs/LPDG/EMBP). PCR efficiency was counted as a ratio between the amount of PCR products in the cycle n and in the cycle (n-2). As the amount of PCR products is after two PCR cycles in the exponential phase quadrupled, the PCR is in the exponential phase when the PCR efficiency is approximately four. However, although the *Taq* polymerase was still active until the end of amplification, the amplification efficiency decreased in the later PCR cycles (supplementary Fig. 2*B*, http://www.ibp.cz/labs/LPDG/EMBP). We thus tried to decrease the amount of heteroduplex DNA with the addition of either 2 U of *Taq* DNA polymerase or 2 U of *Taq* DNA polymerase plus 8 pmol of each primer after the 27th cycle (to the standard PCR mixture already containing *Taq* DNA polymerase and 8 pmol of each primer); the goal was to saturate the PCR products with the *Taq* polymerase. PCR was terminated after the 28th, 29th, and 30th cycle and the heteroduplex DNA was detected by the *Sau*96I restriction. We observed a 30 % decrease of the heteroduplex to homoduplex ratio in the second cycle after addition of the *Taq* polymerase (30th cycle; Fig. 2). The addition of the *Taq* polymerase increased the absolute quantity of homoduplex molecules in both cases, and thus decreased the heteroduplex to homoduplex ratio (Fig. 2*B, s*upplementary Fig. 3, http://www.ibp.cz/labs/LPDG/EMBP). The analysis of the heteroduplex to homoduplex ratio clearly shows that the addition of *Taq* polymerase decreased the heteroduplex to homoduplex ratio in comparison with the experiment where no extra *Taq* polymerase was added.

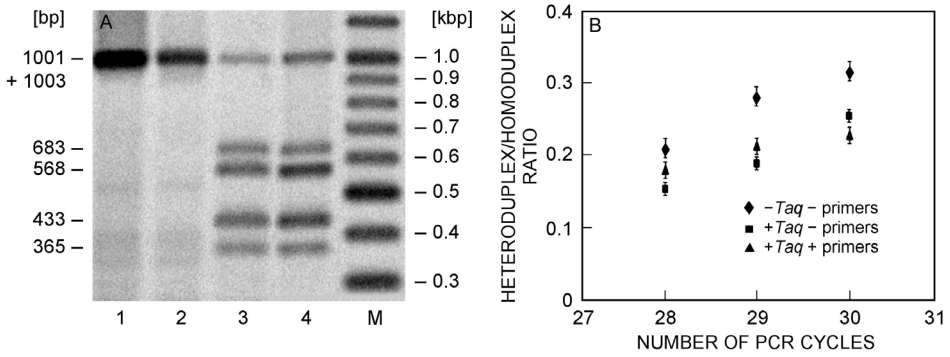

Fig. 2. Heteroduplex formation after reconditioning PCR (*A*) and PCR products after the supplementary addition of *Taq* polymerase and/or primers after the 27th cycle (*B*). *A* - Heteroduplex formation after reconditioning PCR. *Lane 1* - PCR product after 35 cycles; *lane 2* - PCR product after reconditioning PCR. *Lane 3* - PCR product obtained after reconditioning PCR digested by *Sau*96I. As the 1001 bp + 1003 bp fragment consists of heteroduplexes, the reconditioning PCR did not eliminate heteroduplex formation. *Lane 4* - digested PCR product after 31 PCR cycles shows approximately the same amount of homo- and heteroduplex DNA as in the case of the reconditioning PCR (*lane 3*). *Lane M* - DNA marker (*GeneRuler*™ 100 bp plus DNA ladder, *Fermentas*). *B* - The graph shows that the addition of *Taq* polymerase decreased the heteroduplex to homoduplex ratio in comparison with the experiment where no extra *Taq* polymerase was added. Means ± SE, $n = 2$.

We also studied heteroduplex formation using the gene *ScolXY4* (Mrackova *et al.* 2008) from *S. colpophylla*, and found that as in the case of *S. diclinis* spermidine synthase, we were not able to eliminate heteroduplexes using reconditioning PCR; we were, however, able to diminish the level of heteroduplex formation by the addition of *Taq* polymerase or by a decrease in the number of PCR cycles (data not shown).

Our results seem to contradict the results presented by Thompson *et al.* (2002), who observed that the PCR primer to PCR product ratio strongly influenced the heteroduplex formation. However, their experimental design indicates that they probably achieved the elimination of heteroduplexes due to sub-optimal PCR conditions. Sub-optimal PCR conditions tend to decrease the quantity of PCR products, and therefore mimic a decrease in the number of PCR cycles. We have observed that the heteroduplex to homoduplex ratio is low after a few PCR cycles and that the ratio rapidly increases in the later PCR cycles. A similar phenomenon was also observed by Qiu *et al.* (2001) and discussed by Kanawaga (2003). To summarize, we have shown that heteroduplex formation is most likely dependent on the ratio of *Taq* polymerase molecules to PCR product. Therefore, reconditioning PCR may be a suitable method to eliminate heteroduplex formation when the quantity of PCR products is not very high. We have found that the heteroduplex to homoduplex ratio can be diminished by an addition of *Taq* polymerase and by a decrease of the number of PCR cycles. However, this solution limits the PCR yield and thus is not very attractive in general, but it avoids the formation of heteroduplex molecules. A solution that eliminates heteroduplex formation under the standard PCR conditions consisting of a higher number of PCR cycles is based on the addition of *Taq* polymerase in the later PCR cycles to the standard PCR mixture already containing *Taq* polymerase. This approach is much more attractive and could have broad application. Appropriate combination of both (a higher number of PCR cycles + the addition of *Taq* polymerase in the later PCR cycles) can be a method generally applicable to avoid, or at least decrease the formation of heteroduplexes.

References

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., Polz, M.F.: PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. - Appl. environ. Microbiol. **71**: 8966-8969, 2005.

Erhart, M.A., Kim, T., Crews, G.M., Pandya, A.: The use of unilateral PCR to identify prominent heteroduplexes formed during PCR of the mouse microsatellite locus D17Mit23. - Mol. Biotechnol. **33**: 37-48, 2006.

Filatov, D.A.: Substitution rates in a new *Silene latifolia* sex-linked gene, *SlssX/Y*. - Mol. Biol. Evol. **22**: 402-408, 2005.

Gu, Y., Mao, Y.W., Wang, C.G., Zhao, Q.C., Sun, D.L., Song, W.Q.: Cloning of differential expression fragments in cauliflower after *Xanthomonas campestris* inoculation. - Biol. Plant. **52**: 462-468, 2008.

Hayward-Lester, A., Oefner, P.J., Sabatini, S., Doris, P.A.: Accurate and absolute quantitative measurement of gene expression by single-tube RT-PCR and HPLC. - Genome Res. **5**: 494-499, 1995.

Kanawaga, T.: Bias and artifacts in multitemplate polymerase chain reactions (PCR). - J. Biosci. Bioeng. **96**: 317-323, 2003.

Kurata, S., Kanagawa, T., Magariyama, Y., Takatsu, K., Yamada, K., Yokomaku, T., Kamagata, Y.: Reevaluation and reduction of a PCR bias caused by reannealing of templates. - Appl. environ. Microbiol. **70**: 7545-7549, 2004.

Marais, G.A., Nicolas, M., Bergero, R., Chambrier, P., Kejnovsky, E., Monéger, F., Hobza, R., Widmer, A., Charlesworth, D.: Evidence for degeneration of the Y chromosome in the dioecious plant *Silene latifolia*. - Curr. Biol. **18**: 545-549, 2008.

Mrackova, M., Nicolas, M., Hobza, R., Negrutiu, I., Monéger, F., Widmer, A., Vyskot, B., Janousek, B.: Independent origin of sex chromosomes in two species of the genus *Silene*. - Genetics **179**: 1129-1133, 2008.

Negrutiu, I., Vyskot, B., Barbacar, N., Georgiev, S., Monéger, F.: Dioecious plants. A key to the early events of sex chromosome evolution. - Plant Physiol. **127**: 1418-1424, 2001.

Qiu, X., Wu, L., Huang, H., McDonel, P.E., Palumbo, A.V., Tiedje, J.M., Zhou, J.: Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. - Appl. environ. Microbiol. **67**: 880-887, 2001.

Ruano, G., Brash, D.E., Kidd, K.K.: PCR: the first few cycles. - Amplifications **7**: 1-4, 1991.

Ruano, G., Kidd, K.K.: Modeling of heteroduplex formation during PCR from mixtures of DNA templates. - PCR Methods Appl. **2**: 112-116, 1992.

Speksnijder, A.G., Kowalchuk, G.A., De Jong, S., Kline, E., Stephen, J.R., Laanbroek, H.J.: Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. - Appl. environ. Microbiol. **67**: 469-472, 2001.

Thompson, J.R., Marcelino, L.A., Polz, M.F.: Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. - Nucl. Acids Res. **30**: 2083-2088, 2002.