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Notes & Tips

# Self-priming arrest by modified random oligonucleotides facilitates the quality control of whole genome amplification

Ivan Brukner [a,*], Bruno Paquin [b], Majid Belouchi [b], Damian Labuda [a,c], Maja Krajinovic [a,c]

[a] Centre de Recherche, Hôpital Sainte-Justine, 3175 Cote Sainte-Catherine, Montréal, Que., Canada H3T 1C5
[b] Galileo Genomics Inc., 880 McCaffrey, St. Laurent, Que., Canada H4T 2C7
[c] Département de Pédiatrie, Université de Montréal, 3175 Cote Sainte-Catherine, Montréal, Que., Canada H3T 1C5

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Whole genome amplification, WGA[1] [1,2], is widely used in forensic analyses but also extends its application to pathology, molecular diagnostics, and environmental microbiology. Recently, two new methods for WGA have been introduced. The first is based on strand displacement synthesis [3] and termed multiple-displacement amplification. It uses the highly processive *phi 29* DNA polymerase [4] and random primers in an isothermal amplification reaction [5]. The second, OmniPlex method, converts randomly fragmented genomic DNA into a library of amplifiable DNA fragments using ligation of linkers, followed by amplification with linker-specific oligonucleotides [6,7]. The *phi 29*-based WGA procedure is technically more convenient.

Random hexamers are used in this WGA reaction [5,8] to prime DNA polymerization and produce micrograms of DNA from nanograms of the input template. However, high-molecular-weight DNA (>10 kb) is also produced in reactions without addition of the template. This spurious product is electrophoretically indistinguishable from that obtained in the presence of the template. Thus, when the added template fails to be amplified, the spurious product leads much too often to false impression of a successful WGA. To be able to assess the success of WGA merely by the presence of its product, it is necessary to eliminate the false amplification from the control reaction without added template. Toward this end we designed new primers that lead to a complete removal of the background amplification while preserving the standard performance of WGA reaction as judged by subsequent successful PCR of a variety of genomic segments.

To test the performance of the WGA reaction we conducted PCR of five different genomic segments in duplicate, using 1 μl from 10-fold dilutions of WGA reactions. DNA segments, 100–250 bp long, located in genes of methylene tetrahydrofolate dehydrogenase (MTHFD1) [9], glucocorticoid receptor (GR) [10], thymidylate synthase (TS) [11], methionine synthase (MTR) [12], and dihydrofolate reductase (DHFR) [13] were amplified in a mixture containing 0.5 μM each of the relevant primers, 100 μM dNTPs (each), 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 U of *Taq* polymerase (Platinum, InVitroGene) in a volume of 50 μl for 35 cycles consisting of 30 s at 94 °C, 30 s at temperature ranging from 52 to 60 °C (depending on the locus amplified), and 45 s at 72 °C. The sequence of forward and reverse primer pairs used for amplification of the gene regions were 5′-atgggacccaaacgggcgca-3′ and 5′-aaaaggggaatccagtcgg-3′ for DHFR, 5′-cactccagtgtttgtccatg-3′ and 5′-gcatcttgagagccctgac-3′ for MTHFD1, 5′-gtggctcctgcgtttccccc-3′ and 5′-ccaagcttggctccgagccggccacaggcatgcgcgg-3′ for TS, 5′-gaactagaagacagaaattctcta-3′ and 5′-catggaagaatatgaagatattagac-3′ for MTR, and 5′-aaattgaagcttaacaattttggc-3′ and 5′-gcagtgaacagtgtaccagacc-3′ for GR. The product of WGA obtained by varying concentrations of input DNA (1000, 500, 50, and 5 pg) was tested in PCR amplification of described loci.

* Corresponding author. Fax: +1 514 345 4731.
*E-mail address:* ibrukner@justine.umontreal.ca (I. Brukner).

[1] *Abbreviations used:* WGA, whole genome amplification; MTHFD1, methylene tetrahydrofolate dehydrogenase; GR, glucocorticoid receptor; TS, thymidylate synthase; MTR, methionine synthase; DHFR, dihydrofolate reductase.

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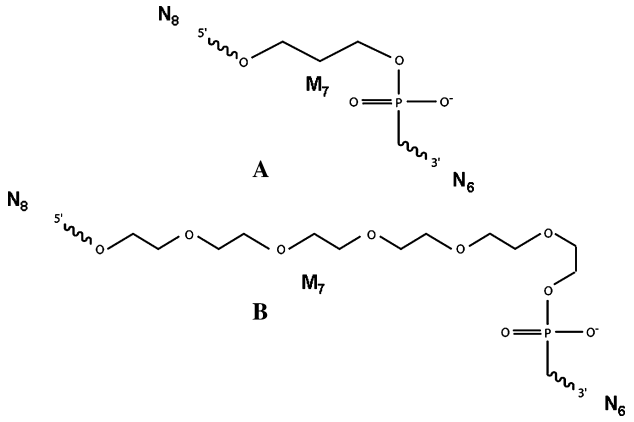


Fig. 1. Structure of modification ($M_7$) of random primers used in WGA. $M_7$ is either internal C3 phosphoramidite (A), or internal 18-atom hexa-ethyleneglycol spacers (B), placed at seventh position from the 3′ end of the random primers, where N denotes degenerative bases.

New WGA oligonucleotides, denoted "modified," were designed to contain additional modification at the seventh position from the 3′ end composed of C3 phosphoramidite (C3) and 18-atom hexa-ethyleneglycol (C18) internal spacers (Fig. 1). These spacers were followed by additional random nucleotide at the eighth position from the 3′ end of the oligonucleotide primer. All oligonucleotides were desalted prior to use. The oligonucleotides containing internal C3 and C18 spacers were also assayed separately after additional purification by HPLC (Integrated DNA Technologies, IA). Random oligonucleotides (6-mers), denoted "standard," were purchased separately (Integrated DNA Technologies; Qiagen, CA). They have two phosphodiester bonds next to their 3′ end, replaced with phosphorothioates [8], to confer resistance to the proofreading activity of phi29.

Template denaturation was performed for 3 min at 95 °C (followed by cooling on ice) with 100 μM random primers and in 10 μl of PCR buffer (Invitrogen, Ontario) containing 20 mM Tris–HCl, pH 8.4, and 50 mM KCl. If modified primers were tested, equimolar mixtures of C3 and C18 oligonucleotides (0.5 nmol of each) were used. The enzymatic amplification was started by adding 10 μl of the second solution, containing 2 μl of 10X reaction buffer (400 mM Tris–HCl, pH 7.5, 500 mM KCl, 100 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, and 40 mM dithiothreitol), 1.25 mM dNTP, and 0.1 μg of Phi29 (Epicentre RepliPHI Phi29 DNA polymerase), and was carried out at 30 °C for 18 h, unless otherwise indicated. Following heating at 65 °C for 10 min to arrest the reaction, a 10 μl aliquot of a 1:10 dilution of the WGA product was analyzed by electrophoresis in 1% agarose gel. After staining using ethidium bromide, the bands were visualized by Chemi-Imager 5500 (Alpha Innotech).

To eliminate self-priming in WGA reactions, we conducted a series of experiments with the primers that were modified to eventually prevent polymerization of the primer–primer duplexes while still allowing template-directed polymerization. In general, DNA polymerases are sensitive to the structural changes of template DNA molecule. For example, polymerization stalls at the abasic site [14–17] and this could likely occur at other modifications also, preventing further chain elongation. The C3 phosphoramidite (C3) and the internal 18-atom hexa-ethyleneglycol (C18) spacers seem to have the required properties. They are longer than the abasic site and structurally more different from DNA backbone (Fig. 1). While designing oligonucleotides to keep the random hexamer segment compatible with the currently used WGA protocols, we placed modifications at and extending beyond the 5′ end of the primer. This preserves the ability of oligonucleotides to prime but not to act as templates in the self-priming reaction.

WGA products obtained using modified and standard primers are illustrated in Fig. 2. Contrary to the standard WGA, there were no visible products of self-priming in the negative control reaction (with no template added) when modified primers are used. We have found out that modified primers are not stable in water (Nanopure) and therefore they should be dissolved in TE buffer (pH 7.6). For longer storage, we lyophilized primer aliquots and kept them dry. Also, we have noticed variations in the WGA yield, using different batches of desalted modified primers from different syntheses and/or different suppliers (Integrated DNA Technologies; Qiagen). This suggests that the ratio of modified to nonmodified primers is critical for WGA performance. Nevertheless, modified desalted primers successfully suppressed self-priming if the incubation time was 18–20 h long. Moreover, while using HPLC-purified modified primers, WGA did not give visible self-priming product, even after 72 h of incubation (see Supplementary Fig. 1).

PCR of the five genomic segments analyzed performed similarly to WGA products obtained with

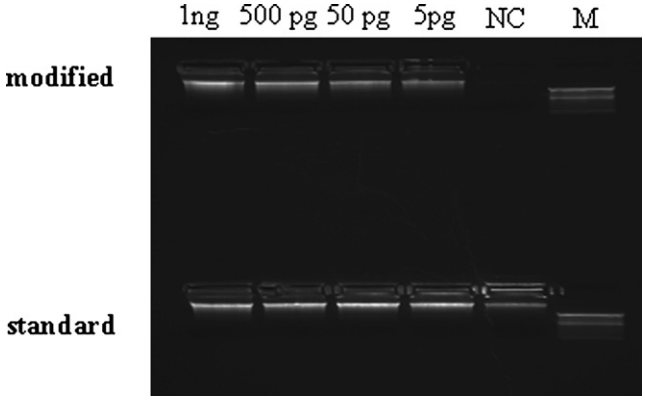


Fig. 2. Modified and standard WGA reactions performed with varying concentrations of template DNA. The quantity of input template used to obtain WGA products using standard or modified oligonucleotides are indicated in each lane. NC, negative control (no input DNA); M, 1-kB ladder.

standard and with modified WGA primers. In fact, the WGA products from 1 ng input genomic DNA using either standard or modified protocol produced repeatedly the expected PCR products for all five loci. However, if the input genomic DNA in WGA reactions was lower than 1 ng, the performance of PCR was variable. This variability might result from a combination of factors: (1) the primers degradation, (2) differences in modification yield during primer synthesis, (3) the enzyme phi29 activity decay caused by repeated transitions from −20 to 4 °C, and (4) the experimental variability when using a low number of genome equivalents. The latter is in accordance with the suggestions made by others [5] that the input template should represent at least 300 (human) genome equivalents.

In summary, we described a modification of random oligonucleotides that efficiently blocks self-priming. By eliminating self-priming and thus spurious DNA products in the reaction with no template added, the success of WGA can be estimated directly by assessing the amount of the product on agarose gels. The resulting removal of self-priming not only facilitates interpretation of WGA results but also could extend the utility of WGA-generated DNA for other downstream applications, such as whole genome hybridization experiments.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2005.01.005.

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