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Notes & Tips

Phi29-based amplification of small genomes

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Whole genome amplification (WGA),[1] based on the isothermal reaction of the enzyme Phi29 and random primers, is one of the most popular methods currently used in genomic research [1–3]. However, there is one major drawback limiting applicability of this technique in certain fields such as forensics, microbiology, and environmental monitoring. The WGA method is hardly applicable to small genomes and/or degraded DNA, especially if the number of genome copies is approaching 100–1000 molecules. Here, we describe a simple procedure in which WGA amplifies 1 fg of the linear pUC19 DNA sufficiently for hundreds of downstream (i.e., post-WGA) PCR reactions. Thus, the amplification of limited quantities of other unknown small genomes or genomic fragments should be possible as well.

It was observed previously that self-priming of degenerate 6-mers might compete with the priming of the template DNA, especially if the quantity and/or length of the DNA template are small [2–5]. However, resolving self-priming problem did not remove performance limitations of the Phi29-based WGA procedure [5]. For example, in our laboratory, 300 copies of a single copy gene could be WGA amplified in the context of human DNA but not as an isolated plasmid DNA. It seemed that the technical bottleneck was due to a low hybridization of the degenerate 6-mers to the template, suggesting a better WGA performance with an equivalent number of copies of longer templates or embedded within longer templates, thereby favoring a higher number of priming events to occur. Therefore, we decided to ligate a small template, such as pUC19 of 2686 bp, with a long carrier DNA, such as poly(dA–dT) or human DNA, before performing Phi29-based WGA.

We compared WGA performance of serial dilutions of (i) *Hind*III-cleaved, blunt end, religated pUC19; (ii) *Hind*III-cleaved, blunt end pUC19 (no ligase); (iii) *Hind*III-cleaved, blunt end pUC19 ligated with a high-molecular DNA; and (iv) *Hind*III-cleaved, blunt end pUC19 with a high-molecular DNA (no ligase). Briefly, 10 pg of plasmid DNA was linearized by 1 U of *Hind*III (NEB, Beverly, MA, USA) for 3 h at 37 °C in 10 µl. The 3′ overhang was removed by incubation for 30 min at 37 °C in 10 µl total volume of reaction buffer (40 mM Tris–HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, and 4 mM dithiothreitol [DTT]) with 1 U of T4 DNA Polymerase (Amersham Biosciences, Canada), and the 5′ overhang fill-in was performed in the presence of 0.25 mM dNTPs by incubation for another 30 min at 37 °C. Six tubes of 10-fold serial dilutions, starting from 10 pg of pUC19 per reaction, were prepared, and 1 U of T4 Ligase and 1× Ligation Buffer (USB, Cleveland, OH, USA) were added in the total volume of 5 µl (12 h at 37 °C). When ligation of the template DNA with high-molecular weight DNA preceded WGA, the samples were prepared as follows: lyophilized poly(dA–dT), approximate average length 8778 bp (Amersham Biosciences), was dissolved in 1× Ligation Buffer to a final concentration of 500 ng/µl. The 500 ng of poly(dA–dT) was treated in the same mode as cleaved plasmid DNA to create blunt ends. The equivalent dilutions of blunt end pUC19 samples (starting from 10 pg) were religated or ligated with 10 ng of blunt end poly(dA–dT) by incubation with 1 U of T4 DNA Ligase in 5 µl of total volume. The reaction steps, which do not include T4 DNA ligation, were done as a control. The WGA was performed for each pUC19 dilution as described previously [1] or using primers with 5′ attached C3 and C18 spacers (Integrated DNA Technologies, Coralville, IA, USA) [5].

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[1] *Abbreviations used:* WGA, whole genome amplification; DTT, dithiothreitol.

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A similar type of experiment was performed using *Hind*III-cleaved pUC19 and 10 ng of *Hind*III-cleaved human DNA as a universal carrier (data not shown), thereby eliminating the need for the creation of blunt ends. Of note is that 10 ng of high-molecular weight carrier DNA always produces WGA product (even if there is no input of additional template DNA) and that downstream PCR must be used to identify the presence or absence of the amplified small template.

Two control PCR reactions were performed with pUC19 and 1 μl of 10-fold diluted WGA product using the following pUC19-specific primer pairs: (A) 5′-gagc cggaagcataaagtgta-3′ and 5′-aactctgtagcaccgcctac-3′, producing a 750-bp amplicon (Fig. 1), and (B) cagg ctgcgcaactgttgggaa and 5′-ccgtcgttttacaacgtcgta-3′, producing a 139-bp fragment using pUC19 (data not shown). The PCR reaction contained 1 μM of each of the primers, 100 μM of each dNTP, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, and 0.5 U of *Taq* polymerase (Platinum, Invitrogen, Carlsbad, CA, USA) in a volume of 50 μl (30 cycles consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C). The identity of PCR product from the WGA reaction was confirmed by cleavage with *Hae*II (Fig. 1C). As shown in Fig. 1B, the PCR from the corresponding WGA was successful when using 100 times less template (0.1 pg) than the current minimal template requirement [6], but only if template ligation with 10 ng of high-molecular weight DNA was performed before WGA reaction.

Blunt end ligation at a limited concentration of pUC19 (<0.1 pg/reaction) did not improve WGA performance, as estimated by specific downstream PCR. Although blunt end ligation of small template DNA improves WGA performance and overcomes terminal underrepresentation of Phi29-based amplification of linear DNA [7] (apparent K_d of T4 Ligase for DNA is in the low-micromolar to high-nanomolar range [8]), it seems that efficiency of T4 ligation below attomolar concentrations of the substrate might compromise the ligation step of template alone and subsequent success of WGA reaction.

The technique presented here goes one step further, allowing the amplification of only 1 fg of linear pUC19, corresponding to 3.4×10^2 plasmid copies. We believe that our procedure increases the applicability of Phi29-based amplification by extending the repertoire of amplifiable templates to include small genomes and/or fragments of degraded DNA.

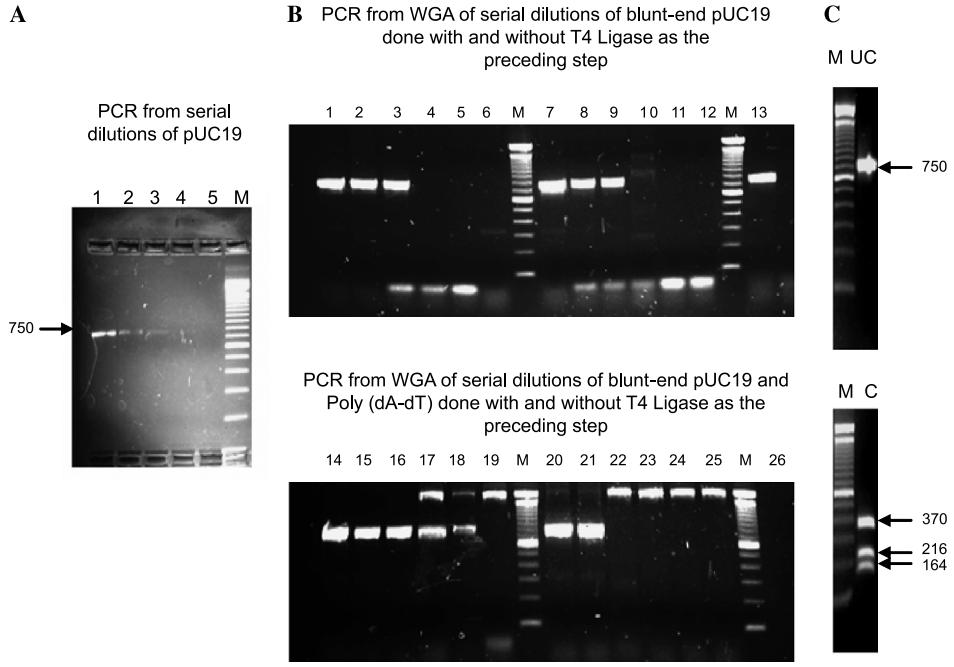

Fig. 1. (A) PCR reactions from pUC19. (B) PCR reactions from WGA of pUC19 with or without religation and/or ligation to poly(dA–dT). (C) Digestion of PCR by *Hae*II, confirming amplicon identity. In (A) lanes 1–4 are PCR products from serial dilutions of pUC19 (starting from 0.1 pg), and lane 5 is negative control. In (B) lanes 1–6 are PCR products performed on 10-fold serial dilutions of plasmid DNA (*Hind*III-cleaved, blunt end, and religated, starting from 10 pg of pUC19), lanes 7–12 are PCR products of WGA of serial dilutions of pUC19 cleaved with *Hind*III and blunt-ended (starting from 10 pg), and lane 13 is PCR positive control (from plasmid DNA). Lanes 14–19 are PCRs from WGA of serial dilutions of pUC19 (starting from 10 pg) ligated with 10 ng of poly(dA–dT), lanes 20–25 are PCRs from WGA of serial dilutions of pUC19 (starting from 10 pg) done in the presence of 10 ng of poly(dA–dT) where the ligation step was omitted, and lane 26 is PCR negative control. (C) PCR product amplified from WGA (lane UC) and cleaved by *Hae*II (lane C), thereby confirming amplicon identity by expected digestion pattern. Numbers on the left and right are length of DNA in base pairs (bp); M is 100-bp ladder (Invitrogen).

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