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Usefulness of repeated GenomiPhi, a phi29 DNA polymerase-based rolling circle amplification kit, for generation of large amounts of plasmid DNA

Masahiro Sato [a,*], Masato Ohtsuka [b], Yuhsuke Ohmi [b,1]

[a] *The Institute of Medical Sciences, Tokai University, Bohseidai, Isehara, Kanagawa 259-1193, Japan*
[b] *Department of Molecular Life Science, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-1193, Japan*

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Abstract

The GenomiPhi™ DNA Amplification Kit employs rolling circle amplification (RCA) using phi29 polymerase, dNTPs, and random hexamers. We demonstrated that repeated RCA (at least three times) is useful for high-fidelity amplification of large amounts of plasmid DNA.

Keywords: GenomiPhi; TempliPhi; Rolling circle amplification (RCA); Plasmid; PCR; Amplification; phi29 DNA polymerase

1. Introduction

A novel method for whole genome amplification using bacteriophage phi29 DNA polymerase has been developed [1]. It is based on rolling circle amplification (RCA) [2,3] and can reliably amplify large circular DNA templates such as plasmid and bacteriophage DNA as well as genomic DNA from clinical samples [4–6]. The commercially available GenomiPhi™ DNA Amplification Kit (Amersham Biosciences Co., Piscataway, NJ, USA) was developed to amplify the entire genome with phi29 enzyme and random priming by hexamers. For example, with this kit, a small quantity of plasmid or genomic DNA (i.e., 10 ng) can be amplified in vitro into 4–7 μg with one-step, overnight incubation.

The final goal of this study was to demonstrate the advantages of the GenomiPhi™ DNA Amplification Kit in amplifying the entire genome of a single sperm or oocyte or fertilized egg. If this kit can be applied to these cells and genomic DNA can be successfully amplified with it, large numbers of assays can be performed on a single sample. In this case, repeated reactions using the kit may result in generation of large amounts of DNA from samples with very small amounts of DNA. In this study, we demonstrated that repeated RCA reactions improved amplification yield greatly without affecting the quality of DNA itself.

2. Materials and methods

For repeated RCA, 1 μl of solution containing 11-kb circular pCETZ-17 plasmid (10 ng; [7]) was subjected to amplification. The components used were all from the GenomiPhi™ DNA Amplification Kit (#25-6600-01; Amersham Biosciences Co.), which includes phi29 enzyme, dNTPs, and random hexamers. The reaction conditions were the same as described in the manufacturer's protocol. A total of 10 tubes were subjected to the reaction simultaneously. After reaction, the mixtures were heat-inactivated, ethanol-precipitated, and then redissolved in 20 μl of water. This solution was designated the result of the ''1st round of RCA''. One microlitre of this solution was subjected to DNA quantification, as described below. In some cases, 1 μl of the solution was directly subjected to electrophoresis in a 0.8%

* Corresponding author. Tel.: +81 463 93 1121x2692; fax: +81 463 94 8524.
E-mail address: masasato@is.icc.u-tokai.ac.jp (M. Sato).
[1] Present address: Department of Biochemistry II, Nagoya University School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan.

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agarose gel. The control circular pCETZ-17 DNA (50 and 200 ng) was concomitantly electrophoresed. For the 2nd round of reaction, the solutions in ten tubes containing the 1st round of RCA products were combined and again subjected to DNA quantification. An aliquot was diluted with water to a concentration of 10 ng/μl. One microlitre of the diluted solution was subjected to the 2nd round of RCA under the same conditions as for the 1st round of RCA. A total of ten tubes were reacted at the same time. After reaction, DNA was quantified for each sample, and in some cases 1 μl of the solution was directly subjected to agarose gel electrophoresis. For the 3rd round of RCA, the samples containing the 2nd round of RCA products were treated in the same fashion as for the 2nd round of RCA.

We used a spot DNA assay [8] to quantify amounts of DNA in the amplified products. 1 μl (600 ng) of the RCA products was subjected to digestion with *Eco* RI, which generated 1.3-, 4.4-, and 5.3-kb fragments of pCETZ-17 [7]. When digested with *Spe* I, 3.0- and 8.0-kb fragments were released [7]. One microlitre (200 ng) of the control pCETZ-17 plasmid was also digested with each enzyme. The digests were electrophoretically separated in 0.8% agarose gels. After electrophoresis, the gels were photographed under UV illumination following staining with ethidium bromide (EtBr). In some cases, the gels were transferred to GeneScreen Plus™ nylon membranes (#NEF976, NEN™ Life Science Products, Inc., Boston, MA). The filters were hybridized as described by Sato et al. [9]. ^{32}P-labelled *Not* I-linearized pCETZ-17 was used as a probe.

The 1st to 3rd round RCA products (approximately 40 μg) were digested with *Eco* RI and size-fractionated. Then 1.3-kb fragments were inserted into the *Eco* RI site of pBluescript SK(-) cloning vector (Stratagene, La Jola, CA) for sequencing. Sequence homology analysis was carried out using the Genetyx-Mac 10.0 software package (Software Development Co., Ltd., Tokyo, Japan).

3. Results

One microlitre of a solution containing pCETZ-17 (10 ng) was subjected to amplification in a total volume of 20 μl. As shown in Fig. 1A, quantification of DNA by spot DNA assay revealed that the average DNA concentration was 813 ng/μ1 of DNA, although the quantity of DNA amplified varied among reactions. A 1626-fold amplification of plasmid DNA was achieved with this system. The electrophoretic pattern of the amplified products revealed that plasmid DNA was in fact amplified, but exhibited a more smeary pattern than the purified pCETZ-17 DNA (Lane 1 versus Lanes C1 and C2 in Fig. 1B), although both peaked at about 11 kb. The smeary pattern of the amplified products indicates that they included a mixture of intact pCETZ-17 molecule and other low-molecular-weight fragments of various sizes.

We next examined whether repeated RCA could amplify more DNA than single RCA without affecting DNA quality. The 1st round of RCA products from pCETZ-17 (10 ng) was collected and diluted with water to a concentration of 10 ng/μ1. When 1 μl of this solution was subjected to the 2nd round of RCA, the average DNA concentration of amplified products was 787 ng/μl, with a range of 500–1260 ng/μl (Fig. 1A), indicating 1574-fold enrichment of DNA. The gel electrophoretic pattern of the 2nd round of RCA products was similar to that of the 1st round of RCA (Lane 1 versus Lane 2 in Fig. 1B). Furthermore, the 2nd round of RCA products was collected and diluted to 10 ng/μl. The diluted solution was subjected to the 3rd round of RCA using the same conditions as for the 2nd round of RCA. DNA quantification revealed that the amplified products had DNA concentrations ranging from 500 to 1370 ng/μl, with an average of 908 ng/μl (Fig. 1A), indicating 1816-fold enrichment of plasmid DNA. The products from the 3rd round of RCA exhibited a pattern similar to those of the 1st and 2nd rounds of RCA on gel electrophoresis (Lanes 1 and 2 versus Lane 3 in Fig. 1B).

These RCA products (600 ng) and the control pCETZ-17 (200 ng) were digested with *Eco* RI or *Spe* I prior to electrophoresis in 0.8% agarose gels. Digestion of pCETZ-17 with *Eco* RI yielded three expected DNA fragments (1.3, 4.4, and 5.3 kb) (Lane C in the upper panel of Fig. 1C). Digestion with *Spe* I yielded two expected DNA fragments (3.0 and 8.0 kb) (Lane C in the lower panel of Fig. 1C). These bands were also discernible for each of the RCA-derived products in the gels that had been stained with EtBr (“EtBr” column in Fig. 1C). Southern blot hybridization using ^{32}P-labelled *Not* I-linearized pCETZ-17 probe confirmed this finding (“Southern” column in Fig. 1C). As expected, low-molecular-weight molecules of various sizes were also hybridized with the probe in a smeary fashion in the RCA-derived samples.

To further confirm that the sequence of the amplified DNA products was the same as that in the authentic plasmid, products from the 1 st to 3rd round of RCA together with purified pCETZ-17 plasmid were digested with *Eco* RI, and the resulting 1.3-kb fragments were isolated. After cloning of the isolated inserts into pBluescript SK(-) vector, plasmid DNA was isolated from 5 randomly picked bacterial clones and subjected to sequencing. We obtained greater than 600 bp of sequence data with 100% identity between the RCA products and the authentic pCETZ-17 sequence for all clones tested (data not shown). These observations indicate that GenomiPhi-based re-amplification is accurate.

4. Discussion

The GenomiPhi™ DNA Amplification Kit is based on amplification of circular DNA as a template. It might be claimed that optimal amplification of circular plasmids is mechanically different from that of eukaryotic linear