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Phi29 DNA Polymerase Based Rolling Circle Amplification of Templates for DNA Sequencing

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Abstract

The generation of DNA sequencing templates is typically an inconsistent and labor-intensive procedure, especially in a high-throughput facility. Purification of recombinant plasmids from *E. coli* and PCR amplification of inserts have generally been employed, but require many laborious/time-consuming steps and do not always yield suitable amounts of high-quality templates to be used in downstream applications. Replication by a rolling-circle mechanism is common among bacteriophages in nature. Recently, rolling-circle amplification (RCA) with Phi29 DNA polymerase has been applied *in vitro* to marker DNA sequences (using specific primers) and to circular cloning vectors (using random hexamer primers) to achieve their exponential amplification via DNA strand displacement. The US DOE Joint Genome Institute has successfully implemented random-

primed RCA into their high-throughput process for production of sequencing templates. Here, we describe the RCA-based plasmid amplification protocol, as well as several practical applications for using amplified DNA in sequencing and related procedures.

Background

Strand-displacement process allows a DNA polymerase to synthesize a new complementary DNA strand while displacing the previous one lying upstream. In contrast to 'DNA-unproductive' nick translation, which is accomplished during the reparative replication of double-stranded DNA by DNA polymerases having associated 5'-3' exonuclease activity (*e.g. E. coli* DNA Pol I), replication by strand-displacing DNA polymerases results in the net DNA synthesis. This is particularly useful when the polymerase is being used for rolling-circle DNA replication (Kornberg and Baker, 1992). Once the enzyme has completed replication of the circular single-stranded DNA template, strand-displacement activity would be required to begin the 'rolling' mode of amplification.

Indeed, DNA circles smaller than 100 nt can readily be 'rolled' by most DNA or RNA polymerase due to strong flexional stress created by requiring the newly made DNA duplex to bend sharply. This stress causes the 5' end of the replicated DNA strand to unwind and dangle at some distance behind the polymerase; hence no actual strand displacement is necessary in case of small circular DNA templates (Kool, 1996; Demidov, 2002). But as the size of the input template circle exceeds ~100 nt, the strand-displacement activity of DNA polymerase becomes an absolute requirement for the RCA reaction to proceed (Kool, 1996). To this end, Phi29 DNA polymerase has no upper limit on the size of DNA being replicated, and in fact performs the strand-displacement replication on circular DNA templates as efficiently as with linear DNAs (Blanco *et al*., 1989; Lizardi *et al*., 1998; Dean *et al*., 2001). This robust ability can be used both for signal amplification in DNA diagnostics with the use of DNA minicircles (Lizardi *et al*., 1998; Demidov, 2002) and for generation of DNA sequencing templates from plasmids, as described in this chapter.
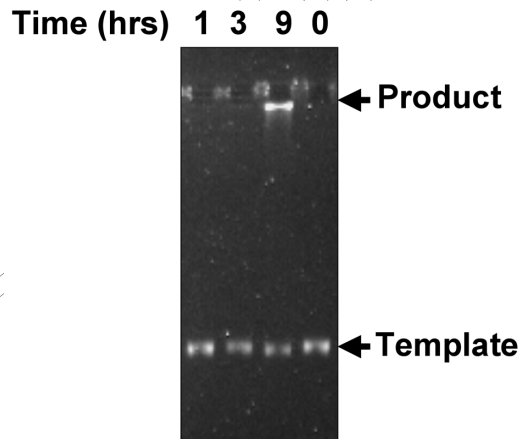
Time (hrs) 1 3 9 0

Figure 1. Strand-displacement activity and processivity of Phi29 DNA polymerase in the single-primed RCA reaction with single-stranded circular M13 DNA template. Reaction samples containing 25 ng of M13mp18 DNA and 5 pmoles of –40 universal primer in 10 µl TE buffer were heated to 95°C for 3 min and slow cooled to room temperature for annealing the primer. These were then chilled, reaction buffer and a sub-saturating amount of Phi29 DNA polymerase was added, and reactions were incubated at 30°C for the indicated times. The RCA products were then resolved by electrophoresis in 0.6% agarose gel (1× TBE buffer).

Phi29 DNA polymerase is a highly processive enzyme that could incorporate ≥70,000 nucleotides per binding event (Blanco *et al.*, 1989; see also the chapter by Salas and Blanco in this book). In a processivity assay performed by us using M13 single-stranded DNA as circular template, the single-primed DNA replication effectively proceeds via rolling-circle mechanism even with limiting amounts of Phi29 DNA polymerase (Figure 1). Phi29 DNA polymerase does not apparently pause when encountering double-stranded template. Thus, it can perform the strand-displacement synthesis in the absence of DNA helicase or SSB protein, which normally assist DNA polymerase in performing this reaction. In Figure 1, high-molecular-weight amplification products are observed after 9 hrs. These products are so large that they fail to migrate into the gel and it is difficult to size them. Note that fragments of intermediate size are not observed, indicating the product has been synthesized during a single binding event. Phi29 DNA polymerase is known to incorporate nucleotides

at 25-50 nt/second (Blanco *et al.*, 1989; Lizardi *et al.*, 1998), which would suggest the potential size of these RCA amplicons is actually over 800,000-nt-long.

Accordingly, a high-throughput strategy for isothermal DNA amplification that employs Phi29 DNA polymerase and rolling-circle DNA synthesis has been developed to generate high-quality templates for DNA sequencing. The RCA-based method, called TempliPhi™ DNA Amplification (Amersham Biosciences), utilizes the random-primed replication by this enzyme to exponentially amplify circular DNA templates consisting of plasmid vector with cloned DNA inserts (Dean *et al.*, 2001; Detter *et al.*, 2002). One can see that using a random set of hexamer primers for initiation of multiple simultaneous replication forks, tandem (concatenated) double-stranded copies of the circular DNA could be generated in a hyperbranched reaction, as depicted schematically in Figure 2. Phosphorothioate modification between the last three nucleotides at the 3' end of the primer prevents the DNA polymerase from degrading the primers via intrinsic 3'-5' exonuclease activity and dramatically accelerates the amplification kinetics (Dean *et al.*, 2001).

This RCA format is a very powerful approach that requires little starting material. Typically there is a brief (~0.5 hr) lag period followed by rapid synthesis of DNA products until the supply of nucleotides is exhausted (Figure 3). The method can isothermally produce the microgram quantities of DNA from the picograms inputs in a few hours, allowing greater than $5 \cdot 10^6$-fold amplification. We utilized the electron microscopy to visualize and examine the amplification products (Figure 4). With this technique, products of ≥100 kb have been observed after 4 hr of amplification using a 2.7-kb circular DNA template and certain RCA amplicons consisted of >50 end-to-end tandem repeats of the input material.

Importantly, our method of DNA amplification should replicate DNA with high fidelity: Phi29 DNA polymerase has an associated 3'-5' exonuclease 'proofreading' activity (Blanco and Salas, 1996) and has a reported error rate of $5 \cdot 10^{-6}$ (Esteban *et al.*, 1993), about 100-fold lower than that of Taq DNA polymerase (Dunning *et al.*,
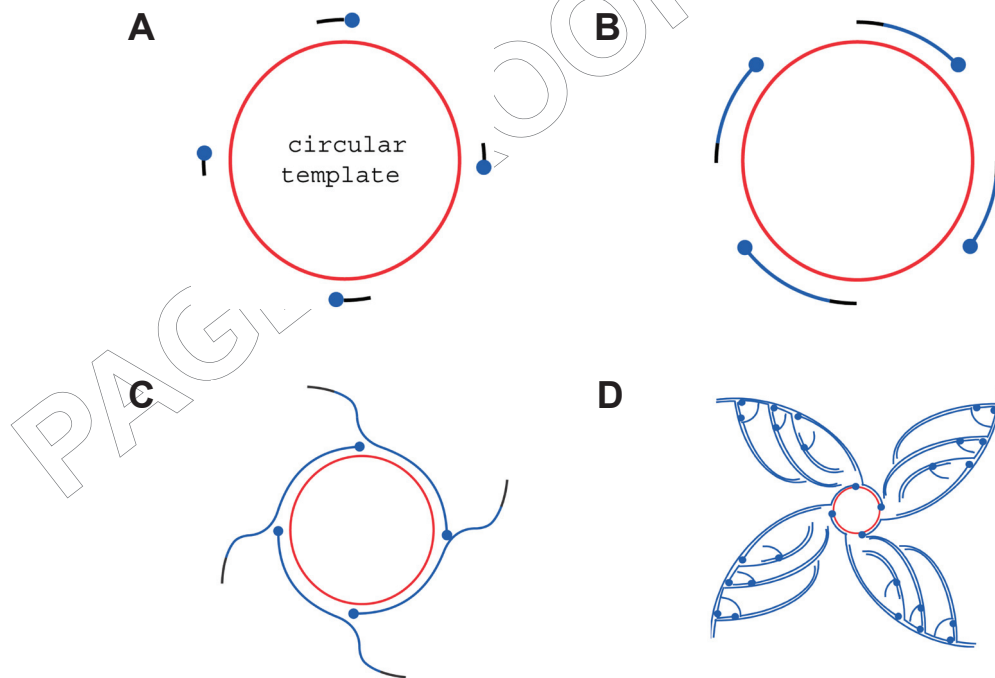
Figure 2. Schematics of the random-primed RCA reaction with circular vector template employing strand displacement. A: Random primers hybridize to multiple locations on DNA circle (single-stranded or denatured plasmid or phage DNA). B: Phi29 DNA polymerase (black sphere) initiates replication from many primers simultaneously synthesizing multiple end-to-end copies. C: Strand displacement allows the polymerase to continue the RCA-type replication. D: Random-priming events subsequently occur on the displaced DNA strands yielding the highly-branched amplification products. This series of displacement and replication events continues until the nucleotide pool is depleted.

1988). In agreement with this expectation, an error rate of $3 \cdot 10^{-6}$ was determined for TempliPhi DNA amplification following a modified Kunkel method (Kunkel, 1985; Nelson *et al.*, 2002). In addition, multiply repeated sequencing experiments indicated that Phi29 DNA polymerase faithfully replicates input DNA as no amplification errors were detected. In these experiments (data not shown), DNA sequence data were obtained from primer walks for TempliPhi-amplified M13mp18 and pUC18 DNA (10,000-fold amplified; approximately 20,000 nt of sequence) using 24 pairs of forward and reverse vector-
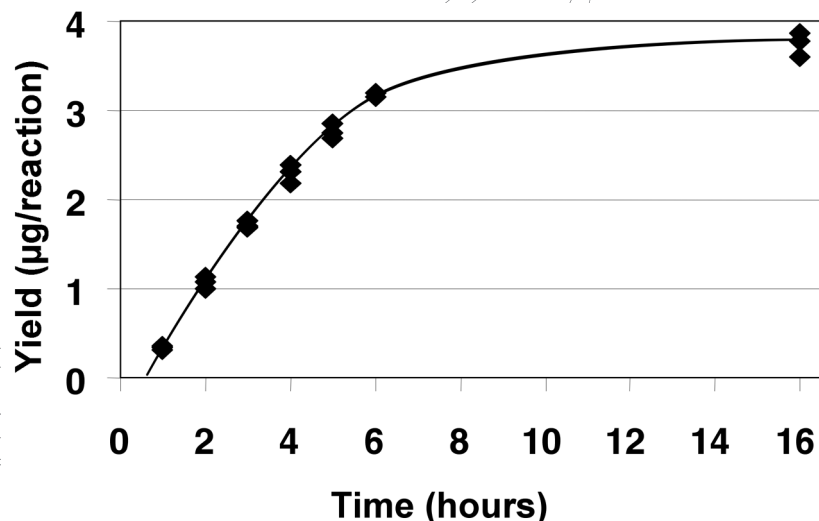
Figure 3. Exemplary TempliPhi DNA amplification kinetics. Supercoiled pUC18 DNA (1 ng) was added to 10 μL of Sample Buffer (TempliPhi™ DNA Sequencing Template Amplification Kit; Amersham Biosciences), heated at 95°C for 3 min to denature the template and chilled to 4°C. Then, 10 μL of TempliPhi Premix from the kit was added and reaction tubes were held at 30°C for the indicated times. To stop the amplification reaction, samples were heated to 65°C for 10 min to inactivate the Phi29 DNA polymerase and then quantified.

specific primers in a total of eleven 96-well-plate sequencing runs on MegaBACE™ 1000 sequencing complex.

The actual steps of the method are quite simple and it can be used to amplify the recombinant plasmid or M13 phage DNA directly from cell culture, colonies or plaques. Interestingly, while chromosomal DNA isolated from bacterial host can also be efficiently amplified by this method, TempliPhi reactions performed on bacterial cells containing medium to high copy number of plasmid DNA show no evidence for the amplification of host DNA, yet contain up to 2-3 μg of newly made plasmid DNA. The presumed reason for this is that the initial heat treatment of bacterial cells (95°C for 3 min) is harsh enough to release plasmid DNA but it does not totally lyse the bacterial cells. Therefore, only plasmid DNA is free to be amplified in this reaction. There is also some evidence that a difference in amplification kinetics of plasmids versus chromosomal DNA (the former could be more
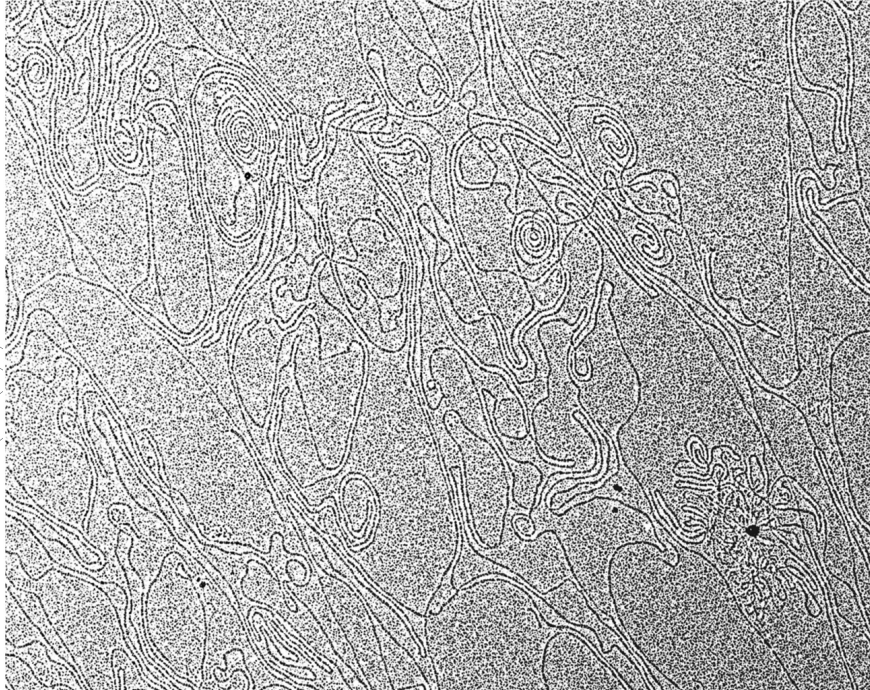
Figure 4. Electron microscopy of the amplification product (with Daniel Collins assistance). Plasmid DNA (pUC18) was amplified for 4 hr using the TempliPhi method. An aqueous basic protein film technique was used (Kleinschmidt method) to analyze the products by transmission electron microscopy using a JEOL JEM 1230 equipment. The sample was shadowcast under vacuum using palladium/platinum wire as the source metal for deposition and the image was captured on Kodak SO-163 film. Exact product length measurements were not possible to make as long DNA strands could not be found, which were entirely contained in the picture. However, many linear double-stranded DNA products greater than 50-120-kb-long, representing RCA products of 20-50 concatenated repeats of the input template circles were identified.

rapidly amplified) may result in a bias for plasmid amplification over small amounts of chromosomal DNA, which can be present at the start of the reaction.

The RCA reaction products obtained do not need to be processed prior to DNA sequencing. As a matter of fact, the completed amplifications are depleted of nucleotides and residual random hexamers will not anneal under typical DNA sequencing conditions. Consequently, the

amplified samples can be sequenced directly after the amplification procedure without any purification using any of the commercially available dideoxynucleotide DNA sequencing kits. In addition, since the amplification self-terminates once the supply of nucleoside triphosphate is exhausted, each reaction contains approximately the same concentration of DNA product. This inherent normalization provides a consistency that can improve DNA sequencing success rate, which is strongly influenced by variability in template DNA concentration. This is especially true in high-throughput settings with capillary sequencers where even slight differences can negatively affect pass rates and read lengths, thus reducing throughput and increasing costs. Besides, the sequenced samples that are processed after the sequencing reaction by G-50 spin columns or precipitation on carboxylate modified magnetic beads to remove reaction components typically have a significant portion of the template DNA selectively removed. This can also lead to improved results on capillary sequencing instruments, which are known to be less tolerant to high concentrations of template DNA than slab gel instruments.

Experimental Protocols and Exemplary Applications

Materials

Amplification

Source of double-stranded plasmid DNA (either purified recombinant plasmid or *E. coli* colony and saturated culture (with or without glycerol) containing plasmid); denaturation buffer (10 mM Tris-HCl/pH 8.2; 0.1 mM EDTA); RCA reaction mixture (reaction buffer, dNTPs, thiophosphate protected random hexamers and phi29 DNA polymerase; all from the Amersham Biosciences TempliPhi DNA Sequencing Template Amplification Kit).

Sequencing

Aliquot of amplified plasmid as sequencing template; sequencing primer; dH$_2$O; DYEnamic^{TM} ET terminator sequencing kit for MegaBACE sequencers (Amersham Pharmacia Biotech) or BigDye^{TM} sequencing kit for ABI sequencers (Applied Biosystems).

Clean-Up of Sequencing Reaction

The microbead-based method uses the following: dH$_2$O, 100% ethanol, tetraethyleneglycol (Aldrich), and magnetic beads (Seradyn); standard ethanol-precipitation method uses the following: dH$_2$O, 100% ethanol, 7.5 M ammonium acetate.

Restriction Digest

Aliquot of amplified plasmid as digestion template; desired restriction endonuclease with corresponding buffer.

Core Procedure: Plasmid Amplification

Plasmid DNA present in bacterial colonies can be used directly as template for TempliPhi amplifications. This is a great convenience since clones can be screened at the colony stage without having to wait for liquid cultures to grow and without the need for DNA plasmid preparation. As little as one part in 10,000 of a single colony can act as a good template for RCA using the high copy-number plasmid pUC18 and generating more than 1 µg of product in 4-6 hr at 30°C (data not shown). In case of the lower copy-number plasmids, such as pBR322, pCUGIblu21 and pMCL200, amplifications should be performed for 8-16 hr to achieve maximal yields. Note that presence of host chromosomal DNA does not affect the efficiency of plasmid amplification, as mentioned above (see the concluding part of Background).

M13-derived plaques are also excellent starting material for amplification. Post-lytic supernatants from M13-infected bacterial culture may be used, too. We have taken 0.02-1 μL of these supernatants in 4-hr RCA reactions to successfully generate DNA sequencing templates (data not shown). Since the resultant amplicons are double stranded, both strands of M13 clones can be sequenced.

Liquid cultures provide good templates for RCA reactions, as well. This is particularly useful to researchers who prefer to grow small overnight cultures for archival storage. There is no significant difference in amplification yield when using 0.001-1.0 μL of a saturated overnight *E. coli* culture as initial template for RCA reactions. A small portion of frozen glycerol stock can be amplified directly using this method. We use this process at the Joint Genome Institute in our high-throughput sequencing production line and in doing so have significantly increased throughput, read lengths, and quality of data, while reducing costs and manpower (Detter *et al.*, 2002).

Below, detailed plasmid amplification steps are presented followed by an example of the process along with quantification results. For additional information, including high-throughput scaling of the process, see research protocols at www.jgi.doe.gov.

Step 1: Sample Heat Denaturation

The sample can be in the form of bacterial liquid culture (typically 2 μL), 7.5% glycerol stock (typically 2 μL), colony (use small to medium picked piece with pipette tip, not tooth pick) or purified plasmid DNA (use <100 ng, down to as little as 1 pg). Mix the above amounts of plasmid-containing sample with denaturation buffer (1 mM Tris-HCl/pH 8, 0.05 mM EDTA) in 10 μL volume total. Heat sample(s) to 95°C for 3-5 min (to avoid release of bacterial chromosomal DNA when amplifying from bacterial cells, do not exceed the 5 min limit). Place samples on ice or keep them at 4°C for no less than 5 min.

Step 2: RCA Reaction

Add 10 μL of RCA reaction mixture (*e.g.* RCA mix from TempliPhi kit) to 10 μL of denatured sample(s). Carry out the RCA reaction at 30°C for 12-24 hr. In our experience, an overnight incubation (~16 hr) yields more consistent and reliable results in a high-throughput setting although shorter (down to 4-6 hr) incubation may also give the adequate results. Note that alternative (larger or smaller) reactions volumes can be used with success.

Step 3: Enzyme Inactivation

Heat inactivate the DNA polymerase at 65°C for 10 min and place sample on ice or keep it at 4°C for at least 5 min. This step inactivates the exonuclease activity of the enzyme and prevents it from potentially interfering with the sequencing reaction. The final product can be stored for ~7 days at 4°C, for ~30 days at –20°C or for much longer time at –80°C before being used as template for sequencing without prior purification.

Exemplary Results

The first use of the RCA-based TempliPhi technology at the Joint Genome Institute (JGI) was to replace the existing plasmid DNA isolation method (Hawkins *et al*., 1997; Elkin *et al*., 2001) used in our high-throughput sequencing production process. Conditions were optimized for amplification of arrayed shotgun libraries in a 384-well-plate format from 2 μL of bacterial glycerol stocks containing randomly cloned fragments in the pUC18 cloning vector. This amplification procedure (detailed in the protocol section above) produces a uniform quantity of DNA for each well of a plate (Figure 5a; see also Detter *et al*., 2002). When run out on an agarose gel, most of the amplified DNA remains in the gel-loading wells due to the high-molecular-weight nature of the highly-branched and concatenated amplification products. A portion of the amplified DNA co-migrates with the 23 kb λ/HindIII fragment regardless of the size of the initial starting material. This

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material is of high enough molecular weight so that it migrates at the limit of resolution of the gel system. When these samples are heated to 95°C and then quickly cooled, the amplified concatemeric DNA forms a complex crosslinked structure that cannot migrate in a non-denaturing gel system and remains in the wells of the agarose gel (data not shown). Using a Fluor-S™ MultiImager with Quantity One 4.2.3 software (BioRad, Hercules, CA), we quantified the relative abundance of the amplified DNA for each 20 μL sample to average 137 ng/μL with a standard deviation of ±20 ng/μL. In contrast, relative abundances in 50 μL DNA samples isolated according to our previous, magnetic bead-based protocol (solid-phase reversible immobilization or SPRI; Hawkins *et al.*, 1997; Elkin *et al.*, 2001) were half as concentrated, averaged 72 ng/μL with a two times higher standard deviation of ±46 ng/μL, as can be seen in Figure 5b.

Application 1: Plasmid Sequencing

The RCA-based generation of templates for plasmid sequencing provides the user with high-quality and mass-uniform DNA samples. Here, sequencing and clean-up steps will be outlined followed by exemplary results obtained with both DYEnamic ET and BigDye v3.1 terminator sequencing chemistries. If necessary, see research protocols at www.jgi.doe.gov for additional information.

Step 1a: DYEnamic ET Terminator Chemistry Sequencing

Each reaction contains 1 μL amplified product, 4 pmoles of primer, 5 μL of dH$_2$O and 4 μL DYEnamic ET terminator sequencing mix for a 10 μL total reaction volume (smaller volumes can also be used with success). The reaction(s) are cycled 30 times: 95°C for 25 s, 50°C for 10 s, 60°C for 2 min with a hold at 4°C.

Step 1b: BigDye Terminator Chemistry Sequencing

Each reaction contains 1 μL amplified product, 4 pmoles of primer, 2 μL of dH$_2$O, 1 μL 5×sequencing buffer and 1 μL BigDye v3.1
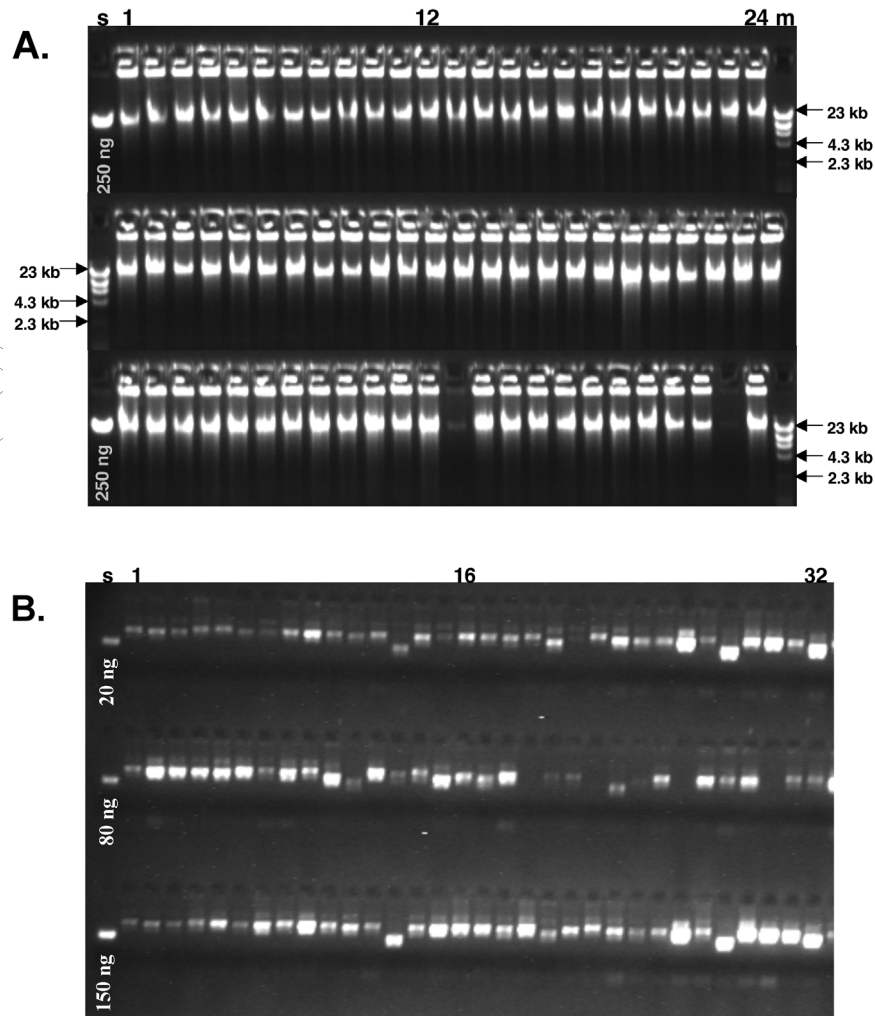
Figure 5. Comparison of the TempliPhi-amplified plasmid subclones and SPRI (solid-phase reversible immobilization)-isolated plasmids.

A: Strand-displacement RCA of plasmids in production. 2 μL aliquots of saturated *E. coli* culture were heat lysed and amplified with TempliPhi for 12 hr at 30°C (see experimental protocol). 3 μL of products from 96 wells of a 384-well plate were run on a 1% agarose gel and stained with ethidium bromide (to conserve space, only 72 wells are shown). Lanes: (s) 250-ng λ mass standard; (1-72) amplified products; (m) λ/HindIII size marker with 23, 4.3, 2.3 kb marked.

B: SPRI isolation of plasmid DNA in production. Plasmid DNA was isolated from 185 μL aliquots of saturated *E. coli* cultures in 96-well format using SPRI protocol (Elkin *et al*., 2001; Hawkins *et al*., 1997). 5 μL of the isolated DNA from 96 wells was run on a 1% agarose gel and stained with ethidium bromide. Lanes: (s) 80- and 150-ng λ mass standard; (1-96) isolated products.

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terminator sequencing mix for a 5 µL total reaction volume (smaller volumes can also be used with success). The reaction(s) are started at 95°C for 1 min, then they are cycled 25 times: 95°C for 30 s, 50°C for 20 s, 60°C for 4 min with a hold at 4°C.

Step 2a: SPRI-Based Clean-Up of Sequencing Reaction

Make stock of washed Seradyn beads as follows. Add 15 mL of thoroughly resuspended Seradyn beads to a 50 mL Falcon tube. Fill tube with dH$_2$O and place it on a magnet for ~15 min or until almost clear. With tube on magnet, pour off water. Perform the wash step thrice. Then add 15 mL dH$_2$O to the Falcon tube. Invert the tube until its content completely mixed (keep well suspended before aliquoting for use).

Prepare fresh BET (bead, ethanol, tetraethyleneglycol) mixture just before use. To process ten 384-well plates make the following. In a 50 mL flask or bottle, mix 32 mL 100% ethanol, 3.5 mL dH$_2$O, and 3.2 mL tetraethyleneglycol. Cover and mix by inverting several times. Then add 1.0 mL of washed beads from above, cover and mix. Additionally, prepare a 70% ethanol wash solution.

To clean up the sequencing reaction(s) do the following: Transfer 5 µL of the sequencing reaction(s) to a clean PCR plate with either 96 or 384 wells. Add 10 µL of the properly mixed BET solution (made as above). Thoroughly mix each well on a plate with a pipette or cover this plate and shake it gently on a vortex. Incubate covered with a lid plate in dark area (closed drawer, *etc.*) for 15 min at room temperature. Place PCR plate on a high-field plane magnet for 1 min. Aspirate out all liquid with a pipette or Hydra device. Remove plate from a magnet and add 15 µL 70% ethanol. Once again, place PCR plate on a magnet for 1 min and aspirate out all liquid. Air-dry samples in a closed drawer or cabinet (dark, ventilated area) for 10 min.

Resuspend purified sequencing reaction with 15 µL dH$_2$O. Seal PCR plate and mix its wells with medium vortexing for ~2 min on vortex plate. Quick spin PCR plate and incubate at room temperature for

10 min. If needed, purified reaction samples can be stored at –20°C for up to two weeks before loading onto sequencer.

Step 2b: Reaction Clean-Up by Ethanol

To each sequenced well add the following. For a 10 μL reaction, add 1/10th volume 7.5 M ammonium acetate (1 μL) and 2.5 volumes 100% ethanol (28 μL). Cut amount added in half for 5 μL sequencing reactions. Seal reaction plate and vortex it at medium setting for 2 min on vortex plate. Centrifuge reaction plate for 30-60 min at $3000 \times g$. If spinning at lower relative centrifugal force, increase time. Dump waste and invert spin on paper towel for 1 min at $\sim 400 \times g$. Repeat process with 70% ethanol to wash pellet if desired. Dump waste and tap lightly on paper towel. Place plate in dark drawer to air dry for ~15 min.

Resuspend purified sequencing reaction with 15 μL dH$_2$O. Seal reaction plate and mix wells with medium vortexing for ~2 min on vortex plate. Quick spin reaction plate and incubate it at room temperature for 10 min. If needed, purified reaction samples can be stored at –20°C for up to two weeks before loading onto sequencer.

Exemplary Results

Using the uniformly amplified plasmid DNA, sequencing conditions were optimized for capillary electrophoresis instruments, including the MegaBACE 1000 and 4000 (Amersham Biosciences, Piscataway, NJ) and the ABI PRISM® 3700 (Applied Biosystems, Foster City, CA) sequencers (Detter *et al*., 2002). We have found that such a high level of uniformity in DNA template concentration we may achieve (see Figure 5a) significantly increases success rates for capillary sequencing. As described in the protocol section above, the protocol we designed utilizes 1 μL of the amplified product and consistently gives the high-quality (Phred Q20 score) sequence reads >600 bases (Ewing *et al*., 1998a,b). Figures 6a and 6b illustrate the sequencing results we commonly observe using either the DYEnamic ET or the BigDye terminator chemistries.

Most DNA isolation processes rely heavily on manpower and reagents, while producing large quantities of waste material and significant lag times between inoculation and sequencing. In contrast, our new RCA-based process has been largely automated, requires fewer reagents and process steps, and produces little to no waste (Detter *et al*., 2002). The major advantages of this technology have been the complete elimination of labor-intensive steps of bacterial lysis and DNA purification as well as streamlining of the production process from 10 steps down to 4, which has lead to cutting the time it requires to go from inoculation to sequencing in half. This process also facilitated moving from a 96-well plate format to 384-well plates, and opens the prospect for even higher density and alternative formats.

Remarkably, the production results from the RCA-based approach have been extremely encouraging. Now that the amplification method has been fully implemented, the average pass rate (% reads/96 with >50 Q20 bases) for this process has been 92% with read-lengths greater than 600 Phred Q20 bases per passed lane. Since being phased into production at the JGI in early 2001, the process has yielded over 25 billion high quality raw bases from a variety of different Prokaryotic and Eukaryotic organisms (including Human, Mouse, Chicken, Xenopus, Fugu, Ciona, Poplar, as well as many microbes). Furthermore, an additional 1-2 billion high quality bases of various genomic sequences are added each month using this process.

Application 2: Plasmid Restriction Digest

Digestion of plasmids has many uses, including insert size verification, subclone library quality control and insert excision/manipulation. Standard plasmid-prep methods are satisfactory for small scale, but in a larger scale they become laborious, time consuming and

Figure 6. Sequencing traces obtained from the RCA-generated DNA templates (3 kb inserts from the TempliPhi amplified pUC18 plasmids). Both DYEnamic™ ET terminator chemistry (A) and BigDye terminator chemistry (B) were used. Sequencing reactions were cleaned up using the BET SPRI method and run on either the MegaBACE 1000/4000 (A) or ABI3730 (B) capillary sequencer.

A

B

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expensive. Since use of amplified plasmids overcomes all these issues, we employed the RCA-based high-fidelity TempliPhi amplification for this purpose. Here, the general steps will be outlined (see plasmid amplification protocol) followed by results.

Plasmid Digestion Protocol

Aliquot 5 μL of amplified plasmid into tube or well of a plate (depending on throughput desired). Add 1 μL of 10× enzyme buffer (use buffer that goes with enzyme of choice). Add ~1 μL of desired enzyme that cuts your plasmid and/or your insert. Bring total volume up to 10 μL with dH$_2$O. Incubate at recommended enzyme reaction temperature (usually 37°C) for ~2-4 hr. Heat kill the enzyme at 65°C (usually) for 20 min. Store between +4 and –20°C.

Volumes of the above reaction are dependant on end use and concentration of enzyme. The above conditions are standard for our purposes. We use high concentrations of enzymes and are digesting the plasmid to check the insert size of 8-10 kb libraries. Reaction volumes can be customarily scaled to fit end user's needs.

Exemplary Results

For our purposes, we became interested in using the RCA-based approach for shotgun library quality control to qualify sizes of our large insert (8-10 kb) plasmid libraries and our high GC-content small insert (3 kb) plasmid libraries that were poorly amplified by PCR due the nature of their inserts. We generally test between 24 and 96 colonies per library in order to determine the average insert size. Standard DNA isolation procedures were not feasible for our quality control digest because of the time and effort they require. We were already using the in-house RCA-based process for our plasmid sequencing line, so we investigated its usefulness for creating template for restriction digests. Typical plasmid preps require an overnight growth period followed by several hours of cell lysis, centrifugation and DNA purification. Our approach utilizes a similar overnight incubation but has no additional

steps. Furthermore, the resulting RCA products are very consistent in concentration, contrary to the plasmid-prep procedure (see Figures 5a and 5b for comparison). This makes the digestions very reliable and easy to qualify. As seen in Figure 7, the vector-related band as well as the insert-related band(s) thus obtained can easily be identified after the gel electrophoresis. As this is typical with restriction digests, rare-cutting enzyme (SwaI in Figure 7a) allows more discriminative size estimates than do frequent cutters (BamHI and HindIII in Figure 7b).
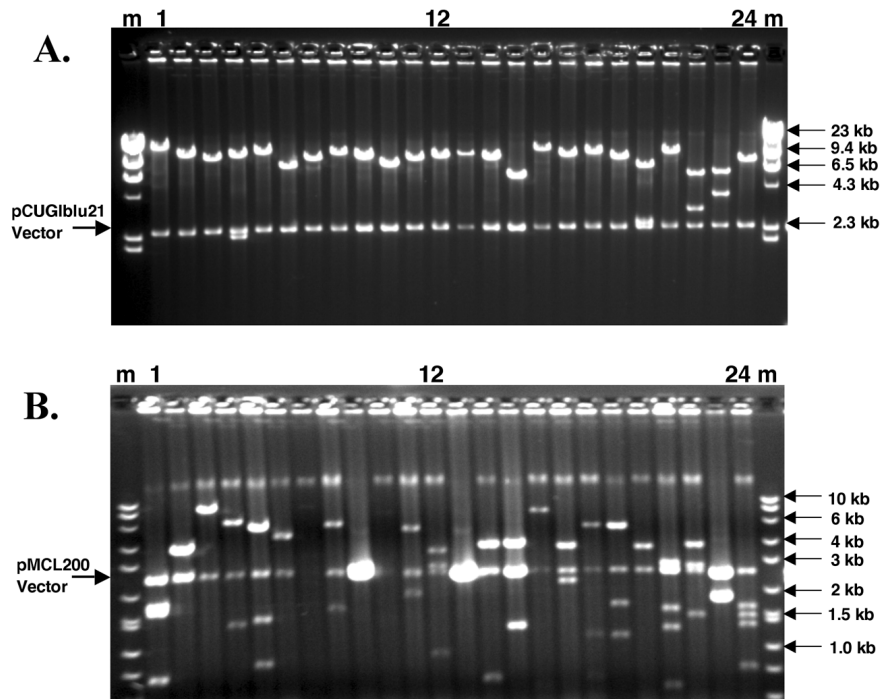

Figure 7. Quality control of shotgun library by restriction digest of RCA products. *E. coli* colonies containing low-copy plasmids with 8-10 kb inserts were amplified with the use of TempliPhi. 5-µL aliquot of amplified products was taken for digestion with either SwaI enzyme (A), which cuts DNA insert out of pCUGIblu21 vector, or BamHI and HindIII enzymes (B), which cut DNA insert out of pMCL200. Both the vector-specific band and the insert-specific bands are clearly seen. The DNA marker (m) identifies the size of inserts.

In addition to digestion of amplified plasmids, the products of this high-fidelity amplification can also be used for cloning purposes, *in vitro* transcription and translation, the products can been spotted on microarray and can be used in both Southern and Northern blot analysis. The only applications, in which these amplification products cannot be used interchangeably with intact plasmid DNA, are those that require supercoiled plasmids (*e.g.* in transformation procedures). However, we have found that decatenation/linearization of amplification products by the restriction enzyme digestion and their subsequent ligation/circularization can be used to make the transformation-proficient DNA from the amplification products.

Summarizing Discussion

The use of random hexamer-primed isothermal strand-displacement amplification with Phi29 DNA polymerase offers many benefits in a variety of molecular biology applications compared to traditional techniques. We have utilized this robust RCA format to optimize conditions for amplification of plasmids from standard glycerol stocks to increase the production efficiency of the sequencing process at the US DOE Joint Genome Institute (JGI) (Detter *et al.*, 2002). Our plasmid amplification method has significant advantages over traditional template generation by greatly simplifying the process and will be helpful for genome centers as well as for core labs, service centers and academic researchers that isolate and sequence DNA from a variety of vectors. This approach has few simple steps, can be highly automated, is completely scalable and is already giving excellent, cost-effective results at our production genomics facility. The collection of plasmid amplification and sequencing protocols we developed based on this approach are available on the web (www.jgi.doe.gov) and are included in the technical literature with the TempliPhi kit.

Our amplification technology was extended for use in the end-sequencing of a variety of other vector constructs. Most high-copy *E. coli* plasmids (pGEM, pET, pSMART, *etc.*) that were attempted produced results similar to those obtained in production with pUC18. Additionally, several low copy plasmids (pCUGIblu21 and pMCL200)

containing inserts up to 10 kb also produce results similar to those obtained in production with pUC18. In fact, these low-copy, medium size-insert constructs now work well enough to have been phased into the standard production amplification sequencing line at the JGI. We have also demonstrated that this process can be used to sequence plasmids cloned into yeast cells (*e.g.* pGADT7 was cloned into yeast cells Y187 for the purpose of a yeast two-hybrid assay; CLONTECH). We have found that a simple lysis procedure similar to that used for amplification of plasmids from *E. coli* also can be used for amplification and sequencing of plasmids from yeast colonies or cultures.

Preliminary experiments with the large-insert vectors, such as cosmids, fosmids and BACs, have been less successful. In our experience, low-copy, large-insert clones do not sequence robustly using this amplification process. Amplified DNA from these clones often contain variable amounts of contaminating *E. coli* DNA and, as a result, yield poor sequencing results (data not shown). Experiments with improved lysis and specific primers are currently underway to reduce the *E. coli* chromosomal DNA amplification and optimize the RCA-based process for these large-insert constructs.

Acknowledgements

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