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Isothermal DNA amplification using the T4 replisome: circular nicking endonuclease-dependent amplification and primase-based whole-genome amplification

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ABSTRACT

In vitro **reconstitution of the bacteriophage T4 replication machinery provides a novel system for fast and processive isothermal DNA amplification. We have characterized this system in two formats: (i) in circular nicking endonuclease-dependent amplification (cNDA), the T4 replisome is supplemented with a nicking endonuclease (Nb.BbvCI) and a reverse primer to generate a well-defined uniform double-stranded linear product and to achieve up to 1100-fold linear amplification of a plasmid in 1 h. (ii) The T4 replisome with its primase (gp61) can also support priming and exponential amplification of genomic DNA in primase-based whole-genome amplification (T4 pWGA). Low amplification biases between 4.8 and 9.8 among eight loci for 0.3–10 ng template DNA suggest that this method is indeed suitable for uniform whole-genome amplification. Finally, the utility of the T4 replisome for isothermal DNA amplification is demonstrated in various applications, including incorporation of functional tags for DNA labeling and immobilization; template generation for *in vitro* transcription/translation and sequencing; and colony screening and DNA quantification.**

INTRODUCTION

In vitro DNA amplification is essential for a wide range of experiments in modern medical and biological research, including applications such as forensic analysis, disease diagnostics, cloning, sequencing and genotyping. The polymerase chain reaction (PCR) is the most commonly used method for DNA amplification (1). Thermal cycling between three temperatures for DNA melting, primer annealing and DNA synthesis requires a thermostable DNA polymerase. The amplified sequence between the two oligonucleotide primers acts as the template in the next cycle, leading to exponential amplification. However, a number of features limit the utility of PCR. For example, whole-genome amplification (WGA) (2–5) by PCR is handicapped by the requirement for thermal cycling instrumentation, size constraints on the amplified DNA and the preferential amplification of some sequences compared to others (amplification bias).

These limitations have spurred the development of a range of isothermal DNA amplification strategies as alternatives to PCR-based methods (6,7). For example, strand displacement amplification (SDA) (8,9) and loop-mediated isothermal amplification (LAMP) (10,11) are widely used for the detection of pathogens. However, their inability to efficiently amplify sequences longer than 200 bp limits the scope of possible applications. Rolling circle amplification (RCA) (12) generates, in analogy to the *in vivo* process, multiple copies of a circular template. The *Bacillus subtilis* bacteriophage Phi29 DNA polymerase is normally used for RCA due to its high strand-displacement activity and processivity. This DNA polymerase is able to synthesize DNA strands longer than 70 kbp (13). However, the observed amplification factor for templates of the size of a plasmid is very low (~20 times per hour), limiting linear RCA to very small circular templates (14). To broaden its scope for amplification of whole plasmids, multiple random hexameric primers have been used (multiply primed RCA) (14). Up to 10 000-fold amplification can be achieved and the hyperbranched product is on

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average >40 kbp (14). Multiply primed RCA is not strictly isothermal as it requires an initial heat denaturation step to facilitate primer annealing.

Based on the same principle—random primers that are extended by the Phi29 DNA polymerase—linear genomes can also be amplified. The method adapted for WGA was termed multiple displacement amplification (MDA) (2) and was the first isothermal DNA amplification method used for WGA. In contrast to PCR-based WGA methods using degenerate oligonucleotide primers (DOP-PCR) (3,4) or 15-mer random primers (primer extension preamplification, PEP) (5), MDA has the advantages of low amplification bias and increased product length (2). An additional method, GenomePlex (15,16), has an amplification bias comparable to MDA (17), but employs a relatively complicated workflow involving cleavage of genomic DNA into fragments with an average size of 400 bp, ligation to adaptor sequences and PCR amplification. In addition, the short products are unsuitable for applications such as restriction fragment length polymorphism analysis and subcloning.

To create a truly isothermal method, the initial DNA melting step can be obviated by including a helicase for separating the two complementary DNA strands. The helicase also eliminates the need for the strand displacement activity of the DNA polymerase. Several helicase-dependent amplification (HDA) systems (18–20) have been reported and fragments of up to 1500 bp can be amplified (20). The bacteriophage T7 replication machinery (consisting of the T7 helicase, T7 DNA polymerase plus the *Escherichia coli* thioredoxin and T7 single-stranded DNA binding protein) makes it possible to amplify fragments in the kilobase pairs range from circular templates (cHDA). Here, a fragment defined by two specific primers is amplified as well as high-molecular-weight concatemers of the circular template DNA by a rolling circle mechanism (21). By also including the T7 primase function (which is present in the same protein as the helicase activity), the replisome can synthesize its own RNA primers that then do not have to be supplemented. This method is not only used for amplification of plasmids, but also of genomic DNA. Primase-based whole-genome amplification (pWGA) is characterized by amplification factors and amplification bias comparable to MDA (22).

In this study, we describe the use of the T4 replisome as an alternative system for isothermal DNA amplification. The complete T4 replication machinery consists of eight proteins (23) (Figure 1). Gp43 is one of the fastest polymerases known with a rate of ~250 nt/s when complexed with its sliding clamp, gp45 (24,25). Its exonuclease-deficient variant gp43[D219A] has similar activity (26). The clamp loader proteins (gp44/62) are required for loading gp45 onto the replication fork in an adenosine triphosphate (ATP)-dependent process. The helicase (gp41), assembled by the helicase loader (gp59), unwinds DNA at a rate of ~400 nt/s (27) using the energy from ATP hydrolysis. Gp32 interacts with the exposed ssDNA and is involved in multiple protein–protein interactions in the T4 replisome. The T4 replisome reconstituted with these seven proteins is highly processive and can thus synthesize leading-strand DNA larger than 20 kbp (25). The primase (gp61) that forms the primosome together with the helicase recognizes the 5′-GTT-3′ and 5′-GCT-3′ priming recognition sites and synthesizes 5 nt RNA primers (28) for initiation of lagging-strand DNA synthesis.

We characterize this system for isothermally amplifying plasmids as well as whole genomes. We also describe a new mechanism for isothermally amplifying whole plasmids avoiding the generation of high-molecular-weight concatemers in a circular nicking endonuclease-dependent amplification (cNDA). The high synthesis rate of the T4 replisome allows a whole plasmid (4.7 kbp) to be amplified up to 1100-fold in 1 h, although the amplification is linear and not exponential. In pWGA human genomic DNA was amplified up to 210-fold in 1 h using the T4 replisome (T4 pWGA), exhibiting low amplification biases (i.e. the ratio of the highest to the lowest amplification factor) between 4.8 and 9.8 among the eight loci tested for three different amounts of input DNA (0.3, 1 and 10 ng).

MATERIALS AND METHODS

Protein expression and purification

The T4 replisome proteins gp43[D219A] (26), gp59 (29), gp44/62 (30), gp45 (31), gp41 (31) and gp61 (28) were expressed and purified as previously described. Typical protein yields (in mg purified protein/l culture) were 50 mg/l for gp45; 13 mg/l for gp44/62; 5 mg/l for gp43, gp59 and gp61; and 1 mg/l for gp41. Gp32 and the nicking endonuclease Nb.BbvCI were purchased from New England BioLabs (NEB).

Plasmids for cNDA

The following circular plasmids were used as templates for cNDA:

(i) pIVEX-GFP (4429 bp) was constructed from pIVEX2.6d (Roche Applied Science) and contains the gene coding for GFP as described in Courtois *et al*. (32). A recognition site for the nicking endonuclease Nb.BbvCI was inserted into the NheI site: Two complementary 5′-phosphorylated oligonucleotides (5′-PO_3-CTAGCATTCTGTACCTAATGAG CTGAGGAGAACTTGAGACG-3′ and 5′-PO_3-CT AGCGTCTCAAGTTCTCCTCAGCTCATTAGG TACAGAATG-3′, Invitrogen) were annealed and ligated into the plasmid pIVEX-GFP previously digested with NheI and dephosphorylated with calf intestinal alkaline phosphatase (CIP). The selected clone was sequenced and contained the nicking site once in the sense strand.

(ii) pIVEX-RFP (4289 bp) was constructed from pIVEX2.6d and contains the gene coding for the monomeric red fluorescent protein mRFP1 (33,34) between the restrictions sites NdeI and SalI. A recognition site for the nicking endonuclease Nb.BbvCI was inserted into the NheI site as described for pIVEX-GFP. The selected clone was sequenced and contained the nicking site twice in the anti-sense strand.

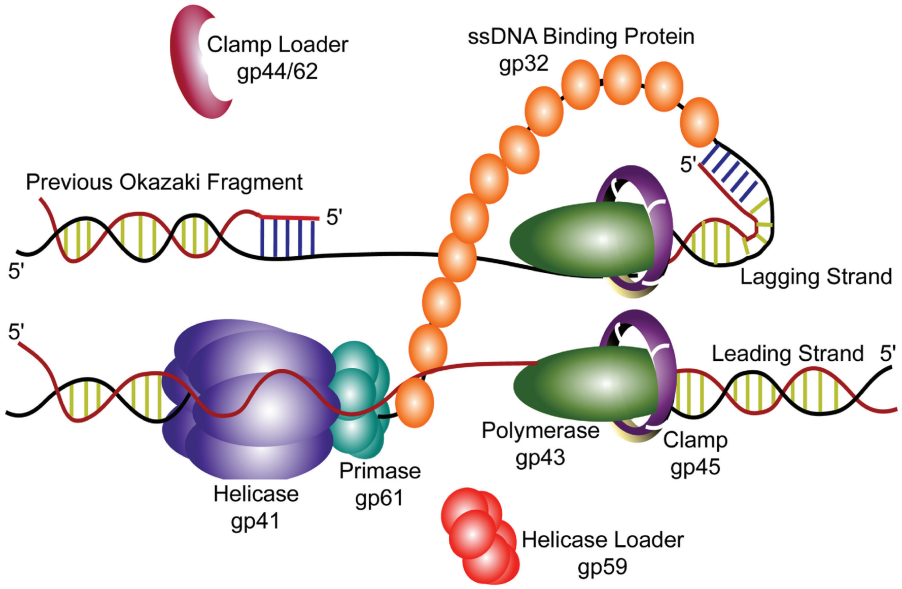

Figure 1. The bacteriophage T4 replisome. The bacteriophage T4 replisome is composed of eight proteins. The hexameric helicase (gp41) and oligomeric primase (gp61) constitute the primosome. Gp41 requires the helicase loader (gp59) for effective loading on the replication fork. Double-stranded DNA is unwound by the helicase. The single-stranded DNA thus generated is prevented from reannealing by binding of gp32. Gp61 synthesizes pentaribonucleotide primers (blue) that are then extended by the DNA polymerase (gp43). The polymerase, the clamp gp45 (trimer) and clamp loader proteins (gp44/62) form the holoenzyme. Gp45 increases the processivity of the polymerase and is loaded by gp44/62.

(iii) pIVEX-AGT-GST (4731 bp) was constructed as described in Stein *et al.* (35). A recognition site for the nicking endonuclease Nb.BbvCI was inserted into the EcoRI site: Two complementary 5′-phosphorylated oligonucleotides (5′-PO_3-AATTC TGTACCTAATGACCTCAGCATACGAACTTG ATGCAGACG-3′ and 5′-PO_3-AATTCGTCTGCA TCAAGTTCGTATGCTGAGGTCATTAGGTAC AG-3′, Invitrogen) were annealed and ligated into the plasmid pIVEX-AGT-GST previously digested with EcoRI and dephosphorylated with calf intestinal alkaline phosphatase (CIP). The selected clone was sequenced and contained the nicking site once in the sense strand.

(iv) pIVEX-PTE (4597 bp) contains the phosphotriesterase (PTE) gene from *Pseudomonas diminuta* between the restriction sites BglII and BamHI in the pIVEX backbone (36). A recognition site for the nicking endonuclease Nb.BbvCI was inserted into the EcoRI site: two complementary 5′-phosphorylated oligonucleotides (5′-PO_3-AATTC TGTACCTAATGAGCTGAGGATACGAACTTG ATGCAGACG-3′ and 5′-PO_3-AATTCGTCTGCA TCAAGTTCGTATCCTCAGCTCATTAGGTAC AG-3′, Invitrogen) were annealed and ligated into the plasmid pIVEX-PTE previously digested with EcoRI and dephosphorylated with calf intestinal alkaline phosphatase (CIP). The selected clone was sequenced and contained the nicking site once in the anti-sense strand.

cNDA reaction

Each reaction contained the following proteins: 2 µM gp32 (NEB), 200 nM gp43, 200 nM gp45 (trimer), 200 nM gp44/62 (4:1 complex), 100 nM gp59, 900 nM gp41 and 0.2 U/µl Nb.BbvCI. The proteins were normally added individually, but if desired, all the proteins can be combined first into a master mix and then added at once to reduce the number of pipetting steps. The buffer contained 1 mM each dNTP, 2 mM ATP, 20 mM Tris-acetate (pH 7.5), 150 mM potassium acetate, 10 mM magnesium acetate and 0.4 µM reverse primer (Invitrogen) (Table 1). Unless otherwise stated, primer b1 (Table 1) was utilized with 10 ng of covalently closed circular plasmid as the template in a reaction volume of 20 µl. Reactions were incubated for 1 h at 37°C. Aliquots of 15 µl were resolved by a 1% agarose gel and stained with SYBR safe (Invitrogen; marker: Hyperladder I, Bioline).

Table 1. Sequences of reverse primers used for cNDA reactions

Name	Sequence (5′-3′)
b1[a]	GCTCATTAGG
b2[b]	GAGCACTGTC
b3[a]	GCTCATTAGGTACAGAAT
b4[a]	GCATACGAACTTGATGCAGA
Nb1[c]	CATGCACATC
Nb2[c]	TCTAGGGCGG
biotin-b1[a]	Biotin-GCTCATTAGG

[a]Complementary to the inserted cassette for the nicking endonuclease.
[b]Complementary to a constant region in pIVEX.
[c]Without a complementary sequence in the templates used.

Real-time monitoring of cNDA

cNDA reactions were prepared as described above and contained a 1× concentration of EvaGreen [Jena Bioscience; ~1.33 µM (37)]. The real-time cNDA reactions contained 0, 0.25, 1 or 4 ng of pIVEX-GFP. They were

carried out in a Rotor-Gene 6000 qPCR (Corbett) machine at 37°C for 6 h recording green fluorescence ($\lambda_{excitation}$: 470 nm, $\lambda_{emission}$: 510 nm) every 3 min.

Determination of the amplification factor in cNDA

cNDA reactions with primer b4 were prepared as described above and included 8 ng, 0.8 ng, 80 pg or 8 pg of pIVEX-AGT-GST. Aliquots of 15 µl were resolved by a 1% agarose gel and stained with ethidium bromide. Aliquots were diluted 100-fold and analysed by quantitative PCR (qPCR) in a Rotor-Gene 6000 instrument (Corbett) using SYBR Green I as the detection dye. DNA amounts were determined relative to a standard ladder of pIVEX-AGT-GST. The experiment also included controls with no template. A SensiMix SYBR No-ROX Kit (Quantace) was used according to the manufacturer's instructions. The total reaction volume was 25 µl and included 2 µl diluted samples. The primers (5′-TCCA AAAGAGCGTGCAGAGAT-3′) and (5′-TGCAATTCT CGAAACACCGTAT-3′) were used at a concentration of 0.8 µM. The thermal cycling conditions were 95°C for 10 min followed by 40 cycles of 10 s at 95°C, 30 s at 60°C and 10 s at 72°C. At the end of the qPCR, a melting curve analysis (60–95°C) was performed. qPCR measurements were performed in duplicate for each sample. Data were analysed with Rotor-Gene 6000 software 1.7 in the dynamic tube normalization mode.

cNDA from single colonies

Electrocompentent *E. coli* DH5α cells (Invitrogen) were transformed with the plasmid pIVEX-RFP and plated on agar plates containing 100 µg/ml ampicillin. A colony of ~60 000 cells (determined by serial dilution and plating) was picked and lysed at 95°C for 3 min in the presence of all cNDA components except the proteins. After chilling on ice, the proteins were added to the reaction to a final volume of 20 µl. Then the cNDA reaction was carried out as described above.

cNDA prior to *in vitro* transcription and translation

cNDA reactions with primer b3 were prepared as described above and included 0, 0.066 or 0.66 ng of pIVEX-PTE. Amplification was allowed to proceed for 1 h at 37°C and then stopped by incubation at 80°C for 20 min. Aliquots of 2 µl were then directly added to 8 µl of *in vitro* transcription and translation (IVTT) mixture (RTS 100, *E. coli* HY kit, Roche Applied Science), prepared according to the manufacturer's instructions. IVTT reactions were incubated at 30°C for 3 h. The assay for PTE activity was performed as described previously (38). Briefly, 30 µl of incubation buffer (50 mM Tris-HCl, 10 mM K_2CO_3, 25 µM $ZnCl_2$, pH 8.5) were added to each of the 10 µl IVTT samples followed by overnight incubation at 16°C. Thereafter, 2 µl of these samples were assayed in triplicate with 0.25 mM paraoxon in 50 mM Tris-HCl (pH 8.5) in a total volume of 200 µl. The release of the 4-nitrophenolate product was monitored at 405 nm in a Spectramax M5 plate reader (Molecular Devices) at 25°C. For the controls without amplification, the same amount of DNA (0, 0.066 or 0.66 ng) was added to 1× T4 buffer (1 mM each dNTP, 2 mM ATP, 20 mM Tris-acetate, 150 mM potassium acetate, 10 mM magnesium acetate, pH 7.5). All the other steps were carried out identically as described above. Linear parts of the time courses (<20% of the total reaction) were fitted to compare initial rates.

Labeling and immobilization of cNDA products

cNDA reactions (40 µl) were prepared as described above and included either reverse primer b1 or biotin-b1. pIVEX-PTE was used as the template at a concentration of 20 ng/40 µl reaction (165 pM) or 2 ng/40 µl reaction (16.5 pM). Amplification was allowed to proceed for 1 h at 37°C and then stopped by incubation at 80°C for 20 min. Aliquots of 15 µl were resolved by a 1% agarose gel and stained with SYBR safe (Invitrogen). Aliquots of 20 µl were immobilized on 5 µl streptavidin-coated magnetic beads (10 mg/ml, Dynabeads M-270 Streptavidin, Invitrogen) using the Dynal kilobaseBINDER Kit (Invitrogen) according to the manufacturer's instructions. After immobilization, beads were resuspended in 10 µl Milli-Q water of which 5 µl were then added to 20 µl of IVTT mixture (RTS 100, *E. coli* HY kit, Roche Applied Science), prepared according to the manufacturer's instructions. IVTT reactions were incubated at 30°C for 3 h. The IVTT sample was separated from the magnetic beads with the aid of a magnet; 30 µl of incubation buffer (50 mM Tris-HCl, 10 mM K_2CO_3, 25 µM $ZnCl_2$, pH 8.5) were added to 10 µl aspirated sample; and after 2 h at room temperature the assay was performed as described in the previous paragraph.

T4 pWGA

Each reaction contained the following proteins: 2 µM gp32 (NEB), 800 nM gp43, 800 nM gp45 (trimer), 800 nM gp44/62 (4:1 complex), 100 nM gp59, 900 nM gp41 and 900 nM gp61. The buffer contained 1 mM each dNTP, 100 µM each rNTP, 2 mM ATP, 20 mM Tris-acetate (pH 7.5), 150 mM potassium acetate and 10 mM magnesium acetate. Unless otherwise stated, 10 ng of human genomic DNA (Promega) was used as the template in a total reaction volume of 20 µl. Reactions were incubated at 37°C for 1 h and stopped by incubation at 80°C for 20 min. Aliquots of 15 µl were resolved by a 1% agarose gel and stained with SYBR safe (marker: Hyperladder I, Bioline).

Sequencing of amplification products

pIVEX-GFP, pF3A WG (BYDV) Flexi Vector (Promega) and pDONR221 (Invitrogen) were amplified as described in the previous section, with the exception that 4 ng of plasmid were used in a 100 µl reaction volume. The products were purified with the QIAEX II kit (Qiagen) and eluted with 50 µl of 1 mM Tris-HCl (pH 8.5). Each amplification product was sequenced with two different primers (Supplementary Table S1). Sequencing (see SI) was performed by the DNA sequencing facility of the Biochemistry Department at the University of Cambridge with an Applied Biosystems 3730xl DNA Analyzer. The longest stretch of sequence that matched

the reference sequence and did not contain any unassigned bases (N) was classified as 'accurately sequenced', even if a longer stretch could be identified by inspection of the chromatogram to identify bases originally labeled as N by the automatic sequencer. This value provides a conservative estimate of the accurately sequenced length.

Determining amplification factor and bias

The amplification factors and biases of T4 pWGA were compared with WGA by illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) and Rapisome pWGA Kit (BioHelix Corporation). Eight SNP loci from different chromosomes were chosen to survey the genome coverage. The sequences of the primers were taken from Hosono *et al*. (39) and are given in Table 2.

The T4 replisome was used to amplify 0.3, 1 or 10 ng of purified human genomic DNA (obtained from Promega, containing DNA of which 90% is longer than 50 kb) in triplicate 25 µl reactions as described above in 'T4 pWGA'. These amounts of DNA correspond to 90, 300 and 3000 genome copies, respectively. Rapisome pWGA reactions were performed according to the manufacturer's instructions. Briefly, 0.3, 1 or 10 ng of purified human genomic DNA (in 5 µl Milli-Q water) were mixed with 20 µl Rapisome master mix and incubated for 1 h at 37°C. The reactions were stopped by incubation for 20 min at 65°C. Illustra GenomiPhi V2 reactions were performed according to the manufacturer's instructions. Briefly, 0.3, 1 or 10 ng of purified human genomic DNA (in 1.25 µl Milli-Q water) were mixed with 11.25 µl sample buffer and heated to 95°C for 3 min. After cooling to 4°C 12.5 µl master mix consisting of 11.25 µl reaction buffer and 1.25 µl enzyme mix were added and incubated for 90 min at 30°C. The reactions were stopped by incubation for 10 min at 65°C. Reactions were stored at −20°C until analysed.

The amplification factors were determined by qPCR on a Roche Light Cycler 480 instrument using SYBR Green I as the detection dye. DNA amounts were determined relative to a standard ladder of human genomic DNA (Promega) for each primer pair. The qPCRs also included controls with no template. A SensiMix SYBR No-ROX Kit (Quantace) was used according to the manufacturer's instructions. The total reaction volume was 25 µl and included samples at final dilutions of 125- to 313-fold. Primers (Table 2) were used at a concentration of 0.5 µM. The thermal cycling conditions were 95°C for 10 min followed by 45 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C. At the end of each reaction, a melting curve analysis (60–95°C) was performed. qPCR measurements were performed in triplicate for each sample. Data were analysed with the LightCycler 480 Software, version 1.5.0.39. Absolute quantification analysis was performed with the second derivative maximum method (high-confidence algorithm).

Amplification factors and standard deviations (SDs) were calculated from triplicates of the WGA reactions for each locus (Supplementary Table S2). SDs from the qPCR triplicates of each sample were insignificant compared to the SDs from the WGA reactions. The averaged amplification factors in Table 3 represent the mean of the amplification factors over the eight loci analysed and the SDs represent the pooled standard deviation of the SDs in individual loci. The amplification bias was calculated as the ratio of the highest to the lowest amplification factor among the eight loci.

Quantitative T4 pWGA

T4 pWGA reactions were prepared in duplicate as described above and contained a 1× concentration of EvaGreen [Jena Bioscience; ~1.33 µM (37)] and 0, 1 pg, 10 pg, 100 pg or 1 ng of human genomic DNA. They were carried out in a Rotor-Gene 6000 qPCR instrument (Corbett) at 37°C for 120 min recording green fluorescence ($\lambda_{excitation}$: 470 nm, $\lambda_{emission}$: 510 nm) every 2 min. Analysis was carried out using the Rotor-Gene 6000 software 1.7 in the dynamic tube normalization mode.

RESULTS

Circular nicking endonuclease-dependent amplification: cNDA

We present an approach for isothermal DNA amplification of whole plasmids termed cNDA (Figure 2A). cNDA yields a single well-defined product, in contrast to other isothermal DNA amplification methods capable of amplifying whole plasmids (i.e. multiply primed RCA and cHDA). We utilized the T4 replisome for cNDA, because its high processivity and DNA synthesis rate result in substantial amplification, despite a linear (rather than exponential) amplification scheme.

Table 2. Primers used for amplification factor and locus bias studies

Chr	WIAF[a]	forward primer (5′-3′)	reverse primer (5′-3′)
1p	984	TTTGATGGAGAAATCCGAGG	TCCCTAAATGACAAAAAGAGAAGG
2p	1004	GTCTTTAGCTGCTGAGGAAATG	AGCAGAATTCTGCACATGACG
3q	979	AGAACCTGACCAGATGTGGC	CAAACGTCAAGCTCAGTTTCC
5p	1331	GGGTAAGATCCAGAGCCACA	CCTCATTCCTTCTCGAAGCA
10q	723	CTTCCTGACCTGTTTGCAGT	CTTCAGTGCACAGAATGCAG
15q	244	GAGTTTAATGAATCCTGTTCCCC	CTGTAATATAACCGTTTCATTCAGC
17q	699	TGCTCCCTGTCCCATCTG	AGACAGTATGCCTTTATTTCACCC
21p	683	TGAGGCTATGATTGCAGATTT	TCCCTAAATGACAAAAAGAGAAGG

[a]Refers to the Whitehead Institute-Affymetrix SNP database numbering of loci in the human genome.

For cNDA, the T4 replisome (without primase) is complemented with a nicking endonuclease (e.g. Nb.BbvCI) and a reverse primer. The nicking endonuclease recognizes a specific sequence on the template and introduces a nick into the double-stranded DNA. At this nick, the T4 replisome can initiate DNA amplification using the 3′-OH group exposed at the nick as a primer. In a rolling circle fashion, the replisome replaces the nicked strand with a newly synthesized one, while displacing the old strand. The nicking endonuclease remaining in the reaction mixture also nicks the newly synthesized strand. If the nicking occurs before the replisome completes one cycle around the template, the displaced strand is released from the template. This released single-stranded copy of the template is made double stranded by extending a reverse primer. The result is a linear copy of the circular plasmid (Figure 2B). A product of twice the size of the template makes up <10% of the total amplified DNA. This observation is consistent with a fraction of DNA not being nicked when the T4 replisome arrives at the nicking endonuclease recognition site.

Product identity, reaction components, specificity and template preference of cNDA

To show that the cNDA product is indeed composed of linear copies of the circular template, the template (pIVEX-RFP) as well as the cNDA product were individually and simultaneously digested by two restriction enzymes, BbvCI and NdeI. The restriction endonuclease BbvCI has the same recognition and cutting site as the nicking endonuclease Nb.BbvCI. Therefore, no effect is expected when the cNDA product is treated with BbvCI (Figure 3B), whereas the template plasmid can be linearized by BbvCI as well as by NdeI (Figure 3A). If the cNDA product is digested with NdeI, two fragments of 1 and 3.3 kbp are expected. The same fragments are expected if the template plasmid is digested with BbvCI and NdeI. Gel electrophoresis of these digests reveals fragments of the expected sizes (Figure 3C). This observation is consistent with the reaction mechanism for cNDA that is proposed in Figure 2A.

Protein components required for cNDA were determined by omitting one protein at a time from the reaction mixture. All proteins included in the reaction mixture were necessary for efficient amplification by the T4 replisome (Supplementary Figure S1). No product could be detected on an agarose gel when any one of these proteins was absent (suggesting at least a 20-fold decrease in reaction efficiency), except for gp59 (the omission of which resulted in a smaller, ~10-fold decrease). These observations are consistent with previous *in vitro* studies of the T4 replisome on small circular substrates (40). The failure to amplify DNA in the absence of the nicking endonuclease (Supplementary Figure S1) suggests that the nick is essential for initiating the DNA synthesis and that the low percentage of naturally occurring nicks is not sufficient to support efficient cNDA.

In cNDA, specificity is provided by the reverse primer and by the nicking endonuclease (Supplementary Figure S2). Different forms of circular DNA were tested as templates for cNDA (Supplementary Figure S3). No product was formed in the absence of template DNA. Covalently closed circular plasmid, nicked circular plasmid and heat-denatured plasmid were all amplified with comparable efficiency.

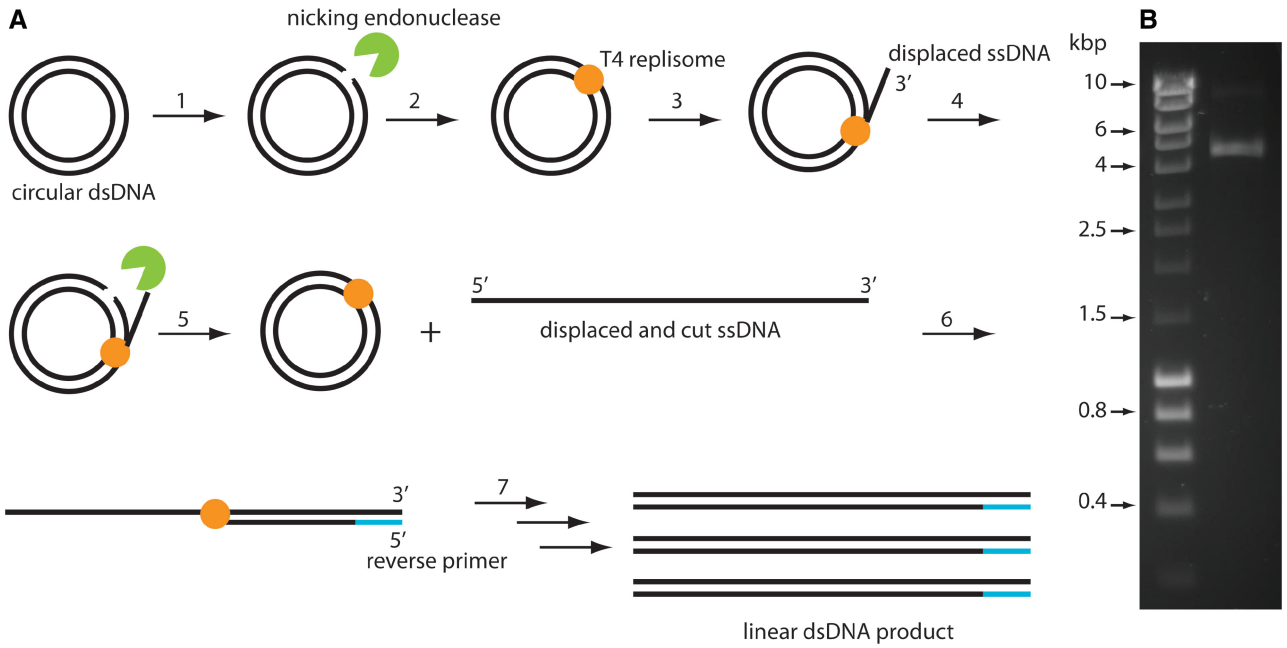

Figure 2. Mechanism of cNDA. (**A**) One strand of the circular template is cut by the nicking endonuclase (1). The nick serves to initiate amplification by the T4 replisome (2). A new DNA strand is synthesized and single-stranded DNA is displaced (3). The nicking endonuclease also nicks the newly synthesized strand (4). The displaced strand is released from the template as it is cut at the nicking site (5). The ssDNA is primed by the reverse primer and the T4 replisome makes it double stranded (6). Linear copies of the circular template DNA result (7). (**B**) One single product of cNDA after amplification of the plasmid pIVEX-RFP.

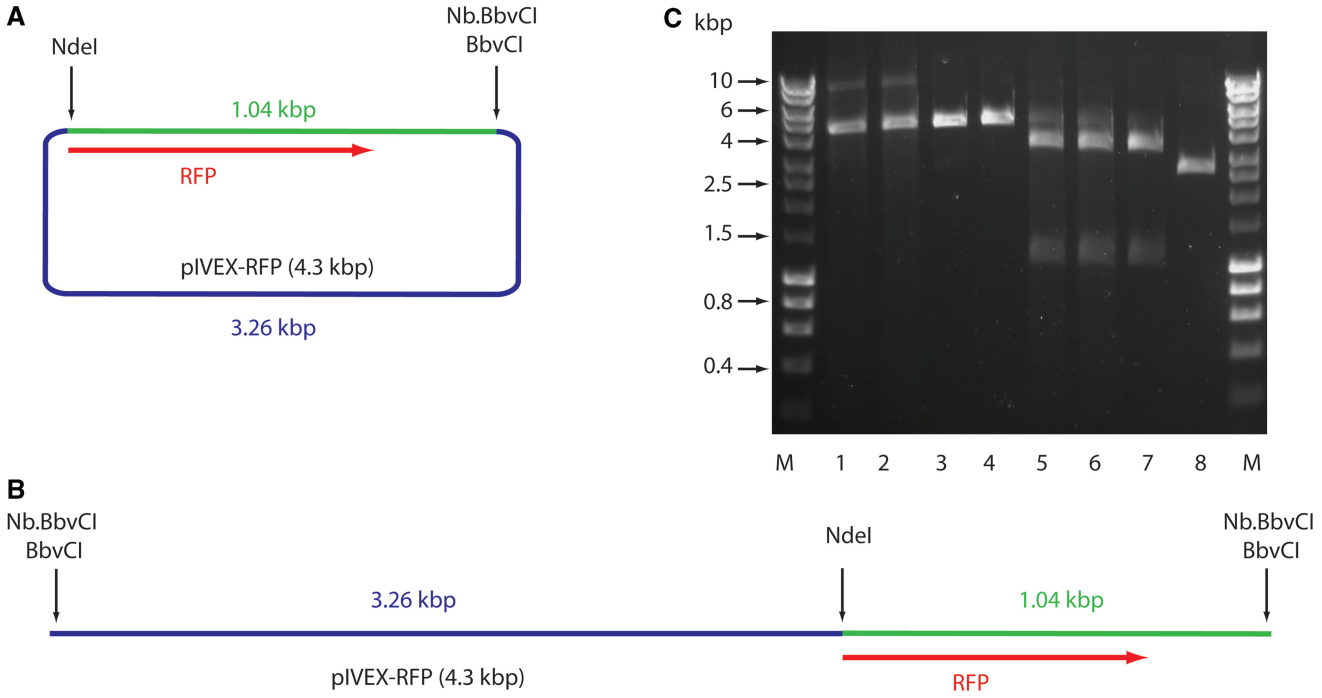

Figure 3. Restriction digests of cNDA product. (**A**) The plasmid pIVEX-RFP (4.3 kbp) was used as the template for amplification. The recognition site for the nicking endonuclease Nb.BbvCI used in cNDA is cut by the restriction enzyme BbvCI. If cut additionally with NdeI, two fragments with the lengths of 1.04 kbp and 3.26 kbp are generated. (**B**) Expected product of cNDA. (**C**) The product of cNDA (lane 1) was cut with BbvCI (lane 2), NdeI (lane 5) or BbvCI and NdeI (lane 6). The plasmid pIVEX-RFP (lane 8) was also digested with BbvCI (lane 3), NdeI (lane 4) or BbvCI and NdeI (lane 7).

Real-time monitoring of cNDA and amplification factors

The progress of cNDA reactions was continuously monitored for 6 h. For this purpose, the dsDNA intercalating dye EvaGreen (37) was added to cNDA reactions and the reactions were carried out in a quantitative PCR (qPCR) machine. Four cNDA reactions containing covalently closed circular template DNA were monitored in real time (Figure 4A). The progress curves were initially linear with the slopes depending on the DNA substrate concentration followed by flattening to a plateau as one of the reaction components became depleted. The lag preceding the linear phase can be ascribed to the time required to perform the following processes before the fluorescent signal crosses the detection threshold: loading of the polymerase and clamp at the nick, slow strand-displacement synthesis to create a ssDNA tail for the helicase to load after which rapid synthesis can take place, priming of the displaced ssDNA and loading of another polymerase and clamp to convert the ssDNA to dsDNA to which the EvaGreen binds resulting in a fluorescent signal.

Amplification factors were determined by qPCR for different amounts of template DNA (8 pg to 8 ng) after 1 h of cNDA. The amplification products were also analysed on a gel (Figure 4B). Amplification factors between 170 and 1100 were detected: The lower the starting DNA concentrations, the higher the amplification factors were. The amplification product from 8 pg template could not be detected on an agarose gel (Figure 4B), but a 1100-fold amplification was determined by qPCR.

Amplification from single colonies

The ability to amplify plasmids by cNDA directly from single colonies of bacteria was tested. Individual colonies of *E. coli* cells carrying the plasmid pIVEX-RFP were lysed in cNDA reaction buffer for 3 min at 95°C. After addition of the proteins, cNDA was performed. All three colonies picked were amplified with similar efficiency, yielding a typical cNDA band (Figure 5).

cNDA prior to *in vitro* transcription and translation

The ability of cNDA products to serve as templates for *in vitro* transcription and translation (IVTT) and to increase IVTT yields was assessed. We performed cNDA of a plasmid (pIVEX-PTE) coding for a phosphotriesterase (PTE) (36), expressed PTE following the addition of an IVTT extract derived from *E. coli* and assayed for degradation of the organophosphate pesticide paraoxon by PTE (Figure 6). Three different amounts of template DNA (0, 0.066 ng and 0.66 ng) were tested. For comparison, the procedure was also performed without prior DNA amplification. Although no increase in the background organophosphate hydrolysis rate was observed for the no template controls (data not shown), significant increases in enzymatic rates of 14- and 67-fold for the cNDA-amplified samples (for 0.066 ng and 0.66 ng template, respectively) were observed compared to the controls without prior cNDA.

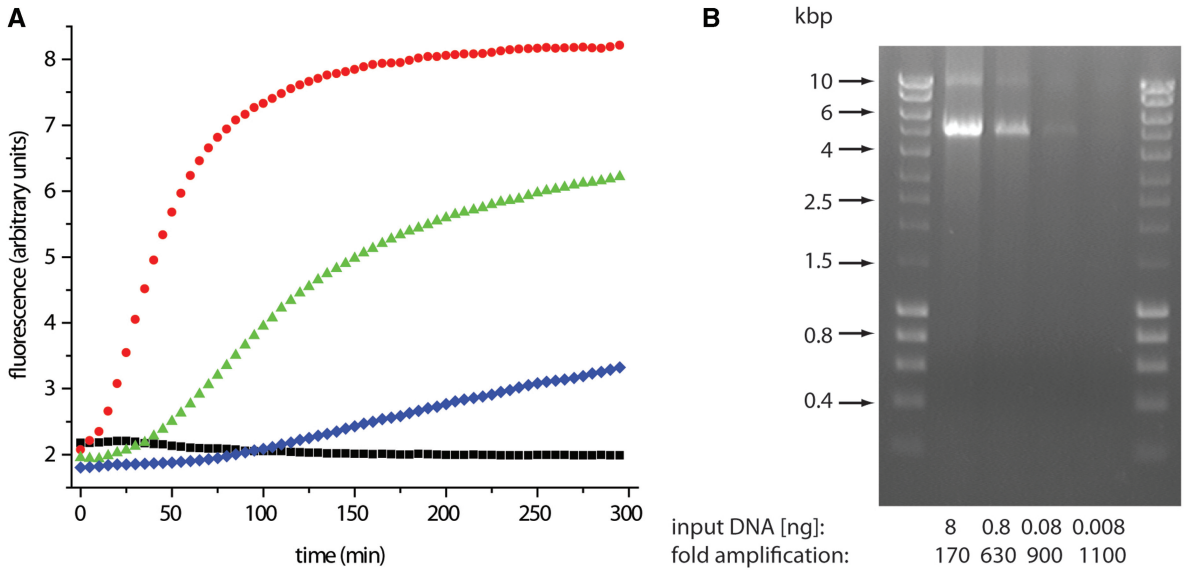

Figure 4. Real-time monitoring and amplification factors of cNDA. (**A**) Twenty microlitres of cNDA reactions containing 4 ng (red circles), 1 ng (green triangles), 0.25 ng (blue diamonds) and 0 ng (black squares) of template plasmid (pIVEX-GFP) were supplemented with the dsDNA intercalating EvaGreen dye. The reactions were carried out in a qPCR machine (Rotor-Gene 6000, Corbett) at 37°C and the fluorescence was recorded every 3 min for 6 h. (**B**) Twenty microlitres of cNDA reactions containing 8 ng (lane 1), 0.8 ng (lane 2), 80 pg (lane 3) and 8 pg (lane 4) of template plasmid (pIVEX-AGT-GST, 4.7 kbp) were incubated for 1 h. The products were run on an agarose gel (1%) and the amplification factors [shown at the bottom of (B)] were determined by qPCR.

Labeling and immobilization of cNDA products

One advantage of the cNDA reaction scheme is that labels such as fluorescent dyes or biotin can be introduced into the product at a defined position with a labeled reverse primer. To demonstrate this principle, a reverse primer carrying a biotin label was used to immobilize the cNDA products on streptavidin-coated beads. cNDA reactions with a template coding for a PTE (10 ng or 1 ng pIVEX-PTE) were performed. The cNDA yield was not affected by the presence of a label on the primer (Figure 7A). The labeled products were then immobilized on streptavidin-coated magnetic beads and added to an IVTT reaction. After protein expression, a PTE activity assay was performed. PTE activity was detected only when a biotinylated primer was used (Figure 7B). These observations suggest that cNDA products were successfully biotinylated, immobilized on streptavidin-coated beads and from there transcribed and translated without significant binding of non-biotinylated DNA to the beads. Alternatively, labeling throughout the whole amplicon is also possible by incorporating modified nucleotides. Supplementary Figure S4 shows a fluorescent cNDA product of a reaction including fluorescein-dCTP (equalling 5% of total dCTP).

Primase-based WGA with the T4 replisome

The T4 replisome is able to amplify DNA by random priming in a similar way to multiply primed RCA and MDA with Phi29 DNA polymerase (Supplementary Figure S5). In addition, the ability of the T4 replisome to amplify linear genomic DNA employing its primase gp61 (T4 pWGA) was assessed. In contrast to cNDA, the nicking endonuclease and primers were omitted and

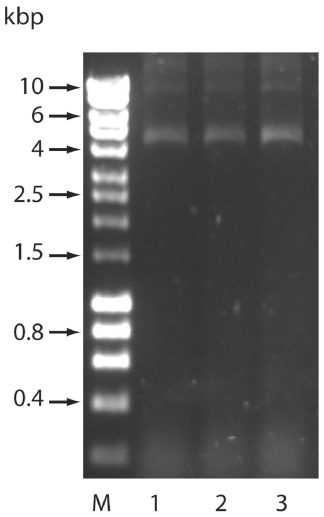

Figure 5. Amplification from crude *E. coli* lysates. cNDA was carried out on three colonies (each containing ~60 000 cells) transformed with pIVEX-RFP. No band was detected from crude lysates without amplification (data not shown).

gp61 and all four rNTPs were added instead. Amplification occurred when the concentration of the holoenzyme (gp43, gp45 and gp44/62) was increased to 800 nM to ensure efficient primer transfer from the primosome to the holoenzyme (41). The products of T4 pWGA of human genomic DNA were run on a 1% agarose gel that indicates amplification products of different lengths (Figure 8).

Previous mechanistic studies of the T4 replisome showed that the length of the Okazaki fragments

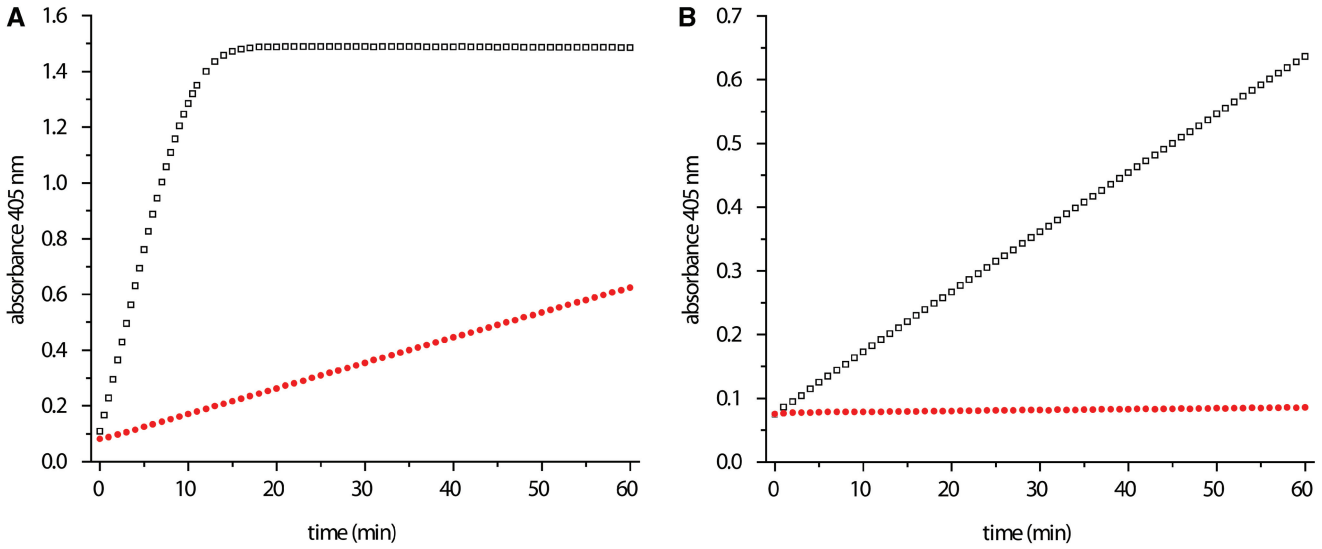

Figure 6. cNDA followed by IVTT. The plasmid pIVEX-PTE [0.66 ng in (**A**) or 0.066 ng in (**B**)] was amplified by cNDA. Then, an IVTT mixture was added and PTE was expressed. Its activity was assayed by hydrolysis of the substrate paraoxon giving 4-nitrophenolate. The black progress curves (open squares) resulted from amplified DNA, whereas the red progress curves (closed circles) were from controls without DNA amplification.

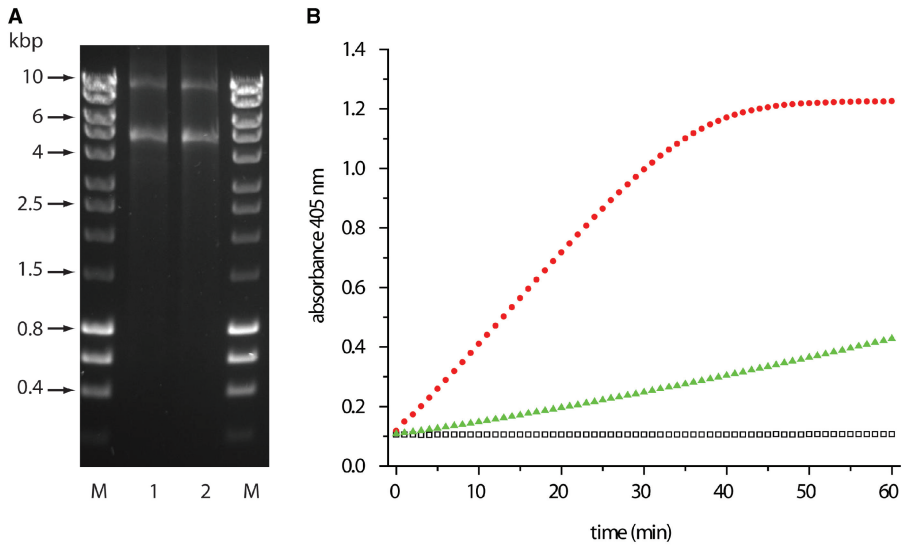

Figure 7. Immobilization of cNDA products. (**A**) cNDA reactions of pIVEX-PTE were performed with a non-biotinylated (b1, lane 1) and a biotinylated primer (biotin-b1, lane 2). (**B**) Reaction products from cNDAs with 10 ng or 1 ng pIVEX-PTE as template using primers b1 or biotin-b1 were immobilized on streptavidin-coated beads and added to IVTT mixtures. The activity of the expressed phosphotriesterase was assayed by hydrolysis of the substrate paraoxon. Red closed circles: 10 ng template DNA and biotin-b1 primer; green closed triangles: 1 ng template DNA and biotin-b1 primer; black open squares: 10 ng template DNA and untagged b1 primer.

increased with decreasing rNTP concentration (40). Consistent with this observation the average product length appears to increase when the rNTP concentration is decreased in T4 pWGA (Supplementary Figure S6). A concentration of 100 µM for each rNTP was used in subsequent T4 pWGA reactions along with 2 mM ATP as an energy source for clamp loader and helicase. Successful product formation was observed when T4 pWGA was carried out with a variety of templates: a covalently closed circular plasmid, a nicked plasmid and human genomic DNA (Supplementary Figure S7). The T4 replisome cannot initiate DNA synthesis on a covalently closed circular plasmid. Although purified plasmid DNA is mainly in the covalently circular closed form, a small fraction is also nicked. This fraction seems to be the template for the exponential amplification by T4 pWGA. This assumption is consistent with the observation that more product was obtained when the fraction of nicked plasmid was increased by heating the plasmid or by deliberately nicking the plasmid with a nicking endonuclease. In contrast to covalently closed circular templates, linear DNA can be

unwound and primed at its ends. No increase in amplification efficiency was observed when linear genomic DNA was heated prior to T4 pWGA.

Sequencing of T4 pWGA products

The suitability of T4 pWGA products for sequencing was assessed. Three commercially available plasmids (Supplementary Table S1) were amplified from 4 ng DNA in 100 µl reaction mixture without prior nicking or heating. Amplification products were purified with a purification kit for long DNA fragments (QIAEX II kit, Qiagen) and sequenced with standard sequencing primers (Supplementary Table S1). Each plasmid was sequenced in the two opposite directions. A representative sequencing result is shown in Supplementary Figure S8. The data display the typical pattern of a sequencing chromatogram, suggesting that the T4 pWGA product is of suitable quality. The lengths of accurate sequencing reads ranged from 724 to 819 bp and are comparable to sequence reads typically achieved in the sequencing of purified plasmids (Supplementary Table S1).

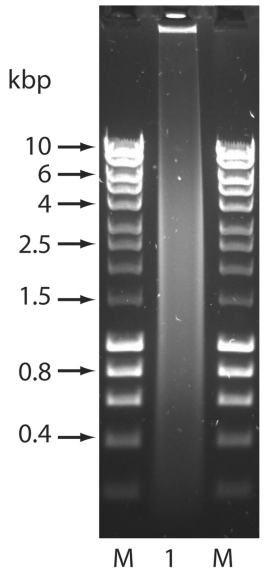

Figure 8. T4 pWGA product. Human genomic DNA (10 ng) was used as the template. The amplification yields products of different lengths.

Amplification bias of T4 pWGA

An important benchmark of every WGA system is its ability to represent the complete genome with minimal amplification bias. The amplification bias of T4 pWGA was examined by qPCR for eight loci that cover single-nucleotide polymorphisms (SNPs), each located on a different chromosome (Table 2) (39). For comparison, the same experiments were performed for pWGA by the T7 replisome and for MDA by the Phi29 DNA polymerase (using the commercial Rapisome pWGA Kit and illustra GenomiPhi V2 DNA Amplification Kit, respectively). With each system, 0.3, 1 or 10 ng of purified human genomic DNA were amplified. T4 pWGA and Rapisome pWGA reactions were incubated at 37°C for 60 min, whereas MDA reactions were incubated at 30°C for 90 min. The measured amplification biases of T4 pWGA were 4.8, 9.8 and 6.1 for 0.3, 1 and 10 ng of input DNA, respectively (Table 3 and Supplementary Table S2). This ratio varied only 2-fold for three different amounts of input DNA (0.3, 1 and 10 ng). In contrast, the amplification biases for MDA ranged between 5.7 and 20 and between 8.4 and 22 for Rapisome pWGA. Average amplification factors of T4 pWGA increased with decreasing input DNA reaching 210-fold for 0.3 ng input DNA. The same trend was observed for MDA, whereas the amplification factor of Rapisome pWGA decreased with decreasing input DNA amplification under these experimental conditions.

Quantitative T4 pWGA

As for cNDA it is also possible to monitor T4 pWGA in real time. T4 pWGA reactions containing 1, 0.1, 0.01 and 0.001 ng of genomic DNA and EvaGreen (37) were performed and the fluorescence was recorded. Figure 9A shows the normalized real-time amplification signal of T4 pWGA. In analogy to qPCR, a fluorescence threshold was defined. The times at which the T4 pWGA reactions crossed the threshold (T_T) were plotted against the amount of input DNA in log scale and fitted to a linear equation (Figure 9B). The squared correlation coefficient (R^2) of 0.991 indicates a good correlation between the amount of input DNA and the time to cross the threshold over four orders of magnitude. The product of the reaction accumulated exponentially and the slope of the linear fit indicates that the product increased 10-fold every

Table 3. Amplification factors and biases

Amplification system	T4 pWGA	Rapisome pWGA	MDA	T4 pWGA	Rapisome pWGA	MDA	T4 pWGA	Rapisome pWGA	MDA
Input DNA (ng)	0.3	0.3	0.3	1	1	1	10	10	10
Amplification factor ± SD[a]	210 ± 55	170 ± 110	3000 ± 550	80 ± 7	250 ± 110	1700 ± 920	20 ± 1	310 ± 42	190 ± 37
Amplification bias[b]	4.8	8.4	9.2	9.8	22	20	6.1	11	5.7

[a]DNA amounts were measured by qPCR.
Amplification factors and standard deviations (SDs) were calculated from triplicates of the WGA reactions for eight loci (Tables 2 and S2).
The averaged amplification factors represent the mean of the amplification factors over the eight loci analysed and the SDs represent the pooled standard deviation of the SDs in individual loci (Supplementary Table S2).
[b]The amplification bias was calculated as the ratio of the highest to the lowest amplification factor among the eight loci.

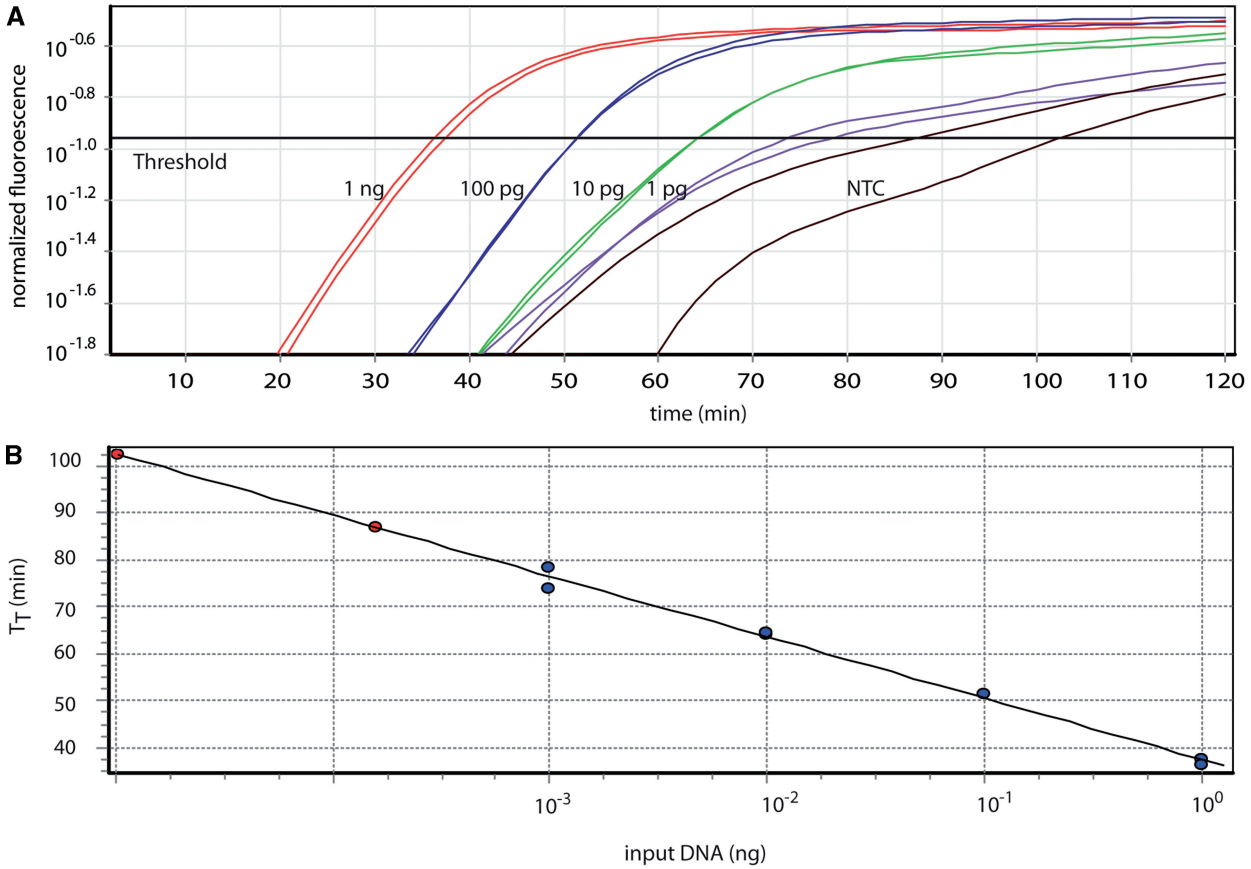

Figure 9. Real-time quantitative T4 pWGA. (**A**) Normalized real-time amplification signal of T4 pWGA containing 1 ng (red), 100 pg (blue), 10 pg (green), 1 pg (purple) and 0 (NTC; no template control; brown) human genomic DNA on a logarithmic scale. The threshold used for the quantification is drawn as a black line. (**B**) The time it took to cross the threshold (T_T) was plotted against the amount of input DNA. The blue circles are the samples containing input DNA and the red circles correspond to controls with no template. The linear fit (black line) corresponds to the following equation: $T_T = -13.01 \log_{10}[\text{input DNA}] + 37.47$ ($R^2 = 0.991$).

13 min. This is identical to the amplification efficiency reported for the T7 replisome (22). The detection limit was 1 pg, as lower amounts of DNA could not be distinguished from controls with no added template. Minute contamination of the T4 replisome proteins with DNA is the likely source of contamination acting as alternative template.

DISCUSSION

A new isothermal amplification system

The complete T4 replication machinery consists of eight proteins (Figure 1) that perform the multistep process of DNA amplification, namely, the assembly of accessory factors and the polymerase into a functional holoenzyme, the separation of the double-stranded template DNA by the helicase and RNA primer synthesis by the primase to initiate DNA synthesis by the DNA polymerase. This process had previously been reconstituted *in vitro* for detailed mechanistic studies (23). In this study, the reconstituted T4 bacteriophage replication machinery was assessed as a truly isothermal DNA amplification method for various biotechnological applications.

cNDA

The cNDA amplification mechanism (Figure 2) produces a well-defined uniform product as the cNDA reaction involves a sequence-specific primer and a nicking endonuclease. This mechanism is similar to the rolling circle replication of phages and plasmids (42,43) where an initiator protein nicks the circular template to initiate amplification. Other isothermal DNA amplification methods such as strand displacement amplification (SDA) (8,9), isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (44) and primer generation–rolling circle amplification (PG–RCA) (45) also involve a nicking endonuclease, but are not suitable for amplifying whole plasmids. Methods for amplifying whole plasmids reported thus far generate a mixture of products. The products of multiply primed RCA (14) and pWGA (22) are branched and of multiple lengths, whereas cHDA (21) yields concatemers of the circular DNA template in addition to a specific product flanked by two primers. In cNDA, specificity is provided by the reverse primer and the nicking endonuclease, whereas in multiply primed RCA (14) and pWGA (22) any plasmid may be amplified. Although the mechanism of cNDA does not involve

exponential amplification, the high synthesis rate and processivity of the T4 replisome [up to 250 nt/s (24,25) and longer than 20 kbp (25)] leads to a substantial amount of amplification as evidenced by a plasmid (4.7 kbp) being amplified up to 1100-fold within 1 h. This is only 3.3-fold less than the highest reported amplification factor of cHDA after a 6-h incubation with 156 pg input DNA (21). Replacement of the T4 replisome by the Phi29 DNA polymerase (13) in cNDA did not yield product detectable by gel electrophoresis, consistent with the low linear amplification of plasmids (20 copies per hour) reported by Dean *et al*. (14). As no high-molecular-weight concatemers are formed in cNDA, the amplicon is directly accessible without the need for a post-amplification restriction digest.

An exonuclease-deficient variant of the T4 DNA polymerase (gp43 [D219A]) (26) was used in this study. This variant was chosen because the wild-type enzyme has a strong $3'\rightarrow5'$ exonuclease activity. The wild-type T4 DNA polymerase has a very low error rate of 9.4×10^{-7} per nucleotide incorporated, while the fidelity of the [D219A] mutant is slightly reduced at 4×10^{-5} misincorporations per nucleotide added (46). Still, this error rate compares well to the error rates of 4.4×10^{-5} for the exonuclease deficient T7 DNA polymerase (Sequenase) (47), 7.2×10^{-5} for *Taq* DNA polymerase (47) and 10^{-5} to 10^{-6} for Phi29 DNA polymerase (48). As cNDA does not involve exponential amplification, any error introduced is not propagated. However, to further increase fidelity it might be possible to add a DNA polymerase with proofreading activity to the reaction. This could, for example, be the wild-type T4 DNA polymerase. In fact, the Rapisome pWGA mixture also includes wild-type T7 DNA polymerase as well an exonuclease-deficient variant (22).

Potential applications of cNDA

A number of applications could benefit from rapid amplification of plasmids producing a well-defined product.

DNA amplification prior to IVTT. Protein expression by IVTT is widely used for high-throughput screening formats including phenotyping of genes of interest, directed evolution, protein truncation tests for mutation detection and proteome analysis (49,50). However, preparing sufficient amounts of pure template DNA for the IVTT can be cumbersome, especially for high-throughput applications with many different templates. A DNA amplification step prior to IVTT could reduce the amount of input DNA required and increase protein yields of IVTT. An increase of up to 67-fold in the detected enzyme activity was observed after IVTT from cNDA products compared to IVTT from non-amplified DNA (Figure 6). This observation suggests that this method could be applied prior to IVTT in high-throughput screening formats. Such high-throughput screening approaches are increasingly miniaturized, for example, in water-in-oil emulsion droplets handled in microfluidic devices (51). The avoidance of thermal cycling and an initial heat denaturation step offer the prospect of less complex microfluidic devices when cNDA is used.

Screening bacterial colonies. cNDA can be carried out directly on plasmids from single colonies of bacteria. This suggests that cNDA could be used for screening bacterial colonies for the presence of a specific plasmid without prior purification.

Labeling strategies based on cNDA. Functional tags such as fluorescent dyes or biotin can be readily introduced into the amplified DNA product at defined positions by using a labeled reverse primer. Here, we demonstrated the incorporation of a biotin into the amplification products and the immobilization of the products on streptavidin-coated microbeads (Figure 7). Immobilization of the amplification product is also a convenient way for purification of the cNDA product. For example, tagged DNA could be purified from bacterial genomic DNA when the reaction is performed from crude cell lysate. The T4 DNA polymerase also allows incorporation of modified nucleotides such as fluorescein-dCTP (Supplementary Figure S4). Simultaneous amplification and labeling could provide a convenient way for the generation of fluorescent probes, for example, for fluorescence *in situ* hybridization (FISH) (52).

Primase-based WGA using the T4 replisome

DNA amplification is necessary for genotyping of samples where the amount of available DNA is limited, as is frequently encountered in diagnostics, forensics, clinical pathology and archaeology. The amplification step must capture the 3×10^9 bp of the human genome uniformly to avoid loss and under-representation of loci or alleles. Biased amplification reduces representation of the original template and distorts subsequent testing results (53).

Degenerate oligonucleotide primers (DOPs) (3,4) or primer extension preamplification (PEP) (5) PCR-based WGA methods have amplification biases as high as 10^6 and 10^4, respectively (2). Isothermal DNA amplification has been shown to yield more representative copies of the genome (2,22,39) with typical amplification biases between 3- and 40-fold (2).

Two isothermal methods for WGA have been reported so far: MDA (2) achieves exponential amplification of genomic DNA via a cascade of priming events by random hexameric primers extended by the Phi29 DNA polymerase. The use of random primers usually requires an initial heat or chemical DNA denaturation step to facilitate primer annealing (54,55). This additional step adds labour to the protocol as well as an additional source of DNA contamination and damage (2). In addition, artifactual DNA synthesis and increased amplification bias have been observed in samples that have been heat denatured (2).

In nature, RNA primers are synthesized by primases and are subsequently elongated by DNA polymerases (23). The mechanism of pWGA has recently been demonstrated with the T7 replisome in which the gene 4 protein (gp4) has both primase and helicase activities (22).

Thus, the requirement for synthetic primers and a DNA denaturation step to facilitate their annealing can be overcome by synthesizing the primers on template DNA. In this study, we showed that the T4 replisome with its primase (gp61) can also support exponential primase-based amplification of genomic DNA and plasmids resulting in products of different lengths (Figure 8). To probe the utility of this method for DNA analysis, the products of three T4 pWGA reactions with plasmids as templates were sequenced. The read lengths of 724–819 bp were comparable to those typically achieved by sequencing plasmids (Supplementary Table S1). This demonstrates that the T4 pWGA products are long enough for Sanger sequencing applications.

Amplification factors and biases of T4 pWGA. T4 pWGA reactions with human genomic template DNA resulted in low amplification biases between 4.8 and 9.8 among eight loci for three different amounts of input DNA (0.3, 1 and 10 ng) (Table 3), suggesting that amplification biases of this system are at least as good as the amplification biases of the two commercial isothermal WGA systems (Rapisome pWGA kit, Biohelix and illustra GenomiPhi V2 DNA Amplification Kit, GE Healthcare) tested here. For the lowest amount of input DNA (0.3 ng) the 210-fold amplification achieved by T4 pWGA was comparable to the 180-fold amplification by the Rapisome pWGA, whereas at the higher input DNA conditions tested, the Rapisome pWGA and the illustra GenomiPhi V2 DNA Amplification Kit had better yields than the T4 pWGA. Finally, in our hands, the T4 pWGA showed the highest reproducibility of the three amplification systems with the lowest SDs over the three replicates.

DNA quantification. Both cNDA and T4 pWGA reactions could be monitored in real time by recording the fluorescence of EvaGreen (37) when bound to the amplified DNA (Figures 4A and 9). For the exponential T4 pWGA amplification mechanism a good correlation between the amount of input DNA and the time to reach an arbitrary fluorescence threshold was obtained by treating the data in analogy to qPCR data. These observations suggest that T4 pWGA could be used for sequence-independent quantification of genomic DNA, as also has been shown for Rapisome pWGA (22).

Further prospects for adaptation. Further improvement of T4 pWGA amplification factors may be achieved by careful adjustment of the concentrations of the T4 replisome proteins and possible additional factors such as pyrophosphatase (to prevent accumulation of inhibitory pyrophosphate). Previous optimization of MDA gives an idea of the potential: initial publications (2,39) used incubation times of 16–18 h, whereas a currently available commercial kit can achieve similar yields within 90 min (54). A system composed of eight proteins might seem daunting, but robust amplification in a variety of different buffers was observed (data not shown). The segregation of the different functions necessary for DNA replication into eight proteins also provides the potential to differentially adjust individual components and optimize this system for different applications. This will be less likely if only a polymerase with strand-displacement activity (such as Phi29 DNA polymerase) is used. For example, different concentrations of the holoenzyme (gp43, gp45 and gp44/62) were used for the cNDA reactions and the T4 pWGA reactions. Moreover, these adjustments can be guided by the mechanistic insights that have been gained during the extensive research on this model system for DNA replication (23).

Taken together, these data characterize DNA amplification by the T4 replisome system as suitable for a variety of applications. In addition to one of the fastest DNA polymerases known, seven additional proteins constitute the replisome, allowing sufficient flexibility for further adaptation. Two different amplification mechanisms have been demonstrated: linear amplification of plasmids in the presence of a nicking endonuclease to yield well-defined products (cNDA) and an exponential amplification of genomic DNA that is primed by the T4 primase (T4 pWGA). This robust, versatile and fast system for isothermal DNA amplification will be a useful alternative to the current commercially exclusive systems.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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