

Duplexing method

The present invention relates to an improvement to cell-free, *in vitro* processes that involve the generation of single-stranded nucleic acid concatemers, where these concatemers favour folding into structures such as nanoflowers, due to the presence of stretches of self-complementary sequence. Single stranded concatemers may be produced via rolling circle amplification of a template nucleic acid under certain conditions. The self-annealing and folding of these single-stranded concatemers can frustrate the use of these concatemers for further isolation or product production, particularly where it is desired to make double stranded DNA. Such difficulties may be due to the inaccessibility for entities such as primers and DNA polymerases. The present invention addresses this need by providing a simple and elegant way to ensure that the single stranded nucleic acid concatemer can effectively be duplexed, even if it forms complex self-annealed structures.

Background to the invention

Single stranded DNA concatemers are the initial product made via rolling circle amplification of double or single stranded circular templates. Single-stranded DNA concatemers may be produced when amplifying a single stranded circular DNA template via rolling circle amplification (RCA), particularly if, for example, the method used for priming the rolling circle amplification only works on the DNA template (for example a specific primer sequence).

If the DNA template contains sequences which are self-complementary, inherently any DNA copied therefrom will also have self-complementary sequences. Since DNA prefers to be in a duplex, these self-complementary sequences will anneal to each other, resulting in complex structures such as nanoflowers forming in the resultant amplified single strand of DNA. DNA templates that are in an alternative configuration, for example, minicircles and plasmid DNA, can contain regions of self-complementary sequence, for example the presence of palindromic sequences or inverted repeats. In particular, the template may contain a recognition sequence for a protelomerase, which include inverted repeat sequences. DNA templates that are effectively a circular single stranded DNA and that contain self-complementary sequences includes closed linear DNA (cIDNA).

Denatured cIDNA is effectively circular, comprising both forward (sense or plus) and reverse (antisense or minus) sequences. cIDNA may be described as a duplexed DNA molecule with closed ends. Other terms used in the art may be neDNA, ministring DNA, celid-DNA, hp DNA or dbDNA™. The ends may be hairpin ends or may include some non-complementary single-stranded loop sections, or even comprise more complex arrangements at each end. cIDNA can be almost entirely self-complementary,

depending on the sequence of the closed ends of the duplexed section. If the cDNA is completely duplexed, the sense and antisense sequences can be described as side by side or juxtaposed.

The continuous nature of the cDNA lends itself to the use of RCA to create multiple copies of the template or "amplify" it. The amplicons of this process comprise single stranded, concatemeric,
5 alternating copies of sense and antisense strands of the template, together with any intervening sequence for the closed ends, if present.

In practice, the RCA amplicon derived from such as process folds and predominantly comprises an extensive secondary structure believed to be the result of hybridisation or annealing between forward and reverse copies of the template. In other words, the sense and antisense sequences are self-
10 complementary and anneal or hybridise to each other, to form a duplex.

One secondary structure formed are so called "nanoflowers". These occur by the hybridisation of sense and antisense copies as shown in figure 4, in which sense and antisense sequences are adjacent to each other. This limits downstream processing because the compact, folded DNA structure prevents efficient priming and extension required for synthesis of the complementary strand, as it is difficult to
15 denature sufficiently to permit access for primers or enzymes. It effectively prevents duplexing of the single-stranded DNA concatemer.

WO2018/033730 recognises this issue and proposes introducing into the template, sequences configured to form stem-loop structures in the single-stranded amplicon or concatemer. In these structures the single stranded loop remains free of the major secondary structure and primers
20 directed to sequences within the loop may then be used to direct synthesis of the complementary DNA strand.

This approach provides useful yields of duplexed DNA, as required by that method, but the process remains sub-optimal from an industrial perspective for several reasons. It requires a specialised template, the design of which is complex. Further, synthesis of the stem loop portion of the structure
25 during amplification is a source of nucleotide depletion, lowers yield and contributes to waste, which the present invention seeks to avoid. As soon as the reaction is primed in the stem-loop, and the concatemer duplexed, the reaction is completed.

Industrial production of this type of molecule requires the process to be cost effective, controllable, and produce minimal waste. Further, particularly where the product has potential to be used in
30 pharmaceutical preparations the product should contain as little unnecessary sequence as possible. Additionally, elements that can increase the amplification potential are desirable.

If the processing of single-stranded DNA concatemers into duplexed DNA can be resolved, this would permit the ready use of cDNA as a template to provide further cDNA products. This would be advantageous because it avoids the preparation of a double stranded circular template, which is notably amplified in bacteria, allows direct scale up of new templates and therefore considerably
5 simplifies the process.

It would be desirable therefore, to provide a process for permitting the effective duplexing or double-stranding of a single-stranded DNA concatemer or amplicon that overcomes at least some of the issues above.

The cell-free production of closed linear DNA has previously been described by the applicant in
10 WO2010/086626 and WO2012/017210; which are hereby incorporated by reference. The method described in these applications relates to the production of linear double stranded DNA covalently closed at each end (closed linear DNA) using a DNA template, wherein the DNA template comprises at least one protelomerase recognition sequence, and where the template is amplified using at least one DNA polymerase and processed using a protelomerase enzyme to yield closed linear DNA. The
15 closed ends of the closed linear DNA each include a portion of a protelomerase recognition sequence. The use of a closed linear DNA as a template is envisioned in the listed applications, and the use of such a template is advantageous, since it means that the minimum amount of reagents are wasted during production. Small-scale experimental production of closed linear DNA works well with a closed linear DNA template using these methods. However, the yield is lower than expected, and not
20 sufficient for preparation of commercially viable amounts of closed linear DNA.

The formation of structures due to self-annealing can also be problematic when other templates are amplified, if sections of sequence are present that are complementary. For example, the inventors have identified that rolling circle amplification from a circular template can result in polymerase “strand skipping” or “template switching”. This results in the extension of the single stranded
25 concatemer using the concatemer itself as a template (Figure 15). This structure, described by the inventors as “lollipops” results in a single stranded concatemer wrapped around the circular template (which could be single or double stranded). It will be understood that such a structure is incapable of separation under amplification conditions, and effectively ceases the amplification of the template, resulting in loss of product and removal of the template for further amplification. The single stranded
30 concatemer has self-annealed around the template. Thus, the formation of these structures can result in a yield lower than expected. Resolving this by-product caused by template switching is desirable.

Summary of the Invention

The present invention therefore provides:

An *in vitro*, cell-free method of generating a complementary DNA strand for a single-stranded DNA concatemer, said single-stranded concatemer comprising at least a first and a second sequence
5 wherein said first and second sequences are complementary to each other, said method comprising contacting said single-stranded concatemer with a modified RNA polymerase in the presence of one or more deoxyribonucleotides.

The *in vitro*, cell-free method is, therefore, an effective method of duplexing a single-stranded DNA concatemer or amplicon which contains self-complementary sequences. Such a method does not rely
10 upon the use of oligonucleotide primers, which tend to be ineffective in the duplexing of such DNA.

The single-stranded concatemer is a linear, single strand of DNA, comprising at least a first and a second sequence, wherein said first and second sequences include complementary nucleotide sequences. Complementary nucleic acid sequences are sequences which anneal or hybridise to each other due to base pairing.

15 The single-stranded concatemer may comprise one or more repeats of the first and second sequences. These repeated sequences may be in parallel. These repeated sequences may be in tandem, i.e., directly next to each other or adjacent. Alternatively, or additionally, the repeated sequences may be separated by a third or further sequence.

The first and second sequences of the single-stranded concatemer, since they are complementary,
20 can anneal or hybridise in the concatemer. This can lead to the concatemer folding into secondary structures such as nanoflowers. The concatemer can anneal around the circular template into a "lollipop" shape, or can form various secondary structures which inhibit binding of primers and polymerases.

The single-stranded concatemer may be an amplicon, such that it is the result of amplification of a
25 suitable template. Preferably the template is a DNA template. Optionally the template is a circular template. If the template is circular, or contiguous, the template may preferably be amplified by rolling circle amplification.

If the single-stranded concatemer is the result of amplification (an amplicon), it may be the product
30 of rolling circle amplification from a closed linear DNA. If the single-stranded concatemer is the result of amplification (an amplicon), it may be the product of rolling circle amplification from a double stranded circular DNA, such as a plasmid or minicircle. The template may be any appropriate template.

Closed linear DNA is a duplex DNA molecule, closed at each end. The closed ends may be any suitable sequence, such that it joins each end of the duplex, such that when denatured, the closed linear DNA is a single stranded circular DNA. The duplex of a closed linear DNA is formed of a sense sequence and antisense sequence, which are complementary in sequence.

- 5 The closed ends of a closed linear DNA may be any appropriate sequence and may or may not be self-complementary. For example, the closed ends may be a loop, which includes a sequence which has no complementary sequence in the closed linear DNA. The closed end may assume a simple or complex structure, ranging from a hairpin to a quadruplex. The closed end may be a hairpin, which requires that the closed end is complementary in sequence, or very nearly so. An example of a
- 10 sequence used to close the end can include a portion of a protelomerase sequence. Such a sequence is self-complementary.

The template may therefore be a closed linear DNA closed at each end with a portion of a protelomerase sequence. This can be viewed as a continuous/contiguous single stranded circular DNA when the base pairs are disrupted, such as under denaturation conditions.

- 15 The template may be a double stranded circle, such as a plasmid or a minicircle.

Rolling circle amplification requires a contiguous or circular template, and the resultant amplified DNA is a continuous linear single-strand, comprising multiple repeats of the template, herein noted as a concatemer. Rolling circle amplification may be carried out by any appropriate polymerase enzyme. Usually, the polymerase selected is a strand displacing polymerase, such as phi29 DNA polymerase.

- 20 The method is carried out with a modified RNA polymerase. The RNA polymerase of the present invention has been modified, changed, mutated, altered or engineered. The RNA polymerase may be described as a variant or mutant polymerase. It is preferred that the RNA polymerase is modified such that it will generate DNA from a DNA template, therefore accepting the use of deoxynucleotide triphosphates (dNTPs) as a substrate, as well as or instead of the usual substrate of ribonucleoside triphosphate (rNTP). Thus, the modified RNA polymerase may act as a DNA-directed DNA polymerase.
- 25

The modified RNA polymerase may have one or more mutations that increase the selectivity of the RNA polymerase for deoxyribonucleotides over ribonucleotides.

- The method is carried out in the presence of one or more deoxynucleotides, preferably deoxynucleotide triphosphates (dNTPs). These are the building blocks of DNA, and may be natural
- 30 deoxynucleotides, modified deoxynucleotides, or a mixture thereof. There are four main types of dNTPs, with each using a different DNA base: adenine (dATP), cytosine (dCTP), guanine (dGTP), and

thymine (dTTP). The present method may use a mixture of appropriate dNTPs and/or derivatives thereof.

It may be preferred that the method is carried out in the absence of ribonucleotides, preferably ribonucleotide tri-phosphate molecules, or with a maximum of 5% ribonucleotides present in relation to the concentration of deoxynucleotides. This is because a pure DNA product is desirable. Pure DNA products are more suitable for therapeutic applications, including DNA vaccines, gene therapy and the like.

The modified RNA polymerase may be any suitable RNA polymerase. It may be preferred that the RNA polymerase is a member of the single-subunit DNA dependent RNA polymerases (ssRNAP). The ssRNAP may be any one or more of T3, T7, T8, K11, KP34, SP6 and N4 RNA polymerase. The ssRNAP may be any one or more of T7, KP34 or SP6.

The modified RNA polymerase may be a viral RNA polymerase, including a phage-type RNA polymerase. This means that the RNA polymerase may be isolated from a bacteriophage. The phage may be any suitable phage, including but not limited to, T7, T3, T8, SP6, 13a, 285P, BA14, EcoDS1, Yepe2, K11, phiSG-JL2, Berlin, Vi06, gh-1, K1f, phiA1122, phiYe03-12, Kvp1, MmP1, N4, Syn5, ΦI, ΦII, W31, H, Y, A1, 122, cro, C21, C22, and C23.

As mentioned previously, the RNA polymerase is modified or mutated such that it functions as a DNA-dependent DNA polymerase. For example, it is known that a T7 mutant with a mutation in the active site, Tyr639 to Phe639 is known to accept deoxynucleotides, but maintain the same preference for its cognate promoter sequence. Mutating residue 639 can therefore alter the preference of T7 for dNTPs as a substrate (Sousa and Padilla, EMBO Journal, Vol 14 (18), p4609 – 4621, 1991, incorporated by reference). The wild type sequence nucleotide for T7 RNA polymerase is presented as SEQ ID No. 36.

It is preferred that the single-stranded concatemer includes at least one promoter sequence for an RNA polymerase. The modified RNA polymerase has been altered with the aim of permitting it to use deoxynucleotides as a substrate. In general, this modification does not alter the preference for its cognate promoter sequence. A promoter is a specific sequence relevant for the particular RNA polymerase, which it recognises in a DNA template, and binds to. This is the sequence at which it would initiate synthesis of nucleic acid. In nature, it would tell the RNA polymerase to initiate transcription. However, in the present invention, it will instruct the modified RNA polymerase to initiate synthesis of a DNA strand. The relevant promoter sequence for a specific RNA polymerase is termed the cognate sequence. Modifications to the active site may alter the sequence specificity of the promoter. It is noted that some enzymes can recognise several promoter sequences, for example KP34 recognises two unrelated promoter sequences.

The present invention also provides an *in vitro*, cell-free method of generating a double-stranded DNA concatemer from a closed linear DNA template, said method comprising contacting said closed linear DNA template with a modified RNA polymerase in the presence of one or more deoxyribonucleotides.

Closed linear DNA (clDNA) may be as described previously.

- 5 Applicable to any relevant template, the modified RNA polymerase may be as described previously. It is modified to be able to act as a DNA dependent DNA polymerase.

The deoxyribonucleotides may be as described previously.

These methods may generate a single-stranded DNA concatemer as an intermediate product, thus the method may proceed as described previously.

- 10 It may be preferred that any method described herein is performed in the absence of additional or exogenous primers, such as oligonucleotide primers, and/or without a primase enzyme.

It may be preferred that any method described herein further includes the use of a DNA polymerase, optionally a strand displacing DNA polymerase such as phi29.

Figures

- 15 The present invention will now be described in more detail with reference to the following non limiting figures.

- Figure 1. Gel photographs from Example 1. A gel image of the closed linear DNA (dbDNA) products following Protelomerase (TelN) digestion. RCA was performed to examine the effectiveness of T7 RNA polymerase PLUS initiating DNA synthesis with different buffers and additives. TelN was added to samples to indicate the presence of double stranded DNA. The digested DNA was then added to the gel and the resultant gel electrophoresis images captured. The gel image shows the different combinations of buffers and additives used in lane 1-8: lane 1: 1M Betaine, lane 2: 0.5x Betaine, lane 3: 2x Betaine, lane 4: 14% PEG, lane 5: 14% PEG + Bet, lane 6: 30mM Tris + Bet, lane 7: 2 x dNTPs, and lane 8: 4 x T7 RNA pol. This initial results indicate that the combination of 1 M betaine and 30 mM Tris (pH 7.9) buffer has resulted in a large band as illustrated by the arrow, indicating successful DNA amplification. No bands appeared in the lane with PEG alone, thus indicating that PEG might not be useful in the DNA amplification;
- 20
- 25

- Figure 2A-C. Gel photographs from Example 2. Gel images of the closed linear DNA (dbDNA) products. Figure 2A. shows the gel image of modified RNA polymerase-initiated DNA synthesis processed to dbDNA using protelomerase (TelN) digestion. Figure 2B. is a gel image showing that unwanted
- 30

material were removed from the dbDNA following exonuclease digestion. Figure 2C. shows the gel image of dbDNA following final purification indicated by the decreased band sizes;

Figure 3A-B. Gel photographs from Example 3. Gel images of the closed linear DNA (dbDNA) products. Figure 3A. shows the gel image of the modified RNA polymerase-initiated DNA synthesis processed to dbDNA using protelomerase (TelN) digestion, following an initial purification post-processing. Figure 3B. shows the gel image of the produced closed linear DNA (dbDNA) following an exonuclease digestion;

Figure 4A-C. These images depict one aspect of the described previous process, and the generation of nanoflowers. Closed linear DNA (dbDNA) amplification. Figure 4A shows that the dbDNA template contains a sense strand and an anti-sense strand. Figure 4B shows the template as a single stranded circles following denaturation, which may be required for oligonucleotide primers to access the template. Figure 4C shows the result of rolling circle amplification to produce a single stranded concatemer. The single stranded concatemer contains multiple repeats of the sense/antisense sequences of the dbDNA template. These sequences are complementary and therefore anneal to each other, permitting the single strand to fold into a nanoflower;

Figure 5A-C. These figures provide a depiction of a method described herein. Closed linear DNA (dbDNA) is amplified using modified T7 polymerase to initiate synthesis of DNA. Figure 5A shows that the dbDNA contains a sense strand and an anti-sense strand. Figure 5B shows an optional denaturation step, this is not required if modified T7 polymerase is used to initiate synthesis of DNA from the dbDNA itself. Figure 5C shows the result of rolling circle amplification, the single-stranded DNA concatemer. Again, the self-complementary sequences (sense and antisense) anneal, and the concatemer folds into a nanoflower. However, the modified RNA polymerase can access the nanoflower and initiate synthesis of the complementary strand. Figure 5D depicts the promoter sequence for the modified RNA polymerase in more detail. A DNA bubble (100) is formed, and the RNA polymerase initiates the synthesis of a complementary DNA strand (101) in the bubble. The sequences shown are exemplary;

Figure 6. This is a diagrammatic representation of the protelomerase recognition sequence protelomerase TelN in a linear format, without the hairpin structures. It can be seen that the first portion of the protelomerase recognition sequence (A) has a sequence which is complementary to the second portion of the protelomerase recognition sequence (B). At the centre (in this example) of the protelomerase recognition sequence (D) is the site at which the protelomerase will cleave the sequence, which is in the centre of the telO sequence (E). The complete protelomerase recognition sequence (C) is composed of TelRL for the enzyme TelN;

Figure 7 shows what happens to the sequence of Figure 6 once protelomerase TelN catalyses the reaction at the recognition sequence. The sequence is cleaved at the point indicated (D on Figure 6) and the cleaved ends are re-ligated with the opposing strand to form two separate hairpin structures;

Figure 8 shows the whole native recognition sequences for a selection of protelomerase enzymes, showing the sequences of both strands of the complementary DNA. Shown are the target sequences: the sequence of SEQ ID NO: 15 (Escherichia coli N15 TelN protelomerase), the sequence of SEQ ID NO: 16 (Klebsiella phage Phi K02 protelomerase), the sequence of SEQ ID NO: 17 (Yersinia phage PY54 protelomerase), the sequence of SEQ ID NO: 1 (Halomonas phage PhiHAP-1), the sequence of SEQ ID NO: 18 (Vibrio phage VP882 protelomerase), the sequence of SEQ ID NO: 19 (Borrelia burgdorferi protelomerase), the sequence of SEQ ID NO: 21 (Vibrio parahaemolyticus plasmid Vp58.5 protelomerase), and the sequence of SEQ ID NO: 20 (Agrobacterium tumefaciens TelA protelomerase). Where the minimum sequence length requirement for the cognate protelomerase is known, this has been indicated by shading the sequence grey, although the enzyme may accept some variation in sequence within this core recognition sequence. Nucleotides represented in bold and underlined indicate imperfections in the palindrome sequence. The vertical line through the sequences represents the centre of the perfect inverted sequence and the point at which the protelomerase cleaves and joins its specific recognition sequence;

Figure 9 is a photograph of the results of Example 4. The amplified DNA was separated by gel electrophoresis on a 1.0% agarose gel. A gel imager was used to capture an image. Lane 1 is DNA amplified with modified T7 polymerase, lane 2 is with modified SP6 polymerase and lane 3 is with modified KP34 polymerase. A size ladder was included in lane 4 for calibration;

Figure 10 is a gel photograph. Samples from the DNA generated in Example 5 were run by gel electrophoresis on a 1.0% agarose gel to visualise the amplified DNA. A gel imager was used to capture an image. Lane 1: WT T7 polymerase, lane 2 phi29. Lane 3 ladder was included for calibration;

Figure 11 is a gel photograph. Samples of amplified DNA from Example 6 were run by gel electrophoresis on a 1.0% agarose gel to visualise the amplified DNA. A gel imager was used to capture an image. Lane 1 is a ladder for calibration, lane 2 is modified T7 polymerase without dNTPs or rNTPs, lane 3 is modified T7 polymerase plus rNTPs, Lane 4 is a reaction performed with the addition of dNTPs and rNTPs, and lane 5 is the reaction performed in the presence of dNTPs only;

Figures 12A and 12B are photographs of the gel electrophoresis of the DNA amplified in Example 7. Samples of amplified DNA were run by gel electrophoresis on a 1.0% agarose gel to confirm the presence of DNA of interest. A gel imager was used to capture an image. The lanes are as follows:

12A lane 1: Ladder, lane 2: starting template for RCA. 12B: Lane 1: ladder, lane 2: DNA obtained post processing of RCA reactions with TelN and enzyme digestion, incubated with a restriction enzyme that nicks within the sequence of interest, lane 3: DNA obtained post processing of RCA reactions with TelN and enzyme digestion;

5 Figure 13 is a photograph of the gel electrophoresis on the amplified DNA from Example 8. Samples were run by gel electrophoresis on a 1.0% agarose gel. These were performed to visualise the cleaned-up, amplified DNA to confirm the presence of duplexed DNA of interest. A gel imager was used to capture an image. The lanes were included as follows: Lane 1: Template 1 amplification, Lane 2: Template 2 amplification, Lane 3: ladder;

10 Figure 14A and 14B are photographs of the gel electrophoresis results of Example 9. Samples of the produced DNA were run by gel electrophoresis on a 1.0% agarose gel. These were performed to visualise the cleaned-up, amplified DNA to confirm the presence of duplexed DNA of interest. A gel imager was used to capture an image. The lanes contain as follows: 14A and 14B: lane 1: concatemers without denaturing conditions, lane 2: concatemers with denaturing conditions, lane 3: 15 concatemers and modified T7 RNA polymerase (no primers), L is ladder, lane 4: 50x dilution, minimal reaction. The DNA in Figure 14B is the same as Figure 14A, except the produced DNA has been processed with TelN and enzymes to provide evidence that the DNA was duplexed appropriately, as processing only occurs for dsDNA; and

Figure 15 is a diagrammatic representation of the generation of a single stranded concatemer by 20 rolling circle amplification of a circular DNA template. The rolling circle amplification of the template is started using primers, and a single strand is generated. However, template switching occurs and the DNA polymerase switches from using the template for amplification, to extending the single stranded concatemer, using the concatemer itself as a template. This results in unwanted secondary structure, which is hard to resolve under usual amplification conditions. The single strand is 25 wrapped around the template but also self-annealed due to sections (at least a first and second sequence) generated that have complementary sequences. The present invention seeks to resolve this unwanted secondary structure using modified RNA polymerases to initiate duplexing of the ssDNA concatemer.

Detailed Description

30 In previous approaches to the amplification of templates such as closed linear DNA (clDNA), amplification steps are carried out in the presence of one or more species of oligonucleotide primers, dNTPs and a DNA polymerase, such as Phi29 DNA polymerase. clDNA is denatured and a

primer is hybridized to the single stranded circle produced. The DNA polymerase extends the primer by incorporation of dNTPs, in an RCA process to provide a single stranded amplicon, or concatemer, which itself should then be primed and the primer extended to provide the double stranded DNA. However, this process is frustrated by the presence of self-complementary sequences in the single-stranded DNA. This inhibits the access of primers and DNA polymerase. The second strand fails to be synthesised. Furthermore, in an industrial process, denaturation of the template can cause unnecessary complications to the process.

Similar considerations may apply to the amplification of double stranded circular templates where the single stranded concatemer gets “wrapped” around the template, halting further amplification.

10 In the presently disclosed method, both the optional amplification step from a cDNA template and synthesis of the complementary strand to the single-stranded DNA concatemer may be initiated by *de novo* DNA synthesis. As a result, the process may occur in the absence of an exogenous primer. The primer is instead synthesised *in situ*, taking advantage of the modified RNA polymerase’s ability to initiate DNA synthesis *ab initio*. The inventors have found that modified RNA polymerase can initiate and synthesise the complementary strand of the concatemer despite the presence of regions of double stranded secondary structure, such as nanoflowers. The inventors have found that this process may also apply to more conventional double stranded templates, since these also generate single stranded concatemers that can anneal due to self-complementary sequences.

Moreover, in the present methods, the modified RNA polymerase may include the substitution of one or more amino acids, such that it retains the ability to initiate nucleic acid synthesis, but is able to use dNTPs as a substrate, either as well as, or instead of, ribonucleoside tri-phosphates (rNTPs). In this way, the process does not require exogenous primers or, indeed, the use of rNTPs. The avoidance of rNTPs is desirable, since it is desired that the product is double stranded or duplex DNA, not an RNA/DNA hybrid.

25 The modified RNA polymerase may be capable of synthesising the entirety of the complementary strand for the single-stranded DNA concatemer. Alternatively, the single stranded DNA concatemer may also be contacted with a DNA polymerase, such as a strand displacing DNA polymerase, for example phi29. The ability of the modified RNA polymerase to initiate the production of double stranded DNA allows any strand displacement amplification step to be carried out without first denaturing a double stranded DNA template, which further simplifies the process.

30 The modified RNA polymerase may have only 1, 2, 3, 4 or 5 modified amino acid residues. Such residues may be modified in any part of the enzyme but are more likely to form part of the active site of the enzyme. Structural studies support the view that the similarities between polymerases go

beyond arbitrary divisions into classes on the basis of the use of nucleotides, for example. It is thought that most polymerases belong to a polymerase superfamily and have closely related active sites similarly positioned within a polymerase cleft whose shape has been compared with that of a half open right hand. Thus, the active site architecture required to carry out the phosphoryl transfer
5 reaction may be shared, and that subtle modifications to this module achieve the substrate specificities characteristic of each polymerase class.

Single-stranded DNA is a DNA molecule that consists of only a single strand contrary to the typical two strands of nucleotides in helical or duplex form. Single-stranded DNA (ssDNA) is inherently less stable than double-stranded DNA (dsDNA) due to its lack of base pairing. This is because there is a much
10 larger thermodynamic driving force for being duplexed or double-stranded. Thus, a single-stranded DNA molecule will seek to form secondary structures using any self-complementary sequences.

The single-stranded DNA concatemer comprises at least two sequences - a first and a second sequence. These first and second sequences contain complementary sequences. Thus, the single-stranded DNA concatemer contains sequences that are complementary, and thus can be described as
15 "self-complementary" since these sequences are present on the same strand of DNA. Self-complementarity refers to the fact that a sequence of DNA may fold back on itself, creating a duplex. Depending on how close together the parts of the sequence are that are self-complementary, the strand may form numerous structures including hairpins, hairpin loops, junctions, bulges or internal loops. These structures may be formed of a third or further sequence.

20 The single-stranded concatemer may include multiple repeats of the first and second sequences, if present, third or further sequences, usually in parallel. Thus, the self-complementary sequences may form multiple hairpins, hairpin loops, junctions, bulges or internal loops in parallel, leading to the formation of secondary structures such as nanoflowers. Such structures are generally hard to denature, and they can prevent the access of various primers or enzymes via steric hindrance. Thus,
25 the production of a double stranded DNA or duplex is frustrated by the folding of the single-stranded DNA concatemer. The structure prevents DNA polymerase and primers from accessing the single-stranded DNA.

The first, second, third and further sequences, as relevant, may be any appropriate length or sequence. For example, the first and/or second sequences might be as small as 3 nucleotides (bases)
30 in length. 3 nucleotides (bases) from each sequence are sufficient to form a duplex if they are exactly complementary to each other. However, the sequences may be longer than 3 nucleotides (bases), such as 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or 70 nucleotides (bases) in length, or any value between these points. The first and/or second sequences may be greater than 70 nucleotides (bases)

in length, or greater than 80, 90, 100, 150, 200, 250 or 300 nucleotides (bases) in length (bases). Since the present invention is concerned with *in vitro* enzymatic DNA synthesis, the first and second sequences can be any suitable length, up to many thousands of bases. For sequence longer than the minimal 3 bases, the first and second sequence may exhibit imperfect complementarity to each other, for example, some mismatches may be tolerated in a longer sequence. Thus, the sequences may be substantially complementary to each other. The first and second sequences thus include a portion of nucleotide sequence which are complementary to each other. This results in the first and second sequence binding to each other via base-pairing. This may also be described as annealing or hybridisation. It is known that adenine pairs with thymine, and guanine with cytosine. The bases pair using hydrogen bonds and are also termed "Watson Crick" pairs.

The single-stranded concatemer may be any appropriate length and may be synthesised by any appropriate method.

It may be preferred that the single-stranded concatemer is an amplicon – i.e., it is a product of amplification of a template, for example using RCA. RCA permits the amplification of a template into a single-stranded DNA product that comprises multiple repeats of the sequence of the template. This template may be any appropriate template, including but not limited to: double stranded circular templates, such as minicircles or plasmids, or closed linear DNA. Particularly preferred may be the amplicon generated by RCA performed on a cDNA template. In this instance, the first sequence may be the sense sequence from a cDNA, and the second sequence may be from the antisense sequence (or *vice versa*).

The production of the single-stranded DNA concatemer may involve amplification of a DNA template. For simplicity, this can be performed at effectively the same time as the duplexing of the single-strand. Thus, a modified RNA polymerase and deoxynucleotides can be used to create and double strand a single-stranded DNA concatemer as defined herein. Thus, the generation of the single-stranded DNA concatemer may also require the use of a modified RNA polymerase and deoxynucleotides. Optionally, the method may also include the use of a DNA polymerase, preferably a strand displacing DNA polymerase, such as phi29. Such a combined method is advantageous, since it reduces waste, simplifies steps and increases efficiency. The template may be any suitable template. Preferably, the template used to generate the single-stranded DNA concatemer as defined here is a closed linear DNA.

The present invention may be described as relating to an improved, cell-free process for amplifying DNA from a double stranded DNA template, particularly where that template contains inverted repeat sequences. The method may involve generating single stranded DNA concatemers as an intermediate, along with a complementary strand for this intermediate.

The present invention may therefore be described as relating to an improved, cell-free processes for amplifying DNA from a closed linear DNA template. The method generates a single stranded concatemer as an intermediate, together with a complementary strand for this single-stranded concatemer.

- 5 Closed linear DNA, i.e., linear double stranded covalently closed DNA molecules; typically comprise a linear double stranded section of DNA with covalently closed ends, i.e., hairpin ends. The hairpins join the ends of the linear double DNA strands, such that if the molecule was completely denatured, a single stranded circular DNA molecule would be produced. The DNA can therefore be described as contiguous – there are no free ends (3'OH or 5'OH are absent).
- 10 The covalently closed ends or hairpins typically contain internally complementary sequences for example in the case where the ends are closed by a portion of a target sequence for a protelomerase. The bases within the apex (end or turn) of the hairpin may not be able to form base pairs, due to the conformational stress put onto the DNA strand at this point. The exact conformation of these regions is likely to be subject to fluctuations depending on the conditions in which the DNA is maintained, and
- 15 the exact sequences around the hairpin. Thus, 2 or more bases may not be able to form pairs given the structural distortion involved, despite their complementary nature.

Some "wobbles" of non-complementary bases within the length of a hairpin may not affect the structure. A wobble may be a break in the palindrome, but the sequences may remain complementary. It is, however, preferred that the sequence of the hairpin is entirely self-

20 complementary. In the case where the ends are closed by a portion of a protelomerase, each protelomerase enzyme, working on its appropriate protelomerase recognition sequence, will generate two different hairpins at the end of the closed linear DNA if there are 'wobbles' in the palindrome.

However, other closed ends are envisioned which are not complementary, such as stem loops, bulges

25 and the like.

The term "complementarity" means that the bases of each polynucleotide in a sequence (5' to 3') are in a hydrogen-bonded pair with a complementary base, A to T (or U) and C to G on an anti-parallel (3' to 5') strand, which may be the same strand (internal complementary sequences) or on a different strand. This definition applies to any aspect or embodiment of the invention.

- 30 It may be preferred that the sequences in the closed end of the cldNA are at least 90% complementary, preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 98%, 99% or 100% complementary.

Thus, in the case where the ends are closed by a portion of a protelomerase recognition sequence for example, each end may be formed of a portion of a protelomerase recognition sequence for the same or different protelomerase enzymes. These portions may be named as the first and second protelomerase recognition sequences, and these form the ends of the closed linear DNA template.

- 5 A protelomerase recognition sequence is any DNA sequence whose presence in a DNA sequence allows for its conversion into a closed linear DNA by the enzymatic activity of protelomerase. In other words, the protelomerase recognition sequence is required for the cleavage and re-ligation of double stranded DNA by protelomerase to form covalently closed linear DNA. Typically, a protelomerase recognition sequence comprises a palindromic sequence i.e., a double-stranded DNA sequence having
- 10 two-fold rotational symmetry, also described herein as an inverted repeat. The length of the inverted repeat differs depending on the specific organism from which the protelomerase is derived. The palindrome or inverted repeat may be perfect or imperfect. A complete protelomerase recognition sequence preferably comprises a double stranded palindromic (inverted repeat) sequence of at least 14 base pairs in length. In more detail, a complete protelomerase recognition sequence is recognised
- 15 and cleaved by its cognate protelomerase, and can be presented as a duplex of a first DNA sequence comprising a forward (or sense) portion of a protelomerase recognition sequence and a complementary second DNA sequence containing the reverse (or antisense) portion of the protelomerase recognition sequence.

- Once the recognition sequence has been cleaved and ligated, what is left behind is effectively the
- 20 sequence of a portion of the protelomerase recognition sequence (that is to say, one strand of the recognition sequence). Thus, the portion is preferably a single strand of the entire sequence (at least 14 bases in length), which when paired with its complementary sequence, forms a complete recognition sequence in a double stranded format. The portion may be the forward (or sense) portion of the protelomerase recognition sequence or the reverse (or antisense) portion. The length of the
- 25 first or second portion of the protelomerase recognition sequence is determined by the minimum sequence recognised by the cognate protelomerase in order to bind, cleave and re-join the free ends. Several complete protelomerase recognition sequences are depicted in Figure 8, and each strand represents a portion of the recognition sequence for the cognate protelomerase. The length of the portion of the protelomerase recognition sequence for a cognate protelomerase may be the same or
- 30 nearly so, since they are capable of annealing to form a duplex. Each portion of a protelomerase recognition sequence may be 20 to 100 bases in length, more particularly 30 to 100 bases in length.

The presence of the protelomerase target sequence in the duplexed or double-stranded DNA provides a complete target sequence for its cognate protelomerase. Thus, since the target sequence is double stranded, the double stranded DNA can be processed with at least one protelomerase. The resultant

product would be closed linear DNA molecules. If the template is a closed linear DNA, this provides maximum efficiency, since closed linear DNA templates can be amplified into numerous closed linear DNA products, with no waste products. As a comparison, the production of closed linear DNA molecules from a plasmid template inherently produces amplified backbone DNA, which is unwanted.

5 Thus, amplifying backbone wastes nucleotides and other reactants, and is ultimately discarded.

The single-stranded concatemer may further comprise any sequence within the double stranded sequence, either naturally derived or artificial. This sequence may form the duplex of the cDNA template, if appropriate.

The single-stranded DNA concatemer and/or DNA template such as a cDNA template may comprise
10 at least one processing enzyme target sequence, such as one, two, three, four or more processing enzyme target sites. Such a target sequence is to allow for the DNA to be optionally processed further following synthesis. A processing enzyme is an enzyme that recognises its target site and processes the DNA. The processing enzyme target sequence may be a target sequence for a restriction enzyme. A restriction enzyme, i.e., a restriction endonuclease, binds to a target sequence and cleaves at a
15 specific point. The processing enzyme target sequence may be a target for a recombinase. A recombinase directionally catalyses a DNA exchange reaction between short (30-40 nucleotides) target site sequences that are specific to each recombinase. Examples of recombinases include the Cre recombinase (with loxP as a target sequence) and FLP recombinase (with short flippase recognition target (FRT) sites). The processing enzyme target sequence may be a target for a site-
20 specific integrase, such as the phiC31 integrase. The processing enzyme target sequence may be a target sequence for a RNA polymerase, such that the DNA becomes a template for polypeptide synthesis. In this instance, the processing enzyme targeting site is a promoter, preferably a eukaryotic promoter.

Preferably, the single stranded DNA concatemer and/or DNA template such as cDNA may comprise a
25 promoter sequence for a modified RNA polymerase, for example a DNA dependent RNA polymerase.

The single-stranded DNA concatemer and/or DNA template such as a closed linear DNA template may comprise an expression cassette comprising, consisting or consisting essentially of a eukaryotic promoter operably linked to a sequence enclosing a protein of interest, and optionally a eukaryotic transcription termination sequence. A "promoter" is a nucleotide sequence which initiates and
30 regulates transcription of a polynucleotide. "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably linked to a nucleic acid sequence is capable of effecting the expression of that sequence when the proper enzymes are present. The term "operably linked" is intended to

encompass any spacing or orientation of the promoter element and the DNA sequence of interest which allows for initiation of transcription of the DNA sequence of interest upon recognition of the promoter element by a transcription complex.

5 The DNA template for producing the single stranded DNA concatemer may also be a double stranded circular template, such as a plasmid or minicircle. Such may also include the recognition sequence for a protelomerase enzyme as discussed above. As such, any single stranded concatemer produced from this template will comprise a first and a second sequence which are complementary to each other, due to the nature of the recognition sequence.

10 The DNA template for producing the single-stranded DNA concatemer may be of any suitable length. Particularly, the cDNA template may be up to 100 kilobases, or up to 50 kilobases, or up to 40 kilobases, or up to 30 kilobases. Preferably the DNA template may be 100 bases to 100 kilobases, 200 bases to 40 kilobases, more preferably 200 bases to 30 kilobases, most preferably 1 kilobases to 15 kilobases. Double stranded circular templates may be any appropriate length, ranging from 250 base pairs for the smallest minicircles to hundreds of kilobases for the largest plasmids. This, circular
15 templates may range from 250 base pairs to 900 kilobases, 500 base pairs to 800 kilobases, 1 kilobase to 700 kilobases, more preferably 1 to 500 kilobases.

The DNA template for producing the single-stranded DNA concatemer may be provided in an amount sufficient for use in the process of the invention by any method known in the art. For example, the template may be produced by the polymerase chain reaction (PCR), Recombinase polymerase
20 amplification (RPA), Helicase-dependent amplification (HDA), template extension or any synthetic means of making DNA. Alternatively, standard molecular cloning techniques can be employed for double stranded circular templates.

DNA dependent RNA polymerases are ubiquitous enzymes that play a central role in the transcription of DNA. Classically, they recognise a specific promoter sequence, within a double stranded DNA, for
25 which they have a high degree of specificity. Unlike DNA Polymerases, which extend a pre-existing primer, hybridized to a single stranded portion of DNA, these enzymes initiate transcription *de novo* by incorporating the two nucleoside triphosphates that are directed by the sequence at the start site of the promoter, incorporating further NTPs by coupling them through the free 3' hydroxyl of the previous NTP in the chain with nucleotides base-paired to the template DNA strand according to
30 Watson-Crick base-pairing interactions. As the chain elongates, the two strands of DNA are separated by the polymerase at the leading edge of transcription and re-annealed at the trailing edge, forming a transcription "bubble" that moves along the strand enclosing an 8 bp RNA:DNA heteroduplex (Figure 5).

The modified RNA polymerase may be any suitable RNA polymerase. It may be preferred that the RNA polymerase is a member of the single-subunit DNA dependent RNA polymerases (ssRNAP). Such RNA polymerases are generally of viral origin, including bacteriophages. The ssRNAP may be any one or more of T3, T7, T8, K11, KP34, SP6 and N4 RNA polymerase. The ssRNAP may be any one or more of T7, KP34 or SP6.

The modified RNA polymerase may be a viral RNA polymerase, including a phage-type RNA polymerase. This means that the RNA polymerase may be isolated from a bacteriophage. The phage may be any suitable phage, including but not limited to, T7, T3, T8, SP6, 13a, 285P, BA14, EcoDS1, Yeye2, K11, phiSG-JL2, Berlin, Vi06, gh-1, K1f, phiA1122, phiYe03-12, Kvp1, MmP1, N4, Syn5, ΦI, ΦII, W31, H, Y, A1, 122, cro, C21, C22, and C23.

Wild type RNA polymerases are many-fold selective for rNTPs (as measured by specificity constant K_{cat}/K_m values). Specificity (or discrimination) for an RNA polymerase may be determined according to US5849546 (which incorporated herein by reference in its entirety - see for example col 7). For wild-type T7 RNA polymerase, for example, the average of the K_{cat}/K_m values for the four common rNTPs (ATP, CTP, GTP & UTP) is about 120-fold larger than the average K_{cat}/K_m values for the four common dNTPs (dATP, dCTP, dGTP and dTTP); i.e., the wild-type enzyme discriminates by a factor of 120 for rNTPs vs. dNTPs.

Certain modified RNA polymerases have a much-increased ability to use dNTPs as a substrate. The enzyme can be modified to allow use of both rNTPs and dNTPs, or so that it exclusively accepts dNTPs as a substrate (Kostyuk *et al* 1995).

Y639 mutants of T7 RNA polymerase (wild type sequence presented as SEQ ID No. 36), in which the tyrosine is replaced by a conservative substitution, such as phenylalanine, display K_{cat}/K_m values for the four common rNTPs that is only about 6-fold larger than the average K_{cat}/K_m values for the four common dNTPs. Thus, using the average K_{cat}/K_m values for these substrates, the K_{cat}/K_m of the Y639F mutant, for example has about a 20-fold reduced discrimination between dNTPs and rNTPs. Similar effects have been shown for the equivalent conservative substitutions in bacteriophage T3 RNA polymerase (wild type sequence SEQ ID No. 37) at the equivalent tyrosine, and for bacteriophage SP6 RNA polymerase (Y631) (Wild type sequence SEQ ID No. 34), Klebsiella bacteriophage K11 RNA polymerase (Y660) (wild type sequence SEQ ID No. 38) and Klebsiella bacteriophage KP34 RNA Polymerase (Y603) (Wild type sequence SEQ ID No. 35) (see US5849546, Kostyuk *et al* 1995, Mutants of T7 RNA polymerase that are able to synthesize both RNA and DNA, FEBS Letters, 369, doi: 10.1016/0014-5793(95)00732-O; Lu *et al* 2019 Front. Microbiol. Sec. Evolutionary and Genomic Microbiology Volume 10. <https://doi.org/10.3389/fmicb.2019.02487>).

In T7 RNA polymerase, the mutation S641A confers a change in substrate specificity from rNTP to dNTP, whilst the double mutant Y639F, S641A allows use of both rNTPs and dNTPs

At least some of these mutants are available commercially, e.g., T7 R & DNA™ (which is a Y639F mutant of T7 RNA polymerase able to incorporate both dNTPs and rNTPs - LGC Biosearch technologies, UK).

Various mutant DNA dependent RNA polymerases with altered affinity for various substrates are described in WO2021/228905A2, herein incorporated by reference. These are postulated for use in the synthesis of short oligonucleotides from a template, such as RNA synthesis.

In the present invention, preferred modified RNA polymerases include modified single subunit RNA polymerases, particularly those of viral origin. Particularly preferred modified RNA polymerases are those capable of using dNTPs as a substrate either in addition to rNTPs or instead of rNTPs.

Additionally, the inventors have generated modified RNA polymerases in-house with an altered substrate specificity, resulting in the acceptance of dNTPs in preference to rNTPs. This demonstrates that it is possible to design and produce appropriate enzymes to perform the present invention.

To enable modified enzyme production, standard techniques may be used. Notably these can include the steps taken by the present inventors as described here. The modified RNA polymerases were designed by rational design based on scientific literature. The wild type polymerases were subject to mutagenesis PCR with commercially available QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). PyMOL molecular visualization system and Missense 3D-PPI were used to further assess the effect of mutations on substrate binding. The mutations primarily were designed using the structure of T7 RNA polymerase, with alignment tools (Needleman-Wunsch algorithm) to identify the corresponding residues and implement the mutations for the other RNA polymerases. Where the structure was unavailable PHYRE2 Protein Fold Recognition Server was used to predict the protein structure.

For T7, several residues were identified in the wild type sequence based upon the literature that would be of interest when designing modified enzymes. These residues included (as numbered in SEQ ID No. 36) : 555, 639, 641, 644, 667, 689, 783, 784. Combinations of various substitutions at these positions are of interest. Alignment tools can identify the corresponding residues in other RNA polymerase enzymes as described above.

Selected literature reviews may include (and may be incorporated by reference):

Sousa R, Padilla R. A mutant T7 RNA polymerase as a DNA polymerase. EMBO J. 1995;14(18):4609-4621.

- Kochetkov SN. Mutants of T7 RNA polymerase that are able to synthesize both RNA and DNA. *FEBS Lett.* 1995;369:165-168.
- Joyce CM. Choosing the right sugar: How polymerases select a nucleotide substrate. *Proc Natl Acad Sci.* 1997;94(5):1619-1622. doi:10.1073/pnas.94.5.1619.
- 5 Huang Y *et al.* Mechanism of ribose 2'-group discrimination by an RNA polymerase. *Biochemistry.* 1997;36(27):8231-8242. doi:10.1021/bi962674l.
- Gudima SO *et al.* Synthesis of mixed ribo/deoxyribopolynucleotides by mutant T7 RNA polymerase. *FEBS Lett.* 1998;439(3):302-306. doi:10.1016/S0014-5793(98)01393-3.
- Briebe LG, Sousa R. Roles of histidine 784 and tyrosine 639 in ribose discrimination by T7 RNA
10 polymerase. *Biochemistry.* 2000;39(5):919-923. doi:10.1021/bi992324+.
- Temiaikov D, *et al.* Structural basis for substrate selection by T7 RNA polymerase. *Cell.* 2004;116(3):381-391. doi:10.1016/S0092-8674(04)00059-5.
- Svetlov V, *et al.* Discrimination against deoxyribonucleotide substrates by bacterial RNA polymerase. *J Biol Chem.* 2004;279(37):38087-38090. doi:10.1074/jbc.C400316200.
- 15 Brakmann S. Directed evolution as a tool for understanding and optimizing nucleic acid polymerase function. *Cell Mol Life Sci.* 2005;62(22):2634-2646. doi:10.1007/s00018-005-5165-5.
- Ong JL, *et al.* Directed Evolution of DNA Polymerase, RNA Polymerase and Reverse Transcriptase Activity in a Single Polypeptide. *J Mol Biol.* 2006;361(3):537-550. doi:10.1016/j.jmb.2006.06.050.
- Duan B, *et al.* A critical residue selectively recruits nucleotides for T7 RNA polymerase transcription
20 fidelity control. *Biophys J.* 2014;107(9):2130-2140. doi:10.1016/j.bpj.2014.09.038.
- Yoon H, Warshel A. The control of the discrimination between dNTP and rNTP in DNA and RNA polymerase. *Proteins Struct Funct Bioinforma.* 2016;84(11):1616- 1624. doi:10.1002/prot.25104.
- Jessica A. Brown and Zucui Suo. Unlocking the Sugar 'Steric Gate' of DNA Polymerases *Biochemistry.* 2011 February 22; 50(7): 1135–1142. doi:10.1021/bi101915z.
- 25 The modified T7 RNA polymerase used in the Examples comprises a Y639F substitution and is listed as SEQ ID No. 33 (protein) and 32 (nucleic acid). The modified SP6 RNA polymerase used in the Examples comprises a Y631F substitution and is listed as SEQ ID No. 28 (protein) and 39 (nucleic acid).
- The modified KP34 RNA polymerase used in the Examples comprises a Y603F substitution and is listed as SEQ ID No. 30 (protein) and 31 (nucleic acid).

Optionally, the modified RNA polymerase may be a modified phage-type RNA polymerase. Such refers to a polymerase with homology to T7 RNA polymerase and which, in its wild type form, can incorporate ribonucleotides into RNA using a DNA template. Exemplary phage-type polymerases include RNA polymerases from the phages T7, T3, SP6, Φ I, Φ II, W31, H, Y, A1, 122, cro, C21, C22, and C23.

Suitable exemplary phage-type RNA polymerases include, but are not limited to, RNA polymerases from Escherichia phage T7 (T7 RNA polymerase, Genbank: ACY75835.1; SEQ ID NO: 36), Salmonella virus SP6 (SP6 RNA polymerase, GenBank: AAR90000.1; SEQ ID NO:34), Enterobacteria phage T3 (T3 RNA polymerase, GenBank: CAC86264.1; SEQ ID NO: 37), Enterobacteria phage 13a (GenBank: ACF15888.1), Enterobacteria phage 285P (GenBank: ACV32460.1), Enterobacteria phage BA14 (GenBank: ACF15731.1), Enterobacteria phage EcoDS1 (GenBank: ACF15785.1), Yersinia phage Yepe2 (GenBank: ACF15684.1), Klebsiella phage K11 (GenBank: ACF15837.1), Salmonella phage phiSG-JL2 (GenBank: ACD75668.1), Yersinia phage Berlin (GenBank: CAJ70654.1), Salmonella phage Vi06 (GenBank: CBV65202.1), Pseudomonad phage gh-1 (GenBank: AAO73140.1), Enterobacteria phage K1F (GenBank: AAZ72968.1), Yersinia phage phiA1122 (GenBank: AAP20500.1), Yersinia phage phiYeO3-12 (GenBank: CAB63592.1), Kluyvera phage Kvp1 (GenBank: ACJ14548.1), Morganella phage MmP1 (GenBank: ACY74627.1), Vibrio phage N4 (GenBank: ACR16468.1, AAY46276.1), Enterobacteria phage K11 (GenBank: CAA37330.1), and Synechococcus virus Syn5 (GenBank Acc. No: YP_001285424.1).

Optionally, a phage-type RNA polymerase has at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% sequence identity to SEQ ID NO: 36. Further, Table 1 of US9062292, which is incorporated by reference, provides a listing of known RNA polymerases, including the homology of those RNA polymerases to T7 (SEQ ID NO: 36). SP6 and T3 are exemplary phage-type DNA dependent RNA polymerases.

Positions in various DNA dependent RNA polymerases that correspond to positions in T7 RNA polymerase can be determined by alignment of amino acid sequences. Alignment of amino acid sequences can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, ALIGN, Clustal Omega or MultiAlin software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

In some embodiments, the single-stranded DNA concatemer and/or the template, such as the cDNA template includes a promoter sequence to which the wild-type RNA polymerase specifically binds and from which it initiates complementary strand synthesis.

5 The RNA polymerase promoter sequences used in the process depends on the RNA polymerase selected. Any suitable promoter sequence may be used so long as the RNA polymerase binds to the promoter and the RNA polymerase initiates second strand synthesis from the promoter sequence. A selection of promoters for RNA polymerase which may be used in the present invention are given below.

5

Polymerase	Exemplary sequence	SEQ ID NO:
T7	5'-TAATACGACTCACTATA-3'	23
T3	5' AATTAACCCTCACTAAAG 3'	24
K11	5'TCAGGCAATTAGGGCAGACTATAGGGAACCTT-3'	25
KP34	5'-AGCCTATAGCGTCCTACGGGGCGCTATGTGAA-3	26
SP6	5'-ATTAGGTGACACTATAG-3'	27

10

RNA polymerases may be modified not only to change their substrate specificity, but to alter the dependence on the promoter sequence.

15

It may be preferred that the modified RNA polymerase is used to synthesise the entire complementary strand. However, it may be preferred to supplement the activity of the modified RNA polymerase with a specific DNA polymerase.

20

Thus, the single-stranded DNA concatemer and/or template such as cDNA template may be contacted with at least one strand-displacing polymerase in combination with the modified RNA polymerase. One, two, three, four or five different strand-displacing polymerases may be used. The strand-displacing type polymerase may be any suitable polymerase, such that it synthesises polymers of DNA. A DNA polymerase may be highly stable, such that its activity is not substantially reduced by prolonged incubation under process conditions. Therefore, the enzyme preferably has a long half-life under a range of process conditions including but not limited to temperature and pH. It is also preferred that a polymerase has one or more characteristics suitable for a manufacturing process.

25

The DNA polymerase preferably has high fidelity, for example through having proofreading activity. Furthermore, it is preferred that a polymerase displays high processivity, high strand-displacement activity and a low Km for dNTPs and DNA. It is preferred that a polymerase does not display DNA exonuclease activity that is not related to its proofreading activity.

30

The skilled person can determine whether or not a given polymerase displays characteristics as defined above by comparison with the properties displayed by commercially available polymerases, e.g., Phi29 (New England Biolabs, Inc., Ipswich, MA, US), Deep Vent (New England Biolabs, Inc.) and

Bacillus stearothermophilus (Bst) DNA polymerase (New England Biolabs, Inc.). Where a high processivity is referred to, this typically denotes the average number of nucleotides added by a polymerase enzyme per association/dissociation with the template, i.e., the length of primer extension obtained from a single association event. Preferred strand displacement-type polymerases are Phi 29, Deep Vent and Bst DNA Polymerase I or variants of any thereof.

"Strand displacement" describes the ability of a polymerase to displace complementary strands on encountering a region of double stranded DNA during synthesis. The template is thus amplified by displacing complementary strands and synthesizing a new complementary strand. Thus, during strand displacement replication, a newly replicated strand will be displaced to make way for the polymerase to replicate a further complementary strand. The amplification reaction initiates when an annealed strand with a free 3'OH end, such as a primer or the free end of a single stranded template anneals to a complementary sequence on a template (both are priming events). As DNA synthesis proceeds, if it encounters a further primer or other strand annealed to the template, the polymerase displaces this and continues its strand elongation. The strand displacement generates newly synthesised single strands of DNA which can act as a template for more priming events. The priming of the newly synthesised DNA leads to hyper-branching, and a high yield of products. It should be understood that strand displacement amplification methods differ from PCR-based methods in that cycles of denaturation are not essential for efficient DNA amplification, as double-stranded DNA is not an obstacle to continued synthesis of new DNA strands. Strand displacement amplification may only require one initial round of heating, to denature the initial template if it is double stranded, to allow the primer to anneal to the primer binding site if used. Following this, the amplification may be described as isothermal, since no further heating or cooling is required. In contrast, PCR methods require cycles of denaturation (i.e., elevating temperature to 94 degrees centigrade or above) during the amplification process to melt double-stranded DNA and provide new single stranded templates. During strand displacement, the polymerase will displace strands of already synthesised DNA. Further, it will use newly synthesised DNA as a template, ensuring rapid amplification of DNA.

A strand displacement polymerase used in the process of the invention preferably has a processivity of at least 20 kb, more preferably, at least 30 kb, at least 50 kb, or at least 70 kb or greater. In one embodiment, the strand displacement DNA polymerase has a processivity that is comparable to, or greater than phi29 DNA polymerase.

Strand displacement replication occurs during the process of the invention. During strand displacement replication, the template is amplified by displacing already replicated strands, which have been synthesised by the action of the polymerase, in turn displacing another strand, which can be the original complementary strand of a double stranded template, or a newly synthesised

complementary strand, the latter synthesised by the action of a polymerase on an earlier primer annealed to the template (which can be generated using the modified DNE dependent RNA polymerase). Thus, the amplification of the template may occur by displacement of replicated strands through strand displacement replication of another strand. This process may be described as strand displacement amplification or strand displacement replication.

One strand displacement replication process is rolling circle amplification/replication (RCA). The term RCA describes the ability of RCA-type polymerases to continuously progress around a circular DNA template strand whilst extending a hybridised primer (however generated). A closed linear DNA template can be denatured to form a single stranded circular DNA. However, in the case of the present invention, this is not necessary due to the ability of the modified RNA polymerase to "strand invade". *De novo* DNA synthesis, typically beginning at the sequence of the RNA polymerase promoter creates a complementary strand as it progresses, copying the cDNA template directly into a single-stranded DNA concatemer, and then into a double stranded DNA.

Amplification from a cDNA template leads to formation of linear amplicons which are single strands of DNA with multiple repeats of amplified cDNA linked in series. The amplicon is considered to be in the opposite polarity to the original polarity of the closed linear DNA template. However, since each closed linear template includes both the plus and minus strands, the amplicon includes alternate minus and plus strand sequences. These linear amplicons can therefore form duplexed portions in which plus and minus sequences hybridise. The amplicon, nevertheless, serves as the basis for multiple initiations of *de novo* complementary or second strand synthesis, regardless of the presence of these regions of duplexed DNA, resulting in formation of double stranded DNA products comprising multiple repeats of the individual units (templates) amplified by the polymerase. There are thus multiple copies of each amplified "single unit" DNA in the concatemeric double stranded DNA products.

RCA DNA polymerases are particularly preferred for use in the processes of the present invention. The products of RCA-type strand displacement replication processes, which are concatemeric, may require processing to release single unit DNAs. This is desirable if single units of DNA are required.

The contacting of the single-stranded DNA concatemer and/or template such as cDNA template with the modified RNA polymerase in the presence of one or more deoxynucleotides may take place under conditions promoting DNA synthesis. The conditions include the presence of appropriate cofactors for the enzymes, such as in some cases divalent salts such as magnesium or manganese salts. The conditions also include a temperature and buffer allowing for DNA synthesis. An example of preferred conditions used in the present invention include a buffer, 30mM Tris-HCl pH 7.5, 20mM KCl, 8mM MgCl.

Typically, in order to synthesise DNA, the polymerase requires a supply of nucleotides. A nucleotide is a monomer, or single unit, of nucleic acids, and nucleotides are composed of a nitrogenous base, a five-carbon sugar (ribose or deoxyribose), and at least one phosphate group. Any suitable nucleotide may be used. The nitrogenous base may be adenine (A), guanine (G), thymine (T), cytosine (C), and uracil (U). The nitrogenous base may also be modified bases, such as 5-methylcytosine (m5C), pseudouridine (Ψ), dihydrouridine (D), inosine (I), and 7-methylguanosine (m7G).

It is preferred that the five-carbon sugar is a deoxyribose, such that the nucleotide is a deoxynucleotide. The nucleotide may be in the form of deoxynucleoside triphosphate, denoted dNTP. This is a preferred embodiment of the present invention. Suitable dNTPs may include dATP (deoxyadenosine triphosphate), dGTP (deoxyguanosine triphosphate), dTTP (deoxythymidine triphosphate), dUTP (deoxyuridine triphosphate), dCTP (deoxycytidine triphosphate), dITP (deoxyinosine triphosphate), dXTP (deoxyxanthosine triphosphate), and derivatives and modified versions thereof. It is preferred that the dNTPs comprise one or more of dATP, dGTP, dTTP or dCTP, or modified versions or derivatives thereof. It is preferred to use a mixture of dATP, dGTP, dTTP and dCTP or modified version thereof. The deoxynucleotide may be provided as any appropriate salt, with any appropriate counter-ion, as disclosed by the present applicants in WO2020/035698, WO2021/161051 and/or WO2023/021286, incorporated herein by reference.

Other conditions promoting DNA synthesis include the presence of metal ions, suitable buffering agents/pH and other factors which are required for enzyme performance or stability. Suitable conditions include any conditions used to provide for activity of polymerase enzymes known in the art.

For example, the pH of the reaction mixture may be within the range of 3 to 12, preferably 5 to 9 or about 7, such as about 7.9. pH may be maintained in this range by use of one or more buffering agents. Such buffers include, but are not restricted to MES, Bis-Tris, ADA, ACES, PIPES, MOBS, MOPS, MOPSO, Bis-Tris Propane, BES, TES, HEPES, DIPSO, TAPSO, Trizma, HEPPSO, POPSO, TEA, EPPS, Tricine, Gly-Gly, Bicine, HEPBS, TAPS, AMPD, TABS, AMPSO, CHES, CAPSO, AMP, CAPS, CABS, phosphate, citric acid-sodium hydrogen phosphate, citric acid-sodium citrate, sodium acetate-acetic acid, imidazole and sodium carbonate-sodium bicarbonate.

The suitable conditions may also comprise metal ions. The reaction mixture may also comprise salts of metals such as, but not limited to, salts of divalent metal ions: magnesium (Mg^{2+}), manganese (Mn^{2+}), calcium (Ca^{2+}), beryllium (Be^{2+}), zinc (Zn^{2+}) and strontium (Sr^{2+}), or salts of monovalent metal ions, including but not limited to lithium (Li^+), sodium (Na^+) potassium (K^+) or ammonium (NH_4^+) and

derivatives thereof. The salts may include chlorides, acetates and sulphates. Other salts that may be included are ammonium salts, in particular ammonium sulphate.

Detergents may also be included in the amplification conditions. Examples of suitable detergents include Triton X-100, Tween 20 and derivatives of either thereof. Stabilising agents may also be included in the reaction mixture. Any suitable stabilising agent may be used, in particular, bovine serum albumin (BSA) and other stabilising proteins. Reaction conditions may also be improved by adding agents that relax DNA and make template denaturation easier. Such agents include, for example, dimethyl sulphoxide (DMSO), formamide, glycerol and betaine. DNA condensing agents may also be included in the reaction mixture. Such agents include, for example, polyethylene glycol or cationic lipid or cationic polymers.

It should be understood that the skilled person is able to modify and optimise amplification and incubation conditions for the process of the invention using these additional components and conditions on the basis of their general knowledge. Likewise, the specific concentrations of particular agents may be selected on the basis of previous examples in the art and further optimised on the basis of general knowledge. As an example, the amount of polymerase present in the reaction mixture may be optimised. This may involve making further addition of polymerase enzyme to the reaction mixture during the DNA synthesis. As a further example, the amount of DNA template may be optimised. This may involve making further addition of DNA template to the reaction mixture during DNA synthesis.

As an example, a suitable reaction buffer used in rolling circle amplification-based methods in the art is 50mM Tris HCl, pH 7.5, 10mM MgCl₂, 20mM (NH₄)₂SO₄, 5% glycerol, 0.2mM BSA, 1mM dNTPs. A preferred reaction buffer used in the RCA amplification of the invention is 30mM Tris-HCl pH 7.4, 30mM KCl, 7.5mM MgCl₂, 10mM (NH₄)₂SO₄, 4mM DTT, 2mM dNTPs. This buffer is particularly suitable for use with Phi29 RCA polymerase.

The DNA synthesis conditions may also comprise use of one or more additional proteins. Any DNA synthesis reaction may be carried out in the presence of at least one pyrophosphatase, such as Yeast Inorganic pyrophosphatase. Two, three, four, five or more different pyrophosphatases may be used. These enzymes are able to degrade pyrophosphate generated by the polymerase from dNTPs during strand replication. Build-up of pyrophosphate in the reaction can cause inhibition of DNA polymerases and reduce speed and efficiency of DNA amplification. Pyrophosphatases can break down pyrophosphate into non-inhibitory phosphate. An example of a suitable pyrophosphatase for use in the process of the present invention is *Saccharomyces cerevisiae* pyrophosphatase, available commercially from New England Biolabs, Inc.

Any single-stranded binding protein (SSBP) may be used in the process of the invention, to stabilise single-stranded DNA. SSBPs are essential components of living cells and participate in all processes that involve ssDNA, such as DNA replication, repair and recombination. In these processes, SSBPs bind to transiently formed ssDNA and may help stabilise ssDNA structure. An example of a suitable SSBP
5 for use in the process of the present invention is T4 gene 32 protein, available commercially from New England Biolabs, Inc.

It is central to the method of the invention that concatemers which are comprised of two distinct complementary strands of DNA are produced.

The concatemeric single strand of DNA as described herein may comprise multiple repeat units, each unit may be the sequence for a closed linear double stranded DNA as defined herein. Each unit may
10 thus comprise two portions of a protelomerase recognition sequence (which may be the same or different sequences)

It will be understood that each protelomerase recognition sequence is present as a portion as the concatemer is single stranded.

15 An additional step of processing the double stranded DNA, once obtained, may be added.

In addition to the amplification step, a process of the invention for amplification of closed linear DNA also comprises a processing step for production of closed linear DNA. Amplified DNA is contacted with at least one protelomerase under conditions promoting production of closed linear DNA. This simple processing step based on protelomerase is advantageous over other methods used for production of
20 closed linear DNA molecules. The amplification and processing steps can be carried out simultaneously or concurrently. However, preferably, the amplification and processing steps are carried out sequentially with the processing step being carried out subsequent to the amplification step (i.e., on amplified DNA).

A protelomerase is any polypeptide capable of cleaving and re-joining a template comprising a protelomerase recognition sequence in order to produce a covalently closed linear DNA molecule.
25 Thus, the protelomerase has DNA cleavage and ligation functions. Enzymes having protelomerase-type activity have also been described as telomere resolvases (for example in *Borrelia burgdorferi*). A typical substrate for protelomerase is circular double stranded DNA. If this DNA contains a complete protelomerase recognition sequence, the enzyme can cut the DNA at this sequence and ligate the
30 ends to create a linear double stranded covalently closed DNA molecule. The requirements for protelomerase recognition sequences are discussed above. As also outlined above, the ability of a given polypeptide to catalyse the production of closed linear DNA from a template comprising a protelomerase recognition sequence can be determined using any suitable assay described in the art.

The production of closed linear DNA may require the use of at least one protelomerase. The process of the invention may comprise use of more than one protelomerase, such as two different protelomerases, one for each end of the closed linear DNA molecule.

Examples of suitable protelomerases include those from bacteriophages such as phiHAP-1 from
5 *Halomonas aquamarina* (SEQ ID NO: 1 and 2), PY54 from *Yersinia enterocolytica* (SEQ ID NO: 3 and 4),
phiK02 from *Klebsiella oxytoca* (SEQ ID NO: 5 and 6), VP882 from *Vibrio sp.* (SEQ ID NO: 7 and 8),
Vp58.5 from *Vibrio parahaemolyticus* (SEQ ID NO: 13 and 14) and N15 from *Escherichia coli* (SEQ ID
NO: 9 and 10), or variants of any thereof. Use of bacteriophage N15 protelomerase or a variant thereof
is particularly preferred. This enzyme is also referred to as TelN. These enzymes are further described
10 in WO2012/017210, incorporated herein by reference.

The processes of the present invention may be performed with a closed linear DNA template that
comprises portions of protelomerase recognition sequence only at the closed ends of the template.
In this instance, a cognate protelomerase for each end will be required to convert the double stranded
concatemeric DNA produced using the methods of the invention into closed linear DNA products. In
15 some instances, the same protelomerase will be sufficient for this task, since each end is a portion of
the same protelomerase recognition sequence.

The process of the invention may be performed with a double stranded circular template that may
comprise one or more protelomerase recognition sequences. If more than one protelomerase
recognition sequence is present, these may be the same or different recognition sequences.

20 The produced double stranded DNA from the methods described herein is preferably incubated with
at least one protelomerase under conditions promoting production of closed linear DNA. In other
words, the conditions promote the cleavage and re-ligation of a duplex DNA comprising a
protelomerase recognition sequence to form a covalently closed linear DNA with hairpin ends.
Conditions promoting production of closed linear DNA comprise use of any temperature allowing for
25 production of closed linear DNA, commonly in the range of 20 to 90 degrees centigrade. The
temperature may preferably be in a range of 25 to 40 degrees centigrade, such as about 25 to about
35 degrees centigrade, or about 30 degrees centigrade. Appropriate temperatures for a specific
protelomerase may be selected according to the principles outlined above in relation to temperature
conditions for DNA polymerases. A suitable temperature for use with E.coli bacteriophage TelN
30 protelomerase of SEQ ID NO: 15 is about 25 to about 35 degrees centigrade, such as about 30 degrees
centigrade. Conditions promoting the production of closed linear DNA also include the presence of
double stranded DNA concatemers, with both portions of the protelomerase recognition sequence
forming a complete site upon which the protelomerase may act.

Conditions promoting production of closed linear DNA also comprise the presence of a protelomerase and suitable buffering agents/pH and other factors which are required for enzyme performance or stability. Suitable conditions include any conditions used to provide for activity of protelomerase enzymes known in the art. For example, where E.coli bacteriophage TelN protelomerase is used, a
5 suitable buffer may be 20mM Tris HCl, pH 7.6; 5mM CaCl₂; 50 mM potassium glutamate; 0.1 mM EDTA; 1mM dithiothreitol (DTT). Agents and conditions to maintain optimal activity and stability may also be selected from those listed for DNA polymerases.

In some embodiments, it may be possible to use the same conditions for activity of protelomerase as are used for DNA synthesis. In particular, use of the same conditions is described where DNA synthesis
10 and processing by protelomerase are carried out simultaneously or concurrently. In other embodiments, it may be necessary to change reaction conditions where conditions used to provide optimal RNA or DNA polymerase activity lead to sub-optimal protelomerase activity. Removal of specific agents and change in reaction conditions may be achievable by filtration, dialysis and other methods known in the art. The skilled person would readily be able to identify conditions allowing for
15 optimal DNA polymerase activity and/or protelomerase activity.

In a particularly preferred embodiment, for use with phi29, the DNA amplification is carried out under buffer conditions substantially identical to or consisting essentially of 35 mM Tris-HCl, 50 mM KCl, 14 mM MgCl₂, 10 mM (NH₄)₂ SO₄, 4 mM DTT, 1 mM dNTP at a temperature of 25 to 35 degrees centigrade, such as about 30 degrees centigrade.

20 The processing step with protelomerase may then preferably be carried out with TelN, and/or preferably under buffer conditions substantially identical to or consisting essentially of 20mM Tris HCl, pH 7.6; 5mM CaCl₂; 50 mM potassium glutamate; 0.1mM EDTA; 1mM dithiothreitol (DTT) at a temperature of 25 to 35 degrees centigrade, such as about 30 degrees centigrade.

Following production of closed linear DNA by the action of protelomerase, the process of the invention
25 for generation of closed linear DNA may further comprise a step of purifying the linear covalently closed DNA product. The purification referred to above will typically be performed to remove any undesired products. Purification may be carried out by any suitable means known in the art. For example, processing of amplified DNA or linear covalently closed DNA may comprise phenol/chloroform nucleic acid purification or the use of a column which selectively binds nucleic acid,
30 such as those commercially available from Qiagen. The skilled person can routinely identify suitable purification techniques for use in isolation of amplified DNA.

EXAMPLES

The invention will now be described in relation to several non-limiting examples.

MATERIALS AND METHODS (EXAMPLES 1 - 3)**REAGENTS**

- 5 The following reagents supplied were used in the presented examples:
- 200mM dNTPs, stock comprising equimolar amounts of dATP, dCTP, dGTP and dTTP (Thermo Fischer Scientific)
- Phi29 DNA polymerase, stock concentration 250,000 U/mL (NEB)
- Thermostable pyrophosphatase, stock concentration 2000 U/mL (NEB)
- 10 T7 RNA polymerase PLUS, stock concentration 200 U/ μ L (Thermo Fischer Scientific)
- clDNA templates: dbDNA 1 and dbDNA 2 (produced in-house)
- Nuclease free water (Sigma Aldrich)
- 5M NaOH (Sigma Aldrich)
- 5M betaine (Sigma Aldrich)
- 15 Betaine powder (Cayman Chemical Company)
- PEG-8000 (Applchem)
- 57.7 μ M Protelomerase TeIN (Sekisui)
- Tris-Base (Thermo Fisher Scientific)
- Tris-HCl (Sigma Aldrich)
- 20 5M NaCl (Sigma Aldrich)
- 2M MgCl₂ (Sigma Aldrich)
- 20 mg/mL Proteinase K Solution (Ambion)

Aim

The chosen modified RNA polymerase specified in the Reagents was tested in a DNA synthesis reaction to demonstrate whether such an enzyme could be used (a) to initiate DNA synthesis from a cDNA template, (b) permit the synthesis of a complementary strand for a single-stranded DNA concatemer which contains self-complementary sequences, as well as to determine the advantages of using such an enzyme in the synthesis of DNA. The process used to test was the production of closed linear DNA molecules from a closed linear DNA template. Such a process is useful, as the intermediate product, double stranded DNA, must be present for the closed linear DNA to be produced. Protelomerase enzymes need a double stranded recognition sequence to cleave and re-ligate double stranded DNA. Thus, production of cDNA inherently proves that a double stranded DNA is present in the reaction mixture.

EXAMPLE 1**DNA synthesis setup**

- 15 Reactions were set up at a 500 μ L scale as follows: all reagents were added one after the other, to make up the main mix. Samples were gently mixed by pipetting, then the RNA polymerase PLUS, a DNA polymerase and pyrophosphatase were added. Reactions were incubated at a temperature of 30°C for a chosen period of 68 hours, followed by sample processing and quantification as detailed below.
- 20 The experimental protocol reaction setup is shown in Table 1, while the materials and methods for RCA are described above.

The effects of a diverse priming system and buffer addition on DNA synthesis**Table 1** – DNA synthesis by Rolling Circle Amplification (RCA) Reaction components for examining the effectiveness of T7 RNA polymerase PLUS priming with different buffers and additives, and one dNTP/RNA polymerase PLUS concentration variation each.

	Reagent	Stock conc.	Final rxn conc.	Volume							
Condition				1	2	3	4	5	6	7	8
Main Mix	dbDNA Template 1	0.750 g/L	0.5 µg	0.67 µL	0.67 µL	0.67 µL	0.67 µL	0.67 µL	0.67 µL	0.67 µL	0.67 µL
	NaOH	1 M	10 mM	5.0 µL	5.0 µL	5.0 µL	5.0 µL	5.0 µL	5.0 µL	5.0 µL	5.0 µL
	Small water vol	-	to 30 µL	24.33 µL	24.33 µL	24.33 µL	24.33 µL	24.33 µL	24.33 µL	24.33 µL	24.33 µL
	Nucleotide complex	200 mM	Variable	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL	50 µL	25 µL
	Betaine	5 M	Variable	100 µL	50 µL	200 µL	-	100 µL	100 µL	100 µL	100 µL
	Tris buffer (7.9)	1 M	30 mM	-	-	-	-	-	15 µL	-	-
	PEG	50%	14%	-	-	-	140 µL	140 µL	-	-	-
	MgCl ₂	2 M	Variable	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	5.0 µL	2.5 µL
	Large water vol	-	to 470 µL total	342.5 µL	392.5 µL	242.5 µL	302.5 µL	202.5 µL	327.5 µL	315.0 µL	342.5 µL
Enzyme 1	T7 RNA polymerase PLUS	200 U/µL	Variable	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	4.0 µL
Enzyme 2	Pyrophosphatase	2000 U/mL	2 U	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Enzyme 3	Phi29 DNA polymerase	250,000 U/mL	1.6 µg/mL	1.0 µL	1.0 µL	1.0 µL	1.0 µL	1.0 µL	1.0 µL	1.0 µL	1.0 µL

Sample processing procedure

To each aliquot, 0.65 $\mu\text{M}/\text{mM}$ dNTPs of 57.7 μM TelN was added to each sample and mixed by vigorous shaking. The samples were allowed to incubate for 90 min at 30°C. Samples were run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the amplified DNA and confirm the presence of the target closed linear DNA (dbDNA). After confirming the presence and size of the desired the bands, a gel imager was used to capture an image as shown in Figure 1.

Results

This data shows that RCA was successful in using T7 RNA polymerase PLUS alone, suggesting that RCA works can work in the absence of an exogenous primer. As shown in Figure 1, varying types and combinations of buffering agents and additives caused differences in the synthesis of DNA. Nevertheless, this Examples demonstrates that the modified RNA polymerase permits DNA amplification/duplexing.

EXAMPLE 2

Example 1 demonstrates that the present invention can be used for DNA synthesis in the tested conditions with the presence of additional buffering agents. This initial data identifies that the method may be effective in combination with the identified buffer and additive combination (denoted with an arrow in Figure 1).

The effect of buffering agents and additives was therefore further investigated. Additional research suggested and subsequent experiments showed that incubation at a higher temperature may increase initiation efficiency of the RNA polymerase PLUS, and thus total yield. Example 2 was therefore performed in the presence of a new buffer and additive combination as well as an additional pre-incubation step. Furthermore, the experiment was set up with a range of template concentrations to examine the effect on total yield.

DNA synthesis setup

Reactions were set up at a 500 μL scale as follows: all reagents were added one after the other, to make up the main mix. Samples were gently mixed by pipetting, then the RNA polymerase PLUS was added and allowed to incubate for 60 min at 37°C. Subsequently, a DNA polymerase and pyrophosphatase were added. Reactions were incubated at a temperature of 30°C for a chosen period of 68 hours, followed by sample processing and quantification as detailed below.

The experimental protocol reaction setup is shown in Table 2.

Sample processing procedure

- 5 To each aliquot, 0.65 $\mu\text{M}/\text{mM}$ dNTPs of 57.7 μM TelN was added to each sample and mixed by vigorous shaking. The samples were allowed to incubate for 90 min at 30°C. Samples were run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the synthesised DNA and confirm the presence of the target dbDNA. A gel imager was used to capture an image as shown in Figure 2 (A). Proteinase K was then added to digest TelN overnight at 37°C.
- 10 Following, 5% of 50% PEG 8000, 500 mM of 5M NaCl and 100 mM of 2M MgCl_2 were added to each sample. Solutions were mixed by vigorously shaking. The DNA was then pelleted by centrifugation in a microcentrifuge (14,000 x g, 30 minutes). The supernatants were carefully decanted, and the pellets were resuspended in 500 μL of water by positive-displacement pipetting, vigorous shaking and vortexing. This purification step was then repeated once more. Once fully resuspended, initial reaction
- 15 DNA concentrations were quantified from UV absorption measurements on a nanodrop spectrophotometer and recorded.

An exonuclease was added to remove unwanted material from the samples, which were allowed to incubate overnight at 37°C. Samples were then run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the amplified DNA and confirm successful clean-up. A gel imager was

20 used to capture an image as shown in Figure 2 (B).

As a final purification step, the above PEG process was repeated twice more. Once fully resuspended, final reaction DNA concentrations were quantified from UV absorption measurements on a nanodrop spectrophotometer and recorded. Samples were additionally run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the final purified products. A gel imager was used to

25 capture an image as shown in Figure 2 (C).

All concentrations presented in Table 3 are expressed in g/L of original volume vs dNTP concentrations used.

Results – Figure 2

5 **Table 3** – DNA yields achieved at different concentrations of template.

Condition	Template (ug)	Raw Yields (g/L)	Final Yields (g/L)
1	0.005	0.07	0.00
2	0.05	0.01	0.00
3	0.5	0.53	0.02
4	1	0.52	0.03
5	2	0.36	0.04
6	5	0.34	0.05
7	12.5	0.59	0.07
8	50	0.45	0.11

While digestion of amplified DNA into dbDNA requires further optimisation (noted by the high molecular weight band in Figure 2 (A)), this data shows a steady increase in DNA yield with increasing template concentration. All well-trapped material and high molecular weight bands were
10 successfully removed from the samples post-digestion, likely accounting for the majority of the Raw Yield. Final purification then appeared to significantly decrease the overall size of the bands. The best final yield achieved at 0.11 g/L with 50 ug of dbDNA template. It should be noted that the 0.5 ug bands shown in Figures 1 and 2 were produced by different dbDNA templates and therefore cannot be directly compared.

15

This data leads the inventors to conclude that increasing dbDNA template is useful in improving yield. Further conditions are to be explored to further optimise yield.

EXAMPLE 3

20 Example 2 demonstrates that the modified RNA polymerase is functional in initiating synthesis of DNA in the presence of specific buffering agents and additive combinations, with an additional incubation and at an increasing range of template concentrations. Example 3 further investigated the effect of template concentration against RNA polymerase PLUS concentration to determine if such was useful.

DNA synthesis setup

Reactions were set up at a 500 µL scale as follows: all reagents were added one after the other, to make up the main mix. Samples were gently mixed by pipetting, then the RNA polymerase PLUS was added and allowed to incubate for 60 min at 37°C. Subsequently, a DNA polymerase and pyrophosphatase were added. Reactions were incubated at a temperature of 30°C for a chosen period of 68 hours, followed by sample processing and quantification as detailed below.

The experimental protocol reaction setup is shown in Table 4.

The effects of a diverse priming system, template concentrations and RNA polymerase PLUS concentration on DNA synthesis

Table 4 – DNA synthesis by Rolling Circle Amplification (RCA) Reaction components for examining the effectiveness of pre-incubated T7 RNA polymerase PLUS priming with a chosen buffer and additive combination, at a range of template and T7 RNA polymerase PLUS concentrations (N.B.: TLG volumes are adjusted to achieve desired concentration based on quantity of TLG-buffered template).

	Reagent	Stock conc.	Final rxn conc.	Volume		
Condition				1	2	3
Main Mix	dbDNA Template 2	0.266 g/L	Var. range (µg)	18.8 µL	18.8 µL	147.8 µL
	NaOH	1 M	10 mM	5.0 µL	5.0 µL	5.0 µL
	Small water vol	-	to 30 µL	6.25 µL	6.25 µL	-
	Nucleotide complex	200 mM	10 mM	25 µL	25 µL	25 µL
	Betaine	5 M	1 M	100 µL	100 µL	100 µL
	TLG buffer (7.9)	10x	1x	48.2 µL	48.2 µL	35.2 µL
	MgCl ₂	2 M	10 mM	2.5 µL	2.5 µL	2.5 µL
Enzyme 1	Large water vol	-	to 470 µL total	294.3 µL	294.3 µL	184.7 µL
	T7 RNA pol. PLUS (1h, 37°C)	200 U/µL	Var. (diluted to same vol)	0.5 µL	0.5 µL	0.5 µL
	Enzyme 2	Pyrophos-phatase	2000 U/mL	2 U	0.5 µL	0.5 µL
Enzyme 3	Phi29 DNA pol.	250,000 U/mL	2.4 µg/mL	1.5 µL	1.5 µL	1.5 µL

Sample processing procedure

To each aliquot, 0.65 $\mu\text{M}/\text{mM}$ dNTPs of 57.7 μM TelN was added to each sample and mixed by vigorous shaking. The samples were allowed to incubate for 90 min at 30°C. Samples were run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the amplified DNA and confirm the presence of the target dbDNA. Proteinase K was then added to digest TelN overnight at 37°C.

Following, 5% of 50% PEG 8000, 500 mM of 5M NaCl and 100 mM of 2M MgCl_2 were added to each sample. Solutions were mixed by vigorously shaking. The DNA was then pelleted by centrifugation in a microcentrifuge (14,000 x g, 30 minutes). The supernatants were carefully decanted, and the pellets were resuspended in 500 μL of water by positive-displacement pipetting, vigorous shaking and vortexing. This purification step was then repeated once more. Once fully resuspended, initial reaction DNA concentrations were quantified from UV absorption measurements on a nanodrop spectrophotometer and recorded. Samples were run by gel electrophoresis on a 0.8% agarose gel. These were performed to better visualise the amplified DNA post-TelN. A gel imager was used to capture an image as shown in Figure 3 (A).

An exonuclease was added to remove unwanted material from the samples, which were allowed to incubate overnight at 37°C. Samples were then run by gel electrophoresis on a 0.8% agarose gel. These were performed to visualise the amplified DNA and confirm successful clean-up. A gel imager was used to capture an image as shown in Figure 3 (B).

As a final purification step, the above PEG process was repeated twice more. Once fully resuspended, final reaction DNA concentrations were quantified from UV absorption measurements on a nanodrop spectrophotometer and recorded.

All concentrations presented in Table 5 are expressed in g/L of original volume vs dNTP concentrations used.

Results – Figure 3

Table 5 – DNA yields achieved at different concentrations of template and RNA polymerase PLUS.

Condition	Template (ug)	[T7 RNA pol +]	Raw Yields (g/L)	Final Yields (g/L)
1	5 ug	1/10x (10 U)	0.07	0.04
2		1/100x (1 U)	0.03	0.02
3	39.4 ug	1/10x (10 U)	0.32	0.06

As previously noted, dbDNA yield increases with template. Notably, there is only a 2-fold decrease in yield despite a 10-fold decrease in T7 RNA polymerase PLUS concentration at 5 ug of template. A reduction in T7 RNA polymerase PLUS concentration additionally appears to prevent the accumulation of large bands or help better manage digestion of product into dbDNA. Interestingly, the final yield achieved at 5 ug of template with 1x (100 U) of T7 RNA polymerase PLUS in Example 2 (Table 3) is comparable to the final yield achieved at 5 ug of the same template with 1/10x (10 U) of T7 RNA polymerase PLUS in this Example (Table 4): the final yields recorded were 0.05 g/L and 0.04 g/L respectively. This data leads the inventors to conclude that similar yields to standard can be achieved at significantly lower concentrations of T7 RNA polymerase PLUS, with a potential of achieving an increase in yield through further experimentation.

Materials and Methods for examples 4 to 9

REAGENTS

The following reagents were used in these examples:

200mM dNTPs stock comprising equimolar amounts of dATP, dCTP, dGTP and dTTP (Thermo Fischer Scientific)

300mM rNTPs, stock comprising equimolar amounts of rATP, rCTP, rGTP and rUTP (Thermo Fisher Scientific)

Phi29 DNA polymerase, stock concentration 250,000 U/mL (NEB)

Thermostable pyrophosphatase, stock concentration 2000 U/mL (NEB)

T7 RNA polymerase, stock concentration 20U/μl (Thermo Scientific)

cIDNA templates: dbDNA2 (produced in-house)

Nuclease free water (Sigma Aldrich)

57.7 μ M Protelomerase TelN (Sekisui)

Tris-Base (Thermo Fisher Scientific)

Tris-HCl (Sigma Aldrich)

2 M MgCl_2 (Sigma Aldrich)

5 20g/mL Proteinase K Solution (Ambion)

10X stock of TLG buffer pH7.9 (prepared in-house): 300 mM Tris, 300mM KCl, 75 mM MgSO_4 , 50 mM $\text{NH}_4(\text{SO}_4)_2$

T7, SP6 and KP34 modified RNA polymerases (produced in-house according to SEQ ID No 33, 29 and 31))

10 Experimental design and rationale: Examples 4 to 9

RCA DNA amplification reactions starting from a closed linear DNA (clDNA) template such as dbDNA2 will produce long single stranded DNA concatemers.

When closed linear DNA is amplified via rolling circle amplification, the resultant single stranded DNA concatemer contains sections of sequence that are complementary to each other, due to the nature of the template,

15

If the template is circular, such as a minicircle or plasmid, it will be double stranded. However, rolling circle amplification will still produce single stranded concatemers as the initial amplification product. If the double stranded circular template contains any palindromic sites (also called inverted repeats) then there will be sections of sequence that are complementary to each other, within the single stranded concatemer. Exemplary palindromic sequences include recognition sequences for protelomerases.

20

Sections of self-complementary sequence within a single stranded DNA concatemer may result in intramolecular base-pairing. This intramolecular base pairing can cause the single stranded DNA to fold into unwanted structures such as DNA nanoflowers, radiators and the like. Such structures are hard to access with standard DNA amplification techniques to duplex the DNA strand, which is desirable to produce various DNA products.

25

In some of the following examples, the modified RNA polymerases are added at an earlier stage, particularly during the initial amplification of the template to generate the ssDNA concatemer. This is to show that the duplexing reaction can occur in the complete absence of primer, and to demonstrate that the duplexing seen is due to the action of the modified RNA polymerase alone, rather than any “retained” primer presence.

30

In these Examples, various reactions are analysed, to see whether DNA concatemers can be produced using the modified RNA polymerase. In order to corroborate that duplexing of ssDNA has occurred, the concatemers are processed with protelomerase, which only acts upon a double-stranded recognition sequence, producing cDNA only if the correct duplexed concatemer is present. This processing step therefore provides evidence of success of the duplexing of the single strand using the methods developed by the inventors.

EXAMPLE 4

Preparation of template for RCA and duplexing of ssDNA concatemers.

Reaction was set up at a 300ul scale as follows: Reagents were added and mixed gently by pipetting. Reactions were incubated at a temperature of 30°C for a period of 30 mins.

Reagent	Stock concentration	Final concentration	Volume (in µL)
Plasmid DNA harbouring dbDNA2	436 g/L	350 mg/L	240
TLG buffer	10X	1X	30
TelN	57.7 µM	5.77 µM	30

Proteinase K was then added to digest TelN for 5 hours at 37°C. After inactivating proteinase K, a restriction enzyme and exonuclease were added to remove unwanted material from the samples and the reaction was incubated overnight at 37°C. dbDNA2, was thus prepared was used as template for DNA synthesis.

DNA synthesis setup

Reactions were set up at a 250µL scale with components as described in the table below. Samples made up of the non-enzymatic components were gently mixed by pipetting, then the modified RNA polymerase (T7 Y639F, SP6 Y631F or KP34 Y603F) and pyrophosphatase were added. The reactions were mixed gently by pipetting at incubated at 37°C for 2 hours. Following this pre-incubation, phi29 was added to the reactions and they were incubated at a temperature of 30°C for a chosen period of 24 hours. Samples were then run by gel electrophoresis on a 1.0% agarose gel to visualise the amplified DNA. A gel imager was used to capture an image as shown in Figure 9.

The experimental protocol reaction setup is shown in Table 6, while the materials and methods for RCA are described above. Results are depicted in Figure 9.

The utility of different modified RNA polymerases in RCA reactions with primers to amplify DNA

Table 6 – DNA synthesis by RCA Reaction for examining the effectiveness of different modified RNA polymerase priming with one modified RNA polymerase each in a reaction.

	Reagent	Stock conc.	Final rxn conc.	Volume		
Condition				Modified T7 RNAP	Modified SP6 RNAP	Modified KP34 RNAP
Main Mix	dbDNA Template 2	0.33 g/L	2 mg/L	1.44 µL	1.44 µL	1.44 µL
	TLG buffer	10X	1X	25.0 µL	25.0 µL	25.0 µL
	Nucleotide complex	200 mM	5 mM	6.25 µL	6.25 µL	6.25 µL
	MgCl ₂	2M	20 mM	2.5 µL	2.5 µL	2.5 µL
	Large water vol	-	to 250 µL total	211 µL	209 µL	209 µL
Enzyme 1	Modified RNA polymerase (as mentioned in column headers)	Variable	0.2 uM	3 µL	1.5 µL	1.5 µL
Enzyme 2	Pyrophosphatase	2000U/mL	2U	0.5 µL	0.5 µL	0.5 µL
Enzyme 3	Phi29 DNA polymerase	250,000U/mL	1500U/ml	1.5 µL	1.5 µL	1.5 µL

- 5 This data shows that the DNA template was amplified in rolling circle amplification reactions with the 3 modified RNA polymerases - T7 Y639F, SP6 Y631F or KP34 Y603F in the absence of primer.

While previous data in earlier Examples indicated that denaturation of template may be important for RCA based amplification using T7 RNA polymerase PLUS, the current data shows that the in-house generated modified versions of T7, SP6 and KP34 RNA polymerases in combination with phi29 DNA
 10 polymerase are able to amplify non-denatured, closed ended dsDNA utilising dNTPs only in the absence of rNTPs.

Reactions were also successful when the reaction conditions were modified, such as the presence of buffering agents, including minimal components (data not shown).

EXAMPLE 5

5

The modified polymerases can utilise dNTPs for amplification of DNA along with the DNA polymerase as demonstrated in Example 4. Experiments investigating the contribution of phi29 alone to the reaction and a comparative experiment using wild type T7 RNA polymerase in the process were performed.

10 DNA synthesis setup

Reactions were set up at a 250µL scale with components as described in the table below. Samples made up of the non-enzymatic components were mixed by, then the modified T7 RNA polymerase and pyrophosphatase were added. The reactions were mixed gently by pipetting and incubated at 37°C for 2 hours. Following this pre-incubation, phi29 was added to the reactions and they were
15 incubated at a temperature of 30°C for a chosen period of 24 hours. Samples were then run by gel electrophoresis on a 1.0% agarose gel to visualise the amplified DNA. A gel imager was used to capture an image as shown in Figure 10.

The experimental protocol reaction setup is shown in Table 7. Results are shown in Figure 10.

The activity of phi29 alone and Wild type T7 RNA polymerase in RCA reactions

20

Table 7 – DNA synthesis by Rolling Circle Amplification (RCA) Reaction components for examining the activity of phi29 alone and in combination with Wild-type T7 RNA polymerase.

	Reagent	Stock conc.	Final rxn conc.	Volume	
Condition				1	2
Main Mix	dbDNA Template 2	0.33 g/L	2 mg/L	1.44 µL	1.44 µL
	TLG buffer	10X	1X	25.0 µL	25.0 µL
	Nucleotide complex	200 mM	5 mM	-	-
	Ribonucleotide complex	300 mM	30 mM	-	25 µL
	MgCl ₂	2 M	20 mM	2.5 µL	2.5 µL
	Large water vol	-	to 250 µL total	217 µL	191 µL
Enzyme 1	Wild-type T7 RNA polymerase	20000U/ml	1600U/ml	20 µL	
Enzyme 2	Pyrophosphatase	2000U/mL	2 U	0.5 µL	0.5 µL
Enzyme 3	Phi29 DNA polymerase	250,000 U/mL	1500U/ml	1.5 µL	1.5 µL

The data shown in Figure 10 shows that there is minimal DNA amplification in both reactions with phi29 alone (lane 2) and in combination with wild-type T7 RNA polymerase (lane 1). There is no high molecular weight DNA produced in either reaction. While T7 RNA polymerase PLUS and phi29 together can amplify DNA in the absence of rNTPs and primers, this functionality is not present when wild-type T7 RNA polymerase is used.

EXAMPLE 6

- 10 The modified polymerases can utilise dNTPs for amplification of DNA and also duplexing of ssDNA concatemers when used with a DNA polymerase, such as demonstrated in Example 4. However, since these are mutants of RNA polymerases (modified enzymes), the effect of addition of rNTPs alone or in combination with dNTPs in the reaction was investigated.

Additionally, experiments showed that the removal of the pre-incubation step at 37°C for 2 hours did not affect the efficiency of DNA amplification in reactions with modified T7 RNA polymerase, phi29 and dNTPs (Data not shown). This step was therefore omitted in Example 6.

DNA synthesis setup

- 5
- Reactions were set up at a 250 µL scale with components as described in the table below. Samples made up of the non-enzymatic components were mixed by pipetting, then the modified RNA polymerase, pyrophosphatase and the DNA polymerase were added. The reactions were mixed gently, and they were incubated at a temperature of 30°C for a chosen period of 24 hours. Samples were then run by gel electrophoresis on a 1.0% agarose gel to visualise the amplified DNA. A gel imager was used
- 10
- to capture an image as shown in Figure 11.

The experimental protocol reaction setup is shown in Table 8. Results are shown on Figure 11.

The effect of ribonucleotides alone or in combination with dNTPs on DNA synthesis: Table 8 – DNA synthesis by Rolling Circle Amplification (RCA) Reaction components for examining the effect of rNTPs on the reaction.

	Reagent	Stock conc.	Final rxn conc.	Volume			
Condition				1	2	3	4
Main Mix	dbDNA	0.33g/L	2mg/L	1.44µL	1.44µL	1.44µL	1.44µL
	Template 2						
	TLG buffer	10X	1X	25.0µL	25.0µL	25.0µL	25.0µL
	Nucleotide complex	200mM	5mM	-	-	6.25µL	6.25µL
	Ribonucleotide complex	300mM	30mM	-	25µL	25µL	-
	MgCl ₂	2M	20mM	2.5µL	2.5µL	2.5µL	2.5µL
	Large water vol	-	to 250µL total	217µL	191µL	184µL	211ul
Enzyme 1	Modified T7 RNA polymerase	20uM	0.2uM	3µL	3µL	3µL	3µL
Enzyme 2	Pyrophosphatase	2000U/mL	2 U	0.5µL	0.5µL	0.5µL	0.5µL
Enzyme 3	Phi29 DNA polymerase	250,000 U/mL	1500U/ml	1.5µL	1.5µL	1.5µL	1.5µL

The in-house generated modified T7 RNA polymerase was unable to polymerise ribonucleotides to make high molecular weight nucleic acids (Figure 11, lane 3). From previous Examples it is known that RNA polymerase plus, an enzyme that can accept both dNTPs and rNTPs, in combination with phi29 DNA polymerase can catalyse the formation of high molecular weight DNA. Results from figure 11 and other experiments (data not shown) showed that the in-house generated modified T7 RNA polymerase performed best in the presence of dNTPs only (lane 5) and that the addition of rNTPs over a range of concentrations – 30 mM version selected for analysis (lane 4) negatively impacted the efficiency of the reaction in generating high molecular weight concatemeric DNA. Thus, the modified T7 polymerase prefers dNTPs and the presence of significant concentrations of rNTPs are seen to be inhibitory for DNA duplexing and amplification.

EXAMPLE 7

Example 6 demonstrated that the modified T7 RNA polymerase is functional in initiating synthesis of DNA and in combination with a DNA polymerase, can amplify a template to produce high molecular weight DNA. Example 7 investigated for the presence of the double stranded DNA sequence of interest in the amplified concatemeric DNA in the reaction. The presence of the double stranded DNA sequence would indicate that the reaction conditions permitted the duplexing of the single stranded DNA concatemer produced via amplification of the template.

DNA synthesis setup

Reactions were set up at a 250µL scale with components as described in the table below. Samples made up of the non-enzymatic components were gently mixed by pipetting, then the modified RNA polymerase and pyrophosphatase were added. The reactions were mixed gently by pipetting and incubated at 37°C for 2 hours. Following this pre-incubation, phi29 was added to the reactions and they were incubated at a temperature of 30°C for a chosen period of 24 hours, followed by sample processing as detailed below.

The experimental protocol reaction setup is shown in Table 9. Results are shown in Figures 12A and B.

Processing DNA amplified in RCA to query for the presence of dsDNA of interest

Table 9: DNA synthesis by Rolling Circle Amplification (RCA) Reaction components for examining the amplified DNA for the presence of duplexed sequence of interest.

	Reagent	Stock conc.	Final rxn conc.	Volume
Condition				1
Main Mix	dbDNA Template 2	0.33 g/L	2 mg/L	1.44 µL
	TLG buffer	10X	1X	25.0 µL
	Nucleotide complex	200 mM	5 mM	6.25 µL
	MgCl ₂	2 M	20 mM	2.5 µL
	Large water vol	-	to 250 µL total	211 ul
Enzyme 1	Modified T7 RNA polymerase	20 µM	0.2 µM	3µL
Enzyme 2	Pyrophosphatase	2000 U/mL	2 U	0.5 µL
Enzyme 3	Phi29 DNA polymerase	250,000 U/mL	1500U/ml	1.5 µL

Sample processing

0.65µM/mM dNTPs of 57.7µM TelN was added to the sample and mixed by vigorous shaking. The samples were allowed to incubate for 30 min at 30°C. Proteinase K was then added to digest TelN for 4 hours at 37°C. Following inactivation of Proteinase K, 2 reactions of enzyme digestions were setup. One with restriction enzymes and exonucleases added to remove unwanted material from the samples and a second with restriction enzyme that cuts within the DNA of interest, and exonucleases to confirm that the DNA sequence of interest is indeed being amplified and were allowed to incubate overnight at 37°C. Samples were then run by gel electrophoresis on a 1.0% agarose gel. These were performed to visualise the cleaned-up, amplified DNA to confirm the presence of DNA of interest. A gel imager was used to capture an image as shown in Figure 12(A and B).

The starting template for RCA can be seen in Figure 12A, lane 2. Results seen in Figure 12B show that the DNA sequence of interest is being amplified into a double stranded format. DNA at the expected size obtained post processing of RCA reactions with TelN and enzyme digestion is visible on the agarose gel (Figure 12B, lane 3) and this band is not observed upon digestion with a restriction enzyme that nicks within the sequence of interest (Figure 12B, lane 2). Additionally, the DNA post-processing

was sequenced to confirm its identity (data not shown). This demonstrates that the modified RNA polymerase is capable of duplexing the intermediate single stranded DNA concatemer generated from RCA of a closed linear DNA product. Since the modified RNA polymerase can be added earlier in the process, if required, it may also permit the amplification of closed linear DNA template into an identical closed linear DNA product. Such a reaction may proceed with a DNA polymerase and with dNTPs. The reaction may be conducted in the absence of primers and rNTPs.

Example 8:

Previous examples demonstrated the utility of modified T7 RNA polymerase in combination with a DNA polymerase in amplifying DNA from a double-stranded, closed-ended, linear DNA template, thus, the inventors sought to discover if this could be applied to other templates for RCA, such as amplifying DNA of interest from circular DNA templates.

DNA synthesis setup

Two circular DNA containing minimal sequences and harbouring a single protelomerase site were used as templates for the reactions. These varied in sequence. Reactions were set up at a 250µL scale with components as described in table 10. Samples made up of the non-enzymatic components were gently mixed by pipetting, then the modified RNA polymerase, pyrophosphatase and phi29 were added. The samples were mixed, and reactions were incubated at a temperature of 30°C for a chosen period of 24 hours followed by sample processing as detailed below.

The experimental protocol reaction setup is shown in Table 10. Results are shown on Figure 13.

RCA based amplification of DNA from non-plasmid circular templates with varying sequences

Table 10: DNA synthesis by Rolling Circle Amplification (RCA) Reaction

	Reagent	Stock conc.	Final rxn conc.	Volume	
Condition				1	2
Main Mix	Circular template 1	80mg/L	2mg/L	6.24µL	
	Circular template 2	160mg/L	2mg/L		3.11µL
	TLG buffer	10X	1X	25.0µL	25.0µL
	Nucleotide complex	200mM	5mM	6.25µL	6.25µL
	MgCl ₂	2M	5mM	0.63µL	0.63µL
	Large water vol	-	to 250µL total	211ul	211ul
Enzyme 1	Modified T7 RNA polymerase	20uM	0.2uM	3µL	3µL
Enzyme 2	Pyrophosphatase	2000U/mL	2 U	1.0µL	1.0µL
Enzyme 3	Phi29 DNA polymerase	250,000U/mL	500U/ml	0.5µL	0.5µL

Sample processing

0.65µM/mM dNTPs of 57.7µM TelN was added to the sample and mixed by vigorous shaking. The samples were allowed to incubate for 30 min at 30°C. Proteinase K was then added to digest TelN for 4 hours at 37°C. Following inactivation of Proteinase K, 2 reactions of enzyme digestions were setup. One with restriction enzymes and exonucleases added to remove unwanted material from the samples and a second with restriction enzyme that cuts within the DNA of interest, and exonucleases to confirm that the DNA sequence of interest is indeed being amplified and were allowed to incubate overnight at 37°C. Samples were then run by gel electrophoresis on a 1.0% agarose gel. These were performed to visualise the cleaned-up, amplified DNA to confirm the presence of DNA on interest. A gel imager was used to capture an image as shown in Figure 13.

DNA of interest was amplified in reactions using both circular DNA as starting templates for RCA (Figure 13, lanes 1 and 2). This demonstrated that the process was equally applicable to ssDNA concatemers generated from circular templates of varying sequence.

EXAMPLE 9:

- 5 The previous Examples have demonstrated the utility of the modified T7 RNA polymerase in duplexing DNA from ssDNA concatemers. This Example seeks to separate the amplification of the original template from the duplexing step, to show that the mechanism of original amplification of the template can be any appropriate method.

DNA synthesis setup

- 10 Plasmid DNA was used as templates for these reactions. Reactions were set up at a 250µL scale as follows: denaturing mix was made by adding the pDNA, primers, NaOH and water and mixing gently by pipetting. The mix was incubated at room temperature for 15 minutes. This was added to the reaction mix comprising of dNTPs and MgCl₂. Samples were gently mixed by pipetting, then the pyrophosphatase and phi29 were added (as described in table 11). The samples were mixed, and
15 reactions were incubated at a temperature of 30°C for a chosen period of 24 hours.

- An aliquot (2%) of the concatemers from this reaction were then added to a second reaction which was set up as described in table 12. Reaction 1 described in table 12 was set up as follows: denaturing mix was made by adding the concatemers, primers, NaOH and water and mixing gently by pipetting. The mix was incubated at room temperature for 15 minutes. This was added to the reaction mix
20 comprising of dNTPs and MgCl₂. Samples were gently mixed by pipetting, then the pyrophosphatase and phi29 were added. Reactions 2 to 4 were set up as follows: all reagents were added one after the other, to make up the main mix. Samples were gently mixed by pipetting, then the specified enzymes were added, and the reaction was mixed by pipetting. The reactions were incubated at a temperature of 30°C for a chosen period of 24 hours. A small volume of the reactions was resolved on 1% agarose
25 by gel electrophoresis. This was followed by sample processing as detailed below.

The experimental protocol reaction setup is shown in Table 11. Results are shown in Figures 14A and 14B.

Table 11: DNA synthesis by Rolling Circle Amplification (RCA) first reaction to generate ssDNA concatemers from a pDNA template

	Reagent	Stock conc.	Final rxn conc.	Volume
Condition				1
Denaturing mix	pDNA 1	0.8 g/L	2 mg/L	0.56 µL
	Primer	5 mM	12.5 µM	0.62 µL
	NaOH	1M	10 mM	2.5 µL
	water vol			11.32 µL
Reaction mix	Nucleotide complex	200 mM	20 mM	25 µL
	MgCl ₂	2 M	20 mM	2.5 µL
	Large water vol	-	to 250 µL total	216 µL
Enzyme 1	Pyrophosphatase	2000U/mL	2 U	1.0 µL
Enzyme 2	Phi29 DNA polymerase	250,000U/mL	500U/ml	0.5 µL

Table 12: DNA duplexing step

	Reagent	Stock conc.	Final rxn conc.	Volume						Volume	
Condition				1	2	Condition				3	4
Denaturing mix	DNA concatemers			5 μ L	5 μ L	Main Mix	DNA concatemers			5 μ L	5 μ L
	Primer	5mM	12.5 μ M	0.62 μ L	0.62 μ L						
	NaOH	1M	10mM	2.5 μ L			TLG buffer	10X	1X	25 μ L	25 μ L
	water vol			6 μ L	8.5 μ L		Nucleotide complex	200 mM	5mM	6.25 μ L	6.25 μ L
Reaction mix	Nucleotide complex	200mM	20mM	25 μ L	25 μ L		MgCl ₂	2M	5mM	0.62 μ L	0.62 μ L
	MgCl ₂	2M	20mM	2.5 μ L	2.5 μ L		Large water vol	-	to 250 μ L total	200 μ L	200 μ L
	Large water vol	-	to 250 μ L total	216 μ L	216 μ L	Enzyme 1	Modified T7 RNA polymerase	20uM	0.2uM	3 μ L	
Enzyme 1	Pyrophosphatase	2000 U/mL	2 U	1.0 μ L	1.0 μ L	Enzyme 2	Pyrophosphatase	2000 U/mL	2 U	1 μ L	
Enzyme 2	Phi29 DNA polymerase	250,000 U/mL	500U/ml	0.5 μ L	0.5 μ L	Enzyme 3	Phi29 DNA polymerase	250,000 U/mL	500U/ml	0.5 μ L	

Sample processing

0.65 μ M/mM dNTPs of 57.7 μ M TelN was added to the sample and mixed by vigorous shaking. The samples were allowed to incubate for 30 min at 30°C. Proteinase K was then added to digest TelN for 4 hours at 37°C. Following inactivation of Proteinase K, 2 reactions of enzyme digestions were setup. One with restriction enzymes and exonucleases added to remove unwanted material from the samples and a second with restriction enzyme that cuts within the DNA of interest, and exonucleases to confirm that the DNA sequence of interest is indeed being amplified and were allowed to incubate overnight at 37°C. Samples were then run by gel electrophoresis on a 1.0% agarose gel. These were

performed to visualise the cleaned-up, amplified DNA to confirm the presence of DNA of interest. A gel imager was used to capture an image as shown in Figure 14(A) and (B).

5 The data shown in Figures 14A and 14B shows that single stranded concatemers when extracted from a typical RCA amplification process from a circular plasmid template can be duplexed using the modified RNA polymerases as described in the present invention. The duplexing can be evidenced by using an enzyme, TelN, that requires the recognition sequence to be duplexed. As can be seen in Figure 14B, application of TelN resulted in much cleaner bands indicative of a cIDNA product. Further, it can be seen that denaturation conditions are not required for this process.

Claims

- 5 1. An *in vitro*, cell-free method of generating a complementary DNA strand for a single-stranded DNA concatemer, said single-stranded concatemer comprising at least a first and a second sequence wherein said first and second sequences are complementary to each other, said method comprising contacting said single-stranded concatemer with a modified RNA polymerase in the presence of one or more deoxyribonucleotides.
- 10 2. The *in vitro*, cell-free method of claim 1 wherein the single stranded concatemer comprises one or more repeats of the first and second sequences.
- 15 3. The *in vitro*, cell-free method of claim 1 or claim 2 wherein the single-stranded concatemer has folded into a secondary structure such as a nanoflower.
- 20 4. The *in vitro*, cell-free method of any one of claims 1 to 3 wherein said single-stranded concatemer comprises one or more repeats of a closed linear DNA template and said first sequence is the sense sequence of the closed linear DNA template and the second sequence is an antisense sequence of a closed linear DNA template.
- 25 5. The *in vitro*, cell-free method of any one of claims 1 to 4 wherein the single stranded concatemer is generated via rolling circle amplification of a closed linear DNA template.
- 30 6. The *in vitro*, cell-free method of any one of claims 1 to 3 wherein the single stranded concatemer is generated via rolling circle amplification of a double stranded circular template.
- 35 7. The *in vitro*, cell-free method of claim 5 or claim 6 wherein said rolling circle amplification uses a strand displacing DNA polymerase, optionally phi29 DNA polymerase.
- 40 8. The *in vitro*, cell-free method of claim 4, wherein the closed ends of the linear DNA comprise self-complementary sequences.
9. The *in vitro*, cell-free method of claim 4 or claim 8 wherein the closed linear DNA template includes at least one portion of a target sequence for a protelomerase enzyme.
10. The *in vitro*, cell-free method of claim 9 wherein the closed linear DNA comprises a portion of a protelomerase target sequence at each end of the closed linear DNA molecule.
11. The *in vitro*, cell-free method of any one of claims 1 to 10, wherein said single-stranded concatemer is also contacted with a DNA polymerase.

12. The *in vitro*, cell-free method of claim 11 wherein the DNA polymerase is a strand displacing DNA polymerase, optionally phi29 DNA polymerase.
- 5 13. An *in vitro*, cell-free method of generating a double-stranded DNA concatemer from a closed linear DNA template, said method comprising contacting said closed linear DNA template with a modified RNA polymerase in the presence of one or more deoxyribonucleotides.
- 10 14. The *in vitro*, cell-free method of any one of claims 1 to 13 wherein the modified RNA polymerase acts as a DNA-directed DNA polymerase.
- 15 15. The *in vitro*, cell-free method of any one of claims 1 to 14 wherein the modified RNA polymerase has one or more mutations that increase the selectivity of the RNA polymerase for deoxyribonucleotides over ribonucleotides.
- 20 16. The *in vitro*, cell-free method of any one of claims 1 to 15 wherein the method is performed in the presence of a mixture of nucleotides which comprise a maximum of 5% ribonucleotides.
- 25 17. The *in vitro*, cell-free method of any one of claims 1 to 16 wherein the method is performed substantially in the absence of ribonucleotides.
- 30 18. The *in vitro*, cell-free method of any one of claims 1 to 17 wherein the modified RNA polymerase is a member of the single-subunit DNA dependent RNA polymerase (ssRNAP) family.
- 35 19. The *in vitro*, cell-free method of claim 18 wherein the ssRNAP is any one or more of T3, T7, T8, K11, KP34, SP6 and/or N4 RNA polymerase.
- 40 20. The *in vitro*, cell-free method of any one of claims 1 to 12 wherein said single-stranded concatemer comprises at least one promoter sequence for an RNA polymerase.
- 45 21. The *in vitro*, cell-free method of any one of claims 13 to 17 wherein said closed linear DNA comprises at least one promoter sequence for an RNA polymerase.
22. The *in vitro*, cell-free method of claim 20 or claim 21 wherein the promoter sequence is the cognate sequence for the modified RNA polymerase.
23. The *in vitro*, cell-free method of any one of claims 13, 21 or claim 22 wherein the closed linear DNA template is further contacted with a DNA polymerase.
24. The *in vitro*, cell-free method of claim 23 wherein said DNA polymerase is a strand displacing DNA polymerase, optionally phi29 DNA polymerase.
25. The *in vitro*, cell-free method of any one of claims 1 to 24 wherein the method is performed substantially in the absence of exogenous primers and/or primase.

26. The in vitro, cell-free method of any one of claims 1 to 25 wherein the synthesized DNA is processed using an enzyme, preferably a protelomerase.