

There is therefore a need for a process that can be used to synthesise DNA and like molecules at significant scale.

Summary

5 The present disclosure relates to a process for cell-free production of DNA. The process may allow for enhanced production of DNA compared to current methodologies involving closed batch processes. This significantly increases productivity whilst reducing the cost of synthesising DNA, particularly on a large scale. The present disclosure relates generally to isothermal methods of amplifying DNA that do not require temperature to be cycled via heating and cooling.

10 Accordingly there is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of one or more primers and nucleotides to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-displacement replication of another strand, wherein further nucleotides are supplied in a controlled manner to the reaction mixture.

15 The cell-free process thus involves amplification of the template via strand displacement replication. There is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of one or more primers and nucleotides to form a reaction mixture, under conditions promoting amplification of said template by strand displacement replication, wherein further nucleotides are supplied in a controlled manner to the
20 reaction mixture

 In one aspect of the present disclosure, there is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-displacement replication of another strand,
25 wherein further nucleotides are supplied in a controlled manner to the reaction mixture.

 In another aspect of the present disclosure, there is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides and at least one primer to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-
30 displacement replication of another strand, wherein further nucleotides are supplied in a controlled manner to the reaction mixture.

 The cell-free process thus involves amplification of the template via strand displacement replication. There is provided a cell-free process for synthesising DNA comprising contacting a DNA

template with at least one polymerase in the presence of nucleotides to form a reaction mixture, under conditions promoting amplification of said template by strand displacement replication, wherein further nucleotides are supplied in a controlled manner to the reaction mixture.

Thus, the provision of a controlled supply of further nucleotides to the reaction mixture is
5 advantageous, since this rational control strategy can allow for improvements in the rate of synthesis of the DNA and/or an improved DNA yield. These improvements are compared to an analogous reaction mixture where all the nucleotides are supplied in the reaction mixture at the

wherein further nucleotides and metal cations are supplied in a controlled manner to the reaction mixture.

The cell-free process thus involves amplification of the template via strand displacement replication. There is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of one or more primers, nucleotides and metal ions to form a reaction mixture, under conditions promoting amplification of said template by strand displacement replication, wherein further nucleotides and further metal cations are supplied in a controlled manner to the reaction mixture.

There is also provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides and metal cations to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-displacement replication of another strand, wherein further nucleotides and metal cations are supplied in a controlled manner to the reaction mixture.

The cell-free process thus involves amplification of the template via strand displacement replication. There is provided a cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides and metal ions to form a reaction mixture, under conditions promoting amplification of said template by strand displacement replication, wherein further nucleotides and further metal cations are supplied in a controlled manner to the reaction mixture.

The further nucleotides and further metal cations may be supplied together or separately. It is preferred that the nucleotides and metal ions are not mixed together prior to being supplied to the reaction mixture. The further nucleotides and further metal cations may be added as each are required to the reaction mixture independently of each other.

Thus, the provision of a controlled supply of further nucleotides and further metal cations to the reaction mixture is advantageous, since this rational control strategy can allow for improvements in the rate of synthesis of the DNA and/or an improved DNA yield. These improvements are compared to an analogous reaction mixture where all the nucleotides and metal cations are supplied in the reaction mixture at the start. The controlled supply of further nucleotides may allow for increased productivity and/or processivity of the polymerase enzyme.

According to each of several further embodiments of the present disclosure, there is provided apparatus configured to perform a process according to the aspects of the present disclosure. The apparatus may be used to perform a process according to any aspect of the present disclosure.

Further, according to the present disclosure, the DNA template may comprise one or more processing enzyme target sites. The reaction mixture may be contacted at any time with one or more processing enzymes. Said enzymes may be provided to said reaction mixture in a controlled manner. Preferably, the processing enzyme is a protelomerase enzyme.

Independently, optional features of any aspects of the present disclosure may be: The DNA template may be circular. The strand displacement amplification of said DNA template may be carried out by rolling circle amplification (RCA). The DNA polymerase may be Phi29 or a variant thereof. The amplification of DNA may be isothermal amplification. The one or more primers may be random primers. The synthesised DNA may comprise concatamers comprising tandem units of DNA sequence amplified from the DNA template. The DNA template may be a closed linear DNA; preferably the DNA template is incubated under denaturing conditions to form a closed circular single stranded DNA. The DNA template may comprise at least one processing enzyme target sequence, preferably a recombinase target sequence or a protelomerase target sequence. The DNA template may comprise an expression cassette comprising a eukaryotic promoter operably linked to a coding sequence of interest, and optionally a eukaryotic transcription termination sequence. The expression cassette may be flanked on either side by a processing enzyme target sequence, preferably a recombinase targeting sequence or a protelomerase target sequence. The amplified DNA may be contacted with a recombinase enzyme or a protelomerase enzyme, as appropriate.

The quantity of DNA that may be synthesised is equal to or higher than 1 gram per litre of reaction mixture.

The further nucleotides and/or further metal ions may be supplied in a plurality of aliquots to the reaction mixture. The further nucleotides and/or further metal ions may be supplied at regular intervals throughout the duration of the process, optionally at least every 30 minutes. At least 3, 4, 5, 6, 7, 8, 9 or 10 aliquots may be supplied to the reaction mixture. The further nucleotides and/or further metal ions may be supplied in a continuous manner to the reaction mixture. The further metal ions and/or further nucleotides may be fed into the reaction mixture, optionally from an external source using a pump or are supplied to the reaction mixture by use of an osmotic pump.

The nucleotides or further nucleotides may comprise biologically inactive nucleotides; optionally these biologically inactive nucleotides are supplied to the reaction mixture via activation, preferably by a chemical or physical means. The biologically inactive nucleotides may be caged nucleotides that are activated by photolysis.

The further nucleotides and/or further metal ions and/or processing enzyme may be supplied in response to a signal related to the concentration of DNA in the reaction mixture, which

Claims

1. A cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-displacement replication of another strand, wherein further nucleotides are supplied in a controlled manner to the reaction mixture.
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2. A process according to claim 1 wherein said reaction mixture further comprises one or more primers.
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3. A cell-free process for synthesising DNA comprising contacting a DNA template with at least one polymerase in the presence of nucleotides and at least one primer to form a reaction mixture, under conditions promoting amplification of said template by displacement of replicated strands through strand-displacement replication of another strand, wherein further nucleotides are supplied in a controlled manner to the reaction mixture.
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4. A cell-free process for synthesising DNA according to any one of claims 1 to 3 wherein the reaction mixture further comprises one or more metal cations and further metal cations are supplied in a controlled manner to the reaction mixture, optionally independently to the supply of the further nucleotides.
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5. The process according to any one of claims 1 to 4 wherein said DNA template is circular, and amplification of said DNA template is by rolling circle amplification.
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6. The process according to any one of the preceding claims, wherein said DNA template is a closed linear DNA, preferably wherein said DNA template is incubated under denaturing conditions to form a closed circular single stranded DNA.
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7. The process according to any one of the preceding claims wherein said DNA template comprises at least one processing enzyme target sequence, preferably a recombinase targeting sequence or a protelomerase target sequence.

8. The process according to claim 7 wherein a processing enzyme is supplied in a controlled manner to the reaction mixture, optionally in response to a signal related to the concentration of DNA in the reaction mixture.
- 5 9. The process according to any one of the preceding claims wherein said further nucleotides are supplied in a plurality of aliquots to the reaction mixture, preferably said further nucleotides are supplied as aliquots at regular intervals throughout the duration of the process, optionally at least every 30 minutes.
- 10 10. The process according to claim 4 wherein said further metal ions are supplied as aliquots at regular intervals throughout the duration of the process, optionally at least every 30 minutes
11. The process according to any one of the preceding claims wherein said nucleotides or further nucleotides comprise biologically inactive nucleotides, and preferably are supplied to
15 the reaction mixture via activation
12. The process according to any one of the preceding claims wherein said further nucleotides are supplied in response to a signal related to the concentration of DNA in the reaction mixture, preferably wherein the concentration of the DNA in the reaction mixture is
20 monitored by measuring the difference in pressure of the reaction mixture, or a portion thereof, which can optionally generate a signal.
13. The process according to claim 12 wherein the signal indicating the concentration of DNA is generated by measuring a pressure difference created by a reciprocating pump that
25 removes and returns a portion of the reaction mixture, optionally over a pressure sensor.

14. The process according to any one of the preceding claims wherein said process is performed in a synthesis apparatus, said apparatus comprising:

a reaction vessel having at least a first port and a second port;

a temperature control device for controlling the temperature of the reaction vessel;

a plurality of reservoirs for holding reaction components, preferably comprising said further nucleotides and/or further metal ions;

a plurality of conduits, each of the plurality of conduits connecting the first port of the reaction vessel with a respective one of the plurality of reservoirs;

at least one pressure sensor located proximate the first port or second port of the reaction vessel;

supply means for selectively supplying controlled amounts of reaction components held in the reservoirs to the reaction vessel;

agitation means for selectively withdrawing an amount of content from the reaction vessel through the second port thereof and selectively returning the contents of the reaction vessel to the reaction vessel through the second port thereof; and

control means for controlling the supply means and the agitation means, wherein, the pressure sensor detects a change in pressure externally of the reaction vessel and sends a signal to the control means and the control means controls the supply means and/or agitation means in response to a change in observed pressure.

15. The process according to claim 4 or claim 14 wherein said metal cations comprise one or more metals selected from the list consisting of: magnesium (Mg^{2+}), manganese (Mn^{2+}), calcium (Ca^{2+}), beryllium (Be^{2+}), zinc (Zn^{2+}) and strontium (Sr^{2+}), lithium (Li^+), sodium (Na^+) or potassium (K^+), preferably Mg^{2+} .

16. The process according to claim 4, or claim 15, wherein said metal cation is a divalent cation and the ratio between said divalent metal cations and the nucleotides is maintained at about 3:1 in the reaction mixture.

17. The process according to any preceding claim wherein the concentration of nucleotides in the reaction mixture is maintained between 0.001mM and 6mM, optionally at about 3mM.

18. The process according to any one of the preceding claims wherein said nucleotides and/or further nucleotides are deoxyribonucleoside triphosphates (dNTPs), or a derivative or modified version thereof, preferably wherein said nucleotides and/or further nucleotides are one or more of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP),
5 deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and derivatives thereof.
19. The process according to any one of the preceding claims wherein said nucleotides are provided as one or more of free acids, their salts or chelates thereof, optionally wherein said
10 salts or chelates includes one or more of the following metal ions: Mg^{2+} , Be^{2+} , Ca^{2+} , Sr^{2+} , Li^+ , Na^+ , K^+ , Mn^{2+} or Zn^{2+} .