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Method Article

Generating single-stranded DNA circles with minimal resources



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ABSTRACT

Single-stranded circular oligonucleotides are heavily utilized in rolling circle amplification and rolling circle transcription technologies. Although various reported methodologies are available to synthesize circular, single-stranded DNA (ssDNA), the unduly complicated protocols and the associated cost minimize the utility of these methodologies to a non-expert or a beginner in the field. Our protocol provides the simplest yet robust synthesis of circular ssDNA templates to be utilized in various applications, using minimal resources.

- In this manuscript, we describe the most basic approach to synthesize circular ssDNA.
- Our method utilizes the minimal resources, yet it is robust.
- The utility of the methodology is very high for a non-expert or a beginner in the field.

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ARTICLE INFO

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Specifications table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Applied biochemistry to medicinal chemistry
Method name:	Development of single stranded circular DNA templates for rolling circle transcription assays
Name and reference of original method:	Diegelman, A. M.; Kool, E. T., Chemical and Enzymatic Methods for Preparing Circular Single-Stranded DNAs. <i>Current Protocols in Nucleic Acid Chemistry</i> 2000 , 00 (1), 5.2.1–5.2.27
Resource availability:	Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St- Louis, MO). The 5'-phosphorylated linear DNA substrate of Kool™ NC-45™ Universal RNA Polymerase template [11] and splint DNA substrates were purchased from Integrated DNA Technologies (see the supporting information for the specific sequences; the DNA sequence for Kool™ NC-45™ Universal RNA Polymerase template [5,11], was provided by epicentre (Madison, WI) once the company discontinued the product, Kool™ NC-45™ Universal RNA Polymerase template). T4 DNA Ligase and Exonuclease I (<i>E. coli</i>) were purchased from New England Biolabs (Ipswich, MA). S1 Nuclease (<i>Aspergillus oryzae</i>) was purchased from Promega (Madison, WI). The dialysis device (Tube-O-DIALYZER™, Medi, 1 K MWCO, 20) was purchased from G-Biosciences (St. Louis, MO). The purified wild-type <i>E. coli</i> RNA polymerase [6,7,13] was a generous gift from George A. Garcia laboratory of University of Michigan (Ann Arbor, MI).

Method details

One of the simplest yet most widely used methods of nucleic acid amplification is the rolling circle approach that includes rolling circle amplification (RCA) and rolling circle transcription (RCT) [1]. Small single-stranded circular oligonucleotides, considerably smaller than RNA polymerase, can efficiently initiate and elongate RNA sequences, therefore, are utilized in aforementioned technologies [2,10]. The increasing demand for circular, single-stranded DNA (ssDNA) is apparent based on the number of citations on related topics over the last 19 years (see Fig S1 for an elaboration). Development of circular ssDNA with [1–3,10,11] oligo “bridges” (often referred as splints) is heavily studied; however, many of these protocols are documented in a sophisticated manner that minimizes the broader use of this simple technique. Development of circular ssDNA without oligo “bridges” is also reported [4,12], but this process requires costly reagents. Due to the discontinuation of the product Kool™ NC-45™ Universal RNA Polymerase template [5], we were faced with the challenge of generating a circular ssDNA template for an *in-vitro* transcriptional activity assay for *Escherichia coli* (*E. coli*) RNA polymerase [6,13], by adapting previous literature. The sophistication of previously reported circular ssDNA synthesis was quite discouraging and authors' background as bioorganic/medicinal chemists was definitely not availing. Persistent effort of the authors resulted in circular ssDNA using minimal resources with 82% average efficiency of circularization (Step 11 of the method), after common DNA purification steps of ethanol precipitation and dialysis. The basic steps of our simple protocol: heat activation of linear ssDNA and the splint, ligation, and isolation and quantification of circular ssDNA, are presented in Fig. 1 to provide a quick overview.

The circularized DNA template was utilized in rolling circle transcription assays [6,13] with and without purified wild-type *E. coli* RNA polymerase [7] (Fig. 2A) and also compared to the template that was synthesized using the original protocol (Fig. 2B) [3]. It is important to note that circular DNA template that had any utility in the rolling circle transcription assay was nominal, when original protocol was used as described [3]. However, when Exonuclease I was used in place of S1 Nuclease, the amount and the quality of circular DNA template synthesized through our method and the original method were comparable (Fig. 2B). S1 Nuclease from *Aspergillus oryzae* that is used in the original protocol [3] is a single-stranded specific endonuclease; thus, it degrades both linear and circular ssDNA [8,14]. Accordingly, replacement of Exonuclease I with S1 Nuclease in the 6th step of our protocol resulted in nominal amounts of circular DNA (Fig. 2B). In conclusion, we are reporting a minimalistic approach to synthesize circular DNA templates of 45 nucleotides for rolling circle transcription assays. Our simple method utilizes minimal resources, yet it is robust. The simplicity

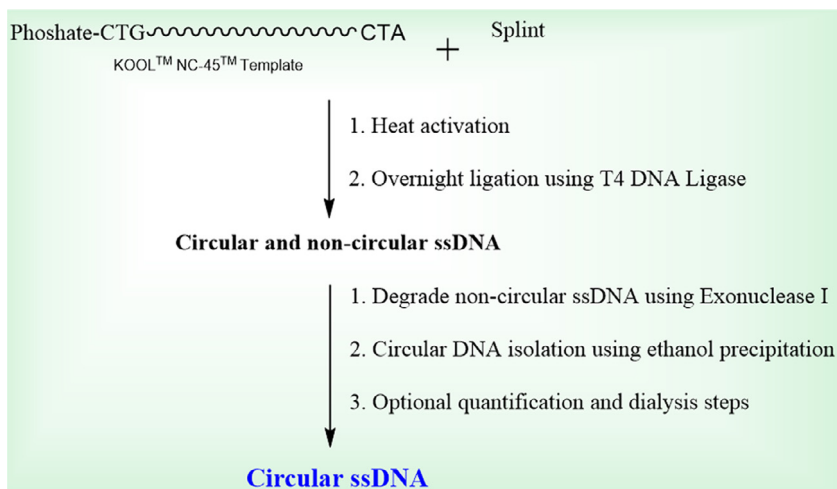


Fig. 1. A flowchart of reported protocol for synthesizing circular ssDNA for rolling circle applications.

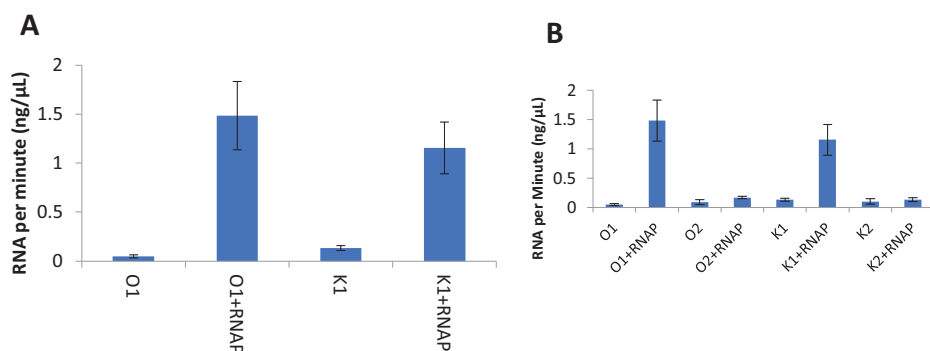


Fig. 2. The results from ten independent rolling transcription assays with circularized DNA templates synthesized using our method (O) and original circularization method (K), with (+RNAP) and without RNA polymerase. (A) The templates O1 and K1 are treated with Exonuclease I. (B) The templates O1 and K1 are treated with Exonuclease I, while the templates O2 and K2 are treated with S1 Nuclease. Notes: The assays were conducted using previously published experimental procedure [6,13]. Each assay contained an exact same amount of DNA template based on the DNA quantification through NanoDrop spectrometric analysis. All DNA templates were isolated, quantified, and utilized in these assays without continuing through the dialysis (Steps 9 and 10 in the method).

of our protocol will provide high utility for non-experts and/or beginners in the field attempting to generate circular ssDNA templates for various applications.

Materials and methods

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St- Louis, MO). The 5'-phosphorylated linear DNA substrate of Kool™ NC-45™ Universal RNA Polymerase template [3] and splint DNA substrates were purchased from Integrated DNA Technologies (see the supporting information for the specific sequences; the DNA sequence for Kool™ NC-45™ Universal RNA Polymerase template [3,5] was provided by epicentre (Madison, WI) once the company discontinued the product, Kool™ NC-45™ Universal RNA Polymerase template). T4 DNA Ligase and Exonuclease I (*E. coli*) were purchased from New England Biolabs (Ipswich, MA). S1 Nuclease (*Aspergillus oryzae*)

was purchased from Promega (Madison, WI). The dialysis device (Tube-O-DIALYZER™, Medi, 1000 Da MWCO, 20) was purchased from G-Biosciences (St. Louis, MO). The purified wild-type *E. coli* RNA polymerase [6,7,13] was a generous gift from George A. Garcia laboratory of University of Michigan (Ann Arbor, MI).

1. The 5'-phosphorylated linear DNA substrate (1 μ L, 100 pmol*), long splint (3 μ L, 300 pmol*) and 2X ligation buffer** (50 μ L) were combined in a polymerization chain reaction (PCR) tube. A batch of ten reactions was run at a given time to collect sufficient circular ssDNA.
2. The final volume of each tube was adjusted to 100 μ L using sterilized water using the following equation.

$$\text{Volume of sterile water} = 100 \mu\text{L} - (\text{total volume used in Steps 1, 4, and 6})$$
3. Using the ARKTIK Thermal Cycler (Thermo Scientific, Waltham, MA), each sample tube was heated to 90 °C for 10 min and cooled to 25 °C within 30 s. The circular DNA synthesis was continued without any further waiting.
4. DTT (10 μ L, 100 mM), ATP (10 μ L, 1 mM), and T4 DNA Ligase (0.6 μ L, 240 units) were added to each PCR tube and incubated at room temperature (~25 °C) for 16 h.***
5. After 16 h, the T4 DNA ligase was deactivated using the manufacture's protocol (heated at 65 °C for 10 min).
6. Exonuclease I (1 μ L, 20 units) was added to each reaction tube and incubated at 37 °C using the ARKTIK Thermal Cycler (Thermo Scientific, Waltham, MA). After 60 min of incubation, Exonuclease I was deactivated using manufacture's protocol (heated at 80 °C for 20 min).
7. All ten reactions in the PCR tubes were combined in a centrifuge tube and ssDNA was purified using ethanol precipitation method****.
8. The resultant DNA residue from the ethanol precipitation followed by the speed-vacuum drying was dissolved in sterilized water and directly utilized in the subsequent transcription assays (the quantity of DNA in each transcription assay was not significant to our work that primarily involved comparison of RNA polymerase activities and inhibitions as long as the same volume of the synthesized DNA batch was utilized in a set of assays that were directly compared to each other).
9. Optional Step 1###: When exact quantification of synthesized DNA was necessary (for preliminary confirmations and reporting purposes in this manuscript), purified DNA was quantified using UV/Vis absorbance protocol specific for nucleic acids through ND-1000 NanoDrop® spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).
10. Optional Step 2###: The resultant ssDNA was further purified using dialysis with MWCO 1000 Da dialysis tubing against 2 L of milliQ water for 3 h at room temperature. The dialysis was repeated with a fresh batch of milliQ water to obtain the purified ssDNA.
11. Optional Step 3###: The resultant ssDNA sample (presumably circular) was dried to a residue using speed-vacuum. The residue was suspended in sterilized water before quantifying the amount of DNA using ND-1000 NanoDrop® spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).
12. The efficiency of the circularization of ssDNA was determined by following calculations based on the DNA quantities from NanoDrop analysis immediately after Step 10. We assumed that Steps 6, 7, and 9 ensured the absence of any linear ssDNA or their remnants and the presence of circular ssDNA only.

$$\text{Percent efficiency of circularization of ssDNA} = \frac{\text{Total amount of DNA recovered after steps 1 through 10}}{\text{Total amount of DNA utilized in step 1}}$$

Notes:

*The DNA quantities provided by the vendor were used for the calculations without further quantification.

**2X ligation buffer recipe: 20 mM MgCl₂ and 100 mM Tris-Cl in milliQ water at pH 7.5.

*** Although authors did not consider utilizing further efficient ligation techniques in place of overnight ligation using T4 DNA ligase, faster ligation is claimed possible using higher concentration

of T4 DNA ligase (ligation in 10 min) or using quick ligation kits available from various manufacturers [9,15,16].

****See supporting information for ethanol precipitation protocol.

##Optional Steps 9, 10, and 11 are required only when exact circular ssDNA quantification is desired.

Additional notes

The rolling circle transcription assays were conducted using previously published assay protocol [6,13]. The exact same quantity of each DNA template was used in the assays shown in Fig. 2 based on the DNA quantities from NanoDrop analysis immediately after Step 8, without following optional purification step of dialysis.

Supplementary material

The Supporting Information is available. Included are Fig. S1: The number of citations on the topic “Circular DNA for Rolling Circle Technologies” in PubMed database, over the last 19 years, DNA sequences for Kool™ NC-45™ Universal RNA Polymerase template, and the splints and ethanol precipitation protocol for the DNA concentration and de-salting.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.mex.2021.101300](https://doi.org/10.1016/j.mex.2021.101300).

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