Rolling Circle Amplification with Chemically Modified Nucleoside Triphosphates

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Modified nucleoside triphosphates (dN*TPs) represent facile and versatile precursors for the introduction of chemical diversity into nucleic acids. While dN*TPs have been utilized in a plethora of practical applications, very little attention has been devoted to the assessment of their compatibility with isothermal amplification strategies. In this context, rolling circle amplification (RCA) is a wide-spread enzymatic replication method in which small single-stranded DNA (ssDNA) circles serve as templates in primer extension reactions yielding very long, ssDNA products. RCA is a pivotal tool for the generation of biosensor and diagnostic devices and is currently evaluated for its usefulness to create novel drug delivery systems. This unit describes the experimental procedures for the synthesis of modified RCA products using dN*TPs bearing chemical alterations at any possible location of the nucleosidic scaffold. Two ligation methods are presented for the generation of the DNA nanocircles that serve as templates for RCA, followed by a description of the RCA method itself and an assessment of the nuclease resistance of the ensuing products.

Keywords: rolling circle amplification • nucleoside triphosphates • polymerases • modified DNA • drug delivery systems

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INTRODUCTION

This unit describes the use of modified nucleoside triphosphates (dN*TPs) in rolling circle amplification (RCA) to generate fully modified, single-stranded, and very long (>1 kb) products. This methodology is general and can be applied to a broad range of modifications (at various locations of the nucleosidic scaffold, see Fig. 7.26.1) and templates of different topologies and lengths. The protocol describes the necessary steps that are required for the generation of the modified RCA products, namely the preparation of the single-stranded DNA (ssDNA) nanocircle template precursors either by application of a standard ligation strategy (see Basic Protocol 1) or by using a ligase that does not require any complementary oligonucleotides (see Alternate Protocol and Support Protocol 1). Lastly, the experimental protocol for the RCA reaction is described, including the assessment of the nuclease resistance of the ensuing modified products (see Basic Protocol 2 and Support Protocol 2).

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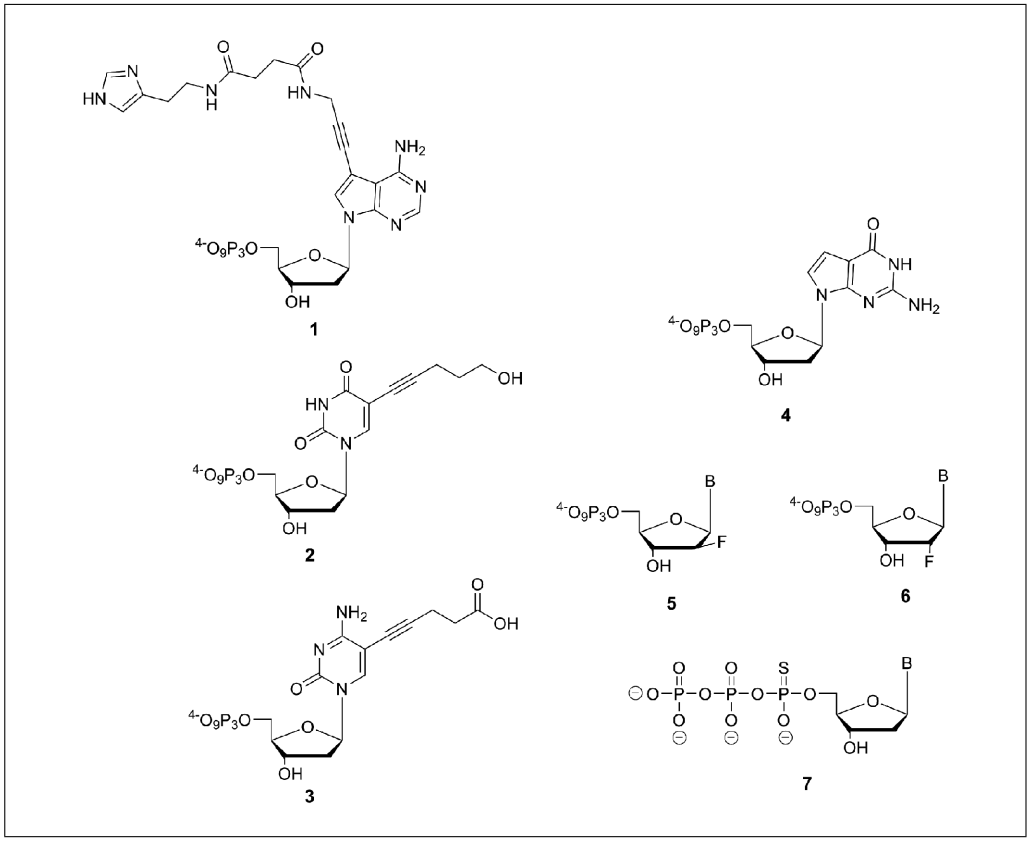

Figure 7.26.1 Chemical structures of the modified nucleoside triphosphates: Base-modified triphosphates **1**-**4**, sugar-modified triphosphates (FANA-NTPs **5** and 2′-fluoro-rNTPs **6**), and phosphate-modified (α-thio-dNTPs **7**). FANA-NTPs, 2′-deoxy-2′-fluoro-β-D-arabinonucleosides-NTPs; B, adenine, cytosine, or thymine.

STRATEGIC PLANNING

Ligation Protocol and Choice of Oligonucleotides

The sequence composition of the template is dictated by the intended application of the RCA products but regardless of its nature, the linear precursor needs to be phosphorylated at the 5′-termini (Lindström and Kool, 2002). The phosphorylation of the linear precursors can be made synthetically (solid-phase synthesis) or enzymatically (T4 polynucleotide kinase [PNK] and ATP). On the other hand, the choice of the ligation protocol will depend on mainly two different considerations: (1) If larger quantities of circular ssDNAs are required along with a minimization of the costs, then the T4 DNA ligation protocol should be selected; (2) if the linear template precursors are not amenable to standard ligation protocols and/or if small to medium scale reactions (up to ~150 pmol) are considered, then the CircLigase protocol should be considered (see Fig. 7.26.2). Other ligation methods have been developed and are available, for instance by using click chemistry (El-Sagheer et al., 2011) or by applying a differential protecting group strategy (Lindström and Kool, 2002), but they are beyond the scope of this unit. Moreover, the primers are usually chosen so as to be partially complementary to both the 3′ and 5′ termini of the template and thus can serve both as splint oligonucleotides in ligation reactions and as primers in RCA.

DNA Polymerase

The choice of the DNA polymerase is crucial and depends on a few important parameters namely (1) the acceptance of the dN*TPs as substrates by DNA polymerases; (2) the compatibility of the polymerase with RCA, i.e., the enzyme needs to display some

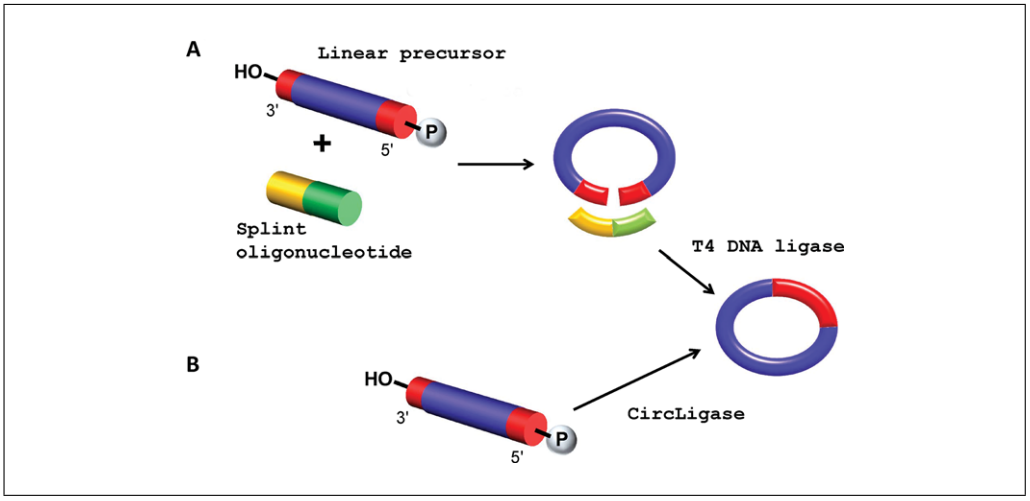

Figure 7.26.2 Ligation protocols for the generation of small circular ssDNA templates: (**A**) Method using a splint oligonucleotide and the T4 DNA ligase: The splint oligonucleotide is annealed to the 5′-phosphorylated single-stranded DNA template. The splint oligonucleotide is complementary to short stretches of both the 3′ and the 5′ ends of the linear precursor. The T4 DNA ligase then catalyzes the formation of the phosphodiester bond between the 5′-phosphorylated end and the free 3′ end to form the expected circular template. (**B**) Use of CircLigase on the phosphorylated linear template: This enzyme ligates the 5′-phosphorylated end and the free 3′ end of the linear precursor to form the expected circular template without the need of a splint oligonucleotide.

strand displacement activity; (3) high processivity of the enzyme. Lastly, it is of crucial importance to assess the nature of the ensuing RCA products since some polymerases such as the Vent (*exo*$^-$) might fulfill the aforementioned criteria but proceed through different mechanisms—in the particular case of Vent (*exo*$^-$) via multiround rolling hairpin replication (Cavalier, 1974; Fire and Xu, 1995). If some of the criteria are unknown at the outset of the experiment, additional tests can be carried out. For instance, the substrate acceptance of dN*TPs by polymerases can be addressed by standard primer extension reactions; the strand displacement activity of the polymerase can be assessed by RCA using unmodified dNTPs; and multiple polymerases can be screened for their efficiency at extending primers under RCA conditions (Liu et al., 1996; Hollenstein, 2015).

Rolling Circle Amplification

Longer reaction times (3 to 12 hr) will obviously lead to a distribution of longer ssDNA products, while the opposite will be observed with shorter reaction times (0.5 to 1 hr). Therefore, the reaction time is an important parameter that needs to be planned in accordance with the desired application. For instance, for the generation of DNA-based hydrogels (Lee et al., 2012; Yata et al., 2015), longer reaction times are often recommended, while for sensing and diagnostic purposes, short reaction times are adequate (Zhao et al., 2008). It is noteworthy mentioning that the nature of the modification might require longer reaction times depending on the substrate acceptance of the dN*TP by the polymerase. Indeed, depending on the nature of the modification, higher concentrations of dN*TPs might be necessary. For instance, α-thio- and α-borano-NTPs (Hollenstein, 2015; Russell et al., 2015) can be used at concentrations usually used for RCA or primer extension reactions (i.e., 100 to 150 μM), while certain base or sugar modifications will require higher concentrations (up to 1 mM). Moreover, RCA is usually carried out at rather low scales (typically 0.5 to 10 pmol quantities of both the primer and template) but can easily be extended to >100 pmol scales (Lee et al., 2012). Lastly, the primer to template ratio is an important parameter that should be maintained close to 1:1 or 1:2 for optimal yields (Liu et al., 1996; Hartig et al., 2005).

BASIC PROTOCOL 1

PREPARATION OF DNA NANOCIRCLES VIA STANDARD LIGATION

The circular ssDNA nanocircle templates can be generated by standard ligation reactions mediated by the T4 DNA ligase (Fig. 7.26.2A). The 5′-phosphorylated linear precursors and the splint oligonucleotides can be either synthesized in house by application of standard automated solid-phase DNA synthesis using phosphoramidite chemistry (see *UNIT 4.67*; Sarac and Meier, 2016) or obtained from commercial sources. In this particular case, a 46-mer long DNA sequence containing a randomized region (N_{20}) is used as a template precursor in order to introduce population diversity into the ensuing RCA products (Fire and Xu, 1995; Baccaro and Marx, 2010; Hollenstein, 2015). Both ends of the template precursor are ligated to form the circular ssDNA template for RCA. This step requires a splint oligonucleotide, which serves at a later stage as the primer for RCA (see Basic Protocol 2).

Materials

40% acrylamide/bis-acrylamide solution (19:1, w/w, electrophoresis purity reagent; Serva Electrophoresis)
10% (w/v) ammonium persulfate (APS) solution
BSA (Promega)
Bromophenol blue
EDTA
Ethanol (EtOH)
Formamide
Elution solution (1 mM in triethylamine [NEt_3], 1 wt. % $LiClO_4$, pH 8: Dissolve 1 g $LiClO_4$ [Sigma-Aldrich] in 50 mL autoclaved Milli-Q water; add 13.9 µL NEt_3 and adjust pH to 8 with HCl or NaOH; make up solution to 100 mL final volume with autoclaved Milli-Q water.)
TEMED
10× TBE buffer (Sigma-Aldrich or preparation by standard recipe; see *APPENDIX 2A*)
T4 DNA ligase (Promega)
10× T4 DNA ligase buffer (300 mM Tris·Cl, pH 7.8; 100 mM $MgCl_2$; 100 mM DTT; and 10 mM ATP)
Sephadex G10 (Sigma-Aldrich)
Xylene cyanol FF
5′-Phosphate-CTTGGTCTACTGGAG-N_{20}-CTACGGATTGC **1** (DNA template, see Strategic Planning; Microsynth)
5′-TAGACCAAGGCAATCCGTA (splint oligonucleotide, see Strategic Planning; Microsynth)
Urea (Apollo Scientific)

Centrifuge
Comb (8 wells)
Gel plates (20 × 20 cm)
Handheld UV lamp (254 nm)
Heating block
PAGE electrophoresis apparatus
50-mL plastic syringe
Power supply
1.5-mL screw-top vials
Spacers (1 mm thick)
ThermoMixer
UV-active thin-layer chromatography plate (0.25 mm, UV_{254}; Macherey-Nagel)
UV/vis spectrophotometer
Vacuum concentrator
Vortex mixing device

Ligation reaction

1. To a 1.5-mL Eppendorf tube, add:

   7 µL 1 mM splint oligonucleotide
   5 µL 1 mM DNA template.

2. Denature mixture of oligonucleotides by incubating tube in a boiling water bath and anneal slowly by allowing the bath to cool down to room temperature over 30 min.

3. Add the following buffers and reagents to the annealed mixture:

   100 µL 10× T4 ligation buffer
   2 µL BSA (20 µg)
   40 U T4 DNA ligase (20 µL)
   866 µL autoclaved Milli-Q H_2O.

4. Incubate reaction mixture in a ThermoMixer at 16°C 12 hr.

5. Deactivate reaction mixture using heat (80°C, 10 min), cool to 0°C, and evaporate to dryness in a vacuum concentrator (at room temperature).

6. Dissolve mixture in 100 µL H_2O and add 1 mL EtOH.

7. Briefly mix sample on a vortex and centrifuge for at least 15 min at 13,000 × *g* at room temperature.

8. Remove and discard supernatant and dry sample briefly in a vacuum concentrator.

Gel purification of the circular template

9. Prepare stop solution by adding the following reagents to a 40-mL tube:

   27 mL formamide
   3 mL EDTA (50 mM)
   0.05 wt. % bromophenol blue
   0.05 wt. % xylene cyanol FF.

10. Prepare a 20% 7 M urea denaturing gel solution by adding and combining the following reagents:

    420 g urea
    500 mL 40% acrylamide/bis-acrylamide solution
    100 mL 10× TBE
    90 mL H_2O.

11. Prepare a PAGE gel set up: Clean plates of adequate size (window cleaning solution followed by EtOH), separate plates by two spacers, and then constrain them with clamps.

12. Prepare urea-PAGE gel solution by combining the following reagents in a beaker in the following order:

    40 mL 20% acrylamide solution
    400 µL 10 wt. % APS
    40 µL TEMED.

13. Briefly mix gel solution with the syringe and insert solution into the gel set up. Insert comb and allow to polymerize for at least 1 hr.

14. Remove comb and preheat urea-PAGE gel 30 min at 10 W (in 1× TBE).

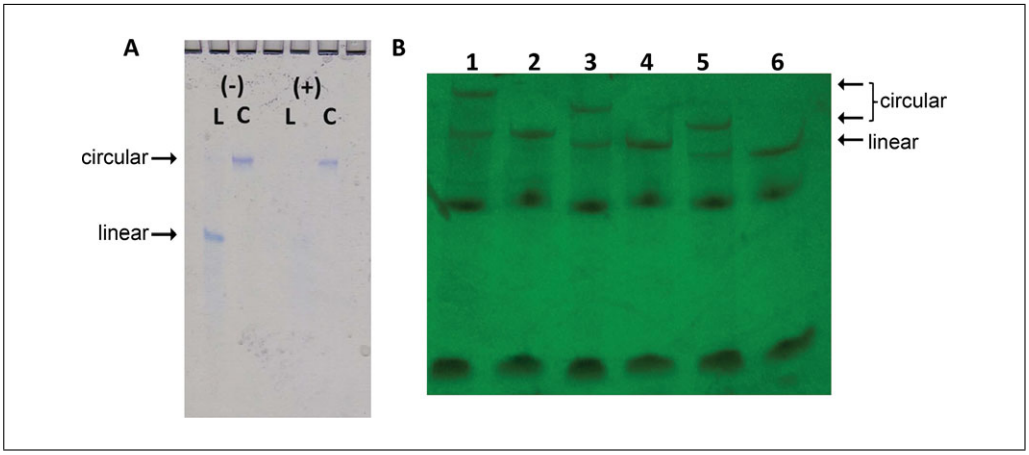

Figure 7.26.3 (**A**) Gel image (20% PAGE) of analysis of the circular ssDNA obtained with template **1** and application of Basic Protocol 1. Lanes L: linear precursor 1; lanes C: circular products; (+): treatment with exonuclease; (−): absence of exonuclease treatment. Visualization was performed with Stains-All as described in Support Protocol 1. (**B**) Gel image (20% PAGE) of synthesis of the circular ssDNAs with templates **2**-**4** by application of Alternate Protocol. Lane 1: reaction with template **4**; lane 2: linear precursor **4**; lanes 3 and 4: identical to lanes 1 and 2 but with template **3**; lanes 5 and 6: identical to lanes 1 and 2 but with template **2**. Visualization was performed by UV shadowing.

15. Add 20 µL H_2O and 20 µL stop solution (see step 9) to the ligation reaction, heat sample (95°C, 5 min), cool to 0°C, and load sample on 20% urea-PAGE (after washing out the wells with 1× TBE).

16. Run gel 1.5 hr at 10 W.

    The bromophenol blue dye should be ~1.5 cm away from the bottom of the gel set up.

17. Stop power supply, remove plates from the gel, and wrap gel in thin foil (or thin plastic wrap is also acceptable). Visualize bands by UV shadowing the gel on the TLC plate with illumination from a UV lamp. Mark position of the band corresponding to the circular template (see Fig. 7.26.3B for an example) and excise gel regions of interest with a razor blade. Place gel slab in 1.5-mL screw-top vials.

    The ssDNA nanocircles always have a significantly reduced gel mobility when compared to their linear precursors (see Fig. 7.26.3B).

Elute circular ssDNA from gel

18. Crush gel slab using a sealed pipet tip. Add 500 µL elution solution consisting of 1 mM NEt_3, 1 wt. % $LiClO_4$, pH 8; briefly vortex and incubate at 72°C 15 min in a ThermoMixer.

19. Briefly vortex sample and centrifuge at least 15 min at 13,000 × *g*, room temperature. Remove top layer and repeat procedure twice. Concentrate combined fractions to dryness in a vacuum concentrator.

20. Dissolve resulting white pellet in 100 µL H_2O and add 1 mL EtOH; briefly mix sample on a vortex and centrifuge at least 15 min at 13,000 × *g*, at room temperature. Remove supernatant and dry in a vacuum concentrator.

21. Prepare Sephadex G10 spin column: Plug a 1-mL tip with silanized glass wool and fill it with a 10 wt % solution of Sephadex G10 in H_2O; centrifuge 1 min at 3000 × *g* to remove H_2O; and wash column three times using 500 µL H_2O per wash. Dissolve sample in 50 µL H_2O and run through Sephadex G10 spin column.

22. Dilute 5 µL sample in 995 µL H_2O and measure the UV/vis spectrum.

The absorbance at 260 nm (A_{260}) serves as the basis for the determination of the concentration of the circular ssDNA template using the Beer-Lambert law.

The shape of the curve of the UV/vis spectrum also gives a hint of the purity of the sample. A strong absorption at 200 to 250 nm is usually indicative of the presence of acrylamide and/or salts and the sample should be desalted a second time.

PREPARATION OF DNA NANOCIRCLES VIA CircLigase LIGATION

ALTERNATE PROTOCOL

This alternate protocol is particularly suited for templates that contain repetitive sequence stretches such as G-rich telomeric repeats, where binding of a splint oligonucleotide at one defined location cannot be guaranteed (see Fig. 7.26.2B). Also, this method does not require any additional splint oligonucleotide since the CircLigase specifically recognizes 5′-phosphorylated single-stranded oligonucleotides and promotes the intramolecular ligation reaction (Blondal et al., 2005). The procedure is exemplified for templates consisting of six to eight G-rich telomeric repeats.

Additional Materials *(also see Basic Protocol 1)*

ATP (10 mM)
Betaine (5 M solution)
CircLigase II ssDNA ligase (Epicentre)
10× CircLigase buffer (0.5 M 3-(*N*-morpholino)propanesulfonic acid [MOPS], pH 7.5; 0.1 M KCl; 50 mM $MgCl_2$; 10 mM DTT)
$MnCl_2$ (100 mM)
Mineral oil
Phenol:chloroform:isoamyl alcohol, 25:24:1 (saturated with 10 mM Tris, pH 8.0, and 1 mM EDTA)
5′-Phosphate-TAGGGTTAG$(GGTTAG)_n$GGTTAGGGT (**2** to **4**, $n = 4$ to 6; DNA templates, see Strategic Planning; Microsynth)

Ligation reaction and purification of the circular ssDNA templates

1. In a 1.5-mL Eppendorf tube, add the following reagents and buffers:

   1 µL 1 mM template DNA
   1 µL 1 mM ATP (diluted 10× from stock solution)
   8 µL 5 M betaine
   4 µL 10× CircLigase buffer
   2 µL 100 mM $MnCl_2$
   200 U CircLigase (2 µL)
   22 µL H_2O
   10 µL mineral oil (to prevent evaporation).

2. Incubate reaction mixture at 60°C 12 hr in a ThermoMixer. Increase temperature to 80°C and heat deactivate 10 min, then cool to room temperature.

3. Add 40 µL phenol:chloroform:isoamyl alcohol solution to the reaction mixture, briefly vortex, and recover top layer.

4. Add 400 µL EtOH, briefly mix sample on a vortex, and centrifuge at least 15 min at 13,000 × *g* at room temperature. Remove supernatant and dry in a vacuum concentrator.

5. Dissolve sample in 20 µL H_2O and add 20 µL stop solution (see Basic Protocol 1, step 9) to the ligation reaction. Purify by urea-PAGE gel electrophoresis as described in Basic Protocol 1 (see steps 10 through 17).

SUPPORT PROTOCOL 1

EXONUCLEASE TREATMENT OF THE CIRCULAR TEMPLATES

Even though the circular ssDNA templates have substantially slower gel mobilities compared to the linear precursors, the circularity needs to be verified in order to exclude that the isolated products are merely linear concatemers or other artifacts. This support protocol will describe a simple method for the assessment of the circularity of the templates based on exonuclease I. Indeed, this enzyme catalyzes the excision of nucleotides of single-stranded DNA sequences in the 3′ to 5′ direction but does not recognize circular oligonucleotides and thus only the desired product will remain unaffected by this treatment (Tate et al., 2012).

Additional Materials *(also see Basic Protocol 1)*

Circular DNA template (prepared in Basic Protocol 1 or Alternate Protocol)
Exonuclease I (*Escherichia coli*; New England Biolabs)
10× Exonuclease I buffer (670 mM glycine-KOH, 67 mM $MgCl_2$, 100 mM 2-mercaptoethanol [β-ME], pH 9.5)
2-Propanol
Stains-All (Sigma-Aldrich)
Tris·Cl (TCI)

Exonuclease treatment

1. Prepare the following reaction mixture in a 1.5-mL Eppendorf tube:

   50 pmol circular (or linear for the control reactions) DNA templates
   2 μL 10× reaction (exonuclease I) buffer
   10 U exonuclease I (0.5 μL).

2. Make up to a total volume of 20 μL with H_2O and incubate at 37°C for 30 min in a ThermoMixer. Heat deactivate (80°C, 10 min) and add an equal volume (20 μL) stop solution. Resolve samples by 15% or 20% urea-PAGE gel electrophoresis as described in Basic Protocol 1 (see steps 10 through 17).

3. Prepare Stains-All stock solution by dissolving 100 mg Stains-All in 100 mL formamide and store in the dark at 4°C.

4. Prepare 20 mM Tris·Cl solution by dissolving 0.8 g Tris·Cl in 300 mL H_2O and adjusting the pH to 8.0 with HCl. Add 100 mL 2-propanol to the Tris·Cl solution to generate a 75% Tris·Cl/25% (v/v) 2-propanol mixture.

5. Prepare working solution by diluting Stains-All stock solution (10 mL; see step 3) in 90 mL Tris·Cl/2-propanol mixture from step 4.

6. Remove glass plates and immerse gel in ~100 mL Stains-All working solution. Stain in the dark, until the bands become clearly visible (typically 2 hr) and remove Stains-All solution. Immerse gel in ~100 mL H_2O and wait until the background turns almost colorless. Scan gel with a normal portable scanner.

   An example of exonuclease treatment of circular and linear templates is shown in Figure 7.26.3A.

BASIC PROTOCOL 2

ROLLING CIRCLE AMPLIFICATION WITH MODIFIED NUCLEOSIDE TRIPHOSPHATES

Using the dN*TPs in RCA is the key step of this unit. The circular ssDNA templates prepared in the previous protocols will be used in conjunction with radiolabeled primers under RCA conditions together with the 9°N_m DNA polymerase since this enzyme readily accepts modified nucleoside triphosphates and is capable of proceeding via an RCA mechanism (see Fig. 7.26.4). In this context, other polymerases can be considered

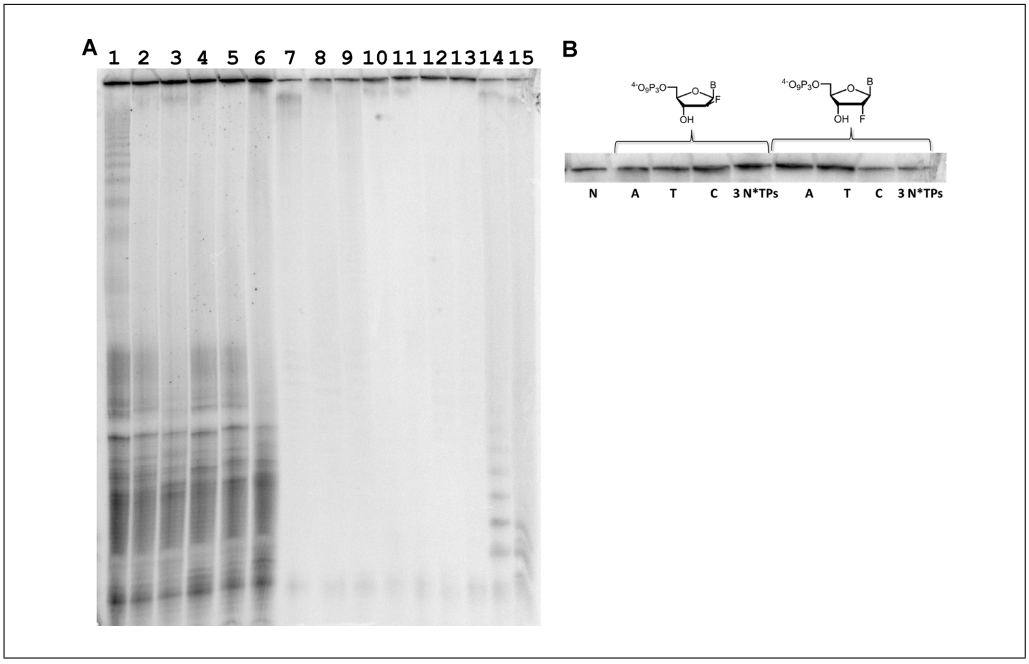

Figure 7.26.4 (**A**) Gel image (7% PAGE) of RCA products with modified dNTPs. Lanes 1 and 7: natural dNTPs; lanes 2-5: single FANA-NTPs **5** (A, C, G, T, respectively) and three natural dNTPs; lane 6: all 4 FANA-NTPs **5** (fully modified product); lanes 8-10: single FANA-NTPs **5** (A, T, C, respectively) and two natural dNTPs; lane 11: all 3 FANA-NTPs **5** (fully modified product); lanes 12-15: same as lanes 8-11 but with 2′-fluoro-rNTPs **6**. For lanes 1-6, circular template **1** was used, while for lanes 7-15, circular template **4** was used. The products were obtained by application of the method described in Basic Protocol 2, using the 9°N_m DNA polymerase. (**B**) Close-up view on the gel showing the relative yields of RCA product formation with FANA-NTPs **5** and 2′-fluoro-rNTPs **6**. RCA, rolling circle amplification; FANA-NTPs, 2′-deoxy-2′-fluoro-β-D-arabinonucleosides-NTPs; B, adenine, thymine, or cytosine.

including the Therminator, DNA polymerase I, Large (Klenow) Fragment, *Bst* DNA polymerase, or the ϕ29 DNA polymerase (Zhu et al., 2013; Ali et al., 2014; Russell et al., 2015). In Basic Protocol 2, two variants of sugar modified dN*TPs were evaluated for their efficiency to serve as substrates for the 9°N_m DNA polymerase under RCA conditions. Indeed, the pucker of the sugar can be controlled for instance by introducing a fluorine atom at position 2′: If the fluorine is positioned upwards (i.e., β) as in 2′-deoxy-2′-fluoro-β-D-arabinonucleosides (FANA, **5** in Figure 7.26.1), a south/east sugar pucker conformation (C2′/O4′-endo) is adopted; on the other hand, when the fluorine atom points down (i.e., in an α position) as in 2′-deoxy-2′-fluoro-β-D-ribonucleosides (2′-fluoro-rNTPs, **6** in Figure 7.26.1), the ribofuranose sugar is coerced into a north (C3′-endo) conformation (Peng and Damha, 2007, 2008). The stereochemistry has an important impact on the substrate acceptance of polymerases under primer extension reactions (Peng and Damha, 2008).

Materials

γ-^{32}P-ATP (Hartmann Analytic)
Circular ssDNA templates (see Basic Protocols 1 and Alternate Protocol)
DNA primers:
5′-TAGACCAAGGCAATCCGTA **3** (for circular template **1**)
5′-CTAACCCTAACCCTAACC **4** (for circular templates **2** to **4**)
Modified dNTPs:
1 to **3** (base-modified triphosphates; synthesized according to Hollenstein, 2013)
2′-Deoxy-2′-fluoro-β-D-arabinonucleosides (FANA) **5** (sugar-modified triphosphates; prepared according to Peng and Damha, 2007)

4 (base-modified triphosphate; TriLink BioTechnologies, cat. no. N-2011)
6 (sugar-modified triphosphates; TriLink BioTechnologies, cat. nos. N-1007 [for 2′-F-dATP]; N-1008 [for 2′-F-dCTP]; N-1055 [for 2′-F-dTTP])
7 (phosphate modified; TriLink BioTechnologies, cat. nos. N-8001 [for 1-thio-dATP]; N-8002 [for 1-thio-dCTP]; N-8004 [for 1-thio-dTTP])
9°N_m DNA polymerase (New England Biolabs)
10× Thermopol buffer
T4 PNK (Thermo Fisher Scientific)
10× T4 PNK buffer (forward reactions; 500 mM Tris·Cl, pH 7.6; 100 mM $MgCl_2$; 50 mM DTT; 1 mM spermidine)
Stop solution (see Basic Protocol 1)
Urea
10× TBE
10% (w/v) ammonium persulfate (APS) solution
TEMED
20% acrylamide solution (see Basic Protocol 1, step 10)

Amersham Hyperscreen intensifying screen (35 × 43 cm, GE Healthcare Life Sciences)
Autoradiography cassette (35 × 43 cm, GE Healthcare Life Sciences)
Gel plates (33 × 42 cm; CBS Scientific)
ImageQuant software (GE Healthcare)
Spacers (0.4 mm thick; CBS Scientific) and combs (16 wells; CBS Scientific)
Storm 820 phosphorimager (GE Healthcare)
ThermoMixer
Sephadex G10 spin column (see Basic Protocol 1)

Radiolabel the primer

1. Prepare the following reaction mixture in a 1.5-mL Eppendorf tube:

   30 pmol appropriate primer (see Basic Protocol 1 and Alternate Protocol)
   3 μL 10× PNK buffer
   3 μL γ-^{32}P-ATP
   10 U T4 PNK (1 μL)
   30 μL H_2O.

2. Incubate mixture at 37°C 30 min in a ThermoMixer.

3. Heat deactivate (95°C, 5 min) and run sample through a Sephadex G10 spin column to remove excess of radiolabel.

Rolling circle amplification

4. Anneal 2 pmol circular templates **1** and **2** ($n = 4$) with 1 pmol radiolabeled primers **3** and **4**, respectively (see Basic Protocol 1).

5. Prepare 10 mM solution of modified or natural dNTPs by diluting the appropriate stock solutions.

6. Cool down each mixture of annealed oligonucleotides to 0°C and add the following reagents in turn in the order listed:

   2 μL 10× Thermopol buffer
   20 μL H_2O
   2 U 9°N_m DNA polymerase (1 μL)
   2 μL dNTP mix (10 mM; 1 mM final concentration).

7. Incubate reactions at 60°C 1 hr (see Strategic Planning) and then add 20 µL stop solution.

8. Prepare gel diluent by combining 420 g urea, 590 mL H_2O, and 100 mL 10× TBE.

9. Prepare 5% urea-PAGE sequencing gel by diluting 20 mL 20% acrylamide solution in 60 mL of diluent and pour mixture, after adding 800 µL 10% APS and 80 µL TEMED, into the glass plates.

10. Resolve reaction products by urea-PAGE gel electrophoresis. Remove gel plates, cover gel with plastic cling wrap, and expose on an erased phosphor screen in an autoradiography cassette.

    The exposure time will depend on the amount and concentrations of products formed during the RCA and the decay state of the radioisotope ^{32}P. Typically, when freshly labeled primers are used (i.e., using γ-^{32}P-ATP, <10 days after the calibration date), an exposure of 3 hr is sufficient.

11. Analyze gel by scanning the exposed screens on a Storm 820 phosphorimager (using the storage phosphor mode) and visualize different products by using the ImageQuant software.

NUCLEASE RESISTANCE

SUPPORT PROTOCOL 2

The robustness of the resulting modified RCA products can be evaluated by treatment with FBS. Alternatively, the ssDNA products can also be incubated with endo- and exonucleases.

Additional Materials *(also see Basic Protocol 2)*

Heat-deactivated FBS (Gibco)

Rolling circle amplification

1. Carry out rolling circle amplification reactions with dN*TPs as described in Basic Protocol 2 and heat deactivate after 1 hr reaction time by heating at 95°C 5 min.

2. Add 20 µL phenol:chloroform solution to the reaction mixture, briefly vortex, and recover top layer.

3. Precipitate with EtOH (400 µL) and dissolve reaction mixture in 20 µL H_2O.

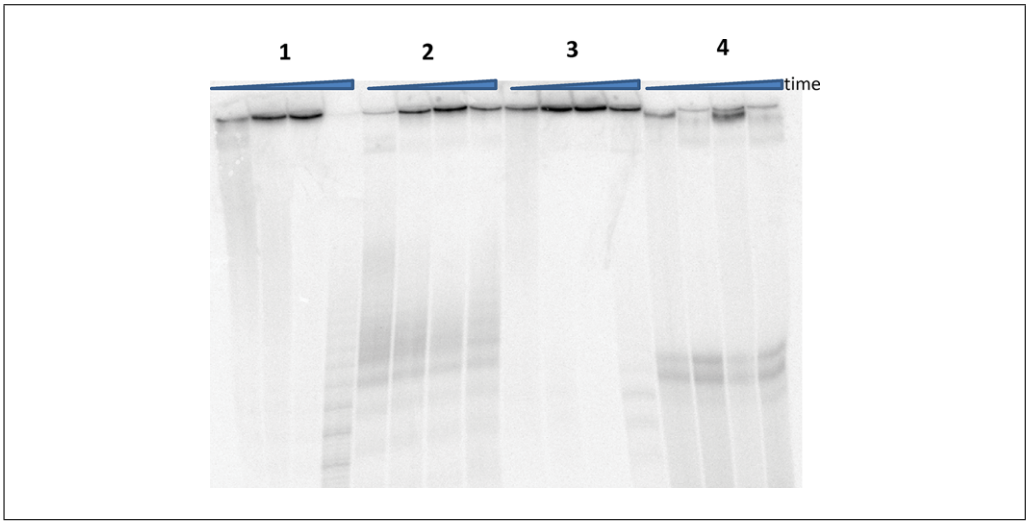

Figure 7.26.5 Gel (10% PAGE) analysis of the FBS treatment of RCA products obtained with circular template **4**. Lane 1: natural dNTPs; lane 2: base-modified dNTPs **1**-**3**; lane 3: α-thio dNTPs **7**; lane 4: 2′-fluoro-rNTPs **6**. Time points were 0, 0.5, 1, and 15 hr. RCA, rolling circle amplification.

FBS treatment and analysis

4. Prepare the following reaction mixture:

   4 μL RCA product
   1 μL FBS
   5 μL H_2O.

5. Incubate at 37°C for given time points (typically 0, 2, 4, 6, 8, 12, and 20 hr) and quench reaction mixture with 10 μL stop solution.

6. Analyze reactions by 10% urea-PAGE gel electrophoresis as described in Basic Protocol 2, steps 10 and 11.

   A representative example of FBS treatment of modified and unmodified RCA products is shown on Figure 7.26.5. Polygons are drawn around the bands corresponding to the RCA products and the progression of the decrease of the RCA products with time was determined by densitometry of band intensity.

COMMENTARY

Background Information

Modified nucleoside triphosphates

The enzymatic polymerization of modified nucleoside triphosphates (dN*TPs) represents a convenient way for the introduction of functional groups into nucleic acids (Peng and Damha, 2007; Hollenstein, 2012; Pinheiro et al., 2012; Hocek, 2014; Hottin and Marx, 2016). This strategy has been adopted in numerous practical applications including the detection of nucleic acids (Hocek and Fojta, 2011) and the selection of modified aptamers (Dunn and Chaput, 2014; Diafa and Hollenstein, 2015) and DNA enzymes (Taylor et al., 2015). A broad variety of functional groups has been shown to be compatible with the method. Not only small moieties such as halogen atoms (Olszewska et al., 2015) but also rather bulky modifications including organic polymers (Baccaro and Marx, 2010) and oligonucleotides (Verga et al., 2015) have been introduced by application of this strategy. The modified triphosphates are generally incorporated into DNA by primer extension reactions or PCR, yielding double-stranded products. However, for most applications the double-stranded products need to be converted into single-stranded modified oligonucleotides, for instance by λ-exonuclease digest or by physical separation (using magnetic particles and biotinylated primers).

Rolling circle amplification and modified nucleotides

In rolling circle amplification (RCA), short, single-stranded circular DNA sequences serve as templates for the extension of primers and the reactions produce long (several kb) ss-DNA products that contain multiple copies of the reverse complement of the templates (Ali et al., 2014). Besides raising interest in the field of biosensing and diagnostics, RCA has been used to develop hydrogels and DNA nanostructures. In this context, RCA-produced DNA is currently being evaluated as scaffolds that could serve as drug delivery systems (Zhang et al., 2013; J.H. Kim et al., 2015; M.G. Kim et al., 2015). For instance, RCA products were converted to amphiphilic nanoparticles by complexation with complementary cholesterol-modified DNA sequences (J.H. Kim et al., 2015). Further functionalization with folate-modified DNA led to spherical nanoparticles that were shown to serve as a potent drug delivery carrier, since the anticancer agent doxorubicin intercalated in the hairpin loops of the RCA nanoparticles. The resulting nanoparticle-doxorubicin complexes were nuclease resistant, selectively bound to folate receptors on cancer cells, and did not induce any cytotoxic side effects (J.H. Kim et al., 2015). This example strongly highlights the potential of RCA products to serve as drug delivery systems.

Surprisingly, very little research effort has focused on the use of modified nucleoside triphosphates in the context of RCA (Hollenstein, 2015; Russell et al., 2015). Indeed, most reports have focused on the use of single modified nucleoside triphosphates to label the ensuing RCA products with fluorescent tags for their detection (Smolina et al., 2005; Zhu et al., 2013) or with biotin moieties for isolation purposes (Yao et al., 2013). Recently, 5′-(α-*P*-borano)-deoxynucleoside triphosphates were shown to be compatible with RCA and fully modified products could be obtained with the

φ29 DNA polymerase. The ensuing products served as the basis for the generation of silver nanoparticles by simple reduction of Ag(I) salts (Russell et al., 2015). Similarly, nucleoside triphosphates bearing modifications at any possible location of the nucleosidic scaffold were shown to be generally compatible with RCA (Hollenstein, 2015). On the other hand, only natural NTPs have positively been evaluated in RCA (Daubendiek et al., 1995), but it is very likely that modified NTPs that are compatible with the T7 RNA polymerase could also yield modified RNA RCA products. Taken together, these results have demonstrated the compatibility of dN*TPs with RCA and bode well for the future development of functionalized nanomaterials (Aldaye et al., 2008) and drug delivery systems based on modified RCA products.

Critical Parameters and Troubleshooting

The gel purification of the circular templates is a crucial step in order to preclude the presence of primer concatemers and other products stemming from template-independent ligation reactions which in turn could be the source of non-RCA products (Kuhn and Frank-Kamenetskii, 2005). The formation of other products could be a severe drawback, especially in diagnostic and biosensing-driven applications. Thus, in addition to (or sometimes instead of) the gel purification, the ligation products can be treated by exonucleases I and III to remove linear ssDNA oligonucleotides and double-stranded DNAs (dsDNAs), respectively. Also, the presence of partially polymerized or non-polymerized acrylamide in the circular ssDNA templates could inhibit polymerases during RCA (Etokebe and Spurkland, 2000) and thus particular attention should be given to the purification and desalting of the templates. The requirement of low percentage PAGE (4% to 7%) for the analysis of the RCA products renders the handling of the gels more difficult and caution should be taken when removing the glass plates to avoid tearing and breaking of the gels. Lastly, if the RCA proceeds with moderate to low efficiency, other parameters such as the reaction time and temperature as well as the addition of $MnCl_2$ in the reaction mixtures should be considered and systematically assessed. Alternatively, other DNA polymerases that have not yet been evaluated for their usefulness in RCA but display a rather large tolerance for chemical modifications (e.g., *KlenTaq*, KOD Dash DNA polymerase, Phusion DNA polymerase) could also be tested.

Anticipated Results

The generation of the circular templates by either of the two ligation protocols is relatively efficient albeit time consuming and typically affords 100 to 300 pmol of products (after gel purification and depending on the scale of the reactions). This represents a rather substantial quantity of material for RCA and circumvents the need of repeated preparations as in other protocols. The experimental procedure for RCA with the dN*TPs does not vary substantially from that for standard primer extension reactions and will lead to high yields (i.e., full conversion of the primers and 20% to 30% of products longer than 1 kb) if an adequate DNA polymerase is used. Fine tuning of the conditions (e.g., longer reaction times) will further improve the yields of long RCA products. Moreover, this method is particularly suited for any type of base and phosphate modifications, while sugar-altered dN*TPs seem to be slightly more refractory to these conditions and additional examples need to be assessed individually. Also, it is worthwhile mentioning that the generation of the modified RCA products is only the first step in the development of new DNA-based materials or drug carriers but controlling all the parameters of the reaction conditions is of paramount importance.

Concerning the fluorinated triphosphates, the yields of the RCA products obtained with the FANA-NTPs **5** are higher than with the 2′-fluoro-rNTPs **6**, especially for the fully modified products (see Fig. 7.26.4B). This stems from the fact that FANA-NTPs are better substrates for the 9°N_m DNA polymerase than their stereochemical counterparts (Peng and Damha, 2008). However, the reactions with three modified triphosphates produce in both cases very long single-stranded 2′-fluoro-RNA and FANA products.

Time Considerations

The preparation of the circular ssDNA templates has a different timeframe depending on the scale and the method that is employed. Typically, the preparation of larger amounts of the templates (~400 to 500 pmol) will require longer handling and reaction times (e.g., 16 hr at 4°C with the T4 DNA ligase and 60°C for 6 to 12 hr with the CircLigase) and can be carried out in 2 to 3 days, including the exonuclease treatment to assess the circularity. On the other hand, smaller scale reactions

(~100 pmol) might reduce the experimental time to 1 day. Lastly, the RCA and the analysis of the resulting products can be carried out within a day. Consequently, the entire protocol can be performed within 1 week.

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