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Modification of 3' Terminal Ends of DNA and RNA Using DNA Polymerase θ Terminal Transferase Activity

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[Abstract] DNA polymerase θ (Polθ) is a promiscuous enzyme that is essential for the error-prone DNA double-strand break (DSB) repair pathway called alternative end-joining (alt-EJ). During this form of DSB repair, Polθ performs terminal transferase activity at the 3' termini of resected DSBs via templated and non-templated nucleotide addition cycles. Since human Polθ is able to modify the 3' terminal ends of both DNA and RNA with a wide array of large and diverse ribonucleotide and deoxyribonucleotide analogs, its terminal transferase activity is more useful for biotechnology applications than terminal deoxynucleotidyl transferase (TdT). Here, we present in detail simple methods by which purified human Polθ is utilized to modify the 3' terminal ends of RNA and DNA for various applications in biotechnology and biomedical research.

Keywords: DNA polymerase, DNA repair, DNA modification, Alternative end-joining, Terminal deoxynucleotidyl transferase, Biotechnology, Nucleotide analogs

[Background] The human *POLQ* gene encodes a large protein that contains an N-terminal superfamily 2 (SF2) type helicase domain and a C-terminal A-family polymerase domain (Sfeir and Symington, 2015; Black *et al*., 2016; Wood and Doublie, 2016). The protein also encodes for a large central domain whose function has yet to be ascribed. Polθ is expressed in metazoans and has been shown to function in multiple aspects of DNA replication and repair (Black *et al*., 2016; Wood and Doublie, 2016). Recent work showed that mammalian Polθ is essential for the error-prone DNA double-strand break (DSB) repair pathway called alternative end-joining (alt-EJ), also known as microhomology mediated end-joining (MMEJ) (Yousefzadeh *et al*., 2014; Kent *et al*., 2015; Mateos-Gomez *et al*., 2015). This essential function of the polymerase is conserved among metazoans (Chan *et al*., 2010; Koole *et al*., 2014).

Interestingly, Polθ mediated alt-EJ results in relatively large deletions and insertions (indels) at DNA repair junctions compared to the more accurate non-homologous end-joining (NHEJ) pathway (Black *et al*., 2016). For instance, alt-EJ typically generates insertions ranging from 1-6 base pairs (bp), and in some cases insertions can exceed 30 bp (Yousefzadeh *et al*., 2014; Mateos-Gomez *et al*., 2015; Black *et al*., 2016; Kent *et al*., 2016). Intriguingly, multiple studies from invertebrates and vertebrate systems show that some insertion tracts are templated by nearby DNA sequences such as those flanking the DSB (Black *et al*., 2016). In other cases, insertion sequences appear to be random (Black *et al*., 2016). These and other studies led to the idea that Polθ might generate insertion tracts at DSBs by both templated and non-templated terminal transferase mechanisms.

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Indeed, in a recent study Kent *et al.* demonstrated that the human Polθ polymerase domain, hereinafter referred to as Polθ, exhibits robust terminal transferase activity preferentially on single-strand DNA (ssDNA) and double-strand DNA containing 3' ssDNA overhangs, referred to as partial ssDNA (pssDNA) (Kent *et al*., 2016). This study also compared the terminal transferase activities of Polθ and terminal deoxynucleotidyl transferase (TdT) using their respective optimal conditions, and found that Polθ is a more versatile enzyme for modifying the 3' terminus of nucleic acids. For example, the authors showed that Polθ is able to modify nucleic acids with a wider variety of nucleotide analogs, such as those containing large fluorophores or attachment chemistries (Kent *et al*., 2016). As a specific example, Polθ was shown to efficiently modify ssDNA with a nucleotide analog containing click chemistry applicability (*i.e.*, a linker attached to an azide group), whereas TdT failed to use the same nucleotide as a substrate (Kent *et al*., 2016). TdT was also unable to use a Texas Red conjugated nucleotide analog that Polθ efficiently utilized to modify ssDNA (Kent *et al*., 2016). Polθ is also capable of modifying the 3' terminal ends of RNA and appears to show a significantly lower discrimination against ribonucleotides compared to TdT (Kent *et al*., 2016). Altogether, this recent report demonstrates that Polθ is a more versatile terminal transferase enzyme than TdT and therefore should be more useful for a wide range of applications in biotechnology and biomedical research that require modification of 3' terminal DNA and RNA ends (Kent *et al*., 2016). Here, we explain in detail step-by-step procedures for using Polθ as a robust terminal transferase enzyme *in vitro*.

Materials and Reagents

A. The following reagents are needed for modifying nucleic acids with Polθ:
   1. Pipette tips (*i.e.*, Fisher Scientific, catalog number: 02-707-432)
   2. Microcentrifuge tubes (*i.e.*, 0.5 ml or 1.5 ml). DNase and RNase free tubes are recommended for reactions (*i.e.*, BioDot Ultra Spin 1.5 ml Microcentrifuge Tubes) (DOT Scientific, catalog number: 711-FTG)
   3. ssDNA or RNA to be modified (typically 10-50 nt in length; desalted, HPLC or PAGE purified)
   4. Purified Polθ (residues 1,792-2,590, MW = 90 kDa) (expression vector and purification methods: Hogg *et al*., 2011)
   5. Nucleoside triphosphate analogs (*i.e.*, TriLink BioTechnologies, catalog number: N-2008-102502 and TriLink BioTechnologies, catalog number: N-5001) or canonical nucleoside triphosphates (*i.e.*, Promega, catalog number: U120)
   6. 1 M Tris buffer pH 8.2 (*i.e.*, DOT Scientific, catalog number: DST60040-10000)
   7. Hydrochloric acid (HCl) (*i.e.*, Thomas Scientific, catalog number: C395L46)
   8. NP-40 detergent *(i.e.*, Thermo Fisher Scientific, Thermo Scientific™, catalog number: 28324)
   9. Bovine serum albumin (BSA) (protease free) (*i.e.*, Fisher Scientific, catalog number: BP9703100)
   10. Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (*i.e.*, Sigma-Aldrich, catalog number: M3634-100G)
   11. 1 M HEPES buffer pH 8.0 (*i.e.*, Oakwood Products, catalog number: 047861-1Kg)

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12. Sodium hydroxide (NaOH)
13. Deionized water (dH_2O) (Autoclaved Nanopure filtered water is recommended for reactions with RNA)
14. 1 M Tris buffer pH 8.2 (see Recipes)
15. Buffer A (see Recipes)
16. 1 M HEPES buffer pH 8.0 (see Recipes)

B. The following reagents are needed if visualization of RNA and DNA modification is desired:
1. Ammonium persulfate (APS) (*i.e.*, Sigma-Aldrich, catalog number: A3678)
2. 40% acrylamide solution (19:1 acrylamide:bis acrylamide) (*i.e.*, Thermo Fisher Scientific, Invitrogen™, catalog number: AM9022)
3. Ethylenediaminetetraacetic acid (EDTA) (*i.e.*, Sigma-Aldrich, catalog number: 03620)
4. Tetramethylethylenediamine (TEMED) (*i.e.*, Thermo Fisher Scientific, Thermo Scientific™, catalog number: 17919)
5. Formamide (*i.e.*, Sigma-Aldrich)
6. 1 M HEPES buffer pH 8.0 (*i.e.*, Oakwood Products, catalog number: 047861-1Kg)
7. Sodium chloride (NaCl) (*i.e.*, Sigma-Aldrich, catalog number: S9888)
8. Glycerol (*i.e.*, Avantor Performance Materials, Macron Fine Chemicals, catalog number: 5092-16)
9. NP-40 detergent (*i.e.*, Thermo Fisher Scientific, Thermo Scientific™, catalog number: 28324)
10. Xylene cyanol (*i.e.*, Fischer Scientific, catalog number: BP125-100)
11. Bromophenol blue (*i.e.*, DOT Scientific, catalog number: DSB40160-25)
12. Dithiothreitol (DTT) (*i.e.*, bioWORLD, catalog number: 40400120)
13. Buffer B (see Recipes)
14. 2x stop buffer (see Recipes)

Equipment

A. The following equipment is needed for modifying nucleic acids with Polθ:
1. Pipettes (*i.e.*, P2, P20)
2. Temperature controlled water bath or incubator

B. The following equipment is needed if visualization of RNA and DNA modification is desired:
1. Large vertical sequencing gel apparatus (*i.e.*, APOGEE ELECTROPHORESIS, model: Model S2) or small vertical gel apparatus (*i.e.*, Bio-Rad Laboratories)
2. Glass plates for large or small gels
Note: Large glass plates for APOGEE ELECTROPHORESIS Model S2 SEQUENCER can be obtained from APOGEE ELECTROPHORESIS, and standard small plates and apparatuses can be obtained from Bio-Rad Laboratories.

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3. Plastic combs for gels
   Note: Plastic combs for large sequencing gels can be obtained from APOGEE ELECTROPHORESIS.
4. Gel spacers 0.4 mm thick (*i.e.*, APOGEE ELECTROPHORESIS)
5. Electrophoresis power supply (standard voltage [*i.e.*, 300 V] source for small gels, high voltage [*i.e.*, 5,000 V] source for large sequencing gels)
   Note: Power supplies may be obtained from Bio-Rad Laboratories.
6. Fluorescence imaging system (for fluorophore labeled nucleic acids)
7. Film developer or phosphorimager (*i.e.*, FUJIFILM, model: FLA7000) (for 5' ^{32}P radio-labeled nucleic acids)

Procedure

A. Modification of the 3' terminal ends of DNA and RNA using Polθ
   1. Purified Polθ is needed for modifying the 3' terminal ends of DNA and RNA. Procedures for expressing and purifying Polθ from *E. coli* are described in previous studies (Hogg *et al*., 2011).

   Notes:
   - *a. Synthetic single-stranded DNA (ssDNA) and RNA typically ~10-50 nt (nucleotides) in length have been routinely modified in our laboratory. Thus, we recommend using nucleic acids of similar length for the following procedure.*
   - *b. All reagents below are listed as final concentrations.*

   2. A typical procedure for modifying ssDNA or RNA is described as follows:
      a. 50-100 nM of the ssDNA or RNA to be modified is mixed with 50-500 μM concentration of the desired nucleotide used for modification in buffer A (see Recipes) along with 5 mM $MnCl_2$ in a reaction volume of 10-20 μl.
      Note: We have not thoroughly tested the effects of different nucleotide concentrations. However, concentration ranges between 50-500 μM have shown efficient terminal transferase activity. Most of our previous reactions included 500 μM nucleotides. Reaction volumes can be varied according to preference and concentrations of ssDNA and RNA used successfully in the reaction in our experience are 50-100 nM. Importantly, the DNA or RNA must include a 3' terminal nucleotide containing a hydroxyl group at the 3' position of the sugar moiety for Polθ terminal transferase activity to occur.
      b. The terminal transferase reaction is then initiated by adding 200-500 nM purified Polθ and incubating at 42 °C for 2 h. Reactions are gently mixed with a pipette. Vortexing is not recommended.
      c. Reactions can be terminated by heating to ≥ 80 °C for 10 min or by the addition of ≥ 10 mM EDTA. Although the amount of Polθ can vary, optimal terminal transferase activity is observed with a 5-10 higher ratio of polymerase to nucleic acid molecule. We note that

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relatively high concentrations (*i.e.*, > 50 mM) of salt (*e.g.*, NaCl) suppress Polθ terminal transferase activity.

3. The 2 h incubation time specified in the above procedure will give rise to multiple (*i.e.*, 3 to > 100) terminal transfer events for most canonical nucleotides. However, in some cases only a single transfer event may occur depending on the particular nucleotide analog used. For example, certain nucleotide analogs may not be efficiently incorporated by Polθ and thus limit the enzyme to a single nucleotide transfer event. For determining the number of terminal transferase events that occur on a given substrate in the presence of particular nucleotides, we recommend visualizing the initial nucleic acid substrate and nucleic acid reaction products in a denaturing sequencing gel as described below in the Data analysis section.

B. Examples of experimental procedures for modifying the 3' terminal ends of DNA and RNA using Polθ

1. As examples of Polθ terminal transferase activity on ssDNA and RNA, reactions were performed as follows. 50 nM of 5' ^{32}P radio-labeled ssDNA oligo (sequence indicated; Figures 1A and 1B) or RNA (sequence indicated; Figure 1C) was mixed with 50 µM (Figures 1A and 1B) or 500 µM.

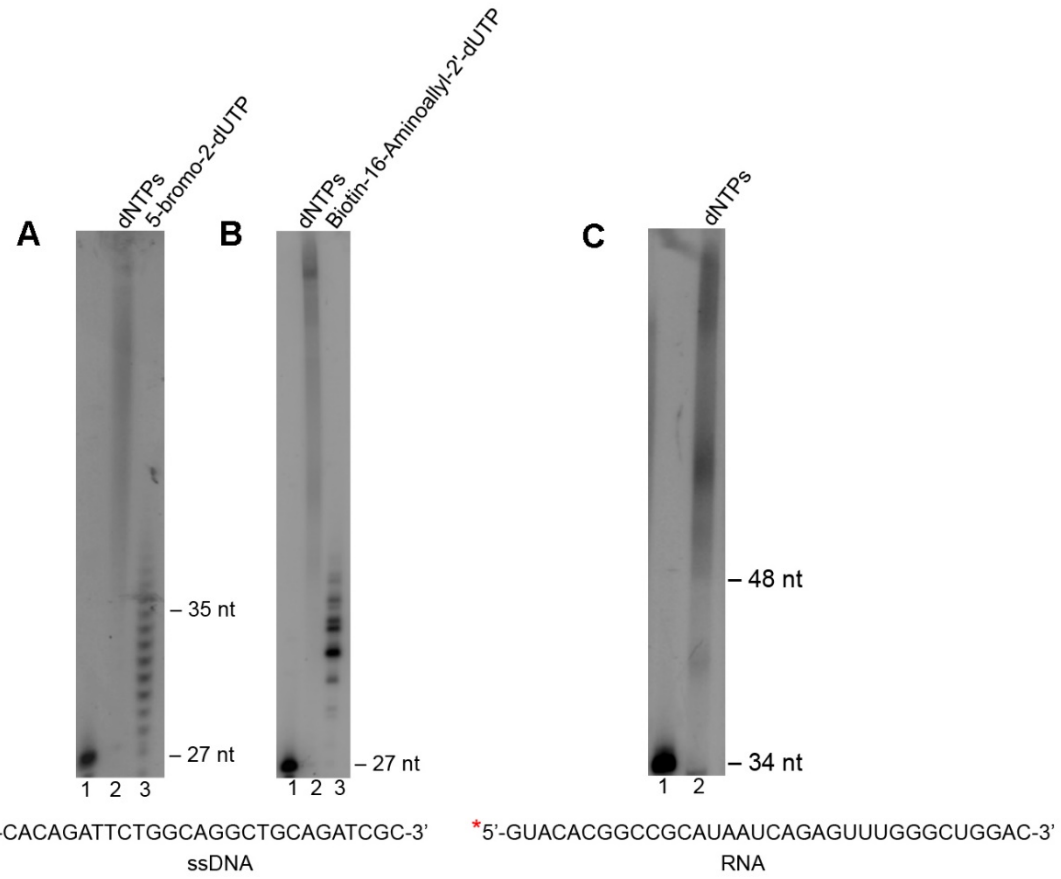

Figure 1. Use of Polθ terminal transferase activity to modify the 3' terminal ends of DNA and RNA. A-C. Denaturing gels showing Polθ terminal transferase activity on the indicated

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ssDNA (A and B) and RNA (C) substrates in the presence of the indicated nucleotides. Lanes 1 lack Polθ and nucleotides. Lanes 2 and 3 include Polθ and the indicated nucleotides. ssDNA and RNA sequences are indicated at bottom. *, ^{32}P radio-label.

2. (Figure 1C) of the indicated nucleotides along with 5 mM $MnCl_2$ in 20 µl of buffer A (see Recipes).
3. Reactions were initiated by adding 200 nM of purified Polθ (stored in buffer B [see Recipes]), then incubating at 42 °C.
4. After 2 h, reactions were terminated by adding 20 µl of 2x stop buffer (see Recipes).
5. Radio-labeled ssDNA and RNA were then analyzed after denaturing gel electrophoresis and autoradiography as described below in the Data analysis section. The data show that Polθ efficiently transfers nucleotides to the 3' terminus of nucleic acid substrates as demonstrated in previous studies (Figure 1) (Kent *et al*., 2016). These experiments also demonstrate the ability of Polθ to efficiently transfer large nucleotide analogs, consistent with recent work (Kent *et al*., 2016). We note that Polθ may also be used to modify double-strand blunt ended DNA, however, fewer nucleotides are transferred to these substrates as demonstrated in previous work (Kent *et al*., 2016). Partial single-strand DNA containing 3' overhangs are most efficiently extended by Polθ (Kent *et al*., 2016).

Data analysis

1. Visualizing modified RNA and DNA in denaturing gels
   a. Reactions should be performed as above with the following modifications. The RNA or DNA should be either 5' radio-labeled, or conjugated with a fluorophore prior to the reaction for their detection in denaturing gels. Nucleic acids can be radio-labeled using T4 polynucleotide kinase in the presence of gamma-ATP. For fluorophore detection, DNA and RNA oligonucleotides can be purchased with 5' fluorophore linkages. We recommend terminating reactions with an equal volume of 2x stop buffer (see Recipes).
   b. Initial nucleic acid substrates and reaction products should be resolved in standard urea denaturing 10-20% polyacrylamide gels. Helpful protocols for pouring and processing urea denaturing polyacrylamide sequencing gels are referenced here (Summer *et al*., 2009; Flett, *et al*., 2013). Large sequencing gels will allow for the highest resolution (*i.e.*, single nucleotide resolution). However, smaller gels may provide enough resolution depending on the particular application. In the case of RNA, we recommend adding 10-15% formamide to urea denaturing polyacrylamide gels to reduce RNA secondary structures that can appear as smears in the gel. Large sequencing gels are typically run at 70-80 W using a high voltage (5,000 V) power supply. The resolved nucleic acids can then be visualized using a fluorescent imager (for 5' fluorophore conjugated oligos) or using a phosphorimager or autoradiography (for 5' ^{32}P labeled oligos). Figure 1 shows examples of 5' ^{32}P radio-labeled

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nucleic acids that were resolved in large sequencing gels, then visualized by autoradiography.

Notes

1. Reproducibility
   In our experience, extension of nucleic acids by Polθ is highly reproducible. However, we note that the precise amount of initial substrates extended may vary. For example, in some cases 100% of nucleic acid substrates are extended, whereas in other cases a small fraction (*i.e.*, ~5-15%) of substrates are not extended. A 4-5 fold higher ratio of Polθ to nucleic acid substrates will usually allow for the majority of substrates to be extended. We note that the number of nucleotides transferred to the 3' terminus of nucleic acids may vary. Thus, the final length of extended nucleic acids will not be identical for all molecules. The respective structures of canonical nucleotides and nucleotide analogs will also give rise to different terminal transferase efficiencies. For example, deoxyadenosine monophosphate is most efficiently transferred by Polθ (Kent *et al*., 2016). Other deoxyribonucleotides are somewhat less efficiently transferred by the polymerase (Kent *et al*., 2016). The initial nucleic acid sequence may also affect Polθ terminal transferase activity. Previous studies compare the efficiency of Polθ terminal transferase activity on different nucleic acid substrates and in the presence of various canonical nucleotides and nucleotide analogs (Kent *et al*., 2016).
2. Additional notes, technical tips and cautionary points
   For optimal Polθ terminal transferase activity, we recommend storing the enzyme in buffer B (see Recipes) at concentrations ≥ 1 mg/ml in small aliquots at -80 °C and limiting freeze thaw cycles to 2-3 times. We note that oligonucleotides relatively short in length (< 10 nt) may not be extended as efficiently as those longer in length (> 10 nt). Oligos containing a high proportion of closely spaced guanosine bases, for example similar to telomere repetitive DNA sequences or those that form G quadruplexes, may exhibit a lower efficiency of extension by Polθ (Kent *et al*., 2016). As noted above, Polθ can also be used to modify double-stranded DNA, however, only 1-3 nucleotides are generally transferred to these substrates (Kent *et al*., 2016).

Recipes

1. 1 M Tris buffer pH 8.2
   Weigh 121.1 g of Tris Ultra Pure and add 800 ml of dH_2O
   Stir until dissolved, then adjust pH to 8.2 with HCl
   Adjust final volume to 1 L with dH_2O
2. Buffer A
   20 mM Tris-HCl pH 8.2
   0.01% NP-40

0.1 mg/ml BSA
10% glycerol

3. 1 M HEPES buffer pH 8.0
   Weigh 238.3 g of HEPES and add 800 ml of dH_2O
   Stir until dissolved, then adjust pH to 8.0 with NaOH
   Adjust final volume to 1 L with dH_2O
4. Buffer B
   50 mM HEPES pH 8.0
   300 mM NaCl
   10% glycerol
   0.01% NP-40
   5 mM DTT
5. 2x stop buffer
   90% formamide
   50 mM EDTA
   0.03% xylene cyanol
   0.03% bromophenol blue

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