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Preparation and Characterization of Internally Modified DNA Templates for Chemical Transcription Roadblocking

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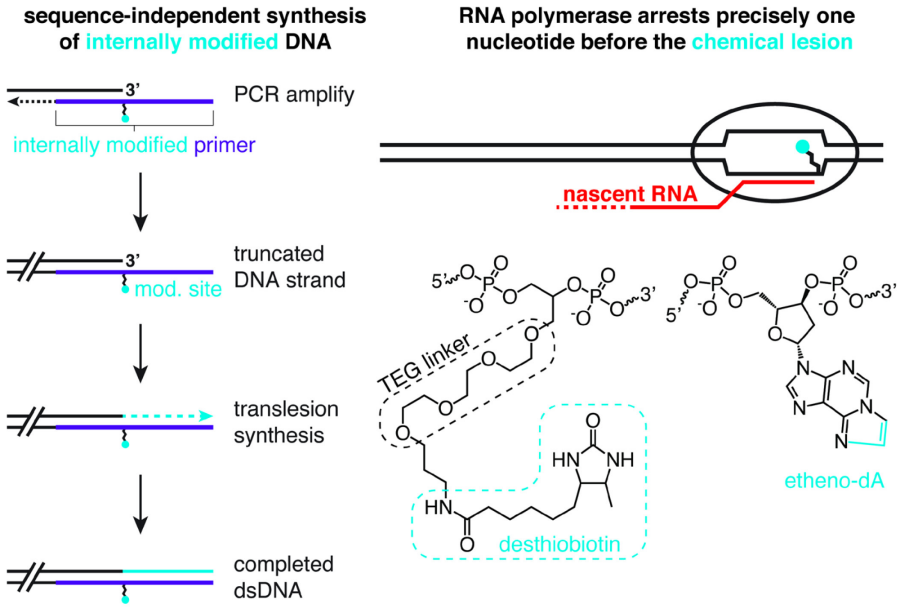
[Abstract] Site-specific transcription arrest is the basis of emerging technologies that assess nascent RNA structure and function. Cotranscriptionally folded RNA can be displayed from an arrested RNA polymerase (RNAP) for biochemical manipulations by halting transcription elongation at a defined DNA template position. Most transcription "roadblocking" approaches halt transcription elongation using a protein blockade that is non-covalently attached to the template DNA. I previously developed a strategy for halting *Escherichia coli* RNAP at a chemical lesion, which expands the repertoire of transcription roadblocking technologies and enables sophisticated manipulations of the arrested elongation complexes. To facilitate this *chemical transcription roadblocking* approach, I developed a sequence-independent method for preparing internally modified dsDNA using PCR and translesion synthesis. Here, I present a detailed protocol for the preparation and characterization of internally modified dsDNA templates for chemical transcription roadblocking experiments.

Graphic abstract:



Precise transcription roadblocking using functionalized DNA lesions

Keywords: Transcription, RNA, DNA lesion, PCR, Translesion synthesis, Cotranscriptional RNA folding

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[Background] RNA begins to fold as it emerges from an RNA polymerase (RNAP) during transcription (Pan and Sosnick, 2006). Our ability to investigate the biological roles of nascent RNA structure and function therefore depends on the development of tools that can assess RNA cotranscriptionally. One successful approach towards this goal has been to leverage the extraordinary stability of the ternary RNAP-DNA template-nascent RNA complex to measure and manipulate nascent RNA in the context of arrested transcription elongation complexes (TECs) (Buenrostro *et al.*, 2014; Tome *et al.*, 2014; Watters *et al.*, 2016; Strobel *et al.*, 2017; Widom *et al.*, 2018).

Multi-subunit RNAPs are remarkably processive enzymes that are capable of transcribing multi-kilobase operons in bacteria and tens of kilobases to megabases in eukaryotic genes (Core *et al.*, 2008; Belogurov and Artsimovitch, 2019). The molecular forces that enable processive transcription elongation (Korzheva *et al.*, 2000; Vassylyev *et al.*, 2007a and 2007b) also render TECs notoriously resistant to dissociation in general. For example, when RNAP encounters a roadblock, such as a DNA-bound protein or a chemical DNA lesion, the TEC typically remains intact until either the roadblock is removed or a termination factor evicts RNAP from the DNA (Selby and Sancar, 1993 and 1994). When constructed *in vitro* by halting RNAP at a transcription roadblock, arrested TECs persist for exceptionally long periods of time. RNAP arrest by transcription roadblocking has consequently become the method of choice for structural and functional studies that require the display of nascent RNA molecules.

The development of high-throughput methods that use arrested RNAPs to display RNA underscores the need for versatile transcription roadblocking tools. Several roadblocking strategies that halt transcription elongation by colliding RNAP with a DNA-bound protein blockade have been developed: Sequence-specific protein roadblocks, such as the catalytically inactive $EcoRI_{E111Q}$ mutant (Pavco and Steege, 1990) and the *E. coli* DNA replication terminator Tus (Tome *et al.*, 2014), are attached to a DNA template by their respective recognition sequences. Streptavidin can be stably attached to template DNA using precisely (Frieda and Block, 2012) or randomly positioned (Strobel *et al.*, 2017) biotin modifications. dCas9 can be reversibly loaded onto arbitrary DNA sequences to halt RNAP sequentially at multiple DNA positions (Widom *et al.*, 2019). In contrast to the abundance of approaches for protein-mediated transcription roadblocking, methods for halting RNAP at DNA lesions have been conspicuously lacking despite the long-established fact that DNA damage causes transcription arrest. I previously addressed this technological gap by developing a sequence-independent method for preparing internally modified linear dsDNA using translesion synthesis and characterizing the transcription roadblocking properties of several DNA modifications (Strobel *et al.*, 2020).

My chemical transcription roadblocking method complements the properties of protein-mediated roadblocking strategies in several ways. First, like biotin-streptavidin roadblocking, chemical transcription roadblocking is sequence-independent and therefore compatible with the preparation of complex sequence libraries. Second, chemical stall sites are fully functional in a minimal *in vitro* transcription reaction and are therefore compatible with diverse experimental conditions. Third, functionalized chemical stall sites can enable TEC manipulations that are not otherwise possible. For example, I previously showed that TECs that are arrested at a desthiobiotin stall site can be enriched by exclusion from streptavidin-coated magnetic particles. The primary disadvantage of chemical

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transcription roadblocking is that *E. coli* RNAP can bypass some DNA lesions over time. Nonetheless, in many cases, lesion bypass is extremely slow and will therefore not be prohibitive for most experiments: in the transcription reactions described below, TECs remain at a desthiobiotin-TEG stall site with a half-life of ~600 min, whereas unimpeded bacterial transcription occurs at 50-100 nt/s. Furthermore, these arrested TECs can persist stably for hours following nucleotide depletion (Strobel *et al.*, 2020).

Here, I provide a detailed protocol for the preparation and characterization of internally modified linear dsDNA templates for chemical transcription roadblocking. I first describe considerations that must be taken into account during oligonucleotide design and provide a set of validated sequences for PCR amplification. I then present a straightforward protocol for the preparation of internally modified DNA using PCR and translesion synthesis and describe how to assess DNA template quality using denaturing and native polyacrylamide gel electrophoresis. Lastly, I present a single-round *in vitro* transcription protocol that can be used to verify the transcription roadblocking activity of the DNA template. Together, these protocols will enable any researcher with basic molecular biology expertise to successfully design and prepare internally modified DNA templates for the display of nascent RNA from arrested TECs.

Materials and Reagents

A. Consumables
1. Low retention 10 µl pipette tips (*e.g.*, Neptune Scientific, catalog number: 2342S3.S)
2. Low retention 200 µl pipette tips (*e.g.*, Neptune Scientific, catalog number: 2102.S)
3. Low retention 1,000 µl pipette tips (*e.g.*, Neptune Scientific, catalog number: 2372.S)
4. 200 µl, 0.4 mm flat gel loading pipette tips (*e.g.*, Laboratory Product Sales, catalog number: L113761)
5. Nuclease-Free 1.7 ml Microcentrifuge Tubes (*e.g.*, Thomas Scientific, catalog number: 1149K01)
6. Nuclease-Free 2.0 ml Microcentrifuge Tubes (*e.g.*, VWR, catalog number: 20170-170)
7. 2.0 ml Screw-Cap Tubes, w/ O-ring (*e.g.*, Laboratory Product Sales, catalog number: L233226)
8. 0.2 ml 8-Strip PCR tube w/ dome cap (*e.g.*, Laboratory Product Sales, catalog number: L216380)
9. Qubit™ Assay Tubes (Invitrogen™, catalog number: Q32856)
10. 15 ml Conical Tubes (*e.g.*, Laboratory Product Sales, catalog number: TR2000)
11. 20 ml Conical Tubes (*e.g.*, Laboratory Product Sales, catalog number: TR2003)
12. 5 ml serological pipette (*e.g.*, Laboratory Product Sales, catalog number: L310500)
13. 10 ml serological pipette (*e.g.*, Laboratory Product Sales, catalog number: L311000)
14. 25 ml serological pipette (*e.g.*, Laboratory Product Sales, catalog number: L312510)
15. Weigh boats (any source)
16. 10 ml Syringe (*e.g.*, BD, catalog number: 302995)
17. 60 ml Syringe (*e.g.*, BD, catalog number: 309653)
18. 20G 1½” Needle (*e.g.*, BD, catalog number: 305176)
19. 0.2 µm Polyethersulfone (PES) syringe filter, sterile (*e.g.*, VWR catalog number: 28145-501)
20. 150 mm Culture Dish (*e.g.*, Corning® catalog number: 430599)

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21. Small 1-Ply Light-Duty Tissue Wipers (*e.g.*, VWR, catalog number: 82003-820)
22. Large 1-Ply Light-Duty Tissue Wipers (*e.g.*, VWR, catalog number: 82003-822)
23. Plastic-backed bench paper (*e.g.*, Fisher Scientific, catalog number: 14-127-47)
24. Nitrile Gloves
25. Vaseline® Petroleum Jelly Original
26. Rain-X® Original Glass Water Repellent
27. Large binder clips (*e.g.*, Staples 2" Binder Clips, Staples, catalog number: 10669)
28. AEP 30510400 ZipSafe Sealwrap, 18" × 2000' (Amazon)
29. Razor blades
30. Aluminum foil
31. Dish soap
32. Plastic brush for cleaning sequencing gel plates

B. Reagents

Note: Chemicals should be Molecular Biology/Biotechnology Grade.

1. Nuclease-Free Water (*e.g.*, Invitrogen™, catalog number: 10977015), store at room temperature (RT)
2. Milli-Q Water, store at RT
3. Deionized Water, store at RT
4. Diethyl pyrocarbonate (DEPC), >97%, (Thermo Scientific™, AAJ14710AC), store at -20°C
   Note: *Handle DEPC in a chemical fume hood.*
5. Tris (1 M), pH 8.0, RNase-free (Invitrogen™, catalog number: AM9855G), store at RT
6. EDTA (0.5 M), pH 8.0, RNase-free (Invitrogen™, catalog number: AM9260G), store at RT
7. Potassium Chloride (KCl) (2 M), RNase-free (Invitrogen™, catalog number: AM9640G), store at RT
8. Magnesium Chloride ($MgCl_2$), 1 M, RNase-free (Invitrogen™, catalog number: AM9530G), store at RT
9. Sodium Acetate (NaOAc) (3 M), pH 5.5 RNase-free (Invitrogen™, catalog number: AM9740), store at RT
10. 1× Tris-EDTA (TE) Solution (10 mM Tris pH 7.5, 0.1 mM EDTA), (*e.g.*, Integrated DNA Technologies, catalog number: 11-05-01-05), store at RT
11. Tris Base (*e.g.*, VWR, catalog number: 97061-794), store at RT
12. EDTA, Disodium Salt, Dihydrate (*e.g.*, Invitrogen™, catalog number: 15576028), store at RT
13. Hydrochloric Acid (HCl) (*e.g.*, Sigma-Aldrich, catalog number: 320331), store at RT with other acids beneath a fume hood.
    Caution: *Hydrochloric acid is highly corrosive and must be handled in a chemical fume hood.*
14. Sodium hydroxide (NaOH) (*e.g.*, Sigma-Aldrich, catalog number: S8045), store at RT with other bases
    Caution: *Sodium hydroxide is highly corrosive.*

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15. Boric Acid (*e.g.*, Fisher Scientific, catalog number: BP168-500), store at RT
16. Sodium dodecyl sulfate sodium salt (SDS) (*e.g.*, Millipore, catalog number: 7910-500GM), store at RT
17. Dithiothreitol (DTT) (*e.g.*, Gold Biotechnology, catalog number: DTT100), store desiccated at -20°C
18. 100% Ethanol (200 Proof) (*e.g.*, Decon Labs, catalog number: 2716), store at RT
19. Isopropanol (*e.g.*, Sigma-Aldrich, catalog number: I9516-4L), store at RT
20. Glycerol (*e.g.*, Sigma-Aldrich, catalog number: G5516-4L), store at RT
21. Phenol:Chloroform + Tris Buffer (*e.g.*, Thermo Scientific™, catalog number: 17909), store at 4°C

    Caution: *Phenol:chloroform should be handled in a chemical fume hood.*
22. OmniPur Formamide, Deionized (*e.g.*, Millipore, catalog number: 4650-500ML), store at 4 °C

    Caution: *Formamide should be handled in a chemical fume hood.*
23. Dimethyl sulfoxide (DMSO) (*e.g.*, Fisher Scientific, catalog number: D128-500), store at RT

    Caution: *Dimethyl sulfoxide should be handled in a chemical fume hood.*
24. Ammonium persulfate (APS) (*e.g.*, Sigma-Aldrich, catalog number: A3678-100G), store at RT
25. TEMED (Tetramethylethylenediamine) (*e.g.*, Bio-Rad, catalog number: 1610801), store at RT
26. Bromophenol blue (*e.g.*, Bio-Rad, catalog number: 1610404), store at RT
27. Xylene cyanole FF (*e.g.*, Bio-Rad, catalog number: 1610423), store at RT
28. Ethidium bromide, 10 mg/ml (*e.g.*, Invitrogen™, catalog number: 15585011), store at RT

    Note: *Handle ethidium bromide in accordance with institutional guidelines.*
29. Quick-Load® Purple 100 bp DNA Ladder (New England Biolabs, catalog number: N0551L), store at RT
30. Low Range ssRNA Ladder (New England BioLabs, catalog number: N0364S), store at RT
31. SYBR Gold Nucleic Acid Stain (Invitrogen™, catalog number: S11494), store at -20°C
32. Deoxynucleotide (dNTP) Solution Mix (New England Biolabs, catalog number: N0447L), store at -20°C
33. dNTP Set (100 mM) (Invitrogen™, catalog number: 10297018), store at -20°C
34. 2-Amino-dATP (TriLink Biotechnologies, catalog number: N-2003), store at -20°C
35. 5-Propynyl-dCTP (TriLink Biotechnologies, catalog number: N-2016), store at -20°C
36. High Purity rNTP Set, 100 mM Solutions (ATP, CTP, GTP, UTP) (Cytiva Life Sciences, catalog number: 27202501), store at -20°C
37. UltraPure™ BSA (Invitrogen™, catalog number: AM2616), store at -20°C
38. Rifampicin (Gold Biotechnology, catalog number: R-120-10), store dessicated at -20°C
39. GlycoBlue™ Coprecipitant (Invitrogen™, catalog number: AM9516), store at -20°C
40. Qubit™ dsDNA HS Assay Kit (Invitrogen™, catalog number: Q32854), some components stored at 4°C
41. Qubit™ dsDNA BR Assay Kit (Invitrogen™, catalog number: Q32853), some components stored at 4°C

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42. SeaKem GTG™ Agarose (Lonza, catalog number: 50074), store at RT
    Critical: *Agarose must be preparatory grade.*
43. UreaGel 19:1 Denaturing Gel System (National Diagnostics, catalog number: EC-833), store at RT
    Caution: *Contains acrylamide. Acrylamide is a neurotoxin and should be handled cautiously.*
44. ProtoGel (30%) (National Diagnostics, catalog number: EC-890), store at RT
    Caution: *Contains acrylamide. Acrylamide is a neurotoxin and should be handled cautiously.*
45. QIAquick PCR purification kit (Qiagen, catalog number: 28106), store at RT
46. QIAquick Gel Extraction Kit (Qiagen, catalog number: 28706), store at RT
47. Internally modified oligonucleotides (Integrated DNA Technologies, see Table 1)
48. UTP, [α-^{32}P]-3000 Ci/mmol 10 mCi/ml (PerkinElmer, catalog number: BLU007H), store at -20°C
    Note: *[α-^{32}P]-UTP must be handled in accordance with institutional radiation safety protocols.*

C. Enzymes
1. Q5® High-Fidelity DNA Polymerase (New England Biolabs, catalog number: M0491L), store at -20°C
2. Vent® (exo-) DNA Polymerase (New England Biolabs, catalog number: M0257L), store at -20°C
3. *Sulfolobus* DNA Polymerase IV (New England Biolabs, catalog number: M0327S), store at -20°C
4. Thermolabile exonuclease I (New England Biolabs, catalog number: M0568L), store at -20°C
5. *E. coli* RNA Polymerase Holoenzyme (New England Biolabs, catalog number: M0551S), store at -20°C
   Note: *E. coli RNA polymerase core and σ^{70} can be purified by well-established protocols (Marr and Roberts, 1997; Svetlov and Artsimovitch, 2015) and used to reconstitute the holoenzyme.*

D. Prepared Solutions (see Recipes section)
1. DEPC-Treated Milli-Q Water
2. 10 mM Tris-HCl, pH 8.0
3. 10 mM Thermostability-enhancing dNTP Solution
4. 1× Tris-Borate-EDTA Buffer
5. 70% Ethanol
6. 10% SDS
7. 6× DNA Loading Dye (+SDS, XC only)
8. 10% APS
9. 1× Formamide Loading Dye
10. 3× Tris-Borate-EDTA Buffer
11. 6× DNA Loading Dye (non-denaturing)
12. 1 M DTT
13. 100 mM DTT

14. 10× Transcription Buffer
15. 20× NTP Solution
16. 5 mg/ml BSA
17. 2 mg/ml Rifampicin
18. 1 M Tris-HCl, pH 8.0
19. 0.5 M EDTA, pH 8.5
20. Stop Solution

Equipment

1. Autoclave
2. Fume Hood
3. -20°C and -80°C freezers (any source)
4. Refrigerator (4°C) (any source)
5. 2 µl, 20 µl, 200 µl, and 1,000 µl micropipettes (any source)
6. Pipette Controller (*e.g.*, Drummond, catalog number: 4-000-101)
7. Plastic tube racks for 1.7 ml, 15 ml, and 50 ml tubes (any source)
8. Lab Timer (*e.g.*, Traceable®, catalog number: 5004CC)
9. Ice bucket (*e.g.*, SCIENCEWARE®, catalog number: M16807-4002)
10. Erlenmeyer flasks (500 ml, 4 L) (any source)
11. Beakers (1 L, 2 L) (any source)
12. Autoclavable media bottles (500 ml, 1 L, and 2 L) (any source)
13. Graduated cylinders (10 ml, 50 ml, 200 ml, 0.5 L, 1 L, and 2 L) (any source)
14. Plastic 250 ml beaker (any source)
15. 4 L polypropylene bottle (any source)
16. 20 L carboy (any source)
17. Microwave (any source)
18. Vortex (*e.g.*, Scientific Industries, catalog number: SI-0236)
19. Thermal cycler (*e.g.*, Bio-Rad S1000 w/ 96-Deep Well Reaction Module, catalog number: 1852197)

    Critical: *100 µl PCRs require a deep well thermal cycler. If 100 µl exceeds the maximum sample volume of your thermal cycler, aliquot the reaction into additional PCR tubes.*
20. Refrigerated microcentrifuge (*e.g.*, Eppendorf 5424 R, catalog number: 5406000046)
21. Mini centrifuge (*e.g.*, VWR, catalog number: 76269-064)
22. Digital Dry Bath (*e.g.*, Thermo Scientific™, catalog number: 88870001)
23. Lab Armor™ Beads (Gibco™, catalog number: A1254301)
24. Aluminum block for 1.7 ml tubes (*e.g.*, Thermo Scientific™, catalog number: 88880130)
25. Aluminum heating/block, holds 96 × 0.2 ml tubes (Sigma-Aldrich, catalog number: Z740270-1EA)

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26. Analog thermometer (*e.g.*, VWR, catalog number: 89095-600)
27. Digital thermometer (*e.g.*, VWR, catalog number: 89094-750)
28. Qubit™ 4 Fluorometer (Invitrogen™, catalog number: Q33238)
29. Rocking platform (*e.g.*, Corning, catalog number: 6703)
30. Rotator (*e.g.*, ATR, Rotamix Rotator RKVSD and end-over end rod, catalog numbers: 10101 and 10110)
31. pH meter (*e.g.*, Mettler Toledo, S220-Bio, catalog number: 30019031)
    Critical: *The pH meter electrode must be compatible with Tris solutions.*
32. Balance (*e.g.*, Ohaus, catalog number: 30253007RM)
33. Analytical balance (*e.g.*, Mettler Toledo, catalog number: 30029076)
34. Flat Spatula (*e.g.*, VWR, catalog number: 82027-532)
35. Weighing Spatulas (any source)
36. Stir Plate (*e.g.*, Corning PC-610D, catalog number: 6795620D)
37. Magnetic stir bars (*e.g.*, VWR, catalog number: 58948-025)
38. Horizontal Gel Apparatus (*e.g.*, Horizon 11-14 Midi Gel System, Gel Company, HZ1114)
    Note: Comb should hold at least 50 µl.
39. Mini-PROTEAN Tetra Cell, 2-gel, for 1.0 mm handcast gels (Bio-Rad, catalog number: 1658003FC)
40. Sequencing Gel Apparatus (*e.g.*, BRL Model S2; Gel Company, catalog number: S2-3040)
    Note: Spacers and comb should be 0.4 mm thickness.
41. Basic power supply (*e.g.*, Bio-Rad, catalog number: 1645050)
42. High voltage power supply (*e.g.*, Bio-Rad, catalog number: 1645056)
43. UV Transilluminator (*e.g.*, UVP catalog number: UV95045901)
    Caution: *Never expose eyes or skin to UV radiation.*
44. Dark Reader Blue light Transilluminator (Clare Chemical, catalog number: DR89X)
45. Cardboard exposure cassette (*e.g.*, Sigma-Aldrich, catalog number: E-9135, discontinued)
46. Storage Phosphor Screen, 35 × 43 cm (Cytiva Life Sciences, catalog number: 28956476)
47. Storage Phosphor Screen Image Eraser (Cytiva Life Sciences, catalog number: 29187190)
48. Typhoon Biomolecular RGB Biomolecular Imager (Cytiva Life Sciences, catalog number: 29187194)
49. Acrylic shielding for high-energy beta radiation
    Note: I have found the items below useful when performing experiments with ^{32}P.
    a. Vertical Bench-Top Beta Shield, 3/8” Thick (*e.g.*, RPI, catalog number: BR-201)
    b. 24-place Beta Block (*e.g.*, Laboratory Product Sales, catalog number: 172644)
    c. Sample Beta Box w/ ice compartment (*e.g.*, Fisher Scientific, catalog number: S29137)
    d. Acrylic Waste Box (*e.g.*, ThermoScientic™, catalog number: 67459024)
    e. Large Vertical Acrylic Shield (Denville Scientific, catalog number S0260-S)
    f. Medium Vertical Acrylic Shield (Denville Scientific, catalog number S0180-S)
    g. Large Acrylic Storage Container (*e.g.*, Mitchell Plastics, catalog number: RP-400)

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h. Small Acrylic Storage Container (*e.g.*, IBI Scientific, catalog number: RB-100)

i. Pipette tip disposal box (Thomas Scientific, catalog number: 1216J05)

Procedure

A. Overview of the procedure

My procedure for preparing internally modified linear dsDNA comprises three primary steps (Figure 1): First, an internal modification is introduced into the DNA template by PCR amplification (Procedure C). PCR amplification yields a truncated DNA product because DNA modifications that halt transcription elongation are also likely to halt elongation by the DNA polymerases that are typically used for PCR. Next, the remainder of the truncated DNA strand is synthesized using the translesion polymerase *Sulfolobus* DNA Polymerase IV (Procedure D). Finally, the completed dsDNA product is purified to remove excess oligonucleotides and the template DNA that was used for PCR amplification (Procedure E or Procedure F). The purified DNA is then subjected to three quality control steps to verify the efficiency of translesion synthesis (Procedure G), the purity of the dsDNA (Procedure H), and the activity of the internal modification as a transcription roadblock (Procedure I). The resulting internally modified linear dsDNA is then ready to be used to cotranscriptionally display nascent RNA from arrested TECs.

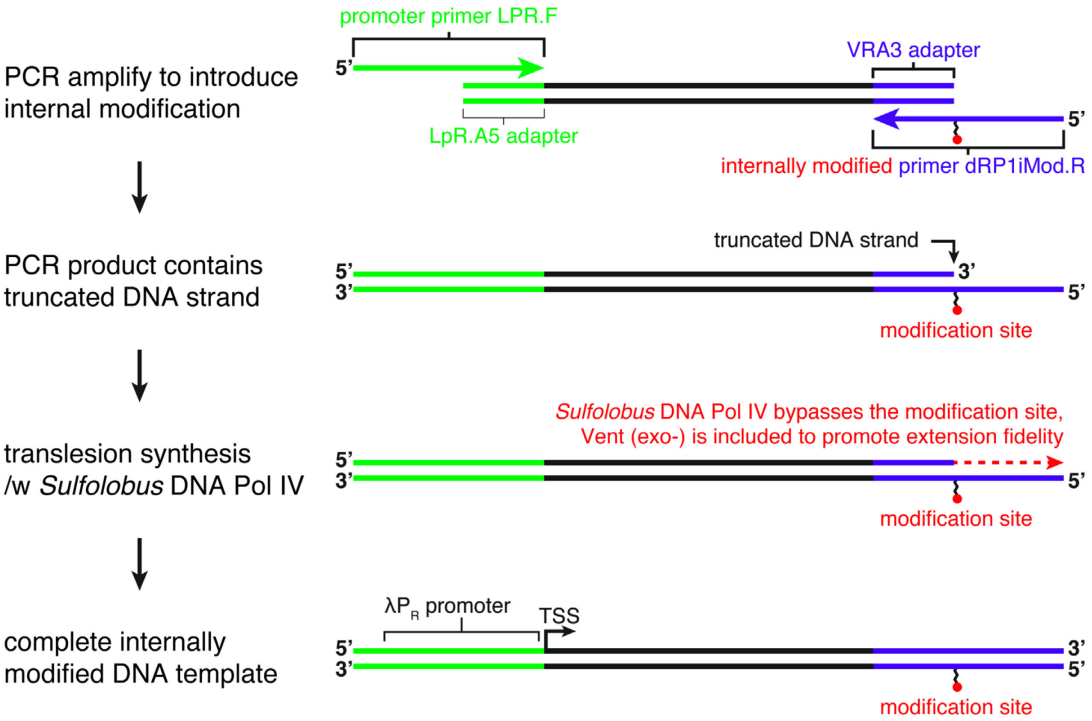

Figure 1. Overview of the procedure for enzymatically preparing internally modified dsDNA. PCR with an internally modified primer yields a DNA product with one truncated strand. The truncated strand is then completed using translesion DNA synthesis with *Sulfolobus* DNA Polymerase IV, which bypasses the internal modification site, and Vent® (exo-). The resulting

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internally modified dsDNA is then purified and assessed for quality prior to use as a DNA template for *in vitro* transcription experiments.

B. Priming site and oligonucleotide sequences

Successful DNA template preparation depends on the careful design of PCR priming sites. My standard protocol uses two constant DNA regions that have been validated for efficient amplification without detectable side products (Table 1).

Table 1. Priming sites and primers used to prepare linear DNA templates. The region of each oligonucleotide that anneals to its corresponding adapter is underlined. '/iMod/' indicates the position of an internal DNA modification. Validated modifications include desthiobiotin-TEG, biotin-TEG, etheno-dA, and UniLink™ Amino-Modifier (Figure 2).

Name	Sequence	Description
LPR.A5	5-ACCTCTGGCGGTGATAATGGTTGCAT-3	Upstream adapter comprising a λP_R promoter fragment.
VRA3	5-GATCGTCGGACTGTAGAACTCTGAAC-3	Downstream adapter, the reverse complement of the Illumina TruSeq Small RNA RA5 adapter.
LPR.F	5-CTAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCAT-3	Forward primer that anneals to the reverse complement of LPR.A5 to append the full λP_R core promoter.
PRA1.F[1]	5-TTATCAAAAAGAGTATTGACTCTTTTACCTCTGGCGGTGATAATGGTTGCAT-3	Alternate forward primer that anneals to the reverse complement of LPR.A5 to append the full P_{RA1} promoter.
dRP1iMod.R	5-AATGATACGGCGACCACCGAGATCTACAC/iMod/GTTCAGAGTTCTACAGTCCGACGATC-3	Internally modified reverse primer that anneals to VRA3.
dRP1.R	5-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC-3	Unmodified reverse primer that anneals to VRA3. Used for quality control.

[1]The P_{RA1} promoter (Strobel, 2021) is a derivative of the λP_R and T7A1 promoters and can be substituted in place of λP_R by using the PRA1.F primer instead of LPR.F.

Critical: *Order the LPR.F, PRA1.F, dRP1iMod.R, and dRP1.R primers at 100 nmol or greater scale with HPLC purification. Oligonucleotides can be from any source. I use Integrated DNA Technologies.*

1. Design PCR primer sequences.

   I recommend using the sequences described in Table 1, which have been extensively validated for use in the procedures below. If your application requires different sequences, use oligonucleotide design tools such as Integrated DNA Technologies OligoAnalyzer™ to verify that your sequences do not contain significant secondary structure and are unlikely to yield primer dimers due to stable homo- or heterodimers. Ideally, primers should be less than 60 nts in length, and the internally modified oligo should have ~30 nts between the modification and the 5' end to ensure duplex stability downstream of the modification site in the final dsDNA product. The region of each primer that anneals to the PCR adapters should have a GC content of ~50%. Tm

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will vary with the polymerase used; when using Q5® DNA polymerase I aim to anneal primers at 65°C. The New England Biolabs Tm Calculator is useful for assessing annealing temperatures when using NEB polymerases.

Priming sites can be added to the PCR template DNA in several ways depending on the intended application. First, if the PCR template will be plasmid DNA, priming sites can be included in the DNA sequence during cloning. Similarly, if the PCR template will be a synthetic oligonucleotide, the priming sites (which together total 52 nts) can be introduced during oligonucleotide synthesis. It is also possible to append priming sites to DNA or RNA of cellular origin by modifying existing protocols for the preparation of genomic or transcriptomic sequence libraries. However, these applications are beyond the scope of this protocol and their implementation will require application-specific expertise.

2. Select an internal modification. I have validated four internal DNA modifications for use with this protocol (Figure 2) and describe their properties below.

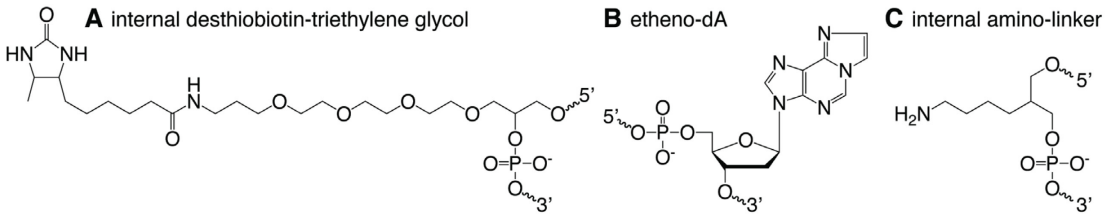

Figure 2. Validated internal DNA modifications. Validated DNA modifications include (A) desthiobiotin-TEG, (B) etheno-dA, and (C) UniLink™ Amino-Modifier. Biotin-TEG has also been validated for use in the protocols below (Strobel, 2021).

a. **Desthiobiotin-TEG/Biotin-TEG:** Desthiobiotin (Figure 2A) is biotin analog that can be eluted from streptavidin by the addition of free biotin. *Sulfolobus* DNA Polymerase IV primarily bypasses desthiobiotin-TEG without incorporating a nucleotide opposite the modification site. Desthiobiotin-TEG is a potent transcription roadblock: ~100% of TECs stall 1 nt upstream of the modification site, and ~87% of TECs remain at the stall site after 32 min in the presence of 500 μM NTPs (Strobel *et al.*, 2020). When stalled at a desthiobiotin-TEG roadblock, RNAP blocks desthiobiotin from binding streptavidin, thereby enabling the enrichment of TECs based on their position along the DNA template. I have also performed the protocols below using an internal biotin-TEG modification (Strobel, 2021).
b. **Etheno-dA:** Etheno-dA (Figure 2B) is an adenosine analog in which an etheno bridge between N1 and N6 of adenine prevents hydrogen bonding. *Sulfolobus* DNA Polymerase IV tends to incorporate a dA nucleotide opposite the modification site when bypassing the etheno-dA modification. Pupov *et al.* (2019) showed that etheno-dA is a potent transcription blockade using a TEC scaffold system. My analysis of etheno-dA-modified DNA templates agreed with the findings of Pupov *et al.* (2019): ~100% of TECs stall 1 nt upstream of the

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modification site, and ~84% of TECs remain at the stall site after 32 min in the presence of 500 μM NTPs (Strobel *et al.*, 2020). Etheno-dA is useful as a functionally inert chemical transcription roadblock.

c. **Uni-Link™ Amino-Modifier:** The Uni-Link™ Amino-Modifier (Figure 2C) comprises a primary amine attached to a six-carbon spacer and is designed to preserve natural nucleotide spacing in the phosphodiester backbone. *Sulfolobus* DNA Polymerase IV incorporates a dA nucleotide opposite the modification site ~50% of the time when bypassing the Uni-Link™ Amino-Modifier. In comparison to desthiobiotin-TEG and etheno-dA, the Uni-Link™ Amino-Modifier is a weak transcription roadblock: although ~100% of TECs initially stall at the modification site, RNAP can bypass the stall site efficiently so that only ~40% of TECs remain at the stall site after 32 min in the presence of 500 μM NTPs (Strobel *et al.*, 2020). Nonetheless, the free amino group may be useful for functionalizing the DNA template in some experiments.

3. Upon receipt of the oligonucleotides described above, dissolve the lyophilized oligonucleotide and prepare working stocks:
   a. Centrifuge the oligonucleotide tubes in a mini centrifuge for several seconds to ensure that the lyophilized oligonucleotide is at the bottom of the tube.
   b. Add 1× TE, pH 7.5, to dissolve the oligonucleotide to a concentration of 100 μM.
      Note: The oligonucleotides used in this protocol are critical and costly reagents. For this reason, I recommend using commercial nuclease-free TE to resuspend primary oligonucleotide stocks so that storage and handling is standardized.
   c. Shake thoroughly and spin down the dissolved oligonucleotide in a mini centrifuge.
   d. Prepare a 10 μM working primer stock by combining 180 μl of 10 mM Tris-HCl, pH 8.0 with 20 μl of 100 μM oligonucleotide stock in a 2 ml screw-cap tube with an O-ring. Vortex and spin down the working primer stock in a mini centrifuge.
      Critical: *Store working primer stocks in a screw cap tube with an O-ring to reduce the possibility of cross-contamination.*
   e. Store both the 100 μM and 10 μM primer stocks at -20°C.
      Critical: *When thawing primer stocks, mix the oligonucleotide solution and then spin the tube in a mini centrifuge so that no liquid remains at the cap.*

C. PCR amplification

The internal DNA modification is incorporated by PCR using the modified primer dRP1iMod.R (Table 1) to yield a dsDNA with a 5' overhang downstream of the DNA modification site (Figure 1).

Note: An unmodified version of the template can be prepared in a separate 500 μl PCR by substituting dRP1iMod.R with the unmodified dRP1.R primer. The unmodified template can be prepared in parallel with the internally modified template using the same procedures except that Procedure D: Translesion DNA Synthesis is omitted. The unmodified DNA template is a useful control for the QC analyses described in Procedures G, H, and I.

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Note: My laboratory typically uses Q5® or Q5U® DNA polymerase for PCR. Other polymerases can be substituted if desired. See Note 2 for additional information.

1. Prepare 620 µl of Q5® PCR master mix (Table 2) in a 1.7 ml microcentrifuge tube on ice. Add each reagent in the order listed in Table 2 and mix as follows:
   Note: When the DNA template will be purified using Procedure F, the PCR can be scaled down if desired because sample recovery is efficient. The smallest scale preparation my laboratory typically performs uses 200 µl of the initial Q5 PCR.
   a. After adding Q5® Reaction Buffer, vortex and spin down the master mix in a mini centrifuge.
   b. After adding every reagent except Q5® DNAP, vortex and spin down the master mix again.
   c. Add Q5® DNA polymerase and gently mix the completed master mix by pipetting.

Table 2. Reaction master mix for Q5® PCR. The Q5® PCR master mix is based on the New England Biolabs protocol for PCR Using Q5 High-Fidelity DNA Polymerase.

Reagent	Stock Conc.	Final Conc.	100 µl Rxn.	6.2 Rxn. Vols.
Nuclease-Free Water	-	-	71.0	440.2 µl
Q5® Reaction Buffer	5×	1×	20.0 µl	124.0 µl
dNTP Solution[1]	10 mM	0.200 mM	2.0 µl	12.4 µl
LPR.F	10 µM	0.250 µM	2.5 µl	15.5 µl
dRP1iMod.R	10 µM	0.250 µM	2.5 µl	15.5 µl
Template DNA[2]	-	-	1.0 µl	6.2 µl
Q5® DNAP	2 U/µl	0.020 U/µl	1.0 µl	6.2 µl
Total Volume			100.0 µl	620.0 µl

[1]Standard dNTP solution containing dATP, dGTP, dTTP, and dCTP at 10 mM each.

[2]The DNA template used for PCR can vary by application. Adjust the volume added accordingly.

2. Aliquot 100 µl reaction volumes into six thin-walled 200 µl PCR tubes.
   Critical: *If the thermal cycler does not accommodate 100 µl reactions, use a smaller aliquot.*
3. Start the thermal cycling protocol shown in Table 3. Place the reactions on the thermal cycler block once the thermal cycler lid is fully heated.

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Table 3. Thermal cycling protocol for PCR amplification. Thermal cycling protocol for the PCR described in Table 2. Always perform the PCR using a heated lid set to 105°C. Adjust the annealing temperature if primers other than those described above or a DNA polymerase other than Q5® High-Fidelity DNA polymerase are used. Adjust the extension time to account for the length of the DNA template. The final hold is at 10°C to reduce energy consumption and extend thermal cycler life.

Step		Temperature	Time
Initial Denaturation		98°C	30 s
30 Cycles	Denature	98°C	10 s
	Anneal[1]	65°C	20 s
	Extend	72°C	30 s/kb
Final Extension		72°C	5 min
Final Hold		10°C	Forever

[1]Adjust the annealing temperature as needed. I recommend using the NEB Tm Calculator to determine annealing temperature.

4. After thermal cycling is complete, place the reactions on ice.
5. Quantify PCR yield using a Qubit™ dsDNA HS assay (Table 4) to ensure that the PCR yielded sufficient DNA for the downstream processing steps. Multiply the sample concentration by 500 (five reaction volumes, representing the five reactions used for translesion synthesis) to estimate the total PCR yield. Yield can vary depending on DNA template length and composition; for reference, a 121 bp DNA template that was used when validating this protocol typically measured ~10 ng/µl by Qubit for an estimated total of ~5 µg of input DNA for the subsequent steps. This is likely an underestimate of DNA quantity because the initial PCR product contains a 5' ssDNA overhang that is not detected efficiently in the Qubit™ dsDNA HS assay.

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Table 4. Protocol for Qubit™ dsDNA assays. The protocol for Qubit™ dsDNA HS and BR assays is presented here for convenience. The protocol is written in general terms to include both assays. Please refer to the Qubit™ 4 Fluorometer, Qubit™ dsDNA HS Assay Kit, and Qubit™ dsDNA BR Assay Kit User Guides for more information.

Step	Action
1	Gather one 2.0 ml microcentrifuge tube and a Qubit™ assay tube for each sample plus two assay tubes for the Qubit™ standards.
2	Dilute the Qubit™ dsDNA Reagent 1:200 in Qubit™ dsDNA Buffer in the 2.0 ml tube. Prepare enough Qubit™ working solution to accommodate both standards and each sample, *e.g.*, for two standards and one sample, prepare four volumes of Qubit™ working solution. Vortex thoroughly and spin down in a mini centrifuge.
3	Aliquot 190 µl of Qubit™ working solution per tube to each standard assay tube. Aliquot 199 µl of Qubit™ working solution to each sample assay tube.
4[1]	Add 10 µl of Qubit™ Standards #1 and #2 to the respective assay tubes.
5	Add 1 µl of sample to the respective assay tubes.
6	Vortex the assay tubes and incubate at room temperature for 2 min.
7	Read each standard and the samples using the Qubit™ 4 Fluorometer.

[1]Qubit™ standards must be prepared fresh for each assay.

D. PCR purification and translesion DNA synthesis

Procedure C yields a truncated dsDNA with a 5′ overhang. Translesion DNA synthesis using *Sulfolobus* DNA Polymerase IV fills this overhang so that the DNA template is fully double-stranded (Figure 1). The PCR product from Procedure C is first purified using a spin-column kit to exchange the reaction buffer and deplete dNTPs. This clean-up step ensures that reaction conditions are optimal for translesion synthesis using *Sulfolobus* DNA Polymerase IV regardless of which DNA polymerase is used for PCR amplification.

1. Purify the six 100 µl PCRs using the QIAquick PCR Purification Kit, which will exchange the reaction buffer and deplete dNTPs (see Note 2):
   a. Transfer each 100 µl PCR into a corresponding 1.7 ml microcentrifuge tube.
   b. Add 500 µl of Buffer PB to each PCR. Vortex thoroughly and spin down in a mini centrifuge.
   c. Apply each sample to a separate QIAquick spin column.
   d. Centrifuge the samples at 18,000 × *g* and 4°C for 1 min. Discard the flow-through.
   e. Apply 750 µl of Buffer PE to each column.
   f. Centrifuge the samples at 18,000 × *g* and 4°C for 1 min. Discard the flow-through.
   g. Centrifuge the samples at 18,000 × *g* and 4°C for 3 min to pull down residual liquid.
   h. Transfer the columns to clean 1.7 ml microcentrifuge tubes.
   i. For five of the six columns, apply 50 µl of Buffer EB. These samples will be pooled for translesion synthesis. For the sixth column, apply 30 µl of Buffer EB. This will be a negative control for denaturing PAGE QC (Procedure G).

      Critical: *Always elute with Buffer EB. Do not elute with water. The DNA must remain buffered. See Note 1b. Buffer EB is 10 mM Tris-HCl, pH 8.5.*

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j. Centrifuge samples at 18,000 × *g* and 4°C for 1 min to elute DNA.

2. Store the sample that was eluted in a 30 µl volume at -20°C for use in Procedure G.
3. Pool the five samples that were eluted in 50 µl volumes into a 1.7 ml microcentrifuge tube.
4. Estimate the volume of the eluted sample by transferring 200 µl to a new 1.7 ml microcentrifuge tube, adjusting the pipette so that it barely aspirates the remaining sample, and transferring the remaining volume. The sample volume will be used in Step D5.
5. Prepare a 500 µl translesion synthesis reaction (Table 5) in a 1.7 ml microcentrifuge tube on ice. Add each reagent in the order listed in Table 5 and mix as follows:
   a. After adding ThermoPol® Buffer, vortex and spin down the master mix in a mini centrifuge.
   b. After adding the purified PCRs, vortex and spin down the master mix again.
   c. Add Vent® (exo-) DNA Polymerase and *Sulfolobus* DNA Polymerase IV and gently mix the completed master mix by pipetting.
      Note: Although Sulfolobus DNA polymerase IV alone is sufficient to complete the translesion synthesis reaction, including Vent (exo-) DNA polymerase may improve the fidelity of DNA synthesis beyond the internal lesion (see Note 3).

Table 5. Reaction master mix for translesion DNA synthesis. The translesion DNA synthesis master mix is used for preparatory scale translesion primer extension.

Reagent	Stock Conc.	Final Conc.	500 µl Rxn.
Nuclease-Free Water	-	-	See footnote 1
ThermoPol® Buffer	10×	1×	50.0 µl
Std./Thermostable dNTP Solution[2,3]	10 mM	0.200 mM	10.0 µl
Purified PCRs	-	-	See Step D4
Vent® (exo-) DNA Polymerase[4]	2 U/µl	0.020 U/µl	5 µl
Sulfolobus DNA Polymerase IV	2 U/µl	0.020 U/µl	5 µl
Total Volume			500.0 µl

[1]Add nuclease-free water so that the total reaction volume including all reagents is 500 µl.

[2]Translesion synthesis can be performed using either standard dNTPs or thermostability-enhancing dNTPs. Standard dNTPs are suitable for most applications. Thermostability-enhancing dNTPs are useful as a safeguard against DNA end-fraying when downstream protocols require prolonged heating to high (>65°C) temperatures.

[3]See Recipe 3 for the composition of thermostability-enhancing dNTP solution.

[4]See Note 3.

6. Aliquot 100 µl reaction volumes into five thin-walled 200 µl PCR tubes.
7. Start the thermal cycler protocol shown in Table 6. Place the reactions on the thermal cycler once the block temperature is 55°C, then advance the thermal cycler to the next step.

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Table 6. Thermal cycling protocol for translesion DNA synthesis. The translesion synthesis reaction proceeds at 55°C for one hour in a thermal cycler with a heated lid set to 105°C. The final hold is at 12°C to reduce energy consumption and extend thermal cycler life.

Step	Temperature	Time
55°C Hold	55°C	Forever
Extension	55°C	1 h
Final Hold	12°C	Forever

8. After translesion synthesis, the completed dsDNA must be purified using either Procedure E (agarose gel purification) or Procedure F (exonuclease I clean-up). If the purification cannot be performed immediately, the reactions can be stored at -20°C.

E. Purification option 1: Agarose gel purification of the completed dsDNA templates

When plasmid DNA is used as the template for PCR Amplification (Procedure C), gel purification is required to remove plasmid DNA and any excess primers. If an ssDNA oligonucleotide was used as the template for PCR amplification, Procedure F (*Purification option 2: Thermolabile exonuclease I clean-up*) can be used to purify the dsDNA transcription template instead of agarose gel extraction.

1. Pool the five 100 µl translesion synthesis reaction aliquots into a 1.7 ml microcentrifuge tube.
2. Add 50 µl of 3M sodium acetate, pH 5.5, and 1 ml of cold 100% ethanol to the pooled sample. Mix the sample thoroughly by vortexing and inverting the tube.
3. Precipitate the DNA by chilling the sample at -20°C overnight or at -80°C for 30 min.
4. When the sample has chilled sufficiently, pour a 1× TBE, 1% agarose gel containing 0.5 µg/ml ethidium bromide. Use a gel comb that holds at least 50 µl per well.
   Critical: *Use preparatory grade agarose (such as SeaKem GTG Agarose) for gel purification.*
   Critical: *The gel must contain ethidium bromide so that the DNA is stained while the gel runs.*
   Note: Ethidium bromide should be handled and disposed of according to institutional guidelines.
5. Centrifuge the sample at 18,000 × *g* and 4°C for 30 min to pellet the precipitated DNA.
6. Aspirate and discard the supernatant by pipetting. Be careful to avoid the pellet.
   Tip: To aspirate large supernatant volumes with precision, a 10 µl pipette tip can be loaded onto the end of a 1,000 µl pipette tip. This enables aspiration of ~1 ml with precise control.
7. Add 1 ml of cold 70% ethanol to the sample and gently invert the tube several times. Do not dislodge the pellet.
8. Centrifuge the sample at 18,000 × *g* and 4°C for 2 min.
9. Aspirate and discard the supernatant by pipetting.
10. Centrifuge the sample for several seconds to spin down residual supernatant.
11. Aspirate and discard the residual supernatant by pipetting.
12. Cover the open tube with a tissue wiper and set it on a bench to dry for several minutes.
13. Assemble and fill a horizontal gel tank with enough 1× TBE to cover the agarose gel.
14. Resuspend the pellet in 30 µl of 10 mM Tris-HCl, pH 8.0.

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Critical: *Always resuspend the pellet in 10 mM Tris-HCl, pH 8.0. Do not resuspend the pellet in water. The sample must remain buffered. See Note 1b.*

15. Add 6 µl of 6× DNA Loading Dye (+SDS, XC only) to the sample and mix by pipetting.
16. Load the sample into the agarose gel and run the gel at 100 V for 1-2 h.
    Note: Because the sample contains several micrograms of DNA, a red band should become visible as the ethidium bromide co-migrates with the DNA (Figure 3A). This enables gel extraction to be performed without exposing the DNA to any UV light.

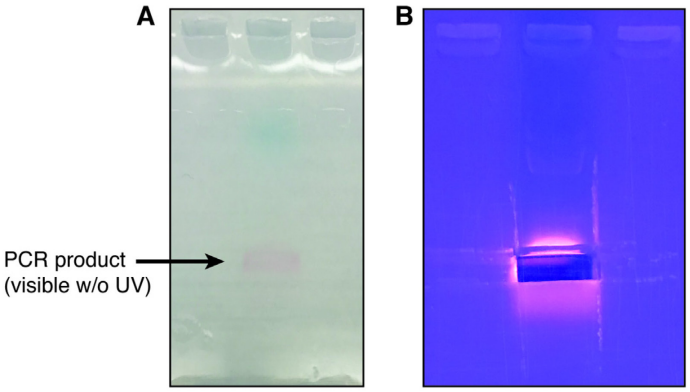

Figure 3. DNA band excision without UV light. A. The PCR product can be visualized as a red band under visible light because ethidium bromide co-migrates with the large quantity of DNA in the gel. B. Visualization of the gel from (A) on a UV transilluminator after the PCR product was excised to assess the quality of the PCR and the band excision. No side products were detected.

17. Without exposing the gel to UV light, excise the PCR product with a clean razor blade (Figure 3).
18. Trim the gel slice to remove as much excess agarose as possible.
    Critical: *Removing excess agarose improves DNA yield and quality.*
19. Tare a balance with a 2.0 ml tube. Add the gel slice to the 2.0 ml tube, weigh the gel slice, and record the mass. The mass of the gel slice will be used below in Steps E21a and E21b.
20. Visualize the gel on a UV transilluminator to confirm that the band was excised precisely and assess whether any side products are present (Figure 3B).
21. Recover the DNA from the excised gel slice using the QIAquick Gel Extraction Kit. Use the following protocol, which includes every optional step recommended by the manufacturer and reduces gel melting temperature to minimize the possibility of DNA end fraying:
    Note: For more information, refer to the Qiagen QIAquick® Gel Extraction Kit Quick-Start Protocol.
    a. If the gel slice weighs more than 400 mg, divide it into several 2.0 ml tubes so that each tube contains <400 mg of gel.
    b. Add 3 volumes of Buffer QG to 1 volume gel (1 mg gel ~1 µl).
    c. Incubate the sample at 30°C and occasionally vortex until the gel slice has dissolved.
       Critical: *The gel slice is melted at 30°C to minimize DNA template end fraying.*

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d. Add 1 gel volume of isopropanol to the sample. Mix by inverting the tube and spin down in a mini centrifuge.
e. Apply 700 µl of the sample to a QIAquick spin column and centrifuge at 18,000 × *g* and 4°C for 1 min. Discard the flow-through and repeat until the entire sample has been applied.
f. Add 500 µl of Buffer QG to the column and centrifuge at 18,000 × *g* and 4°C for 1 min. Discard the flow-through.
g. Add 750 µl of Buffer PE to the column and centrifuge at 18,000 × *g* and 4°C for 1 min. Discard the flow-through.
h. Centrifuge the samples at 18,000 × *g* and 4°C for 3 min to spin down residual liquid.
i. Place the column into a clean 1.7 ml microcentrifuge tube.
j. Apply 30 µl of 10 mM Tris-HCl, pH 8.0 to the center of the QIAquick column.
Critical: *Always elute with 10 mM Tris-HCl, pH 8.0. Do not elute with water. See Note 1b.*
k. Centrifuge the column at 18,000 × *g* and 4°C for 1 min.
l. Transfer the eluted sample to a clean 1.7 ml microcentrifuge tube.

22. Quantify the DNA template concentration using the Qubit™ dsDNA BR Assay Kit (Table 4).
23. Convert the DNA concentration from ng/µl to µM. A typical prep yields 30 µl of DNA at a concentration between 0.75 µM and 1.25 µM.
Tip: Promega has a web-based tool for converting µg of DNA to pmol of DNA. Enter the DNA length in base pairs and the number of µg of DNA in 1 µl of sample to calculate the concentration of your sample in pmol/µl; pmol/µl equals µM.
24. Prepare 200 µl of 50 nM working stock for each template by diluting the primary stock into 10 mM Tris-HCl, pH 8.0. Both the primary stock and the working stock should be stored at -20°C.
Critical: *Always prepare working stocks using 10 mM Tris-HCl, pH 8.0. Do not use water. The DNA must remain buffered to prevent fraying of the duplex downstream of the modification site. See Note 1b.*

F. Purification option 2: Thermolabile exonuclease I clean-up

When an ssDNA oligonucleotide is used as the template for PCR Amplification (Procedure C), the final dsDNA product can be purified by using thermolabile exonuclease I (exoI) to degrade excess primers and a PCR purification kit to exchange the reaction buffer. This alternate purification protocol yields approximately two times more DNA template than agarose gel purification and, consequently, does not require as much input DNA (Note 1c). However, the success of the thermolabile exoI clean-up procedure is contingent upon the specificity of the PCR because it does not select for DNA size.

1. Purify the five 100 µl translesion synthesis reactions using the QIAquick PCR Purification Kit, which depletes dNTPs and enzymes:
Critical: *Sulfolobus DNA polymerase IV is active at 37°C. If substantial amounts of primer remain when the thermolabile exoI reaction is incubated at 37°C, exoI-resistant primer dimers that do not occur at higher temperatures can form. PCR purification before thermolabile exoI treatment removes dNTPs and Sulfolobus DNA polymerase IV, which prevents the formation of*

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primer dimers.

a. Transfer each 100 µl PCR into a corresponding 1.7 ml microcentrifuge tube.
b. Add 500 µl of Buffer PB to each PCR. Vortex thoroughly and spin down in a mini centrifuge.
c. Apply the samples to QIAquick spin columns by adding 600 µl of sample to the column, centrifuging at 18,000 × *g* and 4°C for 1 min, and discarding the flow-through. Multiple samples can be loaded onto one column by repeating this procedure as long as the column capacity (10 µg DNA) is not exceeded.
d. Apply 750 µl of Buffer PE to each column.
e. Centrifuge the samples at 18,000 × *g* and 4°C for 1 min. Discard the flow-through.
f. Centrifuge the samples at 18,000 × *g* and 4°C for 3 min to pull down residual liquid.
g. Transfer the columns to clean 1.7 ml microcentrifuge tubes.
h. Apply 50 µl of Buffer EB to each column.
   Critical: *Always elute with Buffer EB. Do not elute with water. The DNA must remain buffered. See Note 1b. Buffer EB is 10 mM Tris-HCl, pH 8.5.*
i. Centrifuge the samples at 18,000 × *g* and 4°C for 1 min to elute the DNA.

2. Pool the eluted samples into one 1.7 ml microcentrifuge tube.
3. Estimate sample volume by adjusting a pipette so that it barely aspirates the sample and transferring it to a new 1.7 ml microcentrifuge tube. This sample volume is used in Step F4.
4. Prepare a 500 µl thermolabile exoI reaction (Table 7) in a 1.7 ml microcentrifuge tube on ice.

Table 7. Reaction master mix for thermolabile exonuclease I digestion

Reagent	Stock Conc.	Final Conc.	500 µl Rxn.
Nuclease-Free Water	-	-	See footnote 1
ThermoPol® Buffer[2]	10×	1×	50.0 µl
Purified PCRs	-	-	Determined in Step F3
Thermolabile Exonuclease I	20 U/µl	0.1 U/µl	2.5 µl
Total Volume			500.0 µl

[1]Add nuclease-free water so that the total reaction volume including all reagents is 500 µl.

[2]ThermoPol® Buffer is used here because thermolabile exoI is heat inactivated 80°C.

5. Aliquot 100 µl reaction volumes into five thin-walled 200 µl PCR tubes.
6. Set a thermal cycler to 37°C with a heated lid set to 45°C. Place the reactions in the thermal cycler once the block temperature reaches 37°C and incubate for 4 min.
7. Move the reactions to ice.
8. Start the thermal cycler protocol shown in Table 8. Place the reactions on the thermal cycler once the block temperature reaches 80°C, then advance the thermal cycler to the next step.

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Table 8. Thermal cycling protocol for thermolabile exonuclease I heat inactivation. Heat inactivation proceeds at 80°C for 1 min in a thermal cycler with a heated lid set to 105°C.

Step	Temperature	Times
80°C Hold	80°C	Forever
Inactivation	80°C	1 min
Final Hold	12°C	Forever

9. Repeat the PCR purification described in Step F1 with the following modifications to purify the dsDNA template:
   a. Perform the 750 µl Buffer PE wash described in steps F1d/e twice.
   b. In step F1i, elute the samples in 30 µl of 10 mM Tris-HCl pH 8.0 instead of Buffer EB.
10. Quantify DNA template concentration and prepare a working stock as described in Steps E22-E24.

G. Internally modified DNA template quality control by denaturing PAGE

The quality of an internally modified DNA template preparation should be assessed by using denaturing urea polyacrylamide gel electrophoresis (Urea PAGE) to confirm that the translesion synthesis reaction proceeded to completion. Successful DNA template QC requires complete denaturation of the DNA template so that the size of each strand can be assessed. It is therefore critical that the gel is run at a high temperature (~50°C). The protocol below is designed to achieve this using a Bio-Rad Mini-PROTEAN Vertical Electrophoresis Cell.

Note: Experienced readers will be able to perform denaturing PAGE QC and native PAGE QC (Procedure H) in parallel.

1. Thoroughly clean a 1.0 mm spacer plate, a short plate, and a 1.0 mm 10-well comb with deionized water. Rinse the plates with 70% ethanol and allow them to air-dry.
2. Assemble the glass plates in the Mini-PROTEAN casting frame. When securing the plates in the casting frame, be sure that the bottom edges of the plates are flush.
3. Secure the assembled casting frame on a gasket in the Mini-PROTEAN casting stand.
4. Combine 1 ml UreaGel Buffer, 5 ml UreaGel Diluent, and 4 ml UreaGel Concentrate in a 15 ml conical tube. Invert the tube to mix. This mixture will prepare a 10% acrylamide gel.
   Caution: *Acrylamide is a neurotoxin and should be handled cautiously.*
   Note: Adjust the gel percentage as needed depending on DNA template length. My laboratory has used 10% gels to assess DNA templates from 121 to 273 nt in length.
5. Add 80 µl of 10% APS to the UreaGel mixture and invert the tube to mix.
6. Add 4 µl of TEMED to the UreaGel mixture and invert the tube to mix and start polymerization.
7. Pipette the UreaGel mixture carefully but quickly to fill the space between the glass plates.
8. Insert the 1.0 mm 10-well comb and allow the gel to polymerize for at least 30 min.

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9. Once the gel has polymerized, assemble the Mini-PROTEAN Electrode Assembly. Secure the gel plates on one side of the electrode assembly and a buffer dam on the other.
10. Place the electrode assembly in the Mini-PROTEAN gel tank.
11. Fill the chamber between the gel plates and buffer dam with 1× TBE. Add 1× TBE to the outer gel chamber so that it covers the lower gel gasket.
    Critical: *It is important to cover only the bottom of the gel gasket. Using more buffer will disperse heat while the gel is running and result in poor DNA denaturation.*
12. Rinse the wells with 1× TBE by pipetting until the buffer in each well appears homogenous.
13. Pre-run the gel at 480 V for ~30 min to warm the gel.
14. Set a heat block to 95°C once the gel is pre-running.
15. For each sample that will be assessed, combine 15 µl of 1× Formamide Loading Dye with 1 µl of 50 nM DNA template in a 1.7 ml microcentrifuge tube. The negative control and positive control (if performed) should also be prepared this way.
    Critical: *A 16 µl sample volume is used to further dilute the sample so that re-annealing of DNA duplexes after denaturation is reduced.*
16. Combine 19 µl of 1× Formamide Loading Dye with 1 µl of Low Range ssRNA Ladder. Aliquot 5 µl into a separate tube and store the remaining 15 µl at -20°C for future use.
    Note: An ssRNA ladder is used due to the lack of commercially available ssDNA ladders in the required size range. The ladder is therefore intended to be a rough approximation of DNA size.
17. After the gel has pre-run for ~25 min, place all samples on the 95°C heat block for 5 min. Place an aluminum block on top of the samples to prevent the tubes from opening.
18. Remove the samples from the heat block and immediately place them on ice to snap cool. Leave the samples on ice for 2 min.
    Critical: *Snap cooling minimizes re-annealing of DNA duplexes.*
19. Turn off the power supply and quickly rinse the wells with 1× TBE by pipetting.
20. Load the samples onto the gel using gel-loading pipette tips. Take care to fill as little of the well as possible so that the resulting bands are sharp despite the large sample volume.
    Critical: *Load the samples quickly so that the gel remains warm.*
21. Run the gel at 480 V until the bromophenol blue dye band reaches the lower gel gasket.
    Note: Monitor the gel closely. At 480 V, electrophoresis will be complete in ~10 min.
22. Turn off the power supply, disassemble the gel tank, and set the gel plates on a bench to cool.
23. Add 3 µl of SYBR Gold Nucleic Acid Stain to 30 ml of 1× TBE in a 150 mm culture dish. Gently swirl the dish to disperse the stain.
24. Pry apart the gel plates using the Mini-PROTEAN gel releaser.
25. Carefully lift the gel off the plate while touching only the edges. Submerge the gel in the SYBR Gold stain. Cover the dish in aluminum foil and rock gently for 10 min.
26. Visualize and document the gel. Gel visualization can be performed in several ways: I prefer to use a Typhoon Biomolecular Imager or equivalent laser scanner due to its high sensitivity. The Typhoon should be run in fluorescence mode using the settings recommended for SYBR Gold

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(488 nm laser with the 520 BP 40 emission filter on a Typhoon 9400). Set the PMT voltage to 475 V for the first scan and adjust it as needed. If you do not have access to a laser scanner that is capable of imaging SYBR Gold Nucleic Acid Stain, the gel can also be viewed and documented on a Dark Reader Blue Light Transilluminator.

27. Evaluate the quality of the DNA template preparation for the following expected outcomes:
    a. The negative control should have two DNA template bands (Figure 4). The upper (modified) strand should correspond to full DNA template length. The lower (unmodified) band should correspond to the length of the DNA template minus the number of nucleotides downstream of the internal modification site. Some excess primers may be present.
    b. The DNA template preparation should contain a single band that corresponds to the full length of the DNA template (Figure 4). No primers should be present.
    Critical: *Only a single band of the expected length should be present. The translesion synthesis reaction can and should go to completion (see Note 1a).*
    c. The positive control (if performed) should contain a single band that corresponds to the full length of the DNA template (Figure 4). Some excess oligonucleotide may be present if the positive control sample was not gel extracted.
    Note: The positive control is a useful indicator for the effectiveness of DNA duplex denaturation. If a gel-extracted positive control contains multiple bands, this suggests that DNA denaturation is incomplete and should be optimized.

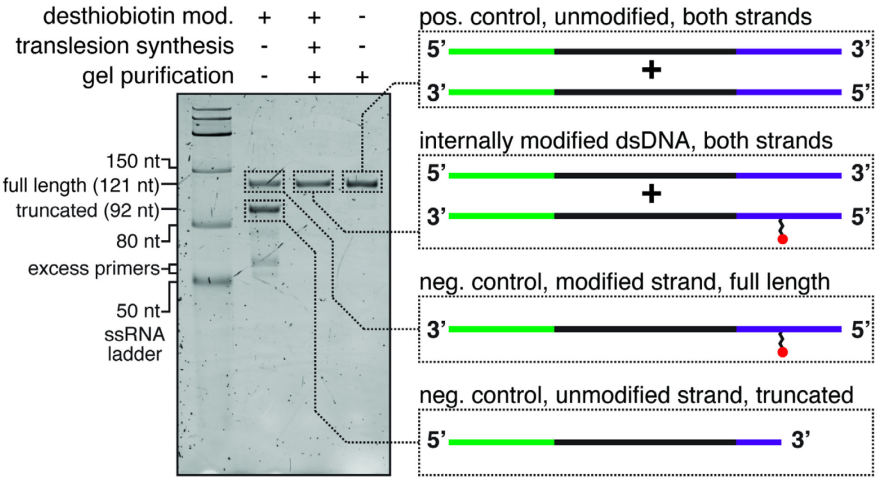

Figure 4. Example of a denaturing PAGE QC gel. Analysis of denaturing gel QC. Denaturation of the dsDNA duplex enables the length of each DNA strand to be assessed. The negative control, which contains a desthiobiotin modification and did not undergo translesion synthesis, contains a truncated DNA strand. In contrast, the internally desthiobiotinylated sample that was treated by translesion synthesis contains only full length DNA and is indistinguishable from the unmodified positive control. The gel image is adapted from Strobel *et al.* (2020) Figure 1E and was acquired using a Typhoon 9400 Biomolecular Imager.

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H. Internally modified DNA template quality control by native PAGE.

After confirming that translesion synthesis went to completion using denaturing PAGE (Procedure G), the internally modified DNA template preparation should be assessed by native PAGE to confirm the purity of intact dsDNA duplexes.

1. Thoroughly clean a 1.0 mm spacer plate, a short plate, and a 1.0 mm 10-well comb with deionized water. Rinse the plates with 70% ethanol and allow them to air-dry.
2. Assemble the glass plates in the Mini-PROTEAN casting frame. When securing the plates in the casting frame, be sure that the bottom edges of the plates are flush.
3. Secure the assembled casting frame on a gasket in the Mini-PROTEAN casting stand.
4. Combine 3.6 ml Milli-Q water, 3 ml 3× TBE, and 2.4 ml ProtoGel (30%), in a 15 ml conical tube. Invert the tube to mix. This mixture will prepare an 8% acrylamide gel.
   Caution: *Acrylamide is a neurotoxin and should be handled cautiously.*
   Note: Adjust the gel percentage as needed depending on DNA template length. An 8% gel will be suitable for DNA up to ~500 bp in length. Use a 6% gel for DNA that is longer than ~500 bp.
5. Add 100 µl of 10% APS to the acrylamide mixture and invert the tube to mix.
6. Add 10 µl of TEMED to the acrylamide mixture and invert the tube to mix.
7. Pipette the acrylamide mixture carefully but quickly to fill the space between the glass plates.
8. Insert the 1.0 mm 10-well comb and allow the gel to polymerize for at least 30 min.
9. Once the gel has polymerized, assemble the Mini-POTEAN Electrode Assembly. Secure the gel plates on one side of the electrode assembly and a buffer dam on the other.
10. Place the electrode assembly in the Mini-PROTEAN gel tank.
11. Fill the chamber between the gel plates and buffer dam with 1× TBE. Add 1× TBE to the outer gel chamber up to the 2-gel fill line.
12. Rinse the wells with 1× TBE by pipetting.
13. For each sample that will be assessed, combine 4 µl of 10 mM Tris-HCl pH 8.0, 1 µl 6× DNA Loading Dye (non-denaturing), and 1 µl of 50 nM DNA template in 1.7 ml microcentrifuge tube. The positive control (if performed) should also be prepared this way. Mix by vortexing and spin down in a mini centrifuge.
    Critical: *The samples must be prepared using 10 mM Tris-HCl, pH 8.0. See Note 1b.*
14. Load the samples onto the gel using gel-loading pipette tips.
15. Load 3 µl of Quick-Load® Purple 100 bp DNA ladder (or a comparable 100 bp DNA ladder).
16. Run the gel at 100 V until the bromophenol blue loading dye reaches the lower gel gasket.
17. Turn off the power supply and disassemble the gel apparatus.
18. Add 3 µl of SYBR Gold Nucleic Acid stain to 30 ml of 1× TBE in a 150 mm culture dish. Gently swirl the dish to disperse the stain.
19. Pry apart the gel plates using the Mini-PROTEAN gel releaser.
20. Carefully lift the gel off the plate while touching only the edges. Submerge the gel in the SYBR Gold stain. Cover the dish in aluminum foil and rock gently for 10 min.
21. Visualize and document the gel using a Typhoon Biomolecular Imager (preferred method) or a

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Dark Reader Blue Light Transilluminator.

22. Evaluate the quality of the DNA template preparation. The DNA template preparation should contain a single band that corresponds to the expected full length dsDNA product. No side products or excess primer should be detectable (Figure 5). If a slow-migrating band is observed by native PAGE after a template preparation passes denaturing PAGE QC, this suggests that the DNA was mishandled during preparation (*e.g.*, diluted into an unbuffered solution; see Note 1b).

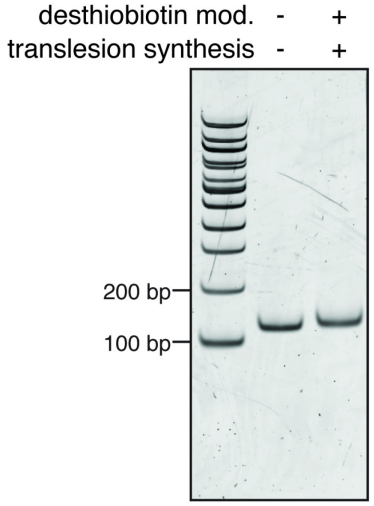

Figure 5. Example of a native PAGE QC gel. The dsDNA template preparation contains no detectable side products or oligonucleotides and is the expected length (121 bp). Note that the internally modified DNA template migrates slightly slower than an unmodified control. The gel image is adapted from Strobel *et al.* (2020) Supplementary Figure S2B and was acquired using a Typhoon 9400 Biomolecular Imager.

I. Assess the efficiency of transcription roadblocking using single-round *in vitro* transcription (optional)
The transcription roadblocking activity of an internally modified DNA template can be evaluated using a single-round *in vitro* transcription time course. **This quality control step is optional when using a previously validated DNA modification**. However, it may be useful to perform this analysis to be confident that the internally modified DNA template has the expected transcription roadblocking activity, particularly if you are new to the protocols described above.
Note: (1) When assessing a new DNA template sequence, prepare an additional transcription reaction using an unmodified version of the DNA template to distinguish halted TECs from run-off transcripts. (2) The experiment described below uses [α-^{32}P]-UTP as a radioactive tracer. Perform this experiment using appropriate radiation shielding and in compliance with institutional protocols. (3) The procedure includes phenol:chloroform extraction and subsequent ethanol precipitation remove protein and concentrate the sample prior to sequencing gel electrophoresis. These steps are not strictly required but ensure that RNA mobility is not influenced by residual protein.

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1. Set a heat block to 37°C at least 30 min before starting the experiment.
2. Prepare *in vitro* transcription Master Mix (Table 9) in a 1.7 ml microcentrifuge tube on ice. Add each reagent in the order listed in Table 9 and mix as follows:
   a. After adding 10× Transcription Buffer, vortex and spin down the master mix.
   b. After adding every reagent except *E. coli* RNAP, vortex and spin down the master mix again.
   c. Add *E. coli* RNAP holoenzyme and gently mix the completed Master Mix by pipetting.

Table 9. Reaction master mix for single-round *in vitro* transcription. Individual *in vitro* transcription reactions have a total volume of 25 µl. $MgCl_2$ is omitted from the master mix so that transcription is only initiated upon addition of 10× Start Solution.

Reagent	Stock Conc.	Final Conc.	25 µl Rxn.	10 Rxn. Vols.
Nuclease-Free Water	-	-	14.85 µl	148.5 µl
Transcription Buffer	10×	1×	2.50 µl	25.0 µl
NTP Solution[1]	20×	1×	1.25 µl	12.5 µl
BSA[2]	5 mg/ml	0.1 mg/ml	0.50 µl	5.0 µl
DNA template	50 nM	5 nM	2.50 µl	25.0 µl
E. coli RNAP holo	1 U/µl	0.016 U/µl	0.40 µl	4.0 µl
[α-^{32}P]-UTP[3]	-	-	0.50 µl	5.0 µl
Total Volume (omits 10× Start Solution)			22.50 µl	225.0µl

[1]NTP concentration is 200 µM ATP, 200 µM GTP, 200 µM CTP, and 50 µM UTP to promote [α-^{32}P]-UTP incorporation. Adjust the NTP mix accordingly if a different radioactive tracer is used.

[2]***Critical:*** *BSA must be molecular biology grade.*

[3]***Critical:*** *Use [α-^{32}P]-NTPs within two weeks of receipt, assuming the stock is from a fresh lot.*

3. Prepare 10× Start Solution.
   Critical: *10× Start Solution must be prepared fresh for each experiment.*
   a. Pipette 200 µl of 100 mM $MgCl_2$ into a 1.7 ml microcentrifuge tube.
   b. Pipette 10 µl of 2 mg/ml rifampicin into the 100 mM $MgCl_2$.
      Note: Rifampicin inhibits bacterial RNAPs by blocking extension of transcripts beyond 2-3 nt. Starting transcription by adding $MgCl_2$ and rifampicin simultaneously permits pre-formed open complexes to initiate but blocks further initiation so that transcription is single-round.
   c. Vortex thoroughly and spin down the 10× Start Solution in a mini centrifuge.
   d. Cover the 10× Start Solution in foil to protect from light and keep at RT.
4. Prepare a 1.7 ml microcentrifuge tube containing 125 µl of Stop Solution for each time point and label the tubes accordingly. Keep these tubes on ice throughout the experiment.
5. Perform the single-round *in vitro* transcription time course. An example time course with eight time points is shown in Table 10.
   Note: In vitro transcription time courses require fast sample manipulation. Inexperienced users

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should practice these manipulations with mock samples before performing the experiment.

Table 10. Single-round *in vitro* transcription time course. Open promoter complexes are formed by incubating the transcription Master Mix at 37°C for 10 min. Single-round transcription is initiated by adding 10× Start Solution, and at each time point, a 25 µl aliquot of Master Mix is transferred to a tube containing 125 µl of Stop Solution, vortexed briefly, and kept on ice.

Time (MM:SS)	Action	Purpose
00:00	Place Master Mix (MM) at 37°C	Form open promoter complexes
10:00	Add 25 µl of 10× Start Solution (2.5 µl/rxn)	Initiate transcription
10:15	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 15 s time point
10:30	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 30 s time point
11:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 1 min time point
12:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 2 min time point
14:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 4 min time point
18:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take an 8 min time point
26:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 16 min time point
42:00	Transfer 25 µl of MM to 125 µl of Stop Solution	Take a 32 min time point

6. Add 150 µl phenol:chloroform:isoamyl alcohol, pH 8.0 to each sample.
7. Vortex the samples thoroughly.
8. Centrifuge the samples at 18,000 × *g* and 4°C for 5 min.
9. For each sample, prepare a 1.7 ml microcentrifuge tube containing 450 µl 100% ethanol, 15 µl 3 M sodium acetate, pH 5.5, and 1 µl GlycoBlue Coprecipitant.
10. Carefully transfer the aqueous phase (upper layer) of each sample to the corresponding 1.7 ml microcentrifuge tube prepared in Step H9. Invert the tube several times to mix.
11. Store the samples at -20°C overnight to precipitate the RNA.
12. The next morning, prepare a 0.4 mm, 12% acrylamide, 7.5 M urea sequencing gel as follows:
    Caution: *Acrylamide is a neurotoxin and should be handled cautiously.*
    Notes:
    i. It is essential to wear a lab coat and safety glasses while pouring sequencing gels to protect against acrylamide splashes – occasionally, inserting the gel comb between the glass plates can cause the acrylamide to splash.
    ii. Gel percentage and running conditions are dictated by the length of the RNA. According to the National Diagnostics UreaGel System Protocol, 12% acrylamide monomer is appropriate for 10 to 50 nt RNAs, 8% for 40 to 100 nt, and 6% for 60 to 150 nts.

    a. Clean the long and short sequencing gel plates thoroughly with a mild detergent and a plastic brush and rinse with deionized water. Rinse the gel plates with 70% ethanol and wipe dry with a light-duty tissue wiper.

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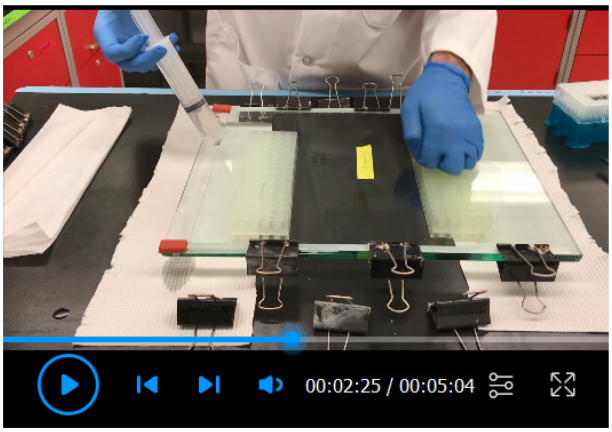
Note: Periodically treat the short plate with Rain-X to assist with releasing the gel plates.

b. Clean a 0.4 mm comb and 0.4 mm spacers with soap and deionized water and dry using a light-duty tissue wiper.
c. Assemble the sequencing gel for pouring. See **Video 1** for a demonstration of the recommended assembly.
d. In a 250 ml plastic beaker, combine 7 ml UreaGel Buffer, 29.4 ml UreaGel Diluent, 33.6 ml UreaGel Concentrate, and 400 µl 10% APS. Swirl the beaker carefully to mix.
Note: This prepares a 12% acrylamide gel. Adjust as needed.
e. Add 25 µl TEMED to the UreaGel mixture and swirl to begin polymerization. Draw the mixture into a 60 ml syringe and carefully expel the mixture once to ensure that it is fully mixed. Draw the UreaGel mixture into the syringe again.
*Note: This protocol uses **less** 10% APS and TEMED than recommended in the National Diagnostics UreaGel System protocol to slow polymerization for easier gel pouring.*
f. Pour the sequencing gel. See **Video 1** for a demonstration of the recommended procedure.
g. Allow the gel to polymerize for 60 to 90 min.



Video 1. Recommended procedure for pouring a sequencing gel. A straightforward and reliable procedure for pouring a 0.4 mm large format sequencing gel is demonstrated.

13. While the gel is polymerizing, centrifuge the samples at 20,000 × *g* and 4°C for 30 min.
14. Aspirate the supernatant from each sample while taking care not to disturb the small blue pellet.
15. Centrifuge the samples for several seconds to spin down residual liquid.
16. Aspirate residual liquid with a 10 µl pipette tip.
17. Add 6.5 µl of 1× Formamide Loading Dye to each sample.
18. Briefly vortex the samples and spin down the liquid.
19. Once the gel has polymerized, assemble the sequencing gel apparatus. Rinse the wells by drawing 1× TBE from the upper buffer chamber into a 60 ml syringe with a bent 20G 1½" needle and expelling the TBE into the wells. See **Video 2** for a demonstration of how to assemble a BRL Model S2 sequencing gel apparatus.

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Video 2. Assembly of the BRL Model S2 sequencing gel system. The procedure for assembling a gel onto the Model S2 sequencing gel system is demonstrated.

20. Close the safety lids, attach the leads, and pre-run the gel at 1,500 V for ~20 min.
21. Place the samples into the 95°C heat block for 5 min. Place a second aluminum block on top of the samples to prevent the tubes from opening.
22. Snap cool the samples by immediately placing on ice.
23. Using a Sharpie, label the sequencing gel plates to indicate what sample each well will contain. Mark any wells that will be left blank between samples.
24. Once the sequencing gel has pre-run for ~20 min, turn off the power supply and rinse the wells a second time by drawing 1× TBE from the upper buffer chamber into a 60 ml syringe with a bent 20G 1½" needle and expelling the TBE into the wells.
25. Carefully load each sample into its corresponding well using a 0.4 mm gel loading tip.
    Tip: Set the pipette volume several microliters higher than the sample volume. This generates an air buffer between the sample and the tip. When loading a sample, dispense the air buffer first and move the tip toward the base of the well as the sample is ejected. The air bubble will either float free of the well or can be gently aspirated.
    Caution: *It is not feasible to load a sequencing gel behind acrylic shielding. Wear a lab coat and safety glasses, and minimize radiation exposure by keeping every sample except the one you are loading shielded. Radiation exposure can be monitored using a dosimetry badge.*
26. Load 6.5 µl of 1× Formamide Loading Dye into any blank wells that are next to sample wells. This helps the sample lanes to run straight.
27. Close the upper buffer chamber lid and reattach the leads.
28. Run the gel at 1,400 V for 2.5-3 h.
29. Blank a storage phosphor screen using the Storage Phosphor Screen Image Eraser.
30. Turn off the power supply and disassemble the Model S2 apparatus. See **Video 3** for a demonstration of how to disassemble the BRL Model S2 sequencing gel apparatus.
    Caution: *The transcription reactions contain free [α-^{32}P]-UTP, which will typically migrate into the lower buffer chamber. The buffer in the lower chamber will therefore be radioactive.*

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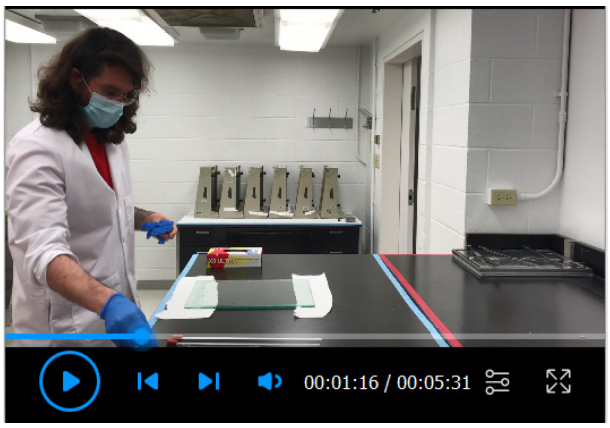
Video 3. Disassembly of the BRL Model S2 sequencing gel system. The procedure for removing a gel from the Model S2 sequencing gel system is demonstrated.

31. Prepare the gel for exposure to a storage phosphor screen by opening the plates and covering the exposed gel in plastic wrap. See **Video 4** for a demonstration of this procedure.
    Critical: *It is* ***absolutely critical*** *that the outer surface of the plastic wrap, which will contact the storage phosphor screen, remains perfectly clean. This is easily achievable by changing your gloves frequently while applying the plastic wrap.*
    Note: It is not typically necessary to dry the gel if the exposure time is short (i.e., <16 h).



Video 4. Preparation of a sequencing gel for storage phosphor screen exposure. The procedure for opening and covering the sequencing gel with plastic wrap is demonstrated.

32. Place the wrapped gel into a cardboard exposure cassette.
33. Put on a clean pair of gloves. Open the storage phosphor cassette and, handling only the edges, set the screen onto the wrapped gel. Close the cardboard exposure cassette and place the storage phosphor cassette on top to prevent the screen from moving during exposure.
    Critical: *The quality of the gel image depends on the condition of the storage phosphor screen. Handle the storage phosphor screen with care.*
34. Expose the storage phosphor screen. Exposure time depends on sample activity. I find that 4-6

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h is usually sufficient for a clear image. It is essential to not overexpose the screen, particularly if you wish to quantify your results. I typically perform a scan after a short exposure (*e.g.*, 4-6 h), blank the storage phosphor screen, and perform a second overnight exposure.

35. Scan the phosphor screen on a Typhoon Biomolecular Imager using phosphorimaging mode.
36. Evaluate the transcription roadblocking activity of the DNA template (Figure 6). Because 100% of TECs are expected to stall at the internal modification site, this analysis can typically be qualitative. For example, in the experiment below run-off transcripts only begin to become visible at the 4 min time point, indicating that the internal DNA modification efficiently halts RNAP.

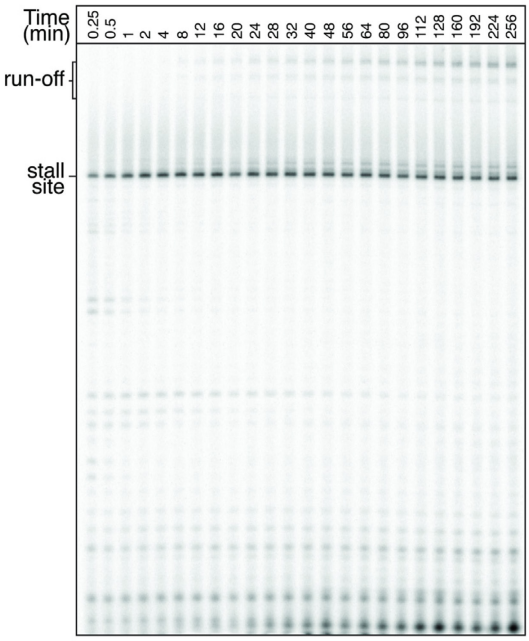

Figure 6. Example of a single-round *in vitro* transcription time course. Single-round *in vitro* transcription time course performed as described in this protocol except with 24 time points for kinetic analysis; the 8 time points suggested in this protocol are more than sufficient to assess transcription roadblocking activity. An unmodified positive control was not performed in this experiment because the precise location of stalled and run-off transcripts had already been determined. Data is adapted from Strobel *et al.* (2020) Figure 4.

Notes

1. **Notes on reproducibility and variability.** At the time of writing, I have performed this protocol numerous times in two laboratory settings, and the procedures described above have become routine in my laboratory. Most preparations have used desthiobiotin-TEG or biotin-TEG as the internal modification, the templates encoded six distinct RNAs, and Q5® High-Fidelity DNA polymerase, Q5U® Hot Start High-Fidelity DNA polymerase, and Vent® (exo-) DNA polymerase have been used for PCR. The sections below describe my general observations.

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a. **Translesion synthesis efficiency.** Translesion synthesis by *Sulfolobus* DNA Polymerase IV is exceptionally efficient and has gone to completion (within the limit of detection of denaturing PAGE QC) in every internally modified DNA template preparation I have performed using the validated modifications described in Procedure B. This is consistent with my assessment of DNA modification bypass by *Sulfolobus* DNA Polymerase IV by primer extension: there was no discernable difference between the efficiency of primer extension across the DNA modifications shown in Figure 2 and an unmodified control (Strobel *et al.*, 2020). The only case in which I have observed detectably incomplete translesion synthesis was during an attempt to construct a DNA template with a complex stall site containing consecutive internal biotin-TEG, 5-propynyl-dC, and biotin-11-dT modifications; even in this case, translesion synthesis was ~99% complete. In such cases, it may be possible to bring the translesion synthesis reaction to completion by substituting Vent® DNA polymerase in place of Vent (exo-) so that stalled lesion bypass attempts can be rescued by 3'→5' exonuclease-mediated degradation. However, my laboratory has not yet tested this procedure extensively.

b. **DNA duplex stability.** In most experiments, the DNA duplex downstream of the modification site will be short (29 and 31 bp in the configurations I have used so far) because it is appended by a primer. Consequently, it is possible for this region of the DNA template to fray if mishandled (*e.g.*, diluted into an unbuffered solution). End fraying is detectable by native PAGE QC as a slow-migrating product despite the observation of only full length product by denaturing PAGE QC (Figure 7). **End fraying can be avoided simply by keeping the DNA template buffered at all times.** As an additional safeguard, translesion synthesis can be performed using the thermostability-enhancing dNTPs 2-amino-dATP and 5-propynyl-dCTP alongside dGTP and dTTP, which I originally described as an alternate protocol in anticipation of end-fraying as a potential issue (Strobel *et al.*, 2020). The thermostability-enhancing dNTPs reduce the occurrence of end-fraying when DNA is diluted in water (Figure 7B) but are not a substitute for proper sample buffering.

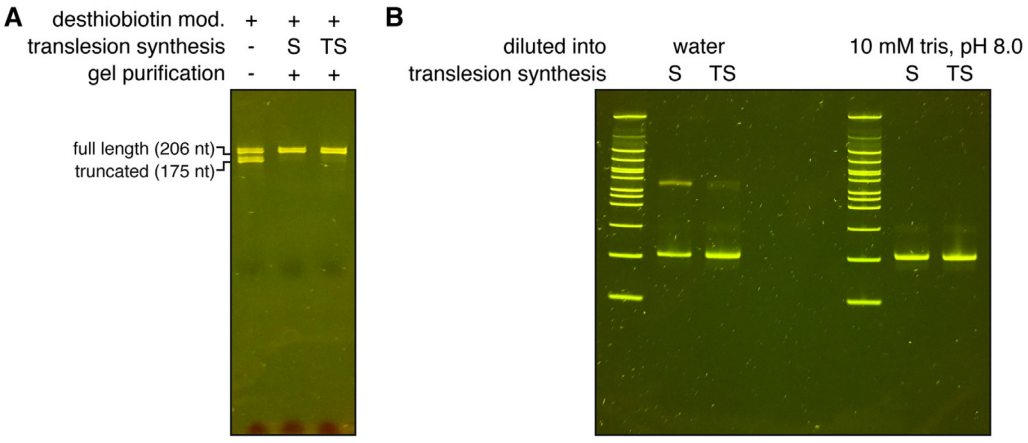

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Figure 7. Occurrence of slow-migrating DNA products. A. After translesion synthesis, only the full length product is detectable by denaturing PAGE QC both when translesion synthesis is performed with standard (S) and thermostability-enhancing (TS) dNTPs. B. Native PAGE QC of the templates shown in (A) after diluting the primary DNA template stock into nuclease-free water or 10 mM Tris-HCl, pH 8.0. Thermostability-enhancing dNTPs reduce the formation of slow-migrating products when DNA is diluted into water, and no slow-migrating products are observed in either preparation following dilution into 10 mM Tris-HCl, pH 8.0.

c. **Yield.** Yield depends on the scale of PCR and the purification procedure used. In my experience, a 500 µl Q5® PCR will yield ~20 to ~40 pmols of DNA template if purified by agarose gel extraction (Procedure E) and ~70 to ~100 pmols if purified by exoI treatment and silica column clean-up (Procedure F).

2. **Choice of PCR polymerase**. The protocol above has been validated for use with Q5® High-Fidelity DNA polymerase, Q5U® Hot Start High-Fidelity DNA polymerase, and Vent® (exo-) DNA polymerase. My laboratory currently uses the Q5® family of enzymes for most applications because of their fidelity. For routine applications that do not require a high-fidelity polymerase, Vent® (exo-) is ideal because it uses the same reaction buffer as *Sulfolobus* DNA polymerase IV. Consequently, for DNA preparations that use Vent® (exo-) for PCR and standard dNTPs for translesion synthesis, it is possible to perform the translesion synthesis reaction simply by adding 1 µl of *Sulfolobus* DNA polymerase IV per 100 µl of PCR, incubating the reaction at 55°C for 1 h, and proceeding with purification and QC as written above. In principle, the DNA polymerase used for PCR should not impact the outcome of the procedures above because, as written, the protocol includes a silica column purification step to exchange the reaction buffer after PCR so that translesion synthesis is always performed under optimal conditions.
3. **Inclusion of Vent (exo-) during translesion synthesis.** Although *Sulfolobus* DNA polymerase IV alone is sufficient to complete translesion synthesis (Strobel *et al.*, 2020), my current protocol includes Vent (exo-) during translesion synthesis to reduce the likelihood of errors in the dsDNA downstream of the modification site: because DNA polymerase IV only incorporates 1-2 nts per binding event, it is possible for Vent (exo-) to complete synthesis of the truncated DNA strand once the internal modification has been bypassed by *Sulfolobus* DNA polymerase IV. The misincorporation frequency of Vent (exo-) was reported to be 1.9×10^{-4} (Mattila *et al.*, 1991), whereas the misincorporation frequency of *Sulfolobus solfataricus* DNA polymerase IV is between 8×10^{-3} and 3×10^{-4}, depending on sequence context (Boudsocq *et al.*, 2001).
4. **Tips for successful execution of the protocol.** The tips below describe several ways in which I maximize precision and minimize variability in sample handling. These suggestions are general and will be familiar to experienced readers but may help less experienced researchers to successfully execute the procedures described above.
   a. Wear nitrile gloves during every step, both for personal protection and to minimize the likelihood of sample contamination. Change gloves regularly to prevent cross-contamination.

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b. Perform all biochemical manipulations in an RNase-free environment.
c. Prepare solutions for small-scale biochemical reactions using commercial RNase-Free water and solutions when possible.
d. Use quantitative pipetting practices. Read the Gilson Guide to Pipetting for more information.
    i. Choose an appropriate pipette for the volume you are pipetting.
    ii. Be sure your pipettes are calibrated.
    iii. Use low retention pipette tips.
    iv. Keep the pipette vertical when aspirating liquid.
    v. Aspirate and dispense liquid smoothly and gently.
    vi. Immerse the pipette tip to an appropriate depth to minimize the accumulation of excess liquid on the outside of the tip – this varies by pipette tip, but ~3 mm is typically appropriate. The exception is when pipetting volumes <1 µl for which the tip should only be immersed ~1 mm into the liquid.
    vii. If excess liquid does accumulate on the outside of the pipette tip, wipe the tip on the edge of the tube you are pipetting from to remove any droplets.
    viii. Most pipette tips have graduations that can be used to approximate the volume of liquid in the tip – visually inspect the tip to confirm that the volume you are pipetting is correct and consistent.
    ix. After dispensing a concentrated reagent into another liquid, pipette up and down several times to rinse residual liquid from the tip.
    x. Change the tip after each sample. Reusing tips reduces accuracy and increases the likelihood of cross-contamination errors.
e. Ensure adequate sample mixing.
    i. With the exception of enzymes, always mix and spin down reaction components once they are thawed.
    ii. After diluting a concentrated buffer into water, vortex and spin down the sample before adding other reaction components.
    iii. Once an enzyme has been added to the reaction, all subsequent mixing should be done by gentle pipetting.
    iv. Visually inspect your samples during handling, particularly when working with small volumes.
f. Ensure adequate sample heating and cooling.
    i. Verify digital heat block temperature using either a high-accuracy digital thermometer with a probe or an analog thermometer before use – do not assume the digital readout on the heat block is correct.
    ii. Perform heat block incubations using Lab Armor™ beads to ensure efficient sample heating. Alternatively, partially fill the wells of an aluminum heat block with deionized water. I prefer Lab Armor™ beads to keep the tubes dry.
    iii. When preparing enzymatic reactions on ice, use an aluminum block as a tube holder to

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minimize the accumulation of liquid on the outside of tubes. This helps to keep gloves dry during sample handling.

g. Set centrifuges to count up if possible. This ensures a tight pellet because your samples will continue spinning until you stop the centrifuge just before you begin processing them.

Recipes

1. DEPC-treated Milli-Q water (makes 1.5 L)
   a. Add 1.5 L of Milli-Q water to a 2 L autoclavable media bottle.
   b. Add a large stir bar to the bottle.
   c. Add 1.5 ml of DEPC the Milli-Q water for a concentration of 0.1% v/v. Close the bottle tightly.
   Caution: *DEPC must be handled in a chemical fume hood.*
   d. Set the bottle on a magnetic stir plate to stir overnight.
   e. The next morning, place the bottle in a chemical fume hood and open the cap to release the carbon dioxide gas that has accumulated.
   f. Autoclave the DEPC-treated water for 30 min using the liquid cycle.
   Caution: *The media bottle cap must be loose when autoclaving.*
   g. Store at room temperature.
2. 10 mM Tris-HCl, pH 8.0 (makes 1 ml)
   a. Pipette 990 µl of nuclease-free water into a 1.7 ml microcentrifuge tube.
   b. Add 10 µl of RNase-Free Tris-HCl, pH 8.0.
   c. Vortex thoroughly and spin down in a mini centrifuge.
   d. Store at RT.
3. Thermostability-enhancing dNTP Solution
   a. Prepare a 1.7 ml microcentrifuge tube containing the reagents in Table 11.
   b. Vortex the solution thoroughly and spin down in a mini centrifuge.
   c. Store at -20°C.

Table 11. Recipe for Thermostability-enhancing dNTP Solution

Reagent	Stock Conc.	Final Conc.	For 100 µl
Nuclease-Free Water	-	-	60 µl
2-Amino-dATP	100 mM	10 mM	10 µl
dTTP	100 mM	10 mM	10 µl
dGTP	100 mM	10 mM	10 µl
5-Propynyl-dCTP	100 mM	10 mM	10 µl
Total Volume			100 µl

4. 1× TBE (tris-borate-EDTA) Buffer (makes 20 L)
   (89 mM tris base, 89 mM boric acid, 2 mM Na_2EDTA, pH 8.3)
   Notes:

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i. TBE will precipitate rapidly if prepared at ≥5× concentration. I recommend preparing TBE at a maximum concentration of 3×. This recipe is for 1× TBE, which will remain in solution.

ii. It is useful to determine and mark a 20 L fill line on the carboy that will hold the TBE solution by filling it with 20 L of deionized water using a 2 L graduated cylinder.

a. Fill a 4 L flask with ~3 L of Milli-Q water.
b. Add a large stir bar and place the flask on a magnetic stir plate.
c. Begin stirring at ~180 RPM.
d. Weigh 215.6 g of tris base (MW 121.14 g/mol) and slowly add it to the flask.
e. Weigh 110 g of boric acid (MW 61.83 g/mol) and slowly add it to the flask.
f. Weigh 14.89 g of $Na_2EDTA{\cdot}2H_2O$ (MW 372.24 g/mol) and slowly add it to the flask.
g. Allow the reagents to dissolve. It may be necessary to add additional Milli-Q water at this time.
h. Transfer the contents of the 4 L flask to a 20 L carboy.
i. Fill the 4 L flask with ~3.5 L of Milli-Q water and pour it into the carboy.
j. Fill the 20 L carboy with Milli-Q water to the previously determined 20 L fill line. Rock the carboy periodically to mix the solution.
k. Store at RT.

5. 70% (v/v) Ethanol (makes 50 ml)
   a. Pipette 15 ml of nuclease-free water into a 50 ml conical tube.
   b. Pipette 35 ml of 100% ethanol into the conical tube.
   c. Shake vigorously to mix.
   d. Store at -20°C.
6. 10% (w/v) SDS (makes 50 ml)
   a. Dissolve 5 g of SDS in ~40 ml of DEPC-treated Milli-Q water in a 50 ml conical tube.
      Note: Slow rotation will help the SDS dissolve without causing excessive bubbles to form.
   b. Transfer the solution to a 50 ml graduated cylinder and adjust the volume to 50 ml using DEPC-treated Milli-Q water.
   c. Transfer the solution back to the 50 ml conical tube and gently mix by inverting the tube.
   d. Store at room temperature.
7. 6× DNA Loading Dye, +SDS, xylene cyanole only (makes 25 ml)
   [10 mM Tris pH 8.0, 30% glycerol, 0.48% SDS, 0.05% (w/v) xylene cyanole FF]
   Notes:
   i. SDS is used to evict protein complexes from DNA.
   ii. Bromophenol blue is not used in this recipe because it typically migrates close to DNA template bands and therefore obscures the visibility of the DNA unless UV light is applied. It is important not to use bromophenol blue as a loading dye so that the DNA template band can be excised without UV exposure.
   a. Weigh 9.38 g of glycerol in a 50 ml conical tube.

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b. Weigh ~12.5 mg of xylene cyanole FF in a 1.7 ml microcentrifuge tube.
c. Dissolve the xylene cyanole FF in 1 ml DEPC-treated Milli-Q water and add to the 50 ml tube.
d. Add 15.05 ml of DEPC-treated Milli-Q water to the 50 ml tube and mix thoroughly by vortexing and inverting the tube.
e. Add 0.25 ml of nuclease-free 1 M Tris pH 8.0 and mix thoroughly by vortexing.
f. Add 1.2 ml of 10% SDS to the conical tube and mix by gently inverting the tube.
g. Transfer 1 ml aliquots to a 1.7 ml microcentrifuge tube as needed.
h. Store at RT.

8. 10% (w/v) APS (makes 10 ml)
   a. Add 1 g of APS to a 50 ml conical tube.
   b. Add 7 ml of autoclaved Milli-Q water and vortex to dissolve.
   c. Raise the volume to 10 ml.
   d. Transfer 410 µl aliquots to a 1.7 ml microcentrifuge.
   e. Store at -20°C. Each aliquot is enough to pour one sequencing gel.
9. 1× Formamide Loading Dye (makes 10 ml)
   [90% (v/v) deionized formamide, 1× transcription buffer, 0.05% (w/v) bromophenol blue]
   Caution: *The formamide stock bottle must be handled in a chemical fume hood.*
   a. Weigh ~5 mg of bromophenol blue into a 1.7 ml microcentrifuge tube.
   b. Dissolve the bromophenol blue with 1 ml of 10× Transcription buffer and transfer to a 50 ml conical tube.
   c. Add 9 ml of deionized formamide and vortex thoroughly.
   d. Store at 4°C.
10. 3× TBE Buffer (makes 4 L)
    (267 mM tris base, 267 mM boric acid, 6 mM Na_2EDTA, pH 8.3)
    a. Fill a 4 L flask with ~3 L of Milli-Q water.
    b. Add a large stir bar and place the flask on a magnetic stir plate.
    c. Begin stirring at ~180 RPM.
    d. Weigh 129.4 g of tris base (MW 121.14 g/mol) and slowly add it to the flask.
    e. Weigh 66 g of boric acid (MW 61.83 g/mol) and slowly add it to the flask.
    f. Weigh 8.93 g of $Na_2EDTA{\cdot}2H_2O$ (MW 372.24 g/mol) and slowly add it to the flask.
    g. Allow the reagents to dissolve. It may be necessary to add additional Milli-Q water at this time.
    h. Transfer 3 L of the solution to 4 L carboy 1 L at a time using a 1 L graduated cylinder.
    i. Pour the remaining solution into the graduated cylinder.
    j. Raise the volume to 1 L with Milli-Q water and transfer to the carboy.
    k. Shake the carboy to mix.
    l. Store at RT.
11. 6× DNA Loading Dye (non-denaturing) (makes 25 ml)

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[10 mM Tris pH 8.0, 30% glycerol, 0.05% (w/v) bromophenol blue]

a. Weigh 9.38 g of glycerol in a 50 ml conical tube.
b. Weigh ~12.5 mg of bromophenol blue in a 1.7 ml microcentrifuge tube.
c. Dissolve the bromophenol blue in 1 ml DEPC-treated Milli-Q water and add to the 50 ml tube.
d. Add 16.25 ml of DEPC-treated Milli-Q water to the 50 ml tube and mix thoroughly by vortexing and inverting the tube.
e. Add 0.25 ml of nuclease-free 1 M Tris pH 8.0 and mix thoroughly by vortexing.
f. Transfer 1 ml aliquots to a 1.7 ml microcentrifuge tube as needed.
g. Store at RT.

12. 1 M DTT (makes 10 ml)
    a. Weigh 1.54 g of DTT (MW 154.25 g/mol).
    b. Dissolve the DTT in 7 ml of nuclease-free water in a 50 ml conical tube.
    c. Transfer the solution to a 10 ml graduated cylinder.
    d. Adjust the volume to 10 ml using nuclease-free water.
    e. Attach a 0.2 µm PES syringe filter to a 10 ml syringe.
    f. Pre-wet the filter by drawing 5 ml of nuclease-free water into the syringe. Discard the water.
    g. Dispense the 1 M DTT stock through the syringe filter into a clean 50 ml tube.
    h. Transfer 1 ml aliquots of the sterile 1M DTT solution into 1.7 ml microcentrifuge tubes.
    i. Store at -20°C.
13. 100 mM DTT (makes 10 ml)
    a. Combine 9 ml of nuclease-free water and 1 ml of sterile 1 M DTT in a 50 ml conical tube.
    b. Vortex thoroughly.
    c. Transfer 1 ml aliquots of the 100 mM DTT solution into 1.7 ml microcentrifuge tubes.
    d. Store at -20°C.
14. 10× Transcription Buffer (makes 10 ml)
    a. Prepare a 50 ml conical tube containing the reagents in Table 12.
    b. Vortex the solution thoroughly.
    c. Transfer 1 ml aliquots of the 10× transcription buffer to 1.7 ml tubes.
    d. Store at -20°C.

Table 12. Recipe for 10× Transcription Buffer

Reagent	Stock Conc.	Final Conc.	For 10 ml
Nuclease-Free Water	-	-	4.48 ml
Tris, pH 8.0, RNase-Free	1 M	0.2 M	2.00 ml
KCl, RNase-Free	2 M	0.5 M	2.50 ml
DTT	100 mM	10 mM	1.00 ml
EDTA, pH 8.0 RNase-Free	500 mM	1 mM	20 ml
Total Volume			10.00 ml

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15. 20× NTP Solution (makes 0.5 ml)

    Critical: *NTP stocks must be prepared using high purity rNTP solutions. I recommend using the High Purity rNTP set from Cytiva Life Sciences (catalog number: 27202501).*

    Note: The 20× NTP solution described below limits UTP concentration to promote internal RNA labeling with [α-^{32}P]-UTP. ***If you use a different nucleotide as a radioactive tracer, you must prepare a separate 20× NTP solution in which the concentration of the nucleotide used for radiolabeling is limited.***

    a. Prepare a 1.7 ml microcentrifuge tube containing the reagents in Table 13.
    b. Vortex thoroughly and spin down in a mini centrifuge.
    c. Store at -20°C.

Table 13. Recipe for 20× NTP Solution

Reagent	Stock Conc.	Final Conc.	For 500 µl
Nuclease-Free Water	-	-	435 µl
ATP	100 mM	4 mM	20 µl
GTP	100 mM	4 mM	20 µl
CTP	100 mM	4 mM	20 µl
UTP	100 mM	1 mM	5 µl
Total Volume			500 µl

16. 5 mg/ml BSA (makes 200 µl)
    a. Add 180 µl of nuclease-free water to a 1.7 ml microcentrifuge tube.
    b. Add 20 µl UltraPure™ BSA (50 mg/ml) to the tube.
    c. Vortex thoroughly and spin down in a mini centrifuge.
    d. Store at -20°C.
17. 2 mg/ml Rifampicin (makes 10 ml)

    Note: Rifampicin is light sensitive. Rifampicin stock solution should be protected from light using aluminum foil during handling.

    a. Weigh 20 mg of rifampicin in a 1.7 ml microcentrifuge tube.
    b. Dissolve the rifampicin in 1 ml of DMSO and transfer to a 50 ml conical tube.

       Caution: *The DMSO stock bottle must be handled in a chemical fume hood.*

    c. Pipette 9 ml of DMSO into the conical tube.
    d. Vortex thoroughly.
    e. Transfer 1 ml aliquots of the 2 mg/ml rifampicin solution to 1.7 ml microcentrifuge tubes.
    f. Store at -20°C protected from light.
18. 1 M Tris-HCl, pH 8.0 (makes 1 L)
    a. Weigh 121.14 g of tris base (MW 121.14 g/mol).
    b. Dissolve the tris base in ~800 ml of Milli-Q water using a magnetic stir plate.
    c. Clean and calibrate the pH meter while the tris dissolves. Rinse the electrode thoroughly with Milli-Q water before inserting it into the tris solution.

Notes:

i. It is crucial to use an appropriate electrode to measure the pH of tris solutions. I use the Mettler Toledo S220-Bio SevenCompact Benchtop pH/Ion Meter (Mettler Toledo, S220-Bio).

ii. It is good practice to calibrate the pH meter using fresh standards immediately before you begin preparing solutions.

d. While measuring the pH of the tris solution, carefully adjust the pH to 8.0 by slowly adding of concentrated HCl while monitoring the pH.

Note: pH is temperature dependent. The solution should be RT when making final adjustments to the pH.

Caution: *Concentrated HCl is highly corrosive and should be handled using appropriate PPE and in a chemical fume hood.*

e. Transfer the tris solution to a 1 L graduated cylinder and adjust the volume to 1 L with Milli-Q water.

f. Transfer the tris solution back to the 2 L beaker and turn the stir plate on so that the solution is fully mixed. Verify that the pH is 8.0 once more.

g. Aliquot 500 ml of 1 M Tris-HCl solution into two 1 L autoclavable media bottles and sterilize by autoclaving for 30 min using the liquid cycle.

Caution: *Media bottle caps must be loose when autoclaving.*

h. Store at RT.

19. 0.5 M EDTA, pH 8.5 (makes 0.5 L)

a. Clean and calibrate the pH meter. Rinse the electrode thoroughly with Milli-Q water.

b. Weigh 93.06 g of $Na_2EDTA{\cdot}2H_2O$ (MW 372.24 g/mol).

c. Mix the $Na_2EDTA{\cdot}2H_2O$ with ~400 ml of Milli-Q water using a magnetic stir plate and insert the pH meter electrode into the solution.

d. Slowly add NaOH pellets while stirring.

Note: $Na_2EDTA{\cdot}2H_2O$ will not dissolve until the solution is ~pH 8.0. Take care not to add too many NaOH pellets at once, or you will overshoot the desired pH.

e. Once the $Na_2EDTA{\cdot}2H_2O$ is fully dissolved and the pH is 8.5, transfer the solution to a 500 ml graduated cylinder and adjust the volume to 500 ml.

f. Transfer the solution back to the beaker, turn the stir plate on so that the solution is fully mixed. Verify that the pH is 8.5 once more.

i. Transfer the solution into a 1 L autoclavable media bottle and sterilize by autoclaving for 30 min using the liquid cycle.

Caution: *The media bottle cap must be loose when autoclaving.*

j. Store at RT.

20. Stop Solution (makes 50 ml)

a. Prepare a 50 ml conical tube containing the reagents in Table 14.

b. Vortex thoroughly.

c. Transfer 10 ml aliquots to 15 ml conical tubes.

d. Store at -20°C.

Table 14. Recipe for Stop Solution

Reagent	Stock Conc.	Final Conc.	For 50 ml
DEPC-treated Milli-Q Water	-	-	18.8 ml
Tris, pH 8.0	1 M	0.6 M	30 ml
EDTA, pH 8.5	500 mM	12 mM	1.2 ml
Total Volume			50 ml

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Competing interests

No competing interests are declared.

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