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Biochemical Analysis of Transcription Termination by RNA Polymerase III from Yeast *Saccharomyces cerevisiae*

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Abstract

Eukaryotic RNA polymerase III (pol III) transcribes short noncoding RNA genes such as those encoding tRNAs, 5S rRNA, U6 snRNA, and a few others. As compared to its pol II counterpart, Pol III has several advantages, including the relative simplicity, stability, and more direct connectivity of its transcription machinery. Only two transcription factor complexes, TFIIB and TFIIC, are required to faithfully initiate and direct multiple rounds of transcription by pol III. Moreover, in contrast to an intricate multipartite mechanism of pol II termination, pol III termination is extremely simple, responsive to a monopartite signal (oligo T stretch on the nontemplate DNA strand) and mediated by a stably associated termination subcomplex of three integral subunits (Arimbasseri et al. *Transcription* 4(6), 2013). This makes pol III a valuable model for dissecting intrinsic molecular mechanisms of eukaryotic transcription termination. In this chapter, we provide protocols we adapted to study the biochemistry of transcription termination by *S. cerevisiae* pol III.

Keywords

Transcription; Termination; RNA polymerase III; C11; C53; C37

1 Introduction

During transcription termination, a very stable transcription elongation complex undergoes changes that lead to its destabilization and release of the RNA product and DNA template [1]. In case of RNA polymerases I and II, extraneous factors are required during the first step of the termination process, i.e., the recognition of a termination signal, to instruct polymerase where to terminate. However, it is largely unknown how these enzymes actually disengage from the transcript and DNA during the second step, which occurs somewhat distant from the termination signal [2]. In contrast, RNA polymerase III has the most direct transcription termination mechanism wherein both steps occur within a simple signal, a stretch of A residues on the template strand, which is sufficient to direct precise and efficient termination. Moreover, no external factors are required [1]. This simple mechanism makes pol III a valuable model for the study of the molecular mechanism of termination by a eukaryotic RNA polymerase [3].

Though the pol III termination signal is monopartite and termination is independent of extraneous factors, prior studies have dissected the mechanism into two components [2]. The core mechanism of pol III termination depends on the sensitivity of the pol III core enzyme (pol III⁺; lacking subunits C53/37 and C11) to the weak rU:dA hybrid generated at the terminator, while the holoenzyme mechanism depends on the core plus the C53/C37 and C11 subunits. The auxiliary components of the mechanism provided by C53/C37 and C11 functions to slow pol III elongation and prevent transcription arrest at the terminator [4]. It is possible to distinguish these two mechanistic components by altering the gene coding for the C11 subunit. When *S. cerevisiae* C11 is replaced with *Schizosaccharomyces pombe* C11, the C53/C37 and C11 subunits are readily lost during purification leading to a 14-subunit pol III (pol III⁻). This property allows one to study the role of individual subunits by adding back the wild type (wt) and/or mutated versions of each of the subunits.

Transcription termination by pol III occurs upon pausing at the terminator followed by release of the transcript [5]. To study the mechanisms of termination, it is important to distinguish these two steps and this requires a means to separate terminator-paused and released transcripts. In this chapter, we describe methods to purify pol III and the termination subcomplex subunits, C53/37 and C11. The pol III purification protocol was developed with the help of George Kassavetis (UCSD). We also provide detailed protocols for the assembly of elongation complexes using RNA-DNA scaffolds as developed by Kashlev group [6].

2 Materials

2.1 Yeast Strains

1. yNZ16 strain that carries an N terminal hexa histidine tag followed by a FLAG tag on the chromosomal RPC128 gene for wild-type (wt) pol III purification [7] (a gift from George Kassavetis, UCSD).
2. yGAKL strain that carries a chromosomal copy of C128 similar to yNZ16 [8]. The chromosomal copy of the RPC11 gene in this strain is deleted, and cell growth is supported by expression of *S. pombe* RPC11 gene under control of Gal promoter cloned into a centromeric plasmid, pRS316. This strain is viable only when galactose is used as the sole carbon source (a gift from George Kassavetis, UCSD).

2.2 Rosetta (DE3) pLysS Competent Cells

1. YPD: 1 % yeast extract, 2 % Bacto peptone, 2 % glucose.
2. YPgal: 1 % yeast extract, 2 % Bacto peptone, 2 % galactose.

2.3 Media

1. LB broth and agar: 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl (1.5 % agar for plates).
2. Antibiotics: 100 µg/ml ampicillin (amp) and 50 µg/ml kanamycin (kan).

2.4 Plasmids

1. RPC11: pGEX4T-1 C11 (wt), pGEX4T-1 C11 D91AE92A, pGEX4T-1 C11HPL (hairpin loop deletion).
2. RPC53: pET28a nH6TEVC53 (wt), pET28a nH6TEVC53 Nt (N-terminal deletion).
3. RPC37: pET21 nFLAG C37 (wt).

2.5 Bead Beater

Bead beater with 350 ml polycarbonate jar, cooling jacket, and sterile 0.5 mm diameter glass beads (all from Biospec products). Keep all components in cold room on the day before purification.

2.6 Chromatography

1. Bio-Rad LP protein purification system with fraction collector and accessories (Bio-Rad), or analogous.
2. HisTrap HP 5 ml prepacked column (we use GE Healthcare chromatography resins and columns, but other suppliers can also be tried).
3. HiTrap 1 ml SP sepharose FF column (GE Healthcare).
4. HiTrap 1 ml Q sepharose FF column (GE Healthcare).
5. Glutathione sepharose 4B resin (GE Healthcare).
6. PD-10 desalting columns (GE Healthcare), or analogous desalting columns.

2.7 Centrifugation

1. A Sorvall RC-5C plus centrifuge with SLA-1500 rotor or equivalent.
2. An ultracentrifuge with rotors and tubes that can hold ~200 samples.

2.8 Buffers

1. Resuspension buffer (RB): 60 mM Na-Hepes pH 7.8, 7.5 % glycerol, 15 mM 2-mercaptoethanol, 0.75 M NaCl, 10.5 mM MgCl₂, 1.5 mM PMSF, 3 µg/ml aprotinin, 0.75 µg/ml chymostatin, 1.5 µg/ml bestatin, 0.75 µg/ml pepstatin A, 3 µg/ml leupeptin.
2. Topping off buffer (TB): 40 mM Na-Hepes pH 7.8, 5 % glycerol, 10 mM 2-mercaptoethanol, 0.5 M NaCl, 7 mM MgCl₂, 1 mM PMSF, 2 µg/ml aprotinin, 0.5 µg/ml chymostatin, 1 µg/ml bestatin, 0.5 µg/ml pepstatin A, 2 µg/ml leupeptin.
3. D500 + xi (x mM imidazole) buffer: 20 mM Na-Hepes pH 8, 10 % glycerol, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 7 mM MgCl₂, 1 mM PMSF. Add Roche complete protease inhibitor (1 tablet per 50 ml buffer); other inhibitor mixtures are available. Add imidazole to final concentration as required in the protocol.

For buffers D500 + 30i and D500 + 300i, increase the glycerol concentration to 20 %.

4. GST lysis buffer (GLB): 50 mM K-Hepes pH 7.8, 0.5 M NaCl, 5 % glycerol, 10 mM 2-mercaptoethanol, 1 % Triton X-100, and Roche complete protease inhibitor (1 tablet/50 ml).
5. GST wash buffer (GWB): 20 mM K-Hepes pH 7.8, 200 mM NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol.
6. Ni lysis buffer (NLB): 50 mM K-Hepes pH 8, 200 mM NaCl, 5 % glycerol, 10 mM 2-mercaptoethanol, Roche protease inhibitor (1 tablet/50 ml).
7. Ni Wash buffer (NWB): 20 mM K-Hepes pH 8, 200 mM NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol, 20 mM imidazole.
8. Ni elution buffer (NEB): Ni Wash buffer with 300 mM imidazole.
9. SP elution buffer (SEB): 20 mM K-Hepes pH 8, 300 mM NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol.
10. Q equilibration buffer (QeqB): 20 mM Tris-HCl pH 8, 200 mM NaCl, 10 mM 2-mercaptoethanol, 10 % glycerol.
11. Q elution buffer (QelB): 20 mM Tris-HCl pH 8, 300 mM NaCl, 10 mM 2-mercaptoethanol, 10 % glycerol.
12. 1× EC buffer: 40 mM Na-Hepes pH 8, 3 mM 2-mercaptoethanol, 5 % glycerol, 100 µg/ml BSA.
13. Transcription stop buffer: 10 mM Tris-HCl pH 8, 10 mM EDTA, 1 % SDS, 0.25 mg/ml proteinase K.

3 Methods

3.1 Purification of RNA Polymerase III

3.1.1 Culturing of Yeast Cells

1. Inoculate 120 ml YPD (for wt pol III) and 120 ml YPgal media (for pol III⁻) with 10 ml respective starter culture grown for 8 h at 30 °C and incubate for 20 h at 30 °C.
2. Inoculate 10 l of YPD or YPgal divided into several large conical flasks with 1 % inoculum and incubate at 30 °C till the OD₆₀₀ reaches 4.
3. Harvest the cells by centrifugation (2,000 × *g*, 15 min at 4 °C).
4. Wash the cells once with 1 mM ice-cold, aqueous solution of PMSF (made fresh from 100 mM PMSF stock in ethanol) in a tared centrifuge tube. Measure and note the weight of the cell pellet.
5. Freeze the cell pellet in ethanol–dry ice mix and store at –70 °C.

6. Continue culturing and harvesting cells till the collective weight of cell pellets reaches 80 g.

3.1.2 S100 Extract Preparation

1. Remove 80 g cell pellet from -70°C and place it in a plastic bag. Use a hammer to break the cell pellet into very small pieces to allow rapid thawing of the pellet.
2. Place the pieces of cell pellet into a cold beaker and pour 2 volumes of RB (160 ml). Stir using a sterile spatula till the pellet is completely thawed. Avoid formation of air bubbles.
3. Remove 0.5 ml of sample and label as pre-lysis sample. Store on ice.
4. Check the temperature of the suspension after thawing. The temperature should be below 0°C .
5. Pour 150 ml of ice-cold glass beads (see Note 1) into the bead beater chamber followed by the cell suspension. After addition of half of the cell suspension, remove the air from the glass beads by stirring with a sterile spatula. Add rest of the cell suspension and fill the chamber with TB.
6. Place the rotor assembly into the chamber. Some suspension will overflow but this makes sure no air bubble is trapped within the assembly.
7. Tighten the cooling jacket over the assembly and fill the cooling jacket with previously prepared ethanol-ice mix (temperature between -15 and -20°C).
8. Bead beat 30 s followed by 3 min 30 s cooling time for 13 cycles. Change the ethanol-ice mix after seventh cycle.
9. After 13th cycle, disassemble the bead beater chamber and check the temperature of the lysate. Ideally, the temperature should be less than 5°C .
10. Filter the lysate through a metal coffee filter. Wash the retained beads with a small volume of topping off buffer (10–20 ml).
11. Measure the volume of the recovered lysate.
12. Take out a 0.5 ml sample from lysate and label as lysis sample.
13. Spin the lysate in a 250 ml centrifuge bottle in Sorvall SLA- 1500 rotor at 12,000 rpm for 30 min.
14. During centrifugation, check the pre-lysis and lysis sample under microscope to analyze the extent of cell lysis. Note down approximate % of lysis (see Note 2).
15. Recover supernatant after centrifugation and centrifuge at $100,000 \times g$ force for 1 h at 4°C in Beckman Ti60 rotor.
16. Recover the supernatant with extra care to avoid the murky loose material above the pellet. Use a 10 ml serological pipette attached to a pipettor that can be set

¹-It is important to keep all glassware and glass beads in a cold room the night before purification.

²-Lysis after 13 cycles is consistently more than 80 %. Thus, checking for lysis at this stage is only for documentation purposes.

for slow suction. Leave the final one-third of the extract in the tube. This step is crucial because the murky layer contains an inhibitor of transcription.

17. Take several 100 µl aliquots of the S100 extract and freeze in ethanol–dry ice mix. Add imidazole to a final concentration of 10 mM and proceed with the column chromatography (*see* Note 3).

3.1.3 Purification of His Tagged pol III

1. Equilibrate 5 ml Hitrap Nickel NTA column with at least 50 ml buffer D + 500 + 10i at 1 ml/min flow rate.
2. Load the S100 extract with added imidazole to the column at a flow rate of 0.5 ml/min and collect the flow-through.
3. Wash the column with 100 ml D + 500 + 10i at a flow rate of 1 ml/min.
4. Wash the column with 50 ml D + 500 + 20i at a flow rate of 1 ml/min.
5. Wash the column with 15 ml D + 500 + 30i at a flow rate of 1 ml/min.
6. Elute the proteins using a 100 ml gradient from 30 mM imidazole to 300 mM imidazole. Collect 1 ml fractions.
7. Freeze the A_{280} peak fractions in ethanol–dry ice mixture.
8. Do activity assay for the peak fractions as detailed below.

3.2 Purification of RPC11

3.2.1 Preparation of Cell Pellet

1. Transform Rosetta competent cells using the pGEX4T-1 RPC11 (wt or mutant) construct and plate on LB amp plates.
2. Inoculate a single colony in 20 ml LB amp and grow at 37 °C overnight.
3. Inoculate 2 l of LB amp with the overnight starter culture and incubate at 37 °C with 250 rpm till the OD600 reaches 0.5.
4. Shift the culture to an incubator set at 18 °C and incubate for 1 h.
5. Induce the culture by adding IPTG to a final concentration of 500 µM.
6. Incubate at 18 °C overnight with 250 rpm.
7. Harvest the cells and store at –20 °C.

3.2.2 Cell Lysis

1. Thaw the cell pellet.
2. Add 20 ml GLB and resuspend the pellet.

³.Alternatively, the extract can be frozen at this stage in ethanol- dry ice mix and stored at –70 °C. Also, freeze aliquots of S100 for in vitro transcriptions after adjusting the glycerol concentration to 20 %. This would help to keep pol III active during multiple freeze–thaw cycles.

3. Add lysozyme to a final concentration of 1 mg/ml, mix well by inverting the tube.
4. Incubate on ice for 30 min.
5. Sonicate six times with 30 s pulse and 2 min cooling time on ice.
6. Make sure the lysate is homogenized and not viscous.
7. Spin the lysate at $13,000 \times g$ for 30 min.
8. Recover the supernatant and save an aliquot for gel analysis (*see* Note 4).

3.2.3 Glutathione Sepharose Purification

1. Wash 0.5 ml glutathione sepharose resin three times with 5 ml GLB.
2. Add the washed resin to the supernatant after centrifugation.
3. Incubate at 4 °C for 2 h with rotation.
4. Spin down the beads at $1,000 \times g$ for 2 min.
5. Save the supernatant as flow-through.
6. Wash the beads four times with 50 ml GWB.
7. Resuspend the beads in 1 ml GWB and transfer to 5 ml tube. Add 20 μ l of thrombin (500 U/ml solution, e.g., from BioPharm laboratories) and incubate overnight at room temperature with rotation.
8. Spin down the beads and save the supernatant. Wash the beads twice with 0.5 ml GWB and save the washes.
9. Add benzamidine hydrochloride to a final concentration of 2.5 mM to the elution samples.
10. Check the lysate, flow-through, washes, and elutions on a 4–10 % acrylamide gel.

3.3 Purification of C53/C37 Heterodimer

3.3.1 Cell Preparation

1. Transform Rosetta cells with pET28a nH6-TEV-C53 and pET21 N-FLAG-C37. Select for transformants on LB amp + kan agar.
2. Inoculate 20 ml LB amp + kan broth with a colony from the transformation plate and incubate overnight in a shaker incubator at 250 rpm and 37 °C.
3. Inoculate 2 l of LB amp + kan broth with 20 ml of overnight starter culture and incubate at 37 °C till OD600 is 0.5.

⁴With all C11 constructs tested, including hexahistidine tagged and GST tagged clones, significant amount of C11 goes into inclusion bodies. But a small fraction that is present in the supernatant can be purified using nickel beads. Also, removal of hexahistidine tag from C11 using TEV protease was not as efficient as GST tag removal using thrombin.

4. Induce the culture with 500 μ M IPTG and incubate for 2 h at 37 °C with 250 rpm.
5. Harvest the cells and store at -70°C .

3.3.2 Cell Lysis

1. Thaw the cell pellet.
2. Add 20 ml NLB and resuspend the pellet.
3. Add lysozyme to a final concentration of 1 mg/ml, mix well by inverting the tube.
4. Incubate on ice for 30 min.
5. Sonicate six times with 30 s pulse and 2 min cooling time on ice.
6. Make sure the lysate is homogenized and not viscous.
7. Spin the lysate at $13,000 \times g$ force for 30 min.
8. Recover the supernatant and add 1 M imidazole to a final concentration of 20 mM.

3.3.3 Nickel NTA Chromatography

1. Connect 1 ml Hitrap Ni-NTA pre packed column to Bio-Rad LP protein purification system and equilibrate with 10 ml NLB.
2. Pass the lysate through the column at a flow rate of 0.5 ml/min.
3. Wash the column with 20 ml NWB.
4. Elute the proteins using NEB.
5. Check the peak fractions on 4–10 % gradient acrylamide gel and pool the peak fractions (*see* Note 5).
6. Add 800 units of TEV protease (e.g., from Eaton biosciences) to the pooled fractions and dialyze the sample against 2 l of Nickel wash buffer.
7. Incubate dialyzed samples with 250 μ l Ni-NTA resin equilibrated in NWB for 30 min at 4 °C.
8. Spin down the resin and save the supernatant.

3.3.4 SP Sepharose Chromatography

1. Equilibrate 1 ml SP sepharose pre packed column with 10 ml Nickel wash buffer (for purification of C53Nt /C37 dimer, *see* Note 6).

⁵.C53 protein is prone to degradation. Using different constructs or growth conditions did not prevent degradation. Degradation products can be separated from the full-length protein by series of ion exchange chromatographic steps. Also, TEV cleavage of hexahistidine tagged C53 is very efficient while thrombin digestion to remove GST tag from C53 resulted in complete degradation of protein.

⁶.The dimer having N terminal domain deletion of C53 does not bind to SP sepharose column. In this case, collect the flow- through and proceed with buffer exchange and Q sepha-rose chromatography.

2. Load the sample on the column using the sample injection valve.
3. Wash the column with 5 ml NWB.
4. Elute the protein using 5 ml SP elution buffer and collect 0.5 ml fractions.
5. Check the fractions on a 4–10 % acrylamide gel.

3.3.5 Q Sepharose Chromatography

1. Pool all the SP sepharose fractions having the dimer and exchange buffer with QeqB using PD10 buffer exchange columns.
2. Equilibrate 1 ml Q sepharose prepacked columns with the QeqB.
3. Load the sample using the sample injection loop.
4. Wash the column with 5 ml QeqB.
5. Elute the C53/C37 dimer using 5 ml QelB and collect 0.25 ml fractions.
6. Check all fractions on 4–10 % acrylamide gel.
7. Pool the fractions with the dimer, aliquot, and freeze.

3.4 Tailed Template Transcription

This protocol details the promoter-independent transcription assay to analyze pol III fractions for activity. A double-stranded DNA template with a 3' overhang is created by annealing the template and non-template strands. Templates can also be created by digestion of appropriate double-stranded DNA with restriction enzymes that leave 3' overhangs such as TspRI or PstI.

3.4.1 Preparation of Template

1. Load 50 µl of 100 µM template strand and non-template strand DNA oligos in formamide dye on a 10 % TBE urea gel (25 cm long, 1.5 mm thick).
2. Run the gel at a constant power of 29 W.
3. When the bromophenol blue is at the bottom of the gel, stop the electrophoresis, remove the glass plates, and place the gel on a TLC plate covered with plastic wrap.
4. Cover the gel with plastic wrap and light a UV lamp over the gel. Mark the position of the DNA band using a marker pen on the plastic wrap. Switch off the UV lamp and cut the gel piece.
5. Take a 0.5 ml centrifuge tube and using a 22-gauge needle, puncture its bottom. Place the 0.5 ml tube in a 1.5 ml tube. Insert the gel pieces into the 0.5 ml tube and close the tube. Spin the assembly at $16,000 \times g$ for 3 min.
6. Pulverized gel pieces will be collected at the bottom of the 1.5 ml tube.
7. Remove the 0.5 ml tube and add 0.4 ml TE buffer to the 1.5 ml tube with gel fragments.

8. Incubate overnight in cold room with rotation.
9. Transfer the gel slurry into a 1 ml syringe fitted with a 0.45 µm filtration unit (do not use PVDF). Insert the plunger of the syringe and push the liquid into a fresh 1.5 ml tube. Wash the syringe and filter using 0.2 ml TE.
10. Add 1 µl 20 mg/ml glycogen, 50 µl 3 M sodium acetate, and 1 ml 100 % ethanol to the sample. Incubate overnight at −70 °C and centrifuge at maximum speed for 30 min at 4 °C. Wash the pellet with 100 µl 70 % ethanol, dry the pellet, and dissolve in 50 µl water.
11. Check concentration of the oligos using a NanoDrop or UV spectrophotometer and adjust the volume so that both template and non-template strands have equal concentration.
12. In a 1.5 ml tube, take 5 µl of 10× TE, 5 µl of 0.5 M NaCl, 20 µl NT strand, and 20 µl template strand. Mix well and incubate on a 90 °C heat block for 10 min. Switch off the heat block and allow the sample to cool gradually.
13. Add 12.5 µl 5× DNA loading dye to the sample and load on a pre-cast 10 % TBE gel at 200 V.
14. Follow **steps 6.1.3–6.1.10** to extract the annealed DNA from the gel.
15. Check the concentration of the annealed template and store at −20 °C.

3.4.2 Transcription Assay

1. Make a master mix according to the list given below per sample. Make master mix for one extra tube to account for the pipetting error. The template to be used is the tailed template prepared as described in Subheading 6.1

5× EC buffer	5 µl
200 mM MgCl ₂	0.875 µl
1 M NaCl	1 µl
20 ng/µl tailed template	1 µl (400 fmol)
100 mM ATP	0.125 µl
100 mM CTP	0.125 µl
100 mM UTP	0.125 µl
1 mM GTP	0.625 µl
α- ³² P-GTP	20 K cpm per picomole of unlabeled GTP
Water	To 22 µl

2. Aliquot 3 µl of each pol III fraction into 1.5 ml tubes.
3. Add 22 µl of the master mix to each tube, mix well and incubate at 25 °C for 30 min.
4. Stop the reaction by adding 100 µl transcription stop mix.

5. Incubate at 55 °C for 15 min.
6. Extract the RNA once with 125 µl phenol–chloroform–isoamyl alcohol.
7. Precipitate the RNA by adding 10 µl 3 M sodium acetate and 350 µl 100 % ethanol.
8. Spin the samples at maximum speed in a microfuge and wash the pellet once with 70 % ethanol.
9. Dry the pellet in a SpeedVac and dissolve in 10 µl formamide dye.
10. Separate the transcription products on a 10 % sequencing gel run at a constant power of 80 W. Run radiolabeled pBR322/MspI digest as a molecular weight marker along with the samples.
11. Dry the gel and expose overnight with a phosphorimager screen.
12. Scan the phosphorimager screen and analyze the data.
13. Pool the peak fractions that give maximum transcription and do buffer exchange with PD-10 columns to remove imidazole.
14. Titrate the pooled pol III in transcription reactions as described above to find optimal amount of pol III to be used in reactions (make sure that all reactions are done at 100 mM salt because salt concentration can greatly affect the transcription output).

3.5 Assembly of Elongation Complexes

This protocol describes the method to assemble pol III elongation complexes using a scaffold of template strand, RNA primer, and a non-template strand. Elongation complexes thus assembled can be immobilized either on Ni-NTA using the hexahistidine tag on RPC2 subunit or on streptavidin beads using a biotinylated non-template strand. Separating the supernatant from the beads separates released transcripts from the arrested transcripts. This assay is used to study transcript release by pol III in detail using mutant versions of pol III. Transcripts or cleavage products are visualized either by radiolabeling the RNA primer at 5' end using ³²P or by incorporating ³²P-labeled NMPs.

3.5.1 Nickel Immobilization

1. Take 50 µl of 50 % NiNTA slurry (25 µl bed volume) per reaction and wash three times with at least 10 column volumes of 1× EC buffer + 100 mM NaCl. 25 µl bed volume of nickel agarose is in excess but it will help to visualize the resin better and will also help to minimize sample loss due to accidental loss of resin during washes.
2. Add the optimized amount of pol III to the washed nickel beads and incubate at room temperature (25 °C) for 30 min.
3. Anneal the template strand and 5' end labeled RNA primer as follows (scale up as required).

5× EC buffer	2 µl
1 M NaCl	1 µl
10 µM template strand	1 µl
4 µM labeled RNA	2.5 µl
SUPERase • In™ RNase inhibitor	0.2 µl
Water	3.3 µl

4. Annealing reaction. Set the following temperature holds in a thermal cycler:
 - 50 °C for 10 min.
 - 48 °C for 2 min.
 - 46 °C for 2 min.
 - 44 °C for 2 min.
 - 42 °C for 2 min.
 - 40 °C for 2 min.
 - 38 °C for 2 min.
 - 36 °C for 2 min.
 - 34 °C for 2 min.
 - 32 °C for 2 min.
 - 30 °C for 2 min.
 - 28 °C for 2 min.
 - 25 °C for 10 min.
5. Wash the beads five times with at least 10 column volumes of 1× EC buffer + 100 mM NaCl.
6. Make up volume to 50 µl.
7. Add 10 µl of annealing mix to the beads and incubate at room temperature for 10 min with very mild shaking. (A Vortex-Genie with adaptor that can hold 1.5 ml tube set at lowest speed works well.)
8. Add 2 µl of 10 µM non-template strand to the tube and continue shaking at room temperature for 10 min.
9. Wash the beads three times with at least 30 column volumes of 1× EC buffer + 100 mM NaCl.
10. Wash the beads once with at least 30 column volumes of 1× EC buffer + 1 M NaCl.
11. Wash the beads once with at least 30 column volumes of 1× EC buffer + 100 mM NaCl.

12. Make up the volume of resin to 50 μ l with 1 $\times$ EC buffer + 100 mM NaCl.

3.5.2 Streptavidin Immobilization

1. Buffer exchange the pol III (wt or) preparation to reduce the salt concentration to 100 mM and to remove MgCl_2 (see Note 7).
2. Annealing reaction: as described in Subheading 3.5.1.
3. Add optimized amount of pol III (wt or) to the annealing mix.
4. Incubate at room temperature for 10 min.
5. Add 2 ml of 10 μ M PAGE purified 5' biotinylated non- template strand to the reaction.
6. Incubate for 10 min at room temperature.
7. Add 50 μ l 50 % streptavidin beads washed with 1 $\times$ EC buffer to the reaction.
8. Incubate at RT with very mild shaking for 20 min.
9. Wash the beads five times with at least 30 column volumes of 1 $\times$ EC buffer + 100 mM NaCl.
10. Make up the volume of resin to 50 μ l with 1 $\times$ EC buffer + 100 mM NaCl.

3.6 Transcription and Cleavage Assays

1. To the elongation complexes assembled as described above, add 20 pmol of the pol III subunits C53/C37 and/or C11 or their mutated versions and incubate for 15 min at RT.
2. For transcription assays, add 10 μ l 6 $\times$ NTP mix(scale up as required).

5 $\times$ EC buffer	2 μ l
1 M NaCl	1 μ l
2 M MgCl_2	0.21 μ l
100 mM ATP	0.3 μ l
100 mM CTP	0.3 μ l
100 mM GTP	0.3 μ l
100 mM UTP	0.3 μ l
Water	5.59 μ l

3. For cleavage reactions, add only MgCl_2 to a final concentration of 7 mM.
4. Incubate at room temperature for 10 min.
5. Spin the beads at 1,200 $\times g$ for 1 min and save the supernatant as a released RNA fraction.

7. Having Mg^{2+} during assembly of elongation complexes using wt pol III yields very low amounts of the EC due to the intrinsic cleavage activity of wt pol III. Presence of Mg^{2+} does not affect EC formation by pol III .

6. Wash the beads with 100 μ l 1 $\times$ EC buffer + 100 mM NaCl and pool the wash with the released fraction.
7. Wash the beads with 40 column volumes of 1 $\times$ EC buffer + 100 mM NaCl and discard the wash.
8. Add 200 μ l of 1 $\times$ EC buffer + 100 mM to the beads.
9. Add 200 μ l transcription stop buffer to beads and supernatant.
10. Incubate at 55 $^{\circ}$ C for 15 min.
11. Extract once with phenol–chloroform–isoamylalcohol.
12. Precipitate the samples by adding 20 μ l sodium acetate and 500 μ l 100 % ethanol. Incubate overnight at -20° C.
13. Spin at maximum speed in a microfuge, wash the pellets with 70 % ethanol, and dry in a SpeedVac.
14. Dissolve the pellet in 10 μ l formamide dye and load 5 μ l on a 15 % TBE-Urea sequencing gel.
15. Dry the gel and expose overnight to a phosphorimager screen.
16. Scan the screen and analyze the data.

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