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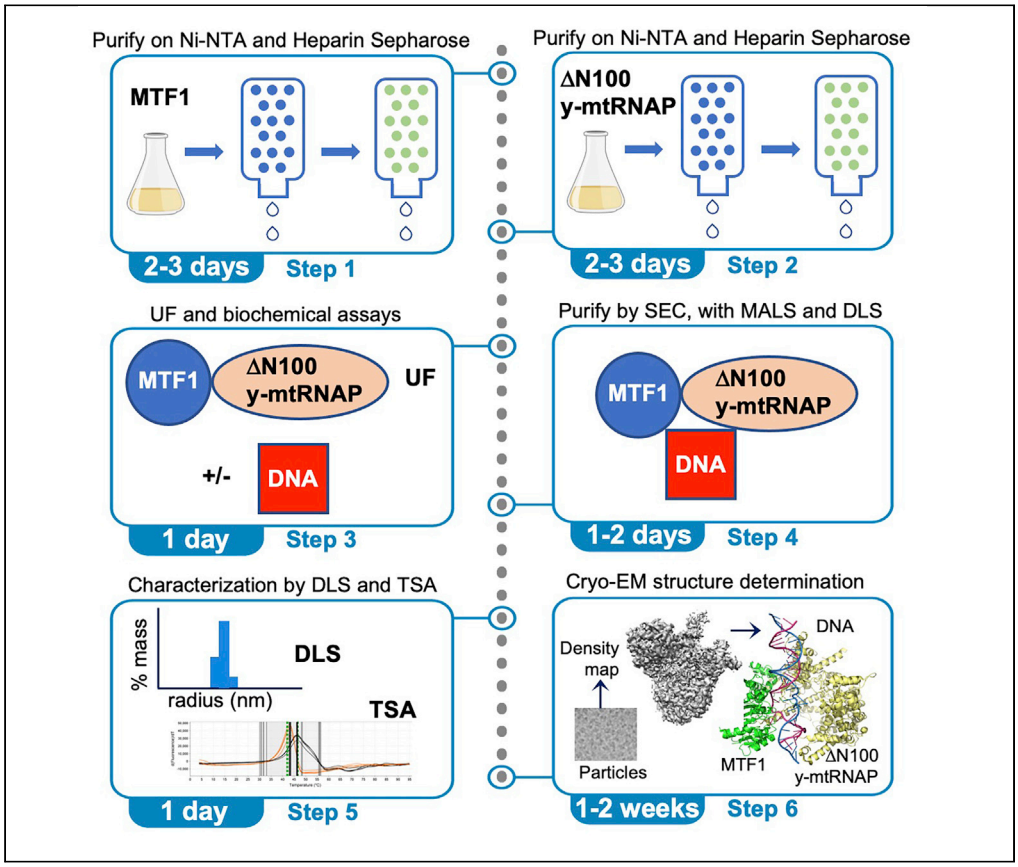
Protocol

Assembly and Cryo-EM structure determination of yeast mitochondrial RNA polymerase initiation complex intermediates



In yeast mitochondria, transcription initiation requires assembly of mitochondrial RNA polymerase and transcription initiation factor MTF1 at the DNA promoter initiation site. This protocol describes the purification of partially melted and fully melted initiation complex states. Both states co-exist in equilibrium in the same sample as seen by cryoelectron microscopy (cryo-EM) and allow elucidation of MTF1's structural roles in controlling the transition into elongation. We further outline how analysis of the complex by light scattering, thermal shift assay, and ultrafiltration assay exhibits reproducible results.

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Highlights

Expression and purification of yeast mitochondrial RNA polymerase (y-mtRNAP)

Transcription initiation complex assembly of y-mtRNAP, MTF1, and a bubble DNA promoter

Biochemical assays validate the transcriptional activity of the complex

Cryo-EM structures reveal two transcription initiation intermediate states

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Assembly and Cryo-EM structure determination of yeast mitochondrial RNA polymerase initiation complex intermediates

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SUMMARY

In yeast mitochondria, transcription initiation requires assembly of mitochondrial RNA polymerase and transcription initiation factor MTF1 at the DNA promoter initiation site. This protocol describes the purification of the component proteins and assembly of partially melted and fully melted initiation complex states. Both states co-exist in equilibrium in the same sample as seen by cryoelectron microscopy (cryo-EM) and allow elucidation of MTF1's structural roles in controlling the transition into elongation. We further outline how analysis of the complex by light scattering, thermal shift assay, and ultrafiltration assay exhibits reproducible results.

For complete details on the use and execution of this protocol, please refer to De Wijngaert et al. (2021).

BEFORE YOU BEGIN

In this protocol, we first detail the procedure for expression and purification of His-tagged MTF1 (Tang et al., 2009) and His-tagged ΔN100 y-mtRNAP (Paratkar et al., 2011; Tang et al., 2009; Matsunaga et al., 2004). We then describe the assembly of the complex on a pre-melted promoter DNA bubble and the isolation of this complex by SEC (Size Exclusion Chromatography). The oligomeric state of the complex is characterized by light scattering, thermal shift assay, and an ultrafiltration assay. The activity is characterized by an *in vitro* transcription initiation assay (Paratkar et al., 2011; Tang et al., 2009) and a 2-aminopurine assay to demonstrate DNA melting and binding of initiating NTPs (Tang et al., 2009). Lastly, we report the cryo-EM structure determination of this complex. To begin, we need to express and purify MTF1 and ΔN100 y-mtRNAP from published plasmids.

Prepare antibiotics and IPTG stocks

1. Make the following antibiotics and IPTG (isopropyl β-D-1-thiogalactopyranoside, Goldbio) stock solutions to use in cell culture and induce expression.
   a. Dissolve 4.76 g IPTG (Goldbio) in 16 mL Milli-Q water. Bring the final volume to 20 mL. Filter the solution through a 0.22 μm syringe filter. Store at −20°C.
   b. Dissolve 5 g ampicillin (Goldbio) in 45 mL Milli-Q water. Bring the final volume to 50 mL. Filter the solution through a 0.22 μm syringe filter. Store at −20°C.

Prepare LB agar plates

2. Weigh out 7.4 g LB Agar, Miller (Thermo Fisher Scientific) and dissolve in 180 mL Milli-Q water.
3. Heat and mix until LB Agar fully dissolves. Cool to 25°C.
4. Add Milli-Q water up to 200 mL and sterilize by autoclaving at 121°C for 15 min.
5. Let agar cool to 50°C–55°C (you should be able to hold the flask without burning your hand).
6. Add 200 μL of 100 mg/mL antibiotic stocks to a final concentration of 100 μg/mL.
7. Pour ~10–15 mL LB Agar into each plate.
8. Let the plates cool down until solid.
9. Seal plate edges with parafilm or foil and store the plates at 4°C.

Expression and purification of MTF1

10. Transform pTrcHisC plasmid, encoding His-6 tagged MTF1 from *S. cerevisiae*, into chemically competent *Escherichia coli* BL21 competent cells. Grow colonies at 37°C overnight on LB agar plates supplemented with 100 μg/mL ampicillin.

Note: MTF1 can be expressed in a number of *Escherichia coli* cell lines including BL21, BL21 (DE3) and XL1-blue.

11. Inoculate a single colony into 100 mL of LB media supplemented with 100 μg/mL ampicillin. Grow the cells for 14–18 h at 37°C with shaking at 200 rpm.
12. Add 10 mL of cell culture to each liter of 10 × 1 L of LB media in 2.8 L baffled flasks. Continue growing the cells at 37°C with 200 rpm shaking until the OD_{600} reaches 0.8. Collect 1 mL of 'uninduced' culture sample.
13. Induce protein expression by adding 1 mL of 1 M IPTG per L of cells to a final concentration of 1 mM. Grow cells for 16 h at 16°C. Collect 1 mL 'induced' culture sample and dilute it with LB media to OD_{600} = 0.8.
14. Harvest the cells by centrifugation at 10,000 × *g* for 15 min, discard the supernatant, and weigh the cell pellet.

Pause point: Pellets can be stored (without flash freezing) at −80°C for several months.

15. Centrifuge 1 mL each of 'uninduced' and 'induced' culture samples at 10,000 × *g* for 15 min at 4°C. Discard the supernatant. Dissolve the cell pellets in 1 mL lysis buffer.
16. Sonicate the samples on ice using microtip for 3 min with 30 s on, 30 s off pulses with amplitude set at 30% (Q700 sonicator, Qsonica LLC, USA). Pipette 100 μL of sample into three microcentrifuge tubes labeled as 'total protein', 'soluble protein' and 'insoluble protein'.
17. Centrifuge the 'soluble protein' and 'insoluble protein' samples at 10,000 × *g* for 30 min at 4°C. Collect the supernatant of 'soluble protein' sample. Discard the supernatant of 'insoluble protein' sample and dissolve the pellet in 2× Laemmli sample buffer to 100 μL final volume. Add 10 μL 2× Laemmli sample buffer to 10 μL 'total protein' and 'soluble protein' samples. Analyze the samples on 4%–20% SDS-PAGE. Assess the induced expression of MTF1 into soluble and insoluble fractions by comparing the respective gel bands.
18. Resuspend cell pellet from step 14 in 5 mL of MTF1 lysis buffer per gram of cells. Sonicate for 3 min on ice with 30 s on, 30 s off pulses with amplitude set at 30% (Q700 sonicator, Qsonica LLC, USA).
19. Centrifuge lysed cells for 30 min at 10,000 × *g* at 4°C.
20. Carry out polyethyleneimine precipitation of nucleic acids. Add solid NaCl to 0.5 M, and gradually add 1/20 the lysate volume of 10% polyethyleneimine (pH 7) while gently stirring at 4°C. Continue stirring at 4°C for 30 min after addition of polyethyleneimine.
21. Centrifuge for 30 min at 10,000 × *g* at 4°C. Discard the pellet.

22. Add a volume of saturated ammonium sulfate to the supernatant to reach 55% of saturation while keeping the solution at 4°C
23. Centrifuge at 10,000 × *g* for 30 min at 4°C. Discard supernatant.
24. Dissolve the ammonium sulfate pellet in buffer M (4 mL buffer/gram of cells) while keeping the solution at 4°C. Pass the dissolved ammonium sulfate pellet through a 0.22 μm syringe filter.
25. Load the dissolved ammonium sulfate pellet on a 5 mL DEAE Sepharose and 5 mL Ni-Sepharose cartridges (GE Healthcare Life Sciences) connected in tandem. Detach the DEAE Sepharose cartridge after loading the sample.
26. Wash the Ni-Sepharose cartridge with 50 mL Ni-Sepharose wash buffer.
27. Elute MTF1 with a 70 mL gradient of 20 mM to 500 mM imidazole. Analyze a 15 μL sample of each peak fraction by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Pool the highest purity fractions.
28. Load the MTF1 containing peak fractions on two, 1 mL Heparin-Sepharose columns (GE Healthcare Life Sciences) connected in tandem and wash with 20 mL Heparin-Sepharose wash buffer.
29. Elute the protein with a 50 mL gradient of 150 mM to 1 M NaCl in Heparin-Sepharose wash buffer. Analyze a 15 μL sample of each peak fraction by SDS-PAGE. Pool the highest purity fractions.
30. Concentrate the pooled MTF1 protein fractions using 10 kDa MW cut-off Amicon ultra-centrifugal filter (Merck Millipore) to 3 mL final volume, flash frozen in liquid nitrogen and store at −80°C.
31. Molar concentration is determined from OD_{280} in 6 M guanidinium hydrochloride and the extinction coefficient of 73,590 M^{-1} cm^{-1} (Tang et al., 2009). The purification protocol yields 3 mL of ~60 μM pure protein.

Note: In the associated article by De Wijngaert et al. (2021), four mutants of MTF1 (E141F, E144F, N211A/K214A and R178A/K179A) were also expressed and purified using the above protocol.

Expression and purification of ΔN100 y-mtRNAP

32. Transform ProEXHTb plasmid, encoding His-6 tagged ΔN100 y-mtRNAP from *S. cerevisiae*, into chemically competent *Escherichia coli* BL21 RIL Codon Plus cells. Grow colonies overnight at 37°C on LB agar plates supplemented with 100 μg/mL ampicillin.
33. Inoculate a single colony into 100 mL of LB media supplemented with 100 μg/mL ampicillin. Grow the cells for 14–18 h at 37°C with shaking at 200 rpm.
34. Add 10 mL of cell culture to each liter of 10 × 1 L of LB media in 2.8 L baffled flasks. Continue growing the cells at 37°C with 200 rpm shaking until the OD_{600} reaches 0.8. Collect 1 mL of 'uninduced' culture sample.
35. Induce protein expression by adding 1 mL of 1 M IPTG per L of cells to a final concentration of 1 mM. Grow cells for 16 h at 16°C. Collect 1 mL 'induced' culture sample and dilute it with LB media to OD_{600} = 0.8.
36. Harvest the cells by centrifugation at 10,000 × *g* for 15 min at 4°C, discard the supernatant, and weigh the cell pellet.

Pause point: Pellets can be stored (without flash freezing) at −80°C for several months.

37. Separately centrifuge 1 mL each of 'uninduced' and 'induced' culture samples at 10,000 × *g* for 15 min at 4°C. Discard the supernatant. Dissolve the cell pellets in 1 mL lysis buffer by vortexing.
38. Sonicate the samples on ice using microtip for 3 min with 30 s on, 30 s off pulses with amplitude set at 30% (Q700 sonicator, Qsonica LLC, USA). Pipette 100 μL of sample into each of the three microcentrifuge tubes labeled as 'total protein', 'soluble protein' and 'insoluble protein'.
39. Centrifuge the 'soluble protein' and 'insoluble protein' samples at 10,000 × *g* for 30 min at 4°C. Collect the supernatant of 'soluble protein' sample. Discard the supernatant of 'insoluble

protein' sample and dissolve the pellet in 2× Laemmli sample buffer to 100 μL final volume. Add 10 μL 2× Laemmli sample buffer to 10 μL 'total protein' and 'soluble protein' samples. Analyze the samples on 4%–20% SDS-PAGE. Assess the induced expression of ΔN100 y-mtRNAP into soluble and insoluble fractions by comparing the respective gel bands.

40. Resuspend cell pellets collected in step 36 in 5 mL of cold ΔN100 y-mtRNAP lysis buffer per gram of cells. Sonicate for 3 min on ice with 30 s on, 30 s off pulses with amplitude set at 30% (Q700 sonicator, Qsonica LLC, USA).
41. Centrifuge at 16,000 × *g* for 50 min at 4°C.
42. Carry out polyethyleneimine precipitation of nucleic acids at 4°C. Add solid NaCl to 0.5 M, and slowly add 1/20 the lysate volume of 10% polyethyleneimine (pH 7) while gently stirring at 4°C. Continue stirring at 4°C for 30 min after addition of polyethyleneimine.
43. Centrifuge at 10,000 × *g* for 30 min at 4°C. Discard the pellet.
44. Take the supernatant and add a volume of saturated ammonium sulfate to 55% of saturation while maintaining the solution at 4°C.
45. Centrifuge at 10,000 × *g* for 30 min at 4°C.
46. Dissolve the ammonium sulfate pellet in buffer N (4 mL buffer/gram of cells). Pass the dissolved ammonium sulfate pellet through a 0.22 μm syringe filter.
47. Load the dissolved ammonium sulfate pellet on a 5 mL DEAE Sepharose and 5 mL Ni-Sepharose cartridges (GE Healthcare Life Sciences) connected in tandem. Detach the DEAE Sepharose cartridge after loading.
48. Wash the Ni-Sepharose cartridge with 50 mL Ni-Sepharose wash buffer.
49. Elute ΔN100 y-mtRNAP with a 70 mL gradient of 20 mM to 500 mM imidazole. Analyze a 15 μL sample of each peak fraction by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Pool the highest purity fractions.
50. Load the ΔN100 y-mtRNAP peak on two, 1 mL Heparin-Sepharose columns (GE Healthcare Life Sciences) in tandem and wash with 20 mL Heparin-Sepharose wash buffer.
51. Elute with a 30 mL gradient of 150 mM to 1 M NaCl. Analyze a 15 μL sample of each peak fraction by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Pool the highest purity fractions.
52. Treat with TEV protease at 100:1 (w:w) ΔN100 y-mtRNAP to TEV protease for 16 h at 4°C.
53. Load the cleaved protein on Ni-Sepharose and collect the flow-through.
54. Concentrate the flow-through using 10 kDa MW cut-off Amicon ultra-centrifugal filter (Merck Millipore) to ~10 mL, flash freeze in liquid nitrogen and store at −80°C.
55. Determine the molar concentration from OD_{280} in 6 M guanidinium hydrochloride and extinction coefficient of 148,670 M^{-1} cm^{-1} (Paratkar et al., 2011). The protocol produces 10 mL of ~70 μM purified ΔN100 y-mtRNAP.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
BL21(DE3)	Sigma-Aldrich	Cat. #CMC0014
BL21(DE3) Codon Plus (RIL)	Agilent	Cat. # 230245
Chemicals, peptides, and recombinant proteins		
Protein Thermal Shift Dye for protein thermal shift assay	Applied Biosystems	Cat. # 4461146
Uridine 5′(α-thio) triphosphate	Jenna Bioscience	Cat. # NU-411S
Acrylamide:bis-acrylamide solution	Bio-Rad Laboratories	Cat. # 161-0154
2× Laemmli sample buffer	Bio-Rad Laboratories	Cat. # 161-0737
Urea	Sigma-Aldrich	Cat. # U5378
Ammonium persulfate	Sigma-Aldrich	Cat. # A3678
TEMED	Sigma-Aldrich	Cat. # T9281

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
ATP, GTP, and UTP	Thermo Scientific	Cat. # R0481
3′-dCTP	TriLink BioTechnologies	Cat. # N-3003
α-^{32}P-GTP	PerkinElmer	Cat. # BLU506H250UC
Poly(ethyleneimine), average M_w ~1300 Da, 50% w/v	Sigma	Cat. # 482595-100ML
Remel™ LB Agar, Miller	Thermo Fisher Scientific	Cat. # R453632
Plasmid pRK793 for expressing tobacco etch virus (TEV) protease	Kapust et al., 2001	Addgene.org plasmid # 8827
Deposited data		
Yeast mitochondrial ΔN100 y-mtRNAP /MTF1/dsDNA, pre-initiation complex	This paper	PDB 6YMV
Cryo-EM map for above	This paper	EMD-10845
Yeast mitochondrial ΔN100 y-mtRNAP /MTF1/dsDNA/RNA/UTPαS, initiation complex	This paper	PDB 6YMW
Cryo-EM map for above	This paper	EMD-10846
Original source data	This paper	https://dx.doi.org/10.17632/vtd45jkx6v.3
Oligonucleotides		
Modified yeast mitochondrial 15S promoter nontemplate strand 5′ -CGAATAAGTATTGATATAAGTAATAGATAATGC	IDT	N/A
Modified yeast mitochondrial 15S promoter template 5′ -GCATTATCTACCGACAATATCAATACTTATTCG	IDT	N/A
Yeast mitochondrial 21S rRNA promoter template 5′ -GGTATTTCAAATCTATTATTCTACTTTTTACTACTT ATATATATAATAATAATAATA	IDT	N/A
Yeast mitochondrial 21S rRNA promoter nontemplate 5′ -TATTATTATTATTATATATATAAGTAG TAAAAAGTAGAATAATAGATTTGAAATACC	IDT	N/A
RNA sequence (primer strand): 5′ pppGpG 3′	TriLink BioTechnologies	Cat. # O-31011
Recombinant DNA		
Plasmid pTrcHisC for expressing yeast MTF1	Tang et al., 2009	N/A
Plasmid ProEXHTb for expressing yeast ΔN100 y-mtRNAP	Paratkar et al., 2011	N/A
Software and algorithms		
PHENIX 1.16	Liebschner et al., 2019	phenix-online.org
RELION 3.0.8	Zivanov et al., 2018	https://www3.mrc-lmb.cam.ac.uk/relion/index.php/Main_Page
CTFFIND-4	Rohou and Grigorieff, 2015	https://grigoriefflab.umassmed.edu/ctffind4
Scipion	de la Rosa-Trevin et al., 2016	http://scipion.i2pc.es
Curves+	(Blanchet et al., 2011)	https://bisi.ibcp.fr/tools/curves_plus/index.html
MotionCor2 (included in RELION 3.0.8)	(Zheng et al., 2017)	https://emcore.ucsf.edu/ucsf-motioncor2
Coot 0.8.2	Emsley and Cowtan, 2004	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
PyMOL	Schrödinger, LLC.	https://pymol.org/2/
Chimera	(Pettersen et al., 2004)	https://www.cgl.ucsf.edu/chimera/download.html
DYNAMICS 7.10.0.23	Wyatt Technology	N/A
ResMap	Kucukelbir et al., 2014	N/A
Other		
Titan Krios 300 keV cryo-electron microscope	FEI/Thermo Fisher	N/A
Titan Glacios 200 keV cryo-electron microscope	FEI/Thermo Fisher	N/A
PELCO easiGlow Glow Discharge Cleaning System	Ted Pella	N/A
EM GP grid plunger	Leica	N/A
Quantifoil® R 1.2/1.3 200 Cu	Quantifoil	Q42272
Whatman™ filter paper, grade 1	GE Healthcare	1001-055
Äkta pure 25 L1	GE Healthcare	29018225
miniDAWN TREOS	Wyatt Technology	N/A
DynaPro NanoStar DLS	Wyatt Technology	N/A
Optilab T-rEX	Wyatt Technology	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NanoDrop™ One UV-VIS Spectrophotometer	Thermo Fisher	N/A
Amicon® Ultra-4 10K centrifugal filter	Merck Millipore	UFC801024
QuantStudio5 qPCR	Thermo Fisher	A28139
Superdex 200 Increase 10/300 GL	GE Healthcare	28990944
HiTrap DEAE FF column (5 mL)	Cytiva	17515401
HisTrap HP His tag protein purification column (5 mL)	Cytiva	17524801
HiTrap Heparin HP affinity column (1 mL)	Cytiva	17040601
Mini dry bath	Greiner Bio-One	848060
Microcon 100 kDa with Biomax membrane	Millipore Sigma	MPE100025

MATERIALS AND EQUIPMENT

Luria-Bertani (LB) media

Dissolve the reagents below in 1 L Milli-Q water and autoclave the media at 121°C for 30 min. 1 L LB media contains 10 g tryptone, 5 g yeast extract, and 10 g NaCl.

Reagent	Final concentration	Amount
LB broth powder	n/a	25 g
ddH_2O	n/a	1 L
Total	**n/a**	**1 L**

Lysis buffer for MTF1

Lysis buffer contains 50 mM sodium phosphate pH 8, 300 mM NaCl, 10% glycerol, 1 mg/mL lysozyme, 0.1 mM PMSF, Roche-Complete Protease Inhibitor Mixture Tablet.

Reagent	Final concentration	Amount
Sodium phosphate, dibasic	50 mM	0.36 g
NaCl	300 mM	0.88 g
Glycerol	10%	5 mL
lysozyme	1 mg/mL	50 mg
PMSF in methanol (50 mM)	0.1 mM	0.1 mL
Roche-Complete Protease Inhibitor Mixture Tablet	n/a	1 tablet
Total	**n/a**	**50 mL**

Adjust pH to 8.0 using phosphoric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

⚠ CRITICAL: Add PMSF, lysozyme and protease tablet just before using the lysis buffer.

Buffer M

This buffer is for dissolving the ammonium sulfate pellet of MTF1. Buffer M contains 50 mM Sodium phosphate pH 8, 300 mM NaCl, 10% glycerol, 0.1 mM PMSF, Roche protease inhibitors.

Reagent	Final concentration	Amount
Sodium phosphate, dibasic	50 mM	0.36 g
NaCl	300 mM	0.88 g
glycerol	10% v/v	5 mL
Roche-Complete Protease Inhibitor Mixture Tablet	n/a	1 tablet
PMSF in methanol (50 mM)	0.1 mM	0.1 mL
Total	**n/a**	**50 mL**

Adjust pH to 8 with phosphoric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

⚠ CRITICAL: Add PMSF and protease inhibitor tablet just before using the wash buffer.

Ni-Sepharose Wash buffer for MTF1 or ΔN100 y-mtRNAP

Ni-Sepharose wash buffer contains 50 mM Sodium phosphate pH 8, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 1 mM PMSF.

Reagent	Final concentration	Amount
Sodium phosphate, dibasic	50 mM	0.36 g
NaCl	300 mM	0.88 g
glycerol	10% v/v	5 mL
imidazole	20 mM	0.068 g
PMSF in methanol (50 mM)	1 mM	1 mL
Total	**n/a**	**50 mL**

Adjust pH to 8 using phosphoric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

⚠ CRITICAL: Add PMSF just before using the wash buffer.

Heparin-Sepharose wash buffer for MTF1 or ΔN100 y-mtRNAP

Ni-Sepharose wash buffer contains 50 mM Sodium phosphate pH 8, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF.

Reagent	Final concentration	Amount
Sodium phosphate, dibasic	50 mM	0.36 g
NaCl	150 mM	0.44 g
glycerol	10% v/v	5 mL
0.5 M EDTA, pH 8.4	1 mM	0.1 mL
PMSF in methanol (50 mM)	1 mM	1 mL
1 M DTT	1 mM	50 µL
Total	**n/a**	**50 mL**

Adjust pH to 8 using phosphoric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

⚠ CRITICAL: Add PMSF and DTT just before using the wash buffer.

Lysis buffer for ΔN100 y-mtRNAP

Lysis buffer contains 40 mM Tris-HCl pH 7.9, 300 mM NaCl, 15% glycerol, 0.1% Tween 20, 1 mM EDTA, 1 mg/mL lysozyme, 0.1 mM PMSF, Roche-Complete Protease Inhibitor Mixture Tablet.

Reagent	Final concentration	Amount
Tris base	40 mM	0.24 g
NaCl	300 mM	0.88 g
glycerol	15%	7.5 mL
Tween 20	0.1%	50 µL
0.5 M EDTA, pH 8.4	1 mM	0.1 mL
lysozyme	1 mg/mL	50 mg
PMSF in methanol (50 mM)	0.1 mM	0.1 mL
Roche-Complete Protease Inhibitor Mixture Tablet	n/a	1 tablet
Total	**n/a**	**50 mL**

Adjust pH to 7.9 using hydrochloric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

⚠ CRITICAL: Add PMSF, lysozyme, and protease tablet just before using the lysis buffer.

Buffer N

This buffer is for dissolving the ammonium sulfate pellet of ΔN100 y-mtRNAP. Buffer N contains 40 mM Tris-HCl pH 7.9, 150 mM NaCl, 15% glycerol, 0.1% Tween 20, 1 mM EDTA, 1 mM DTT, 20 mM imidazole.

Reagent	Final concentration	Amount
Tris base	40 mM	0.24 g
NaCl	150 mM	0.44 g
glycerol	15% v/v	7.5 mL
Tween 20	0.1%	50 μL
0.5 M EDTA, pH 8.4	1 mM	0.1 mL
1 M DTT	1 mM	50 μL
20 mM imidazole	20 mM	0.068 g
Total	**n/a**	**50 mL**

Adjust pH to 7.9 with hydrochloric acid at 25°C. The buffer can be stored at 4°C for a few weeks.

Transcription initiation reaction buffer

Reaction buffer contains 50 mM Tris acetate pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 1 mM DTT, 0.01% Tween 20.

Reagent	Final concentration	Amount
Tris base	50 mM	0.30 g
Potassium glutamate	100 mM	0.93 g
1 M magnesium acetate	10 mM	0.5 mL
1 M DTT	1 mM	50 μL
Tween 20	0.01%	5 μL
Total	**n/a**	**50 mL**

Adjust pH to 7.5 with acetic acid at 25°C. The buffer can be stored at 4°C for a few days.

2-Aminopurine assay buffer

Reaction buffer contains 50 mM Tris acetate pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate.

Reagent	Final concentration	Amount
Tris base	50 mM	0.30 g
Potassium glutamate	100 mM	0.93 g
1 M magnesium acetate	10 mM	0.5 mL
Total	**n/a**	**50 mL**

Adjust pH to 7.5 with acetic acid at 25°C. Pass through activated charcoal to reduce background during fluorescence measurements. The buffer can be stored at 4°C for a few days.

Ultrafiltration assay buffer

Assay buffer contains 50 mM Tris acetate pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 5 mM DTT, 0.01% Tween 20, 5% glycerol.

Reagent	Final concentration	Amount
Tris base	50 mM	0.30 g
Potassium glutamate	100 mM	0.93 g
1 M magnesium acetate	10 mM	0.5 mL
1 M DTT	5 mM	250 μL
Tween 20	0.01%	5 μL
Glycerol	5% v/v	2.5 mL
Total	**n/a**	**50 mL**

Adjust pH to 7.5 with acetic acid at 25°C. The buffer can be stored at 4°C for a few days.

Buffer A

This buffer is for size exclusion chromatography. Buffer contains 50 mM bis-tris propane-HCl pH 7.0, 100 mM NaCl, 5 mM magnesium chloride, 1 mM EDTA, 2 mM DTT.

Reagent	Final concentration	Amount
Bis-tris propane	50 mM	7.06 g
NaCl	100 mM	2.92 g
1 M magnesium chloride	5 mM	2.5 mL
0.5 M EDTA	1 mM	1 mL
1 M DTT	2 mM	1 mL
Total	**n/a**	**500 mL**

Adjust pH to 7.0 with hydrochloric acid at 25°C. The buffer can be stored at 4°C for a few days.

STEP-BY-STEP METHOD DETAILS

In vitro transcription initiation assay

Timing: 1 day

This assay measures transcription runoff and abortive RNA synthesis.

1. Prepare 1:1 mixtures of respective template and non-template DNA oligonucleotides (10 μM each) in standard 1× TE buffer with 50 mM NaCl. Place the tubes containing the mixtures in a heating block set at 95°C for 10 min. Switch off the heating block and allow it to cool to 25°C to promote annealing of the oligonucleotides.
2. Preincubate 2 μM yeast 21S rRNA promoter DNA fragment or the pre-melted 15S initiation bubble promoter with y-mtRNAP (1 μM), and WT or mutant MTF1 (2 μM) in transcription initiation reaction buffer at 25°C.
3. Initiate RNA synthesis by adding 100 μM each ATP, UTP, GTP, and 1.25 mM 3′-dCTP spiked with α-^{32}P-GTP.
4. Terminate reactions after 15 min using 125 mM EDTA and formamide dye mixture (98% formamide, 0.025% bromophenol blue, 10 mM EDTA).
5. Resolve RNA products on a 24% polyacrylamide, 4 M urea gel.
6. Expose the gel image to a phosphor plate, scan, and analyze using ImageQuant to calculate the molar runoff RNA products.

2-Aminopurine assay for DNA melting and initiating NTP binding

Timing: 1 day

This assay measures promoter melting and initiating NTP K_d using 2-aminopurine for both labeling promoter DNA as a fluorescent molecular marker and as a purine analog that can base pair with either thymine or cytosine.

7. Synthesize yeast 21S rRNA promoter DNA fragment with 2-aminopurine (2AP) at position -4 in the non-template strand or -1 in the template strand (IDT).
8. To assay for promoter melting, prepare samples of 2-aminopurine assay buffer alone, -4 2AP DNA alone (200 nM), DNA (200 nM) with 400 nM y-mtRNAP and 400 nM MTF1 WT or mutants.
9. Measure fluorescence intensity (excitation at 315 nm and emission 380 nm) in a FluoroMax spectrometer (Hiroba Scientific) at 25°C.
10. Plot the intensity of each sample.
11. To determine the K_d of initiating NTPs, use the promoter DNA labeled with 2AP at position -1 in the template strand.

12. Add increasing concentrations of initiating nucleotides (ATP+GTP) to a complex of -1 2AP promoter DNA (200 nM), y-mtRNAP (400 nM), and MTF1 WT or mutants (400 nM) and measure fluorescence emission intensity (380 nm after excitation at 315 nm).
13. Plot the titration data and estimate the cumulative K_d of the initiating nucleotides by fitting to a hyperbolic equation Fc = Fm*[N]/K_d+[N], where Fc is the fluorescence intensity at a given concentration of ATP+GTP (N), and Fm is the maximum fluorescence change.

Ultrafiltration assay to monitor y-mtRNAP-MTF1 interaction

Timing: 1 day

This assay analyzes complex formation between y-mtRNAP and MTF1 using ultrafiltration.

14. An equimolar complex of y-mtRNAP and MTF1 (2 μM each) is mixed in ultrafiltration assay buffer in a final volume of 500 μL.
15. Incubate the mixture at 25°C for 15 min.
16. Filter through a 100 kDa MW cut-off Microcon centrifugal filter unit (Millipore Sigma) at 2,000 × *g* until the first retentate volume is about 50 μL (1/10 of initial volume).
17. Dilute the retentate to 500 μL with the above buffer and filter again. Repeat this washing step, and take a sample after each wash. Collect samples consisting of the initial protein complex, first retentate, filtrate, and retentate samples and analyze on a 4%–20% SDS-PAGE.

Assembly and characterization of initiation complex (IC) and partially melted initiation complex (PmIC)

Timing: 2 days

18. Prepare the y-mtRNAP PmIC by incubating ΔN100 y-mtRNAP, MTF1, and promoter DNA in a molar ratio 1:1.2:1.2 for 2 h at 4°C.
19. The complex at a starting concentration of 6 mg/mL in buffer A is purified by size-exclusion chromatography. A Superdex 200 Increase 10/300 GL column is mounted on a GE AKTA Pure 25 FPLC in-line with Wyatt Technology multi-angle light scattering (MALS) device mini-DAWN, differential refractive index (dRI) measuring device Optilab, and dynamic light scattering (DLS) device DynaPro Nanostar. The purification is carried out at 6°C.
20. The purified y-mtRNAP complex sample was concentrated to 2.7 mg/mL in buffer A, aliquoted, and stored at −80°C without flash freezing. The calculated extinction coefficient is 542,608 $M^{-1}cm^{-1}$ at 280 nm. For the contribution from the two DNA strands, this coefficient was approximated by dividing the calculated coefficient at 260 nm by two.
21. The transcription initiation complex (y-mtRNAP IC) is prepared by incubating y-mtRNAP PmIC, 2-mer RNA, and UTPαS at a molar ratio of 1:6:50 for 2 h on ice.
22. For the Dynamic Light Scattering (DLS) experiments, add 8 μL of the y-mtRNAP IC or ymtRNAP PmIC complex at a concentration of ~0.5 mg/mL into a Wyatt disposable cuvette. Place the sample cuvette in the sample chamber maintained at 4°C. Acquire 30 readings for each sample, and analyze the data using Dynamics (Version 7.10.0.23) software (Wyatt Technology).

Thermal shift assay of y-mtRNAP with and without bubble DNA

Timing: 1 day

Thermal shift assay (Huynh and Partch, 2015) show a higher y-mtRNAP PmIC stability compared to that of the y-mtRNAP-only sample.

23. The thermal shift experiment is conducted in quadruplicate using a ThermoFisher QuantStudio5 qPCR setup and Protein Thermal Shift™ dye (Applied Biosystems, Cat. NO. 4461146) in 96 well format. Each well contains 5 μg of protein in a volume of 20 μL of 1× buffer A and 1× Protein Thermal Shift™ dye.
24. The temperature is ramped from 4°C–95°C at a rate of 3°C/min. The fluorescence from the dye is measured at 623 ± 14 nm wavelength while using an excitation wavelength of 580 ± 10 nm.
25. The data are analyzed using Protein Thermal Shift 1.3 software. The T_m of y-mtRNAP PmIC is 47°C compared to 42°C for y-mtRNAP-only sample, showing higher stability of the complex.

Cryo-EM structure determination of y-mtRNAP IC and PmIC

Timing: 1–2 weeks

PmIC and IC complexes were detected in one preparation of the complex of y-mtRNAP, MTF1, bubble DNA, 2-mer RNA, and UTP αS.

26. Prepare vitreous grids of y-mtRNAP IC on Quantifoil R 1.2/1.3 holey carbon grids using a Leica EM GP. Glow-discharge grids for 30 s at 10 mA current with the chamber pressure set at 0.30 mBar (PELCO easiGlow; Ted Pella). Place the grids prior to glow-discharge over a filter paper soaked with chloroform for two hours and air dry for 16 h.
27. Mount the glow-discharged grids in the sample chamber of a Leica EM GP at 8°C and 95% relative humidity, blot, and plunge-freeze in liquid ethane at temperature −172°C.
28. The frozen grids are tested on a JEM-1400 transmission electron microscope (TEM) at VUB Brussels, and the grid preparation conditions are optimized in cycles. The final optimized grids are reproducibly prepared using 5 μL of sample at a concentration of 1 mg/mL in buffer A spotted on Quantifoil R 1.2/1.3 holey carbon grids, incubated on the grid for 30 s, and back blotted for 12 – 14 s using two pieces of Whatman Grade 1 filter paper. The grids of y-mtRNAP PmIC are prepared using the conditions used for y-mtRNAP IC grids.
29. High-resolution datasets for y-mtRNAP IC and y-mtRNAP PmIC are collected at ESRF-Grenoble CM01 facility using a 300 kV Titan KRIOS TEM equipped with a Gatan K2 Summit direct electron detector and a Gatan energy filter (Kandiah et al., 2019). The data collection for y-mtRNAP PmIC is automated using EPU version 2.5 (ThermoFisher). Electron movies are collected in the counting mode at a nominal magnification of 165,000×. The total exposure time was 6 s with a total dose of 65 e-/ Å ^{2} and the movies are recorded as gain corrected MRC files. Images for y-mtRNAP IC sample are collected using EPU software version 2.6.1 (ThermoFisher) in the counting mode again at a nominal magnification of 165,000× yielding a pixel size of 0.827 Å . The exposure time was 5 s for each movie, accumulating to a total dosage of 61 e-/Å^2. The beam-image shift is applied during data collection to increase data throughput. Movies are recorded as compressed MRC files. For both data collections, the energy filter is used with a slit width of 20 eV.
30. All frames in individual movies are aligned using MotionCor2 (Zheng et al., 2017) as implemented in Scipion (de la Rosa-Trevin et al., 2016).
31. Contrast-transfer-function (CTF) estimations are performed using CTFFIND-4 (Rohou and Grigorieff, 2015).
32. A preliminary dataset collected from a cryo-EM grid of y-mtRNAP IC using a 200 kV Glacios/Falcon-3EC setup revealed the existence of IC and PmIC states. A reference set of thirteen 2D classes was obtained from 18,648 particles picked using Xmipp picker.
33. The particles are classified into thirteen 2D classes using Relion 3.0.8 (Zivanov et al., 2018), and the set of thirteen classes is used as the template for picking the particles from high-resolution y-mtRNAP PmIC and y-mtRNAP IC datasets using Autopick routine of Relion 3.0.8

34. For the final map calculations, 1–48 out of 60 frames for y-mtRNAP PmIC and 1–42 out of 50 frames for y-mtRNAP IC are re-aligned using the Relion-implemented MotionCor2.
35. The particles are re-extracted, fitting of CTF parameter and per-particle defocus was applied using Relion 3.0.8 prior to the final map calculation using Refine3D.
36. Maps are B-sharpened using the Postprocessing routine in Relion. The final maps are calculated at 3.1 Å and 3.7 Å (FSC 0.143), respectively, for y-mtRNAP PmIC and y-mtRNAP IC structures. The local resolutions of the maps are calculated using ResMap (Kucukelbir et al., 2014).
37. The model building is done manually using COOT (Emsley and Cowtan, 2004). The structures of h-mtRNAP in hmtRNAP IC (PDB Id. 6EQR) and of T7 RNAP in T7 RNAP IC (PDB Id. 1QLN) are used as references while building the model for y-mtRNAP. The crystal structure of MTF1 (PDB Id. 1I4W) is modeled into its part of the density.
38. The real-space fitting of the model to the density map is carried out using Phenix 1.16 (Liebschner et al., 2019). See Figure 1.

EXPECTED OUTCOMES

The SEC light-scattering measurements (Minton, 2016) confirmed homogeneous complex formation with expected molecular mass of 156 kDa for the complex of MTF1, y-mtRNAP, and DNA bubble. The DLS experiments in stand-alone cuvette mode using Nanostar did not significantly differ in hydrodynamic radius or polydispersity between y-mtRNAP PmIC and y-mtRNAP IC samples. Samples were routinely characterized by the DLS experiments before preparing cryo-EM grids to ensure consistency and reproducibility.

The thermal shift assay indicated an increase in the melting temperature of ΔN100 y-mtRNAP protein by 5°C upon binding to the pre-melted DNA bubble. The 100 kDa cutoff Amicon unit retained the complex.

QUANTIFICATION AND STATISTICAL ANALYSIS

The cryo-EM structures of y-mtRNAP PmIC and y-mtRNAP IC were determined using materials and software listed in the key resources table. Statistics generated by cryo-EM particle data processing, refinement, and structure validation are described (De Wijngaert et al., 2021).

LIMITATIONS

When carrying out thermal shift assay, both protein sample and Thermal Shift™ dye sometimes require some optimization to give the best signal. For different proteins, the optimal number of micrograms may vary, as well as the ratio of Thermal Shift™ dye to protein, and even the kind of dye.

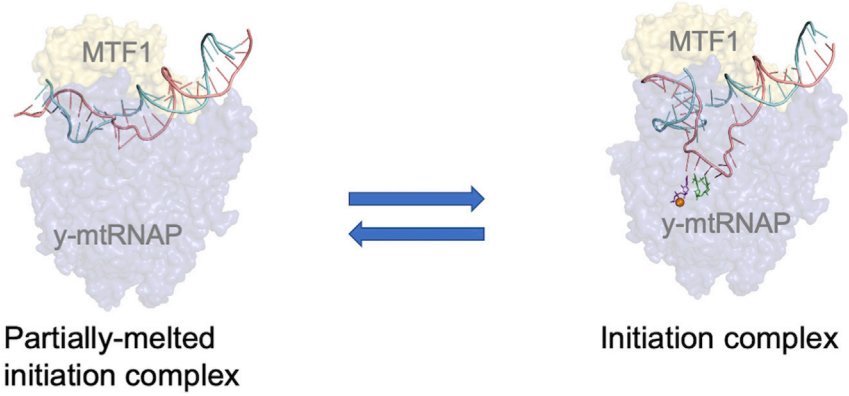

Figure 1. Cryo-EM structures of yeast mitochondrial RNA polymerase (y-mtRNAP) pre-melted initiation complex (PmIC; left) and initiation complex (IC; right)

TROUBLESHOOTING

Problem 1

Protein (MTF1 or ΔN100 y-mtRNAP) expresses but remains in lipid pellet after lysis (lysis steps 18 and 40 in "before you begin").

Potential solution

Check that the pH of the buffers is correct. When the pH is too close to the pI of the protein, it becomes less soluble. Proteins can also precipitate at extreme pH. Increase the sonication time while also making sure that the suspended cells stay ice-cold. The solution can be checked with a thermometer. Sonication is often used because of its low cost and lack of moving parts in the equipment. Other homogenization methods can be tried such as a French press or a microfluidizer. If the protein still misfolds during expression, try a lower concentration of IPTG to ~0.1 mM.

Protein may express better from a fresh transformation of the plasmid.

Problem 2

TEV protease cleavage of His tag on ΔN100 y-mtRNAP is incomplete (step 52 in "before you begin").

Potential solution

The best approach is to try a higher ratio of TEV protease to protein. If not, the cleavage site could be occluded. In that case, try to cleave at a higher temperature, 25°C, instead of 4°C.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kalyan Das (kalyan.das@kuleuven.be).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Coordinates and structure factors are deposited in the Protein Data Bank under PDB and EMBD ID codes 6YMV/EMD-10845 for PmIC (y-mtRNAP:MTF1:dsDNA); and 6YMW/EMD-10846 for IC (y-mtRNAP:MTF1: dsDNA:RNA:UTPαS)

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AUTHOR CONTRIBUTIONS

Investigation and validation, B.D.W., S.S., A.S., H.V., J.S., D.V., S.E.M., and K.D.; conceptualization, B.D.W., S.S., S.S.P., and K.D.; resources, C.D. and E.K.; data curation, B.D.W., E.K., S.S.P., and K.D.; writing and editing S.E.M., S.S.P., and K.D., supervision, visualization, funding acquisition, and project administration, S.S.P. and K.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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