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Purification and Comparative Assay of the Human Mitochondrial Replicative DNA Helicase

Fernando A. Rosado-Ruiz, Minyoung So, and Laurie S. Kaguni

Abstract

The replicative mitochondrial DNA (mtDNA) helicase is essential for mtDNA replication and maintenance of the mitochondrial genome. Despite substantial advances that have been made in its characterization, there is still much to be understood about the functional roles of its domains and its interactions with the other components of the minimal mitochondrial DNA replisome. Critical to achieving this is the ability to isolate the enzyme in a stable, active form. In this chapter we describe a modified, streamlined purification strategy for recombinant forms of the enzyme. We also present assays to assess its helix unwinding activity and the stimulatory effects of the mitochondrial single-stranded DNA-binding protein (mtSSB). Finally, we describe a concentration/buffer exchange method that we have employed to achieve greater enzyme stability and appropriate conditions for biochemical and biophysical studies.

Keywords

Mitochondrial DNA replication; mtDNA helicase; Helicases; Mitochondrial single-stranded DNA-binding protein; Human

1 Introduction

Since the first report of a human mitochondrial DNA helicase belonging to the DnaB family in 2000 [1], substantial advances have been made in characterizing the protein. Both the human protein, also known as Twinkle (for bacteriophage T7 gp4-like protein with intramitochondrial nucleoid localization), and its *Drosophila* analog have shown to serve as the replicative mtDNA helicase, and are essential in animal development [1–3].

Biochemical studies with recombinant mtDNA helicase have demonstrated that it shares many properties of replicative DNA helicases [4]. It unwinds dsDNA in an NTP-dependent reaction with 5' → 3' polarity. Efficient DNA unwinding requires a substrate with a 5'-ssDNA loading site and a 3'-ssDNA tail, which resembles a DNA replication fork, similar to the requirements of T7 gp4 and *E. coli* DnaB [4]. As in other systems, the DNA unwinding activity of the mtDNA helicase is stimulated by its endogenous single-stranded DNA-binding protein [4–6].

Sequence and functional similarities between the mtDNA helicase and T7 gp4 have prompted studies of the architecture of the human mtDNA helicase. Like the T7 gp4, the

mtDNA helicase has a modular architecture comprising two major domains, an N-terminal primase-like domain and a C-terminal helicase domain. These are separated by a flexible linker region [7]. However, despite the overall sequence similarity of the human mtDNA helicase with T7 gp4, the sequence identity between them is relatively low, with the highest variation occurring in the N-terminal domain for which the function remains unclear.

Autosomal dominant progressive external ophthalmoplegia (adPEO) has been shown to be associated with multiple mtDNA deletions, due to mutations in the mitochondrial DNA polymerase, Pol γ , and the mtDNA helicase [8]. In the case of the latter, most of the mutations have been attributed to defects in helicase activity. Recent experiments have shown that mutations in specific residues of the N-terminal domain are directly implicated in helicase function [9, 10]. Comparative sequence analysis of the N-terminal primase-related domain suggests that in many eukaryotic lineages, mtDNA helicase may have primase function, with the exception of most metazoans, which lack critical catalytic amino acids [11, 12].

Despite the substantial advances that have been made in characterizing the human mtDNA helicase, much remains to be learned. Notably, the function of the N-terminal domain of the mtDNA helicase remains elusive. To facilitate further research, we have adopted here a streamlined purification strategy that we have modified from our previous report [13]. We also present here our standard assay to assess the DNA unwinding activity of the enzyme (Fig. 1) and its stimulation by mtSSB. Finally, we describe a concentration (Fig. 2) and buffer exchange strategy for the enzyme that allows versatility in conditions for general biochemical and biophysical studies.

2 Materials

2.1 Purification of Recombinant of Recombinant Human Mitochondrial DNA Helicase

1. 1 M Tris-HCl, pH 7.5, pH 8.0.
2. 2 M Tris-HCl, pH 6.8, pH 8.8.
3. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
4. 5 M Sodium chloride (NaCl).
5. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Aliquots stored at -20°C .
6. 1 M Sodium metabisulfite, 1.0 M stock solutions at pH 7.5, stored at -20°C .
7. 1 mg/mL Leupeptin prepared in 50 mM Tris-HCl, pH 7.5, stored at -20°C .
8. 1 M Dithiothreitol (DTT), stored at -20°C .
9. 14 M 2-Mercaptoethanol (β -Me), stored at 4°C .
10. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4°C .
11. 10 % Sodium dodecyl sulfate (SDS).
12. 4 $\times$ Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8, 0.4 % SDS.

13. 4× Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4 % SDS.
14. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
15. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at –20 °C.
16. 10 % Ammonium persulfate (APS).
17. *N,N,N',N'*-tetramethylethylenediamine (TEMED).
18. Insect-Xpress Protein-Free medium for insect cells with L-Glutamine (Lonza).
19. Fetal Bovine Serum.
20. Amphotericin, penicillin-G, and streptomycin.
21. *Spodoptera frugiperda* (Sf9) cells.
22. Baculoviruses encoding N- and C-terminally His-tagged human mtDNA helicase.
23. Phosphate-buffered saline (PBS), stored at 4 °C.
24. Homogenization buffer: 25 mM Tris–HCl pH 8.0.
25. Buffer A: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 10 mM 2-mercaptoethanol.
26. 1 M Imidazole, stored at 24 °C.
27. Nickel-nitrilotriacetic acid (Ni-NTA) agarose resin.
28. Ni-NTA column buffers:
 - a. Equilibration/Wash buffer: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 10 mM imidazole.
 - b. Elution buffer 1: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 50 mM imidazole.
 - c. Elution buffer 2: 50 mM Tris–HCl, pH 8.0, 0.6 M NaCl, 10 % glycerol, 80 mM imidazole.
 - d. Elution buffer 3: 50 mM Tris–HCl, pH 8.0, 0.4 M NaCl, 10 % glycerol, 250 mM imidazole.
 - e. Elution buffer 4: 50 mM Tris–HCl, pH 8.0, 0.4 M NaCl, 10 % glycerol, 500 mM imidazole.
29. Heparin Sepharose (agarose) resin.
30. Heparin Sepharose column buffers:
 - a. Dilution buffer: 20 mM Tris–HCl, pH 7.5, 20 % glycerol, 0.5 mM EDTA.
 - b. Equilibration buffer: 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 % glycerol, 0.5 mM EDTA.

- c. Wash buffer: 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 % glycerol, 0.5 mM EDTA.
 - d. Elution buffer 1: 20 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 10 % glycerol, 0.5 mM EDTA.
 - e. Elution buffer 2: 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 % glycerol, 0.5 mM EDTA.
31. Polyallomer tubes (14 × 95 mm).
32. SW 40 rotor (Beckman).
33. Glycerol gradient buffers:
- a. 1× gradient buffer: 35 mM Tris-HCl, pH 7.5, 1 mM EDTA.
 - b. 12 % Gradient buffer: 35 mM Tris-HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA, 12 % glycerol.
 - c. 30 % Gradient buffer: 35 mM Tris-HCl, pH 7.5, 330 mM NaCl, 1 mM EDTA, 30 % glycerol.

2.2 Assay of Helicase Unwinding Activity

1. 60-mer oligodeoxynucleotide: (5'-T₍₄₀₎AGGTCGTTTCGCTCCAAGCT-3').
2. pBSKS+ single-stranded plasmid DNA.
3. T4 polynucleotide kinase (PNK).
4. 10× PNK buffer: 700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT.
5. [γ -³²P] ATP.
6. DE-81 chromatography paper cut into 1 × 1 cm pieces.
7. 0.3 M Ammonium formate, pH 7.5.
8. 95 % Ethanol.
9. Micro Bio-Spin P-30 Tris Chromatography column.
10. 200 Proof ethanol.
11. 1 M Tris-HCl, pH 7.5, pH 8.0.
12. 3.5 M Sodium chloride (NaCl).
13. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.
14. 5× Hybridization buffer: 50 mM Tris-HCl, pH 8.0, 1.0 M NaCl, 0.15 M Na citrate, 20 mM EDTA.
15. 3 M Sodium acetate.
16. 3 M Potassium chloride.
17. 1 M Tris-HCl, pH 7.5 and pH 8.0.

18. 1 M Magnesium chloride (MgCl₂).
19. Bovine serum albumin.
20. 5× Helicase buffer: 50 % glycerol, 2.5 mg/mL BSA, 20 mM MgCl₂, 100 mM Tris–HCl pH 7.5.
21. 1 M Dithiothreitol (DTT), stored at –20 °C.
22. 0.4 M Potassium chloride (KCl).
23. 200 mM ATP, aliquots stored at –20 °C.
24. 10 % Sodium dodecyl sulfate (SDS).
25. Recombinant human mtDNA helicase.
26. 10× Stop solution: 6 % SDS, 100 mM EDTA.
27. 10× Sample buffer: 0.25 % Bromophenol blue, 50 % glycerol.
28. 30 % Polyacrylamide (59:1; acrylamide:bisacrylamide), stored at 4 °C.
29. 5× TBE buffer: 450 mM Tris–HCl–borate, 10 mM EDTA.
30. Phosphor Screen.
31. Storm 820 Scanner.

2.3 Assay of Stimulation of DNA Unwinding Activity by mtSSB

1. Recombinant human mitochondrial single-stranded DNA binding protein.
2. All other materials are as described under the materials section of the assay for helicase unwinding activity.

2.4 Concentration and Buffer Exchange of Recombinant Human Mitochondrial DNA Helicase

1. Amicon ultra centrifugal filters, 30 and 100 K cut-off.
2. 0.05 % Tween 20.
3. Milli-Q water.
4. Purified recombinant human mitochondrial DNA helicase (Fr IV) in 35 mM Tris–HCl, pH 7.5, 330 mM NaCl, 20 % glycerol.
5. Purified recombinant human mitochondrial DNA helicase (Fr III) in 50 mM Tris–HCl, pH 7.5, 600 mM NaCl, 250 mM imidazole/10 % glycerol.
6. Dilution buffer for microscopy: 35 mM Tris–HCl, pH 7.5, 330 mM NaCl.
7. Pre-incubation solution for low salt glycerol gradient: 50 mM HEPES, pH 8.0, 10 % glycerol, 4.8 mM MgCl₂, 2.4 mM ATP γ S.
8. Glycerol gradient low salt buffers:

12 % Gradient low salt buffer: 50 mM HEPES, pH 8.0, 100 mM NaCl, 4 mM MgCl_2 , 2 mM $\text{ATP}\gamma\text{S}$, 0.02 % dodecyl maltoside, 12 % glycerol.

30 % Gradient low salt buffer: 50 mM HEPES, pH 8.0, 100 mM NaCl, 4 mM MgCl_2 , 2 mM $\text{ATP}\gamma\text{S}$, 0.02 % dodecyl maltoside, 30 % glycerol.

9. Polyallomer tubes (14×95 mm).
10. SW 40 rotor (Beckman).
11. 30 % Polyacrylamide (29:1; acrylamide:bisacrylamide), stored at 4 °C.
12. 10 % Sodium dodecyl sulfate (SDS).
13. 4× Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8, 0.4 % SDS.
14. 4× Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8, 0.4 % SDS.
15. 5× SDS-PAGE running buffer: 0.125 M Tris base, 0.95 M glycine, 0.5 % SDS.
16. 5× SDS-PAGE loading buffer: 50 % glycerol, 2 M Tris base, 0.25 M DTT, 5 % SDS, 0.1 % bromophenol blue, stored at -20 °C.
17. 10 % Ammonium persulfate (APS).
18. *N,N,N',N'*-tetramethylethylenediamine (TEMED).
19. Silver nitrate.
20. 1 M Arginine buffer: 1 M arginine, 35 mM Tris-HCl, pH 7.5.
21. Slide A-lyzer dialysis device (20 K).
22. Dialysis buffer for mass spectrometry: 10 mM NH_4HCO_3 , 7 mM 2-mercaptoethanol, 12 % glycerol.

3 Methods

3.1 Purification of Recombinant Human Mitochondrial DNA Helicase

3.1.1 Sf9 Cell Growth and Protein Overexpression

1. Grow *Spodoptera frugiperda* cells (Sf9) to a cell density of 2×10^6 cells/mL at 27 °C in Insect-Xpress culture media containing 4.5 % fetal bovine serum. Dilute to a cell density of 1×10^6 cells/mL with Insect-Xpress media containing 4.5 % fetal bovine serum.
2. Infect cells with baculovirus encoding N- or C-terminally His-tagged helicase (cDNA sequence lacking the mitochondrial presequence) at a multiplicity of infection of 5 at 27 °C. Harvest 72 h post infection.
3. Pellet cells at $400 \times g$ for 5 min. Wash with equal volume of ice- cold PBS, repeat the centrifugation and discard the supernatant.
4. Resuspend and combine pellets in PBS using 0.015 volume of original culture. Spin at $400 \times g$ for 5 min, discard supernatant, freeze pellet in liquid nitrogen and store at -80 °C.

3.1.2 Preparation of Soluble Cytoplasmic Fraction (See Note 1)

1. Thaw cells on ice 60 min, resuspend in 1/45 volume of original cell culture in homogenization buffer. Allow to sit for 20 min on ice.
2. Homogenize cells in a Dounce homogenizer with a tight pestle using 20 strokes.
3. Adjust the homogenate to 1 M NaCl, followed by gentle inversion every 5 min for 45 min.
4. Centrifuge at $145,000 \times g$ for 25 min in a 60 Ti rotor. Use buffer A to balance.
5. Pipet off and save supernatant (fraction I) in a pre-chilled beaker.

3.1.3 Nickel-Nitrilotriacetic Acid Chromatography

1. Pack and equilibrate a nickel-nitrilotriacetic acid (Ni-NTA) column (3.5 mL of resin/L of cells) with 10 column volumes (CV) of equilibration buffer at 1 CV/h.
2. Dilute fraction I with equal volume of buffer A and load onto column at a flow rate of 1–1.5 CV/h.
3. Wash column with wash buffer at flow rate of 3 CV/h, and collect 1 CV fractions.
4. Elute column with elution buffers containing 50, 80, 250, and 500 mM imidazole at 3 CV/h. Collect 1/4, 1/4, 1/6, and 1/4 CV fractions, respectively (*see* Note 2).
5. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining, and pool fractions accordingly (fraction II).

3.1.4 Heparin Sepharose Chromatography

1. Pack and equilibrate a heparin Sepharose column at a ratio of 10 mg of total protein in fraction II/mL of resin with equilibration buffer at a flow rate of 1 CV/h.
2. Dilute fraction II to an ionic equivalent of 150 mM NaCl. Load onto column at a flow rate of 2–3 CV/h (*see* Note 3).
3. Wash the column with 4 CV wash buffer at a flow rate of 3 CV/h, collecting 1/2 CV fractions.
4. Elute column with elution buffers containing 600 mM (3CV) and 1000 mM (3CV) NaCl at a flow rate of 3 CV/h, collecting 1/6 and 1/4 CV fractions, respectively (*see* Note 4).
5. Adjust wash and first eight fractions to 1 M NaCl to stabilize the low-salt eluted protein.

¹All buffers contain 1 mM PMSF, 10 mM sodium metabisulfite, 2 µg/mL leupeptin, and 10 mM 2-mercaptoethanol. All steps are to be performed at 4 °C.

²Helicase protein elutes in fractions corresponding to the buffer containing 250 mM imidazole.

³Precipitate will form as ionic strength decreases to 150 mM NaCl. As sample loads onto the column this precipitate will accumulate on the resin bed. Gently swirl fraction II every 30 min while loading.

⁴As ionic strength increases precipitate will go back into solution.

6. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining, and pool fractions accordingly (fraction III).

3.1.5 Glycerol Gradient Sedimentation

1. Prepare 12–30 % glycerol gradients in polyallomer tubes for use in a Beckman SW 40 rotor and chill on ice at least an hour before use.
2. Layer fraction III onto the gradients. Use 1× glycerol gradient buffer to adjust sample and make sure it floats atop the gradients.
3. Centrifuge at $96,600 \times g$ for 40 h at 4 °C, then fractionate by collecting fractions of 200–300 μL .
4. Analyze fractions by SDS-PAGE on 10 % minigels followed by Coomassie staining and pool fractions accordingly (fraction IV).
5. Aliquot fraction IV in an appropriate volume for further studies, freeze in liquid nitrogen and store at $-80\text{ }^{\circ}\text{C}$.
6. Analyze fraction IV by SDS-PAGE on 10 % minigels followed by Coomassie staining to determine purity and yield.

3.2 Assay of Helicase Unwinding Activity

3.2.1 5'-End Labeling of 60-mer Oligodeoxyribonucleotide

1. The kinase reaction (50 μL) contains 1× PNK buffer, $[\gamma\text{-}^{32}\text{P}]$ ATP (0.66 μM , 4500 Ci/mmol), 700 pmol (as nt) of oligonucleotide, and 20 units of T4 polynucleotide kinase.
2. Incubate reaction for 30 min at 37 °C.
3. Spot 0.25 μL of the reaction onto 5 squares of 1×1 cm DE81 chromatography paper. Wash 3 squares in 0.3 M ammonium formate twice for 5 min, then once in 95 % ethanol. Dry all 5 squares under heat lamp for 5 min. Count radioactivity on each square in scintillation fluid, and calculate incorporation (*see* Note 5).
4. Purify labeled oligonucleotide using a Micro Bio-Spin P-30 Tris chromatography column.

3.2.2 Annealing the Labeled Oligonucleotide to pBSKS+ Single-Stranded Plasmid DNA

1. The hybridization reaction (60 μL) contains 4× excess pBSKS+plasmid DNA over labeled oligonucleotide in 1× hybridization buffer.
2. Incubate at 65 °C for 60 min followed by incubation at 37 °C for 30 min. This generates a 20 bp dsDNA region with a 40 nucleotide 5'-tail.

⁵To calculate incorporation: [(average cpm of three washed papers)/(average cpm/pmol of unwashed papers)]/pmol ends in the reaction.

3. Adjust reaction to a final concentration of 0.3 M sodium equivalent with 3 M sodium acetate in a final volume of 100 μ L.
4. Add 2.5 volume of 200 proof ethanol and vortex to mix.
5. Incubate at -20°C for 60 min.
6. Spin at $20,000 \times g$ for 20 min and discard supernatant.
7. Quick-spin dry the pellet and air dry at room temperature for 4 min.
8. Resuspend pellet in 60 μ L of 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM EDTA.
9. Calculate the concentration of substrate using the concentration of the pBSKS+ plasmid DNA in the hybridization reaction (assume 100 % annealing and recovery).

3.2.3 Helicase Unwinding Activity Assay (See Note 6)

1. Prepare a master reaction mix such that each reaction contains $1\times$ helicase buffer, 10 mM dithiothreitol, 3 mM ATP, and 20 fmol of ^{32}P -60-mer/pBSKS+ substrate. Prepare the reaction mixture in a microcentrifuge tube, vortex and centrifuge briefly.
2. Dispense the mix into microcentrifuge tube(s) on ice, adjust salt to desired concentration with addition of 0.4 M KCl and water for a final volume of 50 μ L after considering the addition of desired concentration of the helicase (*see* Note 7).
3. Pre-incubate tube(s) at 37°C for 10 min prior to adding the helicase.
4. Add helicase to desired concentration (in nM as hexamer) and incubate reaction tube(s) at 37°C for 30 min.
5. Stop the reaction(s) with addition of 5 μ L of $10\times$ stop solution.
6. Add 5 μ L of sample buffer, mix and centrifuge sample(s).
7. Load sample(s) onto a pre-run (600 V for 2 h) 22 % PA/TBE gel and electrophorese for 30 min at 600 V in $1\times$ TBE until dye-front migrates to approximately 3 cm. Use a fan for cooling.
8. Dry gel under vacuum with heat and expose to a Phosphor Screen, 4 h to overnight.
9. Scan Phosphor Screen using Storm 820 scanner. Determine the volume of each band and subtract background using computer integration analysis (*see* Note 8).

⁶Set aside two reactions prior to addition of enzyme to be used as controls. One should be boiled for 2 min at 100°C to serve as the positive control, and the other remains unboiled to serve as the negative control.

⁷Take into consideration the salt contribution of the helicase.

⁸DNA unwinding is defined as the fraction of radiolabeled DNA species that is single-stranded: % unwinding = $(V_p/(V_s + V_p)) \times 100$; V_p represents the volume of the product and V_s represents the unreacted substrate in the lane of interest.

3.3 Assay of Stimulation of DNA Unwinding Activity by mtSSB

3.3.1 Substrate Preparation for Helicase Unwinding Activity Assay

1. Substrate for this assay is prepared as described for the helicase unwinding activity assay.

3.3.2 Stimulation of DNA Unwinding Activity of Human mtDNA Helicase by mtSSB

1. Prepare a master reaction mix such that each reaction contains 1× helicase buffer, 10 mM dithiothreitol, 3 mM ATP and 20 fmol of ³²P-60-mer/pBSKS+ substrate. Prepare the reaction mixture in a microcentrifuge tube, vortex and centrifuge briefly.
2. Dispense the mix into microcentrifuge tube(s) on ice, adjust salt to desired concentration with addition of 0.4 M KCl and water for a final volume of 50 µL after considering the addition 3.5 nM of the helicase and the desired concentration(s) of mtSSB (*see* Note 9).
3. Add the desired concentration(s) of mtSSB.
4. Pre-incubate tube(s) at 37 °C for 10 min prior to adding the helicase.
5. Follow **steps 4–9** as described for the helicase unwinding activity assay.

3.4 Concentration and Buffer Exchange of Recombinant Human Mitochondrial DNA Helicase

3.4.1 Concentration

1. Fill an Amicon ultra centrifugal filter (100 K cut-off) with 0.05 % Tween 20. Incubate at room temperature for 1 h. Discard Tween 20 and rinse the device thoroughly with milli-Q water (*see* Note 10).
2. Add appropriate amount of the purified recombinant human mtDNA helicase (Fr III or Fr IV) to the Amicon ultra centrifugal filter device (*see* Note 11).
3. Spin the device at 2000 × *g* at 4 °C until volume of the concentrated protein reaches the desired level. Every 5 min, stop the centrifuge and gently mix the concentrated protein solution with a pipette (*see* Notes 12 and ¹³).
4. To recover the concentrated mtDNA helicase, gently mix the concentrated protein solution and pipette into a new microcentrifuge tube.

⁹Take into consideration the salt contribution of both the helicase and the mtSSB.

¹⁰Treatment with 0.05 % Tween 20 reduces non-specific binding of human mtDNA helicase to the membrane in the device.

¹¹Because the mtDNA helicase is likely to precipitate during centrifugal concentration, the protein should be in a buffer with at least 300 mM NaCl.

¹²Mixing is required in order to disrupt the protein concentration gradient inside the centrifugal filter device because highly concentrated mtDNA helicase (more than 2 mg/mL) is likely to precipitate.

¹³Do not touch the membrane while mixing the concentrated protein.

3.4.2 Dilution—This procedure aims to prepare recombinant human mtDNA helicase stably in less than 5 % (v/v) glycerol for physical studies such as cryo-electron microscopy. All steps should be performed on ice.

1. The purified recombinant human mtDNA helicase should be concentrated to at least 0.5 mg/mL by using the method described above prior to decreasing glycerol amount.
2. Dilute the protein in 4 volumes of dilution buffer for microscopy. Add the buffer drop-wise to the protein solution with occasional tapping (*see* Note 14).

3.4.3 Desalting—This procedure aims to prepare recombinant human mtDNA helicase at near-physiological ionic strength.

1. Prepare a 12–30 % glycerol gradient with glycerol gradient low salt buffers in polyallomer tubes (14 × 95 mm), and chill in the cold room at least 1 h (*see* Note 15).
2. Preincubate 250 µL (total protein amount: 0.1–1 mg) of the purified recombinant human mtDNA helicase Fr III with 1.25 mL of pre-incubation buffer at 21 °C for 5 min. (Final concentrations should be 10 % glycerol, 4 mM MgCl₂, 2 mM ATPγS, and 100 mM NaCl) (*see* Note 16).
3. Layer the protein on the top of the gradient.
4. Centrifuge at 137,500 × *g* for 16 h at 17 °C in SW 40 rotor (*see* Note 17).
5. Fractionate by collecting 300 µL fractions. Analyze 12–15 µL per fraction by SDS-PAGE on 10 % gels and stain them with silver nitrate. Pool the peak fractions.
6. 6. Concentrate the gradient pool with an Amicon ultra centrifugal filter (30 K cut-off) above. To stabilize the mtDNA helicase under physiological ionic strength during concentration, add 1 M arginine buffer at 1/10 volume of the gradient pool (the final concentration of arginine should be 100 mM) (*see* Note 18).
7. Aliquot the concentrated mtDNA helicase and freeze in liquid nitrogen. Store at –80 °C.

3.4.4 Dialysis—This procedure aims to prepare recombinant human mtDNA helicase in a buffer for Q-TOF mass spectrometry.

1. The purified recombinant human mtDNA helicase should be concentrated to at least 0.5 mg/mL using the method in Subheading 3.4.1 prior to change the buffer by dialysis.

¹⁴Make sure that the dilution buffer has at least 300 mM NaCl to avoid precipitation of the recombinant mtDNA helicase.

¹⁵Cofactors (MgCl₂ and ATPγS) and detergent (dodecyl maltoside) are required in the low salt dilution buffer to improve the solubility of the mtDNA helicase at physiological ionic strength [14].

¹⁶Fraction IV of the human mtDNA helicase purification can also be used. In this case, pre-incubation buffer should be prepared differently to achieve the same final concentrations of glycerol, cofactors and salt as for fraction III.

¹⁷The mtDNA helicase is more soluble at 17 °C than at 4 °C [14].

¹⁸100 mM arginine improves the solubility of the human mtDNA helicase in solutions with 10 % glycerol.

2. Add 0.5–1 mg of the protein to a Slide A-lyzer dialysis device (20 K cut-off).
3. Dialyze the protein in dialysis buffer for mass spectrometry at 100 times the volume of sample at room temperature. Replace the buffer every 30 min for 3 h (*see* Note 19).
4. Recover the dialyzed protein from the device with a syringe.
5. Spin the recovered protein at $10,000 \times g$ for 10 min. Transfer supernatant to a new microcentrifuge tube in order to use for mass spectrometry (*see* Note 20).

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¹⁹2-Mercaptoethanol should be added to the dialysis buffer just before use.

²⁰Precipitate will form because ionic strength decreases during the dialysis.

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Fig. 1.

Helicase unwinding activity of *Hs* mtDNA helicase. *Lane 1*: intact substrate; *lane 2*: the substrate was denatured by heating to 100 °C for 2 min. Two fractions of two independent helicase preparations were compared in the assay. The helicase concentration used is indicated above each lane. All reactions were carried out at 100 mM KCl

Fig. 2.

Concentration and dilution of human mtDNA helicase. Protein fractions were electrophoresed in a 10 % SDS-polyacrylamide gel and stained with Coomassie Blue. *Lane 1:* *Hs* mtDNA helicase before concentration (1.2 μ g); *lane 2:* *Hs* mtDNA helicase after concentration (1.2 μ g); *lane 3:* *Hs* mtDNA helicase after dilution (1.2 μ g)