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Comparative Purification Strategies for *Drosophila* and Human Mitochondrial DNA Replication Proteins: DNA Polymerase γ and Mitochondrial Single-Stranded DNA-Binding Protein

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Abstract

The mitochondrion is the eukaryotic organelle that carries out oxidative phosphorylation, fulfilling cellular requirements for ATP production. Disruption of mitochondrial energy metabolism can occur by genetic and biochemical mechanisms involving nuclear-encoded proteins that are required at the mitochondrial DNA replication fork, which often leads to human disorders and to animal lethality during development. DNA polymerase γ (pol γ), the mitochondrial replicase, and the mitochondrial single-stranded DNA-binding protein (mtSSB) have been the focus of study in our lab for a number of years. Here we describe the purification strategies that we developed for obtaining the recombinant forms of pol γ and mtSSB from both *Drosophila melanogaster* and humans. Despite the fact that similar approaches can be used for purifying the homologous proteins, we have observed that there are differences in the behavior of the proteins in some specific steps that may reflect differences in their structural and biochemical properties. Their purification in homogeneous, active form represents the first step toward our long-term goal to understand their biochemistry, biology, and functions at the mitochondrial DNA replication fork.

Keywords

mitochondrial DNA replication; pol γ ; mtSSB; *Drosophila*; human

1. Introduction

Animal mitochondrial DNA (mtDNA) is a circular, compact, double-stranded molecule of about 16 kb, whose gene content is a remnant of the genome of the α -proteobacterium that was incorporated by a nucleated cell early in the evolution of the eukaryotes, according to the endosymbiont theory of mitochondrial origin (1). Because mitochondria retain a genome with genes essential for energy production in eukaryotic cells, they require enzyme systems for mtDNA replication and expression. So far, only three nuclear-encoded proteins have been identified at the mtDNA replication fork in animal cells: DNA polymerase γ , the mitochondrial single-stranded DNA-binding protein and the Twinkle DNA helicase. Together these proteins function to synthesize and proofread new DNA strands, destabilize the DNA helix and protect single-stranded DNA regions, and unwind the duplex DNA, respectively (2, 3).

After the first identification in animal cells and through the development of purification strategies for the enzyme, the biochemical and structural properties of pol γ have been studied in a number of organisms, with an emphasis on *Drosophila melanogaster* and

humans (2, 4–6). The *Drosophila* pol γ holoenzyme consists of a heterodimer of a catalytic subunit, pol γ - α , and an accessory subunit, pol γ - β (2). On the other hand, the human homolog comprises a catalytic core in a heterotrimeric complex with a dimer of the accessory subunit (6). In both cases, pol γ - α retains 5′–3′ DNA polymerase and 3′–5′ exonuclease activities, whereas pol γ - β is responsible for stimulating pol γ - α activity and enhancing holoenzyme processivity and DNA-binding properties (4). Recently, some studies have stressed the importance of pol γ in human diseases and animal development: functional defects due to mutations in human pol γ - α lead to mitochondrial disorders (7, 8); a mutation in *Drosophila* pol γ - α causes mitochondrial and nervous system dysfunction and developmental lethality in the larval third instar (9); site-directed mutagenesis of *Drosophila* pol γ - α and human pol γ - β alter enzyme activity, processivity, and DNA-binding affinity (4, 10); and null mutations of *Drosophila* pol γ - β cause lethality during early pupation, concomitant with loss of mtDNA and mitochondrial mass, and reduced cell proliferation in the central nervous system (11).

mtSSBs share similar physical and biochemical properties with *Escherichia coli* SSB (12, 13), with which they exhibit a high degree of amino acid sequence conservation. Like *E. coli* SSB, mtSSBs are homotetramers of 13–16 kDa polypeptides. It has been demonstrated that *Drosophila* mtSSB can stimulate 15–20-fold in vitro DNA synthesis and the 3′–5′ exonuclease activity of pol γ , in an assay that mimics lagging DNA strand synthesis in mitochondrial replication (13, 14). Furthermore, human mtSSB is able to stimulate specifically the unwinding activity of Twinkle helicase (15) and, along with pol γ and Twinkle helicase, reconstitute a minimal mtDNA replisome in vitro (3). The biochemical data are consistent with an important role for mtSSB in initiation and elongation of DNA strands in mtDNA replication, which has been documented genetically by the fact that an insertion in the third intron of the *Drosophila* gene (*lopo*) results in developmental lethality, concomitant with the loss of mtDNA and respiratory capacity (16). It is important to note the striking conservation of some biological processes from flies to humans. When a *Drosophila* homolog of an essential but poorly understood mammalian gene is identified, as happens with a large number of mitochondrial genes, powerful genetic and molecular techniques available in *Drosophila* can be applied to its characterization (17).

In this chapter, we review the strategies our lab has developed over the years for purifying the recombinant forms of *Drosophila* pol γ and mtSSB (14, 18–21), and we include comparative schemes for purification of the human homologs (4, 8). In Chapter 8, we also address the approaches we have taken to study the human Twinkle helicase (22), the other major component of the mtDNA replication fork. With regard to the purification protocols presented, we have found that the chromatographic methods we employ are generally efficacious for proteins involved in nucleic acid metabolism. In addition, hydroxylapatite, double- and single-stranded DNA cellulose, ATP agarose, and protein affinity chromatography resins, as used for the purification of the native forms of *Drosophila* pol γ and mtSSB (20, 21), are also generally useful. However, the production of a C-terminally histidine-tagged pol γ - α and consequently, the use of nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography have facilitated the purification of the recombinant protein by reducing the number of chromatographic steps required to achieve homogeneity. The order

of use can be varied to good purpose but as a rule of thumb, phosphocellulose as a first step is effective to eliminate ~90% of bulk mitochondrial protein while retaining nucleic acid binding proteins, and velocity sedimentation is useful as a final step to remove adventitious nuclease activities that are invariably of low molecular mass and to remove any small ligands introduced in affinity chromatography steps. Furthermore, different from its *Drosophila* homolog because the human pol γ - β dimer does not require co-overexpression with pol γ - α to obtain a soluble form, we have benefited by use of *E. coli* as the overexpression system, as we have done for overexpression of both fly and human mtSSBs. In principle, after preparation of a soluble fraction, a single chromatographic step combined with a final step of velocity sedimentation is sufficient to obtain highly pure proteins. Velocity sedimentation is of course also useful to link activity and polypeptide profiles and to obtain an *S* value of the protein of interest to help in the determination of subunit structure. Again, these approaches can be used as general guidelines for proteins involved in mitochondrial nucleic acid metabolism. The authors are pleased to address any queries by electronic mail and wish all success in the development of new purification strategies.

2. Materials

2.1. Recombinant *D. melanogaster* DNA Polymerase γ Purification

The ionic strength of all buffers is determined using a Radiometer conductivity meter.

1. Construction of recombinant transfer vectors and baculoviruses encoding the two subunits of *D. melanogaster* pol γ is described in Ref. (19). Briefly, the complete coding sequences were cloned into the baculovirus transfer vector pVL1392/1393 (PharMingen) and viruses were constructed using linearized wild-type baculovirus AcMNPV DNA (BaculoGold) (PharMingen). The recombinant baculoviruses used here, α_{C-HIS} (α with a C-terminal hexahistidine tag inserted between Ser1145 and the stop codon) and β may be obtained from the authors.
2. *Sf9* (*Spodoptera frugiperda*) cells (PharMingen).
3. TC-100 insect cell culture medium and fetal bovine serum (GIBCO-BRL).
4. Insect cell transfection buffer and Grace's medium (PharMingen).
5. Phosphate-buffered saline (PBS): 135 mM NaCl, 10 mM Na_2HPO_4 , 2 mM KCl, 2 mM KH_2PO_4 .
6. Phosphocellulose P-11 (Whatman), prepared according to the manufacturer's directions.
7. Ni-NTA agarose (QIAGEN).
8. 3 M Ammonium sulfate $[(NH_4)_2SO_4]$, ultrapure.
9. 1 M Sucrose, ultrapure.
10. 1 M HEPES-OH, pH 8.0, stored at 4°C.
11. 1 M Tris-HCl, pH 7.5.
12. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0.

13. 1 M Dithiothreitol (DTT). Store aliquots at -20°C .
14. 2-Mercaptoethanol.
15. 1 M Imidazole.
16. 10% Triton X-100.
17. 2 M Potassium chloride (KCl).
18. 1 M Potassium phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.6.
19. 5 M Sodium chloride (NaCl).
20. Phenylmethylsulfonyl fluoride (PMSF) (Sigma) prepared as a 0.2 M stock solution in isopropyl alcohol and stored at -20°C .
21. Sodium metabisulfite is prepared as a 1.0 M stock solution at pH 7.5 and stored at -20°C .
22. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA, and stored at -20°C .
23. 7 ml Dounce homogenizer.
24. Collodion membranes (Schleicher and Schuell).
25. Polyallomer centrifuge tubes (14×89 mm, Beckman).
26. All potassium phosphate buffers are at pH 7.6.
27. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 $\mu\text{g}/\text{ml}$. Where indicated, buffers also contain 0.015% Triton X-100.
28. Homogenization buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 280 mM ultrapure sucrose, 5 mM EDTA).
29. 10 mM Potassium phosphate buffer (10 mM potassium phosphate, pH 7.6, 20% glycerol).
30. 80 mM Potassium phosphate buffer – phosphocellulose equilibration buffer (80 mM potassium phosphate, pH 7.6, 20% glycerol).
31. 100 mM Potassium phosphate buffer – phosphocellulose wash buffer (100 mM potassium phosphate, pH 7.6, 20% glycerol).
32. 150 mM Potassium phosphate buffer – phosphocellulose elution buffer (150 mM potassium phosphate, pH 7.6, 20% glycerol).
33. 350 mM Potassium phosphate buffer – phosphocellulose elution buffer (350 mM potassium phosphate, pH 7.6, 20% glycerol).
34. 600 mM Potassium phosphate buffer – phosphocellulose elution buffer (600 mM potassium phosphate, pH 7.6, 20% glycerol).
35. Ni-NTA agarose equilibration buffer (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 5 mM imidazole).

36. Ni-NTA agarose elution buffer 1 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 25 mM imidazole).
37. Ni-NTA agarose elution buffer 2 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 250 mM imidazole).
38. Ni-NTA agarose elution buffer 3 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 500 mM imidazole).
39. Glycerol gradient buffer (50 mM potassium phosphate, pH 7.6, 200 mM (NH₄)₂SO₄, 0.015% Triton X-100).
40. Stabilization buffer (25 mM HEPES, pH 8.0, 2 mM EDTA, 80% glycerol, 0.015% Triton X-100).

2.2. Recombinant Human DNA Polymerase γ - α Purification

1. Human α_{C-HIS} recombinant baculovirus encoding the catalytic subunit of human pol. γ was obtained from Dr. William C. Copeland, Laboratory of Molecular Genetics, National Institutes of Environmental Health Sciences, Research Triangle Park, NC.
2. All potassium phosphate buffers are at pH 7.6.
3. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 μ g/ml. Where indicated, buffers also contain 0.015% Triton X-100.
4. Homogenization buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM EDTA).
5. 10 mM Potassium phosphate buffer (10 mM potassium phosphate, pH 7.6, 20% glycerol).
6. 80 mM Potassium phosphate buffer – phosphocellulose equilibration buffer (80 mM potassium phosphate, pH 7.6, 20% glycerol).
7. 100 mM Potassium phosphate buffer – phosphocellulose wash buffer (100 mM potassium phosphate, pH 7.6, 20% glycerol).
8. 150 mM Potassium phosphate buffer – phosphocellulose elution buffer (150 mM potassium phosphate, pH 7.6, 20% glycerol).
9. 350 mM Potassium phosphate buffer – phosphocellulose elution buffer (350 mM potassium phosphate, pH 7.6, 20% glycerol).
10. 600 mM Potassium phosphate buffer – phosphocellulose elution buffer (600 mM potassium phosphate, pH 7.6, 20% glycerol).
11. Ni-NTA agarose equilibration buffer (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, 0.1% Tx-100, and 5 mM imidazole).
12. Ni-NTA agarose elution buffer 1 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 25 mM imidazole).

13. Ni-NTA agarose elution buffer 2 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 250 mM imidazole).
14. Ni-NTA agarose elution buffer 3 (20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, and 500 mM imidazole).
15. Glycerol gradient buffer (30 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA).
16. Stabilization buffer (25 mM HEPES, pH 8.0, 2 mM EDTA, 80% glycerol, 0.015% Triton X-100).
17. Other materials are as in **Section 2.1**.

2.3. DNA Polymerase Assay

1. Pol γ , approx. 0.1 unit/ μ l.
2. DNase-I activated calf thymus DNA, 2 mg/ml stock, prepared as described (23).
3. 5X Polymerase buffer (250 mM Tris-HCl, pH 8.5, 20 mM MgCl₂, 2 mg/ml BSA).
4. 1 M DTT (Sigma).
5. 2 M KCl.
6. Stock mix of deoxynucleoside triphosphates containing 1 mM each of dGTP, dATP, dCTP, and dTTP (Amersham Pharmacia Biotech).
7. [³H]-dTTP (ICN Biochemicals).
8. 100% Trichloroacetic acid (TCA).
9. 0.1 M Sodium pyrophosphate (NaPPi).
10. Acidic wash solution (1 M HCl, 0.1 M NaPPi).
11. 95% Ethanol.
12. Glass-fiber filter paper (Schleicher and Schuell).
13. 10 $\times$ 75 mm Disposable culture tubes (Fisher).

2.4. Recombinant Human DNA Polymerase γ - β Purification

1. pQESL encoding the human pol γ - β without the mitochondrial presequence was obtained from Dr. William C. Cope-land, Laboratory of Molecular Genetics, National Institutes of Environmental Health Sciences, Research Triangle Park, NC.
2. *E. coli* XL-1 Blue.
3. Bacterial media (L broth): 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, pH 7.5.
4. 100 mg/ml Ampicillin. Filter-sterilize with a 0.2- μ m syringe filter, aliquot, and store at -20°C.
5. 10 mg/ml Tetracycline in 70% ethanol. Filter-sterilize with a 0.2- μ m syringe filter, aliquot, and store at -20°C.

6. 100 mM Isopropyl- β -D-thiogalactopyranoside (IPTG). Aliquot and store at -20°C .
7. Ultrasonic Processor Model W-225 (Heat Systems, Ultrasonics, Inc.).
8. 1 M Tris-HCl, pH 7.5.
9. 0.5 M EDTA, pH 8.0.
10. 5 M NaCl.
11. 3 M KCl.
12. 1 M Potassium phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.6.
13. 100% Glycerol, anhydrous (J.T. Baker).
14. 80% Sucrose, ultrapure.
15. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at -20°C .
16. 1 M Sodium metabisulfite, prepared as a 1.0 M stock solution at pH 7.5 and stored at -20°C .
17. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and stored at -20°C .
18. 1 M Imidazole.
19. 10% Sodium dodecyl sulfate (SDS).
20. 5 X SDS loading buffer (50% glycerol, 2 M Tris base, 0.25 M DTT, 5% SDS, 0.1% bromophenol blue). Aliquots are stored at -20°C .
21. 10% SDS-polyacrylamide resolving gels (8 cm $\times$ 10 cm $\times$ 1 mm) with 4% stacking gels.
22. Polyallomer centrifuge tubes (14 $\times$ 89 mm, Beckman).
23. Ni-NTA agarose (QIAGEN).
24. All buffers used throughout the purification contain 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, and leupeptin at 2 $\mu\text{g}/\text{ml}$.
25. Tris-sucrose buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose).
26. Resuspension buffer (35 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM imidazole, 0.1% (v/v) Triton X-100).
27. Ni-NTA agarose equilibration buffer (35 mM Tris-HCl, pH 7.5, 500 mM KCl, 25 mM imidazole).
28. Ni-NTA agarose elution buffer 1 (35 mM Tris-HCl, pH 7.5, 500 mM KCl, 25 mM imidazole).
29. Ni-NTA agarose elution buffer 2 (35 mM Tris-HCl, pH 7.5, 500 mM KCl, 250 mM imidazole).

30. Ni-NTA agarose elution buffer 3(35 mM Tris-HCl, pH 7.5, 500 mM KCl, 500 mM imidazole).
31. Glycerol gradient buffer (35 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA).

2.5. Recombinant *Drosophila* mtSSB Purification

1. pET-11a encoding *Drosophila* mtSSB without the mitochondrial presequence, available from this lab.
2. *E. coli* BL21 (λ DE3) pLysS (Stratagene).
3. Bacterial media (L broth): 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, pH 7.5.
4. 100 mg/ml Ampicillin. Filter-sterilize with a 0.2- μ m syringe filter, aliquot, and store at -20°C .
5. 100 mM IPTG. Aliquot and store at -20°C .
6. 1 M Tris-HCl, pH 7.5.
7. 0.5 M EDTA, pH 8.0.
8. 5 M NaCl.
9. 3 M Sodium thiocyanate (NaSCN).
10. 1 M Potassium phosphate ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.6.
11. 80% Sucrose, ultrapure.
12. 100% Glycerol, anhydrous (J.T. Baker).
13. 0.2 M Phenylmethylsulfonyl fluoride (PMSF) in isopropanol. Store aliquots at -20°C .
14. 1 M sodium metabisulfite, prepared as a 1.0 M stock solution at pH 7.5 and stored at -20°C .
15. Leupeptin is prepared as a 1 mg/ml stock solution in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and stored at -20°C .
16. 1 M DTT. Store aliquots at -20°C .
17. 1M Spermidine trihydrochloride (SpCl_3 , Sigma). Store at -20°C .
18. 3 M Ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$, ultrapure.
19. 100% TCA. Store at $0-4^{\circ}\text{C}$.
20. 10% SDS.
21. 5 X SDS loading buffer (50% glycerol, 2 M Tris base, 0.25 M DTT, 5% SDS, 0.1% bromophenol blue). Aliquots are stored at -20°C .
22. 17% SDS-polyacrylamide resolving gels (8 cm $\times$ 10 cm $\times$ 1 mm) with 4% stacking gels.

23. 5% Tween 20.
24. Centricon-30 spin concentrators (Amicon/Millipore).
25. Polyallomer centrifuge tubes (14 × 89 mm, Beckman).
26. Blue Sepharose CL-6B (Amersham Pharmacia Biotech).
27. All buffers used throughout the purification contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, leupeptin at 2 µg/ml, and 2 mM DTT.
28. Tris–sucrose buffer (50 mM Tris–HCl, pH 7.5, 10% sucrose).
29. 5 X Lysis buffer (1.25 M NaCl, 25 mM DTT, 100 mM SpCl₃, 10 mM EDTA, 1.5 mg/ml lysozyme).
30. Dilution buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM EDTA).
31. Blue Sepharose equilibration buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 2 mM EDTA).
32. Blue Sepharose wash buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 800 mM NaCl, 2 mM EDTA).
33. Blue Sepharose elution buffer 1 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 0.5 M NaSCN, 2 mM EDTA).
34. Blue Sepharose elution buffer 2 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 1 M NaSCN, 2 mM EDTA).
35. Blue Sepharose elution buffer 3 (30 mM Tris–HCl, pH 7.5, 10% glycerol, 1.5 M NaSCN, 2 mM EDTA).
36. Glycerol gradient buffer (50 mM Tris–HCl, pH 7.5, 200 mM (NH₄)₂SO₄, 2 mM EDTA).

2.6. Recombinant Human mtSSB Purification

1. pET-11a encoding the human mtSSB without the mitochondrial presequence (beginning with the amino acid sequence: ESETTTSLV), available from this lab.
2. All buffers used throughout the purification contain 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, leupeptin at 2 µg/ml, and 2 mM DTT.
3. Tris–sucrose buffer (50 mM Tris–HCl, pH 7.5, 10% sucrose).
4. 5 X Lysis buffer (1.25 M NaCl, 25 mM DTT, 100 mM SpCl₃, 10 mM EDTA, 1.5 mg/ml lysozyme).
5. Dilution buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM EDTA).
6. Blue Sepharose equilibration buffer (30 mM Tris–HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 2 mM EDTA).

7. Blue Sepharose wash buffer (30 mM Tris-HCl, pH 7.5, 10% glycerol, 400 mM NaCl, 2 mM EDTA).
8. Blue Sepharose elution buffer 1 (30 mM Tris-HCl, pH 7.5, 10% glycerol, 700 mM NaCl, 2 mM EDTA).
9. Blue Sepharose elution buffer 2 (30 mM Tris-HCl, pH 7.5, 10% glycerol, 1.5 M NaCl, 2 mM EDTA).
10. Blue Sepharose elution buffer 3 (30 mM Tris-HCl, pH 7.5, 10% glycerol, 3.0 M NaCl, 2 mM EDTA).
11. Glycerol gradient buffer (35 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA).
12. Other materials are as in **Section 2.5**.

3. Methods

3.1. Purification of Recombinant *Drosophila* DNA polymerase γ

3.1.1. Sf9 Cell Growth and Soluble Cytoplasmic Fraction Preparation

1. Grow Sf9 cells (500 ml) in TC-100 insect cell culture medium containing 10% fetal bovine serum at 27°C to a cell density of 2×10^6 , dilute to a cell density of 1×10^6 with TC-100 containing 10% fetal bovine serum, and then infect with recombinant *Drosophila* pol γ - α_{C-HIS} and - β baculo-viruses at a multiplicity of infection of 5. Harvest 48 h postinfection.
2. Pellet the cells at $400 \times g$ for 5 min, wash with an equal volume of cold PBS, repeat the centrifugation, and discard the supernatant.
3. Resuspend the cell pellet (approx. 1×10^9 cells) using 0.01 culture volume of homogenization buffer.
4. Lyse cells by 20 strokes in a Dounce homogenizer.
5. Centrifuge the homogenate at $1,000 \times g$ for 7 min.
6. Resuspend the resulting pellet using 0.005 culture volume of homogenization buffer and rehomogenize and centrifuge as in Steps 4 and 5 (see **Note 1**).
7. Centrifuge the combined supernatant fractions at $8,000 \times g$ for 15 min to pellet the mitochondria.
8. Remove the supernatant and centrifuge at $100,000 \times g$ for 30 min to obtain the cytoplasmic soluble fraction (fraction I).

3.1.2. Phosphocellulose Chromatography and Ammonium Sulfate Fractionation

1. Adjust fraction I (70–90 mg protein) to an ionic equivalent of 80 mM potassium phosphate and load onto a phosphocellulose column equilibrated with 80 mM potassium phosphate buffer at a packing ratio of 6 mg protein per packed ml of resin, at a flow rate of 0.8 column volume (CV)/h (see **Note 2**).

2. Wash the column with 3 CV of 100 mM potassium phosphate buffer at a flow rate of 2 CV/h.
3. Elute proteins with a 3-CV linear gradient from 150 to 350 mM potassium phosphate buffer at a flow rate of 2 CV/h.
4. Follow the gradient with a 2-CV high-salt wash of 600 mM potassium phosphate buffer at the same flow rate.
5. Assay as described in **Section 3.3.**, and pool active fractions (fraction II, *see Note 3*). Adjust to a final concentration of 10% sucrose.
6. Add solid ammonium sulfate (0.351 g/ml of fraction II) and incubate overnight on ice (*see Note 4*).
7. Collect the precipitate by centrifugation at $100,000 \times g$ for 30 min at 3°C.
8. Resuspend the pellet in 0.05–0.1 volume of fraction II with 10 mM potassium phosphate buffer containing 45% glycerol, and store at –20°C (fraction IIb).

3.1.3. Ni-NTA Agarose Affinity Purification

1. Dialyze fraction IIb (3–5 mg protein) against 10 mM potassium phosphate buffer in a collodion bag (M_r cutoff 25 kDa) until an ionic equivalent of 100 mM KCl is reached.
2. Mix dialyzed fraction IIb with 500 μ l of pre-charged Ni-NTA agarose (QIAGEN) equilibrated in Ni-NTA agarose equilibration buffer.
3. Incubate the suspension for 10 h on ice with gentle shaking.
4. Allow the beads to settle and then wash twice with 1 ml of equilibration buffer for 30 min with gentle shaking.
5. Pack washed beads into a column (0.5 ml), and elute protein retained on the beads successively at 5 CV/h with elution buffer containing 25 mM imidazole (2 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV). Collect 1/4-CV fractions.
6. Assay and pool active fractions (fraction III, approx. 0.9 ml).

3.1.4. Glycerol Gradient Sedimentation

1. Layer fraction III onto two pre-formed 12–30% glycerol gradients prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at $140,000 \times g$ for 60 h at 3°C and then fractionate by collecting five-drop (200–300 μ l) fractions.
3. Assay and pool active fractions, then add an equal volume of stabilization buffer and store the enzyme (fraction IV) at –20°C, –80°C, or under liquid nitrogen.

3.2. Purification of Recombinant Human DNA Polymerase γ - α

3.2.1. Sf9 Cell Growth and Soluble Cytoplasmic Fraction Preparation

1. Grow and dilute Sf9 cells (500 ml) as described in Step 1 of **Section 3.1.1.** and then infect with recombinant human pol γ - α _{C-HIS} at a multiplicity of infection of 5. Harvest 48 h postinfection.
2. Pellet the cells at $400 \times g$ for 5 min and wash with an equal volume of cold PBS, repeat the centrifugation and discard the supernatant.
3. Resuspend the cell pellet (approx. 1×10^9 cells) using 0.03 culture volume of homogenization buffer.
4. Lyse cells by 20 strokes in a Dounce homogenizer.
5. Centrifuge the homogenate at $1,000 \times g$ for 7 min.
6. Resuspend the resulting pellet using 0.01 culture volume of homogenization buffer, rehomogenize and centrifuge as in Steps 4 and 5 (*see Note 1*).
7. Follow Steps 7 and 8 of **Section 3.1.1.** to obtain the cytoplasmic soluble fraction (fraction I).

3.2.2. Phosphocellulose Chromatography and Ammonium Sulfate Fractionation

1. Adjust fraction I (140–240 mg protein) to an ionic equivalent of 80 mM potassium phosphate and load onto a phosphocellulose column equilibrated with 80 mM potassium phosphate buffer at a packing ratio of 6 mg protein per packed ml of resin, at a flow rate of 0.8 CV/h (*see Note 2*).
2. Wash the column and elute proteins as described in Steps 2–4 of **Section 3.1.2.**
3. Assay and pool active fractions (fraction II, *see Note 3*) and adjust to a final concentration of 10% sucrose.
4. Precipitate and pellet proteins as described in Steps 6 and 7 of **Section 3.1.2.**
5. Resuspend the pellet in 0.05–0.1 volume of fraction II in 10 mM potassium phosphate buffer containing 45% glycerol, and store at -20°C (fraction IIb).

3.2.3. Ni-NTA Affinity Chromatography

1. Dialyze fraction IIb (10–20 mg protein) against 10 mM potassium phosphate buffer in a collodion bag (M_r cutoff 10 kDa) until an ionic equivalent of approx. 300–400 mM KCl is reached.
2. Load dialyzed fraction IIb onto an equilibrated Ni-NTA resin at a ratio of approx. 7.5 mg protein per ml of packed resin, at a flow rate of 1–2 CV/h.
3. Wash the column with 2 CV of equilibration buffer and elute successively with elution buffers containing 25 mM imidazole (2 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV), at a flow rate of 5 CV/h. Collect 1/4-CV fractions.

4. Assay and pool active fractions (fraction III, approx. 1.6– 2.3 ml).

3.2.4. Glycerol Gradient Sedimentation

1. Layer fraction III onto two pre-formed 12–30% glycerol gradients prepared in polyallomer tubes for use in a Beckman SW 41 rotor.
2. Centrifuge at $140,000 \times g$ for 60 h at 3°C, then fractionate by collecting five-drop (200–300 µl) fractions.
3. Assay and pool active fractions, then add an equal volume of glycerol gradient buffer containing 80% glycerol or stabilization buffer, and store the enzyme (fraction IV) at –20°C, –80°C, or under liquid nitrogen.

3.3. DNA Polymerase Assay

This section describes an assay of DNA synthesis that utilizes a multiply primed double-stranded DNA substrate and pol γ fractions. DNA polymerase activity can be measured at different salt concentrations to discriminate nuclear (low-salt stimulated) and mitochondrial (high-salt stimulated) DNA polymerase activities. The assay measures the incorporation of [^3H]-labeled dTMP into the DNA substrate, such that 1 unit of activity is the amount that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid insoluble material in 60 min at 30°C.

1. Adjust the water bath to 30°C.
2. Dry down the radioactive substrate by lyophilizing to less than half the original volume.
3. Prepare a master reaction mix using the stock solutions in **Section 2.3** in a microcentrifuge tube on ice such that each reaction (0.05 ml) contains 1 X polymerase buffer, 10 mM DTT, 200 mM KCl (*see Note 5*), 30 µM each of dGTP, dATP, dCTP, and [^3H] dTTP (1,000 cpm/pmol), and 250 µg/ml DNase I-activated calf thymus DNA. Vortex and spin briefly in the microcentrifuge.
4. Dispense the mix, 49 µl, to pre-chilled and numbered 10 $\times$ 75-mm tubes on ice.
5. Add the enzyme, approx. 0.1 unit, to each tube avoiding bubbles and mix gently by flicking the tube three times.
6. Incubate the tubes for 30 min at 30°C, then transfer to ice.
7. Stop the reactions with 1 ml of 10% TCA, 0.1 M NaPPi, mix, and leave on ice 5 min.
8. Filter samples through glass-fiber filters. Wash the tube twice with acidic wash solution, then wash the filtration funnel three times with acidic wash solution and once with 95% ethanol.
9. Dry the filters under a heat lamp for approx. 5 min, then count in scintillation fluid in a liquid scintillation counter.

10. Spot 1–2 μ l of mix directly onto filters, dry, and count in scintillation fluid without filtration, to calculate the specific radioactivity of the mix.

3.4. Purification of Recombinant Human DNA Polymerase γ - β

3.4.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 2l of L broth containing 15 μ g/ml tetracycline and 100 μ g/ml ampicillin with *E. coli* XL-1 Blue containing pQESL (complete human pol γ - β cDNA without the mitochondrial presequence) at $A_{595} = 0.06$ and grow with aeration at 37°C (see **Note 6**).
2. At $A_{595} = 0.6$:
 - (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 μ l of 1X SDS loading buffer. Use a 10- μ l aliquot as control for SDS-PAGE.
 - (b) Induce target protein expression by adding IPTG to 0.3 mM final concentration and continue incubation at 37°C with aeration for 2 h.
3. Harvest cells:
 - (a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5- μ l aliquot in SDS-PAGE.
 - (b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at $3,600 \times g$ for 5 min at 4°C. Decant supernatant.
4. Resuspend cells in 1/10 volume of original culture in Tris– sucrose buffer. First resuspend pellets in one-half of the total resuspension volume, transferring to a chilled plastic beaker. Wash GSA bottles with remaining one-half of volume and combine.
5. Centrifuge washed cells in a Sorvall SS-34 at $3,000 \times g$ for 5 min at 4°C. Aspirate supernatant and freeze cell pellet in liquid nitrogen. Store at –80°C.

3.4.2. Cell Lysis and Soluble Fraction Preparation—The following protocol uses a 2-l induced cell pellet ($A_{595}=1-1.2$) as the starting material with an overall expression of approx. 200 mg pol γ - β /ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (see **Note 1**).

1. Thaw cell pellet (2 l) on ice for at least 30 min.
2. Resuspend cell pellet completely in 1/30 volume of original cell culture in resuspension buffer. Use one-half of the total volume to resuspend the cells and the remaining one-half divided to wash the tubes, combining all samples.
3. Lyse cells by sonication with an Ultrasonic Processor Model W-225 for 20 pulses using microtip at maximum output and 50% usage. Cool the samples for at least 1 min on ice. Repeat sonication and cooling (see **Note 7**).
4. Centrifuge in a Sorvall SS-34 rotor at $20,000 \times g$ for 15 min at 4°C.

5. Collect supernatant by pipetting into a fresh tube. Resuspend resulting pellet in 1/80 volume of original cell culture in resuspension buffer, sonicate, and centrifuge as described in Steps 3 and 4.
6. Collect supernatant and combine with the previous one (fraction I).

3.4.3. Ni-NTA Chromatography and Glycerol Gradient Sedimentation—The following protocol uses a soluble bacterial fraction from 2 l of induced cells loaded onto an equilibrated Ni-NTA column.

1. Load fraction I onto an equilibrated Ni-NTA resin at a ratio of approx. 7.5 mg protein per ml of packed resin, at a flow rate of 2 CV/h.
2. Elute protein by successive washes with elution buffers containing 25 mM imidazole (3 CV), 250 mM imidazole (2 CV), and 500 mM imidazole (2 CV), at a flow rate of 2 CV/h. Collect 1/2-CV fractions for the first elution, 1/6-CV fractions for the second elution, and then 1/3-CV fractions for the last elution.
3. Analyze column fractions, 5 μ l, by SDS-PAGE in 10% gels followed by silver nitrate staining.
4. Pool fractions containing pol γ - β (fraction II, approx. 1.6–2.3 ml).
5. Prepare two (10 ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
6. Layer 1–1.2 ml of Ni-NTA pool of pol γ - β onto each gradient and spin in a SW 41 rotor at $170,000 \times g$ for 60 h at 4°C.
7. Fractionate gradients by collecting 200–300- μ l aliquots. Analyze 5 μ l per fraction by SDS-PAGE in 10% gels followed by staining with silver nitrate.
8. Pool peak fractions of pol γ - β (fraction III). Pol γ - β typically sediments in fractions 10–16.
9. Aliquot pol γ - β , freeze in liquid nitrogen, and store at -80°C (*see Note 8*).

3.5. Purification of Recombinant *Drosophila* mtSSB

3.5.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 1l of L broth containing 100 $\mu\text{g/ml}$ ampicillin with *E. coli* BL21 (λ DE3) pLysS containing pET11a-*Drosophila* mtSSB (complete cDNA without the mitochondrial presequence) at $A_{595} = 0.06$ and grow with aeration at 37°C (*see Note 6*).
2. At $A_{595} = 0.6$:
 - (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 μl of 1X SDS loading buffer. Use a 10- μl aliquot as control for SDS-PAGE.
 - (b) Induce target protein expression by adding IPTG to 0.3 mM final concentration and continue incubation at 37°C with aeration for 2 h.
3. Harvest cells:

(a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5- μ l aliquot in SDS-PAGE.

(b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at $3,600 \times g$ for 5 min at 4°C. Decant supernatant.

4. Resuspend cells in 1/10 volume of original culture in Tris– sucrose buffer. First resuspend pellets in one-half of the total resuspension volume, transferring to a chilled plastic beaker. Wash GSA bottles with remaining one-half of volume and combine.
5. Aliquot washed cells into Sorvall SS-34 tubes, 200 ml cell equivalent per tube, and centrifuge cells at $3,000 \times g$ for 5 min at 4°C. Aspirate supernatant and freeze cell pellets in liquid nitrogen. Store at –80°C.

3.5.2. Cell Lysis and Soluble Fraction Preparation—The following protocol uses 400-ml induced cell pellets ($A_{595}=1-1.2$) as the starting material with an overall expression of 15 μ g mtSSB/ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (*see Note 2*).

1. Thaw cell pellets (2×200 ml) on ice for at least 30 min.
2. Resuspend cell pellets completely in 1/25 volume of Tris– sucrose buffer. Use one-half of the total volume to resuspend the cells and the remaining one-half divided to wash the tubes, combining all samples.
3. Add 5X lysis buffer to 1X final concentration. Incubate for 30 min on ice, mixing every 5 min by inversion.
4. Freeze cell lysis suspension in liquid nitrogen. Thaw partially in ice water, then transfer to ice until thawed completely. Centrifuge in Sorvall SS-34 rotor at $17,500 \times g$ for 30 min at 4°C.
5. Collect soluble fraction (supernatant) by pipetting into a fresh tube. Adjust the sample to 100 mM NaCl by the addition of dilution buffer.

3.5.3. Blue Sepharose Chromatography and Glycerol Gradient Sedimentation—The following protocol uses a soluble bacterial fraction from 400 ml of induced cells loaded onto 10 ml of equilibrated Blue Sepharose resin.

1. Load salt-adjusted soluble fraction (approx. 15–20 mg protein) at a flow rate of 1 CV/h onto a 10-ml column of Blue Sepharose equilibrated with 10-CV equilibration buffer.
2. Wash column with 4 CV of wash buffer at a flow rate of 2 CV/h, collecting 1-CV fractions.
3. Elute column with three steps of increasing NaSCN stringency at 2 CV/h:
 - (a) 4 CV of elution buffer containing 0.5 M NaSCN, collecting 1/2-CV fractions.
 - (b) 4 CV of elution buffer containing 1 M NaSCN, collecting 1/3-CV fractions.

(c) 4 CV of elution buffer containing 1.5 M NaSCN, collecting 1/4-CV fractions.

Drosophila mtSSB elutes in the 1.5 M NaSCN step.

4. Analyze column fractions, 5 μ l, by SDS-PAGE in 17% gels.
5. Pool fractions containing mtSSB and spin-concentrate sample in a Centricon-30 concentrator (pre-treated with 5% Tween 20, *see* **Note 9**) to desalt. Exchange elution buffer for 50 mM potassium phosphate, pH 7.6, 8% glycerol, 200 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM EDTA.
6. Prepare three (10-ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
7. Layer 1–1.2 ml of concentrated mtSSB pool onto each gradient and spin in SW41 rotor at $170,000 \times g$ for 60 h at 4°C.
8. Fractionate gradients by collecting 200–300- μ l aliquots. Analyze 30 μ l per fraction by TCA precipitation followed by SDS-PAGE in 17% gels followed by staining with silver nitrate.
9. Pool peak fractions of tetrameric mtSSB, which typically sediments in fractions 25–31 (*see* **Note 10**). Concentrate gradient pool using a Centricon-30 spin concentrator (pre-treated with 5% Tween 20, *see* **Note 9**).
10. Aliquot concentrated mtSSB, freeze in liquid nitrogen, and store at -80°C (*see* **Note 8**).

3.6. Purification of Recombinant Human mtSSB

3.6.1. Bacterial Cell Growth and Protein Overproduction

1. Inoculate 2 l of L broth containing 100 $\mu\text{g/ml}$ ampicillin with *E. coli* BL21 (λ DE3) pLysS containing pET11a-human mtSSB (complete cDNA without the mitochondrial pre-sequence) at $A_{595} = 0.06$ and grow with aeration at 37°C (*see* **Note 6**).
2. At $A_{595} = 0.6$:
 - (a) Remove a 1.0-ml aliquot of uninduced cells to a microcentrifuge tube, pellet cells, aspirate supernatant, and resuspend cells in 200 μ l of 1X SDS loading buffer. Use a 10- μ l aliquot as control for SDS-PAGE.
 - (b) Induce target protein expression by adding IPTG to 0.2 mM final concentration and continue incubation at 37°C with aeration for 3 h.
3. Harvest cells:
 - (a) Save a 1.0-ml aliquot of induced cells as in Step 2a. Use a 5- μ l aliquot in SDS-PAGE. Stain the gel with Coomassie Blue (*see* **Note 11**).
 - (b) Harvest remaining cells in Sorvall GSA bottles by centrifugation at $3,600 \times g$ for 5 min at 4°C . Decant supernatant.
4. Resuspend cells in Tris-sucrose buffer as described in Step 4 of **Section 3.5.1**.

5. Centrifuge washed cells into a Sorvall SS-34 tube at $3,000 \times g$ for 5 min at 4°C . Aspirate supernatant and freeze cell pellet in liquid nitrogen. Store at -80°C .

3.6.2. Cell Lysis and Soluble Fraction Preparation—The following protocol uses 2-l induced cell pellet ($A_{595}=1-1.3$) as the starting material with an overall expression of approx. 250 μg mtSSB/ml of induced cells. The ionic strength of buffers is determined using a Radiometer conductivity meter (*see Note 2*).

1. Thaw cell pellet (2 l) on ice for at least 30 min.
2. Resuspend cell pellet in Tris–sucrose buffer as described in Step 2 of **Section 3.6.2**.
3. Follow Steps 3–5 of **Section 3.6.2**. to obtain soluble fraction (*see Note 12*). Adjust the sample to 100 mM NaCl by the addition of dilution buffer.

3.6.3. Blue Sepharose Chromatography and Glycerol Gradient Sedimentation—The following protocol uses a soluble bacterial fraction from 2 l of induced cells loaded onto 10 ml of equilibrated Blue Sepharose resin.

1. Load salt-adjusted soluble fraction (approx. 75 mg protein) at a flow rate of 1 CV/h onto a 10-ml column of Blue Sepharose equilibrated with 10-CV equilibration buffer.
2. Wash column with 4 CV of wash buffer at a flow rate of 2 CV/h, collecting 1-CV fractions.
3. Elute column with three steps of increasing NaCl stringency at 2 CV/h:
 - (a) 4 CV of elution buffer containing 700 mM NaCl, collecting 1/2-CV fractions.
 - (b) 4 CV of elution buffer containing 1.5 M NaCl, collecting 1/4-CV fractions.
 - (c) 4 CV of elution buffer containing 3.0 M NaCl, collecting 1/4 CV-fractions.

Human mtSSB elutes between the 1.5 M and 3 M NaCl steps.

4. Analyze column fractions, 10 μl , by SDS-PAGE in 17% gels followed by Coomassie Blue staining.
5. Pool fractions containing mtSSB and spin-concentrate sample in a Centricon-30 concentrator (pre-treated with 5% Tween 20, *see Note 9*) to desalt. Use the dilution buffer to adjust the salt concentration of the sample to 100 mM NaCl.
6. Prepare two (10 ml) 12–30% glycerol gradients and chill on ice for at least 1 h.
7. Layer concentrated mtSSB pool onto each gradient, spin, and collect fractions as described in Steps 7 and 8 of **Section 3.6.3**. Analyze 10 μl per fraction by SDS-PAGE in 17% gels followed by staining with Coomassie Blue.
8. Pool peak fractions of tetrameric mtSSB, which typically sediments in fractions 20–31 (*see Note 10*).
9. Aliquot pooled mtSSB, freeze in liquid nitrogen, and store at -80°C (*see Notes 8 and 13*).

4. Notes

1. An aliquot of the homogenized cells can be analyzed under the microscope to check for cell lysis. If more than 10% of the cells are not lysed, the pellet may be resuspended, rehomogenized, and centrifuged again as in Steps 4–6.
2. All chromatographic and velocity sedimentation operations are performed at 0–4°C.
3. In addition to the DNA polymerase assay, the fractions can be analyzed by immunoblotting and by Bradford determination of protein concentration. These procedures may help to determine the fractions to be pooled.
4. Add 1/10 of the final amount of $(\text{NH}_4)_2\text{SO}_4$ and wait until the salt goes into the solution. Repeat until the total amount has been added. The sample must be stirred during incubation.
5. For the purification of the human pol γ - α , prepare the master mix such that each reaction contains 60 mM KCl.
6. The volume of *E. coli* cell culture may vary from 1 to 2 l. The materials used for cell harvest and lysis may be adjusted proportionally.
7. Each time it is noticed that the samples are getting warm during sonication, stop pulses and cool them for 1 min on ice.
8. Alternatively, samples may be stabilized by the addition of an equal volume of stabilization buffer (20 mM KPO_4 , pH 7.6, 80% glycerol, 2 mM EDTA) and stored at -20°C .
9. Pre-treatment consists of filling up the Centricon-30 tubes with 5% Tween 20 and allowing them to sit overnight at room temperature.
10. Avoid fractions that sediment slowly, trailing toward the top of the gradient. These fractions are likely to contain dimers and monomers of mtSSB.
11. It is important to stain the SDS-polyacrylamide gels used for human mtSSB analysis throughout the purification with Coomassie Blue. We have observed that, especially for the gel analysis of the bacterial cell extract after induction with IPTG, a silver stained gel is not suitable for the detection of human mtSSB.
12. To obtain the soluble fraction of mtSSB-expressing *E. coli* cells, we use 1.5 mg/ml lysozyme in the 5 $\times$ lysis solution (final concentration in cell suspension: 0.3 mg/ml). In the Blue Sepharose chromatography step of the human mtSSB purification, we have observed that the lysozyme has the same elution profile of human mtSSB, constituting the major contaminant of the Blue Sepharose pool. Despite the fact that human mtSSB and lysozyme have similar sizes (approx. 15 and 14 kDa, respectively), we are able to separate them in the glycerol gradient sedimentation step, because human mtSSB is a tetramer in solution and sediments as a 60-kDa protein. As an alternative cell lysis procedure, we have verified that a 5X buffer containing 1.25 M NaCl, 10 mM EDTA, and 10% sodium cholate [prepared as a

20% stock, pH 7.4, and stored at -20°C (cholic acid (Sigma) is dissolved in hot ethanol, filtered through Norit A (J.T. Baker Chemical Co.), and recrystallized twice before titration to pH 7.4 with sodium hydroxide)] is also very efficient (use final concentration of 1X buffer in the cell pellet suspension). Follow up the other steps as described in **Section 3.5.2**. Interestingly, because human and *Drosophila* mtSSBs elute differently from the Blue Sepharose column, most of the lysozymes used in the purification of *Drosophila* mtSSB elute during the wash of the column and does not constitute a major contaminant of the Blue Sepharose pool.

13. The purification procedure presented is suitable for most physical and biochemical experiments involving the human mtSSB, including analysis of quaternary structure, single stranded-DNA binding, pol γ stimulation *etc.* However, we note that on occasion a double-stranded DNA-unwinding contaminant is present in the preparation, which is detected in mtDNA helicase stimulation assays (data not shown). This contaminant can be eliminated by the addition of a phosphocellulose chromatographic step prior to the glycerol gradient sedimentation, which is performed similarly to that described in **Section 3.1.2**. with the following changes: the Blue Sepharose fraction is adjusted to an ionic equivalent of 60 mM potassium phosphate and loaded onto a phosphocellulose column equilibrated with 60 mM KPO_4 , 10% glycerol, 2 mM EDTA, at a packing ratio of 0.5 mg protein/packed ml; the column is washed with the same buffer and protein is eluted with a gradient from 60 to 150 mM KPO_4 , 10% glycerol, 2 mM EDTA, followed by a final elution step of 350 mM KPO_4 , 10% glycerol, 2 mM EDTA.

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