Purification of Recombinant Tubulin from Baculovirus-Infected Insect Cells

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Contents

1. Materials

1. 1. Cell and virus

1. HighFive cells can be purchased from Life Technologies (Invitrogen).

2. Any baculovirus expression system can be used for construction of recombinant baculoviruses. We actually used the Bac-to-Bac Baculovirus Expression System (Life Technologies) with pFastBac Dual transfer vector, and made baculoviruses according to the manual. The following method is for HIS (α-tubulin) and FLAG (β-tubulin) C-terminal tags.

1. 2. Cell culture media

1. Express Five (1X) liquid media can be purchased from Life Technologies (Invitrogen). Supplement the media with 8 % FBS, 2 mM L-glutamine, 10 units/ml heparin sodium and 10 μg/ml gentamicin.

1. 3. Stock solutions for biochemistry

1. **1 M PIPES buffer**, pH 6.8 (Dojindo, 345-02225): Adjust pH with KOH at 25 ˚C and store at 4 ˚C.
2. **0.1 M HEPES buffer**, pH 7.5 (Dojindo, 342-01375): Adjust pH with KOH at 25 ˚C and store at 4 ˚C.
3. **0.2 M $MgSO_4$ solution** (Nacalai Tesque, 21003-75): Store at 4 ˚C.
4. **1 M $MgCl_2$ solution** (Junsei, 19275-0301): Store at 4 ˚C.
5. **4 M NaCl solution** (Nacalai Tesque, 31320-05): Store at room temperature.
6. **0.1 M EGTA**, pH 6.8 (Nacalai Tesque, 15214-92): Adjust pH with KOH at 25 ˚C and store at 4 ˚C.
7. **0.1 M ATP** (Nacalai Tesque, 01072-82) in 0.1 M HEPES (pH 7.5): Adjust pH with KOH at 25 ˚C and store at -30 ˚C in small aliquots.
8. **0.1 M GTP** (Nacalai Tesque, 17450-61) in 0.1 M HEPES (pH 7.5): Adjust pH with KOH at 25 ˚C and store at -30 ˚C in small aliquots.
9. **0.1 M DTT**: Store at -30 ˚C in small aliquots.
10. **BSA stock solution**: Dissolve BSA (Sigma-Aldrich, A4503) in HBS solution at 100 mg/ml, centrifuge at 200,000 x g for 20 min to remove aggregate, freeze in liquid nitrogen, and store at -80 ˚C in small aliquots.
11. **Isopropanol Protease Inhibitor cocktail**: Dissolve PMSF (phenylmethylsulfonyl fluoride, Nacalai Tesque, 27327-52), benzamidine-HCl (Sigma-Aldrich, B6506) and phenanthroline

(Sigma-Aldrich, P-9375) in isopropanol at concentrations of 200 mM, 1 mM and 0.5 mM, respectively, and store the cocktail solution at -30 ˚C in small aliquots.

12. **Aqueous Protease Inhibitor cocktail**: Dissolve antipain (Sigma-Aldrich, A6191), leupeptin (Peptide Institute, 4041), chymostatin (Nacalai Tesque, 09035-74) and aprotinin (Sigma-Aldrich, A1153) in H_2O, each at the concentration of 0.5 mg/ml. Store the cocktail solution at -30 ˚C in small aliquots.
13. **TAME**: Dissolve TAME (Nα-p-Tosyl-L-arginine methyl ester hydrochloride, Sigma-Aldrich, T4626) in H_2O at 100 mM. Store at -30 ˚C in small aliquots.
14. **Pepstatin A**: Dissolve pepstatin A (Peptide Institute, 4397) in ethanol at 0.5 mg/ml. Store at -30 ˚C in small aliquots.
15. **FLAG peptide**: Dissolve FLAG peptide (Sigma-Aldrich, F3290) in HBS at 5 mg/ml and store at -30 ˚C in small aliquots.
16. **CHAPS**: Dissolve CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate) (Nacalai Tesque, 07957-22) in H_2O at 20 % (w/v) and store at 4 ˚C.
17. **Factor Xa**: 1 mg/ml Factor Xa (NEB, P8010) store at -30 ˚C.

1. 4. Working solutions

Note: All solutions must be stored at 4 ˚C. The volume required for one tubulin preparation from 200 ml cell culture (~ 5 g of cells (wet weight)) is indicated in parentheses.

1. **HBS**: 30 mM HEPES-KOH (pH 7.5), 150 mM NaCl (~ 70 ml).
2. **PMI**: 100 mM PIPES-KOH (pH 6.8), 10 mM $MgSO_4$, 2 mM EGTA (30 ml).
3. **PMI with glycerol**: 100 mM PIPES-KOH (pH 6.8), 10 mM $MgSO_4$, 4 mM EGTA, 10 % (v/v) glycerol, 0.04 % (v/v) NP-40 (5 ml)
4. **DEAE WASH**: 100 mM PIPES-KOH (pH 6.8), 60 mM NaCl, 10 mM $MgSO_4$, 10 % (v/v) glycerol (20 ml).
5. **DEAE ELUTION**: 100 mM PIPES-KOH (pH 6.8), 400 mM NaCl, 10 mM $MgSO_4$, 10 % (v/v) glycerol (20 ml).
6. **TALON WASH**: 100 mM PIPES-KOH (pH 7.0), 300 mM NaCl, 5 mM $MgSO_4$, 10 % (v/v) glycerol (40 ml).
7. **TALON ELUTION**: 100 mM PIPES-KOH (pH 7.0), 300 mM NaCl, 5 mM $MgSO_4$, 10 % (v/v) glycerol, 250 mM imidazole-HCl (pH 7.0) (6 ml).

8. **FLAG WASH**: 100 mM PIPES-KOH (pH 7.0), 150 mM NaCl, 5 mM $MgSO_4$, 2 mM EGTA, 10 % (v/v) glycerol, 0.02 % (v/v) NP-40 (~ 60 ml).
9. **Glycine-HCl**: Dissolve glycine powder in H_2O. Adjust pH to 3.5 with HCl. Then, adjust the volume to 100 mM glycine (12 ml).
10. **BRB-2Mg**: 80 mM PIPES-KOH (pH 6.8), 2 mM $MgCl_2$, 1 mM EGTA (~ 5 ml).

1. 5. Resins and centrifugal filters

1. **DEAE**: DEAE Sepharose Fast Flow resin can be purchased from GE Healthcare (Cat No. 17-0709-01). Equilibrate the resin with 100 mM PIPES-KOH (pH 6.8) before use. If the resin will not be used immediately, it can be stored at 4 ˚C for several months with 0.04 % (w/v) NaN_3.

2. **TALON**: TALON Metal Affinity resin can be purchased from Clontech. Wash the TALON resin with (1) 5 bed volumes of HBS (1.4.1) for two times, (2) 0.5 bed volumes of 10 mg/ml BSA in HBS, and (3) 4 bed volumes of TALON WASH solution (1.4.6) for three times. All equilibration steps are done at 4 ˚C, and thus the resin prepared should be used within 1 - 2 days. We usually do this process using Bio-Rad's Econo-Pac columns (Cat No. 732-1010).

3. **FLAG**: Anti-FLAG M2 Affinity Gel can be purchased from Sigma-Aldrich (Cat No. A2220). Wash the Anti-FLAG resin with (1) 4 bed volumes of HBS for two times, (2) 3 bed volumes of glycine-HCl (pH 3.5) (1.4.9), and (3) 4 bed volumes of HBS for two times. Do not expose the resin to low pH for more than 20 min. Block the resin with 0.5 bed volumes of 10 mg/ml BSA in HBS, and equilibrate the resin with 4 bed volumes of FLAG WASH (1.4.8) for three times. All equilibration steps are done at 4 ˚C, and thus the resin prepared should be used within 1 - 2 days. We usually do this process using Bio-Rad's Econo-Pac columns.

4. **Centrifugal filters**: Centrifugal filter (Amicon Ultra 15 - 30k, Millipore) can be purchased from Millipore. The membrane should be pretreated with 1 mg/ml BSA in HBS for 1 hour and wash with HBS for more than three times. Do not dry the membrane.

2. Cell Culture and Protein Expression

1. **Cell culture**: Incubate a 200 ml suspension culture of HighFive cells in the supplemented Express Five medium (1.2.1) in a 500 ml plastic flask (BD Falcon) at 27 ˚C. Set the rotational speed at 140 rpm. To keep the cells in the log phase, maintain the cell density in range of 0.5 x 10^6 - 3.0 x 10^6 cells/ml.

2. **Protein Expression**: Grow HighFive cells to a concentration of 1.0 x 10^6 - 2.5 x 10^6 cells/ml. Infect the cells with the recombinant viruses at a multiplicity of infection of 20, and further culture for 33 - 72 hours.

3. **Collection of the tubulin expressed cells**: Collect the cells by centrifugation (1,000 – 3,400 x g for 5 min at 4 ˚C) 33 – 72 hours after infection. Weigh the collected cells. 3 – 5 g of cells (wet weight) are usually obtained from 200 ml of suspension culture. The cells are either immediately used for DEAE purification, or frozen by liquid nitrogen and stored at -80 ˚C.

3. Purification of Tubulin

Note: The following protocol is for purification from 5 g of cells (wet weight). All procedure should be done in cold room or on ice unless otherwise stated. The containers and columns for protein solution should be plastic (polypropylene). Do not forget to take a sample at each step for SDS-PAGE.

3. 1. Lysis of insect cells

1. **Extraction solution A**: Prepare 15 ml of PMI (1.4.2) with the following supplements just before the use.

Stock solution	Amount to be added
ATP (1.3.7)	1/50 volume of PMI
GTP (1.3.8)	1/50
DTT (1.3.9)	1/100
Isopropanol Protease Inhibitor Cocktail (1.3.11)	1/50
Aqueous Protease Inhibitor Cocktail (1.3.12)	1/250
TAME (1.3.13)	1/50
Pepstatin A (1.3.14)	1/250

2. **Extraction solution B**: To prepare 15 ml of PMI supplemented with detergents, dissolve 3-(1-pyridinio)-1-propane sulfonate (NDSB201, Calbiochem) and CHAPS (1.3.16) in PMI to a final concentration of 1 M and 2 % (w/v), respectively.

3. **Extraction**: Add 15 ml of Extraction solution A (3.1.1) to the cells (5 g wet weight) and suspend thoroughly on ice. Add 15 ml of Extraction solution B (3.1.2) to the suspension, mix well, and let it sit on ice for 15 min.

4. **Ultra-centrifugation**: Centrifuge the cell suspension at 200,000 x g for 25 min. Collect the supernatant, measure its volume (expected to be about 30 ml), and add glycerol to a final concentration of 10 % (v/v). Use polypropylene measuring cylinder for volume measurement.

3. 2. Chromatography on DEAE resin

Note: Tubulin dimer is unstable in solution. In order to obtain tubulin sample with good polymerization / depolymerization property, you should finish each column purification process (DEAE, TALON, FLAG) within a few hours.

1. **Adding DEAE resin**: Add 10 ml DEAE resin (1.5.1) to cell lysate (3.1.4).

2. **Mixing**: Gently mix the solution using end-over-end mixer (~ 10 rpm) for 1 hour in cold room.

3. **Centrifugation**: Centrifuge the mixture at 2,000 x g for 5 min. Carefully discard the supernatant.

4. **DEAE washing solution**: Prepare DEAE WASH (1.4.4) with following supplements just before the use.

Stock solution	Amount to be added
ATP	1/100 volume of DEAE WASH
GTP	1/100
Isopropanol Protease Inhibitor Cocktail	1/100
Aqueous Protease Inhibitor Cocktail	1/500
TAME	1/100
Pepstatin A	1/500

5. **Packing**: Add equal volume of DEAE WASH with supplements (3.2.4) to the pellet, suspend well, and pour it onto a column. Avoid using an uncoated glass column. We usually use Bio-Rad's Econo-Pac columns (20 ml size) for 10 ml DEAE resin. Drain the solution. The flow rate for 10 ml column is 0.5 - 1.5 ml/min.

6. **Washing**: Wash the DEAE resin with 2 bed volumes of DEAE WASH with supplements.

7. **Elution**: Elute the protein with DEAE ELUTION (1.4.5) with 1 mM ATP and GTP. Fractionate the eluent in polypropylene tubes. Collect 0.4 column volumes (4 ml for 10 ml column) for each fraction. The major peak with protein concentration at 5 - 10 mg/ml usually comes in the second and third fractions. This "crude tubulin fraction" can be further processed in TALON affinity column (3.3) or stored in freezer (3.2.8).

8. **Storage**: Measure protein concentration of crude tubulin fraction, and mix 3 fractions around the peak. If necessary, concentrate to > 0.7 mg/ml by using centrifugal filter (Amicon Ultra 15 - 30k, Millipore, see section 1.5.4). Freeze the solution in liquid nitrogen and store at -80 ˚C. The average yield of total protein for crude tubulin fraction (starting from 5 g of cells (wet weight)) is 12 - 18 mg.

3. 3. Chromatography using TALON-affinity column

1. **Adding TALON resin**: Add 2 ml of equilibrated TALON resin (1.5.2) to the crude tubulin fraction (3.2.7). Binding capacity of TALON resin is > 5 - 15 mg/ml.

2. **Mixing**: Gently mix the solution using an end-over-end mixer (~ 10 rpm) for 1 hour in cold room.

3. **Centrifugation**: Centrifuge the mixture at 700 x g for 2 min. Carefully discard the supernatant.

4. **Packing**: Add 2 resin volumes of TALON WASH supplemented with 1 mM each of ATP and GTP to the pellet. Suspend the resin, and load all resin onto the column, drain at a flow rate of 0.2 – 0.4 ml/min.

5. **Washing**: Wash the column with 8 bed volumes of TALON WASH supplemented with 1 mM of ATP and GTP.

6. **Elution**: Elute the column with TALON ELUTION (1.4.7) supplemented with 1 mM each of ATP and GTP. Collect 1 ml fractions in BSA coated polypropylene tubes. (BSA coated polypropylene tubes are prepared as follows; Fill the tubes with BSA (1 mg/ml) in HBS. Leave the tubes for 1 hour at room temperature or several hours at 4 ˚C, and then discard the solution completely.) Quickly measure the protein concentration of each fraction to determine the peak and proceed to the next step as soon as possible.

3. 4. Chromatography using FLAG-affinity column

1. **Dilution**: Combine the fractions with concentration > 0.2 mg/ml together, then dilute the pooled peak fractions (~ 5 ml, total protein is 4 - 7 mg) with an equal volume of PMI with glycerol (1.4.3) supplemented with 1 mM GTP, 1 mM ATP and 0.04 % NP-40 (final 0.02 %).

2. **Mixing with FLAG resin**: Add 2 ml of equilibrated FLAG resin (1.5.3) to the diluted TALON eluent (3.4.1) and gently mix using an end-over-end mixer (~ 10 rpm) for 1 hour in cold room. Binding capacity of FLAG resin is > 0.6 mg/ml.

3. **Centrifugation**: Centrifuge the mixture at 1,000 x g for 5 min. Carefully discard the supernatant.

4. **Packing**: Add 2 resin volumes of FLAG WASH supplemented with 1 mM GTP to the pellet (3.4.3), suspend well, and pour the slurry onto a column (Avoid using an uncoated glass column). Drain the solution at a flow rate of 0.2 – 0.4 ml/min.

5. **Washing**: Wash the column with 8 bed volumes of FLAG WASH with 1 mM GTP.

6. **Elution**: Elute the column with FLAG WASH supplemented with 1 mM GTP and 0.2 mg/ml FLAG peptide (1.3.15). Collect 1 ml fractions in BSA coated polypropylene tubes. Measure protein concentration, combine 4 to 5 fractions with protein concentration > 0.1 mg/ml and proceed to the next step as soon as possible (total protein is about 2 - 3 mg).

3. 5. Concentration and polymerization

1. **Concentration**: Concentrate the pooled FLAG fractions (3.4.6) to a final concentration of 3 - 8 mg/ml by using centrifugal filter at 4 ˚C. Although you can concentrate the tubulin to concentrations higher than 10 mg/ml, more protein would be lost.

2. **Ultra-Centrifugation**: Centrifuge the concentrated tubulin solution at 400,000 x g for 10 min to precipitate denatured protein. Carefully take the supernatant for the next step.

3. **Polymerization**: Polymerize tubulin by adding 33.3 % glycerol and incubate at 30 ˚C for more than 30 min.

3. 6. Removal of tags

1. **Ultra-Centrifugation and re-suspension**: Centrifuge the polymerized tubulin (=microtubule (MT)) at 250,000 x g for 15 min. Re-suspend the pellet in BRB-2Mg (1.4.10) buffer with 2 mM GTP and 33.3 % glycerol such that the final concentration of MT is 10 mg/ml (=100 μM MT).

2. **Digestion of tags**: Add Factor Xa (1.3.17) to a 100 μM MT solution at a final concentration of 0.2 mg/ml and react for 16 hours at 25 ˚C.

3. 7. Depolymerization

1. **Centrifugation and re-suspension**: Centrifuge the MT removed tags at 250,000 x g for 15 min and re-suspend the pellet in BRB-2Mg buffer containing 0.1 mM GTP less than 0.5 vol.

before centrifugation.

2. **Depolymerization**: Put the MT solution on ice-water for 20 min.

3. **Ultra-Centrifugation**: Centrifuge at 400,000 x g for 10 min, and carefully collect the supernatant (= final tubulin).

3. 8. Storage and final yield

1. **Storage**: Measure protein concentration of final tubulin, and if necessary, concentrate to > 5 mg/ml by using centrifugal filter. Freeze the solution in liquid nitrogen and store at -80 ˚C. The average yield of total protein for final tubulin (starting from 5 g of cells (wet weight)) is 0.3 - 0.5 mg.

2. **Final yield**: This method yields 1.5 – 2.5 mg of tag free recombinant tubulin, per a liter of insect-cell culture (~ 25 g of cells (wet weight)).

4. Regeneration of TALON and FLAG Resin

4. 1. Regeneration of TALON resin

1. Wash the TALON resin with 5 bed volumes of 0.1 M NaCl and 20 mM MES (pH 5.0).
2. Wash the resin with 5 bed volumes of distilled water.
3. Wash the resin with 0.1 % NaN_3 and 20 % EtOH and store in the same solution at 4 ˚C.

4. 2. Regeneration of FLAG resin

1. Wash the FLAG resin with 3 bed volumes of glycine-HCl (pH 3.5).
2. Wash the resin with 6 bed volumes of FLAG WASH.
3. Wash the resin with 3 bed volumes of FLAG preservative and store in the same solution at -20 ˚C.

5. SDS-PAGE

Note: Use 95 % SDS (Sigma-Aldrich, L5750) for SDS-PAGE to separate α-tubulin and β-tubulin (Best et al., (1981) *Anal Biochem* 114, 281-284). The concentration of acrylamide in running gel is 7.5 - 8.0 %.