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Large Scale Purification of Brain Tubulin With the Modified Weisenberg Procedure

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Summary

This method is a modification of the initial procedure employed to purify tubulin from mammalian brain. It consists of tissue homogenization, elimination of cell membranes, ammonium sulfate fractionation, and batch anion exchange, followed by selective precipitation with magnesium chloride. Half gram of electrophoretically homogenous, active, concentrated calf brain tubulin is typically purified in 9 h, dialyzed overnight, and stored under liquid nitrogen. Prior to use the protein is equilibrated in the experimental buffer and its concentration measured. This tubulin preparation has been very extensively characterized. Frozen aliquots have been found to retain microtubule assembly activity after 10 yr of storage.

Key Words: tubulin; W-tubulin; purification; assembly; microtubules; brain.

1. Introduction

Tubulin was extracted from sperm tail (**1**), and it was first purified from mammalian brain employing biochemical procedures by Weisenberg, Borisy, and Taylor (**2**) as the colchicine-binding protein proposed to be the subunit of microtubules, found in most eukaryotic cells. Tubulin constituting microtubules is a $\alpha\beta$ -heterodimer of homologous GTP-binding subunits; the GTP bound to β -tubulin is hydrolyzed upon microtubule assembly and GDP-tubulin tends to disassemble, which confers their dynamic properties to microtubules. The single sites of colchicine and taxol binding are at β -tubulin (for a review see **ref. 3**).

Conditions for the in vitro assembly of microtubules were also first discovered by Weisenberg (**4**), and subsequently applied for the preparation of microtubule proteins (tubulin, microtubule associated and motor proteins) by cycles of assembly and disassembly (**5,6**), from which tubulin can be purified from microtubule bound proteins by phosphocellulose ion-

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exchange chromatography (7,8) (known as PC-tubulin) and cycled again to remove the inactive protein fraction (9). Numerous modified assembly cycle procedures have appeared in the literature over the years, including the use of high concentrations of organic acids, such as glutamate, MES, and PIPES buffers to prepare MAP-depleted tubulin (10–12).

The original Weisenberg method of tubulin purification (2) was improved by Weisenberg and Timasheff (13), and subsequently modified to give a very well-characterized preparation of stabilized tubulin (8,14–17) employing the so called modified Weisenberg procedure (W-tubulin). This preparation was employed to first demonstrate microtubule assembly from purified tubulin without the need of microtubule-associated proteins (18). Both W-tubulin and PC-tubulin assemble under appropriate conditions.

Although we use the cycle procedure (6) with modifications (19) to prepare microtubule proteins, we prefer the modified Weisenberg procedure to directly purify active tubulin (instead of PC-tubulin or commercial sources) owing to the relatively large quantities of highly purified W-tubulin, which can be prepared with relatively small manpower (two day, including preparation of materials) and equipment. This method (16,17) is currently in use in the author's laboratory for the purification of bovine brain tubulin (20,21), from which microtubules are prepared for the investigation of microtubule stabilizing drugs binding at the Taxol and other binding sites (22), employing fluorescent competition methods (*see* Chapter 17), which are susceptible of high-throughput application. Bovine W-tubulin has been subjected to in-depth biochemical and biophysical studies. Very similar tubulin has been successfully prepared in other laboratories from pig, dog, lamb (8,23), or chicken brain (24) employing the modified Weisenberg procedure.

Brain remains the main source to prepare large quantities of tubulin for microtubule research, although tubulin has been purified from a variety of other sources for specific purposes, employing diverse methods. Yeast tubulin can be purified in milligram quantities (25). Mammalian tubulin has been expressed as inclusion bodies in bacteria, folded in tens of micrograms quantities and assembled into microtubules (26). Recently, a bacterial tubulin has been discovered; its structure is very similar to eukaryotic tubulin, however, it has not assembled into microtubules (27).

2. Materials

2.1. Centrifuges and Reservations

1. Book four preparative centrifuges, four medium size rotors (1.5-L capacity, six-hole). This is reduced to two centrifuges and rotors after the first 2–3 h (a full preparation can also be started with two centrifuges and rotors and, if necessary

half a preparation can be made with one centrifuge). Prepare 24 centrifuge bottles and a few spares (large mouth, complete with screw caps and sealing inner caps with their O-rings) (*see Note 1*). Prepare two smaller rotors with 16 tubes (*see Note 2*). Carefully inspect the centrifuge bottles, keep separated for tubulin preparation, and do not use any bottles with cracks or other signs of aging; do not oven-dry after washing, because this may damage polycarbonate. Clean rotors and centrifuges after use.

2. Phone the appointed abattoir for the approximate time of slaughter; be there slightly in advance.
3. Make bench space (about 2 m) in the cold room and cover with disposable absorbent paper.
4. Reserve space in a liquid nitrogen tank for about 30 cryogenic vials (1-mL size; not necessarily sterile for tubulin storage).

2.2. Chromatography Gels and Dialysis Membrane

These and the following materials are prepared the day before purification.

1. Swell 15 g DEAE Sephadex A50 in 1.5 L of 10 mM sodium phosphate buffer pH 7.0, using a 2-L beaker or an open bottle, in a boiling water bath during 5 h. Store in the fridge. Equilibrate and wash the day of the preparation (*see Subheading 3.*). Discard after use and wash the containers separate from other materials.
2. Take 450 mL of previously swollen, gravity sedimented Sephadex G-25 medium in water, pack a 2.5 x 80-cm column, wash with 1 L of 10 mM sodium phosphate buffer pH 7.0 and store in the cold room overnight. Equilibrate with PMG buffer (*see Subheading 2.4., item 3*) the day of the preparation. After use, empty the column and wash the G-25 gel three times with distilled water by sedimentation and aspiration; add a bit of sodium azide and store at 4°C.
3. Preboiled and washed dialysis membrane (6-mm diameter) is stored in 10% ethanol in the cold. Rinse well before use and discard afterward.

2.3. Solid Chemicals

1. 800 g very pure ammonium sulfate, 125 g biochemical or analytical grade ammonium sulfate.
2. 300 mg GTP, and 0.5 mL of neutralized 0.1 M GTP stock solution (*see Note 3*). Leave at -20°C the preweighed aliquots required to add to the buffers the following day.

2.4. Buffers and Stock Solutions

Prepared with deionized and freshly purified water (Milli-Q or equivalent).

1. Stock phosphate buffer: (A) 0.2 M NaH_2PO_4 (27.6 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ to 1 L). (B) 0.2 M Na_2HPO_4 (28.39 g Na_2HPO_4 to 1 L). (C) 0.2 M Sodium phosphate buffer stock (0.2 M NaPi): mix equal volumes of A and B. After dilution to 10 mM, its pH is 7.00 +/- 0.05 at 20–25°C. Prepare 1 L.

2. 1 M MgCl₂ (20.33 g of analytical grade MgCl₂ 6H₂O to 100 mL).
3. PMS buffer: 10 mM sodium phosphate, 0.5 mM MgCl₂, 0.24 M sucrose, pH 7.0. (Prepare 8 L: 657.2 g sucrose [biochemical grade], 400 mL 0.2 M NaPi, 4 mL 1 M MgCl₂; check that pH is about 6.95 without adjustment and store in cold room.)
4. PMG buffer: 10 mM sodium phosphate, 0.5 mM MgCl₂, 0.1 mM GTP, pH 7.0. Add the GTP before use. (Prepare 4.5 L buffer: 225 mL 0.2 M NaPi 0.2 M, 2.25 mL 1 M MgCl₂, confirm pH 7.0; store in the cold room; separate 0.5 L of this buffer; to the 4 L buffer left, add 0.214 g GTP Li₂ before use.)
5. 0.8 M KCl-PMG buffer: 10 mM sodium phosphate, 0.8 M KCl, 0.5 mM MgCl₂, 0.1 mM GTP, pH 7.0. Add the GTP before use (prepare 1 L: 59.65 g KCl, 50 mL 0.2 M NaPi, 0.5 mL 1 M MgCl₂ 1 M, adjust to pH 7.0 with NaOH and store in the fridge; separate into two portions of 0.5 L; before use, add 0.027 g GTP Li₂ to 0.5 L).
6. 0.4 M KCl-PMG buffer: 10 mM sodium phosphate, 0.4 M KCl, 0.5 mM MgCl₂, 0.1 mM GTP, pH 7.0. Add the GTP before use. (Prepare 1 L: mix 0.5 L 0.8 M KCl-PMG without GTP with 0.5 L PMG buffer without GTP, adjust to pH 7.0 with NaOH and store in the fridge; add 0.054 g GTP Li₂ before use.)
7. 1 M Sucrose-PMG buffer: 10 mM sodium phosphate, 1 M sucrose, 0.5 mM MgCl₂, 0.1 mM GTP, pH 7.0. Add the GTP before use. Prepare 0.5 L, or 0.25 L if the preparations were coming out small. For 0.5 L: 171.15 g sucrose (biochemical grade), 25 mL 0.2 M NaPi, 0.250 mL 1 M MgCl₂, adjust to pH 7.0 and store in the cold room; before use add 0.5 mL of neutralized 0.1 M GTP.

2.5. Other Items

1. Homogenizer: a 1- to 1.5-L domestic blender (300 W) with a glass jar with bottom blades (Osterizer) or a Waring blender, which is employed only for tubulin preparation (keep a spare jar and blade set).
2. The filtering system for batch ion-exchange consists of a 1-L funnel with a medium-coarse porous plate (keep a spare one), two suction flasks with rubber adaptors for the funnel, and a vacuum trap. After use, the porous glass plate must be cleaned with chromic mixture and thoroughly rinsed with distilled water.
3. After the centrifuge rotors and tubes, the blender, buffers, and the G-25 column, take to the cold room a fraction collector, a powerful magnetic stirrer and assorted magnetic bars, cheese cloth (a coarse gauze), a simple balance to equilibrate centrifuge bottles, forceps, scissors, trays (one covered with aluminum foil for brain dissection), assorted spatula, magnetic bars, Pasteur pipets and rubber bulbs, plastic beakers (two of 5 L, two of 3 L, one of 2 L), glass beakers (250 mL, two of 100 mL, 50 mL, 10 mL), graduated cylinders (two of 2 L, one 500 mL), a washing flask, and wiping paper (put all these in a small cart). Do not forget a copy of the **Subheading 3**.
4. Take a portable refrigerator with plenty of ice, gloves, and plastic bags to the car; drain out the water before use.

3. Methods

The approximate timing at several steps is given in parenthesis. *See Fig. 1* for a scheme. All operations are carried out in the cold room except otherwise noted (it is better to avoid summer for this preparation). Keep rotors in the cold room the night before use and have the centrifuges precooled (*see Note 4*).

3.1. Collection of Bovine Brains ($t = -1$ to -1.5 h)

Eight bovine brains from 15- to 18-mo-old animals (approx 3 kg; better 12 brains from younger calf if they were available) are collected at the local abattoir within 30 min of slaughter (still warm), immediately put into ice within plastic bags in a portable fridge, and transported to the laboratory cold room (*see Note 5*).

3.2. Tissue Homogenization ($t = 0$)

1. Remove meninges, clots, and superficial blood vessels with forceps. Cut and discard any distinctly dark brain portions. Take the whole brains (including the brain stem) and wash them with PMS.
2. Rapidly mince tissue to small pieces with scissors. Wash twice with one volume of PMS, employing two layer of cheese cloth to separate the liquid.
3. Divide tissue into two equal parts of approx 1.4 L each in 5-L beakers, add slightly more than one volume (1.6 L) of PMS, stir and homogenize it (maintaining the tissue to buffer ratio in the several portions) with the domestic blade homogenizer at maximal speed (during 45 s in a Osterizer or 30 s in a Waring blender).

3.3. Removal of Cell Membranes and Debris ($t = 1$ h)

1. Fill 24 centrifuge bottles, to fit into four rotors (excess homogenate not fitting into the bottles will be discarded), equilibrate and carefully close them with sealing caps. (**Caution:** do not fill the bottles to the top, but fill them as if they were uncapped, in order to make sure that liquid will not be spilled at the rotor angle; watch centrifuges accelerate and never over-speed derated rotors.) Centrifuge using two or four precooled medium size six-hole rotors at 19,000g (at average rotor radius, R_{ave}) 30 min at 4°C (*see Note 1*). This step can be done with four centrifuges at a time, or with two centrifuges consecutively while all the tissue is being homogenized; only two centrifuges are required after this step.
2. While the homogenate is centrifuging, add GTP to the buffers, start equilibrating the Sephadex G25 column with 800 mL PMG, aspirate the excess liquid from the settled DEAE-Sephadex, add one volume of PMG and adjust to pH 6.8.

3.4. Fractionation With $(\text{NH}_4)_2\text{SO}_4$

1. Take the supernatant S1, measure its volume (about 2.3–2.5 L) and slowly add 177 g/L of solid $(\text{NH}_4)_2\text{SO}_4$ (32% saturation) with continuous magnetic stirring

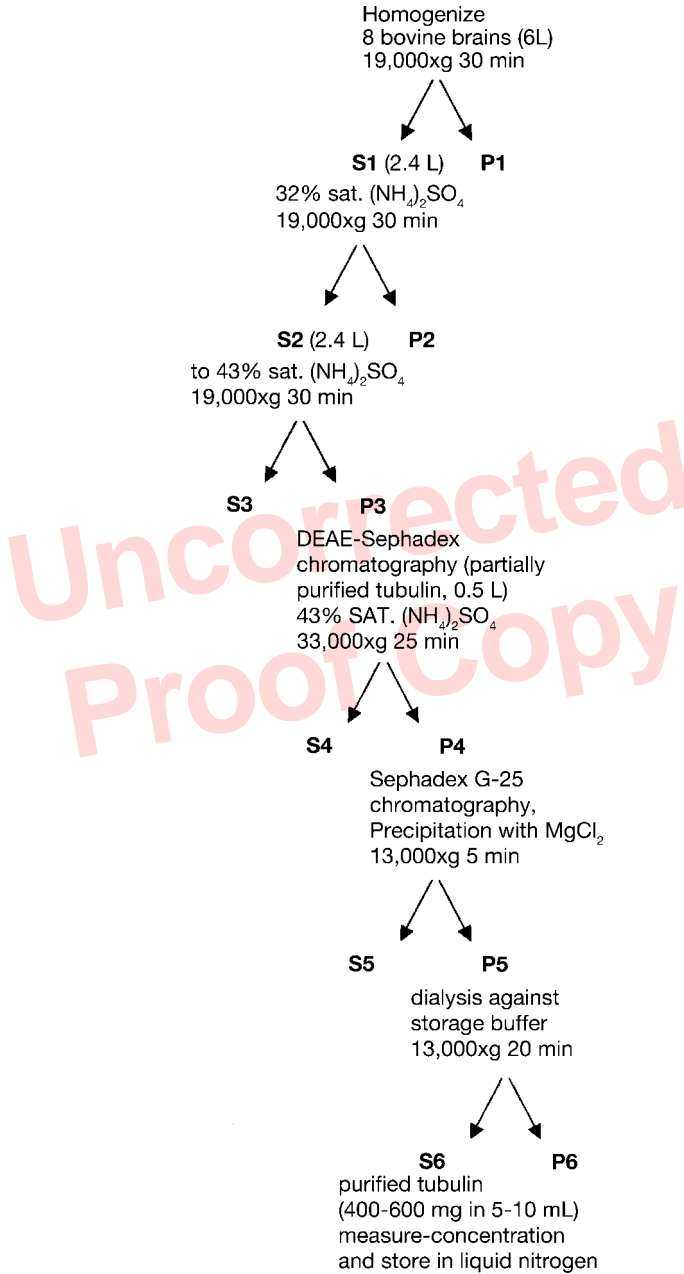


Fig. 1. Scheme summarizing the preparation of tubulin with the modified Weisenberg procedure. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractions during purification (*see refs. 8 and 15*).

($(\text{NH}_4)_2\text{SO}_4$ can be preweighed for 2.2 L and adjusted during addition). When the salt is completely dissolved wait 10 min and centrifuge at 19,000g 30 min at 4°C (this will take two rotors).

2. Wash the DEAE-Sephadex for a second time and adjust pH to 6.8.
3. Take the supernatant S2 (approximately same volume as S1) and slowly add 71 g/L solid $(\text{NH}_4)_2\text{SO}_4$ (this brings the solution to 43% saturation) with continuous stirring, wait 10 min and centrifuge at 19,000g 30 min at 4°C.
4. Wash the DEAE-Sephadex for a third time and check pH (6.8).
5. Discard the supernatant S3, take the tubulin-containing pellet P3 and resuspend it with 50 mL PMG, using a spatula and a magnetic stirrer, avoiding foam formation; use another 50 mL PMG to wash the bottles and mix well to resuspend all precipitate.

3.5. DEAE-Sephadex A50 Batch-Chromatography ($t = 4.5$ h)

1. Most of this step can be performed on ice, out from the cold room. Take the resuspended pellet P3 and mix well with the preequilibrated DEAE Sephadex A50 (about 500 mL settled gel from which the excess liquid has been taken out). Keep 5 min on ice and remove the nonadsorbed material by filtration on a sintered glass funnel on a suction flask, until the wet gel cake cracks.
2. Resuspend the gel with 400 mL of 0.4 M KCl-PMG, keep 5 min on ice and filter as above to remove nontubulin eluting materials. Repeat the resuspension and filtration for a second time.
3. Resuspend the gel with 250 mL of 0.8 M KCl-PMG, keep 5 min on ice and filter with the sintered glass funnel, collecting the eluted tubulin on a clean suction flask. Repeat the resuspension and filtration for a second time with the 250 mL left of 0.8 M KCl. After this ion exchange step the yield is about 1 g of 90% pure tubulin, which is collected by $(\text{NH}_4)_2\text{SO}_4$ precipitation and further purified by selective MgCl_2 precipitation.
4. Slowly add 124 g $(\text{NH}_4)_2\text{SO}_4$ (analytical grade) per 500 mL filtrate, with constant stirring, back in the cold room, wait 10 min after the salt is dissolved and centrifuge at 33,000g (R_{ave} ; see **Note 2**) 25 min at 4°C in two small eight-tube rotors. This will give pellets P4, from which the supernatant should separate well by inverting the tubes.
5. Meanwhile prepare the Sephadex G-25 column and fraction collector for the next step.

3.6. Sephadex G25 Column Chromatography ($t = 6.5$ h)

This is made to remove the $(\text{NH}_4)_2\text{SO}_4$, which would interfere with the MgCl_2 precipitation. Carefully resuspend the precipitate P4 with PMG to a final volume smaller than 80 mL to obtain a turbid solution, through which one can still see. Load the solution into the equilibrated Sephadex G-25 column ($2.5 \times$ about 80 cm) in the cold room and run it to practically maximal gravity flow, collecting 6 mL fractions.

3.7. Tubulin Precipitation With $MgCl_2$ ($t = 8.5$ h)

1. Wait until the protein is coming out from the G-25 column (this can be noticed between tubes 20 and 40, in 10–15 fractions, which tend to make foam). Add 40 mM $MgCl_2$ to each protein-containing fraction (0.24 mL of 1 M $MgCl_2$), cap with Parafilm and mix well by inverting the tubes.
2. Immediately combine the fractions in which a milky precipitate has rapidly (seconds) formed and collect the protein by sedimentation at 13,000g (see **Note 2**) 5 min at 4°C (pellet P5). Tail fractions in which a precipitate is slowly (minutes) formed are not pooled, because they may decrease the overall yield. The supernatant of this $MgCl_2$ precipitation may turn turbid upon standing in the cold room, but it is discarded because it has been observed to contain inactive tubulin.

3.8. Dialysis and Storage

1. Dissolve pellet P5 with a minimal volume (5–10 mL) of 1 M sucrose-PMG (storage buffer) in a small glass beaker, carefully using a magnetic stirrer or a 5-mL syringe with a thick needle, until a cloudy viscous solution is obtained (it will turn more clear upon dialysis).
2. Dialyze overnight at 4°C against 500 mL of 1 M sucrose-PMG, employing prewashed thin dialysis bag, in a Parafilm-capped graduate cylinder, with good stirring.
3. Next morning, centrifuge the dialyzed tubulin and some dialysis buffer at 13,000g (R_{ave}) 20 min at 4°C in Pyrex centrifuge tubes (see **Note 6**). Save the clear viscous protein supernatant S6 and discard aggregates in the bottom of the tube; keep some dialysis buffer for reference.
4. Measure the tubulin concentration. Dilute 5 μ L S6 into 1 mL of 6 M neutral guanidinium chloride (in duplicate), take the ultraviolet spectrum of the diluted samples, employing as reference similar dilutions of buffer, and measure the absorbance at 275 nm. The extinction coefficient of tubulin in this solvent is $E_{275} = 1.09$ g/(Lcm) (17). Tubulin at this final stage of purification is more than 98% homogeneous in polyacrylamide gel electrophoresis with sodium dodecyl sulfate. The tubulin concentration is usually between 50 and 100 g/L. Store in 20- to 30-mg aliquots in cryotubes under liquid nitrogen (with the concentration, operator initials, and preparation number noted). Record the total volume. A typical yield is 400–600 mg tubulin after some training (preparations ranging between 200 and 1200 mg have been obtained).

3.9. Preparation for Use and Activity

1. Rapidly melt the tubulin aliquot(s) and keep the tube(s) in ice. Remove the sucrose and the excess GTP, and equilibrate the protein in the experimental buffer by chromatography in appropriate cooled Sephadex G25 columns. Clarify the protein by centrifugation. Measure the protein concentration as previously listed, or after dilution in neutral 1% sodium dodecyl sulfate ($E_{276} = 1.07$ g/(Lcm); 28), or directly in neutral buffer after scattering correction, employing the extinc-

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tion coefficient of native tubulin, $E_{276} = 1.16 \text{ g/(Lcm)}$ (17). Alternately, the very concentrated tubulin stock solution can be directly diluted >100-fold in experimental buffer, only for experiments in which the residual components of the storage buffer do not interfere, or for less rigorous tests. Unassembled tubulin ages rapidly in most buffers without stabilizers (29), requiring activity controls by the end of experiments; it is normally discarded after 4–6 h on ice. Tubulin without stabilizer should not be frozen again, because upon melting it will precipitate in many buffers.

2. Tubulin polymerization can be easily monitored by turbidity and negative stain electron microscopy, and quantified by pelleting the polymers formed (30). W-tubulin cooperatively assembles into microtubules from a critical concentration (C_r) above which essentially all excess protein goes into the polymers (18,31,32). For an example, in 10 mM sodium phosphate, 3.4 M glycerol, 6 mM MgCl_2 , 1 mM GTP, pH 6.5 buffer at 37°C, the C_r value is 4 μM tubulin. W-tubulin is also fully active in ligand binding. Unassembled W-tubulin $\alpha\beta$ -dimers contain 1.88 ± 0.16 guanine nucleotide (32) and bind 0.97 ± 0.05 colchicine molecules per dimer (17), whereas assembled W-tubulin binds 0.99 ± 0.05 taxol molecules per dimer (33).

4. Notes

1. We use Sorvall SLA 1500 and GSA rotors at 12,000 rpm in Sorvall RC-5C centrifuges (giving 22,000g at R_{\max} , 19,000g at R_{ave} , and 15,000g at R_{\min}), and 250-mL polycarbonate bottles with sealing caps (Sorvall, cat. nos. 03939 and 03278) filled with less than 240 mL each.
2. For example, Sorvall SS34 rotors and unsealed tubes. These give at 19,000 rpm, 43,000g at R_{\max} , 33,000g at R_{ave} , and 23,000g at R_{\min} . At 12,000 rpm, they give 17,000g at R_{\max} , 13,000g at R_{ave} , and 9000g at R_{\min} .
3. For tubulin work, we normally use GTP-Lithium salt (Roche or Sigma), which has a better stability than the sodium salt, and store it dry at -20°C .
4. Tubulin is unstable during purification and the longer the time employed the smaller the yield. Everything should be ready in advance, and the preparation requires a good coordination. Performing several consecutive preparations usually increases the yield. It is recommended (although not necessary) that one or two trained people help the operator with the large volumes of material in the initial steps 3.2 and 3.3. If not enough brains or centrifuges are available, or should the operator find difficult to rapidly handle the 6 L volume of homogenate, the preparation may be downscaled to half, obtaining half or even more tubulin.
5. We have used only bovine brains from veterinary inspected young animals, which comply with human health regulations, and were not subject to any restrictions owing to bovine spongiform encephalopathy. All homogenization and centrifugation procedures are performed wearing disposable gloves, and human contact with the bovine tissue is avoided. Should any local restrictions apply to bovine brain, it is better to switch to pig or other vertebrate brain for tubulin preparation.
6. For example, 12-mL thick wall Sorvall Pyrex tubes with adaptors in a SS34 rotor at 12,000 rpm.

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