CHAPTER

Expression and isolation of recombinant tau

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CHAPTER OUTLINE

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Abstract

In this chapter, we describe methods for the purification of both untagged and polyhistidine-tagged tau protein. These protocols utilize a bacterial expression system to produce the tau isoform of interest, followed by heat treatment and column chromatography to separate tau from impurities. These techniques yield a biochemically pure protein with which to pursue any number of questions regarding the mechanisms of tau action.

1 INTRODUCTION

Preparation of purified tau protein free from contaminants or breakdown products is essential to any investigation of tau action. Tau was originally isolated from bovine and porcine brains after it was observed to copurify with tubulin (Weingarten, Lockwood, Hwo, & Kirschner, 1975). Further separation of tau and tubulin was achieved using phosphocellulose (PC) ion-exchange column chromatography, which takes advantage of tubulin's acidity and resultant inability to adsorb to the column resin to separate it from positively charged, resin-binding microtubule-associated proteins (MAPs) (Weingarten et al., 1975). As a result, a PC column can be employed to deplete MAPs, such as tau, from a tubulin prep to yield biochemically pure tubulin (Sloboda & Rosenbaum, 1982; Williams & Lee, 1982). Subsequent protocols exploited tau's intrinsic heat stability and acid solubility to separate it from other MAPs (Cleveland, Hwo, & Kirschner, 1977; Lindwall & Cole, 1984). It is worth noting that although heat treatment may affect structure and function of other proteins, no evidence of functional alterations to tau's microtubule stabilizing and assembly-promoting activities has been reported to date (Drubin & Kirschner, 1986; Fellous, Francon, Lennon, & Nunez, 1977; Lindwall & Cole, 1984; Weingarten et al., 1975), likely due to tau's intrinsically disordered nature.

Current purification methods generally employ a bacterial expression system to generate the tau isoform of interest. This system can be adapted to the purification of both untagged tau and tau that has been fused to a polyhistidine (His) tag or other tags such as glutathione *S*-transferase (GST). In our lab, both techniques exploit tau's intrinsic hardiness to heat to separate it from heat-labile elements. Finally, column

chromatography is used to isolate tau from contaminants and breakdown products. There are many variations on this basic strategy, several of which are presented elsewhere in this volume (Combs, Tiernan, Hamel, & Kanaan, 2017; Fichou, Eschmann, Keller, & Han, 2017; Melo, Elbaum-Garfinkle, & Rhoades, 2017; Mutreja & Gamblin, 2017; Ramirez-Riosa et al., 2017; Stern, Lessard, Ali, & Berger, 2017). In this chapter, we present in detail our standard protocols for isolation of untagged and His-tagged tau.

In brief, BL21(DE3) *Escherichia coli* cells are transformed with a cDNA plasmid containing the desired tau isoform adjacent to a lac operator and T7 promotor. Protein expression is initiated by addition of IPTG in standard culture medium or by lactose in autoinduction medium (Studier, 2005). Cells are subsequently collected by centrifugation, resuspended, and lysed using a French press. The cell lysate is then heated to 90°C to denature and precipitate heat-labile proteins, which are then removed by centrifugation. Further purification of the cleared lysate is achieved using column chromatography. The type of column utilized is dependent upon the nature of the tau construct. Untagged tau is purified using a PC column followed by a hydrophobic interaction chromatography (HIC) column to remove breakdown products. Poly-His-tagged tau is affinity-purified using a nickel (Ni) column, which binds to the His tag and allows for separation from contaminants. Inclusion of a cleavable sequence between the tag and the protein enables subsequent cleavage and removal of the tag and eliminates the possibility of effects arising from the presence of the positively charged tag. After all chromatography steps, tau-containing fractions are identified using SDS-PAGE/Coomassie Blue staining and then pooled, concentrated, and exchanged into storage buffer (Fig. 1).

2 ISOLATION OF UNTAGGED TAU PROTEINS

2.1 PREPARATION OF TAU-EXPRESSING *E. coli* VIA TRANSFORMATION

1. Prepare LB agar plates containing the appropriate antibiotic and concentration for plasmid selection (for LB/ampicillin (Amp) plates, a final concentration of 50–100 μg/mL Amp is recommended).
2. Transform BL21(DE3)-competent cells with the desired tau plasmid and perform a parallel transformation with pUC19 plasmid, a negative control. See Invitrogen protocol for specific instructions.
3. Pipet 25 and 50 μL of transformed cells onto individual plates for each condition, streaking with sterile inoculation loop to evenly spread bacterial cells.
4. Incubate at 37°C for ∼18 h. Do not allow plates to overgrow.
5. Remove plates from incubator, wrap with parafilm, and store at 4°C until ready to proceed to next step. Plates are best used within 1–2 days.

Notes

- Other *E. coli* cell strains are available, any number of which may be suitable for tau expression. The primary considerations are that cells are specifically

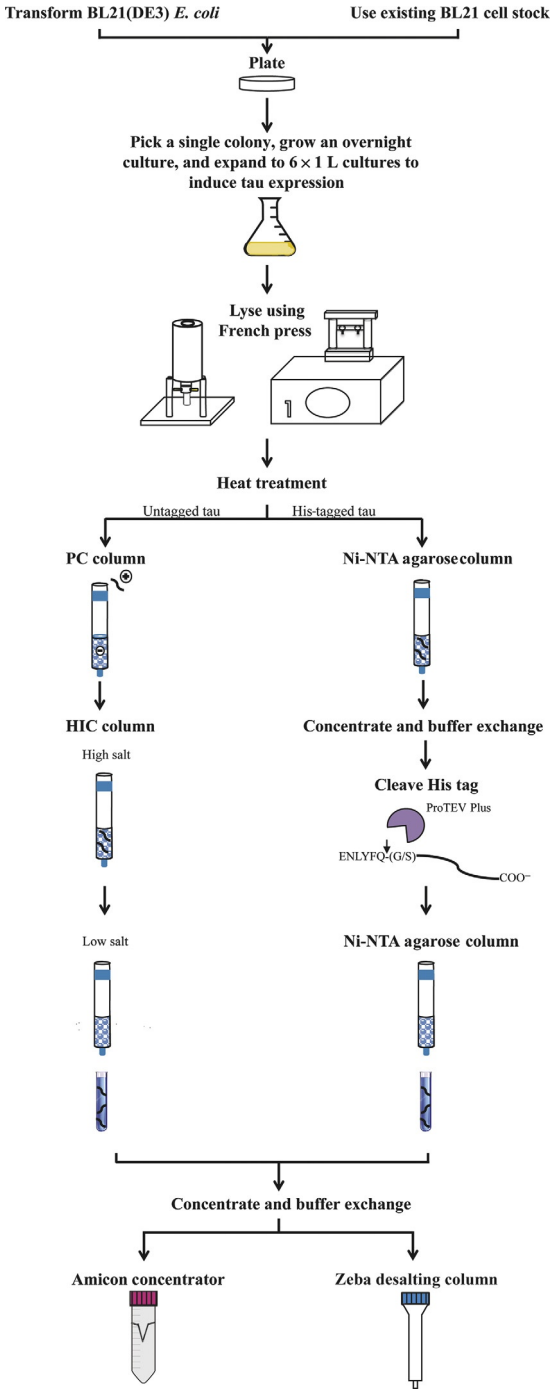

FIG. 1

Isolation of untagged tau and His-tagged tau. Schematic of the protocol for the isolation of untagged and His-tagged tau using a bacterial (BL21(DE3)) expression system. Tau expression is induced in bacteria using lactose or IPTG. Cells are collected, lysed, and treated with heat to denature heat-labile elements. Column chromatography is used to separate tau from contaminants, and the purified protein is concentrated and exchanged into storage buffer.

engineered for recombinant protein expression and that they are compatible with the planned transformation strategy (i.e., are either chemically competent or electrocompetent).
- Place competent cells on ice immediately upon removal from the −80 °C and start the transformation right away.
- Competent cells are susceptible to mechanically induced lysis, so pipetting should not be used to mix competent cells. Instead, mix by gently tapping or swirling the tube.
- Ampicillin is light sensitive, so LB/Amp plates should be stored in the dark. To confirm that the ampicillin is functional, nontransformed cells or cells transformed with a cDNA plasmid that lacks ampicillin resistance (Amp^R) may be plated in parallel as a control.
- Plates should always be stored and incubated lid-side down in order to prevent condensation from dripping onto media surface.

2.2 (ALTERNATIVE) PLATE CELLS FROM PREVIOUSLY PREPARED GLYCEROL STOCK OF TRANSFORMED *E. coli*

1. Prepare LB/Amp plates.
2. On ice, thaw a glycerol stock of BL21(DE3) cells that contain the tau plasmid of interest, as well as a control stock of cells lacking any Amp^R. Glycerol stocks are prepared by mixing 1 mL of an overnight culture with 400 µL of 100% glycerol and are stored at −80 °C in a screw-top vial.
3. Draw a line along the bottom of each plate to divide it into half. One half will be used for the control cells, and the other will be for the tau-expressing cells.
4. Hold each cell stock tube at an angle to prevent contamination, dip a sterile inoculation loop into one of the cell stocks, and streak the appropriate half of the plate. Turn the loop over and streak a second plate with the remaining liquid on the loop.
5. Repeat step 4 with the control cell stock.
6. Place the plates at 37°C for ∼18 h.
7. Remove the plates from the incubator, parafilm, and store at 4°C until ready to proceed to the next step. Plates are best used within 1–2 days.

Notes
- In steps 2–3, cell stocks do not need to be completely thawed for plate streaking, just thawed enough that some liquid can be removed from vial with the inoculation loop.
- The control cell stock may additionally be plated on either: (a) a plate with the appropriate antibiotic for plasmid selection or (b) an antibiotic-free LB plate. Either option confirms that the control cells are alive and verifies that functional ampicillin is preventing growth on the LB/Amp plates.

Materials and equipment

- Invitrogen One Shot BL21(DE3) Transformation Kit (C6000-03) or similar
- Tau plasmid cDNA stock (concentration should be between 1 and 10 ng/μL), or glycerol stock of tau-transformed cells
- Ampicillin sodium salt (Fisher Sci BP1760-5): stock solution is prepared at 50–100 mg/mL (1000 ×) in nanopure water and then sterile-filtered and stored in single-use aliquots at −20°C
- Luria Broth (LB): 10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl in nanopure water
- LB/Amp agar plates: recipe as above, plus 15 g/L agar. Autoclave with a stir bar inside the flask and transfer to a stir plate after autoclaving. Add the antibiotic once the mixture has sufficiently cooled and then pour into sterile petri dishes. Leave lids cracked open until steam no longer builds up when closed, and then leave at room temperature overnight to set. Seal each plate with parafilm, return to petri dish sleeve, and store at 4°C. Plates are best used within 1–2 months
- 37°C Shaking and nonshaking incubators
- 42°C Water bath for heat shock during transformation (or as indicated in manufacturer's protocol)
- Flame-sterilized inoculation loop

2.3 OVERNIGHT STARTER CULTURES

1. Autoclave 240 mL of LB in each of 2 × 500 mL Erlenmeyer flasks.
2. Add Amp to cooled LB (50–100 μg/mL final concentration) and mix thoroughly.
3. Use a sterile method to pick a single colony from a plate prepared in Section 2.1 or 2.2 to inoculate each of the two flasks.
4. Repeat step 3 to transfer a single colony to the second flask. This second colony and flask are backup in case the first does not grow.
5. Place both flasks in a 37°C shaking incubator for 18 h (we use 220 rpm in our Eppendorf model I2500).
6. In the meantime, prepare 6 L of autoinduction media (AIM), leaving room for the 50 × "5052" sugars (20 mL/L) that will be added after autoclaving to prevent caramelization. Divide into 6 × 2 L flasks at 980 mL per flask and autoclave.
7. Autoclave seven screw-top 500 mL centrifuge-compatible bottles for bacterial pelleting (one for 18 h culture and six for 24 h cultures).

Notes

- Molecular biology best practices suggest starting with a smaller overnight culture and then scaling up to 250 mL, but we find this works well.

Materials and equipment

- 500 mL LB/Amp
- Plates containing bacterial colonies

- 2 × 500 mL Erlenmeyer flasks
- 37°C Shaking incubator
- 6 × 2 L Erlenmeyer flasks, baffled for increased aeration (Sigma CLS44232XL)
- 50 × "M" Salts: 1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl, 250 mM Na_2SO_4. Add salts slowly when preparing to ensure complete mixing. Gentle heat may be necessary to encourage dissolution. Filter sterilize
- 50 × "5052" Sugars: 25% glycerol (v/v), 138.8 mM glucose, 292.1 mM α-D-lactose. Filter sterilize
- AIM, 6 L, divided into 6 × 2 L flasks: 10 g/L bacto-tryptone, 5 g/L yeast extract, 1 × "M" salts. After autoclaving and just before use, add 1 × "5052" sugar solution and appropriate antibiotic
- 7 × 500 mL Screw-top centrifuge bottles (ThermoFisher #312110-0016)

2.4 AUTOINDUCTION OF TAU EXPRESSION

1. Remove 18 h cultures from the shaking incubator. Cultures should be cloudy to indicate bacterial growth. Remove 1 mL of culture for later analysis by SDS-PAGE ("preinduction" sample).
2. Make a glycerol cell stock from one or both overnight cultures: 1 mL culture +400 µL 100% sterile glycerol in a screw-top vial. Store at −80°C.
3. Pellet bacteria from one 18 h culture (the same culture used to make cell stock) at 5000 rpm (4200 × *g*) for 15 min at 4°C in an RC-5 centrifuge with a GS-3 rotor. Discard the other 18 h culture.
4. During the spin, add Amp (final concentration 50–100 µg/mL) and 20 mL of 50 × 5052 sugars to each flask of AIM.
5. When spin is complete, resuspend bacterial pellet in AIM as follows:
   a. Remove supernatant and discard.
   b. Take 10 mL of AIM from each 2 L flask (60 mL total) and use it to resuspend the bacterial pellet. Vortex bottle to help start the process and then use a 10 mL serological pipet to create a homogenous, clump-free suspension.
   c. Add 10 mL of resuspended bacteria to each flask. Divide any extra volume evenly between the flasks.
   d. Incubate for 24 h at 37°C, shaking at ~220 rpm.
6. Chill GS-3 and SS-34 rotors for the next day, as well as the French press cell.

Notes

- During resuspension in step 5, pipetting directly at the bottle walls helps to break up clumps of bacteria and achieve homogeneity. Keep the cells cold during this process by performing it on ice.
- Cells preferentially use the glucose in the AIM as their energy source. The glucose is depleted once the culture reaches sufficient density, and the cells switch to using lactose, which induces expression of the recombinant protein (Studier, 2005).

- An alternative to AIM is to induce expression directly with isopropyl-β-D-thiogalactoside (IPTG). In that case, grow cultures in LB and monitor until $OD_{600} = 0.8–1.0$. Add IPTG to 1 mM, incubate 2 h, and then harvest. Note that in our hands this method of induction generally results in decreased protein yield compared to AIM.

Materials and equipment
- 2 × 18 h Cultures
- 100% Glycerol, sterile
- Amp stock (1000 ×), 100 mg/mL
- 2 × 500 mL Screw-top bottles (one sterile for bacteria, one for balance)
- Pipetman and sterile serological pipets
- Shaking incubator
- RC-5 centrifuge and GS-3 rotor or similar (also chill for next day of prep)

2.5 HARVEST BACTERIA

1. Save 1 mL of induced culture to verify induction using SDS-PAGE (compare to preinduction sample taken in Section 2.4).
2. Pellet bacteria from 24 h autoinduced cultures in 6 × 500 mL sterile screw-top bottles at 5000 rpm (4200 × *g*) for 15 min at 4°C, GS-3 rotor, RC-5 centrifuge. Discard supernatant, refill bottles, and repeat until all bacteria are pelleted.
3. Resuspend bacteria in BRB80 + 0.1% βME (1 μL per mL) + AEBSF (5 μL of 100 mM stock per mL). Use 10 mL of buffer per pair of screw-top bottles.
   a. Pour off supernatant and add 10 mL of BRB80/βME/AEBSF to first bottle. To resuspend bacteria, use a metal spatula (cleaned with 70% ethanol) to gently scrape at pellet, layer by layer, while swirling spatula in buffer. Note: It is also possible to skip this step and resuspend with only a serological pipet as described in (b).
   b. Once the pellet is resuspended, pipet mixture with a 10 mL serological pipet until it is a homogenous, clump-free suspension.
   c. Transfer suspension to the paired bottle and repeat steps (a) and (b).
   d. Transfer resuspended bacteria to a 50 mL conical tube.
   e. Repeat steps (a)–(d) with the other two sets of paired bottles.
   f. Rinse bottles with 5 mL BRB80/βME/AEBSF and add to 50 mL conical tube(s) (use same 5 mL for all six bottles).

Notes
- Keep everything on ice throughout these steps and subsequent steps to minimize protease activity.
- During step 2, multiple spins will be needed to get all 6 L of bacterial culture pelleted. Keep cultures in 37°C incubator until ready to pellet. If part of a culture is used, keep it at 4°C until the remainder is used.

- Resuspension may be started during the final spin if some bottles are done earlier.
- There should be 35–40 mL of resuspended bacteria per 50 mL conical tube, as the French press holds 40 mL max.
- BRB80 and all other buffers should be filter-sterilized (using 0.2 μm filters).
- Do not add βME to buffers until just before use, as dilute βME will rapidly lose activity (see product information for more details).

Materials and equipment

– 6 × 500 mL Screw-top bottles sterilized in an autoclave
– 6 × 1 L, 24 h Autoinduced cultures
– Sorvall RC-5 centrifuge and GS-3 rotor or equivalent, chilled
– Pipetman and serological pipets
– 50 mL Falcon conical tubes
– 50 mL BRBB80: 80 mM Pipes, 1 mM $MgSO_4$, 1 mM EGTA, pH 6.8, filter-sterilized
– β-Mercaptoethanol (βME; Sigma M3148)
– 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma A8456): prepare 100 mM stock in nanopure water and store in single-use (~250 μL) aliquots at −20°C

2.6 LYSE BY FRENCH PRESS

1. Assemble the chilled French press cell and set the volume to 40 mL.
2. Run 40 mL of nanopure water for the first press (see manufacturer's guidelines for specific instructions).
3. Reset piston to 40 mL and load bacteria. For optimal results, maintain a steady flow while pressing, around 1200 psi (or as per manufacturer's guidelines). Collect any bacteria that were not pressed from the sample chamber using a transfer pipet.
4. Repeat step 3 until all bacteria have been pressed three times. Samples should become more viscous as they are French pressed. Note the volume of your cell lysate.
5. Run 40 mL of nanopure water for the final press to start the cleaning process.
6. Disassemble the French press cell and clean all parts with soap and water (no scrub brushes). Do a final rinse with deionized water and dry thoroughly before storing at room temperature.

Notes

- If leaking occurs, check: (a) that the nylon ball in the flow valve is present and functional and (b) that the flow valve handle is finger tight.
- During dismantling and refilling, hold the French press cell at an angle to avoid resting weight on the piston.
- Alternative lysis methods include sonication and rapid freeze–thaw cycles. Whichever process is chosen, care should be taken to keep the lysate cold.

Materials and equipment
- Bacterial cell suspension (approximately 80 mL)
- 40 mL Nanopure water
- Plastic transfer pipets
- French press with Glen Mills Manual-Fill 40K Cell (FA-032) or similar

2.7 HEAT DENATURATION AND HIGH-SPEED CENTRIFUGATION

1. Denature heat-labile proteins by transferring 50 mL conical tubes containing pressed lysate to 90–95°C water bath and incubating for 10 min.
2. Transfer lysate to 50 mL polypropylene tubes and seal with parafilm. It may be necessary to push parafilm up to the lip of the polypropylene tube to prevent tubes from getting stuck in the centrifuge rotor.
3. Pellet cell debris at 13,000 rpm (20,200 × *g*) in an RC-5 centrifuge with an SS-34 rotor, 40 min at 4°C.
4. Transfer supernatant to 50 mL conical tubes.
5. Reserve a sample of the supernatant to include in a diagnostic SDS-PAGE gel.

Notes
- Make sure conical tubes are tightly capped during heating.

Materials and equipment
- 90–95°C Water bath
- RC-5 centrifuge with SS-34 rotor or equivalent, chilled
- Several 50 mL polypropylene tubes compatible with centrifuge
- Several 50 mL conical tubes

2.8 PC CHROMATOGRAPHY

2.8.1 Column precycling[a]

1. Weigh 10 g PC into a 1 L glass beaker (final column volume will be approximately 50 mL).
2. Add 500 mL of 0.5 M NaOH and stir gently with a serological pipet tip.
3. Allow resin to settle 5 min.
4. Carefully pour off fines (pulverized resin that will not have settled to the bottom).
5. Add ~400 mL of 0.5 M sodium phosphate buffer (pH 7) and stir. Repeat steps 3 and 4.
6. Repeat step 5 until pH is ~7, measured by pH strips (usually one more time).
7. Pour off final sodium phosphate buffer and add 500 mL of 0.5 M HCl. Repeat steps 3 and 4.

[a]Do this the same day you plan to use the column.

8. Repeat step 5 to exchange into sodium phosphate buffer as above until pH is ~7. It is usually sufficient to exchange 2× with ~400 mL each and then once more with the remaining phosphate buffer.
9. Exchange into BRB80 as in previous steps, 2× with ~400 mL each time. Store the equilibrated resin in a minimal amount of BRB80 buffer at 4°C until use.

2.8.2 Batch bind and run column

1. Add equal volumes of equilibrated PC resin to 50 mL conicals containing equal amounts of postheat treatment, postspin lysate.
2. Add βME (1 μL per mL, 0.1% final) and 0.1 mM AEBSF (1 μL of 100 mM stock per mL) to resin/lysate mixture.
3. Put conical tubes on a rotator at 4°C for at least 30 min (up to 2 h) to allow tau to bind to resin. Set up column during this time.
4. Invert 50 mL conicals containing resin/lysate mixture to ensure mixture is homogenous. Remove a sample for a diagnostic gel and then pour the mixture of resin and lysate into an empty column. Use a peristaltic pump (Rainin Rabbit or equivalent) set to ~3 mL/min and let it run as you pour the mixture into the column. Collect the flow-through in an appropriately labeled beaker and store at 4°C.
5. It is important to minimize resin loss as this will result in protein loss and decreased yield. To collect the last of the resin, rinse the 50 mL conicals with 5 mL of BRB80+0.1% βME and gently pipet it into the column to avoid disturbing the resin bed.
6. Wash column with 100 mL (2 column volumes, CV) of BRB80+0.1% βME. Collect the wash in an appropriately labeled beaker and store at 4°C. Collect the last 1 mL of the wash separately as a sample for a diagnostic gel.
7. Elute tau with a step gradient of ammonium sulfate in BRB80+0.1% βME, collecting ½ CV fractions. Place fractions on ice immediately as they come off the column. Remove a sample of each fraction to run on a gel and store remainder at −20°C.
   a. 50 mL of 50 mM $(NH_4)_2SO_4$ (collect 2× 25 mL fractions).
   b. 100 mL of 150 mM $(NH_4)_2SO_4$ (collect 4× 25 mL).
   c. 50 mL of 250 mM $(NH_4)_2SO_4$ (collect 2× 25 mL).
8. Run a gel to identify tau-containing fractions (Fig. 2A).

Notes

- When making sodium phosphate buffer, the components should be added gradually to a 2 L graduated cylinder with a large stir bar in order to create a fully dissolved and homogenous solution.
- The pH of BRB80 must be adjusted after the addition of ammonium sulfate. We therefore find it convenient to prepare and pH a stock solution of BRB80 +2 M $(NH_4)_2SO_4$ and then combine this with BRB80 to make the PC column elution buffers described above.
- It is important to collect and save the flow-through from all columns throughout the prep in order to detect issues with resin binding (depending on the isoform

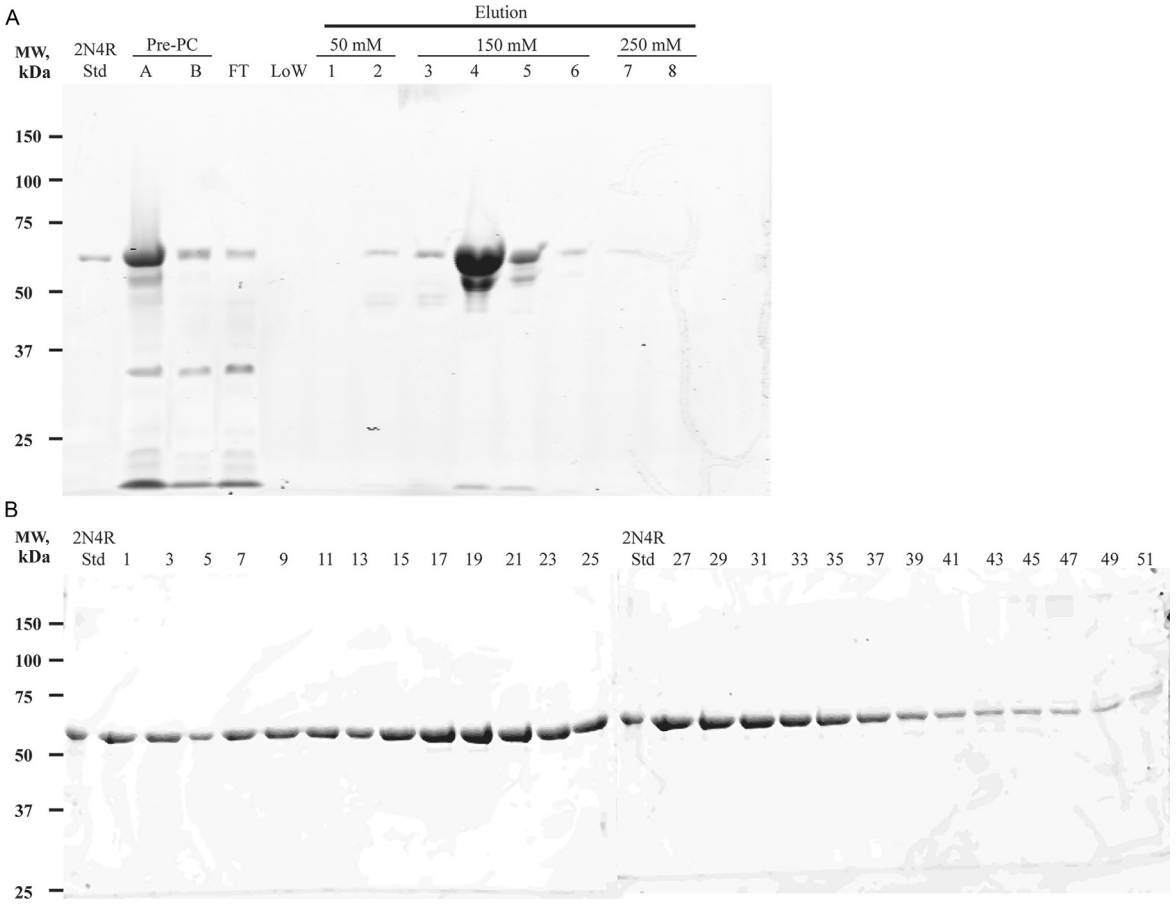

FIG. 2

See legend on opposite page.

being purified, the tau will bind with lower or higher affinity according to its amino acid composition and resultant electrostatic profile).
- Do not let the column resin run dry. Air bubbles can be removed from the top of the solution with a serological pipet tip.
- Whenever you add additional volume to the column, add slowly so as not to disturb the resin. This can be done by setting the pipet to "slow" and pipetting around the top ridge of the column using a slow circular motion, letting the solution run down the sides.
- Adjust the speed of the peristaltic pump as necessary. There is a trade-off between amount of time it takes to run the column and the amount of interaction time samples will have with the PC resin. If a peristaltic pump is not available, the column can be run under gravity flow.
- Following the procedure, running water through the column collection lines will prevent the buildup of salt precipitates.
- The diagnostic PC column gel does not need to be rigorously quantitative, and so the staining process can be sped up in a microwave. Heat the gel in stain for 30 s on full power and then immediately cover the container with aluminum foil and place on a shaker for 20 min before switching to destain.

Materials and equipment[b]

– 500 mL 0.5 M NaOH
– 500 mL 0.5 M HCl

FIG. 2

Phosphocellulose (PC) column and hydrophobic interaction chromatography (HIC) column diagnostic SDS-PAGE gels. (A) Tau ("2N4R" isoform, 441 amino acids) is bound to PC resin and eluted with increasing concentrations of ammonium sulfate. Fractions are run on an SDS-PAGE gel against a regular molecular weight standard and a tau molecular weight standard ("2N4R" isoform) to identify those that contain tau. "Pre-PC" refers to postheat treatment cell lysate collected immediately prior to the column. Lane A is a tau-enriched sample that was incubated with PC resin. Lane B is the same lysate prior to resin addition. "FT" is the flow-through collected during column loading. "LoW" is the last 1 mL of column wash. Elution fractions 1–8 are shown with the indicated concentration of ammonium sulfate (see Section 2.8). (B) Tau-containing PC fractions were pooled and run on a HIC column to separate tau from the lower molecular weight bands (likely contaminants and/or breakdown products) visible in PC elution fractions. Every other fraction collected from the HIC column was run on an SDS-PAGE gel against a regular molecular weight standard and a tau molecular weight standard ("2N4R" isoform, 441 amino acids) to identify fractions containing pure tau protein (see Section 2.9).

[b]All buffers must be passed through a 0.2 μm filter to sterilize them and to remove any particulates; for some chromatography systems degassing is also recommended. See manufacturer's instructions for your specific column/system.

- 2 L of 0.5 M sodium phosphate buffer, pH 7.0. Prepare as 305 mM Na_2HPO_4 and 195 mM NaH_2PO_4. There is no need to adjust the pH
- 10 g PC resin; we use P11 (Whatman 4071200), but cellulose phosphate (Sigma C2258) is a substitute that is currently more readily available
- pH strips
- Serological pipet tips
- β-Mercaptoethanol (βME)
- AEBSF stock
- 100–200 mL Column, empty
- Peristaltic pump (optional)
- 1 L BRB80
- BRB80 + 2 M $(NH_4)_2SO_4$ (pH 6.80), stock solution for preparing elution buffers. Add βME immediately prior to use:
  - 50 mL of BRB80 + 50 mM $(NH_4)_2SO_4$ with 0.1% βME
  - 100 mL of BRB80 + 150 mM $(NH_4)_2SO_4$ with 0.1% βME
  - 50 mL of BRB80 + 250 mM $(NH_4)_2SO_4$ with 0.1% βME

2.9 HIC COLUMN

2.9.1 Equilibrate column 1 day in advance

If this is a new column, follow the manufacturer's instructions for new columns. Then, equilibrate the column for the tau prep using BRB80 and BRB80 + 1.25 M $(NH_4)_2SO_4$ (both filtered, pH 6.80):

a. Rinse column with 10 CV "Buffer B" (BRB80)
b. Rinse column with 15 CV "Buffer A" (BRB80 + 1.25 M $(NH_4)_2SO_4$)

2.9.2 HIC column

1. Thaw protein-containing fractions in a beaker of room-temperature water and then immediately transfer to ice. Minimize time at room temperature.
2. Add $(NH_4)_2SO_4$ to tau-containing fractions to bring the $(NH_4)_2SO_4$ concentration to 1.25 M. Add a little bit at a time to avoid protein precipitation and mix by gently inverting the tube. We do not adjust the pH at this step.
3. Just before loading onto the HIC column, filter protein through a 0.22 μm filter.
4. Load sample onto the column at 1 mL/min. Monitor the conductivity (we use an in-line conductance meter) and spike the solution with BRB80 + 2 M ammonium sulfate if it drops below 150 mS. Take care not to introduce air into the column during this process.
5. Collect the flow-through in an appropriately labeled beaker.
6. Wash column with 10 CV Buffer A (50 min at 1 mL/min). Collect the wash in a labeled beaker or conical tube, keeping the last 1 mL separate. Take samples of the "total wash" and "last of wash" for a diagnostic gel.

7. Elute tau from the column using the following program, collecting 1.4 mL fractions throughout:
   a. 0%–60% Buffer B for 100 min
   b. 60%–100% Buffer B for 10 min
8. Prepare a sample from every second fraction for a diagnostic gel. Cover fractions with parafilm and store at 4°C.
9. Rinse the column with 100% Buffer B for 10 min. Collect in a 15 mL Falcon tube as "last of elution."
10. Run SDS-PAGE gels of the samples prepared in step 8, stain with Coomassie, and then destain overnight (Fig. 2B).
11. Clean column and fraction collector line. We monitor this process with an in-line conductance meter:
    a. Rinse with nanopure water 10–20 CV (conductance <1.00 mS)
    b. Clean with 0.5 M NaOH (conductance $= \sim 115$ mS)
    c. Rinse with nanopure water 10–20 CV (conductance <1.00 mS)
    d. If storing long term, rinse with 5 CV 30% EtOH/nanopure water

Notes

- These conditions work well for wild-type tau isoforms. Truncated or mutant tau constructs may require a higher concentration of ammonium sulfate for efficient binding.
- If spiking with additional ammonium sulfate, note that it may take several minutes to reflect a change in conductance (depending on the placement of the conductance meter). Allow sufficient time before any additional spiking.
- An in-line UV detector can identify fractions carrying the peak of the eluted protein, greatly reducing the number of fractions that need to be examined by SDS-PAGE gels. However, gels are indispensable for detecting the presence of breakdown products and other contaminants.
- For different tau isoforms and constructs the ideal binding and elution conditions may vary. It is therefore prudent to save all washes and flow-throughs, and to take samples throughout the process for diagnostic SDS-PAGE gels.
- A common alternative for this final chromatography step is a sizing column.

Materials and equipment[c]

– HiTrap Phenyl HP hydrophobic interaction column (HIC), 5 mL (GE Healthcare 17-1351-01)
– Chromatography system, LP BioLogic or similar with an in-line conductance meter

[c]All buffers must be passed through a 0.22 μm filter to sterilize them and to remove any particulates; for some chromatography systems degassing is also recommended. See manufacturer's instructions for your specific column/system.

- "Buffer A:" BRB80 + 1.25 M $(NH_4)_2SO_4$, pH 6.8, with 0.1% βME (added immediately prior to use)
- "Buffer B:" BRB80, pH 6.8 with 0.1% βME (added immediately prior to use)
- BRB80 + 2 M $(NH_4)_2SO_4$, pH 6.8
- Nanopure water, filtered
- 0.5 M NaOH, filtered
- 30–50 mL Sterile syringe and 0.22 μm filter
- βME
- Test tubes for fraction collection

2.10 PROTEIN CONCENTRATION AND BUFFER EXCHANGE

1. Pool HIC column fractions that contain clean protein.
   a. Use a 5 mL serological pipet to remove fractions from test tubes, and combine them into a 50 mL conical. Use a p200 to collect any remaining liquid from test tubes.

Option A: Concentration and buffer exchange in Amicon concentrators

2. Concentrate and buffer exchange into BRB80 + 0.1% βME using Amicon Ultra concentrators (10,000 MWCO), spinning at 4000 rpm ($3300 \times g$) at 4°C in an RC-5 centrifuge, SH-3000 swinging bucket rotor.
   a. Rinse concentrators first by doing a 10 min spin with BRB80 + 0.1% βME.
   b. Discard BRB80 + 0.1% βME and load pooled fractions.
   c. Spin for 40 min.
   d. Save the flow-through in case of a concentrator malfunction and then top off concentrators with any additional volume of pooled fractions. Spin for 40 min.
   e. Repeat step (d) until all fractions have been added to the concentrator.
   f. If more than one concentrator was used, pool samples into one concentrator by gently pipetting the solution in the filter to resuspend protein and transferring volume to second concentrator. Try to avoid touching the filter with the pipet tip.
   g. Spin for ~30 min or until total volume is less than 500 μL. Record volume.
   h. Bring the volume up to ~11–12 mL in BRB80 + 0.1% βME, gently pipetting to distribute the protein. Record the new volume and calculate the dilution factor (dilution factor = new volume/original volume). Spin for 60 min or until the volume is less than 500 μL. Record this volume.
   i. Repeat step (h) until the total dilution factor is >10,000 (obtained by multiplying the dilution factors at each step). This will probably take 3–4 rounds. The final volume should be 500 μL or less.
   j. Gently pipet remaining liquid in the concentrator to resuspend the protein. Transfer the solution to a sterile Eppendorf tube. Carefully rinse the filter with ~50 μL BRB80 + 0.1% βME and transfer to same Eppendorf. Pipet total volume gently to homogenize solution.
3. Aliquot protein into sterile tubes and store at −80°C.

Option B: Concentration and buffer exchange with Zeba desalting columns

1. Concentrate protein as described above in Option A, steps 2a–2e to a volume between 700 and 4000 μL (for a 10 mL desalting column).
2. Use a 10 mL Zeba desalting column to exchange into BRB80 + 0.1% βME following manufacturer's protocol.
3. Aliquot protein into sterile tubes and store at −80°C.

Notes

- See product information for Amicon concentrators and Zeba desalting columns to determine appropriate centrifugation speed and time.

Materials and equipment

- 2× Amicon Ultra 15 mL centrifugal filter unit, 10 kDa MWCO (UFC901024)
- BRB80 + 0.1% βME (added immediately prior to use)
- Zeba Spin Desalting Column, 7 kDa MWCO, 10 mL (Thermo #89893/4)
- RC-5 centrifuge and SH-3000 swinging bucket rotor or similar, chilled

3 ISOLATION OF HIS-TAGGED TAU PROTEINS

Our hexa-histidine (6x-His)-tagged tau fusion proteins are engineered to contain an enzymatic cleavage site between the His-tag and tau to enable removal of the tag after tau isolation (Fig. 1). We use the sequence ENLYFQ(G/S), which is cleaved immediately after the glutamine by the tobacco etch virus (TEV), leaving behind a single glycine (or serine; see manufacturer's information on ProTEV Plus for more details). This tag and subsequent purification methods are particularly useful in isolating tau fragments that are not easily isolated using ion-exchange chromatography, such as truncated and/or mutant tau constructs. The linker and tag can be positioned at either the N or C terminus; however, positioning the tag at the C terminus (Tau-linker-6x-His) leaves behind a slightly larger sequence after cleavage.

For preparation of His-tagged tau, the early protocol is identical to those outlined above in Sections 2.1–2.4 and will not be repeated here. During harvest in Section 2.5, the protocols remain identical with the exception of the resuspension buffer. To this effect, we will highlight the differences in purification protocols below.

3.1 MODIFICATIONS TO EARLY STAGES OF THE PROTOCOL

Harvest

Proceed as outlined in Section 2.5, but resuspend bacteria in Ni-Lysis buffer + 1 mM AEBSF. BRB80 can be substituted as long as it does not contain βME, which will reduce the free nickel in the column. We have encountered no difficulties in isolating tau without βME at this step.

High-Speed Centrifugation

Following centrifugation as in Section 2.7, add AEBSF to 1 mM (do not add βME). We find that residual flocculent material in the lysate can interfere with subsequent column steps, so we therefore find it convenient to remove any remaining cell debris by filtering lysate through Whatman filter paper.

Additional Materials

– Ni-Lysis buffer: 5 mM imidazole, 500 mM NaCl, 10 mM Tris, pH to 8.0, filter-sterilized

3.2 AFFINITY CHROMATOGRAPHY WITH NI-NTA AGAROSE

1. Gently invert the 50% Ni-NTA agarose bead slurry to make sure that beads are fully suspended in solution. The binding capacity is 5–10 mg of protein per mL of resin (see manufacturer's information for more details).
2. Pipet 8 mL of Ni-NTA slurry into a capped 10 mL column and allow solution to come to room temperature as the beads settle (final column volume will be ~4 mL).
3. After beads have settled, remove the cap and allow storage liquid to drain. Do not let the column run dry during this or any of the following steps.
4. Equilibrate resin with 100 mL of Ni-Lysis buffer, being careful not to disturb the resin surface. Reduce the liquid level above resin as much as possible without allowing the column to dry.
5. Load lysate onto column, collecting the flow-through. Allow to flow into the column until the level of the lysate is just above the resin. You may pause flow at this point by capping the column or, if ready, immediately begin the wash.
6. Wash column with 50 mL of Ni-Lysis buffer, collecting the first and last mLs to run on a diagnostic SDS-PAGE gel.
7. Wash column with 50 mL of Ni-Wash buffer, collecting the first and last mLs in addition to 10 mL fractions throughout.
8. Elute protein with 50 mL of Ni-Elution buffer, collecting 5 mL fractions.
9. Wash column with 50 mL of 500 mM imidazole to remove any proteins still bound to the resin (save a sample from early in this wash for a diagnostic gel to visualize any protein that remained through elution steps).
10. Take a 20 μL sample of each fraction for diagnostic gels before storing them at −20°C.
11. Run an SDS-PAGE gel to identify tau-containing fractions.
12. Pool desired fractions and buffer exchange into BRB80 as described in Section 2.10, but omit βME. βME and imidazole can both interfere with subsequent cleavage and tag removal.
13. Determine the protein concentration using methods outlined in Section 4. Concentrated samples can be stored at −80°C and aliquots processed for applications as needed.

Notes

- Allowing the resin to come to room temperature prior to column packing prevents bubbles from forming during later steps.
- When adding lysate or performing wash steps, it is important that the column's resin is not disturbed and does not run dry. As such, use caution when adding liquid or modulating flow rate if using a peristaltic pump.
- Resin can be regenerated. See manufacturer's protocols.
- Alternatively, one can use a prepacked Ni column (such as His-Trap HP, GE Healthcare # 17-5248-02) and an automated chromatography system.

Materials and equipment[d]

– 10–20 mL Column with cap
– Peristaltic pump (optional)
– Ni-NTA agarose beads (Qiagen 30230) or similar
– Ni-Lysis buffer: 5 mM imidazole, 500 mM NaCl, 10 mM Tris, pH to 8.0
– Ni-Wash buffer: 10 mM imidazole, 500 mM NaCl, 10 mM Tris, pH to 8.0
– Ni-Elution buffer: 100 mM imidazole, 500 mM NaCl, 10 mM Tris, pH to 8.0
– BRB80: 80 mM PIPES, pH 6.8, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.8
– Amicon Ultra 15 mL centrifugal filter unit, 10 kDa MWCO
– Zeba Spin Desalting Column, 7 kDa MWCO, 10 mL (optional)

3.3 PROTEOLYTIC CLEAVAGE TO REMOVE HIS-TAG

1. Thaw ~1 mg of tau on ice and collect a 20 μL precleavage sample for a diagnostic gel. Assemble the ProTEV reaction according to the manufacturer's protocol (1 mL total reaction volume).
2. Leave reaction on bench-top overnight. Collect a postcleavage sample for a diagnostic gel. Optionally, monitor time course of cleavage by removing samples at several time points.

Notes

- Proteolytic cleavage reaction can be scaled according to volume and amount of protein. To do so, refer to product guidelines.

Materials and equipment

– ProTEV Plus enzyme, reaction buffer, and 100 mM DTT (Promega V6101)

3.4 NI-NTA COLUMN FOR TAG REMOVAL

1. Prepare column as in Section 3.2 and equilibrate with 50 mL of BRB80.
2. Reduce liquid in column to just above the resin without drying.
3. Apply entire cleavage reaction to the column, allowing the sample to move all the way into the resin. Collect 1 mL fractions. Tau will flow through,

[d]All buffers must be filter-sterilized.

while the cleaved tags and the ProTEV enzyme will remain bound to the column.
4. Apply 3 mL of BRB80 when the last of the sample has just entered the resin, taking care not to disturb the resin. Continue collecting 1 mL fractions.
5. Apply 10 mL of BRB80 as above, without disturbing resin. Continue collecting 1 mL fractions.
6. Wash column with 50 mL of Ni-Elution buffer, collecting first and last mLs as 1 mL fractions and the remaining volume in a 50 mL conical.
7. Remove 20 µL samples from all fractions for analytical SDS-PAGE gels to determine tau-containing fractions.
8. Pool desired fractions and add βME to 0.1%.
9. Buffer exchange into BRB80 + 0.1% βME or desired buffer, as in Section 2.10.
10. Determine tau concentration as described below in Section 4.

Materials and equipment as listed in Section 3.2

4 DETERMINATION OF TAU CONCENTRATION BY SDS-PAGE AND COMPARISON TO A TAU MASS STANDARD

Quantification of tau concentration by Bradford analysis and comparison to a BSA standard curve can be off by a factor of up to 2.7 (Barghorn, Biernat, & Mandelkow, 2005; Panda, Samuel, Massie, Feinstein, & Wilson, 2003), likely due to the difference in amino acid composition between tau and BSA. Tau is also less amenable than many proteins to quantification using its extinction coefficient at 280 nm (reviewed in Devred et al., 2010), due to its low content of aromatic residues. Therefore, we have adopted a quantitative SDS-PAGE-based method of concentration determination that relies on comparison to a tau standard of known concentration, as determined by commercially available quantitative amino acid analysis. Once the concentration of the tau mass standard has been established, a dilution series is run against a dilution series of the tau protein of unknown concentration. A standard curve of intensity as a function of protein concentration is then generated and used to interpolate the concentration of the unknown (Fig. 3).

1. On ice, thaw an aliquot of a tau mass standard of known concentration, hereafter referred to as the "tau standard" and an aliquot of purified tau of unknown concentration, hereafter referred to as the "unknown."
2. Create a dilution series of both the tau standard and the unknown in Laemmli sample buffer. For best practice, we have found that loading 0.02–0.2 µg protein/lane is a good working range for our tau standard ("0N4R" isoform, 383 amino acids).
3. Run an SDS-PAGE gel with both standard and unknown dilutions (Fig. 3A). For best practice, we have found that running 10 lanes of standard and 3 lanes of unknown generates a reliable standard curve.
4. Stain in Coomassie Brilliant Blue R stain for 1 h and then destain overnight.

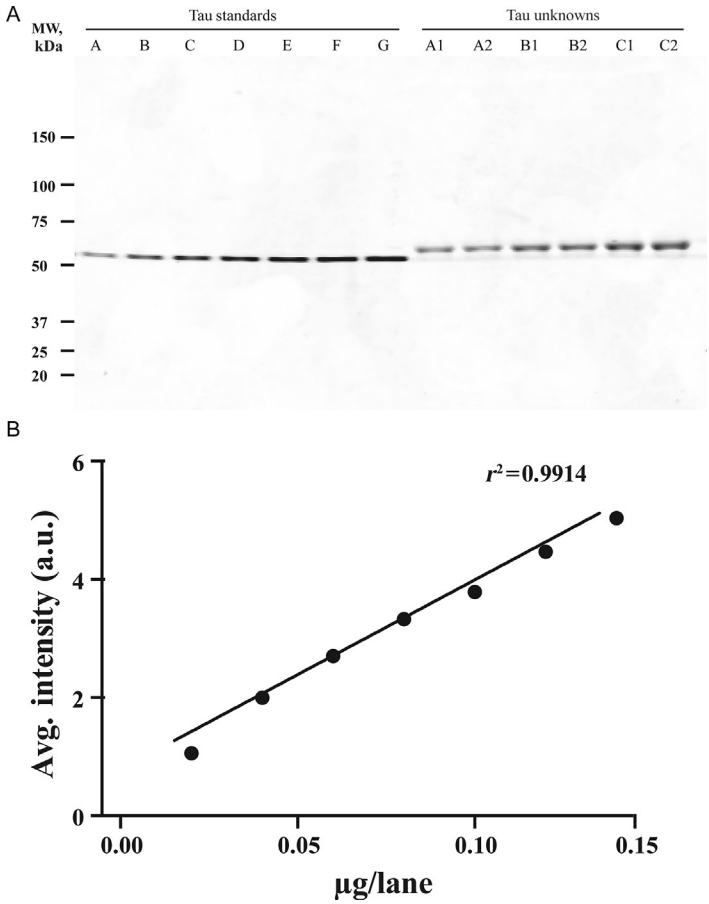

FIG. 3

Determination of tau concentration by SDS-PAGE gel and comparison to a tau mass standard. (A) A dilution series of a tau mass standard ("0N4R" isoform, 383 amino acids) of known concentration, ranging from 0.02 to 0.14 µg tau/lane (lanes A–G), was run against a dilution series of purified tau of unknown concentration ("2N4R" isoform, 441 amino acids; lanes A1–C2, run in duplicate). (B) Band intensities were quantified. Standards were fit by linear regression and then used to interpolate the unknown concentration of the purified tau (see Section 4).

5. For gel imaging and analysis we use a Li-COR Odyssey® infrared scanner and Odyssey® software, respectively (Coomassie Brilliant Blue R is visible in the 700 nm channel). We measure band intensity by drawing a rectangle around the largest band on the gel and then using this same rectangle to measure intensity of all bands on the gel. We measure the intensity of each band three times and then take the average.

6. Generate a standard curve for the tau standard by plotting μg protein/lane vs band intensity (Fig. 3B). Interpolate values for unknowns from the standard curve.
7. Back-calculate the concentration of undiluted unknown sample and average the values obtained from each of the different dilutions of the unknown.
8. Adjust assumed concentration of unknown to the interpolated average and run a confirmatory concentration gel. Three replicates are recommended.

Notes

- We store the tau standard in small aliquots at −80°C in screw-top vials to guard against protein degradation and sublimation. For storage, the standard is diluted in Laemmli sample buffer at 40× the final working tau concentration. Upon thawing, sufficient 1× Laemmli sample buffer is added to bring the standard to the working tau concentration. This working solution of standard can be stored at −20°C in the short term (approximately 1 month) but should be replaced if any breakdown products are noted.
- Coomassie Brilliant Blue R stain binds proportionally to a protein's basic residues (Tal, Silberstein, & Nusser, 1980). As such, it is desirable to run the unknown tau isoform against an identical tau isoform standard if possible, or against one containing a comparable number of basic residues for the most accurate results. It may also be necessary to increase the amount of protein loaded per lane for different tau isoforms or tau fragments, depending on their amino acid composition.
- Linear regression of the tau standard generally produces a reliable standard curve with a high r^2 value, which indicates a good fit (ours are typically $r^2 \geq 0.98$). If a linear fit does not yield an adequate r^2 value, or if it appears to be a poor fit, data can be fit instead with a nonlinear regression to a second- or third-order polynomial. The standard curve should not obviously plateau at higher concentrations; if it does, lower the standard concentration range.
- When doing regression analysis, do not force the line through (0,0). In our experience this results in poorer fits and less reproducibility.
- Discard any unknown intensity values that lie outside the range of the standard curve. The most accurate estimates are obtained within the range where the standards are approximately linear.
- For densitometric quantitation on the Li-COR Odyssey® imaging scanner and software (Li-COR Biosciences), we have found that the "Left–Right, Median" method of background subtraction produces the most reliable results. Practically, this means that the background value for each band is defined by the median intensity of pixels in a 1-pixel border to the left and right side of the selection; pixels above and below the band are ignored. This method is not suitable if the spaces between the bands are not well defined (i.e., if the bands touch each other).
- Some evidence suggests that quick-stain protocols such as those outlined in Sections 2.8 and 2.9 may provide less robustly quantitative data compared to a longer staining procedure such as we describe in Section 4 (Luo, Wehr, & Levine, 2006; Weber, Pringle, & Osborn, 1972; Wilson, 1983).

Materials

- Coomassie Brilliant Blue R stain (Thermo 20278, Sigma 27816) in 10% acetic acid, 10% isopropanol
- Destain solution (same as the staining solution, but omit the dye)
- Tau standard of known concentration (determined by amino acid analysis)
- LiCOR Odyssey Imaging System or similar

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