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In vitro preparation of single filamentous microtubules optimized for dynamic and electrophoretic light scattering measurements.

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We use this protocol and it's working

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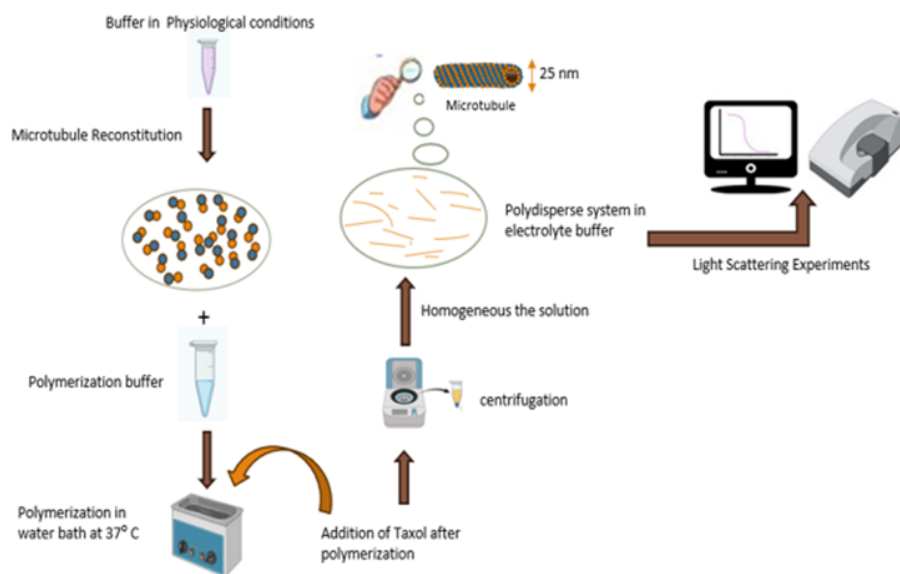
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Abstract

The lack of essential information on sample preparation and the need to characterize and understand microtubules (MTs) in various biological functionalities within the cell have led us to develop a high-quality polymerization protocol to prepare fully formed MTs in vitro, which is particularly designed for Dynamic light scattering (DLS) and Electrophoretic light scattering (ELS) experiments for further analysis. This protocol details the reconstitution of tubulin, GTP, and Taxol, the preparation of PEM, cushion buffer, and polymerization buffers, the step-by-step polymerization process, and the use of Taxol to stabilize the microtubule filaments without precipitation. This polymerization protocol successfully generated samples for light scattering experiments using buffers that replicate physiological conditions. This has provided consistency in preparing stable, diluted, aggregate-free, homogenous microtubule filament samples that could benefit many other scientific research groups currently working in the field. Additionally, it can easily be adapted to prepare samples using other buffers and biological fluids.

Graphical abstract:



This sample preparation protocol requires 3 days.

All the buffers and solutions are prepared to make enough of four samples of fully developed microtubules in 100 millimolar concentration of potassium chloride (KCl), with a total volume of 1 milliliter of solution. (See Materials sections).

Materials

Materials:

■ Laboratory Equipment/List of Equipment

1. Analytical Explorer Pro-Scale (Ohaus Industrial Scales, model: PX84)
2. Pipette+ (Sartorius, Andrew Alliance Stand+)
3. Grant SUB Aqua 12 Plus water bath.
4. Smart electronic pipettes: 5–350 μ L, 10–1,000 μ L, 5–10 mL (Sartorius, Andrew Alliance Stand+)
5. Vortex mixer with standard tube head, 120V (Corning LSE, The Lab Depot, Ref:6775)
6. Orion Star A211 pH meter, accuracy: $\pm$ 0.002 (Thermo Scientific, catalog number: X56954)
7. Allegra 64R benchtop centrifuge machine (Beckman Coulter, product number: 367585) with an F1202 Rotor, 30,000 rpm (Beckman Coulter, catalog number: 19U313)
8. Zetasizer ULTRA, accuracy MW: $\pm$ 10% typical, temperature accuracy: 0.1 $^{\circ}$ C (Malvern Panalytical, model: ZSU5700)
9. 2 mL glass vial (Agilent, catalog number: 20097845)
10. Polypropylene centrifuge tube, 15 and 50 mL (Corning, catalog number: 430791)
11. Polypropylene microfuge tube, 11 $\times$ 38 mm, 1.5 mL (Beckman Coulter, catalog number: 9080511)
12. 12 mm square polystyrene cell cuvettes (Malvern Panalytical, number: DTS0012)
13. 2 mL cryogenic vials (Corning, catalog number: 30721070)
14. Business source stainless steel scissors (Fiskars, catalog number: 01-004250J)
15. Optifit pre-sterilized tips, 10–1,000 μ L (Sartorius, catalog number: PR151159)
16. Optifit pre-sterilized tips, 5–350 μ L (Sartorius, catalog number: PR149531)
17. 5 3/4" pipets (Fisherbrand, Fisher Scientific, catalog number: 13-678-20A)
18. Universal dip cell kit (Malvern Panalytical, catalog number: ZEN1002)

■ Biological Material

1. Tubulin Protein (>99% Pure): Porcine Brain; store at 4 $^{\circ}$ C (Cytoskeleton, catalog number: T240)
2. Guanosine 5'-triphosphate sodium salt (GTP); store at 4 $^{\circ}$ C (Cytoskeleton, catalog number: BST06)
3.  Paclitaxel (Taxol) **Cytoskeleton Inc. Catalog #TXD01** store at 4 $^{\circ}$ C

■ Chemicals and Reagents

1. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: 67-68-5)
2. Water for molecular biology (Millipore, catalog number: H20MB1001)
3. Magnesium chloride, anhydrous, 99% (MgCl₂) (Alfa Aesar, catalog number: W07D102)
4. Potassium chloride (KCl) (VWR, BDH Chemicals, catalog number: 18J2256059)
5. Potassium hydroxide (powder) (KOH) (Sigma-Aldrich, catalog number: 1310-58-3)
6. PIPES (piperazine-N, N'-bis (2-Ethane sulfonic acid)) (Molecular biology reagent, catalog number: 194838)
7. EGTA (ethylene glycol-bis (β aminoethyl ether)-N, N, N', N'-tetra acetic acid) (Sigma -Aldrich, catalog number: E3889-25g)

8. Glycerol, ACS reagent, $\geq 99.5\%$ (Sigma-Aldrich, catalog number: 56-81-5)
9. HCl volumetric standard, 0.1 N solution in water (Sigma-Aldrich, catalog number: 7647-01-0)
10. 70% v/v denatured ethanol solution (Fisher Bioreagents, catalog number: 216731)

Buffers:

1. Orion buffers pH 4.01, pH 7.00, and pH 10.01 (Thermo Scientific, catalog numbers: YX1, YW1, YX1)
2. 100 mM PIPES

	A	B	C
	Reagent	Final concentration	Amount
	PIPES	100 mM	302.4 mg
	H2O ultra-pure	n/a	9.3-9.5 mL
	Total	100 mM	10 mL

3. 10 mM EGTA

	A	B	C
	Reagent	Final concentration	Amount
	EGTA	10 mM	38.04 mg
	H2O ultra-pure	n/a	9.6-9.7 mL
	Total	10 mM	10 mL

4. 50 mM MgCl_2

	A	B	C
	Reagent	Final concentration	Amount
	MgCl_2	50 mM	47.61 mg
	H2O ultra-pure	n/a	10 mL
	Total	50 mM	10 mL

5. 117.47 mM KCl

	A	B	C
	Reagent	Final concentration	Amount



	A	B	C
	KCl	117.47 mM	87.52 mg
	H ₂ O ultra-pure	n/a	10 mL
	Total	117.47 mM	10 mL

6. PEM buffer (pH 7.00)

	A	B	C
	Reagent	Final concentration	Amount
	PIPES	80 mM	4.8 mL
	MgCl ₂	2 mM	240 µL
	EGTA	0.5 mM	300 µL
	H ₂ O ultra-pure	n/a	660 µL
	Total	n/a	6 mL

7. Cushion buffer (pH 7.00)

	A	B	C
	Reagent	Final concentration	Amount
	PIPES	80 mM	3.2 mL
	MgCl ₂	2 mM	160 µL
	EGTA	0.5 mM	200 µL
	H ₂ O ultra-pure	n/a	440 µL
	Glycerol	60 % v/v	6 mL
	Total	n/a	10 mL

8. Polymerization buffer (pH 7.00)

	A	B	C
	Reagent	Final concentration	Amount
	PEM	n/a	1000 µL



	A	B	C
	GTP	n/a	20 μ L
	PEM with glycerol	n/a	166.6 μ L
	Total	n/a	1.186 mL

9. PEM-T

	A	B	C
	Reagent	Final concentration	Amount
	PEM	n/a	212.4 μ L
	Taxol (100 μ M)	15 μ M	37.6 μ L
	Total	n/a	250 μ L

10. Electrolyte (KCl-T) buffer (pH 7.00)

	A	B	C
	Reagent	Final concentration	Amount
	KCl	100 mM	851.2 μ L
	Taxol (50 μ M)	0.5 μ M	10 μ L
	Taxol (100 μ M)	3 μ M	30 μ L
	Taxol (200 μ M)	10 μ M	50 μ L
	Total	n/a	941.2 μ L



First-day procedure: Stock preparations

4m 30s

1

Note

- To maintain the stock buffers biologically active, they must be remade weekly.
- To prepare 100 millimolar (mM) PIPES with a pH close to 6.8, need to add approximately 13-15 drops of 5 Mass Percent KOH solution before adding water.
- To prepare 10 millimolar (mM) GTA with pH close to 6.9, need to add approximately 1-2 drops of 5 Mass Percent KOH solution before adding water.
- All the pipetting and titration are done using the instrument Pipette+ (Sartorius, Andrew Alliace Stand+).

2 100 mM PIPES:

Add 302.4 mg of PIPES to a 15 mL polypropylene centrifuge tube.

2.1 Add 13-15 drops of 5 Mass Percent KOH solution to the tube.

2.2 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10) until there is no residue.

45s

2.3 Add 9.3 mL - 9.5 mL of H₂O ultra-pure to the tube.



2.4 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10).

45s

3 10 mM EGTA:

Add 38.04 mg of EGTA to a 15 mL polypropylene centrifuge tube.

3.1 Add 1-2 drops of 5 Mass Percent KOH solution to the tube.



3.2 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10) until there is no residue.

45s

3.3 Add 9.6 mL – 9.7 mL of H₂O ultra-pure to the tube.



3.4 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10).

45s

4 50 mM MgCl₂ :

Add 47.61 mg of MgCl₂ to a 15 mL polypropylene centrifuge tube.

4.1 Add 10 mL of H₂O ultra-pure to the tube.



4.2 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10).

45s

5 117.47 mM KCl:

Add 87.52 mg of KCl to a 50 mL polypropylene centrifuge tube.

5.1 Add 10 mL of H₂O ultra-pure to the tube.

5.2 Vortex the solution for 00:00:35 – 00:00:45 at low to medium speed (4/10).

45s

Note

Store all the stock buffers at 4 °C .

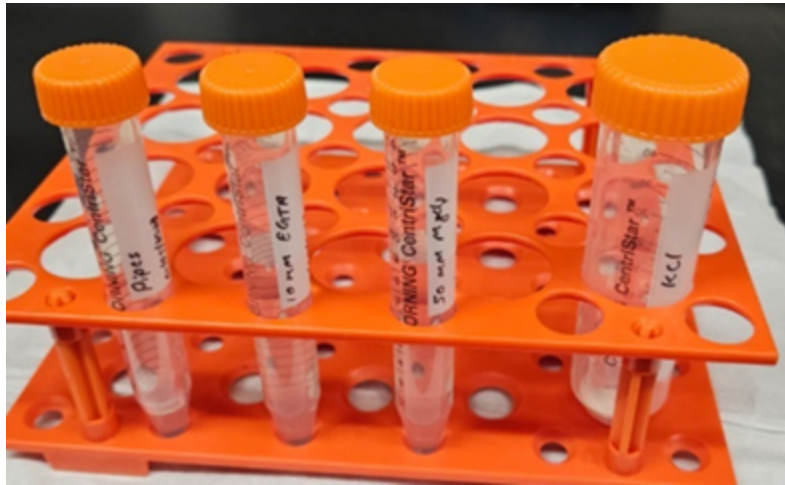


Figure 1: Stock buffers of 100 mM PIPES, 10 mM EGTA, 50 mM MgCl_2 , and 117.47 mM KCl ready for refrigeration.

Second-day procedure: Tubulin buffers preparations

1m 30s

6

[M] 0 Mass Percent

Note

- Calibrate the pH meter at three-point calibration using Orion buffers $\text{pH } 4.01$, $\text{pH } 7.00$, and $\text{pH } 10.01$ before its usage.
- To increase/decrease the pH, add drops of a base ([M] 0.005 Mass Percent KOH) or acid ([M] 0.1 Mass Percent HCl) solution, respectively. Add these drops carefully and constantly and check the pH, as it can rapidly change.
- Using a 50 mL conical tube was vital since it facilitated measuring and adjusting the pH of the buffers. If the tube is smaller in size, the probe of Orion Star A211 pH meter will not be able to fit; also, there will not be enough solution to cover the tip of the probe leading to inaccurate pH readings. We recommend you consider choosing the size of conical tubes and stock buffer volume based on your pH meter probe size, as microprobes can measure smaller sample volumes effectively.
- When adjusting the pH (PEM, and PEM with glycerol and electrolyte buffers) use glass pipettes to carefully add the base and acid solutions drop by drop into the buffers. Too many drops may significantly increase or decrease the pH, so use caution. It is important to mix well by vortexing the solutions after adding these drops; failing to do so will lead to inaccurate pH readings.

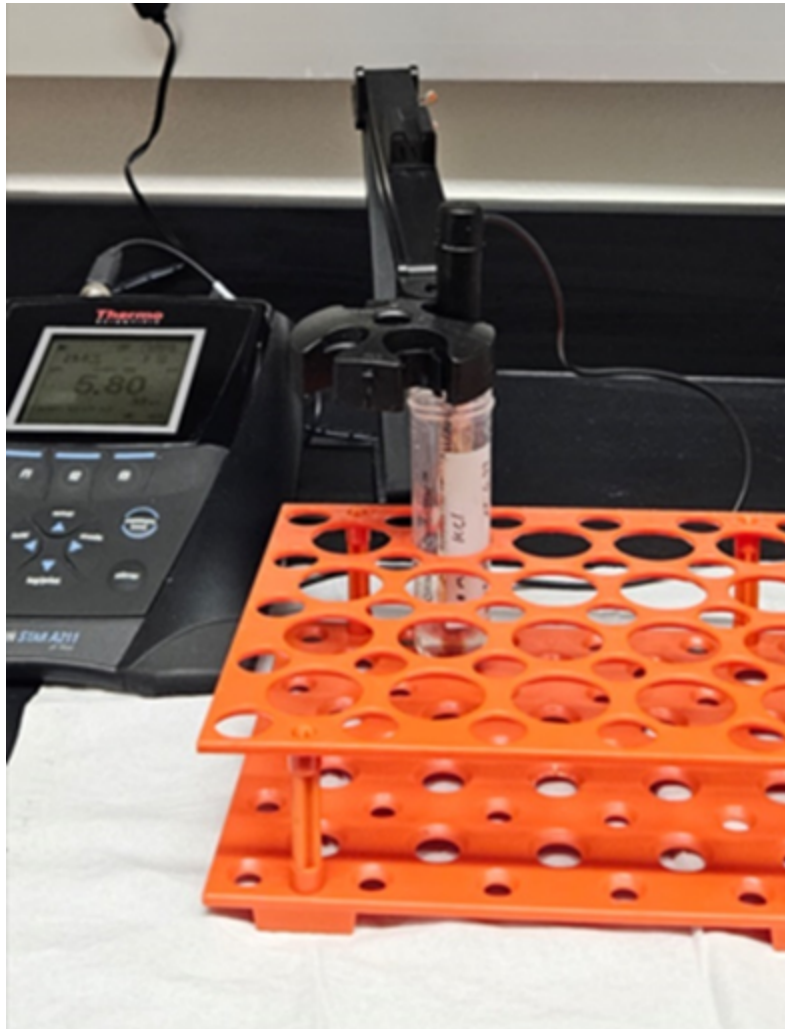


Figure 2: pH meter measuring the pH of [M] 100 millimolar (mM) KCl solution.

7 PEM or BRB80 or general tubulin buffers (pH 6.91):



Add  4.8 mL of PIPES to a 50 mL polypropylene centrifuge tube.

7.1 Add  240 μL of MgCl_2 to the tube.



7.2 Add  300 μL of EGTA to the tube.





7.3 Add  660 μL of H_2O ultra-pure to the tube.



7.4 Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).

45s

8 PEM with glycerol or cushion buffer or tubulin glycerol buffer (pH 6.97):



Add  3.2 mL of PIPES to a 50 mL polypropylene centrifuge tube.

8.1 Add  160 μL of MgCl_2 to the tube.



8.2 Add  200 μL of EGTA to the tube.



8.3 Add  440 μL of H_2O ultra-pure to the tube.



8.4 Add  6 mL of glycerol to the mixture.



8.5 Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).

45s

Note

- Titrate these solutions using the KOH solution with the pH meter to reach the normal pH of  7.00 and record it. A pH value of  6.90 to  7.10 is acceptable for further proceedings.
- Store all these buffers at  4 $^{\circ}\text{C}$.

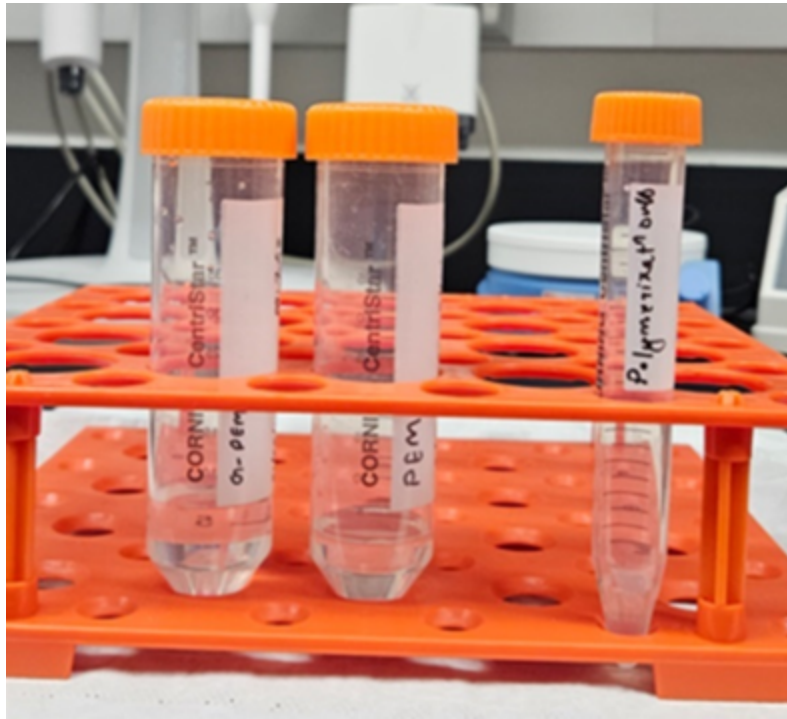


Figure 3: PEM, G-PEM, and polymerization buffer.

Third-day procedure: Polymerization process and suspension buffer

15h 57m 20s

9 Preparations:

Before proceeding with the polymerization buffers and the step-by-step procedure, one must ensure to have enough reconstituted GTP, Taxol, and protein. If you already have these, skip to the 'Tubulin polymerization' steps. If not, follow the procedure given below.

10 a. GTP reconstitution

Note

- The lyophilized GTP (desiccated to <10% humidity) is stable for six months at 4 °C .
- The reconstituted GTP is stable if stored at or below -20 °C for six months.
- All GTP-related steps must be performed with ice in the hood.

10.1 Briefly centrifuge to collect the white powder at the bottom of the storage tube.



10.2 Add  100 μL of ice-cold distilled water for a  100 millimolar (mM) stock solution. 

10.3 Aliquot the GTP into experiment-sized amounts as needed.

10.4 Snap-freeze the GTP with liquid Nitrogen.

10.5 Store at or below  $-20\text{ }^{\circ}\text{C}$.

11 **b. Taxol reconstitution**

Briefly centrifuge to collect the white powder at the bottom of the storage tube.

11.1 Add  100 μL Dimethyl sulfoxide (DMSO) for a  2 millimolar (mM) stock solution. 

11.2 From  2 millimolar (mM) stock solution, pipette  25 μL to a cryotube. 

11.3 Add  475 μL of DMSO to the cryotube to make a total of  500 μL of reconstituted Taxol with  100 micromolar (μM) concentration. 

11.4 For  200 micromolar (μM) , add  50 μL of Taxol ( 2 millimolar (mM)) to  450 μL of DMSO. 

11.5 For  100 micromolar (μM) , add  25 μL of Taxol ( 2 millimolar (mM)) to  475 μL of DMSO. 

11.6 For  50 micromolar (μM) , add  125 μL of Taxol ( 200 micromolar (μM)) to  375 μL of DMSO. 

11.7 For  10 micromolar (μM) , add  50 μL of Taxol ( 100 micromolar (μM)) to  450 μL of DMSO. 



11.8 Store at or below -20 °C .

12 c. Protein reconstitution

Note

1. Tubulin is a delicate protein; to maintain the integrity and functionality of tubulin, it is crucial to handle it gently and avoid conditions that could cause damage or destabilization mainly because of mechanical stress.
2. The 1 mg tubulin protein powder needs to be extracted to begin the protein reconstitution. If the 1 mg of tubulin is stuck to the bottom of the vial, we suggest vortexing the protein vial at a low to medium speed (3/10) for 00:00:30 – 00:00:45 to smooth the protein and allow easy extraction when the protein is still dry.
3. To avoid repeated thawing cycles, consider specific experimental-sized amounts.
4. Some of the steps considered in this section are recommended by the manufacturer (Cytoskeleton, Inc.). In addition, we considered the addition of mixing steps (12.3 and 12.5), due to the rapid agglomeration/aggregation of the tubulin as the buffer is continuously added. Thus, to achieve an aggregate-free solution we implemented the following: the vortex speed is set at low to medium (4 out of 10) for a short amount of time (for 00:00:30 – 00:00:40). This limits the possibility of a mechanical breakdown of the protein.
5. The aliquots containing the protein and tubulin buffers are stable for six months at -80 °C to preserve their biological activity.

12.1 Transfer the protein powder into a 3 mL glass vial.

12.2 Add 100 µL of ultra-pure water into the glass vial containing the protein powder to reconstitute at 10 mg/mL tubulin density.

12.3 Tubulin proteins may concentrate at the solution's surface or walls of the vial. Vortex the solution for 00:00:30 – 00:00:40 at low to medium speed (4/10) to dissolve the protein powder as much as possible. 40s

12.4 Add 1.5 mL of PEM buffer to the protein solution in the glass vial.

12.5 Vortex once more for 00:00:35 – 00:00:45 at low to medium speed (4/10) to dissolve the aggregates as much as possible. If aggregates are still present, pipette and 45s



mix the solution to achieve a homogeneous protein solution.

12.6 Aliquot the solution into experimental-sized amounts according to the number of experiments needed. Transfer the small-size solutions into cryotube vials. We recommend aliquoting the protein solutions into multiples of  400 μL (1×400 μL , 2×400 μL , 3×400 μL , ...).

12.7 Once the wanted protein solutions are aliquoted, snap-freeze the cryotube vials with liquid nitrogen and immediately store them at  -80 °C .

13 Tubulin suspension or polymerization buffer

Note

- Have ice ready in a cooler, as the polymerization buffer must be done on ice.
- Since GTP is toxic to inhale, all steps related to GTP are performed in the fume hood with the sash lowered to ½ of its maximum opening (18 inches).

13.1 Add  1 mL of PEM to a 15 mL polypropylene centrifuge tube. 

13.2 Add  20 μL of reconstituted GTP to the tube. 

13.3 Add  166.6 μL of PEM with glycerol to the tube. 

13.4 Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).  45s

13.5 Keep the solution  On ice .

14 Tubulin polymerization

 1h 35m



Note

1. GTP and Taxol were initially stored at -80 °C and -20 °C respectively. When ready to prepare the solutions, thaw both solutions. We aliquoted these into experimental-sized amounts, particularly for one-time use.
2. Following the manufacturer's recommendation, after the 01:30:00 centrifuge process, remove the top 90% (231.2 µL) of the supernatant from each microcentrifuge tube by following step 14.19. The amount of translucent pellets left in the microcentrifuge will be 25.8 µL .
3. Before adding the reconstituted Taxol to the polymerized tubulin in the water bath, we recommend incubating them in the water bath for 00:05:00 to avoid thermal shock to the microtubule filaments.
4. When homogenizing the microtubule pellet in a water bath after centrifugation (Steps 14.20 – 14.22), keep PEM-T, and KCl-T in the water bath to avoid thermal shock to the filaments.

14.1 Extract one of the cryotube vials containing 1×400 µL of protein solution from the -80 °C freezer.

14.2 De-frost the cryotube at Room temperature for 00:05:00 .

5m

14.3 Incubate the vial On ice for 01:00:00 to depolymerize tubulin oligomers that may form during storage.

1h



14.4 Extract 100 µL from the cryotube vial and transfer to a 1.5 mL polypropylene microcentrifuge tube.

14.5 Add 100 µL of polymerization buffer to the microcentrifuge tube containing the 200 µL protein solution to start polymerization.

14.6 Vortex the solution for 00:00:40 – 00:00:50 at low speed (3–4/10).

50s

14.7 Place the tubulin solution samples On ice for 00:10:00 to bind GTP.

10m

14.8 Incubate the protein solution in the Grant SUB Aqua 12 Plus water bath at 37°C for

00:50:00 .

50m

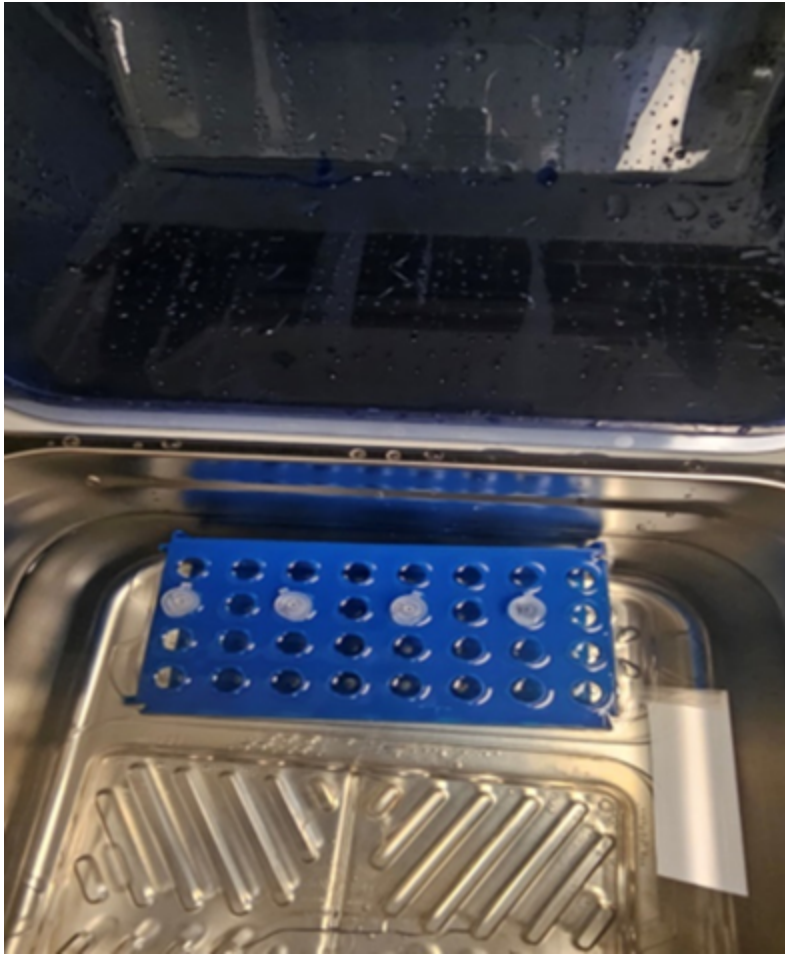


Figure 4: Four tubulin samples immersed in the Grant SUB Aqua 12 Plus water bath at 37°C for polymerization.

14.9 Set the 5–350 μL Pipette+ in pipette mode at very low speed (1/10) and add $11\ \mu\text{L}$ Taxol from $10\ \mu\text{M}$ stock solution.

14.10 Incubate the samples for 00:10:00 in the water bath.

10m



14.11 Set the 5–350 μL Pipette+ in titration mode at very low speed (1/10) and add $20\ \mu\text{L}$ Taxol from $50\ \mu\text{M}$ stock solution.

14.12 Incubate the samples for 00:10:00 in the water bath.

10m



14.13 Set the 5–350 μ L Pipette+ in titration mode at very low speed (1/10) and add 26 μ L Taxol from 100 micromolar (μ M) stock solution.

14.14 Incubate the samples for the last 00:15:00 in the water bath to reach the stabilization of microtubules with Taxol.

15m



14.15 Turn on the centrifuge 00:10:00 – 00:15:00 before the previous step (14.14) is finalized. Set the centrifuge at 25000 rcf, 25°C, 01:30:00 . The acceleration and deceleration should both be set at a very low (2/10) speed.

1h 45m

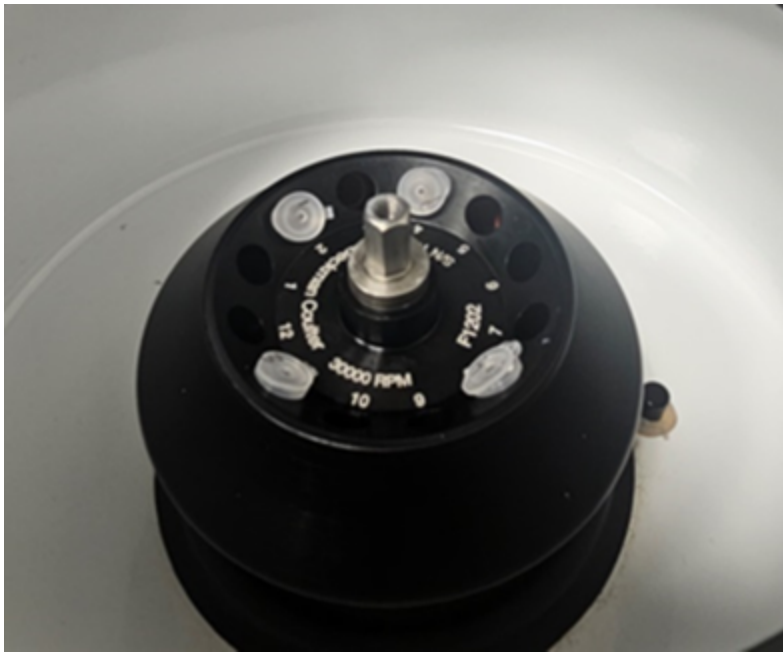


Figure 5: Microtubule samples placed in a centrifuge rotor.

14.16 Centrifuge the protein solutions for 01:30:00 .

1h 30m



14.17 Both PEM-T and KCI-T are prepared during the time of centrifugation.

3m



PEM-T preparation:



1. Add  212.4 μL of PEM buffer to a 1.5 mL polypropylene microcentrifuge tube.
2. Add  37.6 μL of Taxol from  100 micromolar (μM) stock solution.
3. Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).
4. Keep the solution at  Room temperature .

Electrolyte (KCl-T) buffer preparation:

1. Add  851.2 μL of KCl of  117.47 millimolar (mM) mM of stock solution to a 1.5 mL polypropylene microcentrifuge tube.
2. Add  10 μL of Taxol from  50 micromolar (μM) of stock solution to the KCl solution.
3. Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).
4. Add  30 μL of Taxol from  100 micromolar (μM) of stock solution.
5. Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).
6. Add  50 μL of Taxol from  200 micromolar (μM) of stock solution.
7. Vortex the solution for  00:00:35 –  00:00:45 at low to medium speed (4/10).
8. h)Keep the solutions at  Room temperature .

- 14.18 After the centrifugation, remove the tubulin protein solution tubes from the centrifuge and place them in a styrofoam vial rack at  Room temperature .
- 14.19 Set the 5–350 μL Pipette+ in titration mode at a very low speed (1/10) and extract the supernatant from the solution in the following manner to avoid any possible stress in the solution that could lead to the breakage of the microtubule filaments: 100 μL , 100 μL , and 31.2 μL .
- 14.20 Vortex the pellet containing solution for  00:00:20 –  00:00:30 at low speed (3–4/10).  30s
- 14.21 Incubate the pellet samples for  00:10:00 at  37 °C .  10m
- 14.22 Add  33 μL of warm PEM-T to pellet samples. 
- 14.23 Keep the pellet samples at  Room temperature for  00:10:00 .  10m

14.24 Vortex the solution for 00:00:40 – 00:00:50 at low speed (3–4/10) to make the remaining microtubule pellet (25.8 μL) to reach the homogenous form.

50s

14.25 Using the same Pipette+ specifications as in the previous step (14.24), add 941.2 μL of electrolyte buffer to the microtubule protein pellet (58.8 μL).



14.26 Store the solution at Room temperature and leave it Overnight .

8h

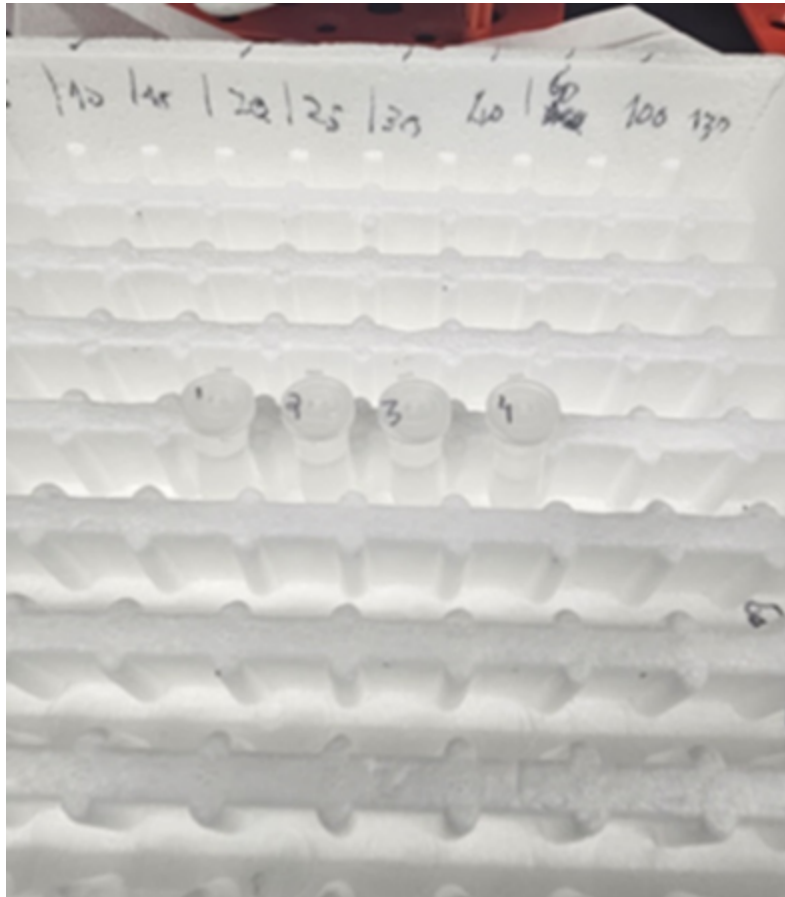


Figure 6: Microtubule samples left overnight to reach the equilibrium with the electrolyte buffer.

14.27 The samples are ready to perform light scattering experiments.



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