Microtubules/Tubulin Biochem Kit

Cat. # BK015

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
To order by e-mail: cserve@cytoskeleton.com
Technical assistance: (303) - 322 - 2254
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MANUAL CONTENTS

Section I: Introduction

Kit Overview

This kit is intended for those researchers who are not accustomed to working with Tubulin protein *in vitro*. The contents of this kit will allow you to reproducibly prepare microtubules of a predetermined mean length. After polymerization the microtubules can be used immediately or stabilized with taxol before use, depending upon the needs of your experimental system.

Kit Uses:

- Polymerize microtubules for motility experiments
- Polymerize microtubules for Microtubule Associate Protein (MAP) binding assays

Tubulin/Microtubule Overview

Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called alpha and beta tubulin. These two proteins are encoded by separate genes, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom. Consequently, tubulin isolated from bovine brain tissue is highly homologous to tubulin isolated from any eukaryotic source. This fact results in the technical benefit that bovine tubulin (in the form of microtubules, see below) can be used to assay proteins originating from many diverse species, e.g. *Saccharomyces cerevisiae* (1, 2, 3) and *Drosophila melanogaster* (4, 5).

Tubulin polymerizes to form structures called microtubules (MTs). When tubulin polymerizes it initially forms proto-filaments, microtubules consist of 13 protofilaments and are 25 nm in diameter, each micrometer of microtubule length being composed of 1650 heterodimers. Microtubules are highly ordered fibers that have an intrinsic polarity, shown schematically in Figure A. Tubulin can polymerize from both ends *in vitro*, however, the rate of polymerization is not equal. It has therefore become the convention to call the rapidly polymerizing end the plus-end of a microtubule and the slowly polymerizing end the minus-end. *In vivo* the plus end of a microtubule is distal to the microtubule organizing center.

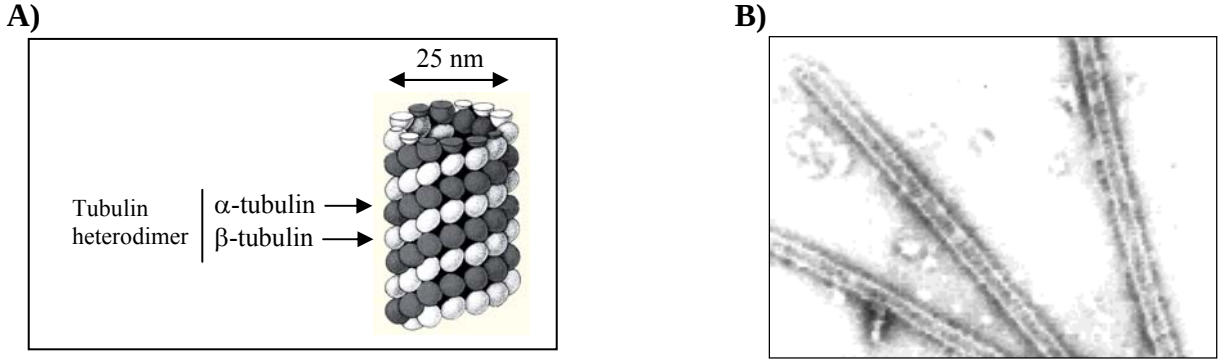

Figure 1. Schematics of a microtubule. A. Diagram showing the organization of tubulin dimers and protofilaments within a typical microtubule. B) Electron micrograph of bovine microtubules assembled *in vitro* (100,000 X magnification).

Example Results

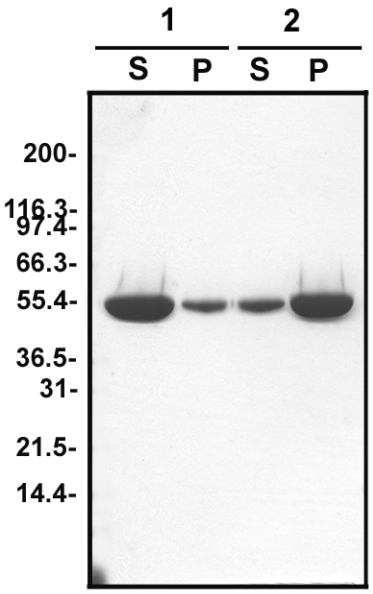

Figure 2. Polymerization of Tubulin into Microtubules. Tubulin was resuspended to 5 mg/ml in G-PEM as described in Section V for the polymerization of microtubules having a mean length of 6.5 µm. Half of the tubulin solution was polymerized at 37°C for 40 min; the remaining half was kept on ice. Both samples were centrifuged at 100,000 x g to separate the pellet fraction (P, containing microtubules) from the supernatant fraction (S, containing unpolymerized tubulin) and run on a 12% SDS-gel for Coomassie staining. Lane 1, unpolymerized tubulin. Note that >90% of the tubulin (55 kDa) remains in the supernatant fraction. Lane 2, polymerized tubulin. Note that >80% of the tubulin (55 kDa) is found in the pellet as microtubules.

Section II: Kit Contents

This kit contains enough reagents for approximately 8 to 200 assays depending on the assay method of choice.
Prior to reconstitution of the components, the kit should be stored (inside the zip-top bag with desiccant) at -70°C where it is stable for 6 months. Warm the kit to room temperature prior to opening. The kit contents should not be allowed to become damp.

Reagent	Cat. # Part #	Quantity	Description
Tubulin protein	Cat. # TL238-A	8 tubes	Lyophilized. 250 µg of protein
General Tubulin Buffer	Cat. # BST01-001	1 bottle	Lyophilized. 80 mM PIPES pH 7.0, 2 mM $MgCl_2$, 0.5 mM EGTA when reconstituted
Tubulin Glycerol Buffer	Cat. # BST05-001	1 bottle	Liquid. 80 mM PIPES pH 7.0, 2 mM $MgCl_2$, 0.5 mM EGTA, 60% v/v glycerol.
GTP stock	Cat. # BST06-001	2 tubes	Lyophilized. 100 mM stock when reconstituted
Taxol stock	Cat. # TXD01	1 tube	Lyophilized. 2 mM stock when reconstituted
Anhydrous DMSO	Part # DMSO	1 tube	Liquid. 1 ml for taxol resuspension. Note: DMSO will freeze at 4°C.

*** Items with Part numbers (Part #) are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.**

Necessary Equipment:
1. Water bath set to 35-37°C

Section III: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as follows:

Component	Reconstitution	Storage
GTP	1) Label 10 tubes "100 mM GTP". 2) For a 100 mM stock solution, reconstitute in 100 µl of ice cold Milli-Q water, aliquot into 10 x 10 µl volumes and store at -70°C.	Store at -70°C.
General Tubulin Buffer	1) For 1X buffer, resuspend in 10 ml of sterile distilled water. Store at 4°C.	Store lyophilized buffer desiccated at -70°C. Stable for 6 months. Store solution at 4°C. Stable for 6 months.
Tubulin Protein	1) Reconstitute as described in Table 1 to polymerize microtubules of a desired length	Store lyophilized protein desiccated at -70°C. Stable for 6 months.
Tubulin Glycerol Buffer	1) No reconstitution required	Store solution at 4°C. Stable for 6 months.
Taxol	1) For a 2 mM stock solution, reconstitute each tube in 100 µl of anhydrous DMSO. Store at -70°C. WEAR GLOVES WHEN HANDLING TAXOL.	Store lyophilized product desiccated at -70°C. Stable for 6 months. Store solution at -70°C. Stable for 6 months.
Anhydrous DMSO	1) No reconstitution required	Store at -70°C. Stable for 1 year. Store at 4°C, stable for 4°C 6 months

Section IV: Important Technical Notes

1. **Temperature.** Tubulin polymerization in this assay is regulated by temperature. At 37°C tubulin will polymerize into microtubules while at 4°C microtubules will depolymerize to the tubulin subunits. There is generally a loss of 5% polymer per degree reduction in temperature. It is critical therefore to pay particular attention to temperature throughout the assay. Tubulin should be kept on ice until transferred to a water bath for polymerization at 37°C.
2. **Glycerol as a Polymerization Enhancer**. The propensity of tubulin subunits to assemble into microtubules is dependent upon their affinity for microtubule ends (termed critical concentration [CC]). In order to achieve polymerization the CC needs to be less than the total tubulin concentration. At concentrations above the CC, tubulin will polymerize until the free subunit concentration is equal to the CC value. The intrinsic ability of pure tubulin to polymerize *in vitro* is very much a function of the experimental conditions. For example, one can manipulate the polymerization reaction to give microtubules of a particular mean length distribution or create conditions under which tubulin will not polymerize significantly until an enhancer component, such as a polymerization stimulating drug or protein, is added. Because of this parameter, pure tubulin in General Tubulin Buffer plus GTP will not generally polymerize significantly at concentrations below 5 mg/ml. If, however, one adds a polymerization enhancer such as 5-20% glycerol to this reaction, tubulin polymerization efficiency will be increased.

3. **Buffer composition.** Microtubules will de-polymerize in buffers containing greater than 10 μM free calcium ions.

Section V: Assay Protocol

Preparation of Microtubules of a Given Length Distribution

1) Decide the mean length of the microtubule (MT) population you will require, see Figure 2, Appendix 1. Generally a mean length distribution of 5-10 μm is used for MT binding assays and MT motility assays (see Table 1).
2) If you are going to stabilize the microtubules with taxol, aliquot 90 μl of General Tubulin Buffer (Cat. # BST01-010) into a clean tube (labeled **TX buffer**) and add 10 μl of 2 mM stock taxol. Mix well by pipetting and keep at 35-37°C.

Note: Each condition for microtubule polymerization described below requires one 250 μg tube of tubulin (supplied in kit, Cat. # TL238-A).

Table 1. Selected Conditions for Microtubule Polymerization

Microtubule length required	Volume of MT Buffer to add to one tube of tubulin	Volume of MT Buffer to make	MT Buffer Formulation		
			Volume of Cushion Buffer to add	Volume of General Tubulin Buffer to add	Volume of 100 mM GTP to add
2 μm	50 μl	1000 μl	500 μl	490 μl	10 μl
6.5 μm	50 μl	1000 μl	160 μl	830 μl	10 μl
16 μm	80 μl	1000 μl	80 μl	910 μl	10 μl

3) Prepare the appropriate MT Buffer formulation as described in Table 1 for your choice of MT length. Keep on ice.
4) Briefly centrifuge (14,000 rpm for 5 s) one 250 μg tube of tubulin (Cat. # TL238-A) to collect the product at the bottom of the tube.
5) Resuspend one tube of tubulin with the required volume of MT Buffer shown in Table 1 column 2.
6) Incubate the tube on ice for 10 min for complete resuspension of the protein.
7) Incubate the tube at 35-37°C for 40 min; this will give you a microtubule stock solution of the required length.
8) To stabilize the microtubules add 1/10th the volume of **TX Buffer** made in step 2 and incubate for a further 10 min at 37°C (optional).
9) The microtubules can now be used directly in your assay of choice.

NOTE: Taxol stabilized microtubules will be stable for several days (Store at room temperature, **do not store at 4°C);** however the length distribution will change over time (the microtubules will become longer). If this is not a problem for your experiments, then these microtubules can be used for 4 days. If however, you require microtubules of a consistent mean length distribution, we recommend using the microtubules within 6-7 h. Anti-microbial agents (50 μg/ml ampicillin and 5 μg/ml chloramphenicol) can be added if the microtubules are going to be kept for longer than one day.

Section VI: Troubleshooting

PROBLEM	POSSIBLE REASON	SOLUTION
Microtubules fail to form	1. Temperature too low. 2. No GTP added. Tubulin requires 1 mM GTP for polymerization 3. In the absence of taxol tubulin requires glycerol for efficient polymerization	1. Polymerize tubulin at 35-37°C. 2. Add GTP stock to the MT Buffer formulation. 3. Add the appropriate volume of Cushion Buffer to the MT Buffer formulation for the required MT population length.

Section VII: References

1) Hyman, A.A., Middleton, K.M., Centola, M., Mitchison, T.J., and Carbon, J. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature* **359**, 533-536 (1993)
2) Jiang,W., Middleton, K.M., Yoon, H., Fouquet, C., and Carbon, J. An essential Yeast protein, CBF5, binds in vitro to centromeres and microtubules. *Mol. Cell. Biol.* August (1993).
3) Barnes, G., Louie, K.A., and Botstein, D. Yeast proteins associated with microtubules in vitro and in vivo. *Mol. Biol. of the Cell.* **3,** 29-47.
4) Walker, R.A., Salmon, E.D., and Endow, S.A. The Drosophila claret segregation protein is a minus-end directed motor molecule. *Nature* **347**, 780-782 (1990).
5) Zhang, P., Knowles, B., Goldstein, L.S., and Hawley, R.S. A kinesin-like protein required for distributive chromosome segregation in Drosophila. *Cell*. **62**, 053-062 (1990).

Section VIII: Example BK015 Citation

1) Van Horck, F.P., Ahmadian, M.R., Haeusler, L., Moolenaar, W.H. and Franenburg, O. Characterization of p190RhoGEF, a RhoA-specific guanine nucleotide exchange factor that interacts with microtubules. *J Biol Chem* 276, 4948-56 (2001).

Appendix I: Mean Length Distribution of Microtubules

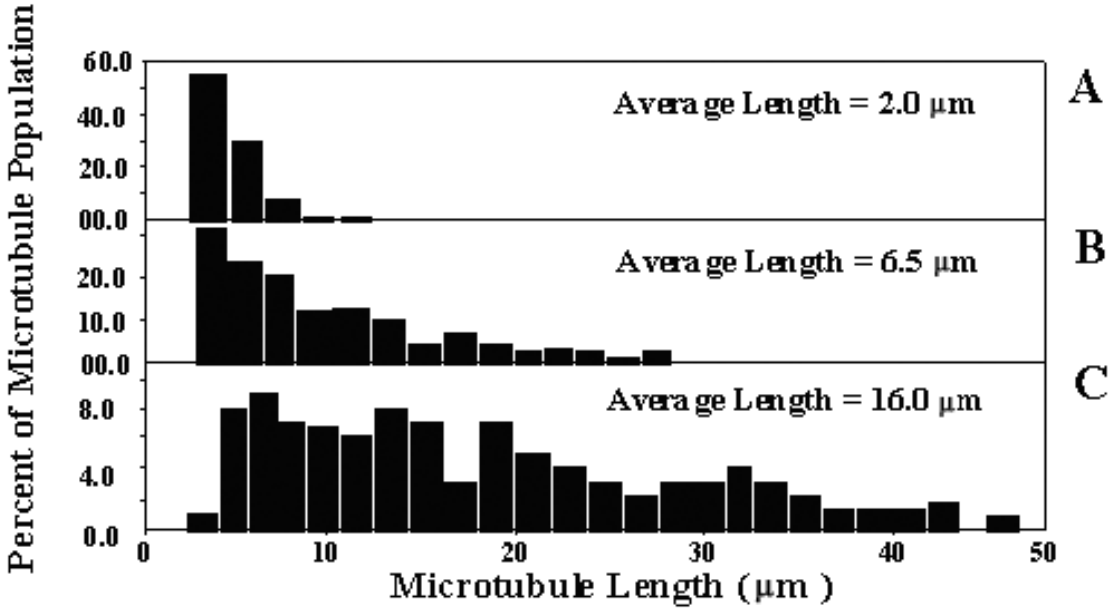

Figure 2: Mean Length Distribution of Microtubules Formed Under Various Conditions. Microtubules were polymerized using the three conditions described in Table 1. A, Microtubule population with a mean length of 2.0 µm, B, microtubule population with a mean length of 6.5 µm, and C, microtubule population with a mean length of 16.0 µm.

Section VIII: Associated Products

Tubulin Related Biochem™ Kits

Cytoskeleton Inc. supplies several alternative tubulin polymerization assays and Biochem™ Kits, consult technical service for advice on application suitability.

Assay	Cat. #	Recommended Uses
Tubulin Polymerization Assay (>99% pure tubulin) Absorbance based	BK006P	This absorbance based assay uses a tubulin preparation that is >99% pure. The assay is designed to give a maximal OD340 between 0.25 – 0.35 under the polymerization conditions given. The use of highly purified tubulin allows researchers to perform accurate IC50 and EC50 measurements on any given tubulin ligand. Kit BK006P contains sufficient reagents for 30 assays. The assay is also available in 96 assay format and HTS quantities.
Tubulin Polymerization Assay (>97% pure tubulin) Absorbance based	BK004P	This absorbance based assay uses a tubulin preparation that is >97% pure. This assay results in a maximal OD340 signal that is approximately half the intensity of the BK006P Polymerization Kit which uses 99% pure tubulin protein. Kit BK004P contains sufficient reagents for 30 assays. The assay is also available in 96 assay format and HTS quantities. This kit provides an economical means for screening large numbers of tubulin ligands and primary libraries.
Fluorescent Tubulin Polymerization Assay	BK011P	This assay incorporates a fluorescent analog that accurately reports microtubule polymer mass. Unlike the absorbance assay, the fluorescence assay performs well at very low reaction volumes (10 ul) and low concentrations of polymer. This equates to a highly sensitive and economical format. The assay is recommended for primary screens to identify tubulin ligands. The kit is sufficient for 96 reactions and HTS quantities are available (inquire)
Pre-Formed Microtubules	MT001	Pre-formed microtubules are extremely convenient for any research that requires a source of stable microtubule substrates. Our stringent quality control results in minimal batch variability and translates to highly consistent behaviour between experiments. This product is recommended for uses such as microtubule binding assays and screening for kinesin inhibitors. Microtubules are available in 2 mg (MT001-A), 10 mg (MT001-XL) and custom sizes (inquire).
Colchicine Competition Assay	CDS15	The Scintillation Proximity Assay (SPA) technology offered by Amersham has been used to create sensitive ligand binding assays for a variety of tubulins (Tahir et al. Biotechniques, 29: 156-160. 2000). Using biotinylated tubulins from neuronal (T333-XL) and cancer cell line (H003) sources, the SPA technology has been optimized to use minimal amounts of tubulin (1 ug per assay). Tritiated colchicine (NEN biosciences) has been tested and quality approved for use in CDS15. Kd and Ki values determined using these assays are similar to published values using other methods. This kit represents a valid, economical alternative for primary screens to identify tubulin ligands.
Cytoskeleton Screening Service	Custom Service	All these assays are available as a screening service performed at Cytoskeleton Inc.'s state of the art screening facility, for more information please contact Technical Service at 303-322-2254 or e-mail tservice@cytoskeleton.com .

Section VIII: Associated Products, continued

Tubulin Related Proteins and Buffers

Cytoskeleton Inc. supplies tubulin protein from a variety of species and tubulins formulated for specific uses, consult technical service for advice on application suitability. Check the web site for new products.

Product	Cat. #	Recommended Uses
Biotin Tubulin	T333	Substrate for tubulin ligands in SPA assays (see CDS15) or in development of immobilized tubulin assays for HTS. Supplied lyophilized in 20 µg or 500 µg sizes.
GTP Stock Solution	BST06	Tubulin polymerization requires GTP. It is generally used at 1 mM final concentration. When reconstituted makes a 100 mM stock. Supplied in 100 µl or 10 x 100 µl final volume sizes.
General Tubulin Buffer	BST01	Used as a general buffer for tubulin proteins. Supplied as 10 ml, 100 ml or 1 L sizes.
HTS Tubulin	HTS02	Tubulin purified from bovine brain to approximately 97% pure. This product is recommended as an economical substrate for tubulin HTS assays. Supplied as 4 mg, 40 mg or bulk quantities.
MAP-rich Tubulin	ML113	Prepared from bovine brain and contains approximately 70% tubulin and 30% microtubule associated proteins. This is an alternative substrate for HTS polymerization assays. Supplied as 1 x 1 mg, 5 x 1 mg, 10 x 1 mg, 20 x 1 mg and bulk quantities.
Microtubules, pre-formed	MT001	Developed by scientists at Cytoskeleton Inc., pre-formed microtubules are extremely convenient for any research that requires a source of stable microtubule substrates. Our stringent quality control results in minimal batch variability and translates to highly consistent behavior between experiments. This product is recommended for uses such as microtubule binding assays and screening for kinesin inhibitors. Microtubules are available in 2 mg (MT001-A), 10 mg (MT001-XL) and custom sizes (inquire).
Paclitaxel Stock Solution	TXD01	Microtubule stabilizing compound, generally used in the 1 – 10 µM range. Supplied lyophilized, when reconstituted gives 10 x 100 µl of 2 mM stock solution.
Rhodamine Tubulin	TL331	Tetramethyl rhodamine labeled tubulin. Useful for examining *in vivo* tubulin dynamics and as a substrate for in vivo or in vitro assays where it is necessary to see microtubules by fluorescence. Supplied as 5 x 20 ug or 20 x 20 ug. Labeled to a stoichiometry of 2 labels per heterodimer.
Tubulin 99% pure from bovine brain	TL238	Highly purified tubulin can be used for polymerization assays or in any situation requiring tubulin substrates. Recommended as a substrate for experiments requiring a high degree of accuracy such as EC50 or IC50 measurements. Available lyophilized (TL238) or frozen (T238) in 4 x 250 µg, 1 x 1 mg, 5 x 1 mg, 10 x 1 mg, 1 x 10 mg, 20 x 1 mg or bulk quantities.
Tubulin 99% pure from porcine brain	T240	Same as TL238 but source of tubulin is porcine brain rather than bovine. The polymerization properties of this tubulin are identical to those of the bovine tubulin.
Tubulin from HeLa cells	H001	Recommended as a more cancer specific tubulin target in tubulin ligand assays. Available in 250 µg sizes and bulk quantities.
Tubulin from MCF-7 cells	H005	Recommended as a more cancer specific tubulin target in tubulin ligand assays. Available in 250 µg sizes and bulk quantities.