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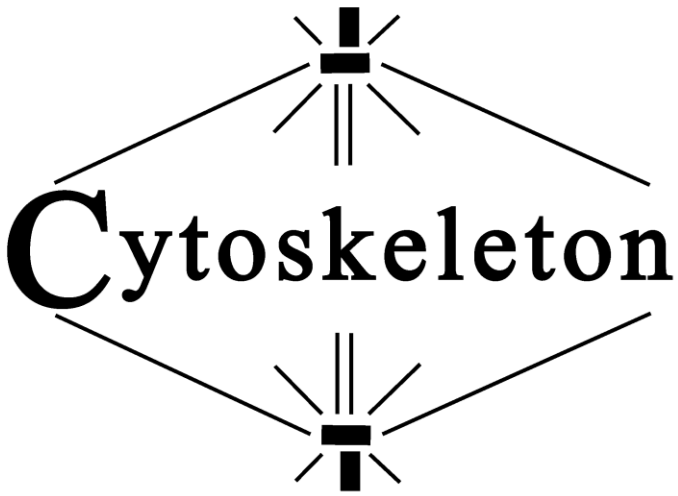

Fluorescent Microtubules Biochem Kit

Cat. # BK007R

ORDERING INFORMATION

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Section I: Introduction

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 fluorescent microtubules of a predetermined mean length and of a predetermined fluorescent intensity. After polymerization the microtubules can be used directly or stabilized with taxol before use, depending upon the needs of your experimental system. This kit is ideal for the preparation of taxol stabilized fluorescent microtubules for use in an *in vitro* motility assay.

Introduction

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 porcine brain tissue is highly homologous to tubulin isolated from any eukaryotic source. This fact results in the technical benefit that porcine 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), Drosophila melanogaster (4, 5).

Tubulin polymerizes to form structures called microtubules (MTs). When tubulin polymerizes it initially forms protofilaments, microtubules consist of 13 protofilaments and are 25 nm in diameter, each μm of microtubule length being composed of 1650 heterodimers. Microtubules are highly ordered fibers that have an intrinsic polarity, shown schematically in Figure 1A. 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.

A) B)

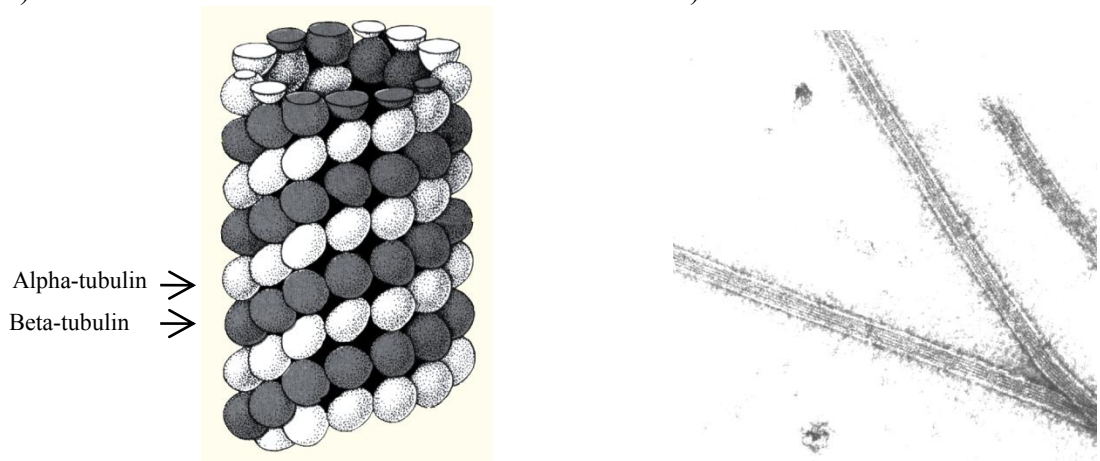


Figure 1. Microtubule structure. A) Schematic representation of a microtubule. B) Electron micrograph of in vitro assembled bovine microtubules (100,000 x magnification).

General Notes On The Handling Of Tubulin Protein And Microtubules

It is very important that you carefully read this section before beginning your experiments;

1) Tubulin protein is highly labile and should not be left at room temperature for longer than necessary. The protein should be stored on ice immediately after it has been thawed at room temperature.

2) Tubulin is not stable to many rounds of freeze/thawing. It is therefore advisable to thaw the protein once and aliquot into "experiment sized" fractions before re-freezing. This applies to both labeled and unlabeled tubulin protein.

3) Microtubules are stable at room temperature when they are in the presence of 10-20 μM taxol solution. In this state the microtubules are stable for at least 24 h.

4) In the absence of taxol microtubules are very sensitive to temperature variations. For example microtubules stored at 4°C in the absence of taxol will depolymerize into tubulin heterodimers very rapidly. Thus if you are performing experiments with non-taxol stabilized microtubules you should keep the temperature at or close to 35°C.

Section II: Kit Contents

Note: BK007R has been shipped at room temperature. Once the kit has arrived it should be stored desiccated at 4°C. Whenstored correctly, the components are stable for a minimum of 6 months. Kit contents are listed in the table below:

Reagent	Cat. # Part #	Quantity	Description
Rhodamine Labeled Tubulin	Cat. # TL590M	5 tubes	Lyophilized. 20 µg of protein per tube. Protein is labeled at a stoichiometry of 1.0 rhodamine per tubulin heterodimer.
Unlabeled Tubulin protein	Cat. # T240	2 tubes	Lyophilized. 1 mg of protein (55 kDa)
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
GTP stock	Cat. # BST06-001	1 tube	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.
Microtubule Cushion Buffer	Cat. # BST05-001	1 bottle	Liquid. 80 mM PIPES pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 60% glycerol
Antifade Solution	Cat. # BSM02	1 tube	Lyophilized. 10X concentrate when reconstituted.

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

Reagents and Components Not Supplied:
100% Glycerol
Beta-mercaptoethanol

Necessary Equipment:
1. Ultracentrifuge capable of centrifuging 50-200 µl volumes at 100,000 x g at 4°C and 24°C. Examples are:
    1. Beckman Airfuge with Ultraclear tubes (Beckman, Cat. # 344718).
    2. SW50 ultracentrifuge rotor with adapters for Ultraclear tubes (Beckman, Cat. # 344718).
    3. Tabletop ultracentrifuge (Beckman) with TLA-100 rotor.
2. Water bath set to 35°C
    3. Fluorescent microscope. The excitation and emission peaks for TL590 tubulin are Ex:547 nm, Em:572 nm.

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:
NOTE: read through this section carefully prior to beginning the preparation of reagents as you will require items readily on hand such as liquid nitrogen. If the proteins in this kit are not aliquoted as specified biological activity may be lost. When reconstituted and stored correctly, the components have a shelf life of at least 6 months.

Component	Reconstitution	Storage
GTP (Cat. # BST06)	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 lyophilized material desiccated at 4°C. • Store reconstituted material at -70°C.
General Tubulin Buffer (Cat. # BST01)	1) For 1X buffer, resuspend in 10 ml of sterile distilled water. Store at 4°C.	• Store lyophilized buffer desiccated at 4°C. Stable for 6 months. • Store solution at 4°C.
Taxol (Cat. # TXD01)	1) For a 2 mM stock solution, reconstitute in 100 µl of anhydrous DMSO. Store at -70°C. WEAR GLOVES WHEN HANDLING TAXOL.	• Store lyophilized product desiccated at 4°C. • Store solution at -70°C.
Antifade (Cat. # BSM02)	1) For a 10X concentrate, reconstitute in 1 ml of 50% glycerol plus 1.5% beta-mercaptoethanol and aliquot into 10 x 100 µl aliquots. Snap freeze aliquots in liquid nitrogen and store at -70°C.	• Store lyophilized product desiccated at 4°C. • Store solution at -70°C.
Rhodamine Tubulin Protein (Cat. # TL590M)	**See reconstitution instructions in Section IV, Step A and B.**	• Store lyophilized protein desiccated at 4°C. • Store resuspended protein frozen at -70°C.
Unlabeled Tubulin Protein (Cat. # T240)	**See reconstitution instructions in Section IV, Step B.**	• Store lyophilized protein desiccated at 4°C. • Store resuspended protein at -70°C.

Section IV: Protocol Description

Preparation of Taxol Stabilized Fluorescent Microtubules

The following protocol describes the formation of taxol stabilized fluorescent microtubules. The microtubules will be an average of 10 μm long and have a stoichiometry of 1.0, 0.33 or 0.17 dyes per tubulin heterodimer. These microtubules are an ideal substrate for *in vitro* motility assays when they are viewed by a CCD camera or similar image enhancing device. This protocol should be taken as a starting point for optimizing your microtubule preparation. You may need to alter the average microtubule length or brightness for optimal results in your experimental system. Details of how to do this are given in later sections.

The preparation of taxol stabilized fluorescent microtubules should take approximately 1 h to complete. The steps involved are;

1. Dilution of tubulin stocks to give diluted fluorescent tubulin (10 minutes).
2. Polymerization of tubulin into microtubules (20 minutes).
3. Stabilization of microtubules with taxol (5 minutes).
4. Separation of microtubules from background fluorescence (35 minutes). This step may not be necessary (see text).

After the microtubules are prepared they are stable for several hours at room temperature.

A detailed protocol is given below:

Step A: Preparing *in vitro* polymerization competent tubulin:

Take one aliquot of rhodamine tubulin (Cat. # TL590M) and place on ice. Resuspend the labeled tubulin to 5 mg/ml by adding 4.0 μl of cold G-PEM (General Tubulin Buffer, Cat. # BST01 plus 0.01 volumes of 100 mM GTP, Cat. # BST06) and mix well. Add 1 μl of cold Microtubule Cushion buffer (60% glycerol, Cat. # BST05) to make a final 12% glycerol solution. The fluorescent tubulin is now at approximately 5 mg/ml in 12% glycerol G-PEM and they are ready to polymerize as full intensity fluorescent microtubules (see Step C). If less intense fluorescence is needed or more economical utilization of the fluorescent tubulin is required then follow the next procedure for diluting the fluorescent tubulin with unlabeled tubulin.

Step B: Diluting fluorescent tubulin:

Perform Step A, and then take one 1 mg vial of unlabeled tubulin (Cat. # T240) and resuspend with 180 μl of cold G-PEM and place on ice. Add 20 μl of Microtubule Cushion Buffer and mix well. Aliquot 39 x 5 μl of this tubulin into separate tubes, freeze in liquid nitrogen and store at -70°C, these can be used for future experiments. With the remaining 5 μl of unlabeled tubulin divide into two tubes 2.5 μl each on ice. To the first tube add 1 μl of fluorescent tubulin tube and mix well, to the second add 0.5 μl fluorescent tubulin and mix well. These dilutions represent your 0.33 and 0.15 labeling ratio stock solutions (dyes per tubulin subunit 110 kDa). These stocks can be made on a larger scale by using a whole tube of fluorescent tubulin at one time then by aliquoting 2

μl per tube, freezing in liquid nitrogen and store at -70°C; these can be used for future experiments.

Step C: Polymerizing fluorescent microtubules:

Polymerize by incubating at 35°C for 20 minutes. While the tubulin is polymerizing, place 500 μl of General Tubulin Buffer (Cat. # BST01) at 35°C. Thaw out the tube containing 2 mM taxol (use DMSO provided for solvent), once taxol has thawed leave at room temperature until required. NOTE: Always wear gloves when handling taxol. After 15 minutes, remove the General Tubulin buffer from 35°C and add 5 μl of taxol (final taxol concentration is 20 μM), this solution should be kept at room temperature and labeled Taxol/Microtubule Buffer. Remove the polymerized tubulin from 35°C and immediately add 100 μl of the Taxol/Microtubule Buffer solution, mix thoroughly but gently. This step should result in a population of taxol stabilized microtubules that average 10 μm in length and are at a concentration of 7×10^{10} per ml.

Step D: Analyzing fluorescent microtubules:

Examine a small aliquot of the microtubules by fluorescence microscopy (1 μl of microtubules diluted into 10 μl of Taxol/Microtubule Buffer containing 1x antifade solution (Cat. # BSM02). Individual microtubules should be clearly visible; however there may also be some noticeable background fluorescence. The background fluorescence results from unpolymerized tubulin and can be removed by passing the microtubules over a glycerol cushion, as described below. NOTE: If background is very low steps E-I can be omitted.

Step E:

Aliquot 400 μl of Microtubule Cushion Buffer (Cat. # BST05 plus 0.01 volumes of GTP, Cat. # BST06) into a microfuge tube and incubate at 35°C for 10 minutes. Remove from incubation and add 4 μl of taxol stock solution. Aliquot the Cushion Buffer (plus taxol) into a 500 μl ultracentrifugation tube, such as a Beckman Ultraclear centrifuge tube (Cat. # 344090). Carefully layer the microtubule solution onto the cushion. Centrifuge at 100,000 x g at 35°C for 45 minutes to pellet the microtubules through the cushion.

NOTE: Several centrifugation systems are suitable for this step. The one recommended in this protocol uses a Beckman SW 50.1 rotor and Ultraclear tubes plus adaptors. This can be substituted by a Beckman Airfuge. The Airfuge the rotor requires tubes capable of holding 200 μl volumes. In this case you should add 30 μl of Taxol/Microtubule Resuspension Buffer in step E. When using an Airfuge the cushion volume should be 120 μl.

Step F:

Immediately after centrifugation, carefully remove the first 100 µl of solution and discard. Wash the cushion / microtubule solution interface with 2 x 100 µl of Taxol/Microtubule Buffer. This is designed to remove the remaining background fluorescence (unincorporated tubulin) from the cushion interface, therefore allow a little mixing of the cushion interface and the buffer. This step should be carried out quickly as you do not want to leave the microtubule pellet at the bottom of the tube for too long as it will loosen and may dislodge during removal of the cushion.

Step G:

Remove the remaining cushion buffer and discard. Again, this step should be carried out as soon as possible after centrifugation and care should be taken so as not to dislodge the microtubule pellet. The pellet may be visible due to its faint pink coloration; however you should not be unduly worried if you can not see the pellet at this stage.

Step H:

Resuspend the microtubule pellet in 100 µl of Taxol/Microtubule Buffer. Resuspension should be gentle and thorough (pipette up and down 7-10 times and gently scrape the bottom of the tube with a pipette tip that has had the first 2 mm of its tip cut off). This taxol stabilized microtubule population will remain stable at room temperature for several hours.

Step I:

The microtubule population can be viewed by fluorescence microscopy as described in step D. At this point you can substitute the General Tubulin Buffer for a buffer of your choice. All buffers must however be supplemented with 10-20 µM taxol and 1x antifade solution. The antifade is present to minimize the photo-damage that occurs to fluorescent microtubules when illuminated under the fluorescent microscope. It is recommended that all experiments be carried out with the minimum of fluorescence intensity. For prolonged periods of viewing (>3 minutes) it is recommended that the microtubules are viewed by time lapse microscopy. Microtubules should be approximately 10 µm long and there should be no background.

Section V: Altering The Ratio Of Fluorescent Dye In The Microtubules

In some cases the microtubules resulting from the conditions described in the previous section are not optimal for a particular experimental system. For example, one may require microtubules that are slightly brighter or dimmer than those resulting from the standard 0.33 labeling ratio stocks described above. The following table gives the dilutions that are required to make stocks of varying fluorescence intensity. The final column in this table indicates the number of 2 µl aliquots that can be made from one kit when all of the fluorescent tubulin is aliquoted.

Table 1. Dilution Table for Fluorescent Tubulin

Fluorescent Tubulin at 5 mg/ml (µl)	Unlabeled Tubulin at 5 mg/ml (µl)	Stoichiometry of Label	Number of 2 µl Aliquots from one Kit
4	-----	1.00	10
4	4	0.50	20
4	8	0.33	30
4	12	0.25	40
4	16	0.20	50
4	20	0.17	60

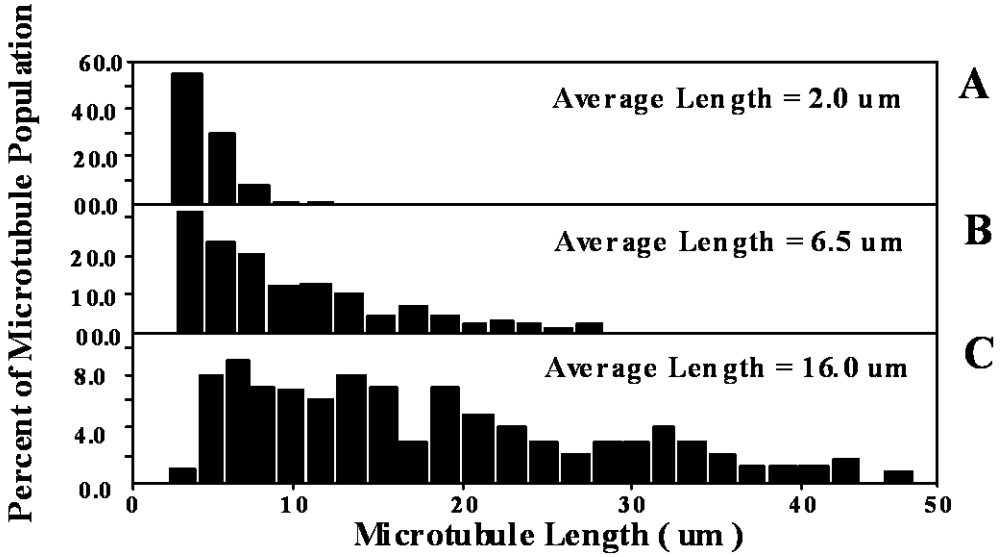
Section VI: Altering The Average Length Distribution Of The Microtubules

In some cases the microtubules resulting from the conditions described in the previous section are not optimal for a particular experimental system. For example, one may require microtubules that are longer or shorter in average length than those resulting the standard polymerization conditions described above. Table 2 gives the polymerization conditions that are required to create microtubules of a given average length. Components of the reaction should be added in the order that they appear reading from left to right. Figure 2 shows actual length distributions of microtubule populations under the various polymerization conditions described in Table 2. It should be noted that all tubulin stock solutions that you are working with (including those that you create of a given stoichiometry) are at an initial protein concentration of 10 mg/ml.

Table 2. Selected Conditions For Microtubule Polymerization

Average Microtubule Length Required (μm)	10 mg/ml Tubulin Stock (μl)	General Tubulin Buffer (μl)	Microtubule Cushion Buffer (ul)	Time of Incubation at 35°C (min)
2.0	2.0	0	2.0	10
6.5	2.0	0.6	1.4	20
16	2.0	1.7	0.3	30

Figure 2. *Mean Length Distribution Of Microtubules Formed Under The Three Conditions Shown Above*



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