HTS-Tubulin Polymerization Assay Biochem Kit™

(*>97% pure tubulin, Porcine Tubulin*)

Cat. # BK004P

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Manual Contents

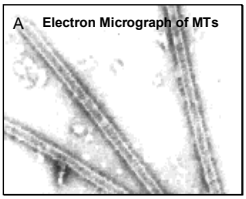
I: Introduction

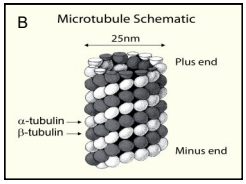
About Tubulin

Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called alpha and beta tubulin. The proteins are highly conserved throughout the eukaryotic kingdom. Consequently, tubulin isolated from porcine brain tissue can be used to assay proteins originating from many diverse species. Porcine (and bovine) tubulin has also been used extensively in the identification of human therapeutics (see below).

Figure 1: Microtubule Structure

Tubulin polymerizes to form structures called microtubules (MTs) (**Figure 1A**). When tubulin polymerizes it initially forms proto-filaments, MTs consist of 13 protofilaments and are 25 nm in diameter, each µm of MT length is composed of 1650 heterodimers (1). Microtubules are highly ordered fibers that have an intrinsic polarity, shown schematically in **Figure 1B**. Tubulin can polymerize from both ends, however, the rate of polymerization is not equal. The rapidly polymerizing end is termed the plus-end of a microtubule and the slowly polymerizing end the minus-end.





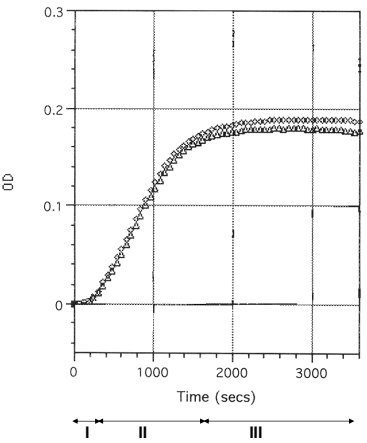
In vivo, microtubules, along with actin microfilaments and intermediate filaments form the structural basis of the eukaryotic cytoskeleton. This highly dynamic structure is essential to many cellular functions including cell shape, motility and intracellular transport. Regulation of microtubule dynamics is orchestrated via a plethora of proteins and is an active area of study. Microtubules are also the major structural component of the mitotic spindle and are critical to cell division. Tubulin is therefore the target of several clinically important anti-mitotics such as taxol and vinblastine (2,3). These drugs work by directly suppressing microtubule dynamics during mitosis (4). Specificity towards dividing cells is favored due to the fact that microtubule dynamics are much greater in mitotic cells than quiescent ones. Drug treatment results in a mitotic block during which time the cells enter into the apoptotic pathway and die (5).

Tubulin polymerization assays are a powerful tool for characterizing MT/drug and MT/protein interactions.

About the BK004P Polymerization Assay

The tubulin polymerization assay is based on an adaptation of the original method of Shelanski et al. and Lee et al. (6,7) which demonstrated that light is scattered by microtubules to an extent that is proportional to the concentration of microtubule polymer. The resulting polymerization curve is representative of the three phases of microtubule polymerization, namely nucleation (**I in Figure 2**), growth (**II in Figure 2**) and steady state equilibrium (**III in Figure 2**). The tubulin used in this assay (Cat. # HTS03) has been purified from porcine brain and consists of approximately 97% tubulin and 2% microtubule associated proteins (MAPs). This assay has been designed to give a standard polymerization yielding approximately 40-45% polymer mass (see **Figure 1**). This allows the polymerization reaction to be highly sensitive to both polymerization enhancers (e.g. paclitaxel, MAPs) and polymerization inhibitors (e.g. nocodazole) (see **Figure 3**).

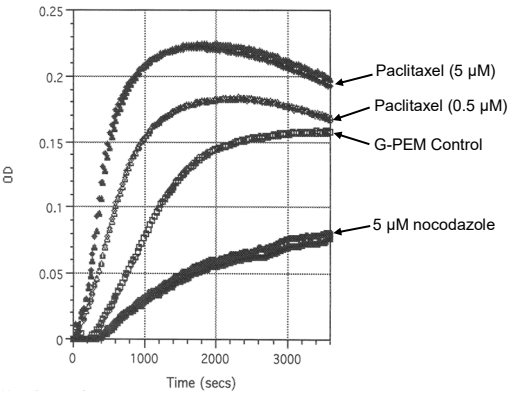
Figure 2: Standard Tubulin Polymerization Curve



Legend: Standard polymerization reactions (minus tubulin ligands) were carried out as described in the Polymerization Protocol (Section VI). Briefly, the standard polymerization reaction contains 100 µl volume of 4 mg/ml tubulin in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM MgCl2, 1 mM GTP, 5% glycerol. Polymerization was started by incubation at 37° C and followed by absorption readings at 340 nm. Under these conditions polymerization will reach a maximal OD340 between 0.15 – 0.25 within 30 minutes. The three phases of polymerization are shown: I (nucleation), II (growth), III (steady state). In this experimental set up (100 µl volume in a spectrophotometer with a 0.5 cm pathlength) an OD340 of 0.1 is approximately equal to 1 mg per ml of polymer mass. Thus under the conditions described, approximately 45% of the tubulin is polymerized, leaving flexibility for detecting enhancers and inhibitors of polymerization. Reaction conditions can be altered to make the assay more sensitive for either enhancers or inhibitors of tubulin polymerization (see **Section VII**).

The BK004P polymerization assay is suitable for screening large numbers of tubulin ligands and primary libraries. Compounds or proteins that interact with tubulin will often alter one or more of the characteristic phases of polymerization. For example, **Figure 3** shows the effect of adding the anti-mitotic drug paclitaxel to a standard polymerization reaction. At 5 µM paclitaxel the nucleation phase is eliminated and the growth phase is enhanced. Therefore, one application of this assay is the identification of novel anti-mitotics. BK004P has been used to identify novel compounds which are potentially useful in anti-cancer applications (8,9). **Figure 3** also shows the effect of adding the microtubule depolymerizing drug, nocodazole. At 5 µM nocodazole the Vmax is a 2.2 fold reduction in the Vmax and a significant reduction in the final polymer mass.

Figure 3: Tubulin Polymerization in the Presence of Tubulin Ligands



Legend: Standard polymerization reactions alone (G-PEM plus 5% glycerol standard assay control) and in the presence of 5 µM paclitaxel, 0.5 µM paclitaxel and 5 µM nocodazole. The Vmax value is enhanced 4 fold in the presence of paclitaxel and decreased 2.2 fold in the presence of nocodazole.

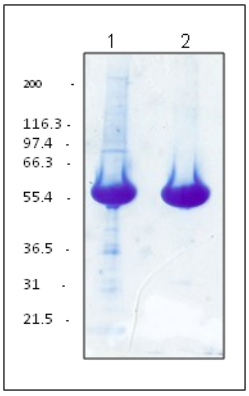
Each kit contains sufficient reagents for 24 - 30 standard assays (see **Section VI**). Generally, using a multichannel pipette results in 24 assays due to some wastage of tubulin protein and single channel pipettes give 30 assays. The use of lyophilized tubulin allows the kit to be stored at 4°C (<10% humidity) prior to use.

Comparison of Polymerization Assays

Cytoskeleton Inc. offers several tubulin polymerization Biochem™ Kits. **Table 1** outlines the differences between the assays. The information below is also useful for comparative purposes:

1) **Nomenclature**: BK004, BK006 contain tubulin derived from bovine brain while BK004P, BK006P and BK011P contain tubulin derived from porcine brain. Tubulins from either source are indistinguishable in all assays tested (see **Appendix 1**).
2) **Glycerol**: Glycerol is utilized in many *in vitro* tubulin polymerization assays and acts as an enhancer of polymerization. The inclusion of glycerol in a standardized assay allows one to use less tubulin protein per assay. In rare cases glycerol may interfere with the binding of a given tubulin ligand and glycerol must be omitted (see **Table 1**).
3) **Tubulin purity**: Tubulin in BK004/BK004P is approx. 97% pure and contains approx. 2% microtubule associated proteins (MAPs) (**Figure 4**). MAPs are advantageous in that they promote efficient polymerization at low tubulin concentrations even in the absence of glycerol. The tubulin in BK006/BK006P and BK011P is 99% pure and is the preferred substrate when unambiguous identification of tubulin as the target protein is required and for IC50/EC50 determinations (**Figure 4**).
4) **Assay optimization for polymerization enhancers:** The standard assay described in each kit aims to create conditions that can identify enhancers and inhibitors. Generally lower tubulin concentrations and/or lower glycerol concentrations can be used for specific characterization of enhancers (see **Section VII**).
5) **Assay optimization for polymerization inhibitors**: Generally higher tubulin and/or glycerol concentrations are required for characterization of inhibitors (see **Section VII**).

Figure 4: Tubulin Purity



Legend: Lane 1, 97% pure tubulin from BK004P; Lane 2, 99% pure tubulin from BK006P and BK011P. Each lane shows 50 µg of protein stained with coomassie blue dye. Tubulin protein runs at 55 kD.

Table 1: Comparison of Tubulin Polymerization Assays

Assay Characteristics	BK004/BK004P	BK006/BK006P	BK011/BK011P
Assay detection method	Absorbance (340 nm)	Absorbance (340 nm)	Fluorescence (Ex 340-360 nm; Em 410-460 nm)
Tubulin purity	>97%	>99%	>99%
Tubulin used per assay	400 µg (total protein)	300 µg	100 µg
Volume of reaction	100 µl	100 µl	50 µl
Coefficient of variation (cv)*	14%	13%	11%
Glycerol required for standard assay conditions	Yes	Yes	Yes
Can assay conditions be adjusted to omit glycerol	Yes	Yes	Yes
Number of standard assays per kit**	24 - 30	24 -30	96
Relative cost per standard assay	Approximately 3X more expensive per assay than BK011P	Approximately 4X more expensive per assay than BK011P	Most cost effective assay

* Duplicate samples, under standard assay conditions

**Standard assay conditions are described in the Assay Protocol for each kit.

II: Purchaser Notification

Limited Use Statement

The products in this kit are based on technology developed at Cytoskeleton Inc. and are the subject of patents assigned to Cytoskeleton Inc. (Patent# 6,750,330). The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

III: Kit Contents

This kit contains sufficient reagents for 24 - 30 standard assays. Generally, using a multi-channel pipette results in 24 assays due to some wastage of tubulin protein and a single channel pipette give 30 assays. The use of lyophilized tubulin allows the kit to be stored at 4°C. When properly stored and reconstituted, kit components are guaranteed stable for a minimum of 6 months. **Table 2** summarizes the kit contents (see **Table 3** for reconstitution).

Table 2: Kit Contents

Reagents	Cat# or Part#*	Quantity	Storage Conditions
HTS Porcine Tubulin Protein, >97% pure	Cat. # HTS03-A	3 tubes, 4.0 mg per tube, lyophilized	Desiccated 4°C
Tubulin Glycerol Buffer (Cushion Buffer)	Cat. # BST05-001	1 bottle, 10 ml	4°C
GTP Stock	Cat. # BST06	2 tubes, lyophilized	Desiccated at 4°C
Paclitaxel Stock	Cat. # TXD01	1 tube, lyophilized	Desiccated at 4°C
General Tubulin Buffer	Cat. # BST01-001	1 bottle, lyophilized	Desiccated at 4°C
DMSO	Part # DMSO	1 tube, 1ml	4°C; product will freeze at 4°C
Half area 96-well plate	Corning Part # 3697	1 plate	Room temperature

*Items with part numbers (Part #) are sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.

The reagents and equipment that you will require but are not supplied:

- Spectrophotometer: capable of reading a 96 well plate in kinetic mode and temperature regulated to 37°C.
- Tubulin ligands of interest (note paclitaxel is supplied as a positive control ligand)
- Multichannel or repeat pipettor (see **Section V**)

IV: Reconstitution and Storage of Components

Many of the components in this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as shown in **Table 3**.

Table 3: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
General Tubulin Buffer (Cat# BST01-001)	Reconstitute with 10 ml of distilled water.	4°C
Tubulin Glycerol Buffer (Cushion Buffer) (Cat. # BST05-001)	No reconstitution necessary.	4°C
GTP Stock (Cat. # BST06-001)	1. Reconstitute each tube with 100 µl of ice cold distilled water for a 100 mM stock solution. 2. Place on ice. 3. Aliquot into 10 x 10 µl volumes.	- 20°C or - 70°C
Tubulin protein (Cat. # HTS03-A)	Before reconstitution see Standard Assay conditions **Section VI**. Make aliquots of a 10 mg/ml tubulin stock as follows; a) Reconstitute each tube in 400 µl of 4º General Tubulin Buffer. b) Add 4 µl of GTP stock, mix well. c) Aliquot into 200 µl volumes (4 assays) and immediately snap freeze in liquid nitrogen and store at –70ºC or –80ºC.	
Paclitaxel Stock (Cat. # TXD01)	Reconstitute the tube of paclitaxel with 100 µl of DMSO for a 2 mM stock solution.	-70°C or - 20°C

V: Important Technical Notes

**The following technical notes should be read carefully prior to beginning the assay.*

A) **Notes on Updated Manual Version**

The following updates from version 7.0 should be noted:

The manual has been re-written to clarify the assay protocol and the use of glycerol in polymerization assays.

B) **Spectrophotometer Settings**

Polymerizations are followed by an increase in absorbance at 340 nm over a 60 minute period at 37°C. A temperature regulated spectrophotometer capable of achieving 37°C and reading at 340nm in kinetic mode is required. The assay is designed for a 96 well microtiter plate format and therefore the spectrophotometer should be able to handle 96 well plates. An example of the settings using a Molecular Devices SpectraMax 250 instrument are given in the following table. This machine uses a monochromatic light source.

Instrument Settings for SpectraMax 250:

Parameter	Setting
Measurement Type	Kinetic, 120 cycles of 1 reading per 30s
Absorbance Wavelength	340 nm If a filter based system is being used then filters between 340—405 nm will work. Signal is optimal at 340 nm and will decrease by 50% at 405 nm. Filters should preferably have a bandwidth less than 20 nm.
Temperature	37°C
Shaking	Once at start of reaction, 5 sec medium, orbital. Do not shake before or after each read.
Designation of Blank	Blanks are not needed. The SpectraMax 250 will automatically read zero at the beginning of the reactions. Other plate readers may require data to be exported into Excel for data processing. Contact tservice@cytoskeleton.com for a free Excel template file.

C) **Spectrophotometer Pathlength**

When using a microtiter plate compatible spectrophotometer the readings are taken from the top of the plate and therefore the volume of your reaction will directly influence the pathlength. The assay volume in this kit is 100 µl and assumes a spectrophotometer pathlength of 0.5 cm when used with a half area plate, provided in kit.

D) **Temperature and Time Dependence of Polymerization**

Tubulin polymerization in this assay is regulated by temperature. At 37°C tubulin will polymerize into microtubules. At 4°C microtubules will depolymerize to tubulin subunits.

The polymerization reaction is started by the increase in temperature from 4°C to 37°C upon transfer of the protein from ice to a pre-warmed plate (see **Assay Protocol Section VI**). The spectrophotometer must therefore be temperature regulated and set at **37°C**. Temperatures cooler than 37°C will decreased the rate of polymerization (longer nucleation phase, shallower growth phase, see **Figure 2**) and the final OD reading (generally 5% loss of steady state polymer per degree reduction in temperature). Also, if temperature is not uniform across a plate, variation between samples will be high. It is essential to PRE-WARM plates for reproducible results.

E) **Recommended Pipetting Technique**

I. Because the tubulin in this kit (Cat. # HTS03-A) contains some microtubule associated proteins (MAPs), it has a rapid nucleation phase under the standard conditions given in this manual (approx. 3 minutes, see **Figure 2**). Pipetting into the microtiter plate wells therefore needs to be rapid. In cases where more than 4-5 samples are being analysed, we strongly recommend the use of a multichannel pipette.

II. Each standard polymerization assay utilizes 100 µl of tubulin. For efficient pipetting, determine the number of assays required then for each assay place 120 µl of tubulin solution into the well of a microtiter plate on ice. Aliquot 100 µl of the tubulin from the 4°C to a 37°C plate using an 8-channel pipettor. Alternatively, use a multi-dispensing pipettor that will dispense 8 x 100 µl from a single tip.

III. It is important to avoid bubbles forming in the wells after pipetting. This leads to incorrect baseline referencing at time = zero, when the bubbles later burst, the optical density decreases rapidly which will create false positive readings. Bubbles form when incorrect pipetting height or pipetting technique are used. Use a low pipette tip height and a quick to medium pipetting out-flow rate and do not “blow out” at the end of the pipette motion.

F) **Tubulin Protein Stability**

I. Tubulin is a labile protein and should be used immediately after resuspension or snap frozen into appropriate aliquots (see Reconstitution and Storage of Components **Section IV**).

II. Frozen tubulin stock should be at a protein concentration above 5 mg/ml and preferably at 10 mg/ml.

III. Lyophilized tubulin should be stored at 4°C and kept dry in a desiccant chamber.

IV. Freeze/thaw cycles should be avoided.

V. Keep tubulin on ice prior to beginning a polymerization reaction.

VI. Any buffer containing GTP should be kept on ice and used within 1-2 h after addition of GTP as GTP will hydrolyze over time. Unused GTP supplemented buffer should be discarded.

G) **Test Compound or Protein Preparation**

Dimethyl sulphoxide (DMSO) is the recommended solvent for stocks of small molecule tubulin ligands. A 2 mM solution of your compound in DMSO is the optimal starting material. The stock should then be diluted in aqueous solution to the desired 10X concentration. If it is not possible to solubilize your compound at this concentration, then you can substitute ethanol for DMSO, or try 200 µM solution directly in 80 mM PIPES pH 6.9.

For tubulin binding proteins a 10X final concentration in General Tubulin Buffer (80 mM PIPES pH 6.9, 0.5 mM EGTA, 2.0 mM MgCl2, Cat. # BST01) is recommended. General guidelines for tubulin compatible buffers are given below:

a. Keep pH between 6.5—7.0.

b. Do not use calcium containing buffers as calcium is a potent depolymerizer of tubulin.

c. Try to avoid using sodium chloride in the buffer. If this is necessary, keep concentrations below 30 mM.

Polymerization Assay Method: Standard Reaction Conditions

The assay takes approximately 1.5 h to complete. Tubulin polymerization is controlled by temperature so pay particular attention to this parameter during the assay and read all instructions carefully. The Standard Polymerization Assay described below results in an assay that is sensitive to polymerization enhancers and inhibitors (see also **Figure 3**). The assay is divided into a three step process; Step 1: Preparation of Assay Reagents, Step 2: Recommended Control Reactions, Step 3: Polymerization Step. Read all three steps before beginning the assay.

STEP 1: Preparation of Assay Reagents

1. Turn on spectrophotometer and enter experimental parameters as described in **Section V.B**: Spectrophotometer Settings.
2. Place the 96-well plate supplied in this kit into the spectrophotometer and allow to warm to 37°C for 30 minutes prior to starting the assay. A warm plate is essential for high polymerization activity and reproducible results.
3. Warm the required amount of General Tubulin Buffer to room temperature. Warm buffer is needed for tubulin ligand dilutions (see STEP 1: 6 and STEP 2: 2).
4. Make the required amount of COLD (4°C) G-PEM buffer plus 5% glycerol, sufficient to dilute the tubulin stocks (see STEP 1: 5). The recipe is given below.

For 1 ml G-PEM Buffer plus 5% Glycerol:

Component	Volume	Final Concentration
General Tubulin Buffer	906.7 µl	80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 0.5mM EGTA
GTP Stock (100mM)	10 µl	1 mM GTP
Tubulin Glycerol Buffer	83.3 µl	5% glycerol

*NOTE: G-PEM buffer is labile due to hydrolysis of GTP; it should be kept on ice and used within 1-2 hours or preparation. Any unused buffer should be discarded.

5. Preparation of Tubulin

a) From lyophilized powder:

If all of the tubulin in a 4 mg vial is to be used in one experiment, then the tubulin can be resuspended to 4 mg/ml as shown below and used immediately;

I. Resuspend each 4 mg tube of tubulin (HTS03) with 1 ml of ice cold G-PEM buffer plus 5% glycerol to give a final protein concentration of 4 mg/ml. This amount of tubulin is sufficient for 8-10 standard polymerization reactions.

II. Place the tube on ice and allow 3 min. for the complete resuspension of the protein. **Place the tubulin on ice and use immediately.** It is not recommended to freeze 4 mg/ml tubulin: frozen stocks should be a minimum of 5 mg/ml and optimally 10 mg/ml (see **Section IV** and below).

b) From frozen tubulin stock:

I. Preparation of frozen tubulin stock is described in **Section IV**: Reconstitution and Storage of Components. The frozen stock is at a protein concentration of 10 mg/ml.

II. Thaw out the required amount of tubulin protein stock in a room temperature water bath. Once thawed, place immediately on ice.

III. Dilute the tubulin stock to 4 mg/ml using ice cold reagents as follows:

200 µl	Tubulin stock (10 mg/ml) in G-PEM buffer
41.7 µl	Tubulin Glycerol Buffer (to give 5% final glycerol)
255.3 µl	General Tubulin Buffer
3 µl	GTP Stock

6. Prepare your compound or protein of interest at 10X strength in G-PEM or another uitable buffer (see **Section V**: Important Technical Notes; Section G).

STEP 2: Recommended Control Reactions: Standard Reaction

Recommended control reactions are shown below. They can be performed as single reactions or in duplicate:

1. Tubulin in G-PEM buffer only plus 5% glycerol. This gives the standard assay control polymerization.
2. Tubulin plus 10 µM paclitaxel. This gives an enhancer control polymerization. Preparation of the paclitaxel stock is described below:

Dilute 5 µl of the 2 mM paclitaxel stock solution with 95 µl of ROOM TEMPERATURE General Tubulin Buffer (100 µM final). Note: the taxol stock must be diluted into room temperature buffer as dilution into 4°C buffer will cause the paclitaxel to precipitate out of solution. Diluted paclitaxel should be kept at room temperature and used within 6 h. Unused paclitaxel should be discarded.

STEP 3: Polymerization Step: Standard Reaction

1. Pipette 10 ul of room temperature General Tubulin Buffer into the wells of the pre-warmed plate that represent the standard assay control polymerization.
2. Pipette 10 µl of room temperature paclitaxel control (100 µM) into the wells of the pre-warmed plate that represent the enhancer control polymerization.
3. Pipette 10 µl of your 10x strength compound or protein into the wells of the pre-warmed plate representing your experimental samples.
4. Incubate the plate for 2 min. at 37°C.
5. Pipette 100 µl of tubulin (4 mg/ml in G-PEM/5% glycerol) into the required number of wells. Immediately place the plate in the spectrophotometer at 37°C and start recording using the kinetic settings described in **Section V**: Spectrophotometer Settings.

Interpretation of Data

Under standard reaction conditions the standard assay control polymerization (minus tubulin ligands) should achieve a maximal OD_{340} between 0.15 – 0.25 within 30 min at 37°C (see **Figures 2 and 3**).

Several parameters can be used to quantitate the response of tubulin to a given compound or protein. For example the addition of paclitaxel to 5-10 µM final concentration will reduce the nucleation phase, enhance the Vmax approximately four fold and increase maximum OD of the reaction. The microtubule destabilizing drug, nocodazole, will reduce nucleation and the Vmax 2.2 fold and decrease polymer mass approximately two fold. Any or all of these parameters can be quantified to compare different samples.

For screening applications, we recommend using the Vmax value as this generally changes to a greater extent and offers the most sensitive indicator of tubulin / ligand interactions.

VII: Assay Optimization

The polymerization reaction conditions of 4 mg/ml tubulin in G-PEM buffer plus 5% glycerol creates a polymerization reaction that is sensitive to de-polymerizing agents such as nocodazole and to polymerization enhancers such as taxol (see **Figure 3**).

To further sensitize the reaction to polymerization enhancers such as taxol and to create a more cost effective assay, one might consider polymerizing tubulin at 3 mg/ml in G-PEM buffer minus glycerol.

Tubulin Glycerol Buffer (Cat. # BST05-001, contains 60% glycerol) has been included in this kit. It can be used to enhance polymerization of very low HTS03 tubulin concentrations, e.g. 2mg/ml in G-PEM plus 10% glycerol. This would be a cost effective way to identify polymerization inhibitors. It should be noted , however, that the presence of glycerol may decrease sensitivity of the assay to inhibitors.

VIII: References

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IX: Troubleshooting Guide

Problem	Possible Solution
No polymerization curve is seen for the tubulin plus paclitaxel sample	1. Polymerizations should be read at 340nm, make sure you have your spectrophotometer set to these wavelengths. 2. To measure polymerization the spectrophotometer needs to be set in kinetic mode to read once every 30 seconds to 1 minute for 1 h. 3. Your tubulin may be inactive. This can be caused by incorrect freezing of the protein. The tubulin stock should be rapidly snap frozen in liquid nitrogen at 10 mg/ml in general tubulin buffer plus 1 mM GTP (G-PEM) . Tubulin stocks should not be frozen/thawed more than once. 4. Your tubulin protein may be inactive. If you have allowed the lyophilized tubulin to become damp, it will rapidly denature. You should store the tubulin desiccated at 4°C. 5. The tubulin may have already polymerized in the tube. Tubulin prior to addition to the 96 well plate must be kept at 4°C, otherwise it will begin to polymerize. This is particularly true before the protein is diluted as high tubulin concentrations favor polymerization; particular care should therefore be taken ensuring the thawing step for tubulin stock protein is rapid and that the thawed tubulin stock is IMMEDIATELY transferred to ice and diluted in ICE COLD polymerization buffer. Polymerized tubulin will appear opaque. 6. The tubulin polymerization may be completed before you begin reading of the plate. Once tubulin is added to the plate begin reading immediately. Taxol causes rapid tubulin polymerization (See Figure 3). Readings should be taken once every 30 s to 1 min. 7. The paclitaxel may not be active. This can happen if you dilute the paclitaxel stock into cold buffer as it will precipitate out of solution. ALWAYS dilute the paclitaxel into room temperature or 37°C buffer.
No polymerization or long nucleation phase is seen in the standard assay control polymerization samples.	1. See 1-5 above. 2. The polymerization of this tubulin reaction is far more sensitive to temperature than the paclitaxel reaction. It is therefore critical to polymerize at 37°C. 3. Make sure the 96 well plate is warmed to 37°C BEFORE addition of 4° C tubulin. If the plate is cold or at room temperature, the polymerization will have a very long nucleation phase.
Polymerization curves appears erratic	1. Air bubbles in the reaction can cause erratic looking polymerization curves. Careful attention to pipetting accuracy is essential. When using a multi-channel pipette it is necessary to aliquot 120 µl of tubulin into 5 wells of a 96 well plate on ice. Only 100 µl of the tubulin is then transferred to the 37°C polymerization assay leaving 20 µl unused. With this pipetting technique, extra tubulin is needed to prevent uneven aliquoting and air bubble introduction into the assay (see Section V: part E). 2. Use of a multi-dispensing pipette can overcome the problem of adding air bubbles to the samples as there is no air behind each volume pipetted.

Introduction

Tubulin purified from bovine and porcine brains are widely recognized as interchangeable (9). The following report has been generated by scientists at Cytoskeleton Inc. and substantiates the comparable nature of the two tubulins in several biochemical tests, including:

1. Polymerization assay measured by turbidometry.
2. Interaction with motors and their inhibitors measured by microtubule stimulated ATPase.
3. Interaction with drugs, efficacy of microtubule inhibitor drugs during polymerization.

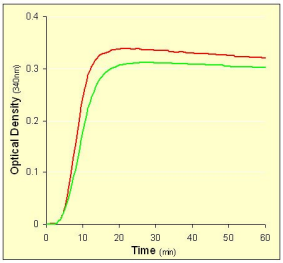
Test 1: Polymerization Assay

Aim: Compare the rate and extent of polymerization of Cat.# TL238 (bovine) and Cat.# T240 (porcine) tubulins under standard conditions.

Assay conditions:	3.0 mg/ml tubulin	
	80 mM Pipes buffer pH 6.90 +/-0.05	
	2 mM MgCl2	
	0.5 mM EGTA	
	10 % glycerol	
	Temperature:	37 °C
	Volume:	100 µl
	96-well plate :	3696 or 3697 from Corning Costar (half area plate)
	Wavelength:	340 nm
	Readings:	Kinetic, 60 readings, one per minute.

Assay description: Optical measurement of microtubule formation relies on light scattering by microtubule polymer. Light scatter is equivalent to light absorbance as detected by a normal spectrophotometer, and light scatter is proportional to the concentration of microtubules in the light path. Using this knowledge one can use the regular 96-well plate reader (with 340nm and temperature control capability) to follow the formation of microtubules from tubulin heterodimers. Examples of this assay provided by Cytoskeleton Inc. are BK004P and BK006P.

Figure 1: **Polymerization Kinetics of Bovine (red) and Porcine (green) brain tubulin**



Results:

Both bovine and porcine tubulins follow a similar profile of increasing optical density over time. They each have a nucleation phase between 0 to 6 min, a polymerization phase 6 to 14 min, and steady state 18 to 60 min.

Conclusions:

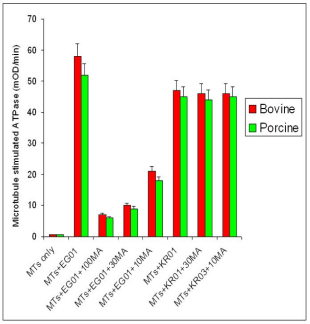
As both tubulins follow a similar time profile of optical density under conditions that promote polymerization, it can be concluded that both tubulins nucleate, polymerize, and remain at steady state to a similar extent. Thus experiments which utilize this assay format can interchange bovine for porcine tubulin without need for reassessing porcine tubulin characteristics.

Test 2: Interaction With Motors

Aim: Compare the activity of Eg5 and KHC kinesin motor proteins on microtubule stimulated ATPase activity using microtubules made from Cat.# TL238 (bovine) and Cat.# T240 (porcine) tubulins.

Assay conditions:	4 µg Eg5 / assay (Cat.# EG01) or 0.2 µg KHC / assay (Cat.# KR01)	
	20 µg tubulin as microtubules / assay	
	15 mM Pipes buffer pH 6.90 +/-0.05	
	5 mM MgCl2	
	1 mM ATP	
	0.5 units phosphonucleotide transferase (detection rea gent)	
	70 µg MESEG (detection reagent)	
	Temperature	24 °C
	Volume	200 µl
	96-well plate	269620 Nunc (regular 96-well plate)
	Wavelength	360+/-2nm monochromatic (360nm filter will not work)
	Readings	Kinetic, 40 readings, one per 30s

Figure 2: **Bovine and porcine microtubule stimulated ATPase of EG5 and KHC in the presence of monastrol**



Results:

Two kinesin proteins were compared for microtubule stimulated ATPase activity. Eg5 (Cat.# EG01) is a human mitotic aster associated motor and KHC (Cat.# KR01) is a ubiquitous vesicle transporting motor. The ATPase activity of both these motors was stimulated by the presence of 1µM tubulin as microtubules. Both bovine (red bars) and porcine (green bars) tubulin derived microtubules stimulated the ATPase activity of these kinesins equally. In addition the presence of monastrol, an Eg5 inhibitor, reduced the activity of Eg5 only, not KHC, in the presence of either bovine or porcine microtubules.

Conclusions:

Microtubules composed of either bovine or porcine tubulin stimulated two different kinesin ATPase activities. The amount of stimulation was identical between both microtubule species indicating that porcine microtubules can be a direct replacement for bovine microtubules without extensive studies.

The ATPase activity of Eg5 but not KHC can be inhibited with monastrol, this was the same in the presence of either bovine or porcine microtubules which indicates again that porcine microtubules can replace bovine microtubules in kinesin ATPase assays.

Test 3: Interactions with drugs

Aim: To compare tubulin polymerization kinetics in the presence of vinblastine or taxol using either Cat.# TL238 (bovine) and Cat.# T240 (porcine) tubulin.

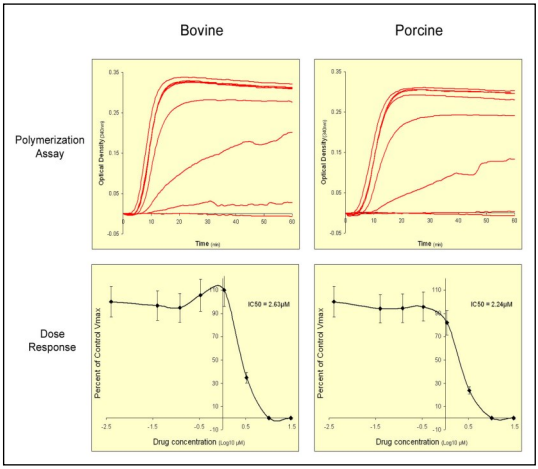
Assay conditions:	0 to 30 µM vinblastine	
	3.0 mg/ml tubulin	
	80 mM Pipes buffer pH 6.90 +/-0.05	
	2 mM MgCl2	
	0.5 mM EGTA	
	10 % glycerol	
OR	0 to 30 µM paclitaxel	
	1.0 mg/ml tubulin	
	80 mM Pipes buffer pH 6.90 +/-0.05	
	2 mM MgCl2	
	0.5 mM EGTA	
	Temperature	37 °C
	Volume	100 µl
	96-well plate	3696 or 3697 from Corning Costar (half area plate)
	Wavelength	340 nm
	Readings	Kinetic 60 readings, one per minute

Assay description:

Optical measurement of microtubule formation relies on light scattering by microtubule polymer. Light scatter is equivalent to light absorbance as detected by a normal spectro-photometer, and light scatter is proportional to the concentration of microtubules in the light path. Using this knowledge one can use the regular 96-well plate reader (with 340nm and temperature control capability) to follow the formation of microtubules from tubulin heterodimers. Examples of this assay provided by Cytoskeleton Inc. are BK004P, BK006P and the fluorescence version BK011P.

In the presence of tubulin ligands the kinetics of this reaction are altered, an inhibitor will prolong nucleation times, slow polymerization rate and reduce the extent of steady state. Conversely an enhancer such as paclitaxel will shorten nucleation times, increase polymerization rate and increase the extent of steady state.

Figure 3: **Effect of vinblastine on polymerization kinetics of bovine and porcine brain tubulins**



Results:

Both bovine and porcine tubulins follow a similar profile of increasing optical density over time. They each have a nucleation phase between 0 to 6 min, a polymerization phase 6 to 14 min, and steady state 18 to 60 min. Both tubulins are inhibited by vinblastine to the same extent, with IC50 values of 2.63 and 2.24 µM respectively. The dose response curves have similar structure which indicates both low, medium and high concentrations of drug interact with both tubulins in a similar manor across the concentration range tested.

Conclusions:

The effect of vinblastine on tubulin polymerization showed that bovine and porcine tubulin were affected equally. Thus experiments which utilize these tubulins for drug discovery and development (e.g. using Cat.# BK004P, BK006P and the fluorescence version BK011P) can interchange bovine for porcine tubulin without need for re-assessing porcine tubulin characteristics.

NOTES

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