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HTS-Tubulin Polymerization Assay Kit

CytoDYNAMIX Screen 01

Cat. # BK004P

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Manual Contents

About the Polymerization Assay

CytoDYNAMIX Screen™ 1 is an economical (one step) tubulin polymerization assay for screening large numbers of tubulin ligands and primary libraries. Tubulin polymerization assay is based on an adaptation of the original method of Shelanski et al. and Lee et al. (1, 2) 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 1A), growth (II in Figure 1A) and steady state equilibrium (III in Figure 1A). The tubulin used in this assay (Cat. # HTS03) will not appear as active as other purified tubulins (Cat. # T240 or ML116) because it is optimized to give equal signals for polymerization enhancing agents and for compounds that inhibit microtubule polymerization.

Figure 1: Typical Tubulin Polymerization Curves for CytoDYNAMIX Screen™ 1

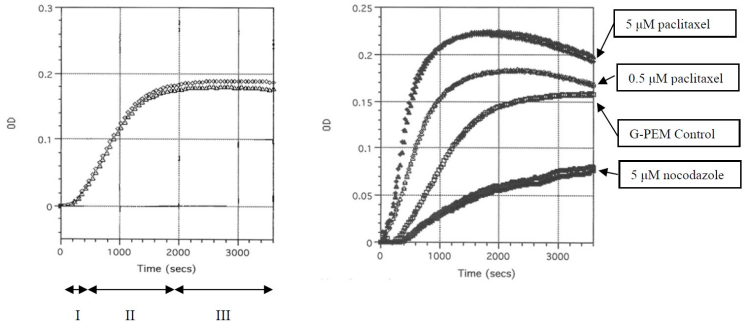


Figure 1 A: Standard polymerization reactions (minus tubulin ligands) were carried out as described in the Polymerization Protocol (Section V). Briefly, the standard polymerization reaction contains 100 µl volume of 4 mg/ml tubulin in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM GTP. Polymerization 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 40% 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 polumerization. Figure 1B: Standard polymerization reactions alone and in the presence of 5 µM paclitaxel, 0.5 µM paclitaxel, G-PEM control 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.

Compounds or proteins that interact with tubulin will often alter one or more of the characteristic phases of polymerization. For example, Figure 1B shows the effect of adding the anti-mitotic drug paclitaxel to the 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. CytoDYNAMIX Screen™ 1 has been used to identify novel compounds which are potentially useful in anti-cancer applications (3, 4). Figure 1B 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. For a detailed discussion of assay conditions and assay optimization, see Section V .

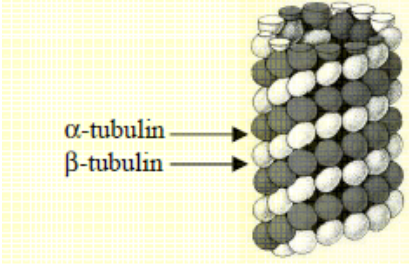
Each kit contains sufficient reagents for 24 - 30 assays. Generally, using a multichannel pipette results in 24 assays due to some wastage of tubulin protein and single channel pipettes give 30 assays. The use of lyophilized tubulin (5) allows the kit to be stored at 4° C (<10% humidity) prior to use.

About Tubulin Protein

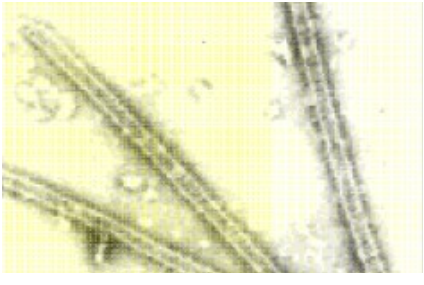
Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called alpha and beta tubulin. The proteins are encoded by separate genes, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom. Consequently, tubulin isolated from porcine brain tissue 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.

Figure 2: Microtubule Structure

A) Schematic of a microtubule



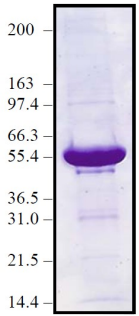
B) Electron micrograph of microtubules (100,000x)



Tubulin polymerizes to form structures called microtubules (MTs) (see Figure 2B). When tubulin polymerizes it initially forms proto-filaments, MTs consist of 13 protofilaments and are 25nm in diameter, each um of MT length is composed of 1650 heterodimers (8). Microtubules are highly ordered fibers that have an intrinsic polarity, shown schematically in Figure 2A. 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.

This assay uses tubulin purified from porcine brain, and consists of approximately 97% tubulin and 3% microtubule associated proteins (MAPs), see figure 3.

Figure 3: Tubulin Purity



Porcine brain tubulin (Cat. # HTS03) was run on a 4-20% SDS PAGE system and stained with 0.1% Coomassie Blue. The gel in shows 50 µg of tubulin protein. Densitometry measurements determined the protein to be greater than 97% pure tubulin. Molecular weight markers are from Invitrogen

Unit Definition

One unit (U) of HTS-tubulin (Cat. # HTS03) in the CytoDYNAMIX Screen is defined as 400 µg of protein (as determined by the Advanced Protein Assay, Cat. # ADV02). When 100 µl of reconstituted HTS-tubulin is pipetted into one well of a Corning Costar 96-well plate (Cat. # 3697) it will polymerize efficiently at 37°C to reach a maximum OD340 nm between 0.12-0.25 in 30 min.

There are three different tubulin product sizes which are optimized for different HTS assay formats as shown below, please inquire if you need other vial sizes (Technical assistance 303-322-2254):

Cat. #	Number of assays	Amount of protein / tube	Reconstitution volume
HTS03-A	8 (½ area 96 well format)	4.0 mg protein / 2 ml tube	1.0 ml
HTS03-B	96 (½ area 96 well format)	40 mg protein / 30 ml tube	10 ml
HTS03-C	384 (384 well format)	100 mg protein / 100 ml tube	25 ml

II: Important Technical Notes

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

Instrument requirements

Spectrophotometer

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 reading at 340 nm in kinetic mode is required. The assay is designed for a 96 well microtiter plate format and therefore your spectrophotometer should be able to handle 96 well plates. The multiwell plate format will also result in lower cv values when multiple samples are to be screened at a time.

It should be noted that temperature is a critical parameter for tubulin polymerization, temperatures cooler than 37°C will significantly decrease the rate of polymerization and the final OD reading (generally 5% loss of polymer per degree reduction in temperature). Also, if temperature is not uniform across a plate, variation between samples will be high.

Multi-channel Pipettor

For HTS applications it is optimal to pipette into all wells at the same time using a 96-channel pipettor. If this is not possible, then pipetting with an 8 or 12 multi-channel hand held pipettor is an alternative. Finally, if a few samples are being assayed use a single channel pipettor and aim to finish all tubulin pipetting within one minute. The more familiar the pipette operator is with the pipettor the lower the variability between samples, so it helps to practice with a BSA protein solution before using the tubulin. The second important point to avoid is 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. The exact technique for using each vial type varies with apparatus and the through-put required, the operator is advised to use a solution of BSA at 3.0 mg/ml to set up their particular apparatus and then the transition to tubulin will be easier.

For 8 or 12 multi-channel pipettes we recommend the Proline series (50-1200 µl) (Biohit. Inc. Neptune, NJ). For 96-channel micro-dispensers we recommend the Hydra-96 (Robbins Scientific Corp. Sunnyvale. Ca).

Pipettor set up:

a. **Equilibration of tips with G-PEM buffer.** If the pipettor has fixed tips then three washes of G-PEM will equilibrate the tips with buffer. If the tips are disposable it is not necessary to wash them.

b. **Filling the pipette tips.** If your application requires multiple pipetting of the same solution this can be performed by loading the appropriate volume in to the tip at the same time e.g. ten plates requires 10 x 100 µl + 100 µl surplus = 1100 µl per tip. However for HTS, it is more usual to fill pipette tips with one well volume in the aspirate and dispense mode.

c. **Pipette placement.** This is the most critical step; care must be taken to set up the height of the dispensing pipette tip so that the likelihood of bubble formation is reduced to a minimum. Failure to do this will lead to more false positives. The optimal pipette tip height is 2 mm above the bottom of the plate well. It is important that the tip heights are equal across the 96-channels, if they are not within 0.5 mm across the 96-channels, this will also lead to an increased rate of false positives.

d. **Liquid dispensing**. Set the pipette dispense mode to “quick to moderate” dispense to allow the greatest mixing to occur. Be careful not to form bubbles with this procedure. If pipetting up and down is used be sure to use only 80% of the total volume for pipette mixing, if 100% is used this can sometimes lead to bubble formation by air being pipetted. Plates should be shaken for 5 seconds prior to reading the first time point only to make all the menisci similar.

Assay Conditions

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 the 96 well plate for polymerization at 37°C.

III: Kit Contents

This kit contains sufficient reagents for approximately 24 - 30 assays of 100 µl volume. Some tubulin is lost in a multichannel pipetting resulting in only 24 assays; single channel pipetting will give 30 assays. Prior to reconstitution the kit should be stored desiccated at 4°C (stable for 6 months) or desiccated at -70°C (stable for 6 months). The kit contents should not be allowed to become damp.

KIT COMPONENT	DESCRIPTION
HTS tubulin protein (Cat. # HTS03-A)	Three tubes, lyophilized. 4.0 mg protein/tube, 8 U minimum per tube. Tubulin is purified from porcine brain and is >97% pure.
GTP Stock (Cat. # BST06-001)	Two tubes, lyophilized. Each tube gives 100 µl of a 100 mM stock solution when reconstituted.
General Tubulin Buffer (Cat. # BST01-001)	One bottle, lyophilized. Gives 10 ml of 1x buffer when reconstituted. Buffer composition is 80 mM Piperazine-N,N'-bis[2-ethanesulfonic acid] sequisodium salt; 2.0mM Magnesium chloride; 0.5mM Ethylene glycol-bis(b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, pH 6.9.
Tubulin Glycerol Buffer (Cushion Buffer) (Cat. # BST05-001)	One bottle of 10 ml 1x buffer. Buffer composition is 80 mM Piperazine-N,N'-bis[2-ethanesulfonic acid] sequisodium salt; 2.0mM Magnesium chloride; 0.5mM Ethylene glycol-bis(b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, 60% v/v glycerol, pH 6.9.
Paclitaxel (Cat. # TXD01)	One tube, lyophilized. Gives a 100 µl of a 2 mM stock solution when reconstituted.
DMSO	One tube containing 1 ml of DMSO. Used for paclitaxel resuspension.
96 well plate	One half area plate

IV: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to reconstitute several components as shown below:

Kit Component	Reconstitution	Storage Conditions
General Tubulin Buffer (Cat. # BST01-001)	Reconstitute with 10 ml of sterile distilled water.	Store at 4°C. Stable for 6 months.
Tubulin Glycerol Buffer (Cat. # BST05-001)	No reconstitution necessary, 10 ml of 1x buffer.	Store at 4°C. Stable for 6 months.
GTP Stock (Cat. # BST06-001)	1. Reconstitute each tube with 100 ul of sterile distilled water. 2. Aliquot each tube into 10 x 10 ul volumes and freeze at -70°C.	Store at -70°C. Stable for 6 months.
Tubulin protein (Cat. # HST03)	1. Reconstitute the required number of tubes just before each experiment as described in Section V, Standard Polymerization Assay method. **For already reconstituted protein, store as follows:** 1. Aliquot the protein into 120 µl volumes in labeled cryotubes. 2. Snap freeze in liquid nitrogen. 3. Store at -70ºC. **NOTE: It is very important to snap freeze the tubulin in liquid nitrogen as other methods of freezing will result in reduced protein activity.** **Thawing stored tubulin aliquots:** **1.** Remove tubes from -70°C and place into a room temperature water bath for 1 min. **2.** Immediately after the solution is defrosted place on ice. **3.** Centrifuge at 14,000 x g for 10 min at 4°C 4. Pipette the supernantant into a new tube and place on ice. This tubulin solution is now ready for use in the microtubule polymerization assay.	Store at -70°C. Stable for 6 months.
Paclitaxel Stock (Cat. # TXD01)	1. Reconstitute the tube of paclitaxel with 100 µl of DMSO. 2. Store at -70°C or -20°C.	Store at -70°C or -20°C. Stable for 6 months.

Test Compound or Protein Preparation

A 2 mM solution of your compound in DMSO is the optimal starting material; this is then diluted in water 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. If this is not possible either, call customer service (303-322-2254 or cserve@cytoskeleton.com).

For proteins you require a 10x final concentration in General Tubulin Buffer (Cat. # BST01) or another tubulin compatible buffer such as 30 mM Tris pH 7.0. General guidelines for tubulin compatible buffers are given below:

a. Keep pH between 6.5 – 7.0

b. Do not use calcium containing buffers.

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

V: Assay Protocol

The assay volume is 100 µl and assumes a spectrophotometer pathlength of 0.5 cm (when used with a half area plate, see later). The tubulin concentration in these assays is 4 mg/ml. NOTE: when using a microtiter plate reading spectrophotometer the readings are taken from the top of the plate and therefore the volume of your reaction will directly influence the pathlength. Cytoskeleton Inc. highly recommends the use of a half area well plate for these assays (Corning Cat. # 3696) for optimal polymerization signal. A half area plate is supplied in this kit. The assay should take approximately 1.5 h to perform, including the 1 h polymerization reaction.

Instrument Settings

Tubulin polymerization is followed by an increase in absorbance at 340 nm over time (usually 60 minutes). Your spectrophotometer should therefore be set in kinetic absorbance mode at 340 nm wavelength. The polymerization reaction is started by the increase in temperature from 4°C to 37° C upon transfer of the reaction to pre-warmed plate. The spectrophotometer must therefore be temperature regulated and set at 37°C. Tubulin polymerization will not be efficient if tubulin is pipetted into a cold (or room temperature) microtiter plate. It is essential to PRE-WARM plates for reproducible results.

An example of the settings using a Molecular Devices SpectraMax 250 instrument are given below. This machine uses a monochromatic light source and is one of the more sensitive machines on the market.

Instrument Settings for SpectraMax 250

Parameter	Setting
Measurement type	Kinetic, 61 cycles of 1 reading per minute.
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 s 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.

Polymerization Assay Method

It is recommended to carry out duplicate polymerization assay control reactions (i.e., polymerization reactions minus compound or protein of interest) for each set of experiments performed. 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 uses G-PEM in the polymerization buffer, this condition results in an assay that is more sensitive to a given compound (see also Figure 1). Glycerol (e.g. 10%) can be added to the reaction (see Assay Optimization, Section VI) and will result in enhanced polymerization.

1. Pre-warm the plate to 37ºC for 30 min prior to starting the assay. A warm plate is essential for high polymerization activity and reproducible results.

2. Enter all plate reader parameters (see previous page) so that the machine is ready to go. Once the tubulin is aliquoted into the 37°C wells, reading must begin immediately or the nucleation phase of polymerization will be missed.

3. Warm 500 µl of General Tubulin Buffer to room temperature. Warm buffer is needed for tubulin ligand dilutions.

4. Paclitaxel is included in this kit as a control, dilute 10 µl of the Paclitaxel stock solution with 190 µl of **ROOM TEMPERATURE** General Tubulin Buffer and use 10 µl of this per well (10 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.

5. Make COLD (4°C) G-PEM buffer or G-PEM plus 10% glycerol buffer (see Section VI, Assay Optimization) as follows:

   G-PEM minus glycerol buffer

Component	Volume	Final Concentration
General Tubulin Buffer	990 µl	80 mM PIPES pH6.9, 2 mM $MgCl_2$, 0.5 mM EGTA
GTP Stock (100 mM)	10 µl	1 mM GTP

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

G-PEM plus 10% glycerol buffer

Component	Volume	Final Concentration
General Tubulin Buffer	820 µl	80 mM PIPES pH6.9, 2 mM $MgCl_2$, 0.5 mM EGTA
Tubulin Glycerol Buffer	170 µl	10% glycerol in General Tubulin Buffer
GTP Stock (100 mM)	10 µl	1 mM GTP

NOTE: G-PEM plus 10% glycerol buffer is labile due to hydrolysis of GTP; it should be kept on ice and used within 2 hours of preparation. Any unused buffer should be discarded.

6. Resuspend each 4mg tube of tubulin (HTS03) with 1 ml of the cold G-PEM buffer (or G-PEM plus 10% glycerol buffer, see Assay Optimization, Section VI) to give a final protein concentration of 4 mg/ml. Place the tube on ice and allow 3 min for the complete resuspension of the protein. **Keep the tubulin on ice**.

NOTE: If all of the tubulin is not to be used in the assay then snap freeze aliquots of tubulin in liquid nitrogen and store at -70°C. It is recommended to snap freeze unused tubulin within 5 – 10 minutes of resuspension as it is a labile protein.

7. Prepare your compound of interest at 10x strength in G-PEM or Milli-Q water.
8. Pipette 10 µl of your 10x strength compound into the required number of wells of the pre-warmed plate. Incubate the plate for 2 min at 37°C.
9. Pipette 10 µl of General Tubulin Buffer only into two control wells (tubulin minus compound controls).
10. Pipette 100 µl of tubulin into the required number of wells (two wells should be the zero compound controls). NOTE: use medium pipetting speed and with the tip of the pipettor on the wall of the well. This technique avoids bubble formation which will disrupt absorbance readings. NOTE: due to the relatively rapid polymerization of tubulin under these conditions (see Figure 1A), it is highly recommended to use an 8 channel pipettor for tubulin addition. For efficient pipetting, place 120 ul of the 4 mg/ml tubulin into several wells of a microtiter plate on ice. Aliquot the tubulin from the 4°C plate to the 37° plate using the 8 channel pipettor. Alternatively, use a multi-dispensing pipettor that will dispense 8 x 100 µl from a single tip.
11. Immediately place the plate into the spectrophotometer at 37°C and start recording using the kinetic set up described above.

Interpretation of Data

Under standard reaction conditions the polymerization control (minus tubulin ligands) should achieve a maximal OD340 between 0.15 – 0.25 within 30 min at 37°C (see Figure 1A and 1B).

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 µM final concentration is seen to reduce the nucleation phase, enhance the Vmax approximately four fold and increase maximum OD of the reaction. The microtubule destabilizing drug, nocodazole, is seen to 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.

VI: Assay Optimization

It should be noted that you may wish to optimize your particular assay by either altering the protein concentration and/or the percentage of glycerol included in the reaction.

For example, if you wish to examine polymerization enhancers such as taxol, it is recommended to use tubulin at 4 mg/ml in G-PEM buffer minus glycerol (see Figure 1A & B). These conditions will result in a polymerization curve that has a reduced Vmax and achieves less polymer mass than a polymerization in G-PEM plus 10% glycerol. In this case, efficient polymerization is achieved by addition of an enhancer such as taxol (0.5 – 5 µM final concentration). The polymerization reaction conditions of 4 mg/ml tubulin in G-PEM minus glycerol also creates a polymerization reaction that is sensitive to de-polymerizing agents such as nocodazole (see Figure 1B).

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

VII: References

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VIII: Troubleshooting Guide

Problem	Possible Solution
No polymerization curve is seen for the tubulin plus paclitaxel sample	1. Polymerizations should be read at 340 nm, make sure you have your spectrophotometer set to this wavelength. 2. To measure polymerization the spectrophotometer needs to be set in kinetic mode to read once every 30 s to 1 min for 1 h. 3. Your tubulin protein 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 4 mg/ml in general tubulin buffer plus 1 mM GTP (G-PEM) plus or minus10% glycerol. 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 or desiccated at -70°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 in making sure that 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 you should begin reading immediately. Taxol causes rapid tubulin polymerization (see Figure 1B). 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 (or water).

Problem	Possible Solution
No polymerization or long nucleation phase is seen in the tubulin plus 10% glycerol samples	1. See 1 – 7 above. 2. The polymerization of this tubulin reaction is far more sensitive to temperature than the paclitaxel reaction. It is very important to polymerize at 37°C. 3. Make sure that 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. 4. The glycerol concentration has a large effect upon polymerization. Make sure you are using diluting the tubulin with the 10% glycerol polymerization buffer. 5. Tubulin protein concentration has a large effect on polymerization. Poor polymerization could be the result of diluting the tubulin below 4 mg/ml.
Polymerization curves appear erratic	1. Air bubbles in the reaction can cause erratic looking polymerization curves. Careful attention to pipetting accuracy is essential. When using a multichannel 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. 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.

IX: Comparison of Tubulins from Bovine and Porcine

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.

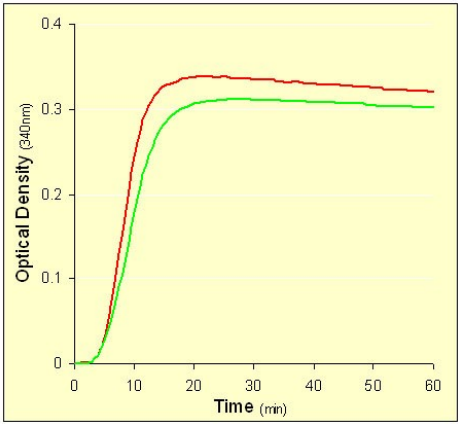
Assay conditions:

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, BK006P and the fluorescence version BK011P.

Figure 1 - Polymerization kinetics of Bovine (red) and Porcine (green) brain tubulin



Results:

Both bovine and porcine tubulin 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, we can conclude 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 re-assessing porcine tubulin characteristics.

IX: Comparison of Tubulins from Bovine and Porcine

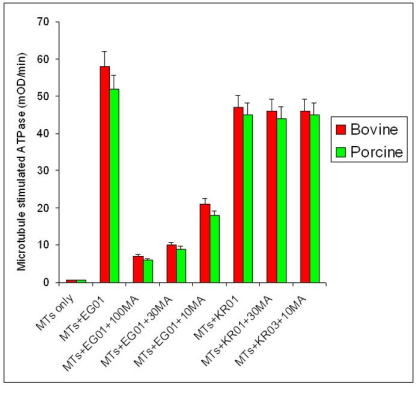
Test 2: Interaction with motors

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 phosphonuleotide transferase (detection reagent)
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.



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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 1uM 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 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, 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.

Results:

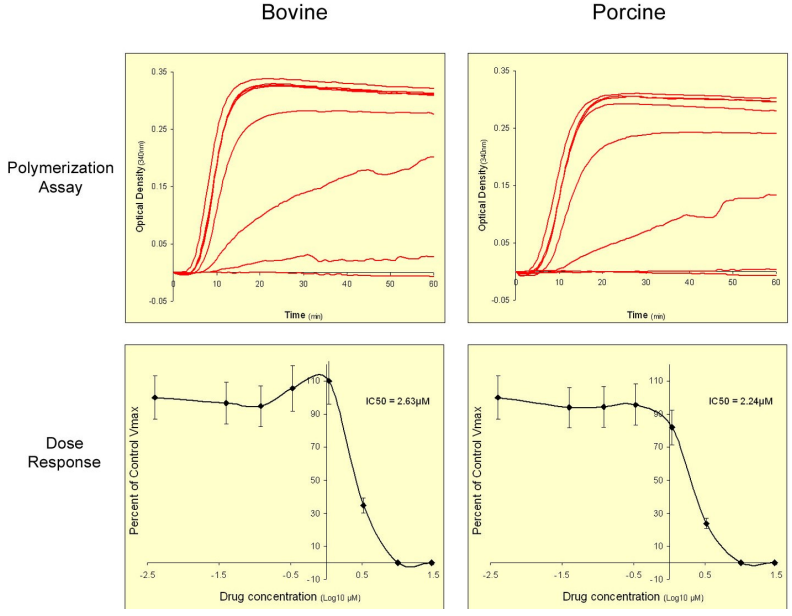
Both bovine and porcine tubulins follow a similar profile of increasing optical density over time. They

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.

Figure 4 – The effects of vinblastine on the polymerization kinetics of Bovine and Porcine brain tubulins



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