



Cytoskeleton, Inc.

Manual

V 3.0

Tubulin Polymerization Assay Kit

Cat. # BK006P

cytoskeleton.com

Phone: (303) 322.2254 **Fax:** (303) 322.2257

Customer Service: cserve@cytoskeleton.com

Technical Support: tserve@cytoskeleton.com

Manual Contents

Section I: Introduction

About the Polymerization Assay	5
About Tubulin	6

Section II: Important Technical Notes

Instrument Requirements	8
Assay Conditions.....	9

Section III: Kit Contents 11

Section IV: Things to do Prior to Beginning the Assay 12

Section V: Assay Protocols

Instrument Settings	13
Standard Polymerization Assay Method	14
Interpretation of Data.....	16
Customization of Polymerization Reaction.....	16
Optimization for polymerization	17

Section VI: References 18

Section VII: Troubleshooting..... 19

I: Introduction

About the Assay

Tubulin polymerization assay (BK006P) 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 1), growth (II in Figure 1) and steady state equilibrium (III in Figure 1).

Figure 1: Typical Tubulin Polymerization Curves for Tubulin Polymerization Assay Kit

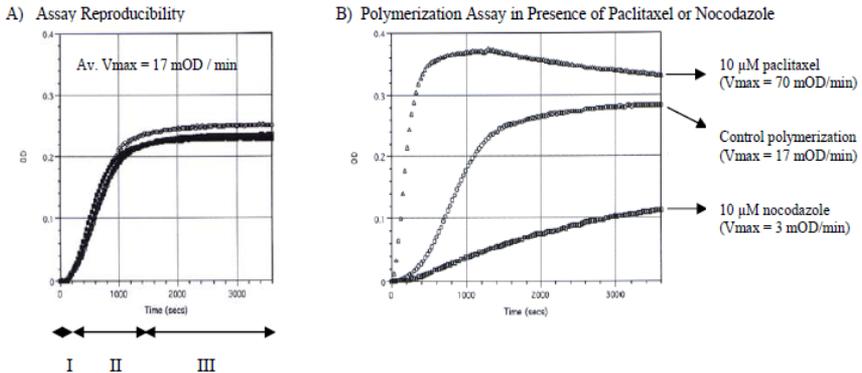


Figure 1 A: Standard polymerization reactions (minus tubulin ligands) were carried out as described in the Polymerization Protocol (Section V). Briefly, the standard polymerization reaction contains 100 μ l volume of 3 mg/ml tubulin in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM GTP, 10% glycerol. Polymerization was started by incubation at 37°C and followed by absorbance readings at 340 nm. Under these conditions polymerization will reach a maximal OD340 between 0.18 – 0.28 within 30 min. In this experimental set up (100 μ l 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 85 - 90% of the tubulin is polymerized. The three phases of polymerization are shown; I (nucleation), II (growth), III (steady state). Polymerization curves from six separate reactions are shown. Figure 1B: Shows standard polymerization reactions alone and in the presence of 10 μ M paclitaxel or 10 μ M nocodazole. The Vmax value is enhanced 4 fold in the presence of paclitaxel and decreased 5.5 fold in the presence of nocodazole. The curves shown represent the average of 4 separate experiments.

I: Introduction (Continued)

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 10 μM final concentration paclitaxel eliminates the nucleation phase and enhances the V_{max} of the growth phase. Thus, one application of this assay is the identification of novel anti-mitotics. BK006P has been used to identify novel compounds which are potentially useful in anti-cancer applications (3, 4). Figure 1 also shows the effect of adding the microtubule depolymerizer, nocodazole. At 10 μM final concentration, nocodazole causes a decrease in V_{max} from 17 mOD/min to 3 mOD/min and a three fold reduction in final polymer mass. For a detailed discussion of assay conditions and assay optimization, see Section V.

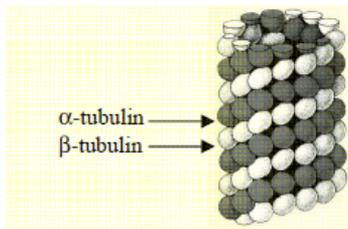
Each kit contains sufficient reagents for 25 - 30 assays. Generally, using a multichannel pipette results in 25 assays due to some wastage of tubulin protein (see method Section V) and single channel pipettes give 30 assays. The use of lyophilized tubulin (5) allows the kit to be stored at 4°C (<10% 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, e.g.; *Saccharomyces cerevisiae* (6) or *Drosophila melanogaster* (7).

Figure 2: Microtubule Structure

A) Schematic of a microtubule



B) Electron micrograph of microtubules

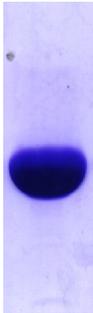


I: Introduction (Continued)

(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 25 nm in diameter, each μm 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 highly purified tubulin from porcine brain. The consistent quality of the protein is critical to dependable and reproducible results. An example of the tubulin used for BK006P is shown in Figure 3.

Figure 3: Tubulin Purity



Porcine brain tubulin (T240) was run on a 4-20% SDS PAGE system and stained with 0.1% Coomassie Blue. The gel in figure 3 shows 50 μg of tubulin protein. Densitometric measurements determined the protein to be >99% pure.

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. You will therefore require a temperature regulated spectrophotometer capable of reading at 340 nm in kinetic mode. 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, a temperature 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. Furthermore, it is essential to avoid forming bubbles 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, then the transition to tubulin will be easier.

II: Important Technical Notes (Continued)

Pipettor set up for 96 well pipettor:

1. **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.
2. **Filling 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 wells requires $10 \times 100 \mu\text{l} + 100 \mu\text{l surplus} = 1100 \mu\text{l}$ per tip. However for HTS, it is more usual to fill pipette tips with one well volume in the aspirate and dispense mode.
3. **Solution dispensing.** 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.
4. **Bubble formation.** Finally, set up your 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.

II: Important Technical Notes (Continued)

Tubulin Protein Stability

Tubulin is a labile protein; the instructions in Section IV specify that reconstituted tubulin should be aliquoted into 200 μ l volumes at 10 mg/ml protein concentration. Aliquoting the protein is VERY IMPORTANT as significant protein denaturation will occur after more than one freeze / thaw cycle. It is particularly important to snap freeze the tubulin aliquots in liquid nitrogen before storing at -70°C , any other freezing method may result in significant protein denaturation. It is also important to keep the tubulin protein concentration above 6 mg/ml prior to freezing; protein left over from a polymerization assay should not be re-frozen. It is also important to keep the lyophilized tubulin dry by storing in a desiccator at either 4°C or -70°C .

Special Considerations When Using 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 glycerol to this reaction, tubulin polymerization efficiency will be increased. The standard polymerization conditions described in Section V of this manual uses 3 mg/ml tubulin in a 10% glycerol buffer. Under these conditions tubulin polymerizes with a V_{max} of approximately 17 milli OD units per minute (mOD/min) and reaches a polymer mass of approximately 280 μg (90% polymerized). The standard reaction conditions create a polymerization reaction that is responsive to the effects of many tubulin ligands. For example, Figure 1B shows that under the standard conditions 10 μM paclitaxel enhances the V_{max} value four fold (17 to 70 mOD/min), while 10 μM nocodazole decreases the V_{max} value by over 5 fold (17 to 3 mOD/min).

IT SHOULD BE NOTED, HOWEVER, THAT IN SOME CASES THE PRESENCE OF GLYCEROL WILL INHIBIT TUBULIN LIGAND BINDING.

III: Kit Components

Microtubule binding proteins such as Tau do not bind efficiently in glycerol containing buffers. It is therefore a good idea to test your ligand binding in a non-glycerol containing reaction buffer. In this case a 5 mg/ml tubulin concentration will give a more robust polymerization curve.

KIT COMPONENT	DESCRIPTION
Tubulin protein (Cat. # T240-DX)	One bottle containing 10 mg of lyophilized protein. The tubulin is purified from porcine brain and is > 99% pure. The protein appears as a white powder.
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; 2.0 mM Magnesium chloride; 0.5 mM 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; 2.0 mM Magnesium chloride; 0.5 mM 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 (kit only)	One tube containing 1 ml of DMSO. Used for paclitaxel resuspension.
Half area 96 well plate (kit only)	One plate

IV: Things to do Prior to Beginning the Assay

This kit contains sufficient reagents for approximately 25 - 30 assays of 100 μ l volume. Some tubulin is lost in a multichannel pipetting resulting in only 25 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. Prior to beginning the assay you will need to reconstitute several components as shown below:

Kit Component	Reconstitution	Storage Conditions
BST01-001	Reconstitute with 10 ml of sterile distilled water.	Store at 4°C.
BST05-001	No reconstitution necessary, 10 ml of 1x buffer.	Store at 4°C.
BST06-001	<ol style="list-style-type: none">1. Reconstitute each tube with 100 μl of sterile distilled water.2. Aliquot each tube into 10 x 10 μl volumes and freeze at -70°C.	Store at -70°C. Stable for 6 months.
T240-DX	<ol style="list-style-type: none">1. Place the 10 mg bottle of T240 on ice, and obtain liquid nitrogen in a dewar.2. Label 5 cryotubes "Tubulin Stock, 10 mg/ml" and place these on ice ready for aliquoting.3. Defrost 10 μl of 100 mM GTP stock.4. Mix 1.1ml of ice cold General Tubulin Buffer with 10 μl of 100 mM GTP stock.5. Completely resuspend the tubulin powder with the 1.1 ml of supplemented General Tubulin Buffer and keep on ice.6. Immediately dispense into 5 x 200 μl aliquots into the labeled cryotubes and drop freeze in liquid nitrogen.7. Store at -70°C. <p>Each aliquot is sufficient for 5 - 6 assays depending upon aliquoting technique used, see Section V.</p> <p>NOTE: It is very important to snap freeze the tubulin in liquid nitrogen as other methods of freezing will give protein with reduced activity.</p>	Store at -70°C.
Paclitaxel Stock (Cat. # TXD01)	<ol style="list-style-type: none">1. Reconstitute the tube of paclitaxel with 100 μl of DMSO.2. Freeze at -70°C or -20°C.	Store at -70°C or -20°C.

V: Assay Protocol

The protocol described below is for 6 reactions. The assay volume is 100 µl and assumes a spectrophotometer pathlength of 0.5 cm (when used with a half area plate). The tubulin concentration in these assays is 3 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. # 3697) 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

The readings are followed by an increase in absorbance at 340 nm over time (usually 60 min). 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 microtiter wells. The spectrophotometer must therefore be temperature regulated and set at 37°C. Tubulin polymerization will not be efficient if tubulin is pipetted into cold (or room temperature) microtiter plates. It is therefore essential to PRE-WARM plates for reproducible results.

An example of the settings using a Molecular Devices SpectraMax250 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 SpectraMax250

Spectrophotometer 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 bandwidths less than 20 nm.
Temperature	37°C
Shaking	5 s medium, orbital
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 an appropriate file set up.

V: Assay Protocol (Continued)

Standard Polymerization Assay Method

It is recommended to carry out duplicate standard polymerization assay control reactions (minus compound / protein of interest) for each set of experiments performed. The assays 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.

1. Pre-warm the plate to 37°C 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. Make COLD (4°C) Tubulin Polymerization (TP) buffer as follows:

Component	Volume	Final Concentration
General Tubulin Buffer	750 µl	80 mM PIPES pH6.9, 2 mM MgCl ₂ , 0.5 mM EGTA
Tubulin Glycerol Buffer	250 µl	15% glycerol in General Tubulin Buffer
GTP Stock (100 mM)	10 µl	1 mM GTP

NOTE: TP buffer is labile due to hydrolysis of GTP; it should be kept on ice and used within 4 h of preparation. Any unused TP buffer should be discarded.

4. Warm 500 µl of General Tubulin Buffer to room temperature. Warm buffer is needed for tubulin ligand dilutions.
5. Pipette 10 µl of General Tubulin Buffer only into two control wells (tubulin minus compound controls). Pipette 10 µl of 10x strength of the compound of interest in General Tubulin Buffer into each well. Incubate the plate at 37°C for 2 min. Paclitaxel solution 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.
6. Defrost one 200 µl vial of tubulin by placing in a room temperature water bath for exactly 1 min, or until thawed, then place on ice. The fast thaw step is critical as the concentrated tubulin stock may begin to polymerize at room temperature.

V: Assay Protocol (Continued)

- Dilute and mix the tubulin with 420 μl of ice cold TP buffer to give a final concentration of 3 mg/ml tubulin in 80 mM PIPES pH 6.9, 2 mM MgCl_2 , 0.5 mM EGTA, 1 mM GTP, 10.2% glycerol.
- Use the diluted tubulin sample immediately.
- Pipette 100 μl of the diluted protein into each of 6 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 3), it is highly recommended to use an 8 channel pipettor for tubulin addition. For efficient multipipetting, place 120 μl of the 3 mg/ml tubulin into each of 5 microtiter plate wells on ice. Aliquot the tubulin from the 4°C plate to the 37° plate using the 8 channel pipettor. It should be noted that this method results in only 5 assays per frozen tubulin stock, however the speed and synchrony of each reaction will result in more accurate recording of polymerization reactions.
- Immediately place in reader at 37°C and start recording using the kinetic set-up described above.

Figure 3: Tubulin Polymerization Curves in the Presence of Polymerization Enhancers and Suppressors

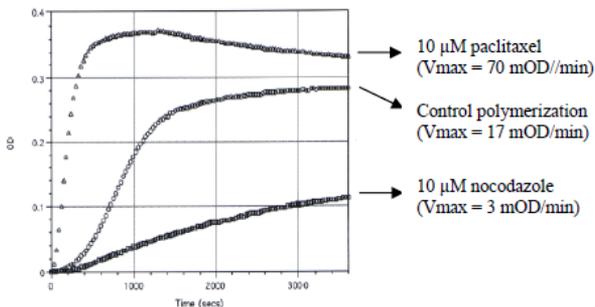


Figure 3: Standard polymerization reactions alone and in the presence of 10 μM paclitaxel or 10 μM nocodazole (not supplied in kit). The V_{max} value is enhanced 4 fold in the presence of paclitaxel and decreased 5.5 fold in the presence of nocodazole. The curves shown represent the average of 4 separate experiments.

V: Assay Protocol (Continued)

Interpretation of Data

Under standard reaction conditions the polymerization control (minus tubulin ligands) should achieve a maximal OD340 between 0.18 – 0.28 within 30 min at 37°C (see Figure 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 10 μ M final concentration is seen to eliminate the nucleation phase, enhance the V_{max} approximately four fold and increase overall polymer mass of the reaction. The microtubule destabilizing drug, nocodazole, is seen to reduce the V_{max} over five fold and decrease polymer mass approximately three fold. Any or all of these parameters can be quantitated.

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

Customization of Polymerization Reaction

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 10% glycerol to this reaction, tubulin polymerization efficiency will approach 90% polymer mass at 37°C after 15 - 20 min. Tubulin polymerization is also a temperature sensitive event, optimal polymerization occurs at 37°C.

Optimization for Polymerization Enhancing Compounds / Proteins

Using less glycerol in the reaction will make the polymerization more sensitive to enhancers such as paclitaxel. By using less glycerol the control polymerizations will have a longer nucleation phase, slower V_{max} and lower final polymer mass. Figure 4 shows tubulin polymerizations at different glycerol concentrations. In situations where zero glycerol buffers are desired, the 3 mg/ml protein concentration will result in little or no polymerization until an enhancer is added.

V: Assay Protocol (Continued)

Optimization for Polymerization Inhibiting Compounds / Proteins

Using less glycerol and higher protein concentrations (5 mg/ml) of tubulin in the reaction will make the polymerization more sensitive to destabilizers such as nocodazole.

Figure 4: Tubulin Polymerization Response to Decreasing Glycerol Concentrations

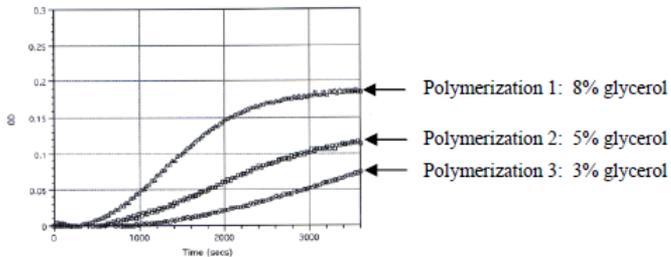


Figure 4: Tubulin polymerizations were carried out as described in the Standard Polymerization Reaction. In all reactions the tubulin concentrations are 3 mg/ml in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM MgCl₂, 1 mM GTP, polymerization volumes are 100 μ l in half area well plates and the reactions occur at 37°C. Polymerization reactions 1-3 differ only in the concentration of glycerol in the buffers; these are 8%, 5% and 3% respectively. Vmax values are 7 mOD/min (8% glycerol), 3.5 mOD/min (5% glycerol) and 2.5 mOD/min (3% glycerol).

VI: References

1. Shelanski ML, Gaskin F. and Cantor CR. 1973. Proceedings of the National Academy of Science, 70, 765-768.
2. Lee JC. and Timasheff SN. 1977. In vitro reconstitution of calf brain microtubules: effects of solution variable. *Biochemistry*, 16, 1754-1762.
3. Jaing JD, Davis AS, Middleton KM, Ling Y-H, Perez-Soler R, Holland JF. and Bekesi G. 1998. 3-Iodoacetamido-benzoylurea: a novel cancericidal tubulin ligand that inhibits microtubule polymerization, phosphorylates bcl-2 and induces apoptosis in tumor cells. *Cancer Research*, **58**, 5389-5395.
4. Davis AS, Middleton KM, Jiang J-D, Wang Y, Weisz I, Ling YH. And Bekesi JG. 1999. Novel suicide ligands of tubulin arrest cancer cells in S-phase. *Neoplasia*, **1**, 498-507.
5. Davis A.S. and Middleton K.M. 1998. Lyophilized Tubulins. USPTO 6750330.
6. Hyman AA, Middleton KM, Centola M, Mitchison TJ. and Carbon J. 1993. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature* 359, 533-536.
7. 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).
8. Amos LA. and Klug A. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Science*, 14, 523-530.

VII: Troubleshooting

Problem	Possible Solution
<p>No polymerization curve is seen for the tubulin plus paclitaxel sample</p>	<ol style="list-style-type: none"> 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 seconds to 1 minute. 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 10 mg/ml in general tubulin buffer plus 1 mM GTP (see Section IV for detailed instructions). Tubulin stocks should not be frozen / thawed more than once. 4. Your tubulin protein may be inactive. This can be caused by re-freezing diluted tubulin from a previous experiment. Tubulin frozen below 6 mg/ml will be largely inactivated. 5. Your tubulin protein may be inactive. If you have allowed the lyophilized tubulin to become damp, it will rapidly denature. You should store the lyophilized tubulin desiccated at 4°C or desiccated at -70°C. 6. 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. 7. 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 1). Readings should be taken once every 30 seconds to 1 minute. 8. 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)
<p>No polymerization or long nucleation phase is seen in the tubulin plus 10% glycerol samples</p>	<ol style="list-style-type: none"> 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 (see Fig. 4). Make sure you are using diluting the tubulin with the 15% glycerol polymerization buffer. 5. Tubulin protein concentration has a large effect on polymerization. Poor polymerization could be the result of diluting the tubulin below 3 mg/ml.
<p>Polymerization appears erratic</p>	<ol style="list-style-type: none"> 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 124 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 24 µl unused. With this pipetting technique, extra tubulin is needed to prevent uneven aliquoting and air bubble introduction into the assay.

