



**Cytoskeleton, Inc.**

**Manual**

V 4.0

# **Tubulin Polymerization Assay Kit**

**(Porcine tubulin and Fluorescence based)**

**Cat. # BK011P**

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# Manual Contents

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## **Section I: Introduction**

About the Fluorescence Polymerization Assay .....	5
Comparison of Fluorescence vs Absorbance Assays .....	6
About Tubulin .....	7

## **Section II: Important Technical Notes**

Instrument Requirements .....	9
Recommended Pipeting Technique & Choice of Pipettor .....	10
Assay Conditions .....	11

## **Section III: Kit Contents** .....

13

## **Section IV: Things to do Prior to Beginning the Assay** .....

14

## **Section V: Standard Assay Protocols**

Method for 1-8 Assays .....	15
Method for >8 Assays .....	16

## **Section VI: References** .....

17

## **Section VII: Troubleshooting** .....

18



# I: Introduction

## About the Assay

This assay is an economical one step procedure for determining the effects of drugs or proteins on tubulin polymerization. It is an adaptation of an assay originally described by Bonne, D. et al. (1). Polymerization is followed by fluorescence enhancement due to the incorporation of a fluorescent reporter into microtubules as polymerization occurs. The standard assay uses neuronal tubulin (Cat. # T240) which generates a polymerization curve representing the three phases of microtubule formation, namely nucleation (Phase I in Figure 1), growth (Phase II in Figure 1) and steady state equilibrium (Phase III in Figure 1). Other tubulins, such as HeLa and MCF-7 tubulin (Cat. # H001 & H005) can also be used in this assay format.

Compounds or proteins that interact with tubulin will often alter one or more of the characteristic phases of polymerization. For example, Figure 1 shows the effect of adding the anti-mitotic drug paclitaxel to the standard polymerization reaction. At 3  $\mu\text{M}$  final concentration paclitaxel eliminates the nucleation phase and enhances the  $V_{\text{max}}$  of the growth phase. Thus, one application of this assay is the identification of novel anti-mitotics. Figure 1 also shows the effect of adding the microtubule destabilizing drug, vinblastine. At 3  $\mu\text{M}$  final concentration, vinblastine causes a decrease in  $V_{\text{max}}$  and a reduction in final polymer mass. For a detailed discussion of assay conditions and assay optimization, see Section V.

Figure 1: Fluorescence Polymerization Assay in Presence of Paclitaxel or Vinblastine

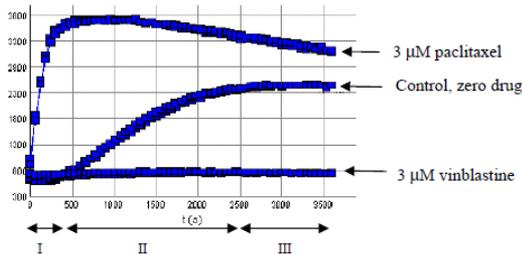


Figure 1: Standard polymerization reactions were carried out as described in the Assay Protocols Section V. Each well of the assay plate contained 5  $\mu\text{l}$  of 10x strength compound which was warmed for 1 min to 37°C, 50  $\mu\text{l}$  of tubulin solution was pipetted into each well. Excitation was at 360 nm and emission at 420 nm. The three phases of tubulin polymerization are shown for the control polymerization curve.

# I: Introduction (Continued)

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Each kit contains sufficient reagents for 96 assays of 55  $\mu$ l volume per assay when using a single channel pipettor. Generally, using a multichannel pipette results in less assays per kit due to some wastage of tubulin protein (see method section V).

## **Comparison of the Fluorescence Based Polymerization Assay with the Classical Absorbance Based Assay**

The classic tubulin polymerization assay uses absorbance readings at 340 nm to follow microtubule formation. It is based upon the fact that light is scattered by microtubules to an extent that is proportional to the concentration of microtubule polymer. This assay is offered by Cytoskeleton Inc. (Cat. # BK006P / CDS03P). The fluorescence based assay has been compared to the absorbance based format and the comparisons are given in Table 1 below. For help in selecting the best assay format for your needs contact [tbservice@cytoskeleton.com](mailto:tbservice@cytoskeleton.com).

Table 1: Comparison of Fluorescence versus Absorbance Based Polymerization Assays

Assay Characteristics	Absorbance Assay	Fluorescence Assay
Tubulin used per assay	300 $\mu$ g	100 $\mu$ g
Volume of reaction	100 $\mu$ l	50 $\mu$ l
Signal to noise ratio (S/N)	2	4
Coefficient of variation (cv)*	13%	11%
Paclitaxel EC50**	1 $\mu$ M	1 $\mu$ M
Vinblastine IC50 **	0.6 $\mu$ M	0.6 $\mu$ M
Possible problems	Glycerol in standard assay format may interfere with drug or protein binding. Assay conditions can easily be altered to test this.	Fluorescent reporter may interfere with drug or protein binding.

\* Duplicate samples

\*\* Under standard assay conditions. Conditions can be optimized for polymerization enhancers or inhibitors.

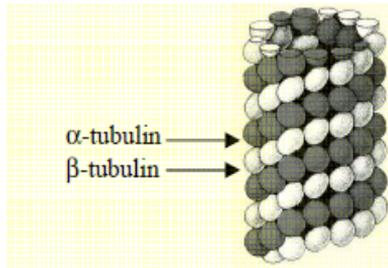
# I: Introduction (Continued)

## About Tubulin Protein

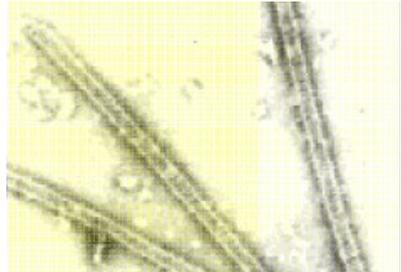
Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called alpha and beta tubulin. The proteins are encoded by separate genes, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom. Consequently, tubulin isolated from Porcine brain tissue 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* (2), *Drosophila melanogaster* (3).

Figure 2: Microtubule Structure

A) Schematic of a microtubule



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



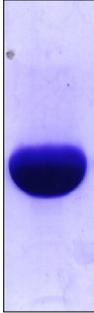
Tubulin polymerizes to form structures called microtubules (MTs) (see Figure 2B). When tubulin polymerizes it initially forms proto-filaments, MTs consist of 13 protofilaments and are 25 nm in diameter, each  $\mu\text{m}$  of MT length is composed of 1650 heterodimers (4). Microtubules are highly ordered 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.

# I: Introduction (Continued)

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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 BK011P (Cat. # T240-DX) is shown in Figure 3.

Figure 3: Tubulin Purity



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

## II: Important Technical Notes

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

### **Instrument Requirements**

#### Fluorimeter

Polymerizations are followed by an increase in fluorescence emission at 410–460 nm over a 60 minute period at 37°C (excitation wavelength is 340 – 360 nm). You will therefore require a temperature regulated fluorimeter capable of reading at 410–460 nm in kinetic mode with excitation filters of 340-360 nm. The assay is designed for a 96 well microtiter plate format and therefore your fluorimeter 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.

The majority of the work in the design of this assay has been based on the TECAN GmbH machine called SpectroFluor Plus. This machine is filter based and is one of the more sensitive machines on the market (pmoles of fluorescein can be detected). The parameters of a Protocol file using the SpectroFluor are given below:

Measurement type:	Kinetic	61 cycles of 1 reading per minute. The time course of the reaction can be reduced to 30 min in some cases.
Fluorescence wavelengths:	Ex. Em.	340-360 nm ± 20 nm 410-460 nm ± 20 nm.
Gain:	80	On a scale of 0-120, where 120 is the highest.
Reads per well:	3	
Fluorescence reading from:	Top	
Integration:	0-40 µs	
Shaking:	5 s	Medium, orbital (first read only)
Plate type	Greiner	GRE96fb (flat, black). (Standard template on TECAN).

The major differences with other plate readers are:

1. If a monochromatic machine is used e.g. Gemini from Molecular Devices Inc. then the wavelengths should be Ex. 360 nm and Em. 450 nm.
2. If the machine is not set-up for Greiner plates then a custom template will have to be created for the Corning Costar 96-well plate provided (Corning Costar, Cat. # 3686). Use 100 µl of ten-fold diluted Buffer 1 pipetted into each well and then activate the New Template scan procedure.

## II: Important Technical Notes (Continued)

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### **Recommended Pipetting Technique & Choice of Pipettor**

#### Single Channel Pipettor (recommended for processing of 1-8 samples)

If a few samples (1 – 8) 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 fluorescence readings fluctuate and 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 operator is advised to use a solution of BSA at 2.0 mg/ml to set up their particular apparatus, and then the transition to tubulin will be easier. Use of a single channel pipettor allows 96 assays to be performed per kit.

#### Repetitive Pipettor or Multichannel Pipettor (recommended when > 8 assays are to be performed at one time)

Tubulin polymerization will begin as soon as the protein is introduced into the warm 96 well plate, it is therefore very important to introduce the protein into reaction wells simultaneously. Working quickly, a single channel pipettor is fine for up to 8 assays. For a greater number of assays we strongly recommend the use of a repetitive or a multi-channel pipettor. Use of these instruments necessitates some loss of tubulin reaction mix and therefore you will only get approximately 75 – 85 assays from this kit.

## II: Important Technical Notes (Continued)

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### **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 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.

#### **Tubulin Protein Stability**

Tubulin is a labile protein; the instructions in Section IV specify that reconstituted tubulin should be aliquoted into 85  $\mu$ 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.

#### **Assay Characterization**

In order to achieve reproducible results the researcher must decide on standard conditions of operation. The recommended standard conditions are 2 mg/ml tubulin in Buffer 1 supplemented with 1 mM GTP and 15% glycerol. Using a higher protein concentration will achieve greater polymerization signal which can be useful for detecting inhibitors. Using lower or zero concentrations of glycerol is useful for detecting polymerization enhancing compounds. In the absence of glycerol, tubulin will not polymerize under the conditions used for this assay except in the presence of an enhancing agent like paclitaxel. Conditions can be modified to suit particular requirements, for example if you require to search for inhibitors that bind hydrophobic pockets of tubulin you may want to use no glycerol and a higher concentration of tubulin or by using MAPs (as in MAP-rich tubulin, Cat. # ML116) which bind ionically rather than in hydrophobic pockets.

## II: Important Technical Notes (Continued)

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### Unit Definition

One unit of Porcine Brain Tubulin (Cat.# T240-DX) in BK011P is defined as 100 µg of protein (as determined by the Precision Red Advanced Protein Assay, Cat. # ADV02). When one unit of reconstituted tubulin in a 55 µl volume is pipetted into one well of a Corning Costar 96-well plate (Cat. # 3686), it will polymerize efficiently at 37°C to reach a maximum fluorescence in approximately 30 min. The fluorescence will increase by 3 to 4 fold over this time.

### 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, 10% glycerol and 10% DMSO. If this is not possible either, call customer service (303-322-2254 or [cserve@cytoskeleton.com](mailto:cserve@cytoskeleton.com)).

For proteins you need a 10x final concentration in General Tubulin Buffer (80 mM PIPES pH 6.9, 0.5 mM EGTA, 2.0 mM MgCl<sub>2</sub>, 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.

### III: Kit Contents

This kit contains sufficient reagents for approximately 80 - 96 assays of 55  $\mu$ l volume. The variability in assay number is due to the fact that the use of a multichannel pipettor results in some tubulin loss, hence less assays can be performed (see Section V). Use of single a channel pipettor results in 96 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
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)	Three tubes, lyophilized. Each tube gives 100 $\mu$ l of a 100 mM stock solution when reconstituted.
Buffer 1 (Part # BP01)	Two bottles, lyophilized. Each bottle 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, 10 $\mu$ M fluorescent reporter.
Tubulin Glycerol Buffer (Cushion Buffer) (Cat. # BST05-001)	One bottle of 10 ml. 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 $\mu$ l 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. Black, flat bottom, Corning Costar Cat. # 3686

## 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
Part # BP01	<ol style="list-style-type: none"><li>1. Reconstitute each bottle with 10 ml of Milli-Q water (20 ml total)</li><li>2. Pool the bottle contents.</li><li>3. Aliquot into 13 x 1.5 ml volumes, freeze at -70°C</li></ol>	Store at -70°C
Cat. # BST05-001	No reconstitution necessary.	Store at 4°C.
Cat. # BST06-001	<ol style="list-style-type: none"><li>1. Reconstitute each of the three tubes with 100 µl of ice cold sterile distilled water (300 µl total).</li><li>2. Place on ice.</li><li>3. Pool contents of the three tubes.</li><li>4. Aliquot into 13 x 20 µl volumes and freeze at -70°C.</li></ol>	Store at -70°C.
Cat. # T240-DX	<ol style="list-style-type: none"><li>1. Place the 10 mg bottle of T240 on ice, and obtain liquid nitrogen in a dewar.</li><li>2. Label 12 cryotubes "Tubulin Stock, 10 mg/ml" and place these on ice ready for aliquoting.</li><li>3. Defrost 20 µl of 100 mM GTP stock.</li><li>4. Mix 1.5 ml of ice cold Buffer 1 with 15 µl of 100mM GTP stock.</li><li>5. Completely resuspend the tubulin powder with the 1.1 ml of the supplemented Buffer 1.</li><li>6. Keep on ice for 2 minutes to allow complete resuspension.</li><li>7. Dispense into 12 x 88 µl aliquots into the labeled cryotubes and drop freeze in liquid nitrogen.</li><li>8. Store at -70°C.</li></ol> <p>Each aliquot is sufficient for 6 - 8 assays depending upon aliquoting technique used, see Section V.</p> <p><b>NOTE: It is very important to snap freeze the tubulin in liquid nitrogen as other methods of freezing will give</b></p>	Store at -70°C.
Cat. # TXD01	Reconstitute the tube of paclitaxel with 100 µl of DMSO. Freeze at -70°C or -20°C.	Store at -70°C or -20°C.

# V: Assay Protocol

## Standard Polymerization Assay Protocol: Method for 1-8 Assays

This standard assay format can detect inhibitors and enhances, the condition are 2 mg/ml tubulin in 80 mM PIPES pH 6.9, 2.0 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 1.0 mM GTP and 15% glycerol. If you want to optimize for inhibitor detection use 20% glycerol, or if you want to detect enhancers use 0% glycerol. Each 88 µl aliquot of tubulin is sufficient for 8 assays when using a single channel pipettor. The assay takes approximately 1.5 hours to complete. Tubulin polymerization is controlled by temperature so pay particular attention to this parameter.

1. Turn on fluorimeter and enter experimental parameters as described in Section II, page 7.
2. Place the 96 well plate (Corning Costar, Cat. # 3686) in the fluorimeter and allow to warm to 37°C for 10 minutes.
3. Thaw out the paclitaxel stock solution and aliquot 5 µl of this into 325 µl of room temperature sterile distilled water. This gives you a 30 µM (10x paclitaxel solution). Keep this at room temperature until use.
4. Prepare 10x stock solutions of your test compounds or protein of interest as described in Section II page 9.
5. Do not continue until the compounds are fully ready.
6. Defrost 1.5 ml of Buffer 1 and place on ice.
7. Defrost 20 µl of GTP stock and place on ice.
8. Remove the Tubulin Glycerol Buffer from 4°C and place on ice.
9. Defrost 88 µl of tubulin (880 µg) in a room temperature water bath until liquid, then IMMEDIATELY place on ice.

**Tubulin left at room temperature will begin to polymerize at 10 mg/ml. It is extremely important to defrost the protein rapidly and immediately transfer to ice.**

Component	Volumes for Standard Conditions	Volumes for Inhibitor Detection	Volumes for Enhancer Detection	Final Concentration*
Buffer 1	243 µl	205 µl	355 µl	1 x
Tubulin Glycerol Buffer	112 µl	150 µl	none	varies
GTP stock (100 mM)	4.4 µl	4.4 µl	4.4 µl	1 mM
Tubulin stock (10 mg/ml)	85 µl	85 µl	85 µl	2 mg/ml

\* This reaction mix is good for up to 1 hour on ice.

## V: Assay Protocol (Continued)

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10. Immediately mix the assay components (choose either Standard, Inhibitor or Enhancer condition) as shown below to make your Tubulin Reaction Mix:
11. Pipette 5  $\mu$ l of control buffer (exact match to your compound or protein buffer) into the first duplicate wells A1 & B1. Then pipette 5  $\mu$ l of the 10x paclitaxel stock into C1 & D1, followed by 5  $\mu$ l of the test compound into E1 & F1 etc, repeat until all compounds are aliquoted.
12. Place plate back into warm plate reader for 1 min, but no longer as the 5  $\mu$ l volume will evaporate quickly.
13. Pipette 50  $\mu$ l of the tubulin reaction mix into each of the eight wells using a single channel pipettor. Work rapidly, use medium pipetting speed and keep the tip of the pipettor on the wall of the well. This technique avoids bubble formation which will disrupt absorbance readings.
14. Start plate reader immediately. If the software crashes in the first 5 min it is worthwhile to restart the protocol file because some data can be rescued in most cases.

**Note:** Five microliters of 5 mM Calcium chloride solution can be used as the 10x stock positive control "inhibitor". You can also use 5  $\mu$ l of vinblastine, nocodazole or colchicine at 30  $\mu$ M concentration as the 10x inhibitor control stock. Paclitaxel solution 2 mM is included as the positive "enhancer control", 5  $\mu$ l of this is used at 30  $\mu$ M for the 10x stock, 3  $\mu$ M final concentration.

### **Standard Polymerization Assay Protocol: Method for > 8 Assays**

When > 8 assays are to be performed, it is recommended to use either a repetitive pipettor or a multi-channel pipettor.

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 fluorescence readings fluctuate and 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 operator is advised to use a solution of BSA at 2.0 mg/ml to set up their particular apparatus, and then the transition to tubulin will be easier. Use of a multi-channel pipettor allows approximately 80 assays to be performed per kit.

## VI: References

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1. Bonne, D., Heusele, C., Simon, C., and Pantaloni, D. 1985. 4', 6-Diamidino-2-phenylindole, a fluorescent probe for tubulin and microtubules. *JBC.* **260** (5): 2819-2825.
2. 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.
3. Walker, R.A., Salmon, E.D., and Endow, S.A. 1990. The *Drosophila* claret segregation protein is a minus-end directed motor molecule. *Nature.* **347**:780-782.
4. Amos LA. and Klug A. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Science.* **14**: 523-530.

## VII: Troubleshooting

Problem	Possible Solution
No polymerization	<ol style="list-style-type: none"><li>1. Polymerizations should be read at 340-360nm (excitation) and 410-460 nm (emission), make sure you have your fluorimeter set to these wavelengths.</li><li>2. To measure polymerization the fluorimeter needs to be set in kinetic mode to read once every 30 seconds to 1 minute.</li></ol>
Variability between experiments	<p>This is the single largest error in the assay. If all steps are strictly adhered to, the assay has a cv of 11%.</p> <ol style="list-style-type: none"><li>1. Re-read instructions paying particular attention to aliquoting technique.</li><li>2. Buffers that require GTP addition should not be kept longer than 4 hours on ice, after this time GTP supplemented buffers should be discarded. <b>DO NOT ATTEMPT TO FREEZE AND RE-USE THESE BUFFERS.</b></li></ol>
Inconsistent polymerization profiles between experiments	<ol style="list-style-type: none"><li>1. 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 Buffer 1 plus 1 mM GTP (see Section IV for detailed instructions). Tubulin stocks should not be frozen / thawed more than once.</li><li>2. 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.</li><li>3. 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.</li><li>4. The polymerization of tubulin is very sensitive to temperature fluctuations. It is very important to polymerize at 37°C.</li><li>5. 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.</li><li>6. The glycerol concentration has a large effect upon polymerization. Make sure you are including Tubulin Glycerol Buffer in the reactions.</li><li>7. Tubulin protein concentration has a large effect on polymerization. Poor polymerization could be the result of diluting the tubulin below 2 mg/ml.</li></ol>
Steadily varying control polymerization profiles across the plate	<p>This is due to slow pipetting resulting in polymerization beginning in early aliquoted wells before the final aliquots are completed. This is more likely to be a problem if you are processing &gt;8 – 10 assays at one time using a single channel pipettor.</p> <ol style="list-style-type: none"><li>1. Speed up pipetting.</li><li>2. Use a multichannel or repetitive pipettor (see important technical notes, Section II).</li></ol>
Polymerization appears erratic	<p>Air bubbles in the reaction can cause erratic looking polymerization curves. Careful attention to pipetting accuracy is essential.</p>



