V. 1.0

Biotin Labeled Tubulin
Source: Porcine Brain
Cat. # T333P

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Background Information

Tubulin is composed of a heterodimer of two closely related 55 kDa proteins called α and β tubulin. The two proteins are encoded by separate genes, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom. Consequently, tubulin isolated from porcine brain tissue is highly homologous to tubulin isolated from the majority of eukaryotic sources. This fact results in the technical benefit that porcine tubulin can be used to assay proteins originating from many diverse species.

Tubulin polymerizes to form structures called microtubules (MTs). When tubulin polymerizes it initially forms protofilaments, MTs consist of 13 protofilaments and are 25 nm in diameter. Each µm of MT length is composed of 1650 heterodimers (1). Microtubules are highly ordered structures that have an intrinsic polarity (see Figure 1).

Figure 1. Microtubule Schematic

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 and the slowly polymerizing end the minus-end. In vivo the plus-end of a microtubule is distal to the microtubule organizing center. 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. 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. Because of this parameter, pure tubulin in General Tubulin Buffer will not polymerize significantly at concentrations below 5 mg/ml. If, however, one adds a polymerization enhancer such as 5% glycerol, tubulin polymerization efficiency will approach 100% polymer mass at 37° C after 15-20 minutes. Tubulin polymerization is also a temperature sensitive event, optimal polymerization occurs at 37°C.

Material

Tubulin protein has been purified from porcine brain by an adaptation of the method of Shelanski et al. (2). The tubulin protein has been covalently linked to a long-chain biotin derivative on random surface lysines. An activated ester of the biotin derivative is used in the labeling procedure. A long-chain biotin derivative was selected for this procedure because it allows the biotin molecules to be spaced far enough away from the tubulin protein so as not to interfere with subsequent detection techniques, e.g. binding to streptavidin based reagents and beads. The labeling stoichiometry was determined to be approximately 1 to 2 biotins per tubulin heterodimer. Biotin-labeled tubulin is supplied as a white lyophilized powder.

Storage and Reconstitution

The recommended storage conditions for the lyophilized material is 4°C and <10% humidity. Under these conditions the protein is stable for 6 months. Lyophilized protein can also be stored desiccated at -70°C for 5 months. To reconstitute, briefly centrifuge the tube to collect the protein product at the bottom of the tube before resuspension. Tubulin protein should be reconstituted to 10 mg/ml with 2 µl of ice cold General Tubulin Buffer (Cat.# BST01, 80 mM PIPES pH 6.9, 2 mM $MgCl_s$, 0.5mM EGTA) supplemented with 1 mM GTP (G-PEM). Gently pipette up and down twice and place back on ice for 2 min to dissolve the protein. Tubulin at 10 mg/ml can be snap frozen in liquid nitrogen for storage. These frozen stocks are stable for 6 months at –70°C. Working stocks of the biotin tubulin can be made by further diluting the protein in G-PEM or G-PEM plus 5% glycerol (5% glycerol is recommended for *in vitro* applications. Reconstituted T333P MUST be snap frozen in liquid nitrogen prior to storage at –70°C; failure to do this will result in significant loss of activity.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% polyacrylamide gel. T333P is determined to be >99% tubulin (mol. wt 55 kDa).

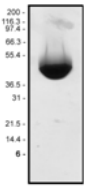

Figure 2. T333P Protein Purity Determination A 50 µg sample of T333P protein was separated by electrophoresis in a 4-20% SDS-PAGE system and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 molecular weight markers are from Invitrogen.

Sensitivity of Biotin Detection

To determine the efficiency of biotin labeling, nanogram amounts of biotinylated tubulin were separated by electrophoresis and electroblotted onto a PVDF membrane. The blot was then probed

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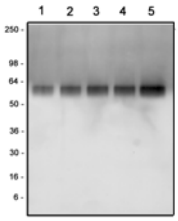
with streptavidin linked alkaline phosphatase. The biotin label on tubulin was detected down to 10 ng of protein (see Figure 3).

Figure 3. Detection of 10 ng of Biotinylated Tubulin.
Serial dilutions of biotinylated tubulin were separated by electrophoresis on a 12% polyacrylamide gel, blotted to PVDF, probed with 1:1000 dilution of streptavidin alkaline phosphatase (Sigma) and detected with 1-Step NBT/ BCIP reagent™ (Pierce). Lane 1, 10 ng, lane 2, 20 ng, lane 3, 40 ng, lane 4, 50 ng and lane 5, 100 ng of biotinylated tubulin. SeeBlue™ molecular weight markers are from Invitrogen.



Biological Activity Assay
The biological activity of biotinylated tubulin can be determined from its ability to efficiently polymerize into microtubules in vitro and separate from unpolymerized protein in a spin down assay. Stringent quality control ensures that >85% of the biotinylated tubulin can polymerized in this assay. This is comparable to the polymerization capacity of unmodified tubulin (Cat. # T240).

Reagents
1) Biotin tubulin (Cat. # T333P)
2) General Tubulin Buffer (Cat. # BST01, 80 mM PIPES pH 6.9, 2 mM MgCl2, 0.5 mM EGTA)
3) Tubulin Glycerol Buffer (Cat. # BST05, 80 mM PIPES pH 6.9, 2 mM MgCl2, 0.5 mM EGTA, 60% glycerol)
4) GTP stock (100 mM, Cat. # BST06)

Equipment
1) 37°C water bath
2) Table top ultracentrifuge capable of centrifuging 200 µl volumes at 100,000 x g at 37°C.

Method
1) Resuspend 500 µg of biotin labeled tubulin to 5 mg/ml in General Tubulin Buffer supplemented with 1 mM GTP and 5% glycerol. NOTE: GTP should be added from a fresh 100 mM stock immediately prior to use.
2) Centrifuge the diluted protein in a 4°C microfuge at 14,000 rpm for 10 minutes to remove any denatured protein aggregates.
3) Remove the 100 µl of supernatant to a fresh 200 µl ultracentrifugation tube and take the protein concentration to determine tubulin concentration in the supernatant prior to polymerization.
4) Incubate at 37°C for 20 minutes to allow tubulin polymerization.
5) Transfer the tube to a 37°C table top ultracentrifuge and pellet the microtubules by centrifugation at 100,000 x g for 45 minutes.
5) Determine the % polymerization by measuring the protein concentration of the supernatant before and after polymerization.

Uses of biotinylated tubulin
Biotin tubulin can be bound to streptavidin beads and used to examine monomer drug binding in scintillation proximity assays (see example 1 below and also Cat. # CDS15P and reference 2). Biotin tubulin can also be used to form paclitaxel stabilized biotinylated microtubules which form a substrate for microtubule binding studies (see example 2).

Methods of use, example 1: Formation of beads containing biotin tubulin monomer
1) Prepare 1 ml of G-PEM buffer by pipetting 1.0 ml of General Tubulin Buffer (BST01) and 10 µl of a 100 mM GTP stock (Cat. # BST06) into a 1.5 ml tube, place this tube on ice for 10 min to be sure its ice cold (this buffer is good for 1 h on ice). Call this buffer TB.
2) Place one vial of T333P on ice for 5 min, then resuspend with 20 µl of ice cold TB. The final protein concentration is 1.0 mg/ml (10 µM) tubulin.
3) Pipette 20 µl of a 50% slurry of streptavidin beads into a 1.5 ml centrifuge tube, add 200 µl of TB and centrifuge for 30 s at 14,000 rpm.
4) Pipette off the excess liquid leaving the beads in the pellet, place on ice.
5) Pipette 20 µl of ice cold 1.0 mg/ml T333P into the beads and rotate at 4°C for 30 min.
6) Centrifuge the slurry at 4°C, 14,000 x g for 30 s to pellet the beads which are now coated with biotin tubulin.
7) Pipette off the liquid leaving the beads in the pellet.
8) Resuspend the beads in 200 µl of TB to wash them free of unbound tubulin.
9) Centrifuge again, and resuspend in 20 µl of TB. This preparation now contains approximately 0.5 µg/µl of T333P bound to beads.
10) Add your protein or compound to the beads in at least 50% by volume of TB, this will retain tubulin's activity. If tubulin denatures it may bind HSP(heat shock proteins) type proteins or acidic proteins non-specifically.
11) Use the binding method in Step 5 and the wash procedures in Steps 6 through 8 to separate bead bound (and hence biotin tubulin bound) proteins or compounds. Use beads alone as a negative control. Analyze proteins by SDS-PAGE and Coomassie or Western detection. Analyze compounds by using tritium labeled versions or mass spectrometry.

Methods of use, example 2: Formation of paclitaxel stabilized biotin microtubules
1) Prepare 1.0 ml of G-PEM buffer as described in Step 1 of the Monomer Biotin Tubulin method.
2) Prepare 100 µl of 200 µM paclitaxel stock (Cat. # TXD01) by pipetting 90 µl of BST01 and 10 µl of TXD01 into a 1.5 ml tube at room temperature. Mix well. Call this 200 µM TX.
3) Place one vial of T333P on ice for 5 min, then resuspend with 20 µl of ice cold TB. The final protein concentration is 1.0 mg/ml (10 µM) tubulin.
4) Pipette 2 µl of 200 µM TX into 20 µl of 1 mg/ml T333P and mix well.
5) Incubate at 37°C for 10 min, then place at room temperature (24°C) or at least 20°C, this is your polymer stock of biotin microtubules, label the tube PBM. These are good for 4 h at 24°C.

References
1) Tahir et al. (2000). *Biotechniques*. **29**: 156-160.
2) Shelanski M.L., et al. (1973). *Proc. Natl. Acad. Science USA*. **70**: 765-768

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