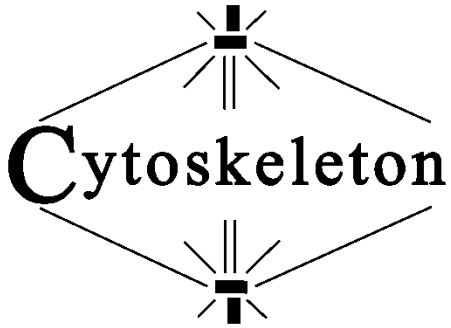
Version 2.1
2/18/13



Microtubules / Tubulin In Vivo Assay Kit

(Cat. # BK038)

ORDERING INFORMATION

To order by phone:	(303) - 322 - 2254
To order by Fax:	(303) - 322 - 2257
To order by e-mail:	cservice@cytoskeleton.com
Technical assistance:	(303) - 322 - 2254 tservice@cytoskeleton.com
Visit the web page:	www.cytoskeleton.com

MANUAL CONTENTS

Section I: Introduction
Overview 4
Uses of the Kit 4

Section II: Kit Contents
Equipment Required 5

Section III: Reconstitution and Storage of Components 6

Section IV: Methods
Quickview 7
Initial Considerations 7
Important Point 7
Detailed Method 8-10

Section V: Interpretation of Results 11

Section VI: Troubleshooting 12

Section VII: Appendix A: Tubulin Immunoblot 13

Section VIII: References 13

Section I: Introduction

Overview

The most reproducible and accurate method of determining the amount of microtubule content versus free-tubulin content in a cell population is to use western blot quantitation of microtubule and free-tubulin cellular fractions. The general approach is to homogenize cells in microtubule stabilization buffer, followed by centrifugation to separate the microtubules from free-tubulin pool. Then the fractions are separated by PAGE and tubulin is quantitated by western blot. The final result gives the most accurate method of determining the ratio of tubulin incorporated into the cytoskeleton versus the free-tubulin found in the cytosol.

Uses of the kit

1. To study the effects of pharmaceutical compounds on the ratio of tubulin to microtubules.
2. To study the effects of mutated cell lines versus their parent cell line for the change in ratio of tubulin to microtubules.
3. To study the effects of physical alterations of environment on the ratio of tubulin to microtubules.

Section II: Kit Contents

Note: BK038 has been shipped at room temperature. Once the kit has arrived it should be stored desiccated at 4°C. When stored correctly, the components are stable for a minimum of 6 months. Kit contents are listed in the table below:

Reagent	Cat. # Part #	Quantity	Description
Lysis and Microtubule Stabilization Buffer	Part # LMS1	1 bottle	100 ml Liquid; 100 mM PIPES buffer pH 6.9, 5mM MgCl2, 1mM EGTA, 30% (v/v) glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% beta-mercapto-ethanol, 0.001% Antifoam.
GTP Stock	Cat # BST06-001	1 tube	Lyophilized; 100 mM stock when reconstituted.
ATP Stock	Cat # BSA04-001	1 tube	Lyophilized; 100mM stock when reconstituted.
Protease Inhibitor Stock (Pepstatin, Leupeptin, Benzamidine, TAME)	Cat# PIC02	1 tube	Lyophilized, 2x concentrate when reconstituted.
Microtubule Enhancing Control Solution (Paclitaxel)	Cat# TXD01	1 tube	Lyophilized. 2 mM stock when reconstituted
Microtubule Depolymerization Control Solution (100X)	Part # BUF01	1 tube	Liquid; 1 ml of 200 mM $CaCl_2$.
Control Tubulin Solution	Cat# TL238	1 tube	Lyophilized. 250 µg of protein.
Tubulin Antibody (Monoclonal)	Part# ATN01	1 tube	Lyophilized; 100 µg
SDS Sample Buffer (5X)	Part # SDS01	1 tube	Liquid, 1.5 ml
Anhydrous DMSO	Part # DMSO	1 tube	Liquid; 1 ml for taxol and PIC02 resuspension. Note: DMSO will freeze at 4°C.

Equipment required

- Centrifuge capable of temperature controlled operation at 100,000 x g with volumes of 100µl to 2ml depending on the cell lyses volume. Suitable set ups are:
    a) Beckman Ultracentrifuge rotor SW50 with split adapters and tubes (Beckman cat# 344718)
    b) Beckman Airfuge with tubes (Beckman cat# 344718)
    c) Table top ultra with 1.5ml tubes.
- Small scale homogenization device, either pestle for 1.5ml tube, 25-Gauge 1ml syringes or fine tip 200µl pipette tip.
- Gel electrophoresis and western blotting equipment.

Section III: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as follows:

NOTE: read through this section carefully prior to beginning the preparation of reagents as you will require items readily on hand such as liquid nitrogen. If the proteins in this kit are not aliquoted as specified biological activity may be lost. When reconstituted and stored correctly, the components have a shelf life of at least 6 months.

Component	Reconstitution	Storage
Lysis and Microtubule Stabilization Buffer (Part# LMS1)	No reconstitution necessary	• Store at 4°C.
GTP Stock (Cat. # BST06)	1) Label 10 tubes "100 mM GTP". 2) For a 100 mM stock solution, reconstitute in 100 µl of ice cold Milli-Q water, aliquot into 10 x 10 µl volumes and store at -70°C.	• Store lyophilized material desiccated at 4°C. • Store reconstituted material at -70°C.
ATP Stock (Cat. # BSA04)	1) Label 10 tubes "100 mM ATP". 2) For a 100 mM stock solution, reconstitute in 1 ml of 100mM Tris-HCl pH 7.5, aliquot into 10 x 100 µl volumes and store at -70°C or -20°C.	• Store lyophilized material desiccated at 4°C. • Store reconstituted material at -70°C or -20°C.
Protease Inhibitor Cocktail (Cat. # PIC02)	Reconstitute with 1ml of dimethyl sulfoxide (DMSO) for a 100x stock solution.	• Store lyophilized buffer desiccated at 4°C. Stable for 6 months. • Store solution at 4°C. (Note: solution will freeze at 4°C)
Taxol (Cat. # TXD01)	For a 2 mM stock solution, reconstitute in 100 µl of anhydrous DMSO. Store at -70°C. WEAR GLOVES WHEN HANDLING TAXOL.	• Store lyophilized product desiccated at 4°C. • Store solution at -70°C.
Microtubule Depolymerization Control Solution (Part# BUF01)	No reconstitution necessary	• Store at 4°C.
Unlabeled Tubulin Protein (Cat. # TL238)	1) Reconstitute to 10 mg/ml with General Tubulin Buffer (80 mM PIPES pH 6.9, 2 mM MgCl2, 0.5 mM EGTA) supplemented with 1 mM GTP. 2) Snap freeze "experiment sized" aliquots in liquid nitrogen and store at -70°C. 3) Reconstituted TL238 is stable for 6 months at -70°C.	• Store lyophilized protein desiccated at 4°C. • Store resuspended protein at -70°C. • Reconstituted TL238 MUST be snap frozen in liquid nitrogen prior to storage at -70°C, failure to do this will result in significant loss of activity.
Monoclonal Tubulin Antibody (Part# ATN01)	1) Reconstitute in 200 µl of 30% glycerol in sterile water for a 500 µg/ml solution. 2) Stable at 4°C for six months. Do not freeze.	• Store lyophilized buffer desiccated at 4°C. Stable for 6 months. • Store resuspended material at 4°C.
SDS Sample Buffer(5x) (Part# SDS01)	No reconstitution necessary	• Store at 4°C.
DMSO (Part# DMSO)	No reconstitution necessary	• Store at 4°C. (Note: solution will freeze at 4°C)

Section IV: Methods

Quickview:

1) Suspend cells in LMS2.

2) Gently homogenize to lyse cells.

3) Centrifuge lysates to remove unbroken cells.

4) Centrifuge supernatants at 100,000xg to separate microtubules from soluble tubulin.

5) Analyze supernatant (tubulin) versus pellet (mictotubules) for tubulin content using western blotting.

6) Scan tubulin bands by densitometry and calculate the ratio of tubulin in the microtubules versus that present as free tubulin.

Initial considerations:

1. The microtubules / tubulin *in vivo* assay requires a constant cells to buffer volume ratio. Essentially the lysis step has to dilute the cellular extract so that the free tubulin does not polymerize onto existing microtubules (MTs). This ratio is roughly 10 volumes of buffer to 1 volume of cell pellet, larger volumes of buffer are fine and in this kit the ratio is aiming at 50 volumes of buffer per volume of cells.
2. The average cell size is important in designing the experiment because of the considerations of #1 above, be sure to estimate the average cell size of your culture so that you can use it to calculate a good estimate for the volume of lysis buffer required.

Important point:

Microtubule populations are very sensitive to temperature; a one degree reduction in temperature at any stage in the following procedure could reduce the MT mass by 5%. If you have five stages that are lower than the culture temperature by 1°C then you could lose up to 25% of your MT mass which would increase errors and make reproducing your results very difficult. Therefore, pay particular attention to temperature detail, for example you can measure the exact temperature inside a centrifuge tube by performing a test run with water in the tube and then placing a thermometer in the water before and after the run. Warm all apparatus rotors and centrifuge tubes to culture temperature before starting the assay.

Detailed method:

1) Place centrifuge rotor in 37°C warm environment (either incubator or centrifuge) and set the centrifuges to 37°C for at least 2h before starting the experiment.

2) Either, estimate the required volume of lysis buffer (LMS2-see recipe below) for one test using the following equation:

   a. (number of cells) x d^2 x 2 x 10^{-5} = lysis buffer in µl,

   b. where d is average diameter of cells in micrometers (µm). Remember to multiply this volume by the number of tests to make the total volume required.

3) Or, use these amounts as a starting point, 4 ml LMS2 per 10 cm plate, 2 ml per 6 cm plate or 1 ml LMS2 per 3 cm well.

4) Make the total required volume of LMS2 by pipetting the total volume required of LMS1 into a clean vessel at room temperature plus the following:

   i. 1 µl BST06 (100mM GTP) per ml of LMS2
   ii. 10 µl BSA04 (100mM ATP) per ml of LMS2
   iii. 10 µl protease inhibitor cocktail.
   iv. Mix all components until homogenous.

5) Warm LMS2 buffer to same temperature as the cells, usually 37°C for 3 min only.

6) Label centrifuge tubes equivalent to the number of samples being tested. Use tube numbers 1, 2 and 3 for "normal", "positive" and "negative" controls respectively. In addition, add $1/100^{th}$ volume of paclitaxel solution to tube 2 (positive control), and $1/100^{th}$ volume of $CaCl_2$ solution to tube 3 (negative control).

7) Harvest the cells by one of the two following procedures:

   a. For cells in suspension:
      i. Pipette cells into culture temperature centrifuge tubes with enough cells per tube for each test.
      ii. Centrifuge cells for 1 min at 7 000xg, in a culture temperature rotor.
      iii. Record time and add the appropriate volume of buffer per tube enough for one test sample (see Step 2).
   b. For cells on a culture dish:
      i. Aspirate media from the bottom of the inclined dish. Add appropriate volume of LMS2 buffer (see Step 2 and/or 3). A larger volume of LMS2 buffer can be used if required to cover the cells. Record time.

ii. Scrape cells off the culture surface and collect them at the bottom of the inclined dish.
iii. Pipette into the pre-labeled tubes.

c. Homogenize cells with the appropriate volume of warm LMS2 (see Step 2 and/or 3) using a 25G syringe with a bent-over tip, or 200µl pipette tip with a fine orifice (syringe or pipette up and down eight times). Homogenize quickly because the samples should be treated for a similar time.

8) Immediately centrifuge for 5 min at 2000 x g at 37°C.

9) Pipette supernatants into ultra centrifuge tubes and centrifuge at 100,000 x g for 30 min at 37°C.

10) Label tubes S1, S2, and S3 etc. for the supernatants and place on ice. Place 10 ml of nanopure water on ice in a 15 ml Falcon tube.

11) When rotor stops, immediately pipette supernatants into appropriate labeled tubes and place on ice.

12) Using ice-cold Microtubule Depolymerization Control solution (Part # BUF01) diluted 1:100 (final solution of 2 mM $CaCl_2$), resuspend pellet to the same volume as the supernatant and leave at room temperature for 15 minutes to depolymerize microtubules. Every 2 min pipette up and down once with a 200 µl pipette tip to shear the pellet.

13) Dilute 10 µl of the supernatant and pellet samples from Steps 11 and 12 with 90 µl of nanopure water, mix well and add 25µl of 5x SDS loading buffer, mix and heat to 95°C for 2 min.

14) Load equivalent volumes (e.g. 20µl) onto a 10 well 10% SDS-polyacrylamide gels in duplicate. Electrophorese to separate the samples according to molecular mass. Use the sample of pure tubulin TL238 to create a standard curve (5, 10, 20 and 40ng per lane).

15) Blot the gel onto a nitrocellulose filter and process as a tubulin immunoblot using the specific protocol in Appendix A with the tubulin antibody provided at $1/500^{th}$ dilution.

16) Determine ratio of tubulin to microtubules by scanning densitometry or light emission from chemiluminescence signal.

Notes:

1. The assays should be performed twice in duplicate to validate results. Experiments should be performed at least on different days and best on different weeks for the highest accuracy. Samples may vary by up to 20% even in the best experiments so the duplicates and two experimental days are crucial for accurate results.

2. The results can be presented either as a percent of total tubulin or as a ratio of Free- versus MT-tubulin, both are valid. In addition the exact tubulin (as Free- and MT-) can be calculated by knowing the amount of total cellular protein loaded onto the wells and the mass of tubulin therein, and by assuming total cellular protein concentration inside the intact cell is 80mg/ml.

3. A useful equation is: Total cellular tubulin = Free-tubulin + Microtubule-tubulin.

   This equation is useful to determine if all the tubulin is being analyzed, if 100% of expected tubulin can be accounted for in the supernatant and pellet fractions then the assay becomes more significant than if you lost 30% in one step for example. To compare fractionate and total tubulin, run a sample of total protein from the lysis step to see if the amount of tubulin in this sample equates with the Free- and Microtubule-tubulin fractions.

4. Absolute quantitation of cellular tubulin can be performed using the Tubulin Standard as a positive control at 40, 20, 10 and 5 ng per lane.

Section V: Interpretation of results:

The paclitaxel control sample is an example of enhanced MT polymerization; you should easily determine that free- to MT-tubulin content has increased from 50:50 to 10:90. Conversely, the calcium treatment is an example of decreased MT content causing a free- to MT-tubulin ratio of 90:10. The remaining free-tubulin in the paclitaxel sample comes from tubulin in complex with other proteins plus the free-tubulin left at the equilibrium of free- to MT-tubulin in the presence of this drug, also called the critical concentration of free-tubulin. In the case of the calcium sample the critical concentration of free-tubulin is very high but there are remnants of MT-tubulin that are associated with Golgi bodies and these are very stable MTs.

These control results are usually extreme with respect to physiological conditional responses. There may be a 30% or 15% difference in MT-tubulin between your samples containing for example different hormones in the culture media, or low concentrations of drug compounds, or mild mutations. The lower the difference between control cells and your test samples the more replicates you will need to show a significant difference. Generally, less than 10% difference cannot be picked out by this or any other presently accepted method.

If there are concerns about your results you can see the Troubleshooting section in this manual or call 303-322-2254 for Technical Assistance.

Section VI: Troubleshooting:

Observation	Possible cause	Correction
Free-tubulin is >80% of the total	1. It could be a valid result some cell lines may have >80% free tubulin. 2. Assay performed at incorrect temperature 3. Excessive proteolysis	1. Try steps 2 and 3. 2. Perform a temperature inventory to see if your equipment and solutions are at the expected temperature. 3. Add extra proteases as required by your samples. (e.g. aprotinin, chymostatin, trypsin inhibitor).
Cannot detect bands on the blot	1. Poor transfer 2. Low activity chemiluminescence system.	1. Use 0.01% SDS in transfer buffer to increase mobility of protein. 2. Use ECL Amersham, or West-Dura Pierce chemiluniescence reagents.
Cells not lysed, this kit will not work on yeast or plant or similar cells that have a hard cell wall.	1. Homogenization not abrasive enough	1. Use a tight fitting homogenization device that shears cells. Do not use sonication which will break-up MTs resulting in rapid disassembly and low MT-tubulin values.

Section VII: Appendix A - Tubulin immunoblot

Method for Western blot procedure

1. Blot proteins on to a nitrocellulose membrane.
2. Block for 20 min in 5% non-fat milk in TBST (20mM Tris-HCl pH 8.0, 50mM NaCl, 0.01% Tween20).
3. Pour block off and probe with 1:500 ATN01 in TBST (no block) for 1h at RT.
4. Wash 3 X with TBST for 5min each.
5. Probe with 1:20,000 anti-mouse-HRP in TBST (no block) for 1h at RT in TBST.
6. Wash 6 X with TBST for 5min each.
7. Detect with Pierce's West Dura chemiluminescent agent for 5sec to 5min to achieve a faint band at the lane containing 5 ng of pure tubulin.

Section VIII: References

1. Pipeleers DG, Pipellers-Marichal MA, Sherline P. and Kipnis DM. 1977. A sensitive method for measuring polymerized and depolymerized forms of tubulin in tissues. *JCB*, **74**, 341-350.
2. Solomon F, Magendantz M. and Salzman A. 1979. Identification with cellular microtubules of one of the coassembling microtubule-associated proteins. *Cell*, **18**, 431-438.
3. Ball R, Carney DH, Albrecht T, Asai DJ. and Thompson WC. 1986. A radiolabeled monoclonal antibody binding assay for cytoskeletal tubulin in cultured cells. *JCB*, **103**, 1033-1041.
4. Thrower D, Jordan MJ. and Wilson L. 1991. Quantitation of cellular tubulin in microtubules and tubulin pools by a competitive ELISA. *J. Immunol. Methods*, **136**, 45-51.
5. Thrower D, Jordan MJ. and Wilson L. 1993. A quantitative solid phase binding assay for tubulin. Methods in Cell Biology, 37, 129-145.