V. 5.3

Microtubules/Tubulin In Vivo Assay Kit

Cat. # BK038

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Manual Contents

I: Introduction

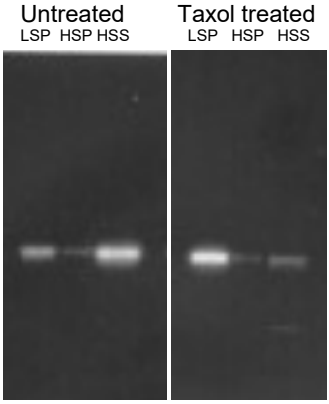
Overview

This kit provides the end user with a method of determining the amount of microtubule content versus free-tubulin content in a cell population. Cells or tissues are lysed in a microtubule stabilization buffer that preserves the integrity of the microtubule to tubulin ratio in the cells and forms the basis of the assay. After lysis, a centrifugation step separates polymerized microtubules (pellet fraction) from non-polymerized tubulin (supernatant fraction). The tubulin in supernatant vs pellet fractions are subsequently analyzed by quantitative western blots. The final result provides a quantitation of 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.

Example results: Microtubule distribution in taxol treated vs untreated 3T3 cells



Legend: Western blot analysis of low speed (LS) and ultra-centrifuged (HS) samples of untreated and paclitaxel treated tissue culture cells.

Untreated or Taxol treated (1 μM taxol,1h,37°C, 5%CO_2) 3T3 cell lysates were centrifuged at low speed (1,000 g), the low speed pellet was saved for western analysis (LSP). The low speed supernatants were centrifuged at 100,000 g and both supernatant (HSS) and pellet (HSP) were saved for western blot analysis. All lanes were loaded with cell lysate that represent an equal number of cells per lane. Western blots were probed with anti-tubulin antibody and tubulin signals were quantitated by densitometry.

The majority of taxol stabilized microtubules appear in the low speed pellet (LSP/Taxol treated), whereas the majority microtubules from the untreated samples appear in the high speed supernatant. The difference in microtubule fractionation reflects the stabilization of taxol treated microtubules in 3T3 cells.

II: Purchaser Notification

Limited Use Statement

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

III. Kit Contents

This kit contains sufficient reagents to process 100 x 1ml lysates. There is sufficient primary antibody to make 100 ml of antibody solution. When properly stored, kit components are guaranteed stable for a minimum of 6 months. Table 1 summarizes kit contents.

Table 1: Kit Contents and storage upon arrival

Reagents	Cat.# or Part#*	Quantity	Storage Upon Arrival
Lysis and Microtubule Stabilization Buffer	Part # LMS01	1 bottle, 100 ml	4°C
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 4°C
ATP	Cat. # BSA04	1 tube, lyophilized	Desiccated 4°C
GTP	Cat # BST06-001	1 tube, lyophilized	Desiccated 4°C
Microtubule Depolymeriz-ing Buffer (100X stock)	Part # BUF01	1 tube 1 ml	4°C
Microtubule Enhancing Solution	Cat # TXD01	1 tube, lyophilized	Desiccated 4°C
Tubulin Protein Standard	Cat # T240	1 tube, 1 mg, lyophilized	Desiccated 4°C
Anti-Tubulin, sheep Poly-clonal Antibody	Cat # ATN02	1 tube, lyophilized	Desiccated 4°C
Anti-sheep HRP conjugat-ed secondary antibody	Part # GL21	1 tube, lyophilized	Desiccated 4°C
SDS Sample Buffer	Part # SDS01	4 tubes, 1.5 ml per tube	4°C
DMSO	Part # DMSO	2 tubes, 1 ml per tube	4°C

* Items with Part numbers (Part #) are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.

The reagents and equipment that you will require but are not supplied;

- Temperature controlled centrifuge capable of reaching 100,000 x g. Ideally accepts 100 –1000 µl sample volumes. The assay can be adapted for larger volumes, however, this may result in less assays per kit (see Section V1: Assay Protocol).
- Small homogenizer suitable for low milliliter volumes or 25G needle and syringe.
- SDS-PAGE & western blot system.

IV: Reconstitution and Storage of Components

Many of the components in this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as detailed in Table 2.

Table 2: Kit Component Storage and Reconstitution

Reagents	Reconstitution	Storage
Lysis and Microtubule Stabilization Buffer	No reconstitution necessary	4°C
Protease Inhibitor Cocktail	1) Reconstitute with 1ml of dimethyl sulphoxide (DMSO) for a 100x stock solution. 2) Aliquot into 10 x 100 µl volumes.	4°C or –20°C
ATP	1) Reconstitute with 1ml of ice cold 100 mM Tris pH 7.5 to make 100 mM stock solution 2) Aliquot into 10 x 100 µl volumes.	-70°C or –20°C
GTP	1) Reconstitute with 100 µl of ice cold water to make 100 mM stock solution. 2) Aliquot into 10 x 10 µl volumes.	-70°C or –20°C
Microtubule Depolymerizing Buffer	No reconstitution necessary	4°C
Microtubule Enhancing Solution	Reconstitute in 100 µl of DMSO. Wear gloves when handling a taxol containing product.	-70°C or –20°C
Tubulin Protein Standard	1) Reconstitute to 10 mg/ml with 100 µl of ice cold water. 2) Aliquot into 20 x 5 µl volumes.	-70°C or –20°C
Anti-tubulin Antibody	1) Reconstitute in 100 µl of 30% glycerol in sterile water 2) Stable at 4°C for 1 month. 3) For long term storage, aliquot into 10 µl volumes and store at -20 or -70°C.	4°C (up to 1 month) -20 or -70°C (up to 1 year)
Anti-sheep HRP conjugated secondary antibody	1) Reconstitute in 20 µl of PBS pH 7.4. 2) Stable at 4°C for 6 months	4°C (up to 6 months)
SDS Sample Buffer	No reconstitution necessary	4°C
DMSO	No reconstitution necessary.	4°C DMSO freezes at 4°C

V: Important Technical Notes

A) Notes on Updated Version 5.3

The following updates from prior versions should be noted:

1. The manual has been changed to a 5.5 x 8.5 format.
2. Resuspension volumes for cell lysates have been reduced to give a more robust tubulin detection while maintaining the endogenous ratio of microtubules to tubulin.
3. The 100,000 x g centrifugation step has been increased from 30 to 60 minutes to give a tighter microtubule pellet fraction.
4. The primary anti-tubulin antibody has been changed from a mouse monoclonal to a sheep polyclonal version. We have found that this Ab has a higher sensitivity. An HRP conjugated anti-sheep secondary Ab has been included in the kit.
5. The control tubulin protein has been changed from a bovine source to a porcine source.
6. The low speed pellet is used as an additional sample for analysis.

B) Assay Temperature

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. Warm all apparatus rotors and centrifuge tubes to culture temperature before starting the assay.

VI: Assay Protocol: Quick Overview

Assay Quick View:

1. Wash cells with 37°C PBS pH 7.4.
2. Suspend cells or tissue in LMS2.
3. Gently homogenize to lyse cells.
4. Centrifuge lysates at 1,000 g to sediment large complexes of microtubules attached to nuclei and Golgi (low speed pellet fraction).
5. Centrifuge supernatants at 100,000xg to separate microtubules (high speed pellet fraction) from soluble tubulin (high speed supernatant fraction).
6. Analyze the high speed supernatant versus high and low speed pellet for tubulin content using western blotting.
7. Scan tubulin bands by densitometry and calculate the ratio of tubulin in the microtubules (pellets) versus that present as free tubulin (supernatant).

Initial Considerations:

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 20 - 50 volumes of buffer per volume of cells.

Detailed Assay Method

PART 1: Assay Preparation

1. The assay requires a low speed centrifuge (1,000 g) and a high speed centrifuge (100,000 g) capable of taking small volumes (0.1-1 ml). Rotors should be warmed to 37°C prior to beginning the assay.

2. Determine the total volume of LMS2 buffer you require per experiment using the volumes given in Table 3 as a guide (see LMS2 recipe below) .

3. Make the required volume of LMS2 buffer as follows;

1 ml	LMS01 buffer (Lysis and Microtubule Stabilization Buffer)
1 µl	BST06 (100 mM GTP stock solution (remaining stock can be re-frozen)
10 µl	BSA04 (100 mM ATP stock solution (remaining stock can be re-frozen)
10 µl	PIC02 (100x protease inhibitor cocktail stock (remaining stock can be re-frozen)

Table 3: Recommended volumes of LMS2 buffer

Culture Vessel (assumes 70-80% cell confluency)	Volume of LMS2 per lysate (ml)
35 mm dish	0.1 ml
60 mm dish	0.3 ml
100 mm dish	0.6 ml
6-well plate	0.1 ml per well
T-25 Flask	0.3 ml
Tissue samples* Per 0.1g of tissue	1 ml

*Tissue density can vary widely. It is recommended to homogenize 0.1g of tissue in 1 ml of LMS01 and determine the approximate tissue volume after centrifugation (10,000 g for 10 minutes). Use 20x this volume for experimental tissue lysates.

4. Warm PBS pH 7.4 to 37°C prior to beginning the assay. The PBS is required to wash tissue culture cells prior to cell lysis. This is particularly important with drug treated cells as residual drugs left in the tissue culture media may affect the tubulin/ microtubule ratio after cell lysis.

5. Warm LMS2 buffer to 37°C prior to beginning the assay.

PART 2: Lysate Collection & Processing

1. Lysis methods for suspension cells (A), adherent cells (B) or tissue samples (C) are given below.

A) **Cells in suspension**

a) Harvest cells by centrifugation at 1,000 x g for 2 minutes.
b) Remove supernatant and discard.
c) Wash once by gently resuspending the cells in 10 ml of 37˚C PBS pH 7.4.
d) Harvest cells by centrifugation at 1,000 x g for 2 minutes.
e) Resuspend cell pellet in 20 x cell pellet volume of 37˚C LMS2.
f) Proceed to step 2.

B) **Adherent Cells**

a) Aspirate media from dish. Incline dish to 30° angle to help remove of as much media as possible.
b) Wash once by gently adding 10 ml of 37˚C PBS pH 7.4.
c) Aspirate PBS from dish. Incline dish to 30° angle to help remove of as much PBS as possible.
d) Add appropriate volume of 37˚C LMS2 (see Table 3).
e) Harvest cells by scraping thoroughly with cell scraper, again keep the plate at a 30° angle to help collect all of the lysate.
f) Pipette cell lysate into tube and proceed to step 2.

C) **Tissue Samples**

a) Add 1000 µl of 37˚C LMS2 per 100 mg (0.1g) of tissue sample.
b) Proceed to step 2.

2. Homogenize samples using a small hand held or motorized homogenizer suitable for low milliliter volumes or a 25G syringe or a 200 µl pipet tip (usually sufficient for cell culture samples).

3. Immediately centrifuge for 5 minutes at 1,000 x g at 37˚C.

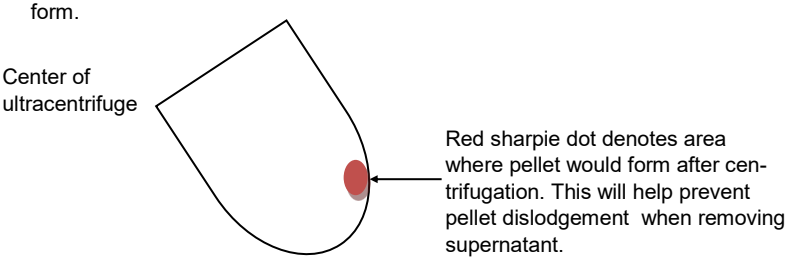
4. Carefully remove the low speed supernatants to fresh tubes and place the low speed pellets on ice.

5. Remove 100 µl volume of low speed supernatant from each sample into a clean ultra -centrifuge tube at 37°C. Any remaining lysate can be discarded or used for other purposes at this point.

   NOTE: Larger volumes of lysate can be processed if required.

6. Centrifuge the low speed supernatant at 100,000 x g for 60 minutes at 37°C. This step will pellet microtubules and leave unpolymerized tubulin in the supernatant.

Tip: Before placing the centrifuge tubes in the ultracentrifuge rotor, mark the outer side of each tube with a sharpie dot to indicate the place where a microtubule pellet will form.



6. After the ultra-centrifuge step is complete, immediately and gently remove the **TOP 80 µl** of supernatants to fresh tubes designated as high speed supernatant samples. Ultra-centrifuge supernatants should be removed gently to avoid disturbing the microtubule pellet. Only 80 µl of supernatant is removed to avoid disturbing the microtubule pellet. The microtubule pellet is not likely to be visible. Add 20 ul of 5X SDS buffer to each 80 µl sample.

7. Very carefully remove the final 20 µl of supernatant, point the pipette away from the position of the microtubule pellet so as to avoid disturbing the pellet. Discard the 20 µl supernatant.

8. Dilute the 100X stock of Microtubule depolymerization Buffer (BUF01) 1:100 to give a 1X BUF01 solution (10 µl of BUF01 into 990 µl of ice cold nanopurewater) and suspend the high speed pellets in a volume equal to the lysate supernatant volume. e.g. if 100 µl of lysate was centrifuged then suspend the microtubule pellet in 100 µl of 1X BUF01. Add 25 ul of 5X SDS buffer per 100 ul of resuspended pellet.

9. Leave the tubes at room temperature for 15 minutes to help disperse the microtubule pellet. Pipette up and down several times with a 200 µl pipette to help shear and homogenize the microtubule pellet.

10. Resuspend the low speed pellets in a volume of 2X SDS buffer that is equivalent to 1.2 volumes of the original lysis buffer e.g. if you lysed the cells in 200 ul of lysis buffer then resuspend the low speed pellet in 240 ul of 2X SDS buffer.

11. The samples are now ready for tubulin quantitation by SDS-PAGE and western blot analysis. Samples can be stored at –20°C prior to moving to PART 3: Tubulin quantification by SDS-PAGE / Western blot analysis.

PART 3: Tubulin Quantification by SDS-PAGE / Western Blot Analysis

1. Prepare the following Tubulin protein standards as follows;

A) Thaw out 1 x 5 µl volume of tubulin stock (10,000 ng/µl tubulin).

B) Add 95 µl of SDS Sample Buffer (SDS01) (500 ng/µl tubulin: Tube 2).

C) Add 10 µl Tube 2 to 90 µl of SDS Sample Buffer (SDS01) (50 ng/µl: Tube 3).

D) Add 3 µl of Tube 3 to 27 µl of SDS Sample Buffer (5 ng/µl: Tube 4).

2. Run lysate supernatant and pellet samples with 50 ng (10 µl of Tube 4) of Tubulin protein standard on an SDS polyacrylamide gel (e.g. a 4-20% gradient gel or a 12% gel).

3. We recommend running 20 µl of each lysate sample as an initial test. In some cases a 20 µl sample of lysate will contain tubulin amounts that are out of the linear range of western blot detection. In these cases either more or less sample will need to be run. It is important not to discard lysate samples until an accurate tubulin quantitation has been obtained. Lysates can be stored at –20°C for up to 12 months.

4. Transfer the proteins from SDS-PAGE to a western blot membrane (nitrocellulose or PVDF) according to the manufacturers instructions.

5. After transfer, block the membrane in TBST/5% non-fat milk (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.01% Tween 20/5% non-fat milk) for 30 minutes at room temperature with agitation, e.g. orbital shaker.

6. Wash the membrane 3 x 10 minutes in TBST at room temperature with agitation.

7. Dilute the anti-tubulin sheep polyclonal antibody supplied in this kit 1:1000 in TBST/1% non-fat milk. This is 5 µl of antibody per 5 ml of TBST/1% non-fat milk.

8. Incubate the membrane in primary antibody for 1h at room temperature or overnight at 4˚C with agitation.

9. Wash the membrane 3 x 10 minutes in TBST at room temperature with agitation.

10. Dilute the secondary anti-sheep HRP antibody 1:10,000 dilution in TBST/1% non-fat milk (1 ul sheep secondary Ab in 10 ml TBST). Incubate for 60 minutes at room temperature with agitation.

11. Wash the membrane 6 x 10 min with TBST at room temperature with agitation.

12. Process the membrane for chemiluminescent detection of tubulin (55kDa). Use the tubulin standard to quantitate the amount of tubulin in your lysate supernatants (tubulin heterodimer) and pellets (microtubules).

Microtubule Enhancing Solution Control

A Microtubule Enhancing Solution (TXD01) is included in this kit. When reconstituted the solution contains 2 mM paclitaxel which is a potent tubulin polymerization enhancer. This can be added to a cell culture to increase microtubule polymer mass. Cells treated with TXD01 should show a greater tubulin signal in the pellet fraction than cells that have not been treated with taxol.

Method

1) Dilute 20 µl of TXD01 stock with 180 µl of room temperature DMSO to give a 200 µM solution.
2) Add 5 µl of the 200 µM solution per 1 ml of tissue culture media and incubate cells at 37˚C in a tissue culture incubator for 1 hour before processing cell lysate as described in the Assay Protocol.

VII: Troubleshooting

Observation	Possible Cause	Possible Solution
No tubulin signal in pellet fractions in the TXD01 control cells	1) Likely cause is dislodgement of pellet during processing. 2) Some cells are more sensitive to paclitaxel than others.	1) Be very careful when removing post centrifugation supernatant and mark the centrifuge tube in the position that the microtubule pellet is expected to form. In this way you can avoid going near the pellet when removing super-natant. 2a) In some cases a titration of TXD01 concentrations are required to deter-mine the responsive window of a given cell line. For example, try 1 µl, 5 µl and 10 µl of TXD01 per 1 ml of tissue culture media. 2b) In cases where a cell line is very responsive to taxol the microtubules formed can be very short and will therefore not pellet well so in some cases longer centrifugation times will give clearer results.
No tubulin signal is ob-served	1) The anti-tubulin antibody is a sheep polyclonal.	1) Make sure that the HRP anti-sheep secondary antibody that is included in this kit is used.

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