V. 5.0

Microtubule Binding Protein Spin-down Assay Kit

Cat. # BK029

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Manual Contents

I: Introduction

Overview

This assay allows the identification of proteins that will bind to microtubules (MTs) *in vitro*. The assay relies on the fact that MTs will pellet when centrifuged at 100,000 x g. Therefore, any protein that is associated with the MTs will pellet with them during centrifugation. A simple SDS-PAGE analysis of the supernatant versus pellet fraction will identify if a protein is able to associate with MTs (see Figure 1, Section VI: Interpretation of Results). The assay description given in this manual is for recombinant "test" proteins; however, the assay can be adapted for cell lysates or *in vitro* translation products (1).

It should be noted that *in vivo* confirmation of MT association should be obtained in order to confirm that the protein can be classified as a microtubule associated protein (MAP). This association need not occur throughout the whole cell cycle and may even be developmentally regulated; indeed transient association of MAPs with microtubules is the norm rather than the exception.

Kit Uses

- Identification of novel Microtubule Associated Proteins (MAPs)
- Confirmation of in vivo data suggesting a given protein is a MAP
- Characterization of known MAPs
- Identification / characterization of MAP regulating proteins
- Identification / characterization of compounds that inhibit MAP binding to microtubules

SDS-Page Protein Detection

This kit does not provide any reagents for the detection of your "test" protein. It is designed to detect proteins by Coomassie or silver staining techniques. The limits of protein detection for Coomassie and silver staining are approximately 200 ng and 1 ng respectively. Sub-ng quantities of test protein will require either labeling of the test- protein directly with radioactivity or use of an antibody for Western blot detection.

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 enough reagents for reagents for approximately 30 to 100 assays depending on the detection method used. When properly stored, kit components are guaranteed stable for a minimum of 6 months.

Reagent	Cat.# or Part#*	Quantity	Storage Upon Arrival
Tubulin protein	Cat# T240	1 tube, 1 mg lyophilized (55 kDa)	Desiccated 4°C
MAP fraction	Cat. # MAPF	2 tubes, 100 µg protein (contains 60% MAP2 at 280 kDa and 40% tau proteins at 40-70 kDa), lyophilized	Desiccated 4°C
BSA protein	Part# BSA	1 tube, 100 µg of protein (68 kDa), lyophilized	Desiccated 4°C
General Tubulin Buffer	Cat# BST01	1 bottle, lyophilized. 80mM PIPES pH 7.0, 2 mM $MgCl_2$, 0.5 mM EGTA when reconstituted.	Desiccated 4°C
Cushion Buffer	Cat# BST05-001	1 tube, liquid, 80mM PIPES pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 60% Glycerol	4°C
Salt Extraction Buffer	Part# SEB12	1 tube, lyophilized. 80mM PIPES pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 500 mM KCl when reconstituted	Desiccated 4°C
GTP Stock	Cat# BST06	1 tube, lyophilized. 100 mM stock when reconstituted	Desiccated 4°C
Taxol stock	Cat# TXD01	2 tubes, lyophilized. 2 mM stock when reconstituted	Desiccated 4°C
Anhydrous DMSO	Part # DMSO	1 tube, liquid. 1 ml for taxol resuspension. NOTE: DMSO will freeze at 4°C	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.

Reagents and Components not Supplied:

- Laemmli reducing-sample buffer (1x stock, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue and 5% v/v β-ME) and (5x stock, 300 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.005% bromophenol blue and 25% v/v β-ME)
- Polyacrylamide gels (12% or 4-20% gradient gels), SDS-PAGE apparatus and buffers
- Reagents for the detection of the "test" protein (Coomassie, silver stain or antibody)
- TCA solution (50% w/v) in Milli-Q water for protein precipitation if necessary (see Appendix I)

Necessary Equipment:

1. Ultracentrifuge capable of centrifuging 50-200 µl volumes at 100,000 x g at 4°C and 24°C. Examples are:

- Beckman Airfuge with Ultraclear tubes (Beckman, Cat. # 344718).
- SW50 ultracentrifuge rotor with adapters for Ultraclear tubes (Beckman, Cat. # 344718).
- Tabletop ultracentrifuge (Beckman) with TLA-100 rotor.

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 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.

Reagents	**Reconstitution**	**Storage**
GTP	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.	-70°C
General Tubulin Buffer	1) For 1X buffer, resuspend in 10 ml of sterile distilled water. Store at 4°C.	4°C (up to 6 months)
Tubulin Protein	1) Defrost one aliquot of 100 mM GTP and place on ice. 2) Pipette 1.0 ml of General Tubulin Buffer into a 1.5 ml tube and place on ice for 10 min. 3) Pipette 10 µl of 100 mM GTP stock solution into the 1.0 ml of General Tubulin Buffer 4) Label 10 x 1.5 ml tubes "5 mg/ml tubulin" and place on ice. 5) Reconstitute 1 mg of tubulin on ice with 200 µl of ice cold General Tubulin Buffer plus GTP. The protein concentration is 5 mg/ml. 6) Immediately aliquot into 10 x 20 µl volumes on ice. 7) Immediately snap freeze the protein in liquid nitrogen and store at -70°C. 8) **It is very important to snap freeze tubulin in aliquots as it responds very poorly to freeze / thaw cycles.**	-70°C (up to 6 months)
MAP Fraction	1) Label 10 tubes "1 mg/ml MAPF" 2) Reconstitute the protein in 100 µl of cold Milli-Q water on ice. 3) Aliquot into 5 x 20 µl volumes on ice 4) Snap freeze in liquid nitrogen and store at –70°C.	-70°C (up to 6 months)
BSA Protein	1) Reconstitute in 20 µl of cold distilled water. 2) Add 2 µl of Salt Extraction Buffer and mix well. The protein is at 5 mg/ml. 3) Aliquot into 10 x 2 µl volumes and snap freeze in liquid nitrogen. Store at –70°C.	-70°C (up to 6 months)
Salt Extraction Buffer	1) For 1X stock solution, resuspend in 1 ml of distilled water. Store at 4°C.	4°C
Taxol	1) For a 2 mM stock solution, reconstitute each tube in 100 µl of anhydrous DMSO. Store at –70°C. WEAR GLOVES WHEN HANDLING TAXOL.	-70°C (up to 6 months)

V: Important Technical Notes

A) **Notes on Updated Version 5.0**

The following updates from version 4.2 should be noted:

1. The manual has been changed to a 5.5 x 8.5 format.
2. The tubulin source has been updated from Cat# TL238, Tubulin Protein (>99% Pure), bovine brain source to Cat#T240, Tubulin protein (>99% Pure), porcine brain source. Cat# T240 is functionally identical to Cat# TL238 when used in this kit.

B) **Considerations before starting your experiments**

1. The "test" protein must be in an appropriate buffer to allow attachment to microtubules. Conditions which do not favor microtubule binding are: high salt (e.g. >25 mM NaCl), high or low pH (i.e. pH of <5.0 or >9.0) and high calcium (greater than 1 mM calcium will depolymerize microtubules). It is often advisable to include 5.0 mM Mg^{2+} in the binding buffer. Appropriate buffers include: HEPES, PIPES and MES all at 80 mM. In some cases the presence of ATP or GTP in the reaction may prevent MAP/microtubule association e.g. kinesin or dynamin respectively (2).
2. If you plan to identify your "test" protein using a general protein stain such as Coomassie, the test protein could be masked by the tubulin band if its molecular weight is between 50 – 60 kDa. A test protein specific detection system (eg. antibody or radiolabeled protein) will overcome this problem. If however the presence of tubulin is going to be a problem then you will need to remove the tubulin before loading the gel (see Appendix I).
3. It is essential to establish conditions in which the "test" protein does NOT pellet in the absence of MTs as pelleting the "test protein only" would invalidate the experiment.

Microtubule assembly

Microtubules (MTs) will be used as the substrate for your microtubule associated protein (MAP) assay. You should use freshly prepared MTs within several hours. MTs should be kept at room temperature, this is very important as MTs will depolymerize on ice.

Method

Defrost the taxol solution; this solution can be kept at room temperature during the course of the experiment, as it will be needed throughout the procedure.

Aliquot 200 µl of General Tubulin Buffer into a labeled centrifuge tube and place at 35°C.

Defrost one 20 µl aliquot of Tubulin Protein by incubating for several minutes in a room temperature water bath. Once thawed IMMEDIATELY transfer to ice, add 2 µl of Cushion Buffer and incubate at 35°C for exactly 20 min. This step allows tubulin to polymerize to microtubules (MTs).

After 20 min incubation, remove the 200 µl of General Tubulin Buffer from 35°C and add 2 µl of 2 mM Taxol stock solution (green cap), mix well.

Immediately remove the MTs from incubation and dilute with the 200 µl of General Tubulin Buffer plus Taxol. Mix thoroughly but gently and leave the MTs at room temperature. The Taxol will stabilize MTs.

You now have a population of stable MTs that are between 5 - 10 µm in length and that are at a concentration of approximately 5.0×10^{11} MT/ml. This is equivalent to 5 µM tubulin dimer or 0.4 nM microtubules.

Microtubule binding

A preliminary assay to determine whether the “test” protein has MAP-like characteristics is described. If the “test” protein shows microtubule binding activity in these preliminary experiments one can proceed to a more detailed analysis described in Section VII.
NOTE: The quantities of protein given in Table 2 assume that you are working with a recombinant protein at >50% purity. If you have a crude extract then higher protein concentrations are required.

Method

1. Aliquot 300 µl of General Tubulin Buffer into a clean centrifuge tube at room temperature. Allow the buffer to warm to room temperature BEFORE proceeding with step 2.
2. Add 3 µl of Taxol stock solution and mix well. This General Tubulin Buffer plus Taxol will be used in the microtubule binding experiments described below.
3. Set up the microtubule binding reactions as outlined in Table 1.

Table 1: Microtubule Spin-down Assay Setup

Tube	Protein	Microtubules (µl)	General Tubulin Buffer plus Taxol (µl)
1	None	20	30
2	MAP fraction (16 µl)	20	14
3	BSA (1.5 µl)	20	28.5
4	MAP fraction (16 µl)	0	34
5	BSA (1.5 µl)	0	48.5
6	"test" protein (5.0 µg)	0	to 50 µl final volume
7	"test" protein (5.0 µg)	20	to 50 µl final volume
8	"test" protein (2.0 µg)	20	to 50 µl final volume

4. Leave the reactions at room temperature for 30 min.
5. While the incubation is proceeding prepare the Cushion Buffer as follows:
   A. To 1 ml of room temperature Cushion Buffer add 10 µl of taxol and mix well.
   B. Do not introduce air bubbles into the Cushion Buffer. If air bubbles are present, centrifuge the buffer for 1 min at top speed to remove them.
6. Label 8 Beckman Ultraclear™ ultracentrifuge tubes 1 through 8. NOTE: Any 200 µl volume ultracentrifuge tubes can be used.
7. Place 100 µl of the Taxol supplemented Cushion Buffer into each tube.
8. Place each reaction from Table 2 carefully on top of the corresponding cushion.
9. Centrifuge each tube at 100,000 x g, at room temperature for 40 min.
10. Label 8 tubes S1 through S8 for the supernatant samples.
11. When the centrifuge has stopped, carefully remove each tube and mark the location where the pellet should be found.
12. Very carefully remove the uppermost layer of supernatant. Note: after the centrifugation step the cushion interface is no longer visible. Thus, remove 50 µl from the top of each solution and place into the appropriately labeled centrifuge tube; add 10 µl of 5X Laemmli sample buffer to each tube.
13. Carefully remove the remaining cushion buffer from each tube. The microtubule pellet is often very difficult to see, however, if you are gentle in removing the Cushion Buffer the pellet will remain safely in the tube. Do not be concerned if you cannot see the pellet. Avoid the area where the pellet should be found (as marked in step 11).
14. If the presence of the tubulin band (55 kDa) is not going to be a problem then the pellet can simply be suspended in 50 µl of 1x Laemmli sample buffer. If you need to remove the tubulin band see Appendix I.
15. Run 20 µl of the supernatant and pellet samples on an SDS-Page gel and visualize the protein of interest by your chosen detection system.

VII: Interpretation of Results

Control Samples

Microtubules Alone (Figure 1, Lanes 1S and 1P):

At least 80-90% of the tubulin sample should appear in the pellet fraction (1P). This demonstrates that microtubules have been formed and are able to pellet when centrifuged at 100,000 x g for 40 min at room temperature. Tubulin migrates at 55 kDa.

MAP fraction plus microtubules (Figure 1, Lanes 2S and 2P):

At least 80% of the MAP2 protein, the major protein found in the MAP fraction, should appear in the pellet (2P). This demonstrates that MAP2 is binding to the microtubules. The MAP2 protein migrates at 280 kDa. A large 55 kDa tubulin band should also be in the pellet fraction.

BSA protein plus microtubules (Figure 1, Lanes 3S and 3P):

At least 80% of the BSA protein should appear in the supernatant fraction (3S). This demonstrates that BSA is not binding to microtubules. BSA migrates at 68 kDa. There should be <10% of total tubulin protein in the supernatant fractions of the controls (3S).

MAP fraction minus microtubules (Figure 1, Lanes 4S and 4P):

In the absence of microtubules at least 80% of the MAP2 should remain in the supernatant (4S). This control demonstrates that microtubules are required to pellet MAP2.

BSA minus microtubules (Figure 1, Lanes 5S and 5P):

In the absence of microtubules at least 80% of the BSA should remain in the supernatant (5S).

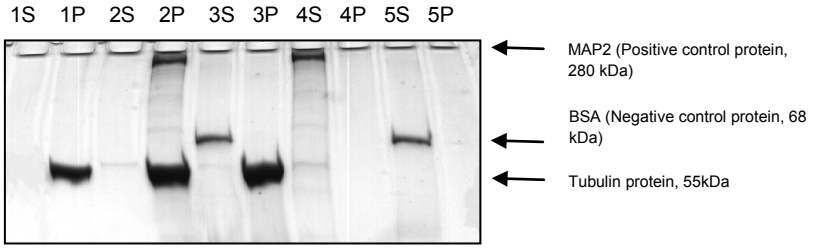

Figure 1. Microtubule Associated Protein Assay Control Samples. *Control reactions to test microtubule binding were performed as described in Section VI: Microtubule Binding. Samples were run on a 12% SDS gel and stained with 1% Coomassie blue.*

Test Protein Samples

Test protein minus microtubules:

In this experiment, the "test" protein should be predominantly in the supernatant fraction. If the "test" protein is pelleted in the absence of microtubules then you MUST find conditions that will allow your protein to remain soluble during centrifugation (eg. changing the buffer composition). Contact technical service at tservice@cytoskeleton.com for assistance.

Notes on Interpreting Results

It should be noted that *in vitro* data alone does not confirm that the “test” protein is a MAP. The final proof of the significance of the *in vitro* data is to show that the protein is associated with microtubules *in vivo*.

Detailed Microtubule Binding Analysis

A detailed analysis, in which the Kd of microtubule affinity is measured can be performed by using a modified version of the assay described in Section VI. Determination of the Kd measurement involves titrating either the test protein or the microtubules in the binding assay described in Section VI. The microtubule binding affinity for a given test protein can be defined in terms of the concentration of either the test protein or microtubules that is/are necessary to co-sediment 50% of the test protein. For more detailed information the investigator is referred to references 1 and 3.

VIII: Troubleshooting

PROBLEM	POSSIBLE REASON	SOLUTION
No “test” protein detectable.	1. Lack of sensitivity of detection method. 2. Proteolytic degradation.	1. Use more sensitive detection method. 2. Increase the amount of test protein used. 1. Use protease inhibitors.
Test protein pellets in the absence of microtubules	1. Aggregation / denaturation of the “test” protein.	1. Pre-centrifuge test protein 2. Add stabilizing agent such as dithiothreitol (DTT). 3. Use a different buffer system (eg. PIPES, HEPES, MES).
Test protein will not salt-off microtubules	1. Lack of dispersal of microtubule pellet. 2. Denatured tubulin results in aggregates	1. Make sure pellet is thoroughly dispersed. 2. Cold spin (14,000 rpm, 10 min at 4°C) tubulin prior to polymerization; keep the supernatant for the polymerization reaction.
Microtubules do not pellet efficiently	1. Taxol not added to buffers. 2. Test protein maybe affecting microtubules.	1. Add taxol to all buffers where MTs are present. 2. Protein may be depolymerizing or severing microtubules.
MAP fraction does not co-pellet with microtubules	1. Denatured tubulin results in aggregates	1. Cold spin (14,000 rpm, 10 min at 4°C) tubulin prior to polymerization; keep the supernatant for the polymerization reaction.
BSA pellets with microtubules and on its own	1. Denatured protein is pelleting on its own.	1. Cold spin (100,000 x g, 20 min at 4°C) and use the supernatant for the binding reactions.

IX: References

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X: Microtubule Spin-down Assay (Cat. #BK029) Citations

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XI: Associated Products

Tubulin Related Biochem™ Kits

Cytoskeleton Inc. supplies several alternative tubulin polymerization assays and Biochem™ Kits, consult technical service for advice on application suitability.

Assay	Cat. #	Recommended Uses
Tubulin Polymerization Assay (>99% pure tubulin) Absorbance based	BK006P	This absorbance based assay uses a tubulin preparation that is >99% pure. The assay is designed to give a maximal OD340 between 0.25 – 0.35 under the polymerization conditions given. The use of highly purified tubulin allows researchers to perform accurate IC50 and EC50 measurements on any given tubulin ligand. Kit BK006P contains sufficient reagents for 30 assays. The assay is also available in 96 assay format and HTS quantities (inquire).
Tubulin Polymerization Assay (>97% pure tubulin) Absorbance based	BK004P	This absorbance based assay uses a tubulin preparation that is >97% pure. This assay results in a maximal OD340 signal that is approximately half the intensity of the BK006P Polymerization Kit which uses 99% pure tubulin protein. Kit BK004P contains sufficient reagents for 30 assays. The assay is also available in 96 assay format and HTS quantities (inquire). This kit provides an economical means for screening large numbers of tubulin ligands and primary libraries.
Fluorescent Tubulin Polymerization Assay	BK011P	This assay incorporates a fluorescent analog that accurately reports microtubule polymer mass. Unlike the absorbance assay, the fluorescence assay performs well at very low reaction volumes (10 ul) and low concentrations of polymer. This equates to a highly sensitive and economical format. The assay is recommended for primary screens to identify tubulin ligands. The kit is sufficient for 96 reactions and HTS quantities are available (inquire)
Pre-Formed Microtubules	MT002	Pre-formed microtubules are extremely convenient for any research that requires a source of stable microtubule substrates. Our stringent quality control results in minimal batch variability and translates to highly consistent behaviour between experiments. This product is recommended for uses such as microtubule binding assays and screening for kinesin inhibitors. Microtubules are available in 2 mg (MT002-A), 10 mg (MT002-XL) and custom sizes (inquire).
Colchicine Competition Assay	CDS15P	The Scintillation Proximity Assay (SPA) technology offered by Amersham has been used to create sensitive ligand binding assays for a variety of tubulins (Tahir et al. Biotechniques, 29: 156-160. 2000). Using biotinylated tubulins from neuronal (T333P-XL) and cancer cell line (H003) sources, the SPA technology has been optimized to use minimal amounts of tubulin (1 ug per assay). Tritiated colchicine (NEN biosciences) has been tested and quality approved for use in CDS15. Kd and Ki values determined using these assays are similar to published values using other methods. This kit represents a valid, economical alternative for primary screens to identify tubulin ligands.
Cytoskeleton Screening Service	Custom Service	All these assays are available as a screening service performed at Cytoskeleton Inc.'s state of the art screening facility, for more information please contact Technical Service at 303-322-2254 or e-mail tservice@cytoskeleton.com .

Tubulin Related Proteins and Buffers

Cytoskeleton Inc. supplies tubulin protein from a variety of species and tubulins formulated for specific uses, consult technical service for advice on application suitability. Check the web site for new products.

Product	Cat. #	Recommended Uses
Biotin Tubulin	T333P	Substrate for tubulin ligands in SPA assays or in development of immobilized tubulin assays for HTS. Supplied lyophilized in 20 µg or 500 µg sizes.
GTP Stock Solution	BST06	Tubulin polymerization requires GTP. It is generally used at 1 mM final concentration. When reconstituted makes a 100 mM stock. Supplied in 100 µl or 10 x 100 µl final volume sizes.
General Tubulin Buffer	BST01	Used as a general buffer for tubulin proteins. Supplied as 10 ml, 100 ml or 1 L sizes.
HTS Tubulin	HTS03	Tubulin purified from porcine brain to approximately 97% pure. This product is recommended as an economical substrate for tubulin HTS assays. Supplied as 4 mg, 40 mg or bulk quantities.
MAP-rich Tubulin	ML116	Prepared from porcine brain and contains approximately 70% tubulin and 30% microtubule associated proteins. This is an alternative substrate for HTS polymerization assays. Supplied as 1 x 1 mg, 5 x 1 mg, 10 x 1 mg, 20 x 1 mg and bulk quantities.
Microtubules, pre-formed	MT002	Developed by scientists at Cytoskeleton Inc., pre-formed microtubules are extremely convenient for any research that requires a source of stable microtubule substrates. Our stringent quality control results in minimal batch variability and translates to highly consistent behavior between experiments. This product is recommended for uses such as microtubule binding assays and screening for kinesin inhibitors. Microtubules are available in 2 mg (MT002-A), 10 mg (MT002-XL) and custom sizes (inquire).
Paclitaxel Stock Solution	TXD01	Microtubule stabilizing compound, generally used in the 1 – 10 µM range. Supplied lyophilized, when reconstituted gives 10 x 100 µl of 2 mM stock solution.
Rhodamine Tubulin	TL590M	Tetramethyl rhodamine labeled tubulin. Useful for examining *in vivo* tubulin dynamics and as a substrate for in vivo or in vitro assays where it is necessary to see microtubules by fluorescence. Supplied as 5 x 20 µg or 20 x 20 µg. Labeled to a stoichiometry of 2 labels per heterodimer.
Tubulin 99% pure from porcine brain	T240	Same as TL238 but source of tubulin is porcine brain rather than bovine. The polymerization properties of this tubulin are identical to those of the bovine tubulin.
Tubulin from HeLa cells	CS-H001B	Recommended as a more cancer specific tubulin target in tubulin ligand assays. Available in 250 µg sizes and bulk quantities. Limited stock available.
Tubulin from MCF-7 cells	CS-H005	Recommended as a more cancer specific tubulin target in tubulin ligand assays. Available in 250 µg sizes and bulk quantities. Limited stock available.

Appendix I:

TCA Precipitation Procedure

It may be necessary to TCA precipitate the supernatant protein before SDS-PAGE analysis. TCA precipitation is achieved as follows;

1. Add 1/10th volume of 100% TCA solution to the sample and mix.
2. Place on ice for 15 min.
3. Centrifuge at 14,000 rpm for 10 min at 4°C.
4. Remove and discard the supernatant.
5. Resuspend the pellet in 10 µl of 1 M Tris-base pH 10.4.
6. Add 50 µl of 1x Laemmli sample buffer.

Note: If the SDS buffer turns yellow, add a further 1 µl of 1 M Tris-base pH 10.4, keep adding Tris until the solution turns blue. The pellet formed after TCA precipitation is often large and clearly visible, this is due to the fact that some of the sucrose from the Cushion Buffer is also precipitated. This will not interfere with protein migration in an SDS gel.

Salting-off Procedure

If you need to remove the tubulin from your pellet this can be achieved as follows:

1. Resuspend the protein pellet in 100 µl of Salt Extraction Buffer plus 20 µM taxol. Make sure that the pellet is completely dispersed; you may have to stir the solution with your pipette tip. This procedure must be thorough but gentle as you wish to disperse any clumped microtubules and dissociate any MAP-like proteins, while at the same time you do not want to disassemble microtubules into free tubulin.
2. Centrifuge the sample at 100,000 x g and save the supernatant which will contain freed MAP -like proteins.
3. TCA precipitate the supernatant as described above.

Note: It should be noted here that while 'salting-off' is a generally recognized procedure for removing MAPs from microtubules there are other possibilities that may help identify the type of MAP that you are dealing with (e.g. ATP will specifically release motor proteins from microtubules, and some proteins such as dynamin can be released by GTP). In these cases it is useful to use non-hydrolyzable analogs of the nucleotides to retain binding.

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