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# ProductInformation

**MICROTUBULES PREPARATION KIT**

Product Number **MI0500**
Storage Temperature -20°C

## TECHNICAL BULLETIN

### Product Description

Microtubules are one of the main elements of the cytoskeleton that are essential for cell division, cell migration, vesicle transport, and cell polarity. The major building block of microtubules is tubulin, an intracellular cylindrical filamentous protein that is present in almost all eukaryotic cells. Except in the simplest eukaryotes, tubulin exists in all cells as a heterodimer of two similar polypeptides, approximately 55 kDa each, designated a and b, that assemble into microtubules. Tubulin polypeptides are arranged head-to-tail in a lattice to form polar microtubules. Tubulin interacts with a large number of proteins in carrying out its activities.[1] Some of these proteins modulate the stability of microtubules (microtubule-associated proteins or MAPs), while some are active motor proteins that carry cargo along microtubules, such as kinesin.[2-3]

The microtubule structure is dynamic and undergoes polymerization (assembly) and depolymerization (disassembly). This is important in cell division, since the microtubules form the mitotic spindle and the kinetochore microtubules, which are responsible for the correct chromosome arrangement and movement during mitosis. The microtubule polymerization process is GTP-dependent.[1]

The Microtubules Preparation Kit contains all the reagents required for efficient preparation of microtubules (tubulin) from brain tissues and cell lines. It was tested on rat, rabbit, and porcine brain, and on HeLa and NIH 3T3 cell lines.

The kit is suitable for:

1. Studying and screening for components and proteins that affect microtubule assembly.
2. Isolation of microtubule-associated proteins (MAPs).

### Components/Reagents

The kit contains reagents sufficient for preparation of microtubules from 50 g tissue.

Homogenization Buffer, 5X 20 ml
(Product Code H 8662)
750 mM sodium glutamate, 150 mM $NaH_2PO_4$, 2.5 mM $CaCl_2$, 5 mM DTT, pH 6.8

Polymerization Buffer 5X 20 ml
(Product Code P 4745)
500 mM MES, 0.5 mM EDTA, 5 mM DTT, pH 6.8

$MgCl_2$-EGTA Solution 20X 10 ml
(Product Code M 8317)
10 mM $MgCl_2$, 20 mM EGTA, pH 7.0

Guanosine-5'-triphosphate (GTP) 100 mg
(Product Code G 8877)

Paclitaxel (Taxol®) 1 mg
(Product Code T 7402)

Glycerol 100 ml
(Product Code G 9012)

Protease Inhibitor Cocktail (PIC) for use with mammalian cell and tissue extracts 1ml
(Product Code P 8340)

### Reagents and Equipment required but not provided

- Stand-mounted overhead electric motor for homogenizer operation, Heidolph electronic stirrer, Product Code Z61,901-9 or equivalent
- Glass test tubes, 16 x 100 mm (Product Code Z28,106-9)
- Water bath
- UV spectrophotometer with temperature-controlled cuvette chamber
- Small quartz cuvette (50 µl or 160 µl) (Product Codes C 9917 and C 9667)

- SDS-Polyacrylamide gels, 7.5%.
- Cuvettes, polystyrene, 3 ml (Product Code C 5291)
- DMSO (Product Code D 8418)
- Dulbecco's PBS (Product Code D 8537)
- 5 N NaOH
- Liquid Nitrogen
- Microcentrifuge

Additional equipment for section A1. For small-scale microtubules preparation (1-2 rat brains, 1.5 - 4 g brain tissue)
- Mechanical 8 ml teflon/glass homogenizer (Product Code P 7859)
- 2 Microcentrifuges: one at 4 °C and one at 25 °C
- Pellet pestle motor (Product Code Z35,997-1) and Pellet pestle (Product Code Z35,994-7)

Additional equipment for section A1. For large-scale microtubules preparation (suitable for more than 2 rat brains or other brain tissues that weigh more than 8 g).
- Mechanical 3-ml teflon/glass homogenizer (Product Code P 7734)
- Mechanical 45 ml teflon/glass homogenizer (Product Code P 7984)
- Sorvall RC-5C centrifuge with SS-34 head
- 50 ml polypropylene centrifuge tubes (Product Code T 4543 or T 2918)
- Ultracentrifuge with suitable rotor
- Ultracentrifuge tubes PC/PPO 16 x 76 mm

Additional equipment for section A2. For preparation of Microtubules from cells
- Sonicator, such as Sonics VCX750 with 3 mm microtip
- Dounce glass tissue grinder set, 7 ml (Product Code D 9063)
- Rubber policeman
- Sorvall RC-5C centrifuge with SS-34 head
- 50 ml PP centrifuge tubes.
- Ultracentrifuge with suitable rotor
- Ultracentrifuge tubes PC/PPO 16 x 76 mm
- Pellet pestle motor (Product Code Z35,997-1) and pellet pestle (Product Code Z35,994-7)

**Precautions and Disclaimer**
This product is for laboratory research use only. Please refer to the Material Safety Data Sheet (MSDS) for information regarding hazards and safe handling practices

**Preparation Instructions**
Note: Use sterile ultrapure (17 MΩ·cm) water for preparation of all reagents.

GTP - 100mM
Add 1.7 ml water to the GTP vial. Adjust the pH to 7.0 by dropwise addition of 5 M NaOH (~16 µl in 2 µl aliquots). Monitor the pH each 2 µl addition in order not to pass the target pH. Mix well, aliquot, and store at –20 °C. The solution is stable for a few months, and it is stable after multiple freeze-thaw cycles.

Paclitaxel (Taxol) - 10 mg/ml
Add 100 µl of DMSO to the 1 mg Paclitaxel vial. Mix well, aliquot, and store at –20 °C. The solution is stable for a few months.

Homogenization Solution (for microtubule preparation)
The homogenization is performed at a ratio of 1.5 ml homogenization solution to 1 g tissue, or 1 ml homogenization solution to 1 ml packed cells. The instructions in the table below are for preparation of 1.5 ml solution. The volume of homogenization buffer prepared may be adjusted to the amount of starting material used.

Combine the following components. Store the Homogenization Solution at 4 °C.

| Compound | Stock solution | Final conc. | Amount per 1 gram tissue |
|---|---|---|---|
| Homogenization buffer | 5X | 1X | 0.3 ml |
| Glycerol* | - | 10% | 150 µl |
| GTP** | 100 mM | 0.2 mM | 3 µl |
| PIC** | - | 1:100 | 15 µl |
| Water | 17 MΩ·cm | | QS to 1.5 ml |

* The glycerol should be completely dissolved in the solution.
** Add the PIC (Protease Inhibitor cocktail) and the GTP just before homogenization.

Polymerization Solution (for microtubule preparation)
Prepare 1 ml of Polymerization solution according to the table below.
Store the Polymerization solution at 37 °C.

| Compound | Stock solution | Final conc. | Amount per 1 ml |
|---|---|---|---|
| Polymerization buffer | 5X | 1X | 0.2 ml |
| Glycerol* | - | 2 M | 0.14 ml |
| Mg-EGTA | 20X | 1X | 50 µl |
| GTP** | 100 mM | 0.2 mM | 2 µl |
| PIC** | - | 1:100 | 10 µl |
| Water | 17 MΩ·cm | | QS to 1 ml |

* The glycerol should be completely dissolved in the solution.
** Add the PIC (Protease Inhibitor Cocktail) and the GTP just before the homogenization.

**Storage/Stability**
Store the kit at –20 °C.

**Procedure**
Principle of Assay
The procedure of microtubules preparation is based on temperature-dependent assembly-disassembly cycles.[4-5] The common source for microtubules is brain tissue but microtubules can also be prepared from cell lines.

The tissue or cells are homogenized at 4 °C and incubated on ice. The homogenate is clarified by centrifugation at 20,000 x*g* (repeated twice). The clarified homogenate undergoes the first assembly step promoted by GTP and glycerol at 37 °C (addition of paclitaxel instead of GTP will result in permanently assembled microtubules). The assembled, or polymerized, microtubules are collected by centrifugation and the first assembly pellet is resuspended in polymerization solution. This suspension can be used immediately or snap-frozen in liquid nitrogen and kept at –70 °C for up to one month for later use. The first assembly microtubules can be disassembled for use in polymerization studies or for an additional assembly step. An additional assembly step will yield more purified microtubules at the expense of yield, and is possible when at least 8 g of brain tissue was used as the starting material.

See Appendix I for a flow-chart of the procedure.

A. Microtubule preparation
A.1 Microtubule preparation from rat brain tissue.
The preparation can be performed on a small scale (1.5 - 4 g, but with not more then 2 rat brains at a time), or a large scale (8 - 50 g). The average weight of a rat brain is 1.5 - 1.9 g, rabbit brain is 9 g, and miniature swine brain is 50 g.

Notes:
- The brain should be stored in ice-cold PBS immediately after removal from the animal. It is very important to start working with the brain within one hour of sacrificing the animal.
- When using brain tissues weighing 8 - 50 g, it is recommended to remove the meninges before homogenizing.
- The assembly/disassembly of microtubules is highly temperature dependent. Make sure the equipment is at the temperature recommended in the instructions.
- For long-term storage of microtubules it is recommended to freeze the microtubules in aliquots.

I. Homogenization
Cool the centrifuge, the mechanical teflon/glass homogenizer, and the glass dish.
1. Keep the brain in ice-cold PBS.

2. Weigh the brain in a pre-cooled dish.

3. Transfer the brain to a pre-cooled 8 ml or 50 ml teflon/glass homogenizer.

For brain tissues weighing up to 8 g use an 8 ml teflon/glass homogenizer and for tissues weighing more than 8 g use a 50 ml teflon/glass homogenizer. For brain tissues weighing about 50 g use a blender.

4. Add 1.5 volumes of ice-cold Homogenization solution per 1 g tissue (e.g. for 1.5 g tissue, add 2.25 ml of Homogenization solution).

*Perform steps 5-8 in a cold room.*

5. Homogenize. Start at low speed and increase to a higher speed until the tissue is completely homogenized.

If the brain weighs around 50 g, homogenize in a blender 3 times for 10 seconds each.

6. Incubate the homogenate for 30 minutes on ice.

7. Further homogenize the crude homogenate by two additional passes at 2000 rpm (the 50 g homogenate should be transferred in 2-3 portions from the blender to a 50 ml homogenizer in order to perform this step).

8. Transfer the homogenate to a single centrifuge tube.

9. Centrifuge the homogenate in a pre-cooled centrifuge at 20,000 x*g* for 30 minutes at 4 °C. Use an Eppendorf centrifuge for brain tissues up to 8 g and a Sorvall centrifuge for tissues above 8 g.

10. Collect the supernatant and repeat step 9 to improve the supernatant clarification.

II. Microtubules first assembly
Note: When permanent microtubules assembly is required, go to section A1-V.
Make sure the water bath is set at 37 °C and the centrifuge and glycerol are pre-warmed.

1. Remove the supernatant with a pipette, note the volume, and transfer it to a glass test tube (glass is used so the microtubules polymerization can be followed visually).

2. Add a volume of pre-warmed glycerol, using a cut tip, equal to 25% of the supernatant volume. Vortex until the solution is homogenous.

3. Add 1/20 volume of 20x $MgCl_2$-EGTA stock solution to the supernatant for a final concentration of 1x $MgCl_2$-EGTA.

4. Add GTP to a final concentration of 1 mM (dilute 1:100). Vortex until the solution is homogenous and cover the tube with a parafilm.

5. Incubate in a water bath for 45 minutes at 37 °C.

6. Spin in pre-warmed microcentrifuge or ultracentrifuge. Volumes less than 6 ml should be centrifuged in a microcentrifuge at 20,000 x*g*, for 1.5 hours at 25 °C. For volumes larger than 6 ml, use an ultracentrifuge in a Ti60 rotor at 100,000 x*g*, for 50 minutes at 25 °C.

7. Decant the supernatant carefully. Note: The pellet can be transparent and difficult to see.

8. Dry the tubes carefully by leaving the tubes upside down on a filter paper for several minutes.

9. Estimate the pellet volume:
   If the volume of the pellet is less than 0.5 ml, add two volumes of pre-warmed Polymerization solution, and homogenize the pellet using Pellet pestle.

   If the pellet volume is larger than 0.5 ml, add two volumes of pre-warmed Polymerization solution, and scratch the residual pellet with a rubber policeman. Transfer the viscous mixture to a 3 ml Teflon/glass homogenizer and homogenize the material twice carefully.

10. The homogenized pellet can be immediately used for further purification and testing. It can also be frozen in one tube or in aliquots by snap freezing in liquid nitrogen, and stored at –70 °C for later use.

Optional: Leave an aliquot at room temperature if you choose to evaluate the preparation.

First assembly evaluation

Take an aliquot of the homogenate for determination of protein concentration. The expected protein yield is 0.9 - 1.6 mg protein/g of brain tissue (by Lowry protein determination).

Run an SDS-PAGE using a 7.5% acrylamide gel, and stain with Brilliant Blue R 250, Product Code. B 7920. Verify that the homogenate contains the tubulin at an apparent size around 50 kDa.

III. Microtubules first disassembly

Cool the centrifuge, the suitable mechanical teflon/glass homogenizer, and the glass dish 4 °C.

1. If the microtubules are frozen (–70 °C), thaw the microtubules aliquot vial in a 37 °C water bath, for 2-3 minutes and transfer it immediately into ice.

2. Incubate the microtubule-containing vial on ice for 90-150 minutes. While incubating on ice, homogenize the solution every 15 minutes using a pellet pestle or 3 ml teflon in glass, according to the volume.

Note: Do not homogenize microtubules prepared from cells.

3. Spin the solution in a pre-cooled microcentrifuge for 40 minutes at 20,000 x*g* (14,000 rpm) at 4 °C.

4. Transfer the supernatant containing the depolymerized microtubules to a new tube. Keep on ice.

Determination of microtubule concentration

1. Determine protein concentration of the supernatant by the Lowry procedure. Expected concentration is >5 mg/ml from brain tissue and 4 mg/ml from cells.

2. Resuspend the pellet with the polymerization solution using the same volume as the supernatant.

Optional:

Run the supernatant and the pellet on a 7.5% SDS-PAGE gel, and stain with Brilliant Blue G. The tubulin distribution between the supernatant and pellet is between 50:50% and 70:30%, respectively.

The depolymerized microtubules can now be used for polymerization studies (see section B) or can undergo a second assembly process (section A1-IV). They can also be used for partial separation of the tubulin from the MAPs using an anion exchange resin such as DE-52. The tubulin and the MAPs that bind the resin, can be separated by a suitable salt gradient.[9]

IV. Microtubules Second Assembly

For brain tissue weighing more than 8 g it is possible to perform an additional cycle of polymerization, in order to further purify the microtubules preparation.

Set the water bath at 37 °C and pre-warm the centrifuge and glycerol.

1. Remove the supernatant with a pipette, note the volume, and transfer it to a glass test tube, in order to visually follow the microtubule polymerization.

2. Using a cut tip, add a volume of pre-warmed glycerol equal to 10% of the supernatant volume. Mix until the solution is homogenous (vortex).

Continue the procedure as in section A1-II, steps 3-10.

V. Paclitaxel (Taxol) induced polymerization

Work according to the procedure described in section A1-II.

In step 4 instead of adding GTP, add paclitaxel to a final concentration of 20 mg/ml (dilute stock solution 1:500.

A.2 Microtubule preparation from cells [6-8]

Tubulin preparation from cells requires a large amount of cells. The procedure below is directed for at least $6 \times 10^8$ cells.

1. Add cold PBS to the cells and, using a rubber policeman, detach them from the vessel surface. Do not use trypsin or EDTA for this step.

2. Collect the cells at 6,800 x*g* for 20 minutes at 4 °C.

Note: The relatively high speed is recommended at this step in order to get a packed pellet.

3. Wash the pellet with cold PBS and spin at 6800 x*g* for 40 minutes, at 4 °C.

4. Estimate the pellet volume.

5. Resuspend the packed pellet in one volume of cold Homogenization solution.

6. Sonicate the cells using a suitable tip (e.g. Sonics VCX750 sonicator, 3 mm diameter microtip for 250 µl-10 ml, at amplitude control of 20% for 1 min., at 10 °C, pulses of 9.9 sec.)

7. Incubate on ice for 30 minutes.

8. Further homogenize the homogenate with a 7 ml pre-cooled Dounce glass tissue grinder (with tight pestle) for 5 times, on ice.

9. At this step check the protein concentration:

a. Spin down a 100 ml sample at maximal speed in a microcentrifuge.
b. Determine the protein concentration in the supernatant. The concentration should be ~15-20 mg/ml.
c. If the concentration is lower, perform step 7 again.

10. Centrifuge the homogenate in a Sorvall centrifuge at 20,000 x*g* for 30 minutes, at 4 °C.

11. Collect the supernatant and repeat step 8 for improved clarification.

12. Continue according to microtubules assembly section A1-II.

13. Continue according to microtubules disassembly section A1-III.

Note: Do not homogenize microtubules prepared from cells.

**B.Microtubule protein assembly (polymerization) assay**

The assay is based on increase in turbidity created by the formation of microtubules. It is performed in a spectrophotometer, which is kept at 37 °C during the entire procedure.

I. Preparation of Polymerization Solution

Note: This solution is not identical to the one used for microtubules preparation.

The example below is for 1 ml buffer, sufficient for 10 assays in 70 µl quartz cuvette.
The Polymerization solution should be kept at 37 °C.

| Compound | Stock solution | Final conc. | Amount per 1 ml |
|---|---|---|---|
| Polymerization buffer 5X | 5X | 1X | 0.2 ml |
| Glycerol | - | 2.5 M | 175 µl |
| $MgCl_2$-EGTA | 20X | 1X | 50 µl |
| Water | 17 MΩ·cm | | QS to 1 ml |

II. Polymerization measurement

Set the water bath and the spectrophotometer at 37 °C and warm the centrifuge and Polymerization solution to 37 °C.

1. Set the spectrophotometer on a kinetics program: Wavelength: 350 nm; Temperature: 37 °C; Read O.D. every 30 seconds from 0 to 60 minutes.

2. Dilute a disassembled microtubules sample (Section A1-III) in a pre-warmed Polymerization solution, to 1-1.5 mg/ml when working with tissue microtubules, or 4 mg/ml when working with cell microtubules. Dilute the material for one test at a time (the volume depends on the size of the cuvette you are using).

3. Pre-warm the cuvette in the spectrophotometer.

4. Transfer the diluted sample into the cuvette and start the measurement.

5. After 2-3 minutes, add GTP to a final concentration of 1mM (i.e. dilute the 100 mM GTP stock solution 1:100). Mix gently by pipetting. Avoid bubbles formation. The absorbance will increase within 30-90 seconds as a function of microtubules polymerization, and will reach a plateau after about 30 minutes. After 40 minutes, add paclitaxel to a final concentration of 20 mg/ml (dilute the paclitaxel stock solution 1:500) in order to stabilize the assembly step. The absorbance will further increase for about 20 minutes due to the polymerization promoted by the paclitaxel.

6. If no increase in O.D. is observed, increase the concentration of the microtubules in the assay (e.g. 2 - 3 mg/ml).

7. As a control, run a test similar to the one describes above but do not add GTP and paclitaxel. The absorbance of this sample will reflect the microtubule self-assembly.

**Results**

Calculation of the degree of polymerization
Subtract the O.D. value of the control reaction measured after 40 minutes from the O.D. value measured for the reaction sample, at the same time.

The increase in O.D. of the test sample over the control, after 40 minutes, (before the addition of Paclitaxel) should be 0.07 - 0.15 O.D. units.

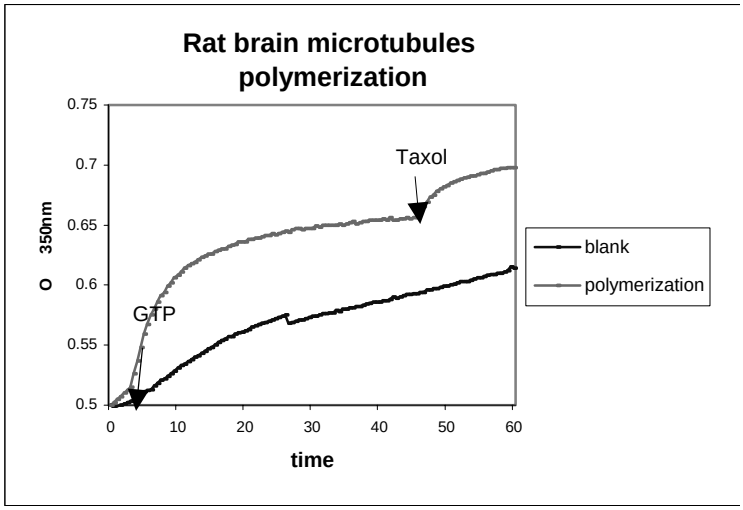


Figure 1: Representative polymerization experiment for rat brain microtubule during 60 minutes.

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**Appendix I**

Process flow chart