In Vitro Tubulin Polymerization Assay Kit (≥99% Pure Bovine Tubulin)

16 Assays

Catalog No. 17-10194

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Not for use in diagnostic procedures.

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Introduction

Microtubules are long, hollow cylinders made up of polymerised α- and β-tubulin dimers. Tubulin dimers polymerize end to end in protofilaments which are the building block for the microtubule structure. Microtubules are part of a structural network (the cytoskeleton) within the cell's cytoplasm. The primary role of the microtubule cytoskeleton is mechanical. However, in addition to structural support, microtubules also take part in many other processes. A microtubule is capable of growing and shrinking in order to generate force, and there are also motor proteins that allow organelles and other cellular factors to be carried along a microtubule.

Tubulin is a highly-conserved major component of eukaryotic cells and the 'building block' of microtubules. Tubulin is a heterodimer of α and β subunits with an apparent molecular mass of ~110 kDa. *In vivo* and *in vitro,* in the presence of GTP, tubulin heterodimers can polymerize (assemble) to form microtubules.

Many proteins or small-molecule compounds affect microtubule assembly. EMD Millipore's ***In vitro*** **Tubulin Polymerization Assay Kit** allows for quick and quantitative determination of tubulin polymerization due to protein and compound-based modulation.

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Materials Provided

Kit Components

1. ≥99% Pure Bovine Tubulin: (Part No. CS210579) 2 vials of 130 µL (240 µM) each of ≥99% pure bovine tubulin stock solution in 1X polymerization buffer (PB) containing 1mM GTP.
2. Polymerization Buffer (PB) 5X: (Part No. CS210569) One vial containing 300µL 5X polymerization buffer.
3. 200mM GTP**, (**Part No. CS210570).1 vial containing 10µL of 200mM GTP.
4. 96-well plate half area**,** (Part No**.** CS210573). One 96-well half area plate.
5. Paclitaxel**,** (Part No**.** CS210567). One vial containing 10µL of 700 µM paclitaxel in DMSO.
6. Nocodazole, (Part No, CS210568). One vial containing 10µL of 700 µM nocodazole in DMSO.

Materials Required But Not Supplied

Reagents

- Distilled water.

Equipment

- 96-well plate spectrophotometer able to read optical densities at 350 nm with temperature control (a must) and shaking (optional).
- Bench top Ultracentrifuge Beckman Optima or equivalent (optional).
- Pipettes, Micropipettes and Tips.
- Microcentrifuge Tubes.

Storage and Stability

Store tubulin stock solution at -80°C, store all other components at -20°C for up to 4 months from date of receipt. Do not freeze and thaw.

Protocol

Background information:

1. Extinction coefficient of tubulin at 280 nm = 115,000 $M^{-1}cm^{-1}$ or 1.15 (mg/mL)-1 (beware that excess of GTP/GDP in the solution can contribute to the optical density [OD] at 280 nm).
2. Tubulin concentration at 1 mg/mL is roughly equivalent to 10 µM (MW of α/β heterodimer is ~110,000 Da).
3. Under the above conditions, control polymerization curve should reach the steady state at an OD between **0.4 and 0.6**.
4. We advise to pre-centrifuge the tubulin sample if it was stored improperly or thawed-frozen before. It enables the removal of tubulin aggregates which act as “seeds” and change the shape of the sigmoid curve by shortening the lag time (LT- see Figure 1), when tubulin heterodimers first polymerize. The presence of the lag time in the control (pure tubulin) reaction is an important indicator of the tubulin quality. The absence of LT can jeopardize the evaluation of experiments where the test substance contributes itself to the formation of seeds (e.g. Taxol™ or some MAPs, for microtubule-associated proteins). Correctly stored tubulin from EMD Millipore does not require the pre-centrifugation step (see **Troubleshooting**).

Assay Instructions

1. Thaw tubulin, GTP, and 5x PB on ice. Assemble all reactions on ice.

   Once tubulin is thawed, use it within one hour to prepare polymerization reactions.

2. Polymerization reactions occur in 70 µL final volumes, of which 60 µL is the 60 µM tubulin in 1x PB-GTP and 10 µl is the test substance dissolved in 1x PB-GTP.

   - Prepare the necessary reactions volume of 1x PB-GTP solution. For this, dilute 5x PB in distilled water and supplement the 1x PB with a 1/200 volume of the 200 mM GTP to obtain 1mM final GTP concentration in 1xPB.
   - Keeping reagents on ice, **dilute 240 µM tubulin stock solution** with 1x PB-GTP solution 4-fold to obtain 60 µM tubulin concentration before adding 10 µL of the test or control compound (~54 µM final tubulin concentration).

     The tubulin stock solution already contains 1 mM GTP.

   - Keeping reagents on ice, combine 9 µL of 1x PB-GTP solution and 1 µL of your test substance (protein/small molecule) or the control molecules (included in the kit, Nocodazole and Paclitaxel are dissolved in anhydrous DMSO and will need a brief warming in order to thaw). Gently pipette the mix, while avoiding the formation of bubbles. The final volume of this mix is 10 µL for 1 reaction.
   - Keep the 96-well plate on ice, combine in one well 60 µL of 60 µM tubulin solution and 10 µL of pre-diluted test (or control) compound.

   Polymerization reaction mix is stable on ice for at least one hour.

Comment: For each experiment, prepare and use at least two control samples (see Troubleshooting, § 9 and 10).

3. Transfer the plate into the pre-warmed spectrophotometer chamber and follow tubulin assembly by measuring the turbidity variation (light scattering) every 30 seconds at 350 nm during 90 minutes. Shake the plate between the measurements if the spectrophotometer is equipped with this option.

Example Data

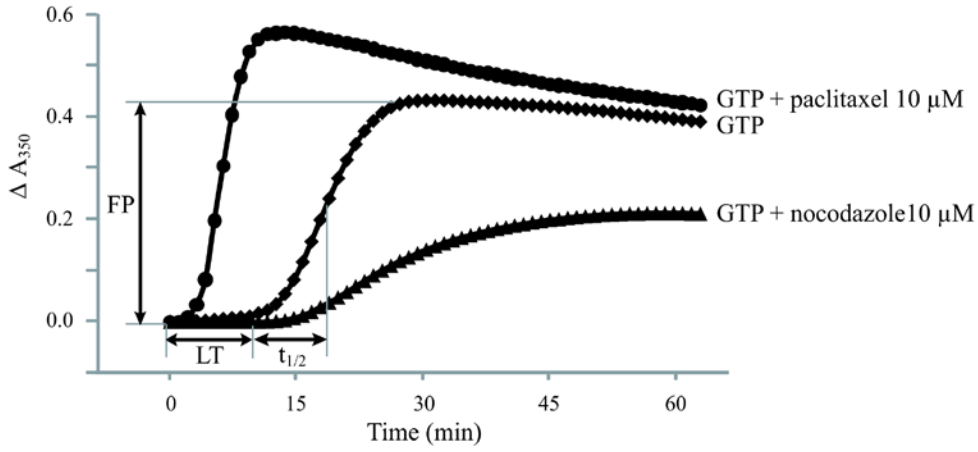

Figure 1: Tubulin polymerization assay in the presence of GTP and control compounds.

Troubleshooting Guide

1. Tubulin stock solution is stable when stored at -80°C for **4 months** after the date of purchase. For longer storage, tubes with tubulin stock solution should be stored at lower temperatures (in liquid nitrogen or in a -150°C freezer).

2. Do not thaw/refreeze tubulin stock. If it was thawed and refrozen by accident before polymerization assay or stored at a temperature higher than -80°C, remove the precipitated (and inactive) tubulin by centrifuging at 60,000 rpm as described below.

   Centrifuge tubulin solution in the TLA100 or TLA100.4 rotor tubes at 60,000 rpm (~140,000 average RCF) for 10 min at 2-4°C. Carefully remove and use the supernatant (pellet contains precipitated and inactive tubulin and should be discarded), but expect lower OD, proportional to the lower tubulin concentration. Alternatively, decrease the dilution factor of the centrifuged tubulin stock solution to increase the OD.

3. Use central wells of the 96-well plate in order to limit temperature variation close to the plate edges.

   Note: Some spectrophotometers are known to have uneven temperature control over the 96-well plates.

4. We recommend keeping the 96-well plate on a metal block put on ice for a better cold transfer.

5. When the 96-well plate is first transferred from ice (or cold metal block) into the spectrophotometer chamber heated to 37°C, some moisture condensation may happen on the bottom of the plate. The condensation may severely affect the readings. To avoid this, insert the cold plate into the warm spectrophotometer chamber for 30-60 seconds, take it out, quickly

wipe the bottom of the 96-well plate with absorbing paper and (re)start the incubation/measurement in the spectrophotometer.

6. The absence of LT in control samples usually indicates the presence of aggregates because of a storage condition problem or the presence of contaminants.

7. Make sure that your test substance does not precipitate on its own in 1x PB-GTP on ice or at 37°C. Precipitation may cause light scattering, mimicking microtubule assembly.

8. If your test substance was initially diluted in 100% DMSO, pre-dilute it in 1x PB-GTP. Note that the maximum DMSO concentration which is compatible with our tubulin polymerization assay is 2%.

9. When running an assay with a protein sample dissolved initially in a buffer different from 1x PB-GTP: the absence of tubulin polymerization or the lower than expected OD values may indicate the presence of certain impurities in the test sample buffer, such as Ca^{2+}.

   Note: adding a chelating agent such as EGTA to "inactivate" Ca^{2+} does not help. To identify/confirm the problem always include control well(s) with buffer alone. Different solutions exist in order to alleviate the contamination problem: (i) dialyze your sample into a pH 6.8 buffer without Ca^{2+} with low salt content (less than 150 mM); (ii) use your protein sample as concentrated as possible to minimize the buffer effect.

10. Some substances may cause tubulin precipitation, which would increase OD without promoting "proper" microtubule assembly. When such a possibility is suspected, at the end of the tubulin polymerization assay transfer the 96-well plate on ice (or cold metal block) for 20 minutes to depolymerize the assembled microtubules. The solution in individual wells should become almost transparent, very slightly opaque. Repeat tubulin assembly as the first time; the second polymerization curve should closely resemble the first one, with final OD at plateau (at ~30 minutes) slightly lower than after the first polymerization.

11. Inaccurate pipetting and/or air bubbles are responsible for most of aberrant readings. Use duplicate or triplicate wells to detect/eliminate experimental errors.

References

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3. A.S. Kiselyov, M.N. Semenova, N.B. Chernyshova, et al. Novel derivatives of 1,3,4-oxadiazoles are potent mitostatic agents featuring strong microtubule depolymerizing activity in the sea urchin embryo and cell culture assays. *European J. Medicinal Chemistry*, 2010, 45:1683-1697.

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