Tubulin Polymerization Assay Biochem Kit.
(Soybean tubulin)
Cat. # BK010-S

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
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Tubulin Polymerization Assay Biochem Kit
Soybean tubulin, Cat.# BK010-S.

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Introduction

Soybean tubulin has been isolated from 6 day old germlings of the Soybean variety 0127562 (Garst Inc. Agripro Round-Up Ready). The protein is greater than 90% pure and hence constitutes a “pure” protein target for compound development. This target protein is ideal for primary screens where large number of compounds have to be screened, or for secondary screens where further development of plant targeted compounds is desired.

The assay utilizes a fluorescent compound (DAPI) which binds to tubulin and microtubules. The quantum yield of fluorescence is increased when the fluorophore is bound to the tubulin and microtubules alike, however there is a ten-fold difference in the affinity of the fluorophore for microtubules compared to tubulin. The result is a fluorescence signal that closely follows microtubule formation. When IC50 values of neuronal tubulin were compared in the fluorescence assay versus the traditional absorbance format, the fluorescent assay closely matched the value of the absorbance assay. One caveat that must be recognized is that the site of fluorophore binding may overlap with your compound’s binding site thereby interfering with the detection of polymer and affecting the IC50 determination. This has not been the case for two well known drug binding sites on tubulin (colchicine and vinblastine) which are not affected when bovine neuronal tubulin is used in both formats. However, the paclitaxel site is competed with DAPI, but the molar amounts of DAPI (10uM, Kd = 10 to 20uM) and paclitaxel (<5uM, EC50 = 1.0uM) mean that the activity of paclitaxel and its analogs can be easily measured in the presence of 20uM (2mg/ml) tubulin.

Using soybean tubulin (Cat.# TP005) to generate the dose response curves, the assay has been used to determine the EC50 or IC50 of four known tubulin ligands, namely Pendimethalin, Triflaran, paclitaxel and nocodazole. In Table 1, the results are compared with bovine brain tubulin using the same microassay. Pendimethalin is >28 times more effective at inhibiting soybean tubulin polymerization than neuronal tubulin polymerization. Whereas Triflaraln is >52 times more effective at inhibiting soybean tubulin polymerization than neuronal tubulin polymerization. These values are represented by the ratio called the Tubulin Ligand Index ratio (TLI ratio; IC50 bovine brain tubulin : IC50 plant tubulin) which compares activities against neuronal and soybean tubulin. These preliminary results indicate that this new microassay may be used to compare known compounds for their efficacy on plant and animal tubulins. For more information about developing other tubulin sources contact customer service at cserve@cytoskeleton.com or call USA-303-322-2254. Custom screening for with various tubulins to create TLI values is also available.
Figure 1: Dose response curves for Pendimethalin and Trifluralin

![Pendimethalin Dose Response](image)

![Trifluralin Dose Response](image)

**Figure 1 legend:** Compounds were assayed in the standard 8% DMSO containing format. Squares are neuronal tubulin (Cat.# TL238) and diamonds are soybean tubulin (Cat.# TP005).

**Table 1: Comparison of IC50 values using Neuronal and Soybean Tubulin.**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC50 or IC50</th>
<th>EC50 or IC50</th>
<th>Tubulin Ligand Index Ratio (Neuronal/Soybean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neuronal Tubulin (uM)</td>
<td>Soybean Tubulin (uM)</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.48</td>
<td>3.60</td>
<td>0.133</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>3.40</td>
<td>&gt;100</td>
<td>&lt;0.034</td>
</tr>
<tr>
<td>Pendimethalin</td>
<td>&gt;100</td>
<td>3.60</td>
<td>&gt;28</td>
</tr>
<tr>
<td>Trifluralin</td>
<td>&gt;100</td>
<td>1.90</td>
<td>&gt;52</td>
</tr>
</tbody>
</table>

* = Values based on the rate of microtubule polymerization, CV +/- 24%.

**Assay Choice**

In order to achieve a sensitive system to measure activity against tubulin polymerization, it is a good idea to choose between measuring enhancers (like paclitaxel), or inhibitors (like pendimethalin) before beginning. In that way the conditions of the tubulin polymerization reactions can be optimized for the respective activities. In practical terms this means choosing between, 2mg/ml tubulin without DMSO (enhancer reaction), or 2mg/ml with 8% DMSO (inhibitor reaction). Both methods can be accomplished with the reagents in this kit. An example of an enhancer reaction is in Figure 2. An example of an inhibitor reaction is in Figure 3.
Example results

Figure 2: Soybean Tubulin polymerization enhanced with paclitaxel.

Legend to Figure 2: Paclitaxel concentrations were, 0.0uM 20% glycerol control = open diamond, 0.1uM = squares, 0.3uM = open squares, 1.0uM = diamonds, 3.0uM = open triangles and 10uM = triangles. Twenty micrograms of TP005 in Buffer 1 at 4°C was pipetted into pre-warmed wells of the 384-well plate containing 2ul of 5X strength compound in Buffer 2 and incubated at 30°C. Tubulin polymerization was detected by measuring the fluorescence of the solution (Ex 360nm, Em 405nm) over time.

Figure 3: Soybean Tubulin polymerization inhibited with trifluralin.

Legend to Figure 3: Trifluralin concentrations were, 0.0uM = diamonds, 1uM = squares, 3uM = triangles, 10uM = open squares, 30uM = open triangles and 100uM = open diamonds. Twenty micrograms of TP005 or T240 in Buffer 1 plus at 4°C was pipetted into pre-warmed wells of the 384-well plate which contained 2ul of 5X strength compound in Buffer 2 and incubated at 30°C. Tubulin polymerization was detected by measuring the fluorescence of the solution (Ex 360nm, Em 405nm) over time. The drop in fluorescence over the first ten minutes of incubation with 10, 30 and 100uM of trifluralin is due to aggregates of tubulin that are dissociated by the drug interactions and hence reduce the fluorescence. This aspect can be minimized if 1.5 or 2.0mg/ml tubulin is used and / or changing to a 20min tubulin solubilizing incubation and performing a microfuge spin (14,000xg, 10min, 4°C) and pipetting the supernatant to a fresh tube on ice.
Material and equipment required

**Material**
100ul of a DMSO solution of 2mM of your compound. If it is not possible to solubilize your compound at this concentration, then you can substitute ethanol for DMSO, or try 200uM solution directly into 80mM Pipes pH6.9, 40% DMSO. If this is not possible either, call customer service (303-322-2254 or tservice@cytoskeleton.com).

**Equipment**
Temperature controlled fluorescence plate reader that measures fluorescence in 384-well plates at Excitation of 360nm+/-20nm and Emission 405nm+/-10nm.

**Kit Contents**
1. 3 x 250ug Soybean Tubulin, >90% pure, 30 to 36 assays (Cat# TP005).
2. 2 x 1.0ml Buffer 1 (5x contains 300mM Pipes buffer pH6.90, 225 µM Dapi).
3. 2 x 1.5ml Buffer 2 (5 x contains 60% PEM buffer (v/v) plus 40% DMSO).
4. 1 x 100ul Paclitaxel (Cat# TXD01).
5. 1 x 1.0ml DMSO for paclitaxel.
6. 1 x plate Fifteen microliter 384-well plate (black, round bottom).

**Tubulin Material and Purity**
Tubulin protein isolated from 6 day old Soybean germlings (Variety: 0127562, Garst Inc. Agripro Round-Up Ready) is presented in lyophilized format. Appearance is a white powder. Purity is determined by scanning densitometry of proteins on SDS-PAGE gels. Samples are >90% pure tubulin.

**Figure 4: Soybean Tubulin analyzed for purity on an 8% PAGE-SDS.**

Legend to Figure 4: Soybean Tubulin was run on a 8% SDS-PAGE gel and stained with 0.1% coomassie blue. Lane 1 - 20ug protein.

**Storage Conditions**
Stable for 6 months when stored at <10% humidity and -70°C. It is not recommended to store reconstituted protein, the polymerization activity is greatly reduced and the IC50 values are altered.
Associated products:

- Tubulin (>99% pure, neuronal source) Cat # T240
- Tubulin Polymerization assay (96 well format, fluorescence based) Cat # BK011P
- CytoDYNAMIX Screen 01 Tubulin Polymerization HTS (Absorbance, Neuronal, 96-well format) Cat # BK004P
- CytoDYNAMIX Screen 03 Tubulin Polymerization Assay (Absorbance, Neuronal, 96-well format) Cat # BK006P
- CytoDYNAMIX Screen 10 MCF-7 Cell Tubulin Polymerization Assay (384-well format) Cat # CDS10M
- CytoDYNAMIX Screen 15 Colchicine binding site HTS (Scintillation Proximity, 96-well format) Cat # CDS15
- HTS-tubulin large quantities Cat # HTS03
Methods:

Introduction

There is a choice to be made before starting operations, that is whether EC50 (polymerization enhancers) or IC50s (polymerization inhibitors) are to be measured. There are two tubulin buffers included in the kit, Buffer 1 and 2. Buffer 1 is used for reconstitution and compound dilution for enhancer reactions, whereas Buffer 2 is used to create the solution for inhibitor assays, and also to create a control reaction for enhancers.

Buffer 1 contains: 300mM Pipes pH 6.90, 225 µM DAPI.

Buffer 2 contains: 50mM Pipes pH 6.90, 1.2mM MgCl2, 0.3mM EGTA, 40% DMSO.

The basic approach to the assay is as follows:

1. Set-up fluorescence plate reader and warm plate in reader to 30°C (soybean tubulin), or 37°C (neuronal tubulin).
2. Prepare compounds to 5x strength in water (enhancers) or Buffer 2 (inhibitors).
3. Prepare 1 ml of 0.1% Triton X-100 in Milli-Q water and place on ice.
4. Reconstitute tubulin with 83 µl of ice cold 0.1% Triton X-100, incubate on ice for 60 min.
5. After 60 min on ice, pipette 20 µl of Buffer 1 into tubulin and mix well.
6. Pipette 2 µl of compound solution from step 2., or Buffer 2 only into each well.
7. Pipette 8 µl of tubulin solution into each well.
8. Start plate reader.
9. After the kinetic protocol has finished, extract V_{10} or V_{max} (IC50) or V_{max} (EC50) from the raw data.
10. Convert V values to Percentage of V_{control}. Inquire to tservice@cytoskeleton.com for an Excel template for this step.
11. Plot Percentage of V_{control} versus Log_{10} of concentration and identify the 50% Percentage V_{control} as the IC50 or EC50 concentration of your compound.

Fluorescence Plate Reader Set-up.

The majority of the work in the design of this assay has been based on the Tecan GmbH machine called SpectroFluor Plus. This machine is filter based and is one of the more sensitive machines on the market (pmoles of fluorescein can be detected). The parameters of a Protocol file in this scenario are:

- Measurement type: Kinetic 41 cycles of 1 reading per minute.
- Fluorescence wavelengths: Ex 360nm +/- 20nm.
- Gain: 100 On a scale of 0-120, where 120 is the highest.
- Reads per well: 10 with 3 flashes per read.
- Fluorescence reading from Top.
- Integration: 0-40us.
- Shaking: 5 s medium, orbital.
- Plate template type: Greiner GRE384fb (flat, black).(Standard template on Tecan).

The major caveats with other plate readers are:

1. If a monochromatic machine is used e.g. Gemini from Molecular Devices Inc., use low light levels wherever possible.
2. If the machine is not set-up for Greiner plates then a custom template will have to be created for the Corning Costar 384-well plate provided (Cat# 3676 from Corning Costar). Use 10ul of ten-fold diluted Buffer 1 pipetted into each well and then activate the New Template scan procedure.

**Preparation of buffers**
1. Defrost Buffer 1 and place on ice, aliquot into 10 x 100ul and freeze at -70°C for storage.
2. Obtain the bottle of Buffer 2 store at RT.

**Preparation of compounds**
This step is performed before the tubulins are rehydrated because if there is an unforeseen error or precipitation occurs, then the experiment can be aborted before rehydrating tubulin. Prepare compound stocks at 0.5, 1.5, 5.0, 15 and 50uM in water (enhancers) or Buffer 2 (inhibitors) as appropriate at RT. The range of concentrations may vary depending on the known IC50 using neuronal tubulin. The necessary control reaction is 2ul of Buffer 2 per well for both enhancer and inhibitor reactions.

**Protocol for determining IC50s with inhibitors (i.e. DMSO containing reactions).**

a) Turn on machine and warm up a 384 well plate (Cat# 3676 Corning Costar), 30°C for soybean tubulin, and 37°C for neuronal tubulin.

b) Prepare compounds as suggested above. Do not continue until the compounds are fully ready.

c) Defrost 100ul of Buffer 1 and place on ice.

d) Remove Buffer 2 from 4ºC and place at RT.

e) Prepare 1 ml of 0.1% Triton X-100 in Milli-Q water and place on ice.

f) Incubate the vial on ice for 60 min (keep this time constant).

g) After 60 min on ice, pipette 20 µl of Buffer 1 into tubulin and mix well. This is your Tubulin Stock.

h) Pipette 2ul of each compound stock into two sets of duplicate wells: Pipette 2ul of Buffer 2 into the first duplicate wells B2,3. Then pipette 2ul of 5uM stock into C2,3, 2ul of 15uM stock into D2,3, 2ul of 50uM stock into E2,3 and 2ul of 150uM stock into F2,3 and 2ul of 500uM stock into G2,3.
i) Using an automatic pipettor, fill with 96ul (12x8ul) of Tubulin Stock. Dispense 8ul of Tubulin Stock into each well B2 thru G3. Start with the control and the low concentrations of compound first as there will be less chance of affecting adjacent wells with carry-over. Place the tip on the side of the well’s wall prior to dispensing as this will aid mixing and reduce bubble formation.

j) Start plate reader as soon as possible thereafter. If the software crashes in the first 5min it is worthwhile to restart the protocol file because some V_{10} data can be rescued in most cases.

Note: The drop in fluorescence over the first ten minutes of incubation with 10, 30 and 100uM of trifluralin is due to aggregates of tubulin that are dissociated by the drug interactions and hence reduce the fluorescence. This aspect can be minimized if 1.5 or 2.0mg/ml tubulin is used and / or changing to a 120min tubulin solubilizing incubation (Step f) and performing a microfuge spin (14,000xg, 10min, 4°C) and pipetting the supernatant to a fresh tube on ice. This supernatant will contain aggregate depleted tubulin.

**Data reduction and analysis for IC50.**

Step 1: Reduce the data to V_{10} (velocity at T = 10min) or Vmax using eight time points with the appropriate command, if this is not possible then import your data into a data analysis software package like Sigma Plot and then perform the same operation. Also average the duplicates in this step.

Step 2: Convert the V_{10} or Vmax data into percent of the 8%-DMSO-control’s V_{10} or Vmax, i.e. using the zero compound concentration wells. Inquire to tservice@cytoskeleton.com for an Excel template for this step.

Step 3: Plot log_{10} of concentration on the X-axis and Percent Vmax on the Y-axis for each compound. Inquire to tservice@cytoskeleton.com for an Excel template for this step.

Step 4: Use the 50% inhibited (IC50) X-axis intercept as the IC50 value respectively.

**Protocol for determining EC50s with enhancers**

The procedure for assaying polymerization enhancers is similar to the inhibitor reactions, with the following notes:

1. Buffer 2 is replaced with water for the tubulin reaction i.e. no DMSO.

2. Prepare Buffer 1 and 2 because they are both needed for this assay, tests and control respectively.

3. Use 2ul of Buffer 2 for the control sample, it is used as the 8% DMSO control sample which will create the V_{max} 100% standard.

4. It is imperative to take readings as soon as possible because some enhancers have a very quick response time (less than 1 min) when they are present at high concentrations (>10uM). In this case the wells containing the high concentrations should receive the TP005 last in the series.

5. Paclitaxel is included as the positive control.

**Data reduction and analysis for EC50.**

Step 1: Reduce the data to V_{max} (Maximum slope) using eight time points with the appropriate command, if this is not possible then import your data into a data analysis software package like Sigma Plot and then perform the same operation. Also average the duplicates in this step.
Step 2: Convert the \( V_{\text{max}} \) data into percent of the 8%-DMSO-control’s \( V_{\text{max}} \). This control can be from another experiment with the same batch of tubulin, or preferably by running the control in the same experiment.

Step 3: Plot \( \log_{10} \) of concentration on the X-axis and Percent \( V_{\text{max}} \) on the Y-axis for each compound. Inquire to tservice@cytoskeleton.com for an Excel template for this step.

Step 4: Use the 50% enhanced (EC50) X-axis intercept as the EC50 value respectively.

**Trouble Shooting**

1. Variation between experiments. This is the largest single error of the polymerization assays, every step must be strictly adhered to and it is essential not to re-freeze tubulin and use only once-frozen buffers. Batch to batch variation is also significant and is best controlled by measuring small groups of compounds (e.g. paclitaxel analogs) with the same batch of tubulin.

2. Poor reproducible TLI ratios. Standardize the neuronal tubulin (T240) by protein assay each time a vial is used. 2mg/ml final concentration is desirable.

3. Consistent use of once-frozen aliquots of Buffer 1.

4. Warm the machine and plate to 30°C (soybean) or 37°C (neuronal) for at least 1h prior to beginning the assays.

**Troubleshooting and more details for first time users**

Pipettor set up:

a) Equilibration of tips with Buffer 1. If the pipettor has fixed tips then three washes of Buffer 1 will equilibrate the tips with buffer. If the tips are disposable it is not necessary to wash them.

b) Filling pipette tips. If your application requires multiple pipetting of the same solution this can be performed by loading the appropriate volume in to the tip at the same time e.g. one IC50 requires 12 x 8ul + 5ul surplus = 101ul per tip.

c) Dispensing. This is the most critical step, care must be taken to set up the height of the dispensing pipette tip so that the likelihood of bubble formation is reduced to a minimum. Failure to do this will lead to more false positives. The optimal pipette tip height is 4mm above the bottom of the plate well and touching the sides of the well. It is important that the tip heights are equal across all wells, if they are not within 0.5 mm across the wells, this will also lead to an increased rate of false positives.

d) Finally, set up your dispense mode to “quick to moderate dispense” to allow the greatest mixing to occur. Be careful not to form bubbles with this procedure. If pipetting up and down is used for mixing purposes (not recommended) be sure to use only 80% of the total volume for pipette mixing, if 100% is used this can sometimes lead to bubble formation by air being pipetted. Plates should be shaken for 5 seconds prior to reading the first time point only, to make all the menisci similar.