Cytoskeleton

KINESIN MOTILITY ASSAY BIOCHEM KIT

BK027

ORDERING INFORMATION

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Manual Contents

Section I: Introduction

BK027 is a Fluorescence based motility assay. To perform this assay effectively you will require a video microscope system with a shutter mechanism for time lapse image acquisition. The following information should be read prior to beginning the assay.

1) The kit is shipped at room temperature. Upon arrival, the kit can be stored at 4°C in a desiccated environment or at -70°C. Many of the components require reconstitution prior to use (Section III). Storage conditions for reconstituted kit components are specified in Section III.

2) Once reconstituted, the tubulin and kinesin proteins are relatively labile. It is imperative to aliquot the proteins as indicated in Section III. Neither of these proteins responds well to repeated freeze / thaw cycles.

3) Microtubules are only stable in the presence of taxol. Therefore remember to include taxol at 20 µM final concentration (i.e., 1 in 100 dilution of your taxol stock) in **ALL** solutions that will come into direct contact with microtubules. NOTE: Taxol is hazardous to health and should be handled with care, always wear gloves.

4) Fluorescent microtubules are sensitive to photo-bleaching and illumination induced breakage. The antifade solution provided in this kit minimizes photo-bleaching and photo-damage. Therefore remember to include 1 x antifade in ALL solutions that are used during microscopic examination of microtubules.

5) The final step in **"Preparation of Taxol Stabilized Microtubules" (section IV)** involves passing the microtubules over a cushion buffer. READ THIS SECTION CAREFULLY AS VOLUMES WILL DIFFER DEPENDING UPON THE CENTRIFUGATION APPARATUS USED. When carrying out high speed centrifugations ALWAYS remember to balance the rotor.

6) This kit uses rhodamine tubulin which requires a microscope filter set of approximately 515 (excitation wavelength) and 590 (emission wavelength).

7) Motor proteins can be very sensitive to minor contaminants that can be transferred from a researchers hands (e.g., grease). It is therefore **VERY IMPORTANT** to wear gloves at all stages of the assay.

8) The recombinant kinesin motor provided with this motility assay moves very slowly (0.5 - 1 µm / min) and motility will not be seen without the use of a time lapse video microscopy system (see references 6 & 7).

9) SECTION III OF THIS KIT TELLS YOU WHAT IS NEEDED TO BE DONE PRIOR TO BEGINNING THE ASSAY. THIS INVOLVES RECONSTITUTION AND STORAGE OF KIT COMPONENTS. IT IS ESSENTIAL TO FOLLOW THESE INSTRUCTIONS CAREFULLY AS INCORRECTLY PREPARED REAGENTS WILL RESULT IN ASSAY FAILURE.

Section II: KIT CONTENTS

Note: Kit BK027 has been shipped at room temperature. Once the kit has arrived it should be stored at either -70°C (as indicated on the Box) or desiccated at 4°C. Kit contents are listed in the Table below;

KIT COMPONENT	DESCRIPTION
RHODAMINE TUBULIN (cat# TL331M)	Three tubes, lyophilized. Contains 20 µg protein per tube. Protein is labeled at a stoichiometry of 1.0 rhodamine per tubulin heterodimer.
UNLABELED TUBULIN (cat# TL238-A)	One tube, lyophilized. Contains 250 µg of protein.
RECOMBINANT KINESIN (cat# KR01)	Two tubes of recombinant human kinesin heavy chain motor domain protein, to be used as a positive control for motility. Supplied as a lyophilized powder, 2 x 25 µg of protein. The kinesin protein contains the amino acid residues 1 - 379 which encompasses the kinesin motor domain. The protein also contains a GST-tag at the amino terminal. The protein is 67 kD in size and should have a velocity of approximately 0.5 - 1 µm per minute.
ANTIFADE (cat# BSM02)	One tube, lyophilized. When reconstituted; 10X stock.
ATP (cat# BSA04-001)	One tube, lyophilized. When reconstituted; 100 mM stock.
MICROTUBULE CUSHION BUFFER (Part # BK027-CB)	One bottle, lyophilized. When reconstituted; 1X stock
GENERAL TUBULIN BUFFER (cat# BST01-001)	One bottle, lyophilized. When reconstituted; 1X stock
BLOCKING SOLUTION (Part # BK027-BL)	One tube, lyophilized. When reconstituted; 1X stock
CHAMBER WASH BUFFER (Part # BK027-WB)	One bottle, lyophilized. When reconstituted; 1X stock
KINESIN BUFFER (Part # BK027-KB)	One tube, lyophilized. When reconstituted; 1X stock
TAXOL (cat# TXD01)	Two tubes, lyophilized. When reconstituted; 2 mM stock.
DMSO (cat# DMSO)	One tube of DMSO, for taxol resuspension.
PERFUSION CHAMBER FILTER PAPER (Part # BK027-FP)	One box of filter paper.
ACID WASHED PERFUSION CHAMBERS (cat# BSM05-04)	One box containing 25 perfusion chambers.

Section III: THINGS TO DO PRIOR TO BEGINNING THE ASSAY

IT IS ESSENTIAL TO FOLLOW THESE INSTRUCTIONS CAREFULLY AS INCORRECTLY PREPARED REAGENTS WILL RESULT IN ASSAY FAILURE. PARTICULARLY, THE RECONSTITUTED TUBULINS AND KINESINS SHOULD BE SNAP FROZEN IN LIQUID NITROGEN FOR OPTIMAL ACTIVITY. ALTERNATIVELY AN ALCOHOL/DRY ICE BATH CAN BE USED. NEVER FREEZE THE RECONSTITUTED PROTEINS BY PLACING THEM AT -70°C AS THEY WILL DENATURE DUE TO THE SLOW FREEZE.

Buffer / Reagent Reconstitution Table

KIT COMPONENT	RECONSTITUTION CONDITIONS
ANTIFADE	Reconstitute in 1 ml of 50% glycerol plus 1.5% beta-mercaptoethanol and aliquot into 10 x 100 µl aliquots. These are now at 10X stock strength and should be snap frozen in liquid nitrogen. Store at -70° C.
ATP	Reconstitute in 1 ml of distilled water and aliquot into 10 x 100 µl aliquots. These are now at 100 mM stock strength and should be snap frozen in liquid nitrogen. Store at -70°C.
MICROTUBULE CUSHION BUFFER	Reconstitute in 10 ml of 50% glycerol. Store at 4°C.
GENERAL TUBULIN BUFFER	Reconstitute in 10 ml of distilled water. Stored at 4°C.
KINESIN BUFFER	Reconstitute in 500 µl of distilled water. Store at 4°C.
BLOCKING SOLUTION	Reconstitute in 1 ml of distilled water. Store at 4°C.
CHAMBER WASH BUFFER	Reconstitute in 5 ml of distilled water. Store at 4°C.
TAXOL	Reconstitute in 100 µl of DMSO. Store at -70°C.

Protein Reconstitution Table

PROTEINS FROM KIT	RECONSTITUTION CONDITIONS
TUBULINS	a) Reconstitute each rhodamine tubulin tube in 4 µl of ice-cold General Tubulin Buffer (12 µl total volume) and immediately place on ice. b) Reconstitute the unlabeled tubulin in 50 µl of ice-cold General Tubulin Buffer and place on ice. c) Mix the labeled and unlabeled tubulins. d) Add 12 µl of ice-cold Microtubule Cushion Buffer and mix well. e) Immediately aliquot 2 µl of the protein into approximately 25 eppendorf tubes and snap freeze in liquid nitrogen. f) This is your tubulin stock protein. g) Store at -70°C.
KINESIN	a) Reconstitute each tube of recombinant kinesin motor domain protein into 6 µl of Kinesin Buffer and pool the contents of each tube. b) Aliquot 1 µl volumes into approximately 10-12 tubes. c) Snap freeze the proteins in liquid nitrogen. d) This is your kinesin stock protein. e) Store at -70°C.

Section IV: ASSAY PROTOCOL

Preparing Taxol Stabilized Microtubules

The preparation of taxol stabilized microtubules should take approximately 1 hr to complete.

The steps involved are: polymerization of tubulin into microtubules (15 minutes), stabilization of microtubules with taxol (5 minutes), separation of microtubules from background fluorescence (40 minutes). After the microtubules are prepared they are stable for several hours at room temperature. A detailed protocol is given below:

a) Aliquot 500 µl of General Tubulin Buffer into a clean eppendorf tube at room temperature.
b) Thaw out one tube of Taxol stock and add 5 µl to the 500 µl of General Tubulin Buffer, leave the tube at room temperature.
c) Immediately thaw one aliquot of tubulin stock protein and add 2 µl of cushion buffer.
d) Incubate at 35°C for 15 minutes.
e) After 15 minutes, remove the polymerized tubulin from 35°C and immediately add 100 µl of the Taxol supplemented General Tubulin Buffer, mix thoroughly but gently.
f) This step should result in a population of taxol stabilized microtubules that average 5 - 10 µm in length and are at a concentration of 7 x 10^{10} per ml. These microtubules are stable at room temperature for several hours.
g) At this point, you may examine a small aliquot of the microtubules by fluorescence microscopy (1 µl of microtubules diluted into 10 µl taxol containing General Tubulin Buffer) . Individual microtubules should be clearly visible; however there will also be some noticeable background fluorescence. The background fluorescence results from unpolymerized tubulin and **MUST** be removed by passing the microtubules over a glycerol cushion, as described below.
h) Aliquot from 100 - 400 µl of Cushion Buffer (depending upon the size of the centrifuge tube used) into an eppendorf tube and incubate at 35°C for 10 minutes. Remove from incubation and add 1-4 µl of taxol stock solution (i.e., 1 in 100 dilution of your taxol stock). Aliquot the cushion buffer (plus taxol) into an ultracentrifugation tube, such as a Beckman Ultraclear centrifuge tube (catalogue # 344090, holds 500 µl volume). Carefully layer the 104 µl of microtubule solution onto the cushion. Centrifuge at 100,000 g at 25°C for 30 minutes to pellet the microtubules through the cushion.
i) Immediately after centrifugation, carefully remove the first 100 µl of solution and discard.
j) Immediately remove the remaining cushion buffer with a fresh pipette tip and discard. The pellet may be visible due to its faint pink coloration; however you should not be unduly worried if you can not see the pellet at this stage.
k) Resuspend the microtubule pellet in 100 µl of Taxol supplemented General Tubulin Buffer. Resuspension should be **GENTLE** and thorough (pipette up and down 7-10 times and gently scrape the bottom of the tube with the pipette tip after 3-4 up/down movements). This taxol stabilized microtubule stock population will remain stable at room temperature for several hours. It is advisable to examine the microtubules by fluorescence before beginning the motility assay. Microtubules should still be approximately 5 µm long and there should be no background.

Section IV: ASSAY PROTOCOL (continued)

Motility Assay Using Recombinant Kinesin Motor Domain as a Positive Control

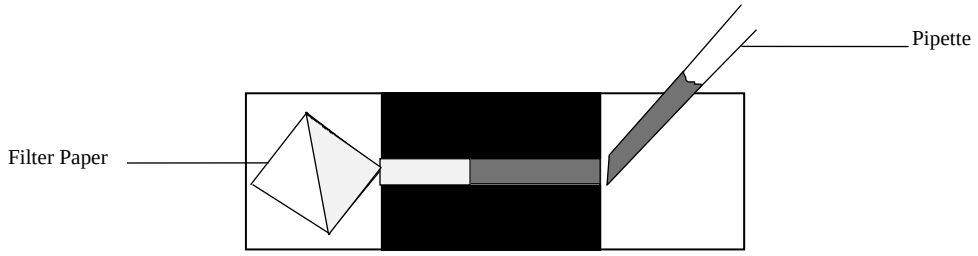
This part of the assay should take approximately 30 - 60 minutes to complete.

a) Aliquot the following into separate tubes at room temperature;

Tube 1:Blocking Solution	100 µl	**Tube 2**:General Tubulin Buffer	495 µl
		Taxol Stock	5 µl
Tube 3:Chamber wash buffer	89 µl	**Tube 4**:General Tubulin Buffer	42 µl
Taxol Stock	1 µl	Chamber Wash Buffer	42 µl
10 x Antifade	10 µl	Taxol Stock	1 µl
		10 x Antifade	10 µl
		100 mM ATP	5 µl

b) Thaw out one tube of kinesin stock protein and add 11 µl of General Tubulin Buffer.
c) Remove two fresh perfusion chambers. Label one "Kinesin Control" and the other "Negative Control".
d) Perfuse the kinesin into the "Kinesin Control" chamber and incubate at room temperature for 5 minutes.
e) You will perform buffer exchange in the perfusion chamber using the provided filter paper (see Figure 1). It is **VERY** important that the perfusion chamber is never allowed to dry out.

Figure 1: How To Exchange Buffers In The Perfusion Chamber



Buffer 2 ▬ *is perfused into the chamber while Buffer 1* ▭ *is removed simultaneously using the provided filter paper. The chamber should never dry out as one buffer is exchanged for another.*

f) Perfuse 11 µl of blocking solution (Tube 1) into the "Kinesin Control" and the "Negative Control" perfusion chamber. Use the Perfusion Chamber Filter Paper to exchange buffers in the chamber (see Figure 1). Incubate at room temperature for 5 minutes. Note: There may be some precipitation in the blocking solution; this is not cause for concern.
g) Perfuse 10 µl of the fluorescent, taxol stabilized microtubule stock into each chamber and incubate for 5 minutes at room temperature.
h) Perfuse in 20 µl of Wash buffer solution (Tube 3) into each chamber. This step removes any microtubules that are not stuck to the motor protein.
i) View the contents of the perfusion chamber by fluorescence microscopy. Focus on the top surface of the inside of the perfusion chamber and note how many microtubules are stuck. There should be virtually no microtubules present on the surface of the "Negative Control" perfusion chamber. Many microtubules will attach to the "Kinesin Control" chamber (see Figure 2).
j) If there are too many microtubules to give a clear view of individual microtubules, the microtubule stock can be diluted 2-4 fold with (Tube 2) buffer.

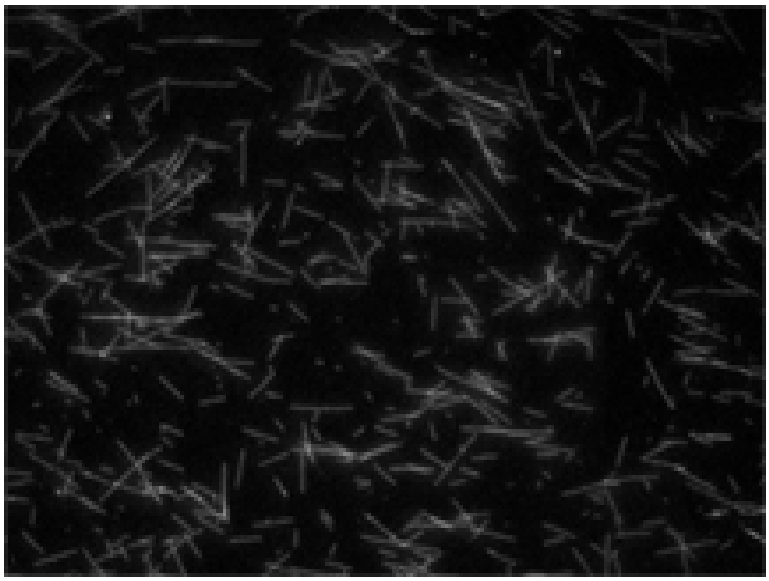
Section IV: ASSAY PROTOCOL (continued)

NOTE: Motor proteins are very sensitive to prolonged light exposure. It is therefore not advisable to observe the microtubules for longer than necessary to ascertain that they are attached to the chamber surface. Light intensity can be kept to a minimum by the use of neutral density filters.

k) The following procedure can be performed whilst keeping the perfusion chamber in place at the microscope. Perfuse 10 µl of motility buffer (Tube 4) into the chamber and observe microtubule movement (see Figure 3).

l) We do not recommend attempting to observe microtubule movement without the use of a video camera and recording device. Also, because of the sensitivity of fluorescent microtubules and motor proteins to light, we recommend the use of a shutter device to generate time-lapsed images. The truncated recombinant kinesin used in this kit moves at a much slower rate than native, full length, kinesin protein. We recommend taking **5 minute** time lapse frames over a total of 30 minutes to 1 hr to confirm motility. The velocity of the recombinant GST-tagged protein should be approximately 0.5 – 1 µm per minute, this velocity is in agreement with published data (12), native or full length recombinant kinesin moves with a velocity of 30 - 40 µm/min. The selected reference list at the end of this section includes several descriptions of suitable microscope layouts.

Figure 2: Microtubule Sticking In The Presence Of Kinesin Control Protein



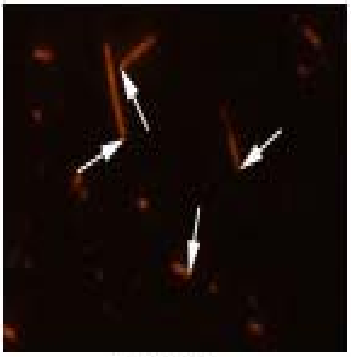
The frame above shows a typical field of view (using a 60X objective) for the Kinesin Control chamber. This field contains >500 microtubules.

Section IV: ASSAY PROTOCOL (continued)

Figure 3: Microtubule Motility Assay

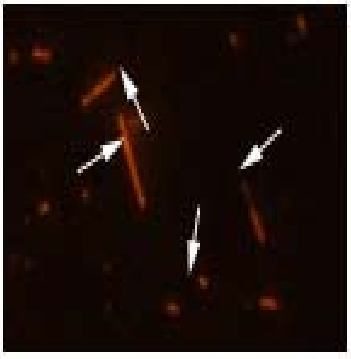
Frames A-D below show a select field of microtubules (MTs). The field has been selected to show a sparse area of MT coverage in order to allow clear visualization of MT motility. Each frame represents a 5 minute time-point, A = time zero (immediately after motility buffer addition), B = 5 minutes, C = 10 minutes, D = 15 minutes. The position of each MT at time zero is marked by an arrow. The arrow position remains identical in each frame to serve as a reference point for MT movement. To obtain an average MT velocity, we recommend measure motility of approximately 30 - 50 individual MTs.

A

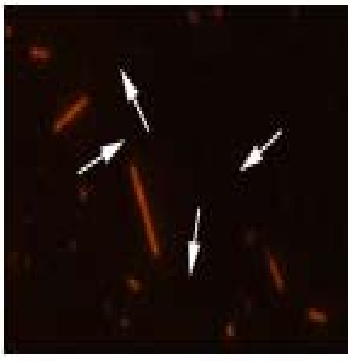


B

C



D



Section IV: ASSAY PROTOCOL (continued)

Motility Assay Using a Motor of Your Choice

As a starting point, we recommend following the procedure outlined in Section IV for the kinesin motor protein. If you have no success from this procedure there are several possibilities;

OBSERVATION	REASON	SOLUTION
No Obvious Microtubule Sticking	There are no stable microtubules	Did you add taxol to the microtubules?
No Obvious Microtubule Sticking	Not enough motor protein in the assay.	Very small amounts of active motor are necessary to observe motility in this assay, <0.2 µg of kinesin will give a high level of microtubule sticking and motility (using a 60X objective). Thus, if your protein of interest is in the low µg range the amount of protein is unlikely to be the limiting factor.
No Obvious Microtubule Sticking	There is an inhibitor in the motor protein solution	Further purification may be necessary.
No Obvious Microtubule Sticking	The motor protein is inactive	There are now many reports of motor protein purifications from both native and recombinant sources, see reference list at the end of this section. They will provide useful information on how to modify your purification procedure.
High Fluorescent background	Did not pass microtubules over a cushion prior to use in the assay	Include a cushion step
High Fluorescent background	The microtubules are more than 8 hrs old	Background fluorescence can increase with time, it is advisable to use the microtubules within 4-6 hrs.
No movement in the presence of ATP motility buffer	There is an inhibitor in the motor protein solution	Further purification may be necessary. Also always use acid washed perfusion chambers and wear gloves.
No movement in the presence of ATP motility buffer	Fluorescence illumination is too high	Motors can be very sensitive illumination intensity, always keep this to a minimum, also keep time of exposure to fluorescence at a minimum by using a time lapse video system.
No movement in the presence of ATP motility buffer	The motor is inactive	There are now many reports of motor protein purifications from both native and recombinant sources, see reference list at the end of this section. They will provide useful information on how to modify your purification procedure.
No movement in the presence of ATP motility buffer	The ATP buffer is old	ATP will hydrolyze over 1-2 hrs and therefore inhibit motility. Make fresh motility buffer every few hours.

Section V: USEFUL REFERENCES

Key Papers

1) Vale, R. D., Schnapp, B.J., Reese, T.S., and Sheetz, M.P. 1985. Movement of Organelles Along Filaments Dissociated from the Axoplasm of the Squid Giant Axon. *Cell*, **40**: 449-454.

2) Vale, R.D., Schnapp, B.J., Reese, T.S., and Sheetz, M.P. 1985. Organelle, Bead, and Microtubule Translocations Promoted by Soluble Factors from the Squid Giant Axon. *Cell*, **40**: 559 - 569.

3) Vale, R.D., Reese, T.S., and Sheetz, M.P. 1985. Identification of a Novel Force-Generating Protein, Kinesin, Involved in Microtubule-Based Motility. *Cell*, **42**: 39-59.

4) Shimizu, T., et al. 1991. Nucleotide Specificity of the Enzymatic and Motile Activities of Dynein, Kinesin, and Heavy Meromyosin. *J. Cell Biol.*, **112**: 1189 - 1197.

5) Hyman, A.A. 1991. Preparation of marked microtubules for the assay of the polarity of microtubule-based motors by fluorescence. *J. Cell Sci., Supp.*, **14**: 125-127.

Video Enhanced Microscopy

6) Video Microscopy. Inoue, S. 1986. Plenum Press. This book is still one of the best information sources available.

7) Methods in Cell Biology. 1993. Volume 39; Motility Assays for Motor Proteins, edited by J.S. Scholey. This book is highly recommended for those working with motor proteins, it is packed with useful tips.

Obtaining Active Motor Protein From Overexpression Systems

8) Methods in Cell Biology. 1993. Volume 39; Motility Assays for Motor Proteins, edited by J.S. Scholey. Chapter 8, Chandra, R and Endow, S.A. Expression of Microtubule Motor Proteins in Bacteria for Characterization in *in Vitro* Motility Assays.

9) Walker, R.A., Salmon, E.D., and Endow, S.A. 1990. The Drosophila claret segregation protein is a minus-end directed motor molecule. *Nature*, **347**: 780 - 782.

10) Yamazaki, H., Nakata, T., Okadad,Y. and Hirokawa, N. 1995. KIF3A/B: A Heterodimeric Kinesin Superfamily Protein That Works as a Microtubule Plus End-directed Motor for Membrane Organelle Transport. *J. Cell Biol*., **130:** 1387-1399.

11) Nangaku, M., et al. 1994. KIF1B, a Novel Microtubule Plus End-Directed Monomeric Motor Protein for Transport of Mitochondria. *Cell*, **79**: 1209-1220.

12) Stewart et al. 1993. Direction of microtubule movement is an intrinsic property of the motor domains of kinesin heavy chain and Drosophila ncd protein. *PNAS*, **90**: 5209-5213.

Purifying Motor Proteins From Native Sources

13) Vale, R.D., Reese, T.S., and Sheetz, M.P. 1985. Identification of a Novel Force-Generating Protein, Kinesin, Involved in Microtubule-Based Motility. *Cell*, **42**: 39-59.

14) Cole, D.G., et al. 1992. Isolation of a sea urchin egg kinesin-related protein using peptide antibodies. *J. Cell Sci.* **101**: 291-301.

15) Cole, D.G., et al. 1993. Novel heterotrimeric kinesin-related protein purified from sea urchin eggs. *Nature*, **366**: 268-270.