CHAPTER 8

In Vitro Assays for Studying *Saccharomyces cerevisiae* Kinetochore Activity

Fedor Severin,[*] Ken Kaplan,[†] Peter Sorger,[†] and Tony Hyman[*]

[*] Cell Biology Program
EMBL
Meyerhofstrasse 1
Heidelberg D-69117, Germany

[†] Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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I. Introduction

Kinetochores are complexes which are required for faithful chromosome segregation: They link centromeric DNA to microtubules of mitotic spindles. Due to its high complexity in higher eukaryote, kinetochore function is best studied in the

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budding yeast *Saccharomyces cerevisiae*. The centromeric DNA in *S. cerevisiae* is only 125 bp and is present in one copy per chromosome. It has been shown that mutations in centromere sequences prevent chromosome segregation (Hyman and Sorger, 1995) and prevent the binding of a kinetochore complex to chromosome 3 centromere (CEN3) *in vitro* (Kingsbury and Koshland, 1991; Lechner and Carbon, 1991; Ng and Carbon, 1987; Sorger *et al.*, 1994, 1995). These findings allowed the development of two assays to monitor kinetochore function *in vitro*. The first assay is a bead-binding assay for studying the interaction of *S. cerevisiae* kinetochores with microtubules *in vitro*. In this assay either wild-type CEN3 or CEN3 with the 3-bp deletion is attached to fluorescent beads. The beads are incubated with yeast extract in the presence of nonspecific carrier DNA and added to microtubules. While the beads with wild-type CEN3 bind to microtubules, the beads carrying CEN3 with 3-bp deletion fail to interact with microtubules. The second assay is a band shift assay for studying the interaction of the kinetochore complex with the CEN3. In this chapter we describe the methods for both of these techniques.

II. Microtubule-Binding Assays for *S. cerevisiae* Kinetochores

A. Preparation of CEN3 Beads

1. Making the DNA

1. For a basic bead-binding assay, amplify two sequences by PCR using a 5′ primer biotin-CCACCAGTAAACGTTTC and a 3′ primer GTACAAATAAGTCACATGATGATATTTG (Sorger *et al.*, 1994). Use the following plasmids: wild-type CEN3 (plasmid PRN505) (Ng and Carbon, 1987) and CEN3 with a 3-bp deletion in the central CCG (plasmid PSF137) (Sorger *et al.*, 1994). To make 300 pmol of each fragment use the following Mastermix for the PCR reaction:

500 μl 10× PCR buffer (Perkin–Elmer, Foster City, CA) 15 m*M* $MgCl_2$, 500 m*M* KCl, 100 m*M* Tris–HCl (pH 8.3), 0.01% gelatin
40 μl of 25 m*M* dNTPs
100 μl of each primer initial concn 100 pmol.
200 μl of 50 m*M* $MgCl_2$
3810 μl H_20

Split the mix in two equal parts and to each add 125 μl of plasmid (initial concn on approximately 5 μg/ml) and 25 μl of taq polymerase (Amplitaq, Perkin-Elmer). Run PCR for 35 amplification cycles (15 sec at 94°C, 15 sec at 56°C, and 15 sec at 72°C) on GeneAmp PCR System 2400 (Perkin–Elmer).

2. Purify the PCR products on a 1-ml MonoQ column (Pharmacia LKB Biotechnology, Piscataway, NJ) using FPLC system (Pharmacia). Load pooled reactions onto the column equilibrated with TE (10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5), wash with 400 m*M* NaCl in TE for 5 column volumes, and elute the DNA with 20 ml 400 m*M*–1 *M* linear gradient of NaCl in TE. The flow rate is 0.5 ml/min. Collect 0.5-ml fractions. The product elutes in fractions 19–21 and the amount of both wild-type and 3-bp-del DNA is 330 pmol.

2. Coupling of CEN DNA to Beads

Prepare the beads as follows:

1. Make biotin–BSA by reaction of BSA with NHS–biotin (Molecular Probes, Eugene, OR, No. B-1606). Dissolve 1 g of BSA in 20 ml of 50 m*M* K-Pipes (pH 7.5). Dissolve 57 mg of NHS–biotin in 1 ml of DMSO. Mix and leave at room temperature for 1 h. Add 1 ml of 1 *M* lysine dissolved in the same Pipes buffer to stop the coupling reaction.

2. Separate biotin–BSA from free biotin on P10 desalting column (BioRad Labs, Hercules, CA). Use a column 10 times bigger than the volume of the load. Collect the peak of the protein (not the trailing fractions) and confirm the presence of biotinylated protein using rhodamine–HABA: Biotin displaces rhodamine from the HABA, thus indicating the presence of the biotinylated protein (Molecular Probes, biotin-labeling kit, No. F-2610; see the instructions provided with the kit).

3. Add 1 ml of biotin–BSA and 0.125 ml of 1 *M* sulfo-NHS (Pierce, Rockford, IL) dissolved in 50 m*M* K-Pipes (pH 7.0) to 1 ml of 0.2-μm beads (carboxylate modified, Molecular Probes) at 4°C. Then bring the tube to room temperature and add 5 mg of 1-ethyl-3-(3-dimethylaminopropil carbomide (EDAC; Sigma Chemical Co., St. Louis, MO) at 1-h intervals for 4 hr while shaking on a time shaker, 5 sec every minute. After the last EDAC addition add 0.1 ml of 1 *M* lysine to stop the reaction.

4. Spin the BSA beads down at 4°C in the Eppendorf centrifuge (14,000 rpm for 10 min), resuspend in the same volume of 0.5 *M* NaCl in 50 m*M* K-Pipes (pH 7.0), spin again, wash again in 100 m*M* K-Pipes (pH 7.0), and finally resuspend in 2 ml of 100 m*M* K-Pipes (pH 7.0). The beads can be stored at 4°C with Na-Azide.

5. Count the number of BSA beads per milliliter under the microscope. Adjust the concentration to 1.2×10^{12} beads/ml.

6. Add 50 μl of beads to 50 μl of 100 m*M* K-Pipes (pH 7.0) and 1 m*M* EDTA. Add 1 mg of streptavidin (Molecular Probes) dissolved in 50 μl of the same buffer. Agitate the mixture for 1 h at 4°C.

7. Dilute the beads with 150 μl of the same buffer and centrifuge in TLX-55 rotor at 20,000 rpm onto 100-μl cushion containing 100 m*M* K-Pipes (pH 7.0), 1 m*M* EDTA, and 20% glycerol for 10 min.

8. Resuspend the bead pellet in 300 μl of 100 mM Pipes 1, mM EDTA and 500 mM NaCl (pH 7.0), and sonicate with a tip sonicator until the beads become monodispersed. To avoid heating perform the sonication on ice with short (0.5-sec) pulses. Spin the beads again under the same conditions and resuspend in 300 μl of 100 mM Pipes and 1 mM EDTA (pH 7.0).

9. Sonicate the beads to make them monodispersed and dilute to a concentration 5×10^7 beads/μl with the same buffer. At this stage split the beads into two equal volumes and add either wild-type or 3-bp-del biotinylated DNA to each tube at the molar ratio 1000 DNA molecules/bead. Incubate the beads at 4°C overnight with agitation.

10. Spin the beads in the Eppendorf centrifuge at 14,000 rpm for 5 min and resuspend in 300 μl of 50 mM Hepes (pH 8.0), 1 mM EDTA, and 500 mM NaCl. Sonicate to disperse the bead aggregates. After the next spin (5 min at 14,000 rpm) resuspend the beads in 50 mM Hepes (pH 8.0), 1 mM EDTA, and 50% glycerol buffer.

11. Sonicate the beads and adjust to the concentration of 3×10^7 beads/μl.

B. Preparation of Carrier DNA

Prepare the carrier DNA from commercial DNA from salmon testis by sonication.

1. Dissolve 1 g of salmon sperm DNA (Sigma, No. D-1626) in 0.5 liters of TE. Sonicate with a tip sonicator—0.1-sec pulses every second for 24 hr. Keep solutions on ice and stirring on a magnetic stirrer to avoid heating.

2. Check the average length of the DNA fragments by running a sample of sonicated DNA on 2% agarose gel. The length should be approximately 200 bp. If the fragments are not short enough, continue sonication.

3. Add 1.25 liters of ethanol and 50 ml of 2.5 NaAc (pH 5.5) to precipitate the DNA. Keep the mixture on ice for 20 min, then spin in J-6B rotor (5000 rpm for 20 min).

4. Resuspend the DNA pellet in 50 ml of TE. Split between two 50-ml Falcon tubes. Add 25 ml of phenol to each Falcon tube and then vortex.

5. Separate the layers by centrifugation at 4000 rpm for 5 min (Megafuge 10R, Heraeus, South Plainfield, NJ). Collect the DNA (top layer).

6. Extract the DNA with chloroform twice. Use the same procedure as for phenol extraction.

7. Collect the DNA (the volume will be about 40 ml). Precipitate the DNA with ethanol: +2.5 vol of ethanol, +1/10 vol of 2.5 M NaAc, 20 min. on ice, spin in a Megafuge at 4000 rpm for 20 min.

8. Finally, resuspend the DNA in TE and adjust to the concentration of 10 mg/ml. Store frozen at −20°C.

C. Preparation of Microtubules

1. Preparation of Polarity-Marked Microtubules

Make all solutions in BRB80 [80 mM potassium Pipes (pH 6.8), 1 mM $MgCl_2$, and 1 mM EGTA]. Prepare and label tubulin with rhodamine and with Oregon green (Molecular Probes) as described (Hyman *et al.*, 1991). Polarity-marked microtubules can be prepared in three ways. To study the total binding of microtubules of kinetochores prepare taxol-stabilized polarity-marked microtubules as described in Howard and Hyman (1993). First, polymerize short, rhodamine-labeled microtubules in the presence of nonhydrolyzable GTP analog, GMPCPP. The commercial source of GMPCPP is not available and must be synthesized (Hyman *et al.*, 1992). Dilute the formed brightly labeled microtubules into lightly labeled tubulin at a concentration of tubulin below the critical concentration for nucleation. Polymerization mostly occurs at the plus ends of the preexisting microtubules. Then stabilize microtubules in taxol.

1. Polymerize 5 μl of bright microtubules in the presence of GMPCPP (10 μM rhodamine-labeled tubulin and 0.2 mM GMPCPP) at 37°C for 10–15 min.

2. Add 40 μl of dimly labeled tubulin (2 μM rhodamine tubulin, 20 μM unlabeled tubulin, 1 mM GTP) to the microtubules. Allow the microtubules to grow for 15 min and then stabilize by the addition of 150 μl of 10 μM taxol.

3. Spin microtubules at 50,000 rpm for 5 min in TLA 100 rotor and resuspended in 50 μl of 10 μM taxol in BRB80.

2. Preparation of Capped Microtubules

To study the interaction of kinetochores with GDP versus GTP types of microtubule lattices, make the capped microtubules as described in Severin *et al.* (1997).

1. Add 1 μM of Oregon green tubulin to 0.5 mM GMPCPP in BRB80 at 37°C. Typical volume is 5 μl.

2. Add 1 μM Oregon green tubulin to the mixture every 30 min for 2 or 3 hr. This creates GMPCPP microtubules with an average length of 6.5 μm. We confirmed that GMPCPP was not hydrolyzed using the method of Field *et al.* (1996).

3. Preincubate 20 μl of 0.1 mM GTP, 10 μM tubulin, and 5 μM rhodamine tubulin in BRB80 for 10 min on ice and prewarm for 30 sec at 37°C. Add 2 μl of the Oregon green microtubules to the mixture and allow GTP tubulin to polymerize for 20 min. Under these conditions more than 90% of the GMPCPP microtubules nucleate GTP microtubules.

4. Preincubate 40 μl of 0.5 μM Oregon green tubulin and 0.5 mM GMPCPP in BRB80 on ice for 10 min, prewarm at 37°C for 1 min, and then mix with 0.5 μl of the GMPCPP–GTP microtubules, giving a final GTP concentration of 1 μM and GMPCPP concentration of 500 μM.

5. Add 0.5 μM of Oregon green tubulin after 1 hr of incubation at 37°C and leave at 37°C for 30 min.

Microtubules are stable only for a few hours at room temperature.

3. Preparation of GMPCPP Polarity-Marked Microtubules

To study the binding of kinetochores to the ends of microtubules versus the microtubules lattice, prepare GMPCPP-stabilized microtubules as described in Severin *et al.* (1997).

1. Add 1 μM Oregon green tubulin to 0.5 mM GMPCPP at 37°C. Add 1 μM Oregon green tubulin to the mixture every 30 min for 3 or 4 hr.
2. Mix 2 μl of the Oregon green microtubules with 20 μl of 0.3 μM tubulin, 0.2 μM rhodamine tubulin, and 0.5 mM GMPCPP. Incubate the mixture at 37°C for 30 min. Add 0.3 μM of unlabeled tubulin and 0.2 μM of rhodamine tubulin every 30 min during 3 or 4 hr. Because tubulin grows approximately three times faster from the plus end compared to the minus end (Walker *et al.*, 1988), the red segments at the minus ends of the microtubules are visibly shorter then the red segments at the plus ends.

We confirmed the polarity of the microtubules in a kinesin-gliding assay. The coverslip was coated with bovine brain kinesin and GMPCPP microtubules were added. Because kinesin is a plus-end directed motor, the microtubules were moving with their short red segments (which correspond to the minus ends) leading.

D. Preparation of Yeast Extracts

Prepare whole cell extracts from cells grown at 30°C in 0.5–11 of YPD medium to a density of 2–5 × 10^7 cells/ml.

1. Pellet the cells by centrifugation in J-6B centrifuge (Beckman) at 4000 rpm for 5 min.
2. Wash cells once by pelting in 50 ml of water in a Falcon tube (Megafuge 10R, Heraeus), weight, and then resuspend in the same weight of two times breakage buffer [200 mM β-glycerophosphate, 100 mM bis–tris propane (pH 7.0), 400 mM KCl, 10 mM EDTA, 10 mM EGTA, and 20% glycerol] with protease inhibitors (final concentrations: 1 mM phenylmethylsulfony fluoride and 10 μg/ml each of pepstatin, leupeptin, and chymostatin).
3. Freeze the cells by dripping the cell suspension in a 50-ml Falcon tube filled with liquid nitrogen.
4. Fragment the cells with porcelain mortar and pestle cooled in liquid nitrogen. It takes 100 pestle strokes to break more than 50% of the cells.

5. Remove the cell debris by centrifugation at 15,000g for 30 min. Typical protein concentration in extracts is 30–40 mg/ml. Freeze the extracts in liquid nitrogen and store at −70°C in aliquots.

E. Microtubule-Binding Assays

1. Add 15–200 μg of yeast extract to a 30-μl reaction containing 3 × 10^7 beads and 10 μg of sonicated salmon sperm DNA in bead-binding buffer [10 mM Hepes (pH 8.0), 6 mM $MgCl_2$, and 10 % glycerol and adjusted to a final KCl concentration of 150 mM].
2. After 30–40 min of incubation at room temperature, take the samples and dilute 1:1 into antifade solution. Antifade contains 40 mM KPipes (pH 6.8), 3.5 mM $MgCl_2$, 0.1% 2-mercaptoethanol, 1.5 mg/ml casein, 0.1 mg/ml catalase, 0.1 mg/ml glucose oxidase, 10 mM glucose, and 10 μM taxol.
3. Perfuse the polarity-marked microtubules into a 5-μl chamber and allow to adsorb for 5 min. Perfuse 6 μl of 3 mg/ml casein into a chamber followed by 6 μl of the bead-binding reaction diluted into antifade.
4. We observe the chambers with a Zeiss Axioskop microscope and a 63× PlanApo 1.4 NA lens. This gives the best field size for counting statistics. A 100× lens gives a higher resolution image of the actual binding event. Count the number of beads/field bound to microtubules in a coverslip focal plane.

To study the binding of the beads to GDP versus GTP parts of microtubules, mix 2 μl of the bead-binding reaction with 2 μl of the capped microtubules. Incubate the mixture for 5 min at room temperature dilute with 40 μl of BRB80, and then observe with a Zeiss Axioskop microscope and a 63× PlanApo 1.4 lens using Colour Cool View camera (Photonic Sciences, East Sussex, UK). To avoid bias, photograph all microtubules containing a bead. To examine which color to which the beads were bound, split the colors into the three components of the RGB signal.

To study the binding of the beads to the microtubule ends versus microtubule lattice, mix 1 μl of the GMPCPP microtubules with 10 μl of the kinetochore beads, allowed to bind for 5 min, then dilute with 200 μl of BRB80 and observe in the same way.

III. Band Shift Assay for the Kinetochore Complex

A. CEN3 Probe Isolation

1. The DNA probe for measuring kinetochore binding *in vitro* consists of an 89-bp fragment from the CEN3 sequence of *S. cerevisiae* and is synthesized by PCR. Probe sequences: 5' TAT TAG TGT ATT TGA TTT CCG AAA GTT AAA AAA GAA ATA GTA AGA AAT ATA TAT TTC ATT GAA TGG

ATA TAT GAA ACG TTT ACT GGT GG 3'. However, it is possible to use as little as 56 bps from this region to detect the kinetochore complex formation (Espelin *et al.*, 1997).

2. Following amplification, the PCR product is purified using anion-exchange column chromatography as described previously.

B. Labeling of CEN3 DNA by T4 DNA Kinase

1. Radiolabeled probe sufficient for approximately 100 reactions is prepared as follows: 7.50 pmol of purified probe DNA is added to a 20-μl reaction in 1X T4 polynucleotide kinase buffer [70 m*M* Tris–HCl (pH 7.6), 10 m*M* $MgCl_2$, and 5 m*M* DTT], 150 μCi of γ-^{32}P-ATP (6000 Ci/mmol; New England BioLabs, Beverly, MA), and 10 units of T4 polynucleotide kinase (New England BioLabs).

2. The reaction is incubated at 37°C for 3 hr.

3. Following the addition of 30 μl TE [10 m*M* Tris–HCl (pH 8.0) and 1 m*M* EDTA] reactions are terminated by phenol extraction and purification over a G-50 sepharose spin column. A yield of 50% is typically recovered and the probe is brought to a final concentration of 40 fmol/μl with TE. A successful labeling is judged to have a $>0.5 \times 10^5$ cpms/μl (Cherenkof counts).

C. Band Shift Gels

1. Kinetochore band shift products are resolved by PAGE using the Hoefer (San Francisco, CA) SE 600 series standard cooled dual gel. These gels are maintained at a more constant temperature due to the buffer surrounding the gel plates (no extra cooling is typically required), giving rise to more consistent results. However, band shift products can be adequately resolved using standard electrophoresis equipment if a cooled apparatus is not available.

2. For a single band shift gel ($16 \times 20 \times 0.1$ cm), a 30% solution of acrylamide/bis (29:1) is diluted to 4% in 1× gel running buffer (0.29 *M* glycine and 0.045 *M* Tris base) and is polymerized by adding 40 μl of ammonium persulfate (20%) and 20 μl of TEMED.

3. After polymerization, band shift gels are prerun for approximately 1 hr before loading reactions in 1× gel running buffer.

D. Kinetochore-Binding Reaction

1. The kinetochore-binding reaction is performed in 1× binding buffer [10 m*M* Hepes (pH 8.0), 6 m*M* $MgCl_2$, and 10% glycerol] with 40 fmol of CEN3 DNA probe in a final volume of 30 μl. Kinetochore binding to CEN3 DNA is extremely sensitive to salt concentrations; therefore, binding reactions are adjusted to a final concentration of 150 m*M* KCl after addition of an extract.

2. To reduce nonspecific binding of proteins to probe DNA, 2.5 μg of sheared salmon sperm testes DNA (ssDNA) is added as a nonspecific carrier DNA. Since the extent of shearing varies with each preparation, we empirically determine the optimal amount of ssDNA for each preparation. To demonstrate CEN-specific binding, excess cold wild-type or mutant CEN DNA (64× molar excess to radiolabeled probe) can be added at the start of the reaction (Espelin *et al.*, 1997). Under these conditions, kinetochore complex binding is easily detected with 40 μg of yeast extract. Reactions are assembled and then incubated at room temperature for 40 min. A small amount of bromophenol blue tracking dye, diluted in 1X binding buffer, is added prior to loading reactions on the prerun band shift gel. Gels are run at 300V, removed when the tracking dye reaches the bottom, and then dried on Whatman 3M paper before exposing to film or using a Phosphorimager (Molecular Dynamics). Screen to detect the radiolabeled DNA.

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