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Probing Kinetochore Structure and Function Using *Xenopus laevis* Frog Egg Extracts

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Summary

Kinetochore are multi-protein machines that initiate mitotic checkpoint signaling and control chromosome movement through interactions with microtubules. Our lab has utilized *Xenopus laevis* frog egg extracts to investigate the requirements for kinetochore assembly and disassembly in vertebrates. Functional kinetochores assemble in egg extracts that are capable of binding microtubules, aligning and segregating chromosomes and sending spindle checkpoint signals. This is the only in vitro system that assembles functional kinetochores, making it particularly well suited for these types of studies. Probing kinetochore assembly using the biochemically tractable egg extract system has elucidated the intricate assembly requirements for numerous vertebrate kinetochore proteins. The following techniques have been used to characterize kinetochore assembly requirements, and to identify factors required for initial kinetochore assembly, as well as those factors that promote maintenance of pre-assembled kinetochores and those that induce kinetochore disassembly.

Keywords

Kinetochore; Mitosis; Aurora B; Protein Phosphatase 1; *Xenopus* Extracts; Ndc80

1. Introduction

The addition of sperm nuclei to Cytostatic Factor (CSF) arrested frog egg extracts leads to the rapid construction of kinetochores onto the centromeres of unreplicated chromosomes. This can be visualized using standard immunofluorescent techniques, with kinetochores appearing as 18 distinct dots on each chromatin mass. Using polyclonal antibodies raised against a large panel of *Xenopus* kinetochore proteins we have dissected outer kinetochore assembly requirements using frog egg extracts. Antibodies are essential to deplete specific proteins from extracts and are also used to immunolocalize proteins to centromeres on mitotic nuclei. Thus, generating high quality antibodies to various kinetochore proteins has been critical to these studies. In addition, the ability to examine the localization of a large panel of kinetochore proteins from a single assembly reaction has provided a semi-high-throughput method for dissecting the kinetochores intricate assembly map.

Our lab has concentrated on assembly and function of the outer kinetochore. It is clear that the entire outer kinetochore rapidly assembles in these extracts (<12 minutes). It is likely that much of the inner kinetochore also assembles. The starting material for these reactions is demembranated sperm, onto which protamines and histones assemble in the first 2–3 minutes

after incubation in egg extract. The centromeres on sperm nuclei act as a template for kinetochore assembly. We have observed the assembly of inner centromere components and an increase in CENP-A earlier than outer kinetochore components in the periods after histone assembly. These data suggest that the *Xenopus* system may be a very useful system for studying the structure and function of the inner kinetochore and deposition of CENP-A nucleosomes.

In this chapter, we describe the generation of high quality, polyclonal antibodies to kinetochore proteins. Since most kinetochore proteins are insoluble when expressed recombinantly, this has been a great challenge. We have devised reproducible methods to generate high quality antibodies and antigen affinity columns under denaturing conditions, which has made antibody production routine. We next describe our standard kinetochore assembly reaction, as we perform it in frog egg extracts. We have improved upon conventional techniques used for preparing mitotic nuclei from extracts for immunofluorescence. These improvements allow us to prepare dozens of coverslips for immunofluorescence, from a single assembly reaction in a quick, easy, inexpensive and reproducible manner. We present methods used to probe maintenance requirements of pre-assembled kinetochores. Finally, we present methods used to examine kinetochore disassembly, as it occurs after inhibition of kinetochore maintenance factors, at exit from M-phase and on isolated nuclei. The production and special handling techniques associated with generating CSF extracts has been described in detail in several excellent reviews and methods chapters, and will therefore not be discussed herein (Murray, 1991; Desai et al., 1999; Maresca and Heald, 2006).

2. Materials

All chemicals used for making buffers were purchased from Sigma-Aldrich, unless otherwise stated.

2.1. Antibody Production

- 6xHis Protein Purification, Lysis Buffer: 20 mM Tris, 500 mM NaCl, 5 mM Imidazole, pH 7.9 (prepared as a 8x Stock, stored at room temperature)
- 6xHis Protein Purification, Wash Buffer: 20 mM Tris, 500 mM NaCl, 30 mM Imidazole, pH 7.9 (prepared as a 8x Stock, stored at room temperature)
- 6xHis Protein Purification, Elution Buffer: 20 mM Tris, 200 mM NaCl, 300 mM Imidazole, pH 7.9 (prepared as a 4x Stock, stored at room temperature)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma Aldrich: Cat #- I5502): Prepared in ddH₂O at as a 1 M stock, and stored at -20°C in 1 ml aliquots.
- Ni-NTA Agarose (Qiagen: Cat #- 30230): Stored at 4°C
- Econopac Columns (Bio-Rad: Cat.#- 732–1010)
- Dialysis Tubing 12,000–14,000 MWCO (Spectra/Por): Stored at 4°C
- Cyanogen Bromide Activated Sepharose 4B (GE Healthcare: Cat #- 17-0430-01): Stored at 4°C
- Guanidine Hydrochloride
- Bradford Reagent (Sigma-Aldrich, Cat # 6916): Stored at 4°C

2.2. Probing Kinetochore Assembly

- *Xenopus laevis* Cytostatic Factor (CSF) arrested frog egg extracts, made from the laid eggs of female frogs, are prepared as described in (Murray, 1991; Desai et al., 1999; Maresca and Heald, 2006)

- Sperm Nuclei isolated from male *Xenopus laevis* frogs, prepared as described in (Murray, 1991)
- CSF-XB: 10 mM HEPES, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 50 mM Sucrose, 5 mM EGTA, pH 7.8
- Nocodazole: Stock prepared in DMSO at 5 mg/ml, stored at −20°C in 5–10 µl aliquots (Sigma-Aldrich: Cat # M1404)
- BRB80: 80mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8 (prepared as a 5x solution and stored at 4°C)
- 80% Glycerol: diluted from 100% glycerol (glycerin) stock. Stored at room temperature protected from light
- 20% Triton X-100: Diluted from Triton-X 100, stored at room temperature, protected from light
- 37% Formaldehyde (by weight) (Fisher Scientific: Cat #- F75P-500ml)
- Extract Fixative: BRB80, 30% Glycerol, 0.1% Triton X-100, 3.7% Formaldehyde
- Extract Glycerol Cushion: BRB80, 40% Glycerol
- Calcium cycling solution: 10 mM HEPES, 100 mM KCl, 1 mM MgCl₂, 5 mM CaCl₂, 50 mM Sucrose, pH 7.8
- Poly-l-lysine, 0.1% w/v in H₂O
- PBSt: 10 mM phosphate buffer, 137 mM NaCl, 0.1% Tween-20, pH 7.5
- TBSt: 10 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.5
- Bovine Serum Albumin, Fraction V (lyophilized)
- Affi-Prep Protein-A Support (Bio-Rad: Cat # 156-0006)
- 12 well tissue culture dishes (COSTAR: Cat #- 3513)
- Antibodies- used for immunodepletion or immunofluorescent analysis of kinetochore assembly
- No. 1 Glass Coverslips, 18 mm, pre-cleaned (Fisher Scientific #12–545)

2.3. Effect of Protein Phosphatase 1 on Kinetochore Disassembly

- Okadaic Acid (Alexis Biochemicals: Cat #- 350-011-C100)
- Purified Protein Phosphatase 1 Enzyme- Purified as described in (Brautigan et al., 1985)
- Phosphatase Buffer- 50 mM MOPS–NaOH, , 0.1 mM EGTA 1 mg/ml BSA, 1 mM dithiothreitol (DTT), 20 mM 2-mercaptoethanol, 0.02% Brij-35, pH 7.0 (DTT and 2-mercaptoethanol are added immediately prior to use)

3. Methods

3.1 Antibody Production

1. *Xenopus* kinetochore proteins are identified by searching Expressed Sequence Tag (EST) databases using BLAST analysis (Altschul et al., 1990). The Gene index databases (<http://compbio.dfci.harvard.edu/tgi/>) of *Xenopus* EST tags are much more

complete than the non-redundant (NR) databases at PubMed (<http://www.ncbi.nlm.nih.gov/>).

2. Genes corresponding to proteins of interest are commonly cloned into a pET-28 vector (Novagen) using standard PCR and molecular biology techniques. PCR amplification is performed from either Stage 11.5–14 *Xenopus* cDNA libraries or directly from ESTs. When possible, cloning is done placing an NdeI site upstream of the gene coding region and a NotI site downstream of the stop codon (Note 1).
3. Protein expression in BL21 (pLysS) bacteria is induced at 20°C by the addition of between 0.1 mM to 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG).
4. Bacterial pellets are frozen in liquid nitrogen, and stored at –80°C overnight. These pellets are stable at –80°C for several weeks.
5. Pellets are thawed and resuspended into ~30 ml of 6xHis protein purification lysis buffer using a dounce homogenizer and a loose fitting pestle.
6. The bacterial suspension is lysed 2–3 times on a French Press and 1,000–1,500 psi.
7. The lysed solution is centrifuged at 17,500 rpm and 4°C in a Sorvall SS-34 rotor for 60–90 minutes.
8. The soluble material (supernatant) is transferred to a 50ml conical tube and stored on ice.
9. The insoluble pellet is resuspended in Lysis Buffer containing 6M Guanidine Hydrochloride at room temperature. The insoluble material is clarified by centrifugation at 17,500 rpm and 23°C in a Sorvall SS-34 rotor for 60–90 minutes.
10. Ni-NTA agarose (1–2 ml of beads per binding reaction) is washed into the appropriate Lysis Buffer (insoluble containing 6 M Guanidine hydrochloride) by resuspension in Lysis buffer and centrifugation at 1,000 rpm, for 2–3 minutes in a Jouan CR-412 centrifuge.
11. The 6xhis tagged proteins are absorbed onto Ni-NTA Agarose from both soluble and insoluble material (soluble purifications are performed at 4°C and insoluble at room temperature). Binding is done in batch in a capped, 50-ml conical tube for 2–4 hours.
12. The beads are washed in batch two times with wash buffer (with or without 6 M Guanidine hydrochloride for soluble and insoluble purifications, respectively). Washing is performed by resuspending beads in 10–20 bed volumes of appropriate buffer, isolating beads by centrifugation (see step 10) and aspirating buffer off of the beads.
13. Beads from the insoluble prep are exchanged into wash buffer containing 6 M Urea, instead of Guanidine Hydrochloride.
14. Beads are transferred to Econopac Columns (soluble and insoluble separately) and 6xHis tagged proteins are released from the beads with Elution buffer (with or without 6 M Urea for soluble and insoluble purifications, respectively). Six to eight 1 ml elutions are normally collected. Soluble protein is kept on ice, and insoluble at room temperature.
15. Protein concentration is determined by Bradford analysis.
16. Eluted protein is analyzed by SDS-PAGE to confirm that the purified product is free of contaminating proteins and is of the appropriate molecular weight.

¹The minimal tags used for expressing recombinant proteins minimizes the generation of antibodies to non-specific antibodies.

17. Small aliquots are frozen in liquid nitrogen at 250 µg/aliquot.
18. Purified proteins are injected into rabbits, 250 µg at a time, for antibody production (Covance). We obtain equally good antibodies from soluble and insoluble protein preps.
19. Whole rabbit serum is affinity purified over columns of recombinant protein covalently coupled to Cyanogen Bromide Activated Sepharose, as described in (Harlow and Lane, 1988). Insoluble preps can be linked to Sepharose as long as the concentration is greater than 0.5mg/ml. Before coupling to the beads, insoluble proteins need to be dialyzed into coupling buffer containing fresh 6M Urea.
20. Affinity purified antibodies are exchanged into PBS or XB buffer using several rounds of dialysis in standard dialysis tubing.
21. Dialyzed, affinity purified polyclonal antibodies are snap-frozen in liquid nitrogen in 100 µl aliquots.

3.2.1 General Kinetochore Assembly Reaction

The following procedure describes our standard kinetochore assembly reaction. In short, CSF arrested extracts are supplemented with sperm nuclei, onto which kinetochores assemble. After assembly, the reactions are fixed and nuclei are pelleted onto coverslips and processed using immunofluorescence. Proteins and enzymes are inhibited in these reactions using either immunodepletion, antibody addition or drug addition.

1. *Xenopus* CSF egg extracts are prepared as described (Note 2 and ³: Murray, 1991; Desai et al., 1999; Maresca and Heald, 2006).
2. To examine a protein's role in the kinetochore assembly, CSF arrested extracts are immunodepleted or supplemented with antibodies or drugs to specific proteins or complexes (Note 4).
 - a. For immunodepletion: polyclonal, affinity purified antibodies are bound to Affi-prep Protein A beads in batch, as follows.
 - i. Affi-prep Protein A beads are first washed with PBSt. Antibodies are bound to beads in a reaction containing 10 bed volumes of PBSt plus antibody (Note 5). Commonly we will use 25 µl of beads and 2–5 µg's of antibody per µl of beads.
 - ii. Binding is done on a rotator in a 1.5 ml micro-centrifuge tube at room temperature or 4°C and proceed for at least one hour. Reactions can be left overnight at 4°C.
 - b. For small molecule inhibitors or antibody addition: these solutions can be added directly to the extracts to inactivate enzymes, proteins or complexes.

²*Xenopus laevis* frog egg extracts, arrested in M-phase by CSF, are very sensitive to handling procedures. They should be handled with the utmost care at all times; this includes gently flicking for mixing, and pipetting through cut off, or wide bore, pipette tips. These handling procedures are discussed more extensively in (Murray, 1991; Desai et al., 1999; Maresca and Heald, 2006)

³We have found that CSF extracts frozen and stored and –80°C perform kinetochore assembly as well as fresh extract. CSF extracts are frozen in liquid nitrogen immediately after being prepared fresh and supplemented with an additional 250 mM Sucrose.

⁴High concentrations of antibodies are normally required to induce inactivation of specific proteins, when being added directly to extracts. We commonly concentrate an antibody to greater than 5 mg/ml, and add it at a dilution of 1:20 to the extract.

⁵For immunodepletion prior to kinetochore assembly, Affi-Prep beads are used because they are less expensive, easier to use and bind more antibody relative to comparable magnetic bead reagents.

- i. Drugs or antibodies are mixed into the extract, which is then stored on ice for ~20 minutes before initiating assembly (Note 6).
3. For immunodepletion, antibody-bead conjugates are transferred to a tube of minimal volume (normally a 200 µl PCR tube), and are washed 3 times with CSF-XB. Immediately prior to immunodepletion, all residual liquid is aspirated from the bead bed with a 27 gauge needle. The bead to extract ratio should not exceed 1:4 at most.
4. Depletions are routinely performed in 1–3 rounds of 20–60 minutes, while rotating at 4°C. The extract is transferred to fresh antibody coated beads after each round. It is critical that control extracts be mock depleted in parallel over similar volumes of beads bound to control IgG (Note 7 and ⁸).
5. Optional: Prior to initiating kinetochore assembly extracts are supplemented with nocodazole (final concentration 5 µg/ml) that has first been diluted in CSF extract (depleted extract should be used when appropriate for dilution) (Note 9).
6. Kinetochore assembly reactions, 50µl in volume, are warmed to room temperature (~22°C) for 1–2 minutes (This and the remaining steps are irrespective of the extract having been immunodepleted, supplemented with drug or antibodies, or treated with nocodazole).
7. Sperm nuclei are added to a final concentration of approximately 1,000 per microliter.
8. Reactions are incubated at room temperature for 45 minutes.
9. Assembly reactions are stopped by diluting 20-fold in approximately 1 ml of extract fixative (BRB80 + 30% Glycerol + 0.5% Triton-X + 3.7% Formaldehyde). Reactions are mixed by gently pipetting with 1 ml pipette tip and tubes are then inverted 10 times.
10. Reactions fix at room temperature for 15 minutes.
11. Fixed and diluted reactions are layered onto a glycerol cushion (BRB80 + 40% glycerol) overlaying a poly-l-lysine coated 18 mm No.1 coverslip (Note 10). Cushions (~3ml) and coverslips are set-up in 12 well culture dishes.
 - a. This allows us to prepare dozens of coverslips from the same reaction simultaneously. Reactions can be split over multiple coverslips. We have found that increasing the concentration of sperm in the extract has no noticeable affect on kinetochore assembly, but allows reactions to be split over increasing numbers of coverslips. Using tissue culture dishes for this purpose is an alternative to that performed by most groups in the past (Note 11).

⁶Care should be taken to dilute the extract with as minimal a volume as possible. Diluting extract in excess of 10% of the starting volume can be detrimental to maintenance of CSF arrest.

⁷Prior to performing a depletion and kinetochore assembly reaction, it important to empirically determine the depletion requirements for each antibody independently. Depletions are done as described above, and aliquots of extract are examined by immunoblot for the removal of specific proteins from the extract supernatant after each round of depletion. The minimal time needed to fully deplete the extract should be used (see Note 8).

⁸The depletion process can degrade the ability of extracts to perform kinetochore assembly or to maintain their mitotic arrest, making it essential that control extracts are treated in an identical manner to those being immunodepleted, diluted with antibodies or drugs, etc.

⁹Since microtubule attachments can strip proteins from kinetochores, we normally include nocodazole in assembly reactions, at a final concentration of 5–10µg/ml

¹⁰Poly-l-lysine coated coverslips are prepared by mixing 1 box of 100-18 mm, No. 1 cover glass in a plastic Petri dish with poly-l-lysine for 1 hour at room temperature on a orbital rotator. The glass coverslips are washed at least 5 times in ddH₂O and are stored in ethanol at room temperature.

12. Culture dishes containing the assembly reactions, layered over cushions and coverslips, are spun on swinging platforms in a Jouan CR-412 centrifuge at 3,000 rpm for 30 minutes at 18°C.
13. After centrifugation the tops of cushions are washed thoroughly with BRB80.
14. Coverslips are rinsed gently, once with BRB80, and are then post-fixed with ice-cold methanol for 5 minutes at 4°C (Note 12).
15. Coverslips are rehydrated with three-10 minute incubations in PBSt or TBSt.
16. Coverslips are blocked, in their culture dish wells, in TBSt + 5% BSA (w/v).
17. Coverslips are processed using standard immunofluorescent staining techniques.

3.2.2 Probing Kinetochore Maintenance

Using a staggered start approach, we have added function blocking antibodies and drugs that affect kinetochore maintenance. An example of a kinetochore maintenance experiment, examining the kinetics of disassembly after the addition of hesperadin, an Aurora B inhibitor which induces kinetochore disassembly, is described below. This experiment has been complemented by adding function blocking antibodies to the Aurora B complex, ensuring specificity of the drug. Any antibody, or drug, could be used in place of hesperadin to examine its affect on kinetochore maintenance.

1. Four-50 μ l reactions (labeled A, B, C, and D) of a fresh CSF extract (that has been supplemented with nocodazole) are aliquoted into 1.5 ml micro-centrifuge tubes and are stored on ice.
2. Kinetochore assembly is initiated with a staggered start approach, on 5 minutes time intervals. Reaction A is started at time zero minutes, B at time five minutes, C at time 10 minutes and D at time 15 minutes.
3. The small molecule Aurora B kinase inhibitor, hesperadin, is added to reaction A at 45 minutes after time zero (Note 13). Subsequently, hesperadin is added to reaction B at 50 minutes and to reaction C at 55 minutes.
 - a. Thus, hesperadin has been added to each reaction 45 minutes after initiating kinetochore assembly.
4. At 60 minutes, all four reactions (A, B, C and D) are stopped by dilution in 1 ml of extract fixative.
5. Reactions fix at room temperature for 15 minutes and are layered onto cushions, spun onto coverslips, and prepared for immunofluorescence, as described above.

3.2.3 Probing Kinetochore Disassembly at M-phase Exit

In higher eukaryotes, outer kinetochore proteins dissociate from centromeres at the end of mitosis. This kinetochore disassembly reaction can be examined at M-phase exit in frog egg

¹¹Standard procedures for spinning nuclei out of extract and onto glass coverslips have utilized glass tubes, with a chock inserted into the bottom of the tube with a cover glass lying atop the chock. Using a standard HB-6 rotor accommodate the preparation of 6 coverslips at a time. Using our methods, we can prepare upwards of 48 coverslips, simultaneously. We have used 24 well dishes, together with 12 mm coverslips, to prepare hundreds of coverslips at one time.

¹²When adding and removing buffers to and from coverslips in multi-well dishes, care should be taken not to dispense liquid directly onto the coverslips. Solutions are added slowly down one side wall of the well and are removed by aspiration from the opposing corner of the well.

¹³We have found that many kinase inhibitors need to be used at higher concentrations in egg extracts than they are normally used in cells. For example, to inhibit Aurora B, hesperadin is used at 2 μ M in extracts. IP-kinase assays of Aurora B suggest that hesperadin has an IC50 value of ~250 nM (Hauf et al., 2003).

extracts, through the addition of exogenous calcium which destroys CSF. The kinetics of kinetochore disassembly can be followed using the staggered start approach described above. An experiment examining disassembly at M-phase exit in egg extracts is described.

1. Four 50 μ l reactions (labeled A, B, C, and D) of a fresh CSF extract (that has been supplemented with nocodazole) are aliquoted into 1.5 ml micro-centrifuge tubes and are stored on ice.
2. Kinetochore assembly is initiated with a staggered start approach, on 5 minutes time intervals, as described above. Reactions are first removed from ice and warmed to room temperature for one minute, after which 1,000 sperm nuclei per microliter are added to initiate assembly.
 - a. Reaction A is started at time zero minutes, B at time five minutes, C at time 10 minutes and D at time 15 minutes.
3. Forty-five minutes after initiating the first assembly reaction, 0.1 volumes of calcium cycling solution is added to reaction A to drive it out of M-phase.
 - a. At 50 and 55 minutes, reactions B and C are supplemented with 0.1 volumes of calcium cycling solution, respectively.
4. Sixty minutes after initiating assembly in reaction A, all four reactions (A, B, C and D) are stopped by dilution in 1 ml of extract fixative.
5. Reactions fix at room temperature for 15 minutes and are layered onto cushions, spun onto coverslips, and prepared for immunofluorescence, as described above.

3.3 Effects of Protein Phosphatase 1 on Kinetochore Disassembly

Using the techniques described below, we have shown that Protein Phosphatase 1 (PP1) is sufficient to disassemble kinetochores on nuclei isolated from *Xenopus* extracts. These experiments utilize purified PP1 enzyme (Note 14). Nuclei with pre-assembled kinetochores are isolated from extracts, onto coverslips, which are subsequently blocked, treated with PP1 and processed for immunofluorescence.

1. A 50 μ l reaction of fresh CSF extract (that has been supplemented with nocodazole) is pipetted into 1.5 ml micro-centrifuge tube and stored on ice.
2. The reaction is thawed to room temperature for one minute, and kinetochore assembly is initiated by adding 1,000 sperm per microliter.
3. Forty-five minutes after initiating the kinetochore assembly reaction, the extract is diluted with 5 ml (100x dilution) of CSF-XB + 1mM Okadaic Acid. The diluted reaction is layered onto a cushion of BRB80 + 40% glycerol + 0.5 mM Okadaic Acid, overlaying poly-L-lysine coated coverslips, in a 12 well culture dish. The reaction is split over the 12 cushions/coverslips.
4. Nuclei are pelleted out of the extract and onto the coverslips in a Jouan CR-412 centrifuge at 3,000 rpm for 30 minutes at 18°C.
5. After centrifugation, the cushions are washed thoroughly with CSF-XB, and after removing the cushion, the coverslips are gently rinsed two times with CSF-XB + 0.1 mM Okadaic Acid.

¹⁴PP1 enzyme purification was done as first described in (Brautigan et al., 1985) and was a generous gift of David Brautigan at the University of Virginia.

6. Coverslips are blocked at room temperature for 45 minutes, in the 12 well dish, with 250 μ l per coverslips of CSF-XB + 1 mM Okadaic Acid + 2.5% BSA (w/v).
7. During the blocking step, solutions of PP1 or BSA in phosphatase dilution buffer are prepared and stored on ice.
8. The BSA or PP1 solutions are aliquoted in 50 μ l spots onto Para film, set-up in a humidified chamber containing wetted paper using a covered, plastic kitchen container.
9. After the 45 minute block, coverslips are overturned into a solution of PP1 or BSA, at various time intervals, between 0 and 20 minutes. Thus, those placed in enzyme (or BSA last) spend the additional time in blocking solution.
10. After incubation with enzyme (or BSA), coverslips are placed in a fresh 12-well dish containing CSF-XB.
11. Coverslips are rinsed two times, by aspirating and re-adding CSF-XB buffer.
12. Fixative of CSF-XB + 4% formaldehyde + 0.1% Triton X is added to the coverslips.
13. Coverslips fix at room temperature for 15 minutes.
14. Coverslips are washed 3 times in PBSt, and are then post-fixed in ice cold Methanol, for 5 minutes at 4°C.
15. Coverslips are rehydrated in PBSt (see above), and processed using standard immunofluorescent techniques.

Reference List

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J. Mol. Biol* 1990;215:403–410. [PubMed: 2231712]
- Brautigan DL, Shriner CL, Gruppiso PA. Phosphorylase phosphatase catalytic subunit. Evidence that the Mr = 33,000 enzyme fragment is derived from a native protein of Mr = 70,000. *J. Biol. Chem* 1985;260:4295–4302. [PubMed: 2984200]
- Desai A, Murray A, Mitchison TJ, Walczak CE. The use of *Xenopus* egg extracts to study mitotic spindle assembly and function in vitro. *Methods Cell Biol* 1999;61:385–412. [PubMed: 9891325]
- Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1988.
- Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol* 2003;161:281–294. [PubMed: 12707311]
- Maresca TJ, Heald R. Methods for studying spindle assembly and chromosome condensation in *Xenopus* egg extracts. *Methods Mol. Biol* 2006;322:459–474. [PubMed: 16739744]
- Murray AW. Cell cycle extracts. *Methods Cell Biol* 1991;36:581–605. [PubMed: 1839804]