Chapter 13

DNA Replication in Nucleus-Free *Xenopus* Egg Extracts

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Summary

Extracts derived from *Xenopus laevis* eggs represent a powerful cell-free system to study eukaryotic DNA replication. A variation of the system allows for DNA replication not only in a cell-free environment, but also in the absence of a nucleus. In this nucleus-free system, DNA templates are licensed with *H*igh-*S*peed *S*upernatant (HSS) and then replicated with a concentrated *N*ucleo*P*lasmic *E*xtract (NPE). This method has the advantage of allowing replication of small plasmids with desired modifications and manipulation of the nuclear environment. This chapter describes the protocols needed to prepare HSS and NPE and how these extracts are used to study DNA replication.

Key words: DNA replication, *Xenopus laevis*, Nucleoplasmic extract, In vitro, Cell-free system, Pre-replicative complex, Origin unwinding.

1. Introduction

Chromosomal replication occurs via a multistep biochemical pathway involving more than two dozen proteins and related complexes *(1)*. Cell-free extracts made from *Xenopus laevis* eggs are a valuable system to dissect those steps, reveal which proteins are responsible, and understand their molecular functions *(2–4)*. In conventional *Xenopus* egg extracts, sperm chromatin is added to a *L*ow-*S*peed *S*upernatant (LSS) of egg cytoplasm *(5, 6)*. ORC, Cdc6, Cdt1, and MCM2-7 sequentially load onto the chromatin to assemble pre-Replicative Complexes (pre-RC). Subsequently, a nuclear envelope forms and critical replication factors, such as Cdk2-Cyclin E, are imported. Cdc45 loads and origins unwind, followed by DNA polymerase recruitment and bidirectional DNA synthesis. All of the input DNA is replicated

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and no second round of replication occurs due to the presence of Geminin and the destruction of Cdt1 *(7–9)*.

Although nuclear assembly extracts have provided insight into the biochemical mechanism of eukaryotic DNA replication, they have several disadvantages. First, perturbing nuclear assembly indirectly inhibits DNA replication because critical factors cannot be imported. Second, efficient plasmid replication is not supported by nuclear assembly extracts. This precludes the use of plasmids with defined sequences or modifications as templates to study DNA replication. Lastly, the contents of the nuclear environment cannot be easily modified since they depend on the capacity of the nuclear import machinery.

In this chapter, we describe a procedure using *Xenopus* egg extracts that bypasses nuclear envelope formation, yet supports efficient replication of added DNA templates *(10)*. In this approach, DNA is incubated in a *H*igh-*S*peed *S*upernatant of egg cytoplasm that lacks nuclear membrane vesicles, leading to pre-RC assembly. To stimulate replication, a highly concentrated *N*ucleo*P*lasmic *E*xtract (NPE) is added. NPE supplies high concentrations of Cdk2-Cyclin E *(11)* and other critical replication factors, thus removing the need for a nuclear envelope. As with nuclear assembly extracts, NPE rapidly and completely replicates DNA using conserved machinery and pathways. Moreover, the NPE system improves on the drawbacks of the nucleus-dependent system. First, a factor's direct involvement in DNA replication can be addressed, regardless of whether nuclear assembly is affected *(12)*. Second, NPE supports 100% efficient replication of circular plasmids. Since plasmids can be easily modified to incorporate a specific sequence or lesion, this quality opens possibilities to study how such modifications influence and are dealt with by replication factors *(13–15)*. Moreover, plasmids can be exploited to study key DNA structural transitions throughout the replication pathway, such as origin unwinding *(16)*. Lastly, since there is no nuclear envelope barrier to overcome, the replication environment can easily be manipulated through simple exchange of the extracts or supplementation with either DNA templates or factors *(17, 18)*.

This chapter describes protocols related to the NPE system. We outline how to make HSS (**Subheading 3.1**), the first extract used in the reaction. HSS is derived from unfertilized eggs crushed through centrifugation. The second extract, NPE, is made by centrifuging a large-scale preparation of growing nuclei. Nuclei are produced with sperm chromatin. Therefore, we describe how to prepare sperm chromatin (**Subheading 3.2**), which is followed by a description of how to derive NPE from nuclei (**Subheading 3.3**). We then provide protocols covering how the NPE system can be exploited to study DNA replication including measuring DNA replication (**Subheading 3.4**), chromatin loading of factors

(**Subheading 3.5**), and removal of proteins from the system through immunodepletion (**Subheading 3.6**).

2. Materials

2.1. Deriving High-Speed Supernatant from Egg Cytosol

1. Aprotinin/leupeptin stock solution (both from Roche): dissolve in water to a final concentration of 10 mg/mL each and store at –80°C.
2. Buckets, 10 L.
3. Cycloheximide (Calbiochem), stock solution: dissolve powder in sterile water to 10 mg/mL and store at –20°C.
4. Cytochalasin B (Sigma), stock solution: dissolve in DMSO to 5 mg/mL. Store at –20°C.
5. Dithiothreitol (DTT), stock solution: dissolve powder in sterile water to 1 M final concentration and store at –20°C.
6. ELB (Egg Lysis Buffer) 10× salts: 25 mM $MgCl_2$, 500 mM KCl, 100 mM HEPES-KOH, pH 7.7, filter sterilize, and store at 4°C.
7. ELB: 1× ELB salts, 1 mM DTT, 50 μg/mL cycloheximide, 0.25 M sucrose.
8. Falcon 2059 15-mL tubes (BD Biosciences) or similar polypropylene tubes.
9. Gel-loading tips.
10. Human Chorionic Gonadotropin (Sigma).
11. l-Cysteine HCl.
12. MMR 1× (Marc's Modified Ringer's): 100 mM NaCl, 2 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES-KOH, pH 7.8. Make 10× stock and store at 4°C.
13. NaCl.
14. Needles, 18- and 27-gauge.
15. Pasteur pipettes, glass.
16. Pregnant Mare Serum Gonadotropin (Calbiochem), 5000 IU.
17. Syringes, 1, 3, and 10 mL.
18. Plastic transfer pipettes.
19. Ultracentrifuge, such as Optima Max-E centrifuge (Beckman) or equivalent that has a vertical axis of rotation, and swinging-bucket rotor, such as the TLS-55 (Beckman) or equivalent. It is important that the ultracentrifuge can be chilled and the configuration can reach and withstand speeds of up to 260,000 × *g*

20. Ultracentrifuge tubes, 2.5 mL thin walled and clear.
21. *Xenopus laevis* females (Nasco).

2.2. Preparing Demembranated Sperm Chromatin

1. Benzocaine (Sigma): prepare a 10% (w/v) stock solution in ethanol. Solution is stable at room temperature for 6 months.
2. Bovine serum albumin (BSA; Sigma).
3. Buffer X, 10× stock solution: 100 mM HEPES-KOH, pH 7.4, 800 mM KCl, 150 mM NaCl, 50 mM $MgCl_2$, 10 mM EDTA. Filter sterilize and store at 4°C.
4. Buffer X/0.2 M sucrose: Add 5 mL of 10×Buffer X to 3.42 g of sucrose. Fill up to 50 mL with water. Sucrose can be dissolved and solution kept at room temperature.
5. Buffer X/2.0 M, 2.3 M, 2.5 M sucrose: Add 1 mL of 10× Buffer X to 6.84 g, 7.87 g, and 8.56 g of sucrose, respectively, in different conical tubes and adjust volume in each to 10 mL with ddH2O. Dissolve sucrose on a rotating wheel at room temperature.
6. Buffer X/0.5 M sucrose/BSA: Add 1 mL of 10× Buffer X to 1.712 g of sucrose and 300 mg of BSA and adjust volume to 10 mL with ddH_2O. Store at 4°C.
7. Buffer X/0.2 M sucrose plus BSA: Add 300 mg of BSA to 10 mL of Buffer X/0.2 M sucrose solution. Store at 4°C.
8. 90° fixed-angle centrifuge, angle fixed at 90° with respect to the vertical axis of rotation. Alternatively, a microfuge that reaches 16,000 × *g* equipped with a swinging-bucket rotor can be used.
9. Falcon 2063 5-mL tubes (BD Biosciences) or similar polypropylene tubes.
10. Glass rods.
11. Hoechst (Sigma), stock solution: 8 µg/mL Hoechst, 7.4% formaldehyde, 200 mM sucrose, 10 mM HEPES-KOH, pH 7.6. Store at room temperature protected from light.
12. Microscope, epifluorescent such as an Eclipse E600POL (Nikon).
13. Petri dishes.
14. Razor blades.
15. Polypropylene tubes, 15-mL screw cap (Corning).
16. Triton X-100. Make a 20% stock solution with sterile water.
17. *Xenopus laevis* males (Nasco).

2.3. Making Nucleoplasmic Extract

1. 0.2 M ATP: dissolve in sterile water and adjust the pH to ~7 with 10 M NaOH using pH indicator strips. Store 50 µL and 1 mL aliquots at –20°C.

2. Creatine phosphokinase (CPK; Sigma), stock solution: dissolve in 50 mM NaCl, 50% glycerol, 10 mM HEPES-KOH, pH 7.5, to a 5 mg/mL concentration. Store 250 µL aliquots at –20°C. These aliquots are stable for 2–6 months.
3. Falcon 2059 15-mL tubes (BD Biosciences) or similar polypropylene tubes.
4. Nocodazole (Sigma), stock solution: dissolve in DMSO to 5 mg/mL. Store 50 µL aliquots at –20°C.
5. Phosphocreatine disodium salt (PC; Sigma), stock solution: dissolve in 10 mM sodium phosphate pH 7 to a 1 M concentration. Store 50 µL and 1 mL aliquots at –20°C.

2.4. DNA Replication in NPE

1. [α-P^{32}]-dATP (3,000 Ci/mmol). Take the necessary radiation safety training and precautions when working with this material.
2. Agarose.
3. ATP regeneration mix: Combine 10 µL of PC (*see* **Subheading 2.3**, **item5**), 5 µL of 0.2 M ATP (*see* **Subheading 2.3**, **item1**), and 0.5 µL of 5 mg/mL CPK (*see* **Subheading 2.3**, **item2**) immediately before use. Store on ice.
4. Diethylaminoethyl (DEAE) paper.
5. Gel dryer.
6. Nocodazole (Sigma), stock solution: dissolve in DMSO to 0.5 mg/mL. Store 50 µL aliquots at –20°C.
7. pBluescript II KS(–).
8. Paper towels.
9. Phosphorimager such as the Personal Molecular Imager (Bio-Rad).
10. Proteinase K (Roche): prepare a 20 mg/mL stock in water. Store 50 µL aliquots at –20°C.
11. Stop Solution (2.5×): 2% (w/v) SDS, 75 mM EDTA, 25 mM Tris-HCl, pH 8. Prepare fresh before use using standard lab stock solutions.
12. TBE-loading dye (6×): 50% glycerol, 30 mM EDTA, 0.25% bromophenol blue (w/v), 50 mM Tris-HCl, pH 8. Store at room temperature for up to 1 year.
13. Whatman paper.

2.5. Chromatin-Loading Assay

1. β-Mercaptoethanol.
2. 1× ELB salts/0.5 M sucrose: Add 1.712 g sucrose to 1 mL of 10× ELB salts (**Subheading 2.1**). Make up the volume to 10 mL. Store at 4°C for up to 1 month.

3. 1× ELB salts/0.25 M sucrose: Add 0.856 g sucrose to 1 mL of 10× ELB salts (**Subheading 2.1**). Make up the volume to 10 mL. Store at 4°C for up to 1 month.
4. 1× ELB/0.2% Triton X-100: Add 0.856 g sucrose to 1 mL of 10× ELB salts (**Subheading 2.1**). Add 100 µL of 20% Triton X-100 stock (**Subheading 2.2**). Make up volume to 10 mL. Store at 4°C for up to 1 month.
5. SDS PAGE gel, precast (Bio-Rad) or lab made.
6. SDS sample buffer: 10% glycerol, 100 mM DTT, 2% SDS (w/v), 0.1% bromophenol blue (w/v), 50 mM Tris-HCl, pH 6.8.
7. Spin-down microfuge polypropylene tubes, 400 µL. The tubes currently used are 44 mm in length and 5 mm in diameter. It is important that the tubes be of similar dimensions.

2.6. Assessing the Role of a Protein in DNA Replication by Immunodepletion

1. Antibody to the protein of interest.
2. Nitex filter membrane (Sefar).
3. Protein A Sepharose Fast Flow beads (GE Healthcare).
4. Siliconized 0.65-mL microtubes.

3. Methods

3.1. Deriving High-Speed Supernatant from Egg Cytosol

This procedure can be divided into three parts comprising (a) inducing ovulation of *Xenopus* females (**Subheading 3.1.1**), (b) collecting crude S-phase extract from frog eggs (**Subheading 3.1.2**), and (c) making HSS from the crude extract (**Subheading 3.1.3**).

3.1.1. Inducing Ovulation of Xenopus Females

Frogs are primed with 75 international units (IU) of Pregnant Mare Serum Gonadotropin (PMSG) 2–8 days before the secondary injection to induce ovulation. The secondary injection is with 625 IU of Human Chorionic Gonadotropin (HCG) and is performed 18–20 h before the eggs are required.

1. Inject 2 mL of sterile water into a 5,000 IU vial of PMSG to give 2,500 IU/mL stock. Mix gently by swirling and inversion. Stock is stable at 4°C for up to 2 weeks.
2. Using a 1-mL syringe, withdraw 200 µL of the PMSG stock and transfer to a 2-mL Eppendorf. Dilute tenfold to 250 IU/mL with sterile water.
3. Draw 1 mL of 250 IU/mL PMSG into each of two 1-mL syringes attached to a 27-gauge needle, taking care to avoid air bubbles.

4. Inject one frog with 300 µL of 250 IU/mL subcutaneously along the leg (*see* **Note 1**). Repeat with the same needle for two other frogs, leaving approximately 100 µL behind in the syringe.
5. Repeat the earlier step for the three other frogs with the second syringe.
6. Between 2 and 8 days after the PMSG injection, fill six 10 L buckets with 2.5 L of chlorine-free water and add 14.6 g NaCl to give 100 mM. Dissolve the salt by stirring.
7. Inject 4.8 mL of sterile water into a 10,000 IU vial of HCG to give a 2,083 IU/mL stock. Mix gently by swirling and inversion. The HCG stock is stable at 4°C for up to 2 weeks.
8. Withdraw up to 2.7 mL of the HCG stock into a single 3-mL syringe attached to a 27-gauge needle taking care to remove any air bubbles.
9. Inject each frog subcutaneously along the leg with 300 µL and place the frog in its own bucket.

*3.1.2. Collecting LSS Extract from Xenopus Eggs (See **Note 2**)*

1. The day after injecting frogs with HCG, make 1 L of 2.2% (w/v) l-cysteine HCl, pH 7.7 (adjusting the pH with 10 M NaOH), 2 L of 0.5× MMR, and 1 L of ELB.
2. 18–20 h after the HCG injection, remove frogs from buckets and decant as much of the water as possible to help collect the eggs.
3. Reject "bad" batches of eggs, i.e., white, variegated, and/or stringy (*see* **Note 3**).
4. Pick out debris and individual bad eggs from otherwise good batches using forceps and/or a plastic transfer pipette.
5. Pool all eggs together and pour off as much water as possible again.
6. Dejelly the pooled eggs with four volumes of l-cysteine, swirling frequently with the wide end of a glass Pasteur pipette. It is critical that eggs be completely dejellied as seen by a dramatic compaction of the eggs and dissolution of the translucent jelly coats. Eggs should be completely dejellied within 5 min; however, this may take up to 7–8 min. When eggs have been fully dejellied, immediately decant the l-cysteine.
7. Gently wash the eggs three times with 0.5× MMR or until the entire 2 L is consumed (*see* **Note 4**). Move rapidly through these washes. Eggs should be fully resuspended during each wash by briefly stirring with the wide end of a glass Pasteur pipette. After the last wash, decant as much MMR as possible.
8. Wash the eggs three times with ELB as earlier. Here it is important to remove bad eggs and this can be done with a Pasteur pipette. After swirling, the bad eggs will collect in the middle of the beaker making this task easier. It is important

to move quickly through these washes, but take the necessary time to remove the bad eggs.

9. Transfer eggs to 15-mL falcon 2059 tubes, allow them to settle, and remove excess buffer by aspiration. Pack the eggs by spinning at 200 × *g* in a swinging-bucket rotor for 1 min. Try to get the tubes as full as possible, even if that means packing twice.
10. Remove excess buffer by aspiration. Add 0.5 µL of aprotinin/leupeptin stock solution and 0.5 µL of Cytochalasin B per mL of packed eggs on top of the packed eggs. The centrifugation in the next step mixes these two supplements with the eggs.
11. Crush eggs via centrifugation at 20,000 × *g* in a swinging-bucket rotor for 20 min at 4°C. The rotor is kept at room temperature until use to ensure that eggs are warm when crushed. This is important to drive them into interphase. After the spin keep extracts on ice.
12. Remove the LSS (**Fig. 1 Subheading 3.1.2**). Put a parafilm seal on top of the falcon tubes containing the crushed eggs. Puncture the side of the tube with an 18-gauge needle

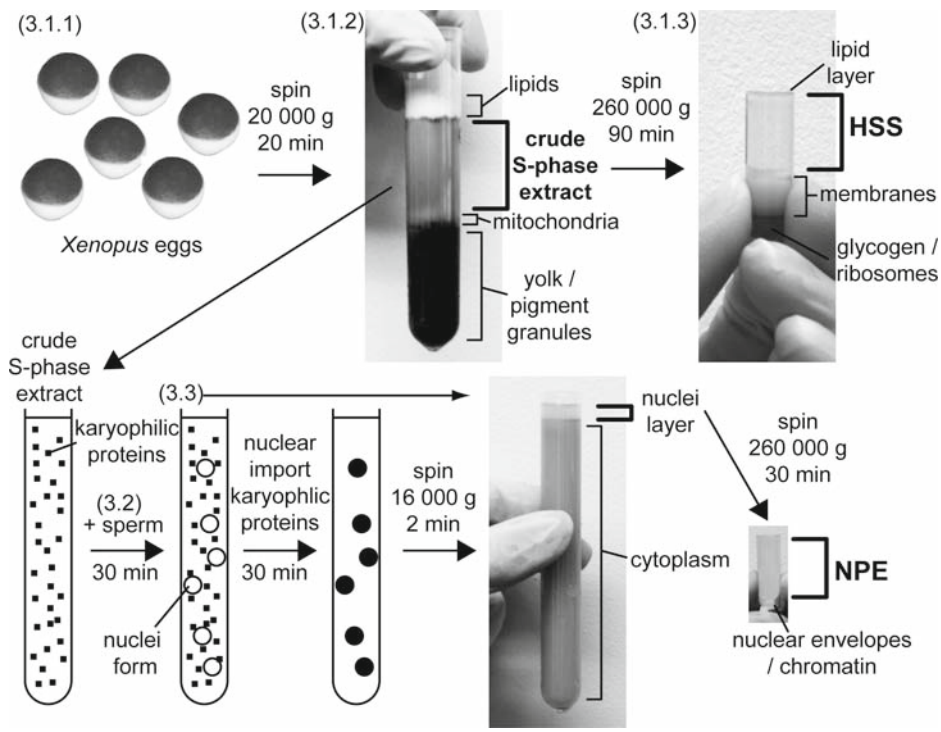

Fig. 1. Making DNA replication extracts from *Xenopus* eggs. Females are induced to lay eggs through hormone injection (**Subheading 3.1.1**). The eggs are crushed through centrifugation to make LSS (**Subheading 3.1.2**). This extract can be used to make *H*igh-*S*peed *S*upernatant (HSS) through ultracentrifugation (**Subheading 3.1.3**). Alternatively, if crude S-phase extract is mixed with sperm chromatin (**Subheading 3.2**), nuclei are grown on a large scale (**Subheading 3.3**). Spinning this extract causes the nuclei to float and *N*ucleo*P*lasmic *E*xtract (NPE) can be derived from these nuclei through ultracentrifugation.

about 3 mm above the layer of mitochondria. Remove the needle. Quickly insert a fresh 18-gauge needle attached to a 10-mL syringe into the hole. Remove the parafilm and, in approximately 30 s, suck out the extract as follows. Start with the needle angled about 45° up, pressed all the way to the opposite side of the tube. The opening of the needle should point upward. When the yellow yolk starts to enter the needle, reposition the needle downward and turn the opening away from the yolk to avoid it, trying to get as much extract as possible. Stop when the remaining cytoplasmic layer in the tube is no less than 3 mm deep or when the extract can no longer be harvested without significant lipid or mitochondrial contamination (*see* **Note 5**). Gently eject the extract into a 50-mL conical tube.

13. Repeat the earlier step for all 15-mL falcon tubes containing crushed eggs.

3.1.3. Making HSS from LSS

1. To the LSS, add cycloheximide to 50 μg/mL from a 10 mg/mL stock solution, DTT to 1 mM from a 1 M stock solution, aprotinin/leupeptin to 10 μg/mL each from a 10 mg/mL each stock solution, and cytochalasin B to 5 μg/mL from a 5 mg/mL stock solution.
2. Mix by gently inverting ten times.
3. Transfer LSS to 2.5-mL thin-walled ultracentrifuge tubes. The rotor is kept at 4°C until use. Spin samples at 260,000 × *g* (55,000 rpm for our setup) at 2°C in the swinging-bucket rotor for 90 min.
4. Collect HSS **(Fig.1**, **Subheading 3.1.3)** Remove the top layer of lipids by gently touching the surface with a bulb of a plastic transfer pipette and lifting it slowly. Then aspirate the rest of the lipids with a very thin gel-loading tip, trying not to remove too much of the extract.
5. Using a cut-off p200 tip, recover the cytoplasmic layer, taking care not to contaminate it with the underlying membrane fraction.
6. Transfer the extract to new 2.5-mL ultracentrifuge tubes.
7. Repeat the centrifugation outlined in **step 3**; however, adjust the spin time to 30 min.
8. To harvest the extract, repeat **step 4**, and then repeat **step 5**. At this point there should be very little lipids and/or membranes. Most of the tube contents should be clear and harvested as HSS.
9. Mix the recovered extract thoroughly by pipetting up and down or by inversion, and snap-freeze 33 μL aliquots in liquid nitrogen. Store at –80°C. HSS is stable at –80°C for several years.

3.2. Preparing Demembranated Sperm Chromatin

Demembranated sperm chromatin is a DNA template for replication assays (*see* **Subheading 3.4–3.6**) and is also used in the production of NPE (*see* **Subheading 3.3**). Starting with six frogs yields approximately 10^9 sperm. All steps are performed at room temperature unless stated otherwise. Begin this protocol by making the sucrose solutions described in **Subheading 2.2** as they take a long time to dissolve.

1. Dilute 5 mL of 10% (w/v) benzocaine in 1 L of water on the day of the procedure.
2. Place one male frog in the benzocaine. After ~ 10 min, the frog is euthanized, which can be determined by the absence of a righting reflex (frog does not turn over when placed on its back) and sucking reflex (frog does not suck on finger placed in its mouth).
3. Remove the frog from the benzocaine and ensure euthanization by pithing the frog at the junction between the spinal cord and the head.
4. Recover the testes by making a midbody incision across the stomach, moving the internal organs to one side of the body, and snipping the testes away from the connective tissue. *Xenopus* testes are white or cream colored, conical in shape, and approximately 5–8mm long and 3–4mm wide.
5. Thoroughly blot off blood from testes and place them in a Petri dish containing 3 mL of Buffer X/0.2 M Sucrose.
6. After collecting all the testes, remove the excess buffer. Mince testes into tiny pieces by extensive chopping with a razor blade (*see* **Note 6**).
7. Transfer the testes to a 15-mL screw cap conical tube with a p1000 tip. If testes pieces are too big to enter the tip, the testes are not minced enough.
8. Wash the Petri dish with 3 mL of Buffer X/0.2 M Sucrose and combine with the testes.
9. Vortex testes for 1 min, which releases additional sperm.
10. Pellet the larger pieces of tissue by centrifugation for 10 s at 200 × *g* in a swinging-bucket rotor.
11. Transfer the supernatant to a new 15-mL screw cap conical tube.
12. To the remaining pellet add 3 mL of Buffer X/0.2 M Sucrose.
13. Repeat **steps 9–10**. Add the supernatant to the supernatant from the previous extraction.
14. Repeat **steps 12** and **13**. At this point if more buffer is added to the pellet and the mixture vortexed, the mixture should not become very cloudy. This indicates that the majority of the sperm has been extracted.

15. To pellet the larger pieces of tissue from the collected sperm, centrifuge the combined supernatants for 50 s in a swinging-bucket rotor at 380 × *g*. Transfer the supernatant to a new 15-mL falcon tube.
16. Add 2 mL of Buffer X/0.2 M Sucrose to the tissue pellet and vortex for 1 min to release any remaining sperm. Centrifuge the tube for 50 s in a swinging-bucket rotor at 380 × *g* to pellet the larger pieces once again. Combine the supernatant with the supernatant from **step 15**.
17. Pellet the sperm in the combined supernatants via centrifugation at 2,600 × *g* in a swinging-bucket rotor at 4°C for 10 min.
18. During this time prepare sucrose gradients in four 2.5-mL thin-walled ultracentrifuge tubes (*see* **Subheading 2.1**). In each tube underlay 1.7 mL of Buffer X/2.3 M sucrose with 0.25 mL of Buffer X/2.5 M sucrose.
19. Upon termination of the centrifugation step, resuspend sperm in 0.8 mL of Buffer X/2.0 M sucrose.
20. Overlay the sucrose gradients with evenly distributed amounts of the sperm mix, i.e., 0.2–0.4 mL per tube.
21. Thoroughly blend the interface between the sperm mix containing the 2.0 M sucrose and the underlying cushion containing the 2.3 M sucrose using a glass rod.
22. Spin the sucrose cushions in an ultracentrifuge (*see* **Subheading 2.1**) cooled to 2°C at 93,000 × *g* in a swinging-bucket rotor for 25 min.
23. Any red blood cells should band on top of the 2.3 M sucrose cushion. The sperm descends to the bottom of the tube creating a whitish or light gray pellet (*see* **Note 7**).
24. Discard the top half of the sucrose gradient via aspiration.
25. Transfer the remaining bottom half with a p1000 tip to a 15-mL falcon tube. In each tube, resuspend the pelleted sperm with 500 µL of Buffer X/0.2 M sucrose by pipetting up and down extensively and transfer the mixture to the 15-mL falcon tube containing the supernatant. Do not touch the upper half of the tube with the tip to avoid contaminating the sperm preparation with red blood cells.
26. To the sperm mix, add Buffer X/0.2 M sucrose to reach a final volume of 12 mL. Mix by inversion.
27. Pellet the sperm by centrifugation at 3,000 × *g* for 10 min in a swinging-bucket rotor at 4°C.
28. To 2 mL of Buffer X/0.2 M sucrose, add 10 mg/mL aprotinin/leupeptin stock solution (*see* **Subheading 2.1**) and 1 M DTT (*see* **Subheading 2.1**) to final concentrations of 10 µg/mL and

1 mM, respectively. Mix well. Using this buffer, resuspend the pelleted sperm and transfer to a 5-mL falcon tube. The initial resuspension can be performed with 1 mL by pipetting up and down with a p1000 tip. The other 1 mL is then added. Add 40 µL of 20% Triton X-100. Gently mix by inversion. Slowly rotate end over end at 4°C for 30–60 min.

29. To Buffer X/0.5 M sucrose plus BSA, add aprotinin/leupeptin and DTT to final concentrations of 10 µg/mL and 1 mM, respectively. Put 500 µL of this buffer in four 1.5 mL Eppendorf tubes. Overlay the sucrose cushions with equal quantities of the sperm mix. Centrifuge for 10 min at 750 × *g* in a 90° fixed-angle centrifuge.
30. To Buffer X/0.2 M sucrose plus BSA, add aprotinin/leupeptin and DTT to final concentrations of 10 µg/mL and 1 mM, respectively.
31. Remove the supernatant and with 0.2 mL of the buffer made in **step 30**, resuspend the sperm pellet in each tube, taking care to avoid the walls of the tube that contain residual Triton. Transfer the sperm mix to four new 1.5-mL Eppendorfs and dilute to 0.7 mL with the same buffer. Centrifuge at 750 × *g* in a swinging-bucket rotor for 10 min.
32. Repeat **step 31** one time.
33. Resuspend the sperm with 1.5 mL of the buffer made in **step 30**.
34. Take 1 µL of the sperm mix, add 10 µL of Hoechst solution, and dilute with sterile water to a final volume of 100 µL. Count the number of sperm using a hemocytometer using the UV/DAPI channel on an epifluorescent microscope, keeping in mind that this is a 1/100 dilution.
35. Dilute the sperm to 220,000/µL (*see* **Note 8**) with the buffer made in **step 29** and snap-freeze in liquid nitrogen 90 µL aliquots in 1.5-mL Eppendorfs. These are used for preparing NPE (*see* **Subheading 3.3**). Some sperm is also diluted to 100,000/µL and snap-frozen in liquid nitrogen as 5 µL aliquots. This is used for replication reactions (*see* **Subheadings 3.4**–**3.6**). All sperm aliquots are stored at –80°C where they are stable for several years.

3.3. Making Nucleoplasmic Extract

Carry out procedures detailed in **Subheadings 3.1.1** and **3.1.2** to make LSS; however, start with 15 female frogs, instead of 6 (*see* **Note 9**). All steps and reagents need to be scaled accordingly, particularly the egg wash and lysis buffers used in **step 1** of **Subheading 3.1.2.** This is followed by a large-scale nuclear assembly reaction using the sperm made in **Subheading 3.2** and isolation of the soluble nucleoplasmic extract contained within the growing nuclei.

1. Perform **step 1** of **Subheading 3.1.3**; however, also include nocodazole to a final concentration of 3.3 μg/mL from a 5 mg/mL stock. If nocodazole is omitted, microtubules will bind to nuclei in the reaction (*19*) and the nuclei cannot be recovered.
2. Transfer extract to new 15-mL falcon tubes.
3. Centrifuge the crude S-phase extract at 20,000 × *g* in a swinging-bucket rotor at 4°C for 10 min.
4. Remove the lipid layer by gently touching the surface with a bulb of a plastic transfer pipette, twisting it, and lifting it. Then completely remove the rest of the lipids via aspiration with a very thin gel-loading tip, trying not to remove too much of the extract. Approximately 0.5–1 mL of extract per tube will be lost at this step.
5. Decant the remainder of the cytoplasm into a fresh tube, taking care not to transfer the brown particles at the bottom of the tube. This will result in the loss of another ~ 1 mL of extract.
6. Collect all decanted extracts into a 50-mL conical tube(s). For each milliliter of LSS, add 10 μL of 0.2 M ATP, 20 μL of 1 M phosphocreatine, and 1 μL of 5 mg/mL creatine phosphokinase to give 2 mM, 20 mM, and 5 μg/mL final concentrations, respectively. Mix gently by inverting at least ten times.
7. Set up nuclear assembly reactions by adding demembranated sperm chromatin to a final concentration of 4,400/μL. This is done by first distributing 4.5 mL of the cold extract with a plastic pipette to 5-mL falcon tubes. The extract is allowed to warm to room temperature, which takes 3–5 min. Take 1 mL of the extract with a p1000 and thoroughly mix with a 90 μL aliquot of 220,000/μL sperm chromatin (*see* **Subheading 3.2**) by pipetting up and down 15 times. Return the extract/sperm mix to the 5-mL falcon tube, close the cap, and invert the whole reaction ten times.
8. Incubate the nuclear assembly reaction at room temperature or in a 22°C incubator. Invert gently five times every 10 min to mix.
9. Check nuclear assembly at 60 min. Mix 1 μL of the reaction with 1 μL of Hoechst solution (*see* **Subheading 2.2**) on a microscope slide and cover with a cover slip. Examine via fluorescence microscopy using the DAPI channel. Repeat every 15 min until nuclei are 25–30 μm in diameter, which usually takes 75–90 min.
10. When nuclei have reached the desired size, reactions are pooled in 15-mL falcon tubes, each tube containing three separate assembly reactions. For any leftover reactions, the entire

5-mL falcon tube can be placed in a new 15-mL falcon tube containing 2 mL of water. The water is included to prevent cracking or crushing of the inner 5-mL falcon tube during the following centrifugation step. From this point onward, the extracts are kept on ice.

11. To collect the nuclei, the reaction is centrifuged for 2 min at 20,000 × *g* in a swinging-bucket rotor. During this step, all the nuclei float to the top of the tube, creating a 4–6 mm thick white clear layer that is very viscous (**Fig.1**, **Subheading 2.3**). This nuclear layer sits on top of the golden cytoplasm (*see* **Note 10**).
12. Harvest the nuclei. Hold the tube to a light to better distinguish the nuclear from the cytoplasmic layer. Remove the layer of nuclei by slowly pipetting with a cut-off p200 tip while simultaneously rotating the tube containing the extracts (*see* **Note 11**). Transfer all the nuclei to a single 1.5-mL Eppendorf tube or a second tube if necessary.
13. In an ultracentrifuge (*see* **Subheading 2.1**), centrifuge the nuclei in a swinging-bucket rotor at 260,000 × *g* and 2°C for 30 min.
14. Any lipids collected at the top surface are aspirated with an ultrathin gel-loading tip, taking care not to suck up too much of the extract underneath.
15. The clear soluble nucleoplasmic extract is harvested. The insoluble pellet consisting of nuclear envelopes and chromatin should be avoided.
16. After mixing the collected nucleoplasm, mix thoroughly, and then snap-freeze the NPE in 20 µL aliquots with liquid nitrogen. The aliquots are stored at –80°C and are stable at this temperature for several years.

3.4. DNA Replication in NPE

After extract preparation, quality control systematically follows. This is done by determining the efficiency with which newly prepared extracts replicate DNA (*see* **Note 12**). Moreover, replication reactions are carried out to test the effect of a depletion of a particular protein on DNA synthesis as outlined in **Subheading 3.6**. The following protocol describes how DNA templates are replicated in the NPE system. Generally, DNA templates are incubated in HSS followed by addition of NPE. We usually use pBluescript II KS(–) (pBS) as the DNA template, but any circular plasmid as well as sperm chromatin can also be used. Radioactive [α-P^{32}]-dATP is added to the reactions, whose degree of incorporation is used to measure DNA replication during the analysis. The following protocol is for three time points. Scale up or down and adjust the time points as needed. All the incubations and/or reactions are performed at room temperature or 20–22°C.

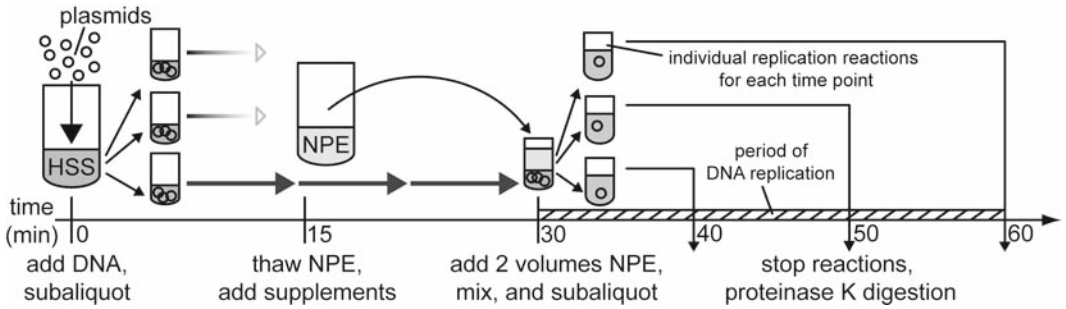

Fig. 2. A general guideline for DNA plasmid replication in NPE. The flow chart outlines a plasmid replication experiment using NPE for three time points. The DNA input, number of time points, and number of conditions can vary significantly. Normally all HSS/DNA subaliquots are used in the replication experiment, which is indicated by the fading arrows. See text for details.

An overview of the DNA plasmid replication in NPE procedure is provided in **Fig. 2**.

1. Make the ATP regeneration mix (*see* **Subheading 2.4**).
2. Thaw a 33 μL HSS aliquot and add 1 μL of the ATP regeneration mix and 0.2 μL of 0.5 mg/mL nocodazole (*see* **Note 13**).
3. Transfer 4 μL of HSS to a fresh tube and add pBS to a final concentration of 10 ng/μL (*see* **Note 14**). Mix by pipetting 8–10 times. This is considered the start of the reaction (T = 0 min). Distribute a 2.5 μL volume of the HSS/DNA mix to a fresh tube. This mix is incubated for 30 min prior to addition of NPE.
4. At T = 15 min, thaw NPE (*see* **Note 15**). Add 0.03 volumes of ATP regeneration mix and supplement with 50 mM DTT (*see* **Subheading 2.1**) to a final concentration of 2 mM. Also, add 0.3 μL of [α-P^{32}]-dATP (*see* **Note 16**).
5. At T = 30 min, transfer 5 μL (or two volumes) of NPE to the 2.5 μL HSS/DNA mix. Mix by pipetting 8–10 times and immediately prepare three 2 μL aliquots of the final reaction mix, one for each time point. Do not discard the leftover reaction mix.
6. At T = 40 min, 50 min, and 60 min, stop an aliquot of the reaction with 2 μL of stop solution and add 1 μL of proteinase K. Vortex for 3 s.
7. To 1 μL of the remaining reaction mix, add 1 μL of stop solution and 0.5 μL of proteinase K. This standard will be used to calculate the replication efficiency in **steps 13–17**.
8. Incubate all the samples at 37°C for at least 1 h.
9. Add 1 μL of TBE-loading dye and vortex vigorously for 3 s. Spin briefly to collect the sample.

10. Load each sample, but not the standard, on a 0.8% agarose gel and run at 4.5 V/cm, cm referring to the distance between the electrodes.
11. When the dye front has moved approximately 6 cm, cut the gel about 1 cm above the dye front. Discard the bottom half of the gel in the appropriate radioactive waste container.
12. Place the gel in between two pieces of DEAE paper. Then put one piece of Whatman paper and approximately 20 paper towels on either side of the DEAE/gel sandwich. Dry the gel by applying a weight of about 1 kg for approximately 1 h. Dispose of the paper towels and Whatman paper, taking care to check whether they are radioactive. Complete drying by placing the gel with the DEAE paper in a gel dryer, surrounded by fresh Whatman paper. The dryer should be heated to 80–90°C to increase the drying speed.
13. Spot 1 μL of the leftover "reaction" from **step 7** onto the filter paper beside the gel. Allow the spot to air dry.
14. Scan the gel using a phosphorimager and determine the counts in the spot and the replicated samples.
15. The endogenous dATP concentration in extracts is approximately 50 μM (*see* **Note 17**) Therefore the total amount of DNA that can theoretically be synthesized per microliter of extract is 50 μM × 1 μL × 330 g/mol × 4 nucleotides, or about 66 ng. If this much DNA is synthesized, all the radioactive dATP should be incorporated in the DNA.
16. To calculate the amount of DNA synthesized, divide the counts in the replicated samples by the total counts in a microliter of extract determined from the spot and multiply by 66 ng.
17. The fraction of the DNA replicated is calculated by dividing the amount of DNA synthesized by the amount of the input DNA.

3.5. Chromatin-Loading Assay

The interaction of a factor with DNA or with other factors already bound to DNA is the basis for many of the molecular pathways involved in DNA replication. To query the interaction of a factor with DNA, Western blotting is performed on proteins bound to sperm chromatin (*see* **Note 18**) recovered from the extract. This protocol describes how to isolate such chromatin using a sucrose cushion (*see* **Note 19**).

1. Prepare an appropriate number of sucrose cushions by placing 180 μL of ELB salts/0.5 M sucrose in a Spin-down tube and store on ice.
2. Repeat **steps 1** and **2** in **Subheading 3.4** (*see* **Note 20**).
3. Thaw out an aliquot of 100,000 per microliter of sperm prepared according to **Subheading 3.2**, and add it to the

HSS at a final concentration of 10,000 sperm per microliter. Subdivide the reaction into 5 μL aliquots, keeping in mind not to exceed six conditions per time point so that samples can be processed quickly. Incubate the aliquots at 20–22°C for the desired time (usually 20–30 min to monitor MCM2-7 loading). To isolate sperm from HSS, go to **step 5**. If it is desired to isolate chromatin after NPE addition, go to **step 4**.

4. Prepare NPE according to **step 4** in **Subheading 3.4**, but omit [α-P^{32}]-dATP. Add 10 μL of NPE per HSS/DNA aliquot.
5. At the desired time, add 60 μL of ice cold ELB/0.2% Triton X-100 to each reaction, either 5 μL of HSS/DNA (from **step 3**) or 15 μL of NPE/HSS/DNA (from **step 4**). Mix the reaction with a p200 tip by pipetting three times.
6. Carefully layer the mixture over the cold 180 μL ELB/0.5 M sucrose cushion.
7. Spin at 16,000 × *g* in a 90° fixed-angle centrifuge (*see* **Subheading 2.2**) cooled to 4°C for 25 s.
8. Aspirate the supernatant with a narrow pipette tip, leaving approximately 3 μL at the bottom of the tube.
9. Add 200 μL of ice-cold ELB salts/0.25 M sucrose on top of the chromatin pellet. Do not mix.
10. Repeat **steps 7** and **8**.
11. Add 12 μL of SDS sample buffer with 10% β-mercaptoethanol, which has been added on the day of experiment, vortex for 5 s, boil for 2 min., vortex for 5 s, and load the sample on an SDS PAGE gel.
12. Carry out Western blotting using antibodies specific to the proteins of interest.

3.6. Assessing the Role of a Protein in DNA Replication by Immunodepletion

A powerful tool to test the function of a factor in DNA replication is to deplete it from *Xenopus* egg extract and measure the effect on DNA replication. The goal of the depletion protocol is to remove as much of the endogenous protein as possible. Some replication proteins are present in a large functional excess, necessitating >99% depletion to see a defect in DNA replication. Notably, the depletion protocol is harsh and often nonspecifically reduces the activity of an extract. Therefore, a balance must be achieved where most of the protein is removed from the extract, yet the extract remains active. This is empirically determined with each antibody (*see* **Note 21**). The following protocol is given as a guideline. The experiments are described for crude rabbit serum. Sometimes, an affinity-purified antibody is available, in which case several micrograms of antibody are generally used for each immunodepletion. All steps are carried out on ice where possible.

1. Wash an appropriate amount of Protein A Sepharose Fast Flow beads (PAS beads) in 1× PBS in 0.65 mL siliconized microtubes (*see* **Note 22**). For each round of depletion, one volume of PAS beads is needed relative to five volumes of extract (*see* **Note 23**). Washing is achieved by adding ten volumes of 1× PBS, mixing by flicking, spinning at 2,800 × *g* for 40 s in a 90° fixed-angle centrifuge (*see* **Subheading 2.2**) at 4°C to pellet the beads, aspirating the supernatant, and repeating the entire procedure two more times.
2. Preclear the serum containing the antibody by spinning at 16,000 × *g* for 5 min in a microcentrifuge cooled to 4°C.
3. To the beads, add 0.5–4 volumes of serum relative to one volume of beads (*see* **Note 24**) and mix well by flicking, making an effort not to spray the beads on the walls of the tube.
4. Incubate at 4°C on a slowly rotating wheel for 30 min to overnight.
5. Repeat the washing procedure five times as outlined in **step 1**. After the last bead collection, aspirate the majority of the supernatant. Then immerse a 27-gauge needle (*see* **Subheading 2.1**) directly into the PAS beads and aspirate the void volume. The beads become white and opaque. Proceed to **step 7** immediately to prevent any drying out of the beads.
6. During **step 5** thaw out the extract and spin at 12,000 × *g* at 4°C for 1 min. Transfer the extract to a fresh tube leaving 3 µL behind. This preclears the extract of sediment. Add nocodazole to a final concentration of 3.3 µg/mL from the 0.5 mg/mL stock (**Subheading 2.4**). Consequently, adding nocodazole to extracts at later points in the procedure, i.e., before replication, is not needed and is omitted. Also, since NPE is sensitive to oxidation, add DTT to a final concentration of 20 mM from the 1 M stock (**Subheading 2.1**).
7. Add five volumes of extract to one volume of PAS beads and place in a rotating wheel at 4°C (*see* **Note 25**).
8. After 2 h, spin the beads in a 90° fixed-angle centrifuge (*see* **Subheading 2.2**) at 4°C at 2,800 × *g* for 40 s. Go to **step 9** if there are additional rounds of depletion. Otherwise proceed to **step 12**.
9. Transfer the extract using a p200 tip to a second batch of beads, keeping in mind that contamination of the second batch with the first batch is not detrimental. Repeat **steps 7** and **8**.
10. Repeat **steps 9**, **7**, and then **8** for up to a total of three rounds of depletion, as needed.
11. Just before the last round of depletion is complete, prepare a nitex spin column. This is done by placing a square piece

of nitex filter that is approximately 2 × 2 cm in size over the opening of a 0.65-mL microfuge tube, followed by inserting the wide end of a cut-off p200 tip into the tube. This creates a flat filter inserted one-quarter to one-half of the way into the microfuge tube.

12. After the last spin in **step 8**, transfer the entire depletion with the beads to the filter. Spin the tube in a 90° fixed-angle centrifuge (*see* **Subheading 2.2**) at 4°C and 2,800 × *g* for 40 s. Recover the extract from the bottom of the tube.
13. The proportion of the protein depleted can be determined by Western blotting.

4. Notes

1. Injecting frogs can be difficult, since they can begin to struggle or scratch with their legs when they are picked up. We find it useful to pick frogs up with fingers pointing to the legs of the frog and the head tucked in the base of the palm. Squeezing does not make the frog still. Conversely, by being as gentle as possible and covering the eyes with the palm of the hand, frog struggling diminishes in intensity and frequency. If certain frogs are problematic, put them back in the water and wait 30 s for them to calm down before trying again.
2. It is important to move through extract protocols as quickly as possible. Where possible, the next step should be prepared while waiting for the current step to finish. This is particularly crucial for the NPE protocol (*see* **Subheading 3.3**), which should not take more than 6 h from dejellying eggs to harvesting NPE. Taking longer can lead to a decrease in NPE activity.
3. Usually frogs either lay entire batches of "good" or "bad" eggs. This means that before taking time to sort individual eggs from within a batch, whole batches can be quickly rejected from the outset. We have found that once a frog starts to lay "bad" eggs, it usually will never lay "good" eggs at a later date and, therefore, should be culled from the population.
4. After the jelly coats have been removed from the eggs, the eggs are fragile. Therefore, when moving through the MMR and ELB washes, it is important to be careful with the eggs. Stir vigorously, but also smoothly. Moreover when new buffer is added to the beaker full of eggs, pour gently to avoid shocking the eggs. This can be assured by slowly pouring the buffers onto the beaker wall and not onto the eggs directly.

5. Avoiding mitochondria is particularly important for NPE preparations, which also starts with crude S-phase extracts. Contamination of cytoplasm with significant quantities of mitochondria can lead to apoptosis due to cytochrome C release (*20*) and nuclear fragmentation during nuclei growth. If apoptosis occurs during a nuclear assembly reaction, no NPE can be harvested.
6. Although sperm is released relatively easily, we have found that mincing the testes into the smallest possible pieces increases the yield significantly. It is recommended to mince testes until a thick mixture with indistinguishable pieces is achieved. 25 min of intensive chopping is not unusual to achieve this level of homogenization.
7. Sometimes not all the sperm will be pelleted leaving behind a cloudy layer at the interface between the 2.3 M and 2.0 M sucrose cushions. Reagitate this interface and spin again.
8. Sperm sediments very easily. Therefore, to ensure that the correct number of sperm is aliquoted, take care to mix by inverting the tube ten times prior to its distribution.
9. The NPE protocol can be scaled down or up as needed. An NPE preparation from 15 female frogs usually yields 800–1200 µL of NPE at the end of the procedure. We do not, however, recommend surpassing 30 frogs as the increased time needed for the preparation will likely compromise extract quality.
10. Thin nuclear layers can be due to several reasons. First, using an epifluorescent microscope, check that the nuclei achieved a size of 25–30 µm in diameter. Second, make sure that the sperm used in the NPE preparation had the correct concentration (*see* **Subheading 3.2**). Third, sometimes ATP, phosphocreatine, and/or creatine phosphokinase go "bad" leading to poor nuclear growth.
11. We have found the following method to be optimal for recovery of nuclei. First withdraw as many nuclei as possible by slowly sucking and rotating the tube at the same time, starting at the outside of the tube and then shortening the distance of the pipette tip to the center of the tube. This is repeated until at some point it is not possible to recover pure (or almost pure) nuclei. The brown particulate matter at the interface between the cytoplasm and the nuclei should be avoided, as should the cytoplasm, because these will dilute the NPE and make it less potent. Please note, however, that contamination of the nuclei with either is not detrimental to NPE activity, so it is not critical to avoid it altogether. Once no more nuclei can be recovered by this procedure, transfer the entire mixture of nuclei and underlying brown viscous material to a new 1.5-mL Eppendorf tube and spin

for 2 min at 16,000 × *g*. Sometimes nuclei that are trapped in the brown matter will go to the surface, appearing as a clear layer that is readily harvested. Also, examine the tube after removal of the brown material. This can also sometimes contain pockets of nuclei, which may be recovered.

12. In our hands, almost any preparation of HSS is able to support DNA replication. On the other hand, NPE preparations can vary significantly in their ability to stimulate replication. Highly active NPE will completely replicate up to 10 ng/μL (final DNA concentration in the reaction mix) of supercoiled plasmid DNA that is less than 5 kb in length in under 15 min. This is the ideal to which any freshly prepared extract should be compared. Whether a less active NPE is acceptable for use depends on the application.
13. Every effort should be made to avoid introducing air bubbles into HSS and NPE at all steps in the protocol. Air bubbles reduce the effective pipetting volume and likely lead to more oxidation of proteins in the extracts.
14. The relative volumes of HSS and DNA can vary. However, the HSS to DNA volume ratio must not drop below 2:1, as this may reduce the efficiency of pre-RC assembly and DNA replication.
15. We have found that NPE incubated at room temperature for 15 min prior to use replicates DNA more quickly than freshly thawed NPE. The reasons for this are unclear.
16. For unexplained reasons, diluting NPE to 80% with ELB (*see* **Subheading 2.1**) often leads to more rapid and complete DNA replication compared to undiluted NPE. Moreover, some NPEs can be diluted with ELB up to 50% without losing activity, allowing one to perform significantly more experiments with a single NPE aliquot. Therefore, prior to using a new batch of NPE, we calculate the replication efficiency at 80%, 50%, and 30% strengths, taking into account the effect of ELB on nucleotide pools in the efficiency calculation described in **Subheading 3.4**.
17. The concentration of endogenous dATP in an extract varies between 35 and 65 μM. This variation will change the theoretical DNA synthesized by a microliter of extract and affect the calculated replication efficiency. The endogenous dATP concentration can be determined empirically for every extract prepared. To do this, add a nonradioactive dATP to a 50 μM final concentration. This will compete with the endogenous dATP and reduce the latter's incorporation. Carry out replication reactions as outlined in **Subheading 3.4** with and without the added nonradioactive dATP. After processing the samples for analysis and scanning with a phosphorimager, multiply

the counts in the nonradioactive dATP samples by the nonradioactive dATP final concentration, i.e., 50 μM. Divide this by the difference in the counts between the samples with and without nonradioactive dATP added. This can be done for each time point. The average, minus any outliers, represents the endogenous dATP concentration.

18. Although plasmids or linear DNA templates can be coupled to magnetic beads, and thereby recovered, these templates do not undergo DNA replication for reasons we do not understand. Nevertheless, they do support pre-RC assembly (*21*).
19. Negative controls should systemically be included in any chromatin Spin-down experiment. To avoid false-positives, it is important to include reactions that lack sperm chromatin to determine whether any of the protein isolated during the experiment has been pulled down nonspecifically. Moreover, in many cases, the origin recognition complex (ORC) can be depleted to document specific chromatin DNA binding of replication factors (*22*).
20. Extracts should be centrifuged at 12,000 × *g* at 4°C for 1 min just before use and any insoluble debris avoided to prevent nonspecific recovery of proteins during chromatin isolation.
21. Generally, two rounds of depletion are performed, 1–2 h each. For each depletion, 1 volume of extracts is mixed with 0.2 volumes of PAS that was previously incubated with 0.6 volumes of antibody serum. If there is no prior information regarding an antibody's use, start with this condition. To modify the depletion, the time can be increased to 8 h total for a depletion, the number of rounds can be varied between one and three, the volume of PAS increased from 0.2 to 0.5 volumes of extract, and the amount of antibody bound to the resin adjusted (*see* **Note 25**). NPE is not as resistant to the depletion protocol as HSS, so this should be taken into account when adjusting the depletion conditions for this extract.
22. Siliconized Eppendorf tubes are used throughout the depletion protocol to minimize nonspecific absorption of antibodies, beads, and/or proteins to the walls of the tube.
23. As a general guide, we deplete 50 μL of extract at a time. Approximately one-third of the extract will be lost during the protocol, and this needs to be taken into consideration when designing the experiment.
24. PAS beads have a capacity of approximately 50 mg/mL IgG and crude serum contains 10–15 mg/mL IgG. Therefore, binding five volumes serum to one volume PAS saturates the beads. In general, limit the IgG to four volumes serum to one volume of PAS to ensure that all the IgG stays on

the resin. Also, for unknown reasons, reducing the serum: bead ratio for particular antibodies, such as anti-ORC2, can improve the depletion efficiency. This must be determined empirically.

25. Tubes are incubated on a rotating wheel to increase the mixing between the PAS beads and the extract; however, care should be taken to ensure that the extract is not running down the walls of the tube, as this will greatly decrease the efficiency of the depletion. Generally, keeping the volume of extract below 50 µL per tube prevents this from happening. Also, sometimes PAS beads aggregate during the depletion. This depends on the antibody and its concentration on the PAS beads. If this becomes a problem, beads can be resuspended every 20–30 min by gently mixing with a p10 tip. Importantly, to increase mixing our lab systemically introduces a tiny air bubble into the depletion reaction. This air bubble acts as a mini stir rod during rotation.

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