9

Chromosomal DNA Replication in a Soluble Cell-Free System Derived From *Xenopus* Eggs

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Summary

Cytoplasmic egg extracts from the frog *Xenopus laevis* represent a powerful cell-free system to study eukaryotic chromosomal DNA replication. In the classical approach, sperm chromatin is added to unfractionated egg cytoplasm, leading to the assembly of transport-competent nuclei that undergo a single, complete round of DNA replication. The need for nuclei in this system has been circumvented. Sperm chromatin or plasmid DNA is first incubated with clarified egg cytoplasm to form chromatin-bound prereplication complexes. Subsequently, a highly concentrated nucleoplasmic extract is added that stimulates initiation from these prereplication complexes, and a single complete round of chromosomal DNA replication ensues. This review describes the preparation of the cytosolic and nucleoplasmic extracts, as well as their use in DNA replication, origin unwinding, and chromatin isolation assays.

Key Words: Cell-free system; DNA replication; geminin; MCM2-7; nucleoplasmic extract; ORC; origin unwinding; prereplication complex; *Xenopus laevis*.

1. Introduction

Cell-free extracts made from the eggs of the frog *Xenopus laevis* represent a powerful system to study the biochemical mechanisms underlying eukaryotic DNA replication. Addition of demembranated sperm chromatin to cytoplasmic extracts of unfertilized eggs leads to the formation of nuclei that undergo a single, complete round of semiconservative DNA replication per cell cycle ***(1–4)***. The use of these "nuclear assembly" extracts has led to many fundamental insights into the mechanism and regulation of eukaryotic DNA replication ***(5)***.

However, nuclear assembly extracts have several drawbacks. First, any manipulations that interfere with the delicate process of nuclear assembly indirectly affect DNA replication because of defects in nuclear transport, a frequent source of artifacts. Second, although nuclear assembly extracts support DNA replication of purified DNA molecules such as plasmids, the efficiency is very low ***(1,4)***. Third, the requirement

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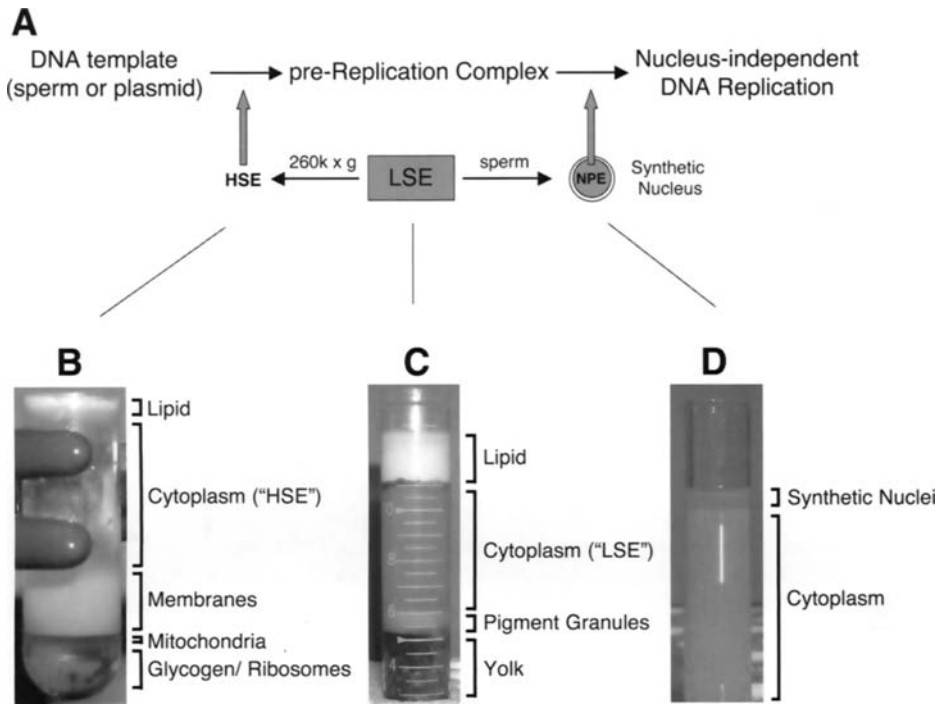

Fig. 1. DNA replication in nucleus-free extracts. (**A**) Schematic representation of the nucleus-free DNA replication assay. Sperm chromatin or plasmid DNA is incubated in HSE to promote the assembly of prereplication complexes (pre-RCs). Addition of NPE after pre-RC assembly converts the pre-RCs to active replication forks, and replication commences. NPE also contains inhibitors that prevent *de novo* pre-RC assembly as well as origin refiring and thus restricts replication to a single round. (**B–D**) Photos of various centrifugation fractions during the production of LSE, HSE, and NPE, respectively.

for a nuclear envelope to initiate DNA replication precludes biochemical fractionation of the factors required for these steps.

In this review, we describe a procedure for DNA replication in *Xenopus* egg extracts that circumvents the need for nuclear assembly ***(6)***. In this approach, DNA replication is carried out using two extracts, both of which are derived from low-speed extract (LSE) of egg cytoplasm (**Fig. 1A**). To make the first extract, LSE is centrifuged at high *g*-force to generate clarified egg cytosol or high-speed extract (HSE). To make the second extract, LSE is supplemented with sperm chromatin to assemble nuclei on a large scale. The nuclei are harvested and centrifuged at high *g*-force to separate the soluble nucleoplasmic extract (NPE) from the chromatin and nuclear envelopes.

To initiate DNA replication, DNA templates are first incubated with HSE to form the prereplication complex (pre-RC). This reaction does not require a specific DNA sequence and involves the sequential loading of the initiation factors ORC, Cdc6, Cdt1,

and MCM2-7. To initiate DNA replication from pre-RCs, two vol of NPE are added. NPE supplies high concentrations of Cdk2/cyclin E *(7)*, Cdc7/Dbf4 *(8)*, MCM10 *(9)*, and possibly other factors. DNA replication initiates, and a single complete round of DNA replication takes place during which MCM2-7 is lost from chromatin *(6)*. Additional rounds of DNA replication in the nucleus-free system are inhibited because NPE blocks the *de novo* loading of MCM2-7 *(6)* to high concentrations of the inhibitor geminin (**ref.** ***10*** and our unpublished results).

The nucleus-free system has several advantages over nuclear assembly extracts. First, the system can be used to resolve whether a particular protein is required for DNA replication directly rather than indirectly because of involvements in nuclear envelope assembly *(11)*. Second, in this system, the nuclear environment can be extensively manipulated. For example, different DNA templates can be replicated simultaneously in the same biochemical environment, a feature that was used to demonstrate the existence of diffusible checkpoint signals *(12)*. Third, the nucleus-free system supports efficient DNA replication of small plasmids, allowing structural analysis of key replication events such as origin unwinding *(13)*. In addition, the effects of DNA sequence on DNA replication can be examined. Finally, the demonstration that DNA replication can take place in a completely soluble environment holds the promise that this process can eventually be reconstituted from purified components. Although the current preparation method for NPE is too cost and labor intensive to allow fractionation, NPE may ultimately be replaced with purified components.

In this review, we describe the protocols that are necessary to carry out DNA replication in the nucleus-free system. We first describe how to isolate *Xenopus* sperm chromatin (**Subheading 3.1.**). We then describe how to induce egg laying in female frogs (**Subheading 3.2.**) and the preparation of LSE (**Subheading 3.3.**), HSE (**Subheading 3.4.**), and NPE extracts (**Subheading 3.5.**). Finally, we present protocols for measuring DNA replication (**Subheading 3.6.**), origin unwinding (**Subheading 3.7.**), and chromatin loading in the nucleus-free system (**Subheading 3.8.**); we describe how to use immunodepletion to remove specific proteins from the system to assess their roles in DNA replication (**Subheading 3.9.**).

2. Materials

1. [α^{32}P]dATP (3000 Ci/mmol).
2. Aphidicolin (cat. no. A0781; Sigma): dissolve in dimethylsulfoxide (DMSO) to 5 mg/mL. Store 5-µL one-use aliquots at −70°C.
3. Adenosine triphosphate (ATP; cat. no. A-7699 disodium salt; Sigma): dissolve in water and adjust to pH 7.0 with NaOH. Prepare as a 0.2 *M* stock and store 50-µL and 1-mL aliquots at −20°C.
4. ATP regeneration mix: combine 10 µL of 1 *M* phosphocreatine (PC), 5 µL of 0.2 *M* ATP, and 0.5 µL of 5 mg/mL creatine phosphokinase (CK). Prepare fresh and keep on ice before use.
5. Benzocaine (cat. no. E-1501; Sigma): prepare a 10% solution in ethanol at room temperature on the day of use.
6. Bovine serum albumin (BSA; cat. no A7906-100G; Sigma).
7. Buffer X: 10 m*M* HEPES, pH 7.4, 80 m*M* KCl, 15 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* ethylenediaminetetraacetic acid (EDTA). Prepare as a 10X stock and filter sterilize.

8. Chloroquine phosphate (cat. no. C-6628; Sigma): dissolve in water to 40 m*M* final concentration. Prepare fresh on the day of use.
9. CK (35,000 U; cat. no. C-3755; Sigma): dissolve in 50 m*M* NaCl, 50% glycerol, 10 m*M* HEPES, pH 7.5. Prepare as a 5 mg/mL stock and store as 1-mL aliquots at −20°C for up to several months.
10. Cycloheximide (cat. no. 239763; Calbiochem): dissolve in water to 10 mg/mL. Prepare fresh at 4°C on the day of use.
11. Cysteine HCL (cat. no. CY115; Spectrum): prepare a 2.2% solution in room temperature water immediately before use and adjust to pH 7.7 with KOH.
12. Cytochalasin B (cat. no. 6762; Sigma): dissolve in DMSO to 5 mg/mL. Store 50-µL aliquots at −20°C.
13. 10X ELB (egg lysis buffer) salts: 25 m*M* $MgCl_2$, 500 m*M* KCl, 100 m*M* HEPES. Adjust pH to 7.7 with KOH, filter sterilize, and store at 4°C.
14. ELB: 1 m*M* dithiothreitol (DTT), 50 µg/mL cycloheximide, 0.25 *M* sucrose, 1X ELB salts. Prepare at room temperature on the day of use.
15. Ethanol.
16. Glycogen (cat. no. 901393; Roche).
17. hCG (human chorionic gonadotropin): 10,000 U (cat. no. CG010; Sigma).
18. Hoechst fix solution: 8 µg/mL Hoechst, 7.4% formaldehyde, 200 m*M* sucrose, 10 m*M* HEPES, pH 7.6. Store at room temperature.
19. MMR (Marc's modified Ringer's): 100 m*M* NaCl, 2 m*M* KCl, 0.5 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 0.1 m*M* EDTA, 5 m*M* HEPES, pH 7.8. Prepare as a 10X stock and store at 4°C.
20. NaOAc: prepare a 3 *M* stock and adjust pH to 5.8. Autoclave and store at room temperature.
21. Hypodermic needles (21, 27 gage).
22. Nocodozole (cat. no. M1404; Sigma): dissolve in DMSO to 5-mg/mL and 0.5-mg/mL concentrations. Store 50-µL aliquots of each concentration at −20°C.
23. PC (cat. no. P-6502; Sigma; disodium salt, hydrate, synthetic): dissolve in 10 m*M* sodium phosphate, pH 7.0. Prepare as a 1 *M* stock and store 50- and 1-mL aliquots at −20°C.
24. Phenol:chloroform (1/1).
25. Pregnant mare serum gonadotropin (PMSG; 5000 U; cat. no. 367222; Calbiochem).
26. Protease inhibitor mix (Aprotinin; cat. no. 981532; Roche; Leupeptin; cat. no. 1034626; Roche): dissolve each part of the mix in water to 10 mg/mL (1000X stock). Freeze 25-µL aliquots in liquid N_2 and store at −70°C.
27. Proteinase K (cat. no. 3115879; Roche): prepare a 20-mg/mL stock in water. Store 50-µL aliquots at −80°C.
28. Replication stop solution: 8 m*M* EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 80 m*M* Tris-HCl, pH 8.0. Store at room temperature.
29. Ribonuclease A (cat. no. R-4875; Sigma): prepare a 2-mg/mL stock in water. Store 50-µL aliquots at −20°C.
30. SDS sample buffer (2X): 50 m*M* Tris-HCl, pH 6.8, 2% SDS, 100 m*M* DTT, 10% glycerol, 0.1% bromophenol blue. Store at room temperature for up to 1 wk.
31. Sybergold (cat. no. S-11494; Molecular Probes).
32. Syringes (1, 3, 5 mL).
33. 6X TBE loading dye: 50 m*M* Tris-HCl, pH 7.9, 50% glycerol, 30 m*M* EDTA, 0.25% bromophenol blue. Store at room temperature for up to 1 mo.
34. Triton X-100 (cat. no. BP151-100; Fisher).
35. Unwinding stop solution: 1% SDS, 20 m*M* EDTA. Store at room temperature for up to 1 wk.

36. *Xenopus laevis* females (cat. no. LM00531M; Nasco).
37. *Xenopus laevis* males (cat. no. LM00713M; Nasco).

3. Methods

The methods described next outline: (1) the preparation of demembranated sperm chromatin, (2) the induction of oocyte maturation and ovulation and the harvesting of eggs, (3) the preparation of interphase LSE, (4) the preparation of interphase HSE, (5) the preparation of NPE, (6) DNA replication in NPE, (7) DNA unwinding in NPE, (8) chromatin loading of replication factors in NPE, and (9) the immunodepletion of proteins from HSE or NPE.

3.1. Demembranated Sperm Chromatin

Demembranated sperm chromatin is the conventional template for DNA replication in *Xenopus* egg extracts and is a necessary reagent in the production of nucleoplasmic extract described in **Subheading 3.5.** The preparation of demembranated sperm chromatin is described in **Subheadings 3.1.1.** to **3.1.4.** This includes: (1) euthanization of *Xenopus* male frogs, (2) surgical extraction of the testes, (3) extraction and purification of sperm from the testes, and (4) determination of sperm concentration. This procedure is designed to yield approx 10^9 sperm and requires four to six male frogs. All steps in **Subheadings 3.1.1.** to **3.1.4.** are performed at room temperature unless otherwise noted.

3.1.1. Euthanization of Xenopus *Males*

Anesthetize one frog at a time by placing into 1 L 0.05% benzocaine (5 mL 10% benzocaine in 1 L water). After about 5 to 7 min, the frog should exhibit no movement and should not be able to right itself when turned over. The frog also should not exhibit a sucking reflex on insertion of a finger into its mouth. Remove the frog from the benzocaine solution and place on several paper towels. Euthanize by pithing.

3.1.2. Extraction of Testes

Open the peritoneal cavity by creating a midline incision through the abdominal wall. Move the organs of the intestinal tract to one side to reveal the testes. They are located in the midbody on either side of the midline. The testes appear grayish-white and almond shaped and are about 5 to 8 mm long. Remove them by cutting them with dissection scissors along the base. Blot excess blood away from the testes and place them in a Petri dish containing about 2 mL of 200 m*M* sucrose in buffer X. Repeat steps in **Subheadings 3.1.1.** and **3.1.2.** for the remaining frogs.

3.1.3. Sperm Extraction and Purification

1. Mince the testes from all frogs in the Petri dish with a fresh single-edge razor blade. Use a repeated chopping motion until the tissue forms a viscous sludge.
2. Transfer the minced testes to a 15-mL screw-cap conical tube using a wide-bore disposable transfer pipet. Rinse the Petri dish with 1 to 2 mL 0.2 *M* sucrose in buffer X and combine with the testes in the 15-mL tube. Vortex vigorously for 1 min and pellet the larger tissue fragments by mild centrifugation for 10 s at 1000 rpm (170*g*) in a clinical centrifuge.

3. Transfer the supernatant to a new 15-mL tube. Add 2 to 3 mL 0.2 *M* sucrose in buffer X to the pellet, vortex vigorously for 1 min, and recentrifuge for 10 s at 1000 rpm (170*g*). Combine the supernatants and repeat the pellet extraction three or four times or until the supernatant is not cloudy.
4. Centrifuge the combined supernatants in a clinical centrifuge for 50 s at 1.5 rpm (380*g*) to pellet the larger tissue fragments. Transfer the supernatant to a new 15-mL tube. Add 5 mL of 0.2 *M* sucrose in buffer X to the pellet, vortex vigorously for 1 min, and recentrifuge for 50 s at 1500 rpm (380*g*). Combine the supernatants.
5. Transfer the combined supernatants into Falcon 2059 tubes and pellet the sperm by centrifugation for 10 min at 4°C in a Sorvall HB4 or HB6 swinging bucket rotor at 4000 rpm (2600*g*).
6. Prepare sucrose step gradients in four 2.5-mL Beckman ultracentrifuge tubes (cat. no. 347356). To each tube, add 0.25 mL 2.5 *M* sucrose in buffer X. Carefully overlay with 1.7 mL 2.3 *M* sucrose in buffer X.
7. Resuspend the sperm pellets thoroughly by pipeting up and down with 0.8 mL 2 *M* sucrose in buffer X.
8. Overlay each gradient with 0.2 to 0.25 mL resuspended sperm. Stir the interface between the sperm and the 2.3 *M* sucrose *extensively* with a flame-sealed Pasteur pipet tip. The interface should no longer be visible.
9. Centrifuge the gradients for 25 min at 33,000 rpm (93,300*g*) at 2°C in a Beckman Optima MAX-E ultracentrifuge using a TLS55 swinging bucket rotor.
10. The sperm pellets to the bottom of the gradient and is visible as a light gray pellet (*see* **Note 1**). Red blood cells from the testes sediment on top of the 2.3 *M* sucrose layer. Aspirate off the top half of the gradient containing the red blood cells. Transfer the remainder of the gradient, including the sperm pellet, to a new Falcon 2059 tube. Completely resuspend the sperm pellet with gentle pipeting with 0.5 mL 0.2 *M* sucrose in buffer X. Avoid contamination with the upper walls of the tube, which may contain residual red blood cells. Transfer the resuspended sperm to the Falcon 2059 tube that contains the lower half of the gradient.
11. Dilute the sperm to 12 mL with 0.2 *M* sucrose in buffer X and mix well. Pellet the sperm by centrifugation for 10 min at 5000 rpm (3000*g*) in the HB4 or HB6 swinging bucket rotor at 4°C.
12. Aspirate the supernatant and resuspend the sperm pellet in 0.8 mL 0.2 *M* sucrose in buffer X supplemented with 0.4% Triton X-100, 1X protease inhibitor mix, and 1 m*M* DTT. Incubate on a rotating wheel for 30 min at 4°C.
13. Prepare four sucrose cushions by placing 0.5 mL 0.5 *M* sucrose in buffer X supplemented with 3% BSA, 1 m*M* DTT, and 1X protease inhibitor mix in 1.5-mL microcentrifuge tubes. Overlay each cushion with 25% of the sperm prep. Centrifuge for 10 min at 2100 rpm (750*g*) in a clinical centrifuge at room temperature.
14. Aspirate the supernatant and resuspend each sperm pellet with 0.2 mL 0.2 *M* sucrose in buffer X supplemented with 3% BSA, 1X protease inhibitor mix, and 1 m*M* DTT. Avoid contaminating the pellet with residual Triton X-100 from the walls of the tube. Transfer the sperm to four new microcentrifuge tubes, dilute to 0.7 mL total with the same buffer, and recentrifuge for 5 min at 2100 rpm (750*g*) in a clinical centrifuge at room temperature. Repeat once.
15. Resuspend and combine the sperm pellets in a total of 2 mL 0.2 *M* sucrose in buffer X supplemented with 3% BSA, 1X protease inhibitor mix, and 1 m*M* DTT.

3.1.4. Determination of Sperm Concentration and Storage

1. Dilute 3 µL of the sperm prep from **Subheading 3.1.3.** into 267 µL water and 30 µL Hoechst fix solution (*see* **Note 2**).
2. Pipet 12 µL into a standard hemocytometer and count the sperm on a fluorescence microscope with a 10× nonoil objective lens. Use the DAPI channel to visualize the sperm and simultaneous low-level bright field illumination to visualize the grid lines on the hemocytometer. Count the sperm in 0.1-µL volume (usually 16 squares) and multiply by 10^3 to calculate the number of sperm per microliter.
3. Dilute the sperm preparation to 200,000/µL with 0.2 *M* sucrose in buffer X supplemented with 3% BSA, 1X protease inhibitor mix, and 1 m*M* DTT. Freeze 90-µL aliquots in 1.5-mL microcentrifuge tubes in liquid N_2. These aliquots will be used in the preparation of NPE, described in **Subheading 3.5.** Save 100 to 200 µL, dilute further to 100,000/µL, and freeze as 5-µL aliquots (in 0.65-mL or similar tubes) in liquid N_2. These aliquots will be used in the assays described in **Subheadings 3.6** to **3.8.** Frozen sperm are stable for up to several years.

3.2. Oocyte Maturation, Ovulation, and Collection

Unfertilized eggs from *Xenopus* females are the source of interphase extracts used in the study of DNA replication in vitro. The injection of *Xenopus* females with hormone and the collection of unfertilized eggs is described in **Subheadings 3.2.1** to **3.2.3.** This includes: (1) the priming of *Xenopus* females by injection of PMSG to induce ovulation, (2) the induction of egg laying by injection of hCG, and (3) the collection and dejellying of the eggs for subsequent extract preparation.

3.2.1. Priming Xenopus *Females With PMSG to Induce Ovulation*

Females are primed with 75 U of PMSG at least 2 but not more than 8 d before the secondary injection with hCG, described in **Subheading 3.2.2.** The number of frogs to be injected (6–15) depends on the extract to be prepared (*see* **Subheading 3.3.**).

1. Prepare a 2500-U/mL stock of PMSG by slowly injecting 2 mL sterile water into a vial of 5000 U of PMSG using a 3-mL syringe and a 21-gage needle. Also, insert a 27-gage needle to relieve pressure. Mix by gentle inversion. This preparation can be stored at 4°C for up to 2 wk.
2. Transfer an appropriate amount of PMSG from the vial to a sterile Falcon 2059 tube using a 1-mL syringe. Dilute 10-fold with sterile water to a final concentration of 250 U/mL.
3. Fill the necessary number of 3-mL syringes with the diluted PMSG and inject each female subcutaneously along the leg with 0.3 mL using a 27-gage needle. You can use the same needle for about five frogs.

3.2.2. Induction of Egg Laying With hCG

1. Prepare a 2000-U/mL stock of hCG by slowly injecting 4.8 mL sterile water into a vial of 10000 U of hCG using a 5-mL syringe and a 21-gage needle. Also, insert a 27-gage needle to relieve pressure. Mix by gentle inversion. This preparation can be stored at 4°C for up to 2 wk.
2. Fill the necessary number of 3-mL syringes with the hCG and inject each female as above with 0.3 mL using a 27-gage needle. Injection is performed 18 to 22 h before the eggs are needed.

3. Each injected frog is placed into a separate bucket containing 100 m*M* NaCl (it is critical that the water not contain chlorine).

3.2.3. Harvesting and Dejellying the Eggs

Eggs are harvested 18 to 22 h after injection with HCG. Eggs from each frog are not combined until after they are dejellied and have been inspected under a dissection microscope.

1. Remove the frogs from the buckets and slowly decant all but about 100 mL of water from each bucket. Decant the remainder of the water and the eggs from each bucket into 250-mL beakers.
2. Decant as much of the water as possible from each beaker. Add 3 vol of 2.2% cysteine and mix every 30 s using a glass rod until the eggs are dejellied. Up to eight batches of eggs are dejellied at a time. Dejellying should be complete within 6 to 8 min. Dejellied eggs form a much more highly compact layer at the bottom of the beaker than eggs with jelly coats. The jelly coats can be seen as clear halos floating above the eggs before they dissolve completely in the cysteine.
3. Decant the cysteine and quickly wash the eggs three times with about 50 mL of 0.5X MMR at room temperature. Decant as much of the MMR as possible between washes.
4. Quickly wash the eggs twice with ELB, decanting as much of the ELB as possible between washes. Before decanting the last wash, remove any debris with a Pasteur pipet. Inspect each batch of eggs under a dissection microscope. Discard batches of eggs if the majority of eggs do not contain a visible germinal vesicle, if the majority of eggs have lysed, or if the pigment appears extensively mottled in appearance (*see* **Note 3**).
5. Combine the eggs and wash them once more with ELB. Eggs are now ready to be crushed for the production of LSE, described in **Subheading 3.3.**

3.3. Low-Speed Interphase Extract

Described next are the steps involved in preparing an LSE from eggs that have been harvested and washed as described in the **Subheading 3.2.3.** Preparation of LSE is an intermediate step in the preparation of HSE (**Subheading 3.4.**) and NPE (**Subheading 3.5.**). For HSE, we first prepare LSE from the eggs of 6 frogs; for NPE, we prepare LSE from the eggs of 15 frogs.

1. Transfer the washed eggs to Falcon 2059 tubes. Allow the eggs to settle and aspirate the supernatant. Pack the eggs by gentle centrifugation at 1100 rpm (200*g*) in a clinical centrifuge for 1 min at room temperature. Aspirate as much supernatant as possible.
2. Add 0.5 µL protease inhibitor mix and 0.5 µL 5 mg/mL cytochalasin per milliliter of packed eggs on top of the packed eggs. Do not put the eggs on ice.
3. Crush the eggs by centrifugation for 20 min at 11,000 rpm (20,000*g*) in a Sorvall HB4 or HB6 rotor at 4°C. Precool the centrifuge to 4°C but keep the rotor and eggs at room temperature until the spin is begun to ensure optimal activation of the eggs. For all subsequent steps, keep the extract on ice.
4. Transfer the tubes to ice. For each tube, puncture the side with a 21-gage needle just above the mitochondrial layer, remove the needle, and insert a fresh needle attached to a 5-mL syringe in the same hole (**Fig. 1C**). Withdraw as much of the cytoplasm or LSE as possible until just before the pigment granules and lipid begin to enter the syringe. Typically, for a Falcon 2059 tube with 13 mL of packed eggs, we recover 4 to 5 mL of LSE. Combine the LSE fractions from each tube in a 50-mL tube on ice (*see* **Note 4**).

5. For each milliliter of LSE, add 5 µL 10 mg/mL cycloheximide, 1 µL 1000X protease inhibitor mix, 1 µL 5 mg/mL cytochalasin, and 1 µL 1 *M* DTT. Mix thoroughly by gentle inversion. The yield of LSE is typically 1 to 2 mL per injected frog.
6. Immediately follow the protocol for HSE (*see* **Subheading 3.4.**) or NPE (*see* **Subheading 3.5.**).

3.4. High-Speed Interphase Extract

This subheading describes how to prepare HSE using freshly prepared LSE (described in **Subheading 3.3.**). All steps are carried out on ice unless otherwise noted.

1. Transfer the freshly prepared LSE (prepared from the eggs of six frogs) from **Subheading 3.3.** into four 2.5-mL thin-walled ultracentrifuge tubes (cat. no. 347356; Beckman) and centrifuge for 90 min at 55,000 rpm [260,000*g*]) in the TLS55 rotor at 2°C. As shown in **Fig. 1B**, the extract separates into a thin layer of lipids, clarified egg cytosol (the HSE), a membrane fraction, mitochondria, and glycogen.
2. Remove the lipid layer by aspiration with a very thin pipet (i.e., a gel-loading pipet tip).
3. Transfer the HSE from the top of the tube to a new ultracentrifuge tube using a cut-off P1000 pipet tip.
4. If the extract is contaminated with membranes or other debris, recentrifuge as in **step 1** for 30 min.
5. Remove the top lipid layer as in **step 2**, if necessary, and harvest the HSE as in **step 3**. Freeze the extract as one-use aliquots (50 µL) by submerging tubes in liquid N_2. Store the extract at −70°C, at which it can be stable for up to several years.

3.5. Nucleoplasmic Extract

Subheadings 3.5.1. to **3.5.4.** describe how to prepare an NPE extract using freshly prepared LSE described in **Subheading 3.3.** This includes (1) preparing the nuclear assembly reactions, (2) monitoring nuclear assembly and growth, (3) harvesting the nuclei, and (4) isolation of the nucleoplasm. All steps are carried out on ice unless otherwise noted.

3.5.1. Preparing the Nuclear Assembly Reactions

1. Note the final volume of the freshly prepared LSE (from the eggs of 15 frogs) from **Subheading 3.3.** and add 0.15 vol of ELB and 1/1500 vol of 5 mg/mL nocodozole. Mix gently yet thoroughly by inversion. Avoid introducing air bubbles.
2. Transfer the extract to new Falcon 2059 tubes and centrifuge for 20 min at 11,000 rpm (20,000*g*) in a Sorvall HB4 or HB6 rotor at 4°C.
3. Carefully and *completely* remove the residual lipids and brown material forming the top layer by aspiration with a P200 tip attached to a vacuum line. It is normal to lose about 0.5 mL of extract during this step.
4. Gently decant the extract into a 50-mL conical tube, taking care not to decant any of the dark granular material from the pellet. About 1 mL will have to be left behind per tube to avoid decanting any of the pellet material.
5. Per 1 mL of LSE, add 10 µL 0.2 *M* ATP, 20 µL 1 *M* PC, and 1 µL 5 mg/mL creatine kinase. Mix gently yet thoroughly by inversion.
6. Transfer the extract to 5-mL Falcon 2063 tubes and allow to warm to room temperature (22–23°C, about 5 min). Add at least 4 mL but not more than 4.7 mL to each tube.
7. To each tube, add 90 µL 200,000/µL demembranated sperm (**Subheading 3.1.**). To thoroughly resuspend the sperm in the extract, first add 1 mL of extract from the 2063 tube to

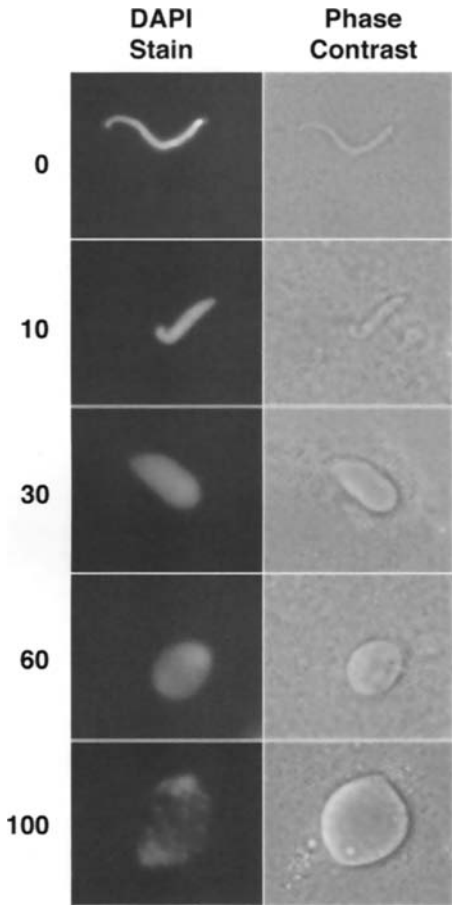

Fig. 2. Nuclear assembly in LSE. 1-µL aliquots of a nuclear assembly reaction are taken at the indicated time-points (in minutes), fixed with Hoechst dye, and visualized at 40× magnification on a fluorescence microscope. As the nucleus matures, the DNA staining pattern changes from diffuse to punctate. The mature nucleus shown at the 100-min time-point is 30 µm in diameter.

the tube with 90 µL of sperm. Pipet the mixture 15 times with a P1000 pipet tip and transfer the mixture back to the Falcon 2063 tube. Cap the tube and invert 10 times to distribute the sperm evenly. Repeat for each tube of extract.

3.5.2. Monitoring Nuclear Assembly and Growth

1. Incubate the nuclear assembly reactions at room temperature (22–23°C), gently inverting the tubes approximately once every 10 min.
2. At 20-min intervals, monitor nuclear assembly by mixing 1 µL of each assembly reaction with 1 µL Hoechst fix solution on a microscope slide. Cover each sample with a cover slip and examine the nuclei using a 40× oil immersion objective in the DAPI channel (**Fig. 2**).

3. Allow nuclei to grow to an average diameter of 25 to 30 μm, which should take about 90 min.

3.5.3. Harvesting the Nuclei

1. Transfer the nuclear assembly reactions from the Falcon 2063 tubes to 13 × 100 mm disposable glass tubes (cat. no. 60825-924; VWR) on ice, combining the contents of two nuclear assembly reactions into each glass tube. Place the glass tubes inside Falcon 2059 tubes containing 3 mL cold water to create a jacket that prevents the glass from cracking. Adjust the level of water in each 2059 tube so that the meniscus is at the same height as the meniscus of the nuclear assembly reactions. Harvest the nuclei by centrifugation for 3 min at 11,000 rpm (20,000*g*) in a Sorvall HB4 or HB6 rotor at 4°C. A gray, viscous layer of nuclei about 2 to 4 mm thick will form at the top of the glass tubes (**Fig. 1D**). Transfer the tubes to ice (*see* **Note 5**).
2. Remove the entire layer of nuclei to a new 1.5-mL tube on ice, combining the nuclei from all the tubes. The nuclei layer is very viscous and is sometimes difficult to separate from the cytoplasmic layer beneath it. It is most effectively removed by using a P200 Pipetman and a cut-off P200 pipet tip, slowly rotating the tube as the layer is very slowly withdrawn from the perimeter of the tube. When no more nuclei can be harvested this way, continue withdrawing from a smaller radius around the center axis of the tube, working inward. Finally, there may come a point when nuclei remain but are inseparable from the underlying cytoplasm. In this case, transfer the uppermost approx 500 μL of the nuclear assembly reaction (which may appear as a viscous, darker brown fraction) to a 0.5-mL microcentrifuge tube and centrifuge at top speed for 2 min. Frequently, this results in a clear layer of nuclei that is easily harvested. Although the underlying cytoplasm is not inhibitory for DNA replication, contamination of the nuclei with this fraction should be minimized because it will dilute the nucleoplasm recovered from the nuclei.

3.5.4. Isolation of the Nucleoplasm

1. Transfer the pooled nuclei to 5 × 20 mm tubes (cat. no. 342630; Beckman) using a cut-off P200 pipet tip. Centrifuge the nuclei in a Beckman Optima MAX-E ultracentrifuge using a TLS55 swinging bucket rotor (Teflon adaptors [cat. no. 358614; Beckman] are required for the 5 × 20 mm tubes) for 30 min at 55,000 rpm (260,000*g*) at 2°C.
2. Remove the tubes to ice. Remove any lipids that may be visible on the top of the tubes by careful aspiration with a narrow-tip gel-loading pipet tip attached to a vacuum line. Care must be taken not to remove a significant volume of the clear nucleoplasm. Using a P200 pipet tip, carefully transfer the nucleoplasm to a new tube, avoiding contamination by the pellet of membranes and chromatin at the bottom of the tube.
3. Freeze the NPE in 20-μL aliquots in liquid N_2 and store at –70°C. Optimal yields of NPE are 30 to 40 μL per milliliter of nuclear assembly reaction. The NPE is stable for several years at –70°C, but it loses approx 50% activity for every freeze–thaw cycle.

3.6. DNA Replication in NPE

Sperm chromatin or plasmid DNA can be used as a template for efficient DNA replication when incubated sequentially in HSE and NPE (**Fig. 1A**). Incubation of the DNA template with HSE leads to pre-RC formation. Subsequent addition of NPE

stimulates replication initiation by providing high levels of several replication factors (*see* **Subheading 1.**), while also limiting DNA replication to a single round because of the presence of inhibitors that block the formation of pre-RCs ***(6)***. Complete DNA replication of a 3-kb plasmid usually takes 20 to 30 min, whereas replication of sperm chromatin takes 45 to 60 min.

1. Rapidly thaw 1 aliquot of HSE (50 µL) and demembranated sperm (5 µL; 100,000 sperm/µL), then transfer immediately to ice.
2. To 30 µL HSE, add 1 µL of ATP regeneration mix, 0.2 µL of 0.5 mg/mL nocodozole, 0.1 to 0.5 µL [a32P]dATP (3000 Ci/mmol), and either 3 µL of 100,000 sperm/µL or 20 ng/µL final concentration of a purified, supercoiled plasmid DNA (3–12 kb). Mix thoroughly by repeated pipeting (10 times).
3. Immediately subdivide the reaction into smaller aliquots (usually 3–5 µL), which will later be stopped at different time-points, and incubate for 30 min at 22 to 23°C to allow the assembly of pre-RCs (*see* **Note 2**).
4. After pre-RC assembly has been initiated, thaw an appropriate amount of NPE and supplement it with ATP regeneration mix (1/30 vol) and DTT to 20 m*M* final concentration. The NPE is incubated at room temperature for 10 min before 2 vol are added to each aliquot of HS/DNA template. Mixing is carried out by flicking the tube or by gentle pipeting (*see* **Notes 6** and **7**).
5. The reaction is transferred to room temperature (22–23°C) and immediately subdivided into several 2.5-µL aliquots, each of which is stopped at different time-points. To stop the reactions, add 5 µL replication stop buffer in 20- to 30-min intervals and vortex briefly to mix.
6. Once all reactions have been stopped, add 1 µL 20 mg/mL proteinase K to each reaction and vortex briefly to mix. Incubate for 30 min at 37°C.
7. If sperm DNA is the template, vortex each reaction vigorously for 30 s to reduce the viscosity of the DNA. Then, load each reaction on a 0.8% agarose gel in 1X TBE. Electrophoresis is carried out at 25 V/cm, until the bromophenol dye migrates at least 2 cm. If the DNA template is plasmid, vortexing is not necessary, and electrophoresis is performed for 6 cm.
8. Cut the gel immediately above the dye front and discard the bottom part of the gel. Reduce the water content of the gel by placing, on both sides of the gel, a single sheet of Whatman paper and a stack of approx 10 paper towels (in the case of plasmid DNA, place a piece of diethylaminoethyl [DEAE] paper between the gel and the Whatman paper). Place a weight on top of the stack for approx 30 min. Remove the paper towels and Whatman paper (but not the DEAE paper) and place the gel on a fresh piece of Whatman paper, transfer to a preheated gel dryer (with the gel facing up), and cover with Saran Wrap. Dry at 80°C under vacuum.
9. The amount of input DNA replicated is calculated by determining the fraction of radioactive dATP that was consumed ***(1)***. To this end, spot 1 µL of the final replication reaction on the filter paper supporting the dried gel and air dry. Expose the gel to a PhosphorImager. Comparing the amount of radioactivity in the replicated DNA with the amount of radioactivity in the replication reaction and assuming 50 µ*M* endogenous dATP concentrations for HSE and NPE, the picomoles of dATP consumed in the reaction can be calculated. An Excel spreadsheet that calculates replication efficiency is available on request.

3.7. Using DNA Structure to Monitor Replication Initiation Events: Origin Unwinding

An important advantage of the soluble DNA replication system is that it supports extremely efficient DNA replication of small circular plasmids, which have a structure that can be used to monitor different steps of DNA replication. For example, origin unwinding can be detected as a substantial increase in negative supercoiling of plasmid DNA *(6)*. Although the assembly of nucleosomes onto plasmids in HSE causes the plasmids to become underwound and therefore negatively supercoiled on protein extraction *(14)*, origin unwinding on addition of NPE leads to further underwinding of the template.

This underwinding is dramatically enhanced in the presence of aphidicolin, an inhibitor of replicative DNA polymerases. Aphidicolin appears to uncouple the activity of the helicase that unwinds the DNA from the replication fork, leading to extensive negative supercoiling. Because plasmid incubated in HSE is already underwound because of the presence of nucleosomes, the further increase in underwinding that results from initiation of DNA replication can only be detected when the extracted DNA is separated on a gel containing an appropriate concentration of chloroquine, an intercalating agent.

The structure of the plasmid can be used to observe other events in DNA replication. Most notably, when DNA replication is allowed to proceed in the absence of aphidicolin, several low-mobility forms of the plasmid are observed that persist after DNA replication has ceased. These likely represent catenated daughter molecules that are only later resolved, presumably by topoisomerase II. In this subheading, we describe the origin unwinding assay.

1. Follow **steps 1-4** in the DNA replication protocol in **Subheading 3.6.**, except that a 3- to 4-kb circular plasmid should be used as the DNA template at a concentration of 40 ng/µL in the HSE. Also, the NPE is supplemented with 50 µg/mL aphidicolin.
2. Immediately before, and at different times after, NPE addition, aliquots containing 40 ng DNA (1 µL before NPE addition, 3 µL after NPE addition) are mixed with 100 µL unwinding stop solution. A potent preparation of NPE will completely unwind all added plasmid within 10 min.
3. After all time-points are collected, add 2 µL of 20 mg/mL proteinase K to each time-point and incubate for 30 min at 37°C.
4. Extract each sample with 100 µL of 1/1 phenol/chloroform. Do not vortex the samples. Mix gently by repeated inversion. Centrifuge at top speed in a microcentrifuge for 1 min.
5. Remove 80 µL of the aqueous layer to a new 1.5-mL tube. Precipitate the samples by adding 1 µL 20 mg/mL glycogen, 9 µL 3 *M* NaOAc, and 230 µL 100% EtOH. Mix well by inversion.
6. Chill on ice for 15 min. Spin at top speed in a microcentrifuge for 15 min. Aspirate the supernatant and allow the pellet to air dry for at least 5 min at room temperature.
7. Resuspend the pellet in 20 µL 1X TBE loading dye supplemented with 100 ng/mL ribonuclease A and incubate for at least 10 min at room temperature.
8. Load one-half of the sample on a freshly poured 0.8% TBE agarose gel supplemented with 1.8 µ*M* chloroquine. Also, add fresh chloroquine to the TBE running buffer to a final concentration of 1.8 µ*M*. Load 25 ng of a 1-kb DNA ladder as a marker.

9. Perform electrophoresis at 10 V/cm until the dye has migrated 9 to 10 cm. Stain the gel for 60 min in 100 mL Sybergold nucleic acid stain diluted 1/10,000 in water, keeping protected from light. Photograph using a yellow filter if available.

3.8. Chromatin Loading of Replication Factors in NPE

Most of the key events in DNA replication result in the loading or unloading of DNA replication proteins onto and off of chromatin. To monitor these events, chromatin that has been incubated in HSE or HSE followed by NPE can be isolated by centrifugation through a sucrose cushion and analyzed by immunoblotting. In HSE, ORC loading occurs within seconds, Cdc6 and Cdt1 loading occurs within 3 to 5 min, and maximal MCM2-7 loading requires 15 to 20 min. On NPE addition, Cdc7 and MCM10 load onto chromatin within 1 min, and Cdc45, RPA, and DNA polymerase-α load within 5 to 15 min. Some proteins, such as RPA, are sometimes isolated nonspecifically during the chromatin isolation step after NPE addition. It is therefore important to include control reactions that lack sperm chromatin.

1. Repeat **steps 1** to **4** in the DNA replication protocol.
2. Transfer the reaction to room temperature and subdivide into several 6-µL aliquots.
3. Prepare as many sucrose cushions as the number of aliquots. To do this, add 180 µL 1X ELB salts containing 0.5 *M* sucrose to 5 × 44 mm microfuge tubes (cat. no. 342867; Beckman) and place on ice.
4. At the desired time, supplement an aliquot with 60 µL cold ELB containing 0.2% Triton X-100 (but lacking cycloheximide or DTT), mix by pipeting up and down three times, and place on ice. Carefully overlay the entire volume (~70 µL) onto a sucrose cushion. Centrifuge for 25 s at 12,000 rpm (16,000*g*) in a Prima 18R horizontal centrifuge at 4°C.
5. Aspirate the supernatant with a narrow gel-loading tip, leaving behind about 3 µL. Do not touch the bottom of the tube with the gel-loading tip.
6. Add 200 µL cold ELB (lacking cycloheximide or DTT), but do not mix, and centrifuge as in **step 4**.
7. Aspirate the supernatant, this time leaving behind about 1 µL. Again, do not to touch the bottom of the tube with the gel-loading tip.
8. Add 12 µL 1X SDS sample buffer, vortex gently, boil for 2 min, collect the condensate with brief centrifugation, vortex gently again, and load the entire sample onto SDS-PAGE (polyacrylamide gel electrophoresis).
9. Perform Western blotting using antibodies against factors of interest.

3.9. Immunodepletion From HSE or NPE

An important tool in the study of DNA replication in *Xenopus* egg extracts is the removal of specific proteins using immunodepletion so that their function in DNA replication can be assessed. This subheading describes immunodepletion of HSE and NPE using crude antisera or affinity-purified antibody. For some factors, a single round of immunodepletion is sufficient to remove the protein, whereas in many cases, two to three rounds are needed. The number of rounds required must be determined empirically. Because of the high concentration of replication factors present in NPE, sometimes greater than 99% of a protein must be removed to observe a defect in DNA

replication. Unlike HSE, NPE is very sensitive to dilution, and it can become inactivated over time. Therefore, it is important first to determine the minimum number of depletions required to remove the factor of interest effectively. Typically, NPE is not depleted for longer than a total of 8 h, although some extract preparations are robust enough to survive 16 h of immunodepletion.

The protocol described next is for depletion from 40 µL HSE or NPE, but all vol in the protocol can be scaled up or down proportionally. The smallest volume of extract that can be practically depleted using this protocol is 20 µL. It is important to perform a "mock" depletion using preimmune serum or an unrelated affinity-purified antibody. In addition, any defects in DNA replication should be reversible by adding back the depleted protein from a heterologous source.

1. Transfer 8 µL packed bed volume of Protein A Sepharose Fast Flow beads (cat. no. 17-1279-01; Amersham) to a siliconized 0.65-mL microfuge tube (cat. no. 3206; Costar). This is the volume of beads required for a single round of depletion of 40 µL of extract. If multiple rounds of immunodepletion are required, multiply 8 µL of beads by the number of rounds to be performed (*see* **step 4**). Wash the beads three times with 10 vol of ELB (lacking cycloheximide and DTT). For each wash, centrifuge for 40 s at 5000 rpm (2800*g*) in a horizontal centrifuge at 4°C to pellet the beads. Aspirate the supernatant with a 27-gage needle attached to a vacuum source.
2. Add 3 vol of crude antiserum to the beads and mix well. If using affinity-purified antibody, add roughly 3 to 6 µg of antibody in 3 to 5 vol of buffer. Incubate for 30 min at 4°C. Mix on a rotating wheel to prevent the beads from settling.
3. Wash the beads five times as in **step 1**. After the last wash, aspirate as much of the supernatant and void volume as possible by sticking the 27-gage needle directly into the settled Sepharose. The Sepharose will become opaque. If multiple rounds of immunodepletion will be performed, subdivide the antibody-coupled beads into multiple 8-µL aliquots before the final aspiration. Keep on ice until use.
4. Rapidly thaw HSE or NPE and transfer to ice. Supplement HSE with nocodozole (3.3 µg/mL final concentration; this obviates the need to add nocodozole during replication). Add 40 µL extract to the tube containing 8 µL of aspirated Sepharose and mix well. Incubate at 4°C on a rotating wheel. Care should be taken to ensure that the extract remains at the bottom of the tube, rather than coating the entire inside of the tube, because this will lead to loss and inactivation of extract. A single round of depletion is performed for 1 to 2 h. If the factor of interest is not completely depleted after a single round, transfer the supernatant from the first aliquot of Sepharose to a new aliquot of aspirated Sepharose (**step 1**) using the spin filter technique described in **Note 8** and incubate at 4°C for an additional 2 h. Repeat if necessary.
5. After the final round of incubation, collect the supernatant in a new tube using the spin filter technique.
6. NPE is frequently inactivated because of oxidation during the immunodepletion procedure. Therefore, after immunodepletion, NPE is supplemented with 20 m*M* DTT.
7. The degree to which the factor was immunodepleted should be assessed for every experiment. This is done by analyzing 1 µL of immunodepleted extract alongside a dilution series of mock-depleted extract using Western blotting with antibodies against the factor of interest.

4. Notes

1. If the interface of the sperm and the 2.3 *M* sucrose layer are not thoroughly stirred prior to centrifugation, a significant amount of sperm may not sediment to the bottom. If after the first centrifugation you see a cloudy layer above the red blood cell layer, remix the cloudy layer and centrifuge for another 20 min.
2. Sperm settles quickly. Thoroughly resuspend sperm by pipeting 10 times before and about once every minute when aliquoting to maintain accurate concentration.
3. Healthy eggs have a uniformly light-to-dark-brown animal hemisphere with a germinal vesicle centered in the middle, appearing as a white spot. Batches of eggs in which the majority of eggs lack a germinal vesicle or with animal hemispheres that are extensively variegated or mottled in pigment should be discarded. It is normal for some eggs to lyse, resulting in an empty sphere, mostly white in appearance. Any batch of eggs in which more than 10% of the eggs have lysed should be discarded.
4. The first needle is used to poke a hole in the side of the tube. This needle cannot be used to withdraw the cytoplasm because it becomes clogged with the plug of polypropylene removed from the tube. Therefore, the second needle, attached to a syringe, is inserted in the hole to withdraw the cytoplasm. To minimize the amount of cytoplasm that flows out of the hole on removal of the first needle, seal the top of the tube with parafilm, which will prevent significant loss of cytoplasm by vacuum. Before withdrawing the cytoplasmic fraction from the crushed eggs, be sure to remove the parafilm or the vacuum created by the syringe will perturb the layers. Avoid withdrawing from the mitochondrial layer immediately above the cytoplasmic layer and from the pigment layer below. Keep the beveled side of the needle tip facing up and centered in the tube. Withdraw slowly to minimize the amount of turbulence generated while withdrawing. Continue withdrawing until the cytoplasmic layer is less than 2 mm thick.
5. If the nuclear layer is too thin to harvest, check the following: first, verify that the average nucleus really grew to the desired size. Second, double check the concentration of the sperm preparation used. Third, examine the nuclei that are in the layer using Hoechst dye. Extracts sometimes undergo apoptosis, which can cause complete, irreversible destruction of nuclei within minutes.
6. Although HSE has never been observed to support DNA replication of duplex DNA in our hands, a control in which the NPE is supplemented with 50 µg/mL aphidicolin, an inhibitor of DNA polymerase-α can be included to verify that any DNA replication observed is caused by replicative DNA polymerases and not to repair synthesis.
7. Depending on the relative concentration of factors between preparations of NPE, NPE may not need to be used at full concentration to achieve efficient DNA replication. For each preparation of NPE, a titration experiment should be performed. Test NPE at 100, 80, and 60% concentrations (diluted with ELB) but always add 2 vol of NPE to 1 vol of HSE. Using NPE at less than 100% concentration effectively increases the usable size of a given preparation. Further, having a preparation of NPE that can support efficient replication even when diluted will facilitate extensive manipulation of the NPE. For example, if you are performing immunodepletion studies with NPE, the procedure (**Subheading 3.9.**) usually causes about 10 to 20% dilution of the NPE. Last, having a dilutable NPE preparation will also facilitate efficient replication if the NPE becomes diluted in experiments by addition of recombinant proteins when performing depletion/add-back studies.
8. To remove extract from a mixture with antibody beads with minimum volume loss, we use a homemade spin filter using a 0.65-mL microfuge tube, a P200 pipet tip, and a Nitex mesh membrane (cat. no. 03-20/14; Sefar). To fabricate the filter, cut the bottom 2 cm off

of a P200 pipet tip with a clean razor blade and discard the bottom portion. Cut the top portion precisely where the protruding ridge along the side ends (this is where the pipet tip is "seated" in the pipet tip box). You now have a top segment of the pipet tip with the ridges and the narrower tapered segment. Cut a 1.5 × 1.5 cm square of Nitex mesh. Direct the mesh square into the upper segment through the top by using the narrow end of the lower segment. The lower segment will fit snugly into the upper segment and, because of its tapered shape, will fit more tightly the farther in it is pushed. The snug fit between the two segments holds the mesh in place. The whole filter "assembly" can hold up to 100 µL above the mesh membrane and fits inside a 0.65-mL microfuge tube. To recover extract from the Sepharose, pipet the mixture into the filter assembly and centrifuge for 40 s at 5000 rpm (2800*g*) in a Prima 18R horizontal centrifuge at 4°C. When another round of depletion follows, place the filter inside a new 0.65-mL tube containing a fresh aliquot of aspirated Sepharose. After the final round of depletion, place the filter assembly in a fresh empty tube. Using this approach, approx 5% of the starting volume of extract will be lost for every round of immunodepletion.

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