Sperm Chromatin Assembly in CSF or Interphase HSS

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Stock Solns:

10x XBE2: (for CSF HSS)
1 M KCl
1 mM CaCl2
20 mM MgCl2
100 mM K+ HEPES pH 7.7
500 mM sucrose
50 mM EGTA

10x XB1: (for interphase HSS)
1 M KCl
1 mM CaCl2
10 mM MgCl2
100 mM K+ HEPES pH 7.7
500 mM sucrose

20x energy mix:
20 mM ATP (Sigma)
20 mM MgCl2
200 mM phosphocreatine (Boehringer Mannheim)
2 mg/ml creatine kinase (Boehringer Mannheim)
store in 10 and 50 ul aliquots; -80 degC.

Frog Fix:
67 ul 80% glycerol
10 ul 10x MMR
23 ul 16% CH2O (Ted Pella, MeOH free)
1 ul 0.1 mg/ml Hoechst 3325

Procedure:

1. For chromatin and chromosome assembly, we dilute our extracts 1:1 with buffer. "XBE5" is XBE2 supplemented with MgCl2 so that final Mg2+ concentration is 5mM. Note that stock energy mix also includes Mg2+, so you must include this. The morphology mitotic chromosomes made from CSF HSS is highly sensitive to MgCl2 concentration. Generally, the right concentration is 5 or 6 mM MgCl2. Extract dilution improves morphology and most importantly, allows spinning of assembly rxn products through cushions. Spindowns without dilution give massive contamination with actin and other extract proteins. Make appropriate dilution buffer from 10x stocks (above). For CSF mitotic chromosome assembly, use 1x XBE5 or XBE6 with ATP regeneration. For interphase chromatin assembly use 1x XB2 with ATP regeneration. An example: 20 ul 10x XBE2 0.2 ul 1 M MgCl2 20 ul 20x energy mix 160 ul dd H2O
2. Quickly thaw aliquot(s) of extract in RT water bath. If necessary (for protein analysis), combine aliquots. Dilute 1:1 with dilution buffer. *Mix by pipetting up & down, but do not introduce air bubbles.*
3. Spin in cold microfuge, highest speed, 10'. Carefully remove supernatant and place in new, pre-chilled 0.65 ml tube. *Unstable pellets are indicative of poor HSS This step is absolutely required for protein analysis, probably unnecessary for morphological analysis. Assembly is most efficient in small tubes, even with large volumes. Go figure.*
4. Add sperm. For morphological assay, use 0.5 ul 3x107/ml sperm for 20 ul of diluted extract. For protein analysis, use 25 ul 3x107/ml sperm for 200 ul of diluted extract. *For protocol for sperm prep, see A. Murray. (1991) Meth. Cell. Biol. 36:581-604. One can also use naked DNA substrates for chromatin asembly. Use no more than 10 ug DNA/ml extract. I usually use 5ug/ml just to be safe. If using lambda DNA decatenate before addition to extract by heating to 65 deg C 10', then immediately put on ice.*
5. Incubate in 20 deg C water bath 2-3 hours *Incubation can probably be done on bench. The temperature in our lab varies alot, so we use the water bath.*
6. For morphological assay: At each timepoint, open tube, stir with pipette tip (but no up & down pipetting), and remove 1 ul sample. Place on slide and overlay with 4 ul fix. Squash with 22 x 22 mm coverslip. Observe on fluorescence microscopy. For biochemical assay: Chill assembly rxn 15' on ice. Take 200 ul sperm assembly rxn and layer on 800 ul 30% sucrose/XBE2.

Spin JS13.1 or HB-4 10k, 15 min, 4oC, brake on. (For naked DNA assembly, layer on 1 ml cushion, spin 40k rpm, 30 min, TLS-55). Wash interface 4-5x with XBE2. Remove all except last ~5ul of cushion. Add Laemlli SB to pellet. Freeze/thaw to break up DNA. Load on 7.5 - 15% Laemmli gel.