Centrosomes are not always very concentrated. This is a problem for assays and especially EM. We tried the last time to be at the final stage going onto one or at the most two sucrose gradients. Just downscale the wash steps of the cells so have according amounts.

Procedure

1. Grow the cells at densities between 1 and 4 x 10^6 cells/ml in RPMI 1640 + 10% FCS + 1:100 pen-strep and 1:100 glutamine. When thawing cells put one tube in 20 or 40 ml depending on cell density. Grow in plastic flasks (5% CO2, 37C, loose cap) till you have at least 1l (more is better). You can use spinner flasks, but the cells won't grow in them so well unless adapted to them for a while. Ideally the cells should be split 1:1 the day before the prep.

2. Cells should be at 1.5 x 10^6 cells/ml the day of purification

3. One hour before the purification add nocodazole to 3.3 x 10^-7 M and cytochalasin D to 1µg/ml.

4. Spin down at 2krpm at 4C for 5 min. The pellet is very loose. Do not decant SN; use aspiration. This is true for the further steps as well.

5. Resuspend cells in 200ml cold PBS, transfer to red-cap 50 ml Falcons, spin 5min, 1.5 krpm.

6. Resuspend in cold 8% sucrose in 0.1x PBS (25ml per tube), spin at 1krpm, 5 min at 4C. Be gentle with resuspension but as fast as possible (no bubbles!).

7. Squirt 10ml Lysis buffer (1mM Tris pH 8.0, 0.1% BME, 0.5% NP-40, 5x10^-4 M MgCl2, 1mM PMSF, 10 µg.ml Aprotinin, 1 µg/ml Leupeptin, 1 µg/ml pepstatin) on the pellets in each tube. This is the key step. If the lysis is too slow, the prep will not work well. We use the old manual pipette aid with the wheel, but you could use mouth blow-out. Be as fast as possible. Apirate 3 times, invert 2 to 3 times. Cells should lyse immediately. Speed of lysis is decisive for the prep. Leave on ice for 5 min, spin 3krpm, 10min, 4C.

8. Filter SN through a nylon mesh, pool SN (100-150ml) and add 2-3 ml of 0.5M K-PIPES pH7.2, 1mM EDTA and 150 µl of 20,000 U/ml DNase I, leave on ice for 15min.

9. Load lysate on 50% sucrose in 10mM K-PIPES pH 7.2, 1mM EDTA, 0.1% BME, 0.1% Triton X-100

10. Spin at 11 krpm (for a SW27 rotor), 4C for 20 min

11. aspirate away SN leaving 2ml lysate above the cushion. Take these 2ml lysate + 2ml of the underlying cushion, mix well

12. layer on discontinuous sucrose gradients in SW40 tubes: 2ml of 70% sucrose, 1.5 ml of 50% sucrose, 1.5ml of 40% sucrose (in 10mM K-PIPES pH 7.2, 1mM EDTA, 0.1% BME, 0.1% Triton X-100), 7ml lysate on top of that.

13. spin 1h15min, 4C, 25 krpm (110,000g)

14. eliminate top of gradient to the 40% solution, elute from bottom of the tube, collect fractions of ca. 400 µl.

15. Read fractions with the refractometer, take 10 µl aliquots for centrosome counting (aster assembly), freeze the remainder and keep at -80C. Centrosomes should be between 40% and 50% sucrose.