REAGENTS:

10x Buffer A

10x final concentration	stock soln	add/250 ml final
150 mM Tris:HCl pH 7.4	1 M	37.5 ml
800 mM KCl	4 M	50 ml
20 mM K-EDTA pH 7.4	100 mM	50 ml
7.5 mM Spermidine	1 M	1.87 ml
3.0 mM Spermine	1 M	0.75 ml

20x Buffer 3

10x final concentration	stock soln	add/100 ml final
100 mM Tris:HCl pH 7.4	1 M	10 ml
40 mM KCl	4 M	1 ml
40 mM K-EDTA pH 7.4	0.1 M	40 ml
7.5 mM Spermidine	1 M	75 μl

10x Buffer 3

10x final concentration	stock soln	add/500 ml final
50 mM Tris:HCl pH 7.4	1 M	25 ml
20 mM KCl	4 M	2.5 ml
20 mM K-EDTA pH 7.4	0.1 M	100 ml
3.75 mM Spermidine	1 M	1.87 ml

10x Buffer 4

10x final concentration	stock soln	add/50 ml final
50 mM Tris:HCl pH 7.4	1 M	2.5 ml
20 mM KCl	4 M	0.25 ml
3.75 mM Spermidine	1 M	187 μl

Sucrose Solutions [15%, 60%, 80%]

Add sucrose to one-half final volume.
Add 1/10 final volume of 10x Solution 3.
Dilute to ~90% of final volume with H_2O.
When all sucrose is dissolved, dilute to final volume with H_20.
Filter with 0.2 μm filter.
Store at 4^oC.

Colcemid (Sigma) - 100 μg/ml. Filter sterile. Store 1 ml aliquots at -20^{o}C.

Protease inhibitors: TRAS/Aprotinin, PMSF, CLAP

10% Digitonin (Sigma) in H_20. This is critical – the whole prep will not work without it.
This requires special digitonin that will form a clear solution upon boiling in H_20.
WCE has bottles of a lot tested by Ciaran in his office.
Take a 5 gm bottle and dissolve in 50 ml boiling H_20. If this does not make an absolutely clear solution, it is no good. Aliquot into 4 x 10 ml and 20 x 0.5 ml and freeze at –20°C. Thaw one 0.5 ml aliquot per experiment and discard it at the end of the experment.

10% AMX [Ammonyx Lo - supplier unknown – Bill E has two bottle of this. That is all there ever will be as the maker is out of business! Ask Bill if you need some.]

RNase A 10 mg/ml in 10 mM Na Acetate pH 4. Boiled 7 min. Store at 4^{o}C.

0.1% SDS in H_2O.

You also need two Wheaton dounce homogenizers:
15 ml with pestle A (the tighter one)
40 ml with pestle B

POLYAMINES (Spermidine & Spermine - both from Sigma) Make 10 ml of each and store at 4^{o}C.

1 M Spermidine (fw 254.6 - stored desiccated in freezer) – 2.54 gm/10 ml
1 M Spermine (fw 348.2 - stored desiccated in freezer) – 3.48 gm/10 ml

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EXPERIMENTAL PROTOCOL:

I. OBTAIN MITOTIC CELLS

Two days before:
125 ml cell stock + 125 ml RPMI 1640 $\Rightarrow$ Incubate in 500 ml bottle with stir bar.

One day before:
In the morning dilute culture to 500 ml with RPMI 1640.
Book the J25 centrifuge with JLA10.5 and JA25.5 rotors.
Check to see that all stock solutions are sufficient.
At 5:00 pm block the culture with colcemid at 0.1 μg/ml.
[1 μl/ml of a 100 μg/ml stock.]

II. CHROMOSOME ISOLATION

Turn on Beckman and Heraeus table top centrifuge to begin cooling.

A. Make sucrose step gradients.

Label three 50 ml cylinders for 15%, 60% and 80% sucrose.
For a 500 ml prep, add 25 ml of each sucrose stock solution + 250 μl 10% AMX + 25 μl each of protease inhibitors Tras/Aprotinin, PMSF and CLAP
Mix sucrose solutions well, tipping cylinders end over end.

For each 500 ml culture, make 2 gradients, each in 50 ml Sorvall tubes.
I. Pipet 10 ml 15% sucrose into each tube.
II. Underlayer 10 ml 60% sucrose with a 10 ml pipette and a pipette pump.
II. Similarly underlay 8 ml of 80% sucrose.

Keep on ice.

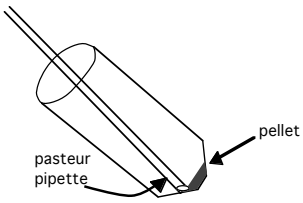
B. Make SOLUTIONS 1 and 2 [swelling solution and lysis buffer].

SOLUTION 1 - **100 ml**/500 ml culture.
5 ml 10x Buffer A + 95 ml H_2O.
Add protease inhibitors (10 μl each) just before use.
Detergent will kill this step. Rinse out the graduate with water before making the solution.
Store at room temperature.

SOLUTION 2 - **50** ml/500 ml culture.
5 ml 10x BUFFER A + 45 ml H_2O.
Pour into a 125 ml Erlenmeyer and put on ice.
Add protease inhibitors (5 μl each) + 500 μl 10% digitonin [***just before use***].

C. Spin cells down in J25 centrifuge.

Turn centrifuge on.
Set temperature to 4^{o}C (but temperature is not critical for the first spin.)
Place JLA 10.5 rotor in centrifuge with drive pins engaged.
Pour culture into two 500 ml tubes.
Balance on pan balance.
Spin at 1600 x g for 5 min. [It is best to time this spin with a lab timer.]
Remove supernatant with a cut 5 ml plastic pipette on aspirator. [Suck off bubbles from surface first.] If pellet is loose, only aspirate down to ~20-30 ml, resuspend using a 25 ml pipette, transfer to a Corning 50 ml tube, and respin in the Heraeus centrifuge @ room temp (3 minutes at 2100 rpm = 900 x g).
Aspirate any bubbles first with a Pasteur pipette, then tilt the tube and completely remove the supernatant.



D. Swell the cells.

[The timing of this step is critical. Know what you are doing. Work as rapidly as you can. Be organized.]

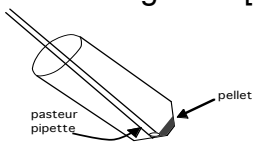
Add protease inhibitors to the Solution 1. [1 μl/ml of Solution 1.]
With a 10 ml pipette and pipettor - take 10 ml of Solution 1.
Gently add to cells while progressively mixing in and out. [Add 2 ml. - Suck back. – Add 4 ml. - Suck back. - etc.] The cells should be resuspended by the time you have added all 10 mls.
Be gentle - mitotic cells are fragile. It should take ~15-20 seconds/tube to resuspend the cells.
When all tubes are resuspended, pour inSolution 1 to 45 ml.
Cap tubes with Parafilm and invert to mix.
Set on bench at room temperature for 5 minutes, ***exactly***.

E. During the 5 min incubation prepare to lyse cells and determine mitotic index.

Remove RNase from refrigerator. Place on ice.
Put the 10 ml Dounce (with B pestle inserted) on ice.
Add protease inhibitors to the SOLUTION 2. (1 μl/ml of SOLUTION 2.)
When 1 minute is left on timer make a slide of the swollen cells.
10 μl droplet on freshly wiped slide - add cover slip very carefully.
Mitotic cells are fragile!
Put tubes in Heraeus Centrifuge.
When timer rings immediately start spin (3 minutes at 2100 rpm = 900 x g).
During spin determine mitotic index.
[Examine swollen cells at 40x under phase. Mitotic index = # mitotics/# total number counted.]
When centrifuge timer rings, stop counting and return to your bench.

F. Lyse the cells.

Aspirate the supernatant with Pasteur pipette as above. [Pellet should be fluffy.]
Add digitonin [1/100 volume of 10% stock] + protease inhibitors [1 μl/ml] to the Soln 2.



Add 50 μl of RNase A to each pellet.
Use pipettor to draw 10 ml SOLUTION 2 into pipette.
Aim pipette at pellet and jet-stream the SOLUTION 2 onto the pellet, disrupting it.
Vigorously and rapidly pipette the solution up and down twice.
The final time, expel the solution into the dounce.

QUICKLY yank up on the pestle to give the first stroke of homogenization.

Dounce with a total of 10 strokes. In each case, snap upward quickly, being careful not to break the surface. (This makes a "popping" noise, and if it happens, then that stroke does not count.)

Pause ~2 seconds between each down stroke (***GENTLE***! or you will have a fountain of chromosomes on the bench) and the next upwards snap. [With the Wheaton dounce, the upwards stroke is the shear stroke. Thedown stroke is simply a recovery.]

After the 10 strokes, carefully pipette the solution and foam into a 15 ml disposable plastic centrifuge tube (Corning equivalent) and place on ice

Discard the 50 ml tube.

THE ABOVE STEPS, FROM ASPIRATION OF THE SOLUTION 1 TO THE FIRST DOUNCE STROKE MUST TAKE ≤30 SECONDS, OR THE YIELD WILL DROP RADICALLY. [WCE can do it in 12 seconds.] This is because the detergent permeabilizes the cells and the chromosomes and cytomatrix begin to clump together. This clump is lost during either the first centrifugation or the Percoll gradient.

From this point on all operations are performed on ice. Keep all solutions chilled. Ice plus protease inhibitors are not enough to prevent degradation of topoisomerase II, but ice helps.

Make slide of lysate (10 μl/coverslip). Examine under phase contrast.

Chromosomes will be seen among the debris.

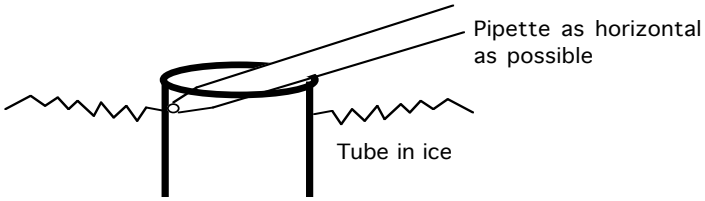
G. Remove nuclei and debris.

Spin tube in Heraeus centrifuge at 1000 rpm for 5 minutes at 4°C [200 x g.]

Using pipettor, **carefully** transfer the supernatant from over the pellet of nuclei and unlysed cells into a new tube. It is better to leave 200-300 μl of supernatant than to contaminate the supernatant by sucking up too much and getting some pellet. The smaller the pellet the fewer nuclei and unlysed cells you have. [This is good.]

H. Sucrose gradients.

Using a pipettor **carefully** layer the supernatant over the sucrose step gradient, holding the pipette tilted as shown to minimize mixing.



Spin 1400 rpm 10 minutes (400 x g).

Set centrifuge timer for 15 minutes, and use a separate lab timer to time the 10 minute pre-spin. i.e. **Don't let it stop here!**

After 10 minutes, reset time dial for 30 minutes and the speed dial to 2800 rpm (1600 x g).

While the gradient is spinning, make the PERCOLL and SOLUTIONS 3 and 4.

PERCOLL: [Note, Percoll costs ≥ £147/liter so be careful.]

For a 500 ml culture, make 50 ml as follows.

To a 50 ml graduate cylinder add:

2.5 ml 20x SOLUTION 3
+ 75 μl of 1 M Spermidine
+ 30 μl of 1 M Spermine)
+ 500 μl 10% AMX
+ In the hood add Percoll to 50 ml. [In the hood to minimize anything falling into the Percoll. Try to keep it sterile. Be careful!]

Pour into a 125 ml Erlenmeyer and place on ice.

SOLUTION 3

For a 500 ml culture, make 200 ml as follows.

20 ml 10x SOLUTION 3 + 2.0 ml 10% AMX + 180 ml H_2O

Pour into a 500 Erlenmeyer and place on ice.

SOLUTION 4

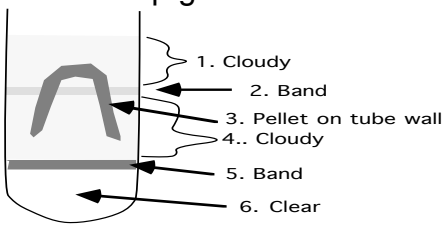
For a 500 ml culture, make 10 ml as follows.

1 ml 10x SOLUTION 4 + 0.1 ml 10% AMX + 8.9 ml H_2O. (Use a pipette.)

Make in a 25 ml Erlenmeyer and chill on ice.

I. Fractionating the step sucrose gradient:

The step gradient will have 6 regions roughly as follows:



a.) With a Pasteur pipette on an aspirator carefully remove the region above the 60% "pellet 3" [**the half moon on the wall of the tube!**]. Use a rotary motion just at the surface (should make a telltale noise if you are at the surface) until you get down near pellet 3, then use a semi-circular motion so that you do not disturb pellet 3.

b.) Use a shortened Pasteur pipette attached to tygon tubing on a 60 ml syringe to carefully withdraw the 80% sucrose (region 6) from underneath band 5. Discard.

c.) Resuspend pellet 3 and band 5 in the residual material (region 4). With the pipettor, suck up all liquid to measure the volume. If necessary add solution 3 to make a total of 10 ml.

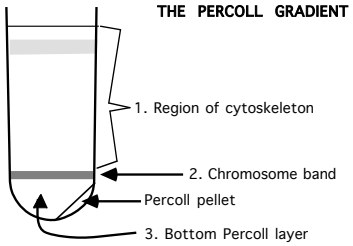
Transfer the resuspended solution to a fresh Sorvall 50 ml centrifuge tube.
In no case transfer > 10 ml !!

J. Percoll gradient.

Add protease inhibitors to the PERCOLL. (1 μl of each per ml PERCOLL solution.)

Add POLYAMINES to each tube with its 10 ml of sucrose gradient.
Add 37.5 μl 1 M spermidine + 15 μl 1M spermine
Swirl to mix.
Forgetting to add the polyamines is a common reason for a failed prep!

Add 10 ml PERCOLL solution to each tube.
Pour the PERCOLL /chromosome mixture into the pre-cooled 40 ml dounce (Pestle B) and homogenize with 10 "medium forceful" up strokes.
[Be careful to be gentle on the down stroke!]
Pour back into the centrifuge tube and repeat for the other gradient.
Add 14 more ml of PERCOLL solution to each tube.
[There is enough PERCOLL solution, but you have to pipette it carefully.]
Mix end over end.
Balance the tubes on pan balance.
Spin 19,000 rpm in the J25 centrifuge (44,000 x g) at 4^{o}C (JA25.5 rotor).
[45 minutes for chicken chromosomes. 30 minutes for chicken chromosomes.]
Set the J25 centrifuge for "**slow deceleration**".
Wash out the 40 ml Dounce homogenizer with H_2O and replace on ice.
The Percoll gradient has three regions as shown:



a.) With aspirator and Pasteur pipette use a circular motion to remove region 1 (cytoskeletal contaminants).
It is important to keep the pipette at or just above the surface to get rid of all contaminants.
b.) With Pasteur pipette and bulb remove band 2 and region 3, carefully avoiding the clear pellet of Percoll.
Place the solution in a fresh Sorvall 50 ml tube.
Put the now empty Percoll gradient tube to one side and add distilled water to soften the pellet.

K. Wash away Percoll.

Add protease inhibitors to SOLUTION 3. (1 μl of each per ml SOLUTION 3).
Pour SOLUTION 3 into each tube with the band from the Percoll gradient up to the level of the line (~40 ml).
Pour into the 40 ml dounce and homogenize with 4 gentle strokes (Pestle B).
Split into 2 Sorvall tubes and fill each to the line with SOLUTION 3 (total of 4 tubes/500 ml culture). Mix end over end.
Spin in Heraeus at 3700 RPM 15 minutes at 4^{o}C (2900 x g) .
Carefully aspirate away the supernatant.
The pellet should look like a ring.

L. Resuspend the chromosomes.

Let the tube sit on ice ~2-3 min.

Gently tap tube with finger to resuspend the chromosomes.

Tap one side, rotate 90^{O} and tap again until the pellet turns into an even slurry.

If you are too vigorous at this stage you destroy the chromosomes! Don't finger-vortex! If you are not vigorous enough you will end up with huge clumps of chromosomes that you will never separate. Be perfect!

Add TRAS/Aprotinin only to SOLUTION 4. (1 μl/ml SOLUTION 4)

Add 0.5 ml SOLUTION 4 to each tube.

Gently mix by repipetting gently with a cut blue pipette tip.

Pool the chromosomes in a polyallomer tube.

M. Characterize the chromosomes.

Look at 5-10 μl in the phase contrast microscope.

Dilute 10 μl with 1 ml 0.1% SDS. (Do in duplicate).

Read the OD_{260}, having first zeroed the spectrophotometer with SOLUTION 4 + 0.1% SDS.

Avoid bubbles, as these will give an inaccurate reading. [Use a fresh Pasteur pipette for each pipetting.]

An OD_{260} of 20 = 1 mg/ml of DNA (and thus 1 mg/ml of chromosomal non-histone proteins).

Expect 2 ml of 10 OD_{260} units/ ml from a 500 ml culture.

N. Clean-up

All Sorvall tubes should be scrubbed out carefully with a test tube brush, taking particular care to remove the Percoll pellet. Store the tubes inverted.

Clean-up is best done immediately upon completion of prep.