HeLa Cell Nuclear Extract Preparation
(Martinson Lab, UCLA, August 2006)

Growing the cells

1) Dilute cells daily to $3X10^5$ cells/ml.
2) To dilute, feed the cells with media made as follows:
Put magnet in flask and rinse with ddH2O.
Then prepare:

For	2L	1.5L	1L	500ml	250ml
ddH2O	1800 ml	1350 ml	900 ml	450 ml	225 ml
S-MEM[1]	20.9 g	15.675 g	10.45 g	5.225 g	2.613g
Dextrose	2 g	1.5 g	1 g	0.5 g	0.25 g

[1] Minimal Essential Medium (S-MEM)(Autoclavable). GIBCO (Invitrogen). Formula No. 00-5163EF.

After mixing these ingredients the solution should be yellow which is indicative of a pH of about 4.
Cover flask with aluminum foil.
Autoclave for 30min (1L) or 1hr (2L). Cool overnight to room temperature.

3) The next day add the following in the tissue culture hood:

For	2L	1.5L	1L	500ml	250ml
7.5% $NaHCO_3$	60 ml	45 ml	30 ml	15 ml	7.5 ml
10N NaOH	600 ul	450 ul	300 ul	150 ul	75 ul
100X L-Glutamine[2]	20 ml	15 ml	10 ml	5 ml	2.5 ml
100X Pen/Strep[3]	20 ml	15 ml	10 ml	5 ml	2.5 ml
Fe Enriched Calf Serum[4]	100 ml	75 ml	50 ml	25 ml	12.5 ml

[2] L-Glutamine 200mM. GIBCO (Invitrogen). Catalog No. 25030-081.
[3] Penicillin/Streptomycin. GIBCO (Invitrogen). Catalog No. 15140-122.
[4] Iron Supplemented Bovine Calf Serum. Omega Scientific. Catalog No. IS-06.

L-glutamine and Penicillin/Streptomycin must be sterile filtered before use.
After mixing these ingredients the solution should be red which is indicative of a pH of about 7. The indicator (phenol red) is present in the S-MEM (acid=yellow, alkali=purple).

3) Incubate cells in 37°C incubator for 24hr with gentle stirring so that a very small vortex is visible.

Counting cells

1) Done before and after cells are passed. In the tissue culture hood use a serological pipet to draw up 2ml of cells and transfer into a test tube.
2) Mix the cells by tapping to avoid collection at the bottom of test tube.
3) Dip in a Pasteur pipette to draw up cells by capillary action.
4) Put the tip of the pipette at the "V" slash at the loading side of each plateau of the hemacytometer (cohesion force will draw the sample into the counting area).
5) Count four of the 1 mm squares on each side of the hemacytometer (50 counts translates to $5X10^5$ cells/ml).

Stock solutions

1) 7.5% $NaHCO_3$
   For 500ml: 37.5g (MW 84.01) ----> add ddH2O to 500ml ---> sterile filtration.

2) 100mM TRIS, pH 7.9
   For 500ml: 6.06g (MW 121.14) ---> ddH2O ---> pH with 1N HCl to 7.9 ---> add ddH2O to 500ml.

3) 1M $MgCl_2$
   For 15ml: 3.05g (MW 203.31) ---> add ddH2O to 15ml.

4) 1M KCl
   For 15ml: 1.12g (MW 74.55) ---> add ddH2O to 15ml.

5) 0.1M EDTA
   For 500ml: 18.61g (MW 372.24) ---> ddH2O ---> pH with 5M KOH to 7.2 --> add ddH2O to 500ml.

6) 3.2M $(NH_4)_2SO_4$
   For 40ml: 16.91g (MW 132.14) ---> add ddH2O to 40ml.

7) 100mM HEPES, pH 7.9
   For 500ml: 11.915g (MW 238.3) ---> ddH2O ---> pH with 5M KOH to 7.9 ---> add ddH2O to 500ml.

8) 0.2M PMSF
   For 10ml: 348mg (MW 174.2) ---> add Isopropanol to 10ml.

9) 0.5M DTT
   For 10ml: 0.772g (MW 154.3) ---> 10mM Sodium Acetate to 10ml.

Autoclaving

1) Autoclave for 30min (wet autoclaving).
   Dialysis membrane (Spectrum Laboratories, Catalog # 132678, MW cut-off 12-14000). Cut several strips and submerge in a 400ml beaker with ddH2O. Cover with aluminum foil.

2) Autoclave for 45min (dry autoclaving).
   1X 400ml beaker with 8 dialysis membrane clips covered with aluminum foil.
   100X cryo tubes and caps.

   Rinsed with ddH20 and covered with aluminum foil:
   2X high neck 1L beaker with stir bar plus 8X 50ml thick wall centrifuge tubes.
   1X 1L bottle for phosphate buffer.
   1X 100ml bottle for buffer A.
   2X 1L bottles for dialysis buffer.
   2X 50ml beakers with small stir bar.
   1X 40ml Dounce Homogenizer (Kontes article no. 885303-0040) and pestle “A” (Kontes article no. 885301-0040)
   2X 40ml Dounce Homogenizer (Kontes article no. 885303-0040) and pestle “B.” (Kontes article no. 885302-0040)
   Metal spatulas

After autoclaving, cool down to room temperature and keep autoclaved materials in the fridge.

Buffers

Prepare the day before making the extract

1) Phosphate buffer: for washing the cells (the only buffer that can be re-used).

Composition: 137mM NaCl, 2.68mM KCl, 4.29mM Na_2HPO_4, 1.47mM KH_2PO_4

Use 1L bottle that was autoclaved.
For 1L (1L measuring cylinder):

8g NaCl (MW 58.44)
200mg KCl (MW 74.55)
1.15g Na_2HPO_4 (MW 268.07)
200mg KH_2PO_4 (MW 136.09)

Check pH. It should be ~7.2 (+NaOH if necessary). Add ddH2O to 1L ----> sterile filtration.

2) Buffer A (hypotonic buffer): for swelling the cells.

Composition: 10mM TRIS (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT.

Use 100ml bottle that was autoclaved.
For 100ml (100ml measuring cylinder):

10ml	100mM TRIS, pH 7.9
150μl	1M $MgCl_2$
1ml	1M KCl

Add ddH2O to 100ml ----> sterile filtration.

Before use add:	100μl	0.5M DTT

3) Buffer C (low salt/nuclei resuspension buffer).

Composition: 25% glycerol, 20mM TRIS (pH 7.9), 1.5mM $MgCl_2$, 20mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, 0.7mg/ml Pepstatin, 0.4mg/ml Leupeptin.

For 10ml (15ml blue cap conical tube):

2.5ml	Glycerol
2ml	100mM TRIS, pH 7.9
15μl	1M $MgCl_2$
200μl	**1M KCl**
20μl	0.1M EDTA

Add ddH2O: up to 9ml, then + 525μl ---> sterile filter with syringe into a 15ml blue cap conical tube.

Before use add:	280μl	25mg/ml Pepstatin[5]
	160μl	25mg/ml Leupeptin[6]
	10μl	0.5M DTT
	25μl	0.2M PMSF

4) Buffer C (high salt/nuclear extraction buffer).

Composition: 25% glycerol, 20mM TRIS (pH 7.9), 1.5mM $MgCl_2$, 800mM $(NH_4)_2SO_4$, 0.2mM EDTA, 0.5 mM DTT, 0.5mM PMSF, 0.7mg/ml Pepstatin, 0.4mg/ml Leupeptin.

For 10ml (15ml blue cap conical tube):

2.5ml	Glycerol
2ml	100mM TRIS, pH 7.9
15μl	1M $MgCl_2$
2.5ml	**3.2M $(NH_4)_2SO_4$**
20μl	0.1M EDTA

Add ddH2O: up to 9ml, then + 525ul ---> sterile filter with syringe into a 15ml blue cap conical tube.

Before use add:	280ul	25mg/ml Pepstatin
	160ul	25mg/ml Leupeptin
	10μl	0.5M DTT
	25μl	0.2M PMSF

[5] Pepstatin A. MP Biomedicals. Catalog No. 195368.
[6] Leupeptin Hemisulfate. MP Biomedicals. Catalog No. 151553.

5) Dialysis buffer: for removing salts.

Composition: 20% glycerol, 20mM HEPES (pH 7.9), 100mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.1mM PMSF

Use 2X 1L bottles that were autoclaved.
For 2 liters (2L measuring cylinder):

400ml	Glycerol
400ml	100mM HEPES, pH 7.9
14.9g	KCl (MW 74.55)
4ml	0.1M EDTA

Add ddH2O to 2L. Measure conductivity (1000ul ddH20 + 20ul sample). This should be between 160 and 180 μMHO. Sterile filter into 2X 1L bottles.

Before use, add (each 1L):	1ml	0.5M DTT
	0.5ml	0.2M PMSF

Making The Extract

KEEP ALL BUFFERS AND HARDWARE THAT WILL BE IN CONTACT WITH CELLS OR EXTRACT ON ICE AT ALL TIMES.

1. Count cells before starting to make sure that they doubled over the 24hr growth period (this procedure is for 16L of cells at a density of 5 to $6X10^5$ cells/ml).
2. Turn on CRU 5000 centrifuge. Put the 276 rotor in.
3. Turn on Sorvall RC2-B centrifuge. Put the HB-4 rotor in.
4. Get ice: 2 buckets, 2 small trays, 1 large tray.
5. Chill with ice: 3 Douncers + pestles, 10 plastic serological pipets and metal spatulas.
6. Take out DTT and PMSF and put on ice.
7. Get 4 big 1L plastic centrifuge bottles and rinse with ddH2O.
8. Fill bottles with cells.
9. Centrifuge in the CRU at 2,000rpm for 15min.
10. Pour off supernatant (keep pellet on ice) and put in more cells for additional spin in same bottle. Any cells that are not spun down must constantly be stirred.
11. Keep centrifuging until all cells are pelleted (last spin = 20min).
12. Take out phosphate buffer and put on ice.
13. Put 200ml of phosphate buffer into one 1L bottle that has pelleted cells. Swirl and transfer cells to another bottle. Do this until all the cells are collected in one bottle.
14. Centrifuge in CRU at 2,000rpm for 10min to collect cells.
15. Parafilm two 50ml centrifuge tubes and put on ice (first weigh each centrifuge tube).
16. Take out buffer A and put on ice.
17. Pour out supernatant and resuspend cells in 70ml of phosphate buffer (first about 40ml to resuspend cells and then 10ml to wash out the remaining cells).
18. Transfer into two 50ml centrifuge tubes. Centrifuge in Sorvall at 2,000rpm (653g) for 15min.
19. Put Dounce in a bucket full of ice.
20. Parafilm two 50ml centrifuge tubes and put on ice (first weigh each centrifuge tube).
21. Add DTT to buffer A.
22. Pour off supernatant. Determine packed cell volume (PCV) for both pellets by measuring the weight of the centrifuge tube plus PCV.
23. Resuspend the cells from one centrifuge tube with 1 PCV of buffer A. Transfer to Dounce. Wash centrifuge tube with 1 PCV of buffer A and transfer to Dounce. If the capacity of the Dounce (40ml) is exceeded than discard the excess. Let sit on ice for 20 min. Mix well every 2min by pipetting up and down with circular motion.
24. Take out pepstatin and leupeptin and put on ice.
25. Dounce 25 strokes with pestle “B” (tight). Make sure you hold the Dounce by the neck for increased stability. Towards the end the strokes become harder.
26. Transfer into a 50ml centrifuge tube and centrifuge at 3,500rpm (1999g) for 15min.
27. Remove supernatant by aspiration. If one looks carefully, three layers should be visible. Aspirate the top two layers and keep the bottom one. Determine packed nuclei volume (PNV) by the same method as when determining the PCV. Typical PNV = 4-5ml. Clean the inside of the centrifuge tube and keep nuclei on ice.
28. Repeat cell lysis with the second batch of cells and determine the PNV.
29. Take out buffer C (low salt and high salt) and put on ice.
30. Put PMSF in 37°C water bath to make sure crystals dissolve.
31. Arrange an ice bath: the cover of a P1000 tip box + ice + 50ml beaker covered with saran wrap.

32. Add DTT, PMSF, pepstatin and leupeptin to both low and high salt buffer C.
33. At this stage there are two batches of nuclei with a combined PNV of 8-9ml.
34. The next steps involve manipulations of nuclei that tend to stick to centrifuge tubes and serological pipettes. This loss of nuclei should be determined by weighing the centrifuge tubes and serological pipette before and after they come into contact with the nuclei.
35. Resuspend the first batch of nuclei with 0.5 PNV of low salt buffer C by pipetting up and down with a serological pipette. Only pipette 3ml at a time.
36. Transfer the resuspended nuclei into the second batch and resuspend again.
37. Transfer the resuspended nuclei to 50ml beaker in ice bath.
38. At this point the amount of nuclei lost should be determined (only 2/3 of this volume are nuclei since 0.5 PNV was added to them). Look at the following example to determine how much high salt buffer C to add.
    Measured PNV before nuclei resuspension = 8.174ml
    Nuclei lost = 1.500ml (0.184 PNV)
    PNV of high salt buffer C to be added = (1PNV – 0.184 PNV)/2 = 0.408 PNV
        If this PNV is **smaller** than **0.425** PNV then:
            Volume of high salt buffer C to be added = 8.174ml * 0.408 PNV = 3.33ml
        If this PNV is **larger** than **0.425** PNV then:
            Volume of high salt buffer C to be added = 8.174ml * 0.425 PNV = 3.47ml
39. Add slowly, drop-wise 0.425 PNV of high salt buffer C with a transfer pipette (add through an incision in the saran wrap covering the beaker). Add 1 drop/10s or after the previous drop has cleared. The stirring should be very slow but fast enough to avoid build up of high salt at the location where it is being added. This step typically takes about 40min. Towards the end of the high salt addition a "blob" starts to form. No alarm as this is expected.
40. Put Dounce in a bucket full of ice.
41. Transfer to Dounce with the help of a spatula. Place a magnet underneath the beaker so that during the transfer the magnetic stir bar inside the beaker remains immobilized.
42. Dounce 1 stroke with pestle "A" (loose).
43. Transfer to a 50ml beaker that has a stir bar and stir on ice bath for 30min. Cover the beaker with saran wrap. Again stirring is slow. As time progresses, the "blob" should become less viscous.
44. Parafilm a 50ml centrifuge tube and put on ice.
45. Transfer to a 50ml centrifuge tube by using a spatula, and a magnet underneath the beaker. Centrifuge at 11,000rpm (19,745g) for 30min in the Sorvall.
46. Put PMSF in 37°C water bath to make sure crystals dissolve.
47. Add DTT and PMSF to each 1L of dialysis buffer.
48. Put high neck 1L beaker on ice and fill with dialysis buffer. Put in dialysis tubing.
49. Take out the supernatant slowly and put into one-end-double clipped dialysis tubing. The supernatant should be clearly visible and very easy to draw out with a Pasteur pipette. Use as much dialysis tubing as necessary to maintain a high surface to volume ratio.
50. Double clip the tubing to seal it and dialyze at 4°C. Stir at a reasonable speed making sure that the tubing does not get sucked to the bottom.
51. Change dialysis buffer after 2.5hr.
52. Dialyze until the conductivity of the extract equals that of the dialysis buffer (about 160μMHO). This should take 4 to 5hr.
53. Aliquot 100μl into pre-chilled cryo tubes (total extract is about 5ml).
54. Quick freeze in liquid nitrogen and store in liquid nitrogen tank.