

**Hirt Supernatant DNA Preparation
(RL version)**

This procedure is based on the method described in Schwartzman *et al.*, MCB 10:3078-3086.

Grow $\sim 8 \times 10^8$ cells for one large prep.

Spin the cells down in 6 separate bottles.

Resuspend each pellet in 23.5 ml of 10 mM Tris-HCl, pH 8, 10 mM EDTA (5.7×10^6 cells/ml).

Transfer to 50 ml tubes. Add SDS to 0.6%, *i.e.* 1.4 ml. Mix gently until evenly lysed. It is important for lysis to be complete, so take the time, but don't be too rough so that chromosomal DNA is sheared excessively.

Add NaCl to 1M, *i.e.* 5 ml. Mix slowly and gently until the NaCl is incorporated. This may take some time but mix gently throughout, again you don't want to shear too much.

Leave the mixture overnight in an ice bucket in the cold room.

Centrifuge 3-4 hr at 12000 rpm ⁽⁵⁾ in the SW28 rotor at 4°C. The Hirt JMB procedure indicated 17,000 x g. Be sure that 12,000 rpm is at least 17,000 x g.

Collect the supernatant carefully into a fresh tube. Add Proteinase K⁽¹⁾ to 100 µg/ml. Incubate at room temperature for 1-2 hrs. Some procedures do this at 37°C or 42°C but RT seemed to work.

Extract with phenol once, with phenol/chloroform/isoamyl alcohol once and with chloroform/isoamyl alcohol once.

[Adjust to 1M ammonium acetate and add 2 volumes of ethanol.; salt is already 1M so addition of AmAc should not be necessary]. Mix well and leave at -20°C.

Centrifuge at 12000 rpm in the SW28 rotor and resuspend the pellet in 6 ml of TE8.

Add RNase A to 100 µg/ml and RNase T1 to 100 units/ml. Incubate at 37°C for 30 min. ⁽²⁾

Extract with phenol/chloroform/isoamyl alcohol once and with chloroform/isoamyl alcohol once.

Add NaCl to 0.3 M ⁽³⁾ and 2 volumes of ethanol.

Centrifuge in the SW41 rotor for 1 hr at 12000 rpm.

Resuspend so that the final volume is 1 ml.(4)

Digest with restriction enzymes, do BND columns and run 2D gels.

The least I have used for one neutral/neutral 2D gel is 0.3 ml of the 1 ml sample. In this experiment, bubbles were visible overnight for p174 which is about 5 copies/cell in Wilson/p174.

Notes

P. Galgano obtained Hirt extracts from J. Hearing. PG did 2D gels 4/19/95. Obtained very strong bubble arc.

See Hearing manila folder and CS lab protocols for Hirt proc. Must do RNase. Paul did RNase A + T1. Then phenol. The plasmid was pHEBo in Raji. Hearing has this cell line. Prepared by electroporation. Hyg. selection. The Hearing procedure does not involve using proteinase K.

Notes

1. The Hearing procedure does not use proteinase K so this is probably optional.
2. At this point CS suggests to raise the salt conc. before the phenol treatment. Make it 1x NET as we do for the total DNA prep. procedure. --Add 10X NET 8 and water to achieve a final concentration of 1X NET 8 in 10 ml , mix gently.

10X NET 8

1.5 M NaCl	87.66 g
0.5 M Tris-HCl [pH 8.0]	60.5 g
0.15 M EDTA [pH 8.0]	55.84 g

3. If you raise the salt conc. using NET before the phenol treatment, then you do not have to add additional NaCl for the pptn.
4. Before you digest, check the DNA conc. The yield could be as low as 70 ug. Too high a yield (more than 500) prob. indicates you have RNA contaminating the prep. RNA is not good since it will overload the BND column. You can compare the OD of the prep and the DNA conc. calculated from the Hoechst test or the DABA test. If the OD is much higher, this indicates RNA (or ribonucleotides) is present.
5. For SW28 rotor at 12,000 rpm, at the avg. radius the force is about 19.700 x g.

Hirt fractionation for isolation of plasmid DNA from transfected BJAB cells

1. Transfect in 5 μg of a plasmid of interest into 8×10^6 BJAB cells and incubate cells 4 days to allow at least two rounds of DNA replication.
2. Count cells and record volume of culture.
3. Harvest cells by centrifugation at 1,200 rpm for 7 min 18 C.
4. Wash with 2x 10 ml of cold 1x PBS+ 1mM EDTA, and spin as above.
5. Resuspend in 4 ml of 20 mM Tris.HCl, pH8.0, 10 mM EDTA.
6. Lyse cells by addition of 0.25 ml of 10 % SDS and mix gently.
7. Add immediately 1.06 ml of 5 M NaCl and mix gently.
8. Incubate on ice 10 hr.
9. Centrifuge at 12,000 x g for 40 min, 4C^o (or 9,000 rpm in JS 13.1 rotor).
10. Transfer supernatant to a new tube. and extract DNA once with 4 ml phenol saturated with TE.
11. Precipitate DNA with isopropanol.
12. Recover DNA and dissolve in 400 μl TE. Extract first with TE-saturated phenol and then with phenol/chloroform.
13. Take DNA from 1×10^7 cells and digest to completion with a single cutter for the plasmid and then with Dpn I to chop down unreplicated plasmid DNA.
14. Resolve digested DNA on a 0.8 % agarose gel.
15. Do Southern blot assay.