Plasmid midiprep protocol – Old-school method (phenol/chloroform)

Hazards: Phenol is toxic and corrosive to skin and eyes. Chloroform is toxic by inhalation and will also damage eyes. NaOH is corrosive to skin and eyes. SDS powder is an irritant in eyes or inhaled.

NOTES

This protocol will work well for plasmids that have a medium to high copy number (say 20-200 copies per cell), and for plasmids that are small to medium in size (say 2-20 kb). For low copy plasmids (1-2 copies per cell) and/or very large plasmids (>20 kb) you need to use special procedures, and the below protocols will not work well, if at all. The solutions needed for the plasmid prep are listed at the end of the protocol – make sure you have these ready before starting the procedure!

1. Inoculate 50 ml of LB broth containing the appropriate antibiotic with a large loopful of your *E.coli* culture from a fresh plate of the same medium ('fresh' here means less than ~2 weeks old).
Grow overnight (16-24 h) at 37°C with shaking.

It is critical to remember to add the antibiotics to the LB broth. If you forget this, you won't get any plasmid. Using the correct type and concentration of antibiotic correct is also essential. Some common antibiotics and their typical concentrations are shown below:

Ampicillin: 100 µg/ml	Chloramphenicol: 25 µg/ml (usually) or 12.5 µg/ml (for fosmids)
Kanamycin: 50 µg/ml	Tetracycline: 10 µg/ml (light-sensitive! wrap plates/broths in foil)
Streptomycin: 200 µg/ml	Gentamicin: 10 µg/ml

2. Aseptically transfer (pour) the cells into a 50 ml Falcon tube. Find or make a balance tube with the same volume of water. these, Estimating equal volumes by eye (+/- 1 ml) is OK for low speed centrifuges (e.g. Centaur /Centurion), but a balance should be used for more sensitive machines or when spinning at higher speeds (>3000g) (for these, need accuracy +/- 0.1 ml).

3. Centrifuge at 4000 rpm (~3000 g in Centaur/Centurion machine) for 10 minutes. This can be done cold or at room temp, it doesn't matter. You can spin faster, up to say 7000 g, but above this point, faster is not better, and cell pellets will be hard to resuspend.

4. Pour off supernatants into culture waste, resuspend the cell pellet in 2 ml TE by vortexing.

5. Add 4 ml "lysis solution" to the cell suspension, mix the tube by rapidly inverting it approx 10 times. Leave at room temp for 10 min. (no longer!).

6. Add 3 ml of ice-cold "precipitation solution". Mix by rapidly inverting the tube 10 times – its essential that the K-Acetate is thoroughly mixed in. Viscosity should disappear, and a white precipitate should appear. Keep the mixture on ice for at least 15 min (up to 30 min).

7.Spin at top speed (4000 rpm) in cold Centaur centrifuge for 15 min. Recover tube immediately and handle gently (pellet is soft and easily resuspended). Pour the supernatant into a new 50 ml Falcon tube. Try to avoid the white junk, but don't worry if little bits of it get transferred. Discard tube with white junk into culture waste.

8. Add an equal volume of isopropanol (~7 ml), and mix by shaking briefly, then put on ice 15 min. This step precipitates the plasmid DNA.

9. Spin at top speed (4000 rpm) in Centaur centrifuge (doesn't need to be cold) for 15 min. Pour off the supernatant into culture waste, and keep the pellet. The pellet contains plasmid DNA, but note that a lot of RNA and salt are also precipitated by the isopropanol, and will contribute to the pellet

10. Add 10 ml of 80% ethanol (vol/vol) to the pellet, and resuspend by brief vortexing or shaking. You don't need to disrupt the pellet into tiny bits, just try to get it off the wall of the tube. If it doesn't come off, don't panic, this doesn't matter too much. Leave the 80% ethanol to soak the pellet for 5-10 min at room temp; this removes some of the excess salt from the pellet.

11. Spin for 15 min in Centaur centrifuge (4000 rpm, doesn't need to be cold). Pour off the supernatant, being careful not to lose the pellet into the culture waste!

12. Give the tube another quick spin to collect residual ethanol at the bottom, then remove this with a pipette, being careful not to disturb the pellet. Then take some paper towel and tube to the 50°C incubator / heat block, lay the tube flat, and allow a further 5 min to remove residual ethanol and isopropanol (inspect tube for traces of liquid, reincubate until dry).

13. Redissolve the pellet in 2 ml TE containing 100 µg/ml RNAse. To assist dissolution, tap the tube vigorously for ~ 30 sec. Don't vortex from this point onward, excessive physical shearing forces can damage the DNA, even with smaller plasmids. You can heat if necessary to help dissolution. (eg. 50°C, 10-30 min). Note that sometimes there will be 'bits' remaining that don't dissolve – these can be chunks of the white junk from the K-acetate precipitation step, or can be chromosomal DNA. Note that plasmid DNA is much more soluble than chromosomal DNA, and dissolves preferentially. The solution at this stage should become slightly viscous (if you tap the tube, it won't behave like water), this is due to traces of chromosomal DNA that are still present.

14. Incubate 37°C for 30 min to allow the RNase to work (its not in its preferred buffer here, but it doesn't matter so much, this is one of the most robust enzymes in the world!)

15. < work in fume hood for this step> Suck up 500 µl phenol: chloroform: isoamyl (PCI) from under the aqueous layer in the reagent bottle, and transfer to a fresh Eppi tube. Repeat with another aliquot of 500 µl of PCI in a second tube. Pipette 1 ml of your plasmid prep. into the first tube of PCI, and another 1 ml into the second tube of PCI. Ensure caps are on tightly. Remove and discard gloves, and replace with fresh pair.

16. Take tubes to vortex mixer, and vortex for ~5-10 sec until a uniform milky white emulsion is obtained. It's important to hold down the caps of the tubes to ensure they remain closed during vortexing. Then centrifuge tubes for 5 min at ~10,000 rpm.

17. <return to fume hood> Place two fresh Eppi tubes in your rack, with lids open. Suck up 500 µl of chloroform:isoamyl alcohol (CI) from under the aqueous layer in its bottle, and add to the first tube, then repeat with another 500 µl into the second tube. Warning: the CI reagent will drip out of the pipette tip spontaneously, so position the CI reagent bottle right next to your eppi tubes to minimise spillage.

18. Transfer ~800 µl of the top phase (aqueous) of the first plasmid prep tube into one of the tubes containing the CI reagent. Discard the bottom phase (PCI) and the white interface layer into the

phenol/chloroform waste. Avoid the white junk at the interface between phases – it's better to leave a bit of DNA behind than to carry over phenol or junk into the next stage of the prep. Repeat with the next tube of plasmid.

19. Ensure caps of tubes are on tightly. Discard gloves, and put on fresh pair. Vortex and then centrifuge the tubes, exactly as described above for the PCI extraction step. Note that at this stage, you won't get a milky white emulsion, the liquids will remain more-or-less as two separate layers.

20. <return to fume hood> Set up four fresh Eppi tubes. To each tube, add 1 ml of cold 100% ethanol (keep this reagent in the -20°C freezer, remove just before use), and 40 µl of 3M Na-acetate. Then add 400 µl of the top phase (aqueous) from one of your plasmid preps to the first tube of ethanol/acetate, close the lid and shake briefly. Then repeat with another 400 µl of the aqueous layer from the same plasmid prep tube into the second tube of ethanol/acetate. Discard the CI-containing tube into phenol/chloroform waste. Repeat these steps for the second plasmid prep tube. At the end, you should have four tubes, each with ethanol / acetate / DNA mixture in them. These should look slightly cloudy as the DNA starts to precipitate.

21. Incubate the tubes >2 hr at -20°C to precipitate the DNA (overnight is fine). You can incubate at -80°C instead for a shorter time (e.g. 20 min), but you risk precipitating more salt at the lower temp.

22. Centrifuge the tubes for 10 min at ~10,000 rpm (cold or room temp is OK). Pour off the supernatants into culture waste. Rinse the pellets with 500 µl of 80% ethanol, then centrifuge, pour off ethanol, drain and dry pellets (exactly the same as the above steps #10-12, just using a smaller volume of 70% ethanol for rinsing). The pellets may look very small and/or be quite hard to see – this is normal. A pellet of *pure* DNA should be near-transparent, any opacity comes from salts. It's a good idea to note/mark which side of the tube is facing the outside of the centrifuge before spinning the tubes, to help localise the pellet.

23. Redissolve the plasmid DNA in one of the tubes in 100 µl of EB. Tap or flick the tube to assist dissolution (~30 sec of tapping should be enough, don't vortex it). Transfer the 100 µl to the next tube, and repeat. Do this two more times, until all four DNA pellets are dissolved into the same 100 µl aliquot of EB. The solution should appear somewhat viscous.

24. Expected yields range from approx 5 µg plasmid per ml culture (eg pUC19/pGEM-T/pSB1C3) down to 0.2 µg plasmid per ml culture (eg. RSF1010) – this all depends on the copy number of the plasmid, and how carefully you performed the procedure! Final expected DNA conc. may range from 10-1000 ng/µl. Digest 2 µl of the plasmid preparation with an appropriate restriction enzyme (eg. an enzyme that cuts once or twice) (see restriction digest protocol), and run on an agarose gel to evaluate yield. The Nanodrop is not accurate for plasmid preps.

25. If your plasmid DNA looks like it is too dilute to use effectively for cloning or sequencing or other purposes, you can repeat the ethanol/acetate precipitation step, and redissolve in a smaller volume (eg. 20 µl). If the plasmid is not performing well in digests (impure DNA), you can repeat the chloroform/isoamyl extraction AND ethanol precipitation steps, and it is also not a bad idea to also dissolve in a smaller volume to make up for losses due to the extra steps performed (e.g. 50 µl EB)

26. Store plasmid DNA cold (4°C) or frozen (-20°C). Cold is better if you are going to use it a lot (repeated freeze/thaw cycles are bad for DNA), but frozen is better for very long-term storage.

PLASMID PREP SOLUTIONS (OLD-SCHOOL METHOD, see Sambrook Appendix 1)

TE : 10 mM Tris, 1 mM EDTA, pH 8. Sterilised by autoclaving.

Its easiest to make this solution by first preparing a 1 M stock of Tris-HCl (adjust pH to 8) and a 0.5 M stock of disodium EDTA (adjust pH to 8), then mix the appropriate volumes of these in RO water, and autoclave (also autoclave the conc. stock solutions!) <wear safety glasses when doing any pH adjustments with acids or bases!>

Lysis solution: 0.2 M NaOH, 1% SDS. <wear safety glasses!>

It is traditional to prepare the lysis solution fresh each time from separate stocks of NaOH (2 M) and SDS (10%). i.e. just before you do the plasmid prep, add 800 µl of 2 M NaOH and 800 µl of 10% SDS to 6.4 ml of sterile RO water. However, if you keep the solution in a tightly-closed bottle and minimize air exposure, it will stay good for a month or two.

The 2 M NaOH stock solution can be sterilized by autoclaving. Note that over time, the 2 M NaOH stock will start to look cloudy due to precipitation of Na2CO3 (it pulls CO2 out of the atmosphere) – this effect reduces the amount of available hydroxide, and the solution should therefore be re-made approx. every 6 months, or whenever it appears cloudy. (note that this cloudiness is not microbial growth).

The 10% SDS stock cannot be autoclaved (tends to precipitate), so the best way to make this is to add the appropriate amount of SDS powder to the appropriate amount of sterile RO water (autoclaved). If you do this while the sterile water is still hot, this helps to dissolve the SDS. This solution will be close-to-sterile, and there are no microbes known that can grow in 10% SDS !

Precipitation solution: 3 M potassium, 5 M acetate, pH 4.8. Can sterilise by autoclaving but not really necessary, nothing will grow in this. If you make it in sterile water in a sterile bottle, this is fine.

This solution is 3 M with respect to potassium, but 5 M with respect to acetate…tricky, eh? The way this works is that you first make a 3 M solution of potassium acetate, then you adjust the pH down to 4.8 using glacial acetic acid <safety glasses!>. Its best to check the appendix of Sambrook if you are not sure about how to make this, there is a detailed description there.

Na-acetate: 3M sodium acetate in RO water, sterilise by autoclaving if you like. (note that you don't need to adjust pH of this solution)

EB (Elution buffer): 5 mM Tris-HCl, pH 8. Autoclaved.

RNAse: Dissolve RNAse at 10mg/mL in 10mM Na-acetate (pH 5.2) and boil (100 degrees) in heat block for 15 min to destroy traces of DNase. Once cooled back to room temperature, neutralise pH with 0.1 volume of 0.1M Tris-HCl (pH 7.5). Store in frozen aliquots at -20°C. (these aliquots can be repeatedly frozen and thawed). Be careful not to spread the RNase around the lab; if other people are doing RNA work, you will create problems for them.