**Good aseptic technique and general lab safety**

Inoculating or transferring cultures

* Swab bench before starting.
* Work close to the Bunsen burner. Use the ‘Hot flame’ (bright blue cone inside lighter blue cone) to get a strong updraft, to flame the necks of bottles, and to flame your loop. The ‘Safety Flame’ (yellow) is only good for lighting the ethanol on the glass spreader when you are doing spread plates, it has no other real use.
* Turn off the Bunsen burner whenever you aren’t immediately at your bench using it.
* Keep the work area clear of clutter and mess
* Don’t do culture work directly on top of your lab book or other paper notes – if there is a spill, you don’t want to have to autoclave your lab book !
* If working with slow-growing organisms, it can be helpful to swab hands or gloves with 80% ethanol. If you do this, don’t get the ethanol near the flame, and wait for it to evaporate off hands before lighting Bunsen!
* Flame the neck of bottles and tubes when opening and closing them (pass thru the Bunsen flame briefly e.g. back and forth 3 times over 3 seconds).
* Be mindful of where you are putting things down – e.g. don’t put down your inoculating loop on the bench with culture in it, flame it first, then put back upright in correct holder
* Secure your lab coat or lab gown sleeves so that they don’t flap around – these are a common source of contamination. Sticky tape them or elastic band them to fit snugly, or roll them up a little bit (don’t do the later if you are working with corrosives or toxics or UV radiation ! in those cases, its important to have the sleeves all the way down)

Sterile bottles, solutions, and gear

* Make sure everything is LABELLED ! With the chemical, the date, and your name.
* Make sure any hazardous reagents are clearly labelled with the chemical name, the concentration, and the nature of the hazard (e.g. flammable/corrosive/toxic)
* When you take lids off, don’t put them down on the bench unless it’s absolutely necessary; and if you have to do this, put them upside down, so the screw thread is facing upwards. SURFACES are by far the biggest problem for microbial contamination
* Don’t leave the lids off bottles for any longer than is necessary.
* Getting sterile eppi tubes out of a sterile beaker:
	+ acceptable way #1. take off foil top, carefully pluck out tube by its base or edge, place carefully in tube rack, repeat if necc., then replace foil
	+ acceptable way #2. take off foil top, shake out some tubes into foil, carefully place them in rack, careful not to touch any part of the inside of the tube, replace foil.
	+ UNACCEPTABLE: plunge your hand into the beaker and rummage around vigorously, then emerge triumphantly with a tube, leave the foil lid off until tomorrow.

 Pipetting with Gilsons

* Swab down your pipettes with 80% ethanol at least once each day, before starting work. Also do this if you change from doing microbiology to DNA work, or when changing from working with one type of microbe to another, or whenever you think it has become contaminated.
* If you have persistent issues with contamination, a more serious cleaning of pipettes is required. Dissemble the pipette and soak in bleach (0.5% hypochlorite = a 1/10 dilution of household bleach, freshly prepared), so that all parts of the pipette are covered. Let them soak for 30 min, then rinse extensively with water, then with 80% ethanol, then allow to dry. Don’t leave pipettes in bleach longer than 30 min, or you will rust the metal parts.
* Remember that ONLY THE TIP IS STERILE ! Even if you have swabbed it with ethanol, the main body of the pipette is still contaminated with microbes and enzymes and DNA etc. The body of the pipette should not come into contact with anything that needs to stay sterile (e.g. it the inner wall of bottles or Falcon tubes when you are pipetting liquids out of these)
* Hold the pipette VERTICAL! ….or as close to vertical as possible. If you hold it sideways, the liquids may run up into the barrel of the pipette, which could be catastrophic for your PCR or your culture, when they run back down into the tip, carrying junk with them.
* Plugged pipette tips are not needed for most kinds of work, if you are careful with your pipetting. The only situations where these are really essential are for RNA work, and for 16S rDNA PCRs – the latter is exceedingly sensitive to all kinds of DNA contamination.
* Be careful when getting the tips out of the box. Open it all the way, put on the tip firmly, move the pipette away, then close the box. Don’t hit the tip on the edge of the lid, and don’t leave the tip box open after you have got the tip out.

Setting up PCRs and other enzyme reactions

* Swab down bench and pipettes before starting. Label all tubes before starting. Read through the procedure carefully before starting.
* Keep all enzymes in the freezer or on ice at all times. This means restriction enzymes, ligase, polymerases, reverse transcriptase, phosphatase, kinase, Gibson assembly mix, etc. The only exceptions here are thermostable polymerases (Taq, Pfu, Phusion); these can be kept on the bench for a little while during setup of a reaction (they are thermostable!).
* Keep all DNAs (plasmids, PCR products, genomic DNAs) either in the fridge or the freezer whenever they are not actively being used. DNA can sit on the bench for a little while (a few minutes up to an hour or so), but don’t leave it at room temp longer than this.
* Double check all buffer and incubation requirements (time? temperature?)
* Make up the buffer first (to 1x conc), \*then\* add the enzymes and/or DNA.
* Handle tubes carefully: don’t touch any part of the inside of the eppi tubes or PCR tubes, this includes the inside part of the lids! This takes some care and some practice. Especially be careful when opening Eppi tubes, its easy to push your thumb up onto the inside of the lid; use your thumbnail (not your whole thumb) to carefully flick open the lid from the outside catch
* Be extra careful during any procedures where you are putting tubes on ice. The ice (or melted ice) must not get into your tubes ! So don’t push the tubes too far down into the ice, and handle them carefully.

**Making agar plates**

1. Figure out the total number of plates you will need, then multiply x 25 to get the approx. amount of agar you will need (in ml). Its better to make more than you think you need ! Alternatively, just make up the agar in multiples of 400 ml, this a standard amount that will make ~16 plates (this is about right for one ‘sleeve’ of sterile petri dishes, which have 20 dishes in them).

2. Add all the required media ingredients (eg. for LB agar, this is 10 g tryptone, 5 g yeast extract and 5 g of NaCl per litre) – make sure you correct these ingredient weights for the amount of agar you are making (e.g. multiply by 0.4 if you are only making 400 ml).

DON’T ADD THE AGAR YET ! (this doesn’t dissolve, it settles out)

DON’T ADD ANTIBIOTICS, TRACE METALS, or TWEEN yet, if these ingredients are needed.

3. Dispense the media into multiple lots of 400 ml in 500 ml media bottles. Make sure that the media bottles have a plastic pouring ‘lip’ on them, not just a naked glass top; the lip is important to be able to pour the agar neatly. You could also make multiples of 200 ml in 250 ml bottles, but don’t make >400 ml in a 1 L bottle – this is too heavy to easily pour plates with.

4. Once you have dispensed the media into the bottles, now you can add the appropriate amount of agar to each bottle. This is 17 grams per litre (=6.8 grams per 400 ml). DON’T USE AGAROSE!

5. Add bottle caps. Don’t screw these on all the way, leave them a bit loose. Hold the caps in place with a small piece of autoclave tape.

6. Arrange bottles of agar in a wire rack, add a piece of masking tape on one corner with your name and the lab number (566), then take to the autoclave. If the agar media will not be autoclaved on the same day, keep it in the cold room until the next day (microbes will start to grow within a few hours, especially in rich media like LB)

7. When the agar comes out of the autoclave, if its still molten and very hot, put it in the hot water bath (~60°C) until it cools down to pouring temperature (~15 min). If you don’t want to use it immediately, leave on the bench until it solidifies (if it hasn’t already), then tighten the cap and it can be stored indefinitely at room temp. If agar has solidified and you want to use it straight away, microwave it (lid loose!) for approx 10 min on 50% power (these settings are for 400 ml of agar at room temp, adjust accordingly!), then put in hot water bath for ~15 min.

8. Prepare laminar flow hood for plate pouring as follows: give it a blast of UV for ~15 min (switch in upper position, with the doors closed!), then turn off UV light (switch in middle position), take out the doors, then turn on the light and fan (switch in bottom position). Swab down with 80% ethanol.

9a (optional). If you are working with very slow-growing microbes and/or you are making rich medium non-selective plates, you can add an extra layer of sterility to the proceedings by wearing gloves, and rinsing the gloves thoroughly in 80% ethanol before you start pouring the plates.

9b. (optional). If you don’t have access to the laminar flow hood, you can make plates on your regular lab bench. This is usually fine, but swab down the bench with ethanol first, and work close to the Bunsen flame. (if you swab your gloves with ethanol, be careful to let this evaporate before turning on the Bunsen burner!)

10. Collect one bottle of molten agar from the waterbath, wipe down the outside with paper towel, and take to your bench. Add any “after-autoclaving” additions at this stage, from filter-sterilised stock solutions (this may be antibiotics, or trace metals solution, or Tween etc). Mix by swirling for 10-20 seconds, don’t shake the bottle as this will cause persistent bubbles. Its good practice to write all the additions on the bottle, then tick them off after each addition. Forgetting these is bad!

11. Take the final agar to the laminar flow hood. Open up one sleeve of plates (at the ‘base’ end), and slide them out. Keep the sleeve! Label all the plates with the type of medium (e.g LB-Cm25). Pour the agar into the first plate; use a single smooth pouring motion. Stop when it reaches all the edges. Put the lid on. Do the next plate. Repeat. Don’t stack up the plates in big towers, they will take longer to cool.

12. Leave the plates for about 10 min to alow the agar to solidify. Then invert the plates with the lids off to allow them to dry for 20-30 min. The setup should look like this:



or this: 

If you don’t dry the plates, you won’t get nice spread-plates, and the risk of contamination is greater from other microbes ‘climbing’ into the plate over the edge.

13. Pack the plates back into the sleeve, then label the sleeve with the type of agar and the date of manufacture and your name. (don’t label the individual plates with the date, this is confusing, since the date written on an agar plate is usually the date it was inoculated).

14. Store plates in the cold room. They should stay good for >6 months if you have been careful !

**Growing bacterial cultures (*E.coli*)**

**NOTES:** It is good practice in most kinds of bacteriology experiments to be sure that the culture you are working with is PURE and FRESH.

To check purity, make a streak plate on non-selective medium (e.g. plain LB or TSA or R2A), and incubate at 30°C for at least a week to check for contaminants. (don’t use this plate for any further work, as it will have lost any plasmids).

To ensure freshness, use a -80°C stock of the culture, or use a streak plate that is <1 week old. Ideally, when starting up an experiment like a plasmid prep or a competent cell prep, you should use a culture that is only 1-3 days old.

1. Inoculation: cultures can be inoculated from broths or plates or glycerol stocks. Agar plates are best because you can see by eye that the culture is pure (anything could be hiding in a broth!). When inoculating from a plate, use a loopful of growth from the streakline (if you are already sure the culture is pure!). If you pick a single colony, there is a small risk that you will pick some kind of mutant in the culture, ie. that the single cell that founded that colony was not representative of the whole culture. You should use enough inoculum to give you ‘just visible’ turbidity; this corresponds to an OD600 of ~0.05. In practice this is a small loopful into ~10 ml broth, or a fat loopful into ~100 ml broth. At this inoculation level, the culture will grow up well overnight (16 hr), and be in early stationary phase the next morning.

2. What kind of culture vessel? One of the nice things about E.coli is that you can grow it in any kind of culture vessel, this can be a ‘proper’ vessel ie. an Erlenmeyer flask, or a ‘makeshift’ vessel, ie. a media bottle. The bacteria will grow fastest and to highest densities if you grow them aerobically, with shaking, in a system where there is a very large headspace to liquid ratio – the Erlenmeyer flask is always better if you can be bothered. ‘Baffled’ Erlenmeyer is even better still, since this gives excellent aeration. If you are trying to overexpress proteins, this stuff matters (since it impacts the cellular physiology), but if you are doing a plasmid prep, it is less important.

What you DON’T want to do is fill the growth vessel all the way to the top with liquid, they will grow very poorly in that case. A nice compromise between convenience and good growth is to use e.g. 50 ml of culture in a 250 ml media bottle (ie. the vessel is only 1/5-full of liquid). You can tightly cap the bottle, since there is plenty of air in the headspace (200 ml) for them to grow aerobically to stationary phase, and this minimises the risk of spills if the bottle falls over in the shaker. Also keep in mind that E.coli is a facultative anaerobe, and can grow by fermentation even when the oxygen runs out. (though it grows much better aerobically).

3. Storage. *E.coli* cultures will remain viable for at least 2 months when kept either at 4°C or at room temp (if kept wrapped in parafilm or plastic bag so they don’t dry out), but plates as old as this are not ideal, and may lose plasmids, or their plasmids may undergo rearrangements etc. Fresh is best ! So either restreak them at approx. monthly intervals, or make glycerol stocks (below).

The best practice for long-term storage is to make a glycerol stock of the culture – do this by growing up a streak plate of the culture on whatever is the appropriate antibiotic agar, then scrape off all the growth from the plate into 500 µl of sterile 20% glycerol (in ROW), vortex well, and then put at -80°C. Label the tube on the top and on the side! Use high-quality labels. Cheap labels or ‘permanent’ marker do not survive freezing very well.

**Plasmid miniprep protocol (5 ml culture) – Spin Column Method**

**NOTES BEFORE STARTING**

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! Note that some of these are different from the “old school” solutions, and also some are different from standard plasmid kit buffers. These should work with any kind of silica-based purification column and could also be adapted to purification using free silica particles (e.g. diatomaceous earth).

1. Inoculate 5 ml of LB broth in a narrow-neck McCartney bottle containing the appropriate antibiotic with a 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. Centrifuge the culture at 4000 rpm (~3000 g in Centaur/Centurion machine) for 10 minutes. You can spin it in the McCartney bottle, there is no need to decant into a centrifuge tube. Make sure you find appropriate balance(s) if you have an odd number of bottles – these should be weighed since it’s hard to estimate volumes in McCartneys by eye, and the bottles may have different weights.

3. Pour off supernatant into culture waste, resuspend the cells in 1 ml of **buffer EB** by vortexing. Transfer to 1.5 ml Eppendorf tube. Centrifuge 1 min at ~10,000 g. Pour off supernatant, keep pellet.

(this step efficiently transfers all the cells into a smaller tube…it is possible to skip this step and directly resuspend in 250 µl buff. P1 in the McCartney bottle, but this risks losing a lot of cells)

4. Resuspend the cell pellet in 250 µl **buffer P1** by vortexing.

 <<put on gloves and safety glasses (hazards: NaOH, SDS, guanidine)>>

5. Add 250 µl **buffer** **P2**. Mix by rapidly inverting the tube 10 times. Incubate 10 min at room temp. (No longer than 10 min!) The mixture should go clear and become viscous as the cells break open.

6. Add 350 µl **buffer** **N3**. Mix by rapidly inverting the tube 10 times. It is essential that the N3 buffer is thoroughly mixed in. Viscosity should disappear, and a white precipitate should appear. Incubate for 5 minutes at room temp. Centrifuge for 5 min at ~10,000 g.

7. Place a spin column (silica-based e.g. ‘Econospin’ brand) into its collection tube (if it isn’t already set up that way), and carefully pipette 750μl of the plasmid extract supernatant onto the column. Avoid the white pellet and any white junk that may be floating on top. Centrifuge 30 seconds at ~10,000 g. Discard flow-through into culture waste.

8. Add 700 μl **buffer PB** to the column, centrifuge 30 sec at ~10,000 x g. Discard flow-through.

 <<can now remove gloves and safety glasses>>

9. Add 700μl **buffer PE** to the column, centrifuge 30 sec at ~10,000 x g. Discard flow-through.

10. Repeat step 9.

11. Spin the ‘empty’ column one more time for 1 min at ~10,000 x g to remove the last traces of PE.

12. Discard collection tube, and place the column part with the lid open on a clean ‘Kimwipe’. Place in 60 C° oven for ~10 min to evaporate residual ethanol. Avoid touching the ‘nipple’ at the bottom of the column with your fingers.

13. Place column into an Eppi tube, and add 30 µl of **buffer** **EB** to each column. (ensure EB goes onto the membrane at the bottom, not the wall). Allow to sit for 2 min. Centrifuge 1 min at ~10,000 g.

14. 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. Note that the Nanodrop may not be accurate for plasmid preps.

**PLASMID PREP SOLUTIONS (SPIN COLUMN METHOD) – based on recipes at openwetware.org**

**Buffer P1:** 5 mMEDTA, pH 8. Sterilise by autoclaving. Then add RNAse to 300 µg/ml.

 Usually this would be prepared by starting with a 0.5 M EDTA, pH 8 stock,

 and a 10 mg/ml aliquot of boiled RNAse.

**Buffer P2:** 1% SDS, 0.2 M NaOH. Not sterilised.

Prepare from stocks of 10% and 2 M NaOH (these don’t need to be sterilised, but its not a bad idea to prepare these and the final buffer in sterile bottles or tubes containing the correct amount of autoclaved RO water). <<safety glasses!>> Keep in a tube or bottle with minimal headspace and tightly closed. Will eventually develop precipitate of Na2CO3 – remake the solution if you see this. Recommend to make fresh approx. monthly.

**Buffer N3:** 4.2 M guanidine HCl, 0.9 M K-acetate, pH 4.8. Not sterilised.

 Adjust the pH using glacial acetic acid <<safety glasses!>>

**Buffer PB:** 5 M guanidine HCl, 30% isopropanol. Not sterilised.

Prepare in a sterile bottle or tube containing the correct amount of autoclaved RO water.

**Buffer PE:** 10 mM Tris-HCl, pH 8, 80% ethanol. Not sterilised.

Prepare in a sterile bottle or tube containing the correct amount of autoclaved RO water.

**Buffer EB:** 10 mM Tris-HCl, pH 8. Autoclaved.

 Usually this would be prepared by diluting a 1M Tris-HCL pH 8 stock.

**Plasmid midiprep protocol (50 ml culture) – Spin Column Method**

**NOTES BEFORE STARTING**

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! Note that some of these are different from the “old school” solutions, and also some are different from plasmid kit buffers. These should work with any kind of silica column and could be adapted to free silica particles (e.g. diatomaceous earth).

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 2.5 ml **buffer P1** by vortexing.

 <<put on gloves and safety glasses (hazards: NaOH, SDS, guanidine)>>

5. Add 2.5 ml **buffer** **P2**. Mix by rapidly inverting the tube 10 times. Incubate 10 min at room temp. (No longer than 10 min!) The mixture should go clear and become viscous as the cells break open.

6. Add 3.5 ml **buffer** **N3**. Mix by rapidly inverting the tube 10 times. It is essential that the N3 buffer is thoroughly mixed in. Viscosity should disappear, and a white precipitate should appear. Incubate for 5 minutes at room temp. Centrifuge 3000 g for 15 min. \*Carefully\* pour off supernatant into a fresh 50 ml tube. Some of the white junk may carry over, but try to minimize this.

7. Place four spin columns (silica-based e.g. ‘Econospin’ brand) in their collection tubes and pipette 750μl of the clarified sample supernatant onto each column. Centrifuge for 30 seconds at ~10,000 x g and discard flow-through into culture waste. Repeat this procedure until all of the supernatant has been used up (ensure the supernatant is evenly distributed among the tubes at the last spin step).

8. Add 700 μl **buffer PB** to each column, centrifuge 30 sec at ~10,000 x g. Discard flow-through.

 <<can remove gloves and safety glasses>>

9. Add 700μl **buffer PE** to each column, centrifuge 30 sec at ~10,000 x g. Discard flow-through.

10. Repeat step 9.

11. Spin the ‘empty’ column one more time for 1 min at ~10,000 x g to remove the last traces of PE.

12. Discard collection tubes, and place the column part with the lid open on a clean ‘Kimwipe’. Place in 60 C° oven for ~10 min to evaporate residual ethanol. Avoid touching the ‘nipple’ at the bottom of the column with your fingers.

13. Place columns into Eppi tubes, and add 30 µl of **buffer** **EB** to each column. (ensure EB goes onto the membrane at the bottom, not the wall). Allow to sit for 2 min. Centrifuge 1 min at ~10,000 x g. Combine all the flow-through liquids into one tube. Note that you will only recover about 25 µl from each, giving a total of approx. 100 µl of plasmid DNA.

14. 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.

**PLASMID PREP SOLUTIONS (SPIN COLUMN METHOD) – based on recipes at openwetware.org**

**Buffer P1:** 5 mMEDTA, pH 8. Sterilise by autoclaving. Then add RNAse to 300 µg/ml.

 Usually this would be prepared by starting with a 0.5 M EDTA, pH 8 stock,

 and a 10 mg/ml aliquot of boiled RNAse.

**Buffer P2:** 1% SDS, 0.2 M NaOH. Not sterilised.

Prepare from stocks of 10% and 2 M NaOH (these don’t need to be sterilised, but its not a bad idea to prepare these and the final buffer in sterile bottles or tubes containing the correct amount of autoclaved RO water). <<safety glasses!>> Keep in a tube or bottle with minimal headspace and tightly closed. Will eventually develop precipitate of Na2CO3 – remake the solution if you see this. Recommend to make fresh approx. monthly.

**Buffer N3:** 4.2 M guanidine HCl, 0.9 M K-acetate, pH 4.8. Not sterilised.

 Adjust the pH using glacial acetic acid <<safety glasses!>>

**Buffer PB:** 5 M guanidine HCl, 30% isopropanol. Not sterilised.

Prepare in a sterile bottle or tube containing the correct amount of autoclaved RO water.

**Buffer PE:** 10 mM Tris-HCl, pH 8, 80% ethanol. Not sterilised.

Prepare in a sterile bottle or tube containing the correct amount of autoclaved RO water.

**Buffer EB:** 10 mM Tris-HCl, pH 8. Autoclaved.

 Usually this would be prepared by diluting a 1M Tris-HCL pH 8 stock.

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

**NOTES BEFORE STARTING**

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 ypical 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. <Put on safety glasses>. 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).

<can take off safety glasses now>.

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. Drain off excess supernatant by leaving the tube inverted on a few layers of paper towel for 5 min or so. Give it a tap on the towel at intervals to assist the removal of liquids. Then take the paper towel and tube to the 50°C incubator, lay the tube flat, and allow a further approx. 10-20 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. <put on gloves+safety glasses, and 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, keep safety glasses and gloves on> 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, keep safety glasses and gloves on> 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.

<can remove safety glasses and gloves now>

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.

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)

**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:** DissolveRNAse enzyme powder at 10 mg/ml in 50 mM Tris-HCl, 10 mM EDTA (pH 8), then boil for 10 minutes to destroy traces of DNase. 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.

**Genomic DNA extraction with CTAB** (Cetyl trimethylammonium bromide)

**Notes:** This protocol can be used on most gram-negative bacteria to obtain high-quality genomic DNA, which is both pure and very high molecular weight. The method needs to be modified for use on Gram-positives or yeast etc, by adding on extra lysis treatments at the ‘front end’ of the protocol. Remember that ‘genomic DNA’ means ‘all the DNA’, so this method extracts both chromosomes and plasmids. Note also that all waste chloroform and phenol arising from this procedure must be discarded as hazardous chemical waste (not down the sink!).

**Hazards:** CTAB\* is a strong detergent, it will damage eyes and irritate skin

Chloroform is toxic by inhalation and will also damage eyes.

 Phenol is toxic and corrosive to skin and eyes.

**Protocol:**

1. Grow culture in 5 ml broth, pellet cells (~3000 g, 10 min), discard supernatant.

2. Add 400 µl TE and 100 µl NaCl (5 M) to pellet, resuspend cells by vortexing.

 <<put on safety glasses and gloves>>

3. Add 50 µl CTAB, vortex, incubate 60C for 20 min, occasionally mixing by inversion of tube.

4. Add 500 µl chloroform, vortex and mix thoroughly, incubate on ice 30 min.

5. Spin at ~10,000 g in microfuge (cold if possible) for 10 min.

6. Slowly pipette out and retain supernatant – avoid interface material and lower phase.

7. Add 500 µl phenol:chloroform, vortex until milky solution obtained (~20 sec), spin at top speed in microfuge for 5 min, retain supernatant.

8. Add 500 µl chloroform, vortex, spin 5 min, retain supernatant.

9. Discard all waste phenol and chloroform into chemical waste bottle in fume hood.

 <<take off safety glasses and gloves>>

10. Add 1/10 volume Na-acetate (50 µl) and 2 vol. ice-cold ethanol (1000 µl) to precipitate DNA – incubate at -20C for at least 1 hour.

11. Spin at top speed in microfuge (cold if possible) 10 min, drain off ethanol, retain pellet.

12. Add 500 µl 70% ethanol, resuspend pellet by flicking, allow to sit for ~5 min at room temp, then spin and drain again.

13. Invert tube on paper towel with lid open for ~5 min to drain last bits of 70% ethanol, then transfer to 60C incubator (OK if tube is lying on its side, but lid open) for 10 min to dry residual ethanol.

14. Redissolve pellet in 100 µl EB. Heating at 50-60C (up to an hour, intermittent mixing) and/or addition of more EB may be required to dissolve all the DNA.

15. Store final DNA preparation at -20C. If you will be using it a lot, consider making multiple smaller aliquots, since repeated freeze/thawing will deteriorate the quality.

**CTAB GENOMIC DNA PREP SOLUTIONS**

**TE:** 10 mM Tris-HCl, 1 mM EDTA. pH 8.0 Autoclaved.

**NaCl:** 5 M NaCl. Autoclaved.

**CTAB:** 10% w/v CTAB. Autoclaved. May need heating (~50C) to dissolve before each use.

**Chloroform:** Mixture of chloroform and isoamyl alcohol (24:1 ratio) equilibrated with a layer of TE buffer (pH 8) on top. Not sterilized.

**Phenol:chloroform:** Mixture of phenol, chloroform, isoamyl alcohol (25:24:1 ratio) equilibrated with a

layer of TE buffer (pH 8) on top. Not sterilized.

**Na-acetate:** 3M sodium acetate in RO water, sterilise by autoclaving (don’t need to adjust pH)

**EB (Elution buffer)**: 5 mM Tris-HCl. pH 8.0 Autoclaved.

**CTAB GENOMIC DNA PREP – further notes and comments**

1. Plate-grown cells are fine also – growth scraped from half of a streak-plate is about the same amount of biomass as a fully-grown 5 ml broth. The procedure can also be easily scaled up tenfold to accommodate 50 ml broths. In this case, it is recommended to add an isopropanol precipitation step after the first chloroform extraction, then do a 70% ethanol rinse, dry pellet, redissolve in 1-2 ml TE. Then the amount of phenol and chloroform used is greatly reduced (you can use 500 µl instead of 5 ml !), and the procedures can be done in microfuge rather than big centrifuge (faster!).

2. Wash cell pellet in TE (resuspend then pellet again) before lysis if nuclease activity is a problem.

3. A lysozyme incubation can be added if cells don’t lyse well with CTAB alone. In this case, after adding TE and NaCl to cell pellet, add lysozyme to 1 mg/ml, and incubate 1 hour at 37C.

4. A proteinase K step could be added at the same point as the lysozyme step, or immediately after the lysozyme step (add prot. K to 0.5 mg/ml, incubate 60C 1-16 h)

5. Increasing the heat and duration of the CTAB step might help with tough cells (eg. 70C, 1 hour).

6. Vortexing steps can be replaced or supplemented by inversion and flicking of tube.

7. RNase A can be added to remove RNA – either add this at the start of the prep, or to the dissolution buffer used at the end of the prep. Remember to boil RNAse before use to kill any DNAse in it (see Sambrook for method).

8. Overdrying of the final DNA pellet is BAD. Its better to leave a trace of ethanol and water in the pellet (and have it dissolve easily), than to remove all moisture, and then have great difficulty dissolving the pellet. However, too much ethanol is also BAD…DNA will float out of wells on gel.

9. Elution buffer (EB) is just Tris-HCl, which gives the DNA some protection due to its pH buffering. EB wont give as much protection as TE, which also protects against nucleases. However, TE can interfere with subsequent enzyme reactions (EB won’t)

10. We have used this procedure successfully with *Pseudomonas stutzeri* strains which did not give good DNA preps with SDS-based DNA extraction methods due to polysaccharide production. The CTAB procedure would likely work with many gram-negative strains without modification, but gram positives would likely require the addition of lysozyme and proteinase K steps, and may also require modification of medium – eg. addition of glycine and/or ampicillin to weaken cells.

**Genomic DNA extraction (via bead beater) – original ‘FastPrep’ method**

**Notes:** This protocol is versatile and can be used for any kind of cells/samples (animal, plant, microbe, soil, faeces etc). Note that the DNA obtained is of lower quality than that obtained via chemical/enzymatic lysis (e.g. above protocol), and it will be sheared to ~ 1-10 kb in size. This is fine for PCR, but may not be ok for making clone libraries. Reference: Yeates and Gillings (1998)

**Hazards:** Binding matrix contains conc. guanidine thiocyanate which is an irritant. Wear gloves and safety glasses for any steps involving binding matrix.

**Protocol**

1. Prepare bead-beater tubes ahead of time: Get glass beads plus the scoop and funnel from the chemicals cabinet (under ‘G’ for Glass) and put 2 large beads and two scoops each of the medium and small size beads into each tube. Sterilise by autoclaving.
2. Add 122 µl of MT buffer and 780 µl sodium phosphate buffer to the bead beater tube, then add your sample (200-500µl volume). Leave a little bit of headspace for good beating action. For pure cultures, the pellet from a 5 ml broth or a large loopful (~half of a streak plate) is good.
3. Place tubes in bead beater and process for 30 sec at speed 5.5
4. Centrifuge ~ 10000 g for 5 min to pellet beads and debris
5. Transfer supernatant to 1.5ml microfuge tube
6. Add 125 µl PPS and mix
7. Centrifuge ~ 10000 g for 5 min to pellet the protein precipitate
8. Transfer 700 µl of supernatant to a new 1.5 ml microfuge tube. If high yield is important, you can transfer the remaining liquid (~300-600 µl) to a second tube, and process in parallel.
9. Add 700 µl Binding Matrix Suspension and mix gently for 5 minutes
10. Centrifuge ~ 10000 g for 1 min to pellet matrix-bound DNA
11. Remove supernatant (avoid Binding matrix)
12. Add 500 µl of salt/ethanol wash solution and resuspend pellet by vortexing
13. Centrifuge ~ 10000 g for 1 min and discard supernatant
14. Repeat steps 12-13
15. Invert tube with lid open on paper towel and drain off excess salt/ethanol (~5 min), then transfer tubes to 60oC incubator for ~20 mins to evaporate residual ethanol. When fully dry, the pellet should look white, not grey/brown.
16. Resuspend matrix by vortexing in 200 µl TE buffer. Incubate 60C for 10 min to dissolve DNA.
17. Centrifuge ~ 10000 g for 1 min and transfer supernatant to new tube. Store at -20oC

**Solutions:**

 **MT buffer\***: 1% SDS, 0.5% Teepol, 5% PVP40, 10 mM Tris (pH 8), 10 mM EDTA (pH 8). Autoclaved.

 **Sodium phosphate:** 0.1M Na2HPO4 (pH 7.0), sterilise by autoclaving

 **PPS:** 7.5M potassium acetate, sterilise by autoclaving

 **Binding Matrix Suspension**: (bought from MPBio = silica in guanidine solution). Can be diluted 2:1 in guanidine thiocyanate (6 M) solution to make stock go further.

 **Salt/ethanol wash solution:** 70% ethanol, 100 mM sodium acetate

(add ethanol to autoclaved 100 mM sodium acetate after cooling)

 **TE buffer:** 10 mM Tris-HCl, 1 mM EDTA. pH 8.0, sterilise by autoclaving

\* This MT recipe is modified from original version. Has more PVP and more EDTA

**Genomic DNA extraction (via bead beater). 2019 version (Neil Wilson mods)**

1. Prepare the beadbeater tubes by adding two scoops\* of tiny beads, two scoops of small beads, and one large bead to each 2 ml screw cap tube. Autoclave with lids off, loosely covered in foil. Autoclave lids separately, also loosely wrapped in foil. Put caps on tubes aseptically once cooled.

\* one ‘scoop’ is approx 100 ul volume. tiny beads are ~100-200 micron, small beads are ~500 micron, and large bead is 5 mm. All ‘beads’ are acid washed glass beads. eg. Sigma G1145-100G

2. Add 300 µl or 300 mg of sample (soil, cells etc.) per tube, then 150 µl of **SH solution**. Mix by vortexing until the sample is evenly mixed with the solution.

3. Add 750 µl of **Lysis Buffer** per tube, vortex again, then freeze at -80C for at least 20 min (prep can be indefinitely stored at this point for later processing).

4. Thaw samples. Ensure fully thawed and cap is on tightly. Bead beat 30 seconds in beadbeater.

5. Incubate samples at 50C for 1 hr with occasional mixing, then freeze again at -80C for >20 min.

6. Add 120 µl of **K-acetate** solution, incubate on ice for 10 min, centrifuge at ~10,000 g for 5 min.

7. Transfer 700 µl of supernatant to a new (labelled) 1.5 ml tube. Find the **‘binding matrix’** and vortex the bottle/tube to resuspend the silica particles, then immediately add 500 μl of matrix to DNA sample. Incubate 5 minutes at room temp, mixing occasionally to keep the matrix suspended.

8. Centrifuge at ~10,000 g for 30 seconds to pellet the matrix-bound DNA.

9. Pour off the supernatant into the discard bottle. Be careful not to back-contaminate your DNA sample from the discard bottle ! Tap gently on paper towel to remove last drops of supernatant.

10. Add 750 μl of **SEWS** and resuspend the pellet by vigorous vortexing/flicking (approx 30 sec). Allow matrix to sit in SEWS buffer ~1 minute, with occasional mixing.

11. Centrifuge at ~10,000 g for 30 seconds, then discard supernatant. Leave the tubes open for 15 min in heating block at 60°C to allow residual ethanol (in SEWS buffer) to evaporate. Its very important to remove all the ethanol or DNA will misbehave later ! When fully dry, the matrix pellet should be a uniform bright white colour, with no darker patches.

12. Add 200 μl **EB** to the pellet, vortex/flick vigorously, heat 60°C for 5 min (lid closed this time!).

13. Centrifuge at ~10,000 g for 1 min, then transfer the DNA-containing supernatant to a new labelled tube. Note that you will only recover ~100 µl liquid. Be careful to avoid the matrix material.

**SH solution:** Sodium hexametaphosphate (200 mM). Sterilised by autoclaving.

**Lysis buffer:** Tris base (50 mM), EDTA (50 mM), guanidine-HCl (1 M), Triton X-100 (0.5% w/v),

adjust to pH 10 with NaOH. Sterilised by autoclaving.

**K-acetate:** 7 M potassium acetate. Sterilised by autoclaving.

**Binding matrix:** MP Biomedicals™ Binding Matrix (silica in 6M guanidine salt). Note: the commercial product can be diluted 1:2 with 6 M guanidine-HCl before use to make it go further. The raw product has more binding capacity than is needed.

**SEWS** (salt/ethanol wash solution): 100 mM sodium acetate in 70% ethanol. Make 30 ml of 333 mM sodium acetate, autoclave this, then after cooling, add 70 ml of 100% ethanol.

**EB:** 10 mM Tris HCl, pH 8

**Protocol for making and running agarose gels**

1. Figure out how many samples you have and choose well-formers and gel volume accordingly: the smallest gel tank uses 60 ml agarose and takes up to 10 samples, the largest tank uses 150 ml agarose and takes up to ~40 samples. Remember to leave a lane for the molecular weight marker! Also read the next section about buffer and staining options \*before starting\*.

2. Decide what agarose concentration and what buffer are appropriate. For many gels, 0.5 x TBE buffer, and 1% agarose will be fine. However, if you are trying to separate very small DNAs (<500 bp), you can increase up to 2% agarose, and if you are trying to separate large DNAs (>5 kb), you can decrease down to 0.7% agarose. Using 1 x TAE instead of 0.5 x TBE will also allow better resolution of large DNAs. If you need to cut out DNA from the gel for cloning or sequencing, you need to use 1 x TAE instead of 0.5 x TBE (borate interferes with later enzyme reactions).

3. Decide whether you need very accurate band-sizing or not. If you do not, you can put the detection dye (GelGreen) into the gel. If you do need high accuracy, instead use post-staining. (*see later sub-section on buffer and staining options for gel electrophoresis)*

4. Set up the gel-casting tray, either by masking-taping the ends of the tray (old black gel box), or placing in the plastic end-formers (newer clear gel boxes). Place the well-former (comb) such that it is level and straight, and sitting ~2 mm above the base of the gel forming unit. It is crucial that the well formers are NOT TOUCHING the casting tray – in that case, your wells will have holes in the bottom and you will lose your samples. See pics. In some cases you can adjust the height of the well formers, in others you can’t.

5. Using a small, clean, dry conical flask, weigh out the appropriate amount of agarose powder (NOT AGAR!) for the correct and % agarose gel that you intend to make, then pour in the appropriate volume of TBE or TAE buffer. Put in microwave, and heat on high for 1 minute. Put on heat resistant gloves, then take out to examine whether all the agarose is dissolved. If not, replace for another 30 sec on high power, and repeat examination and heating until all dissolved.

5. Cool the conical flask down under a stream of cold water, while swirling. Be careful not to get tap water INTO the flask. After about 20 seconds, see if you can hold the flask comfortably in your hand (no glove) – if it is too hot to hold, continue cooling, re-check every 20 seconds or so. When the flask feels warm but not uncomfortably hot, it is ready to pour (this is approx. 45-50°C). If you cool it too much, it may set solid. In this case, microwave again (as above) in 30 sec increments to re-dissolve.

6. (if appropriate): add GelGreen dye to the gel (see later section for details).

7. (if using plastic end-formers): using a p-1000 pipette, pipette a thin line of agarose along the bottom edge of the end-formers, where they meet the base of the casting tray – the aim is to seal this crack through which agarose can potentially leak out. Allow 30 sec or so for this agarose seal to set.

8. Pour the molten agarose into the casting tray. Use a single smooth motion, don’t stop and start. Stop when the agarose is 3/4 of the height of the ‘teeth’ on the well-forming comb. Or alternatively, stop when you judge the wells are deep enough to hold the amount of sample that you need to load. What you DON’T want to do is over-fill the agarose so that it goes over the teeth – this will result in a channel that connects all the wells, and subsequent cross-contamination of the samples.

9. Allow the gel to set. This takes about 15 minutes, but there are a few ways to speed this along. If using the black gel tanks you can fill these with buffer (to half-way up the masking tape height on the casting tray), to get faster cooling. OR with either type of gel box, you can set up the whole casting tray / gel box in the cold room, and do the gel-pouring there.

10. When gel is set, pour a little of the appropriate buffer (TBE or TAE) over the top, then carefully pull out the well formers (straight up, don’t yank them side to side or forwards and back). The reason for the buffer is to stop the wells collapsing on themselves (this can happen with thin wells at lower % agarose). Then pull out the end-formers or take off the masking tape.

11. Fill the gel tank up with the appropriate buffer so that it fills the reservoirs on both sides of the gel, and so that it \*just\* covers the gel (by 1-2 mm).

12. Choose the appropriate molecular weight marker. For DNAs <1 kb, use the NEB 100 bp ladder. For DNAs from 1 kb – 10 kb use the NEB 1 kb ladder. For DNA’s 10 kb-30 kb, use the lambda/HindIII digest ladder. You may need to load two different ladders, one on each side of the gel. For GelGreen staining, load 100-200 ng ladder (= 4-8 µl of a 1/20 dilution of stock at 0.5 µg / ml). This amount may require some tweaking to get a nice looking gel.

13. Prepare a row of spots of loading buffer on a Parafilm strip, of appropriate volume and number. The NEB buffers are 6x conc, so e.g. you will need 2 µl spots if you are loading 10 µl samples. Check whether you need the ‘with SDS’ or ‘no SDS’ version of the buffer first, and whether you should be using GelGreen added to the buffer also – see later section for discussion of these issues.

14. Sketch in your labbook a map of which samples will be loaded in which locations. Avoid the first and last wells if possible, these tend not to run as straight as the more central wells.

15. Mix your first sample with the first blue spot, and load into the first well. Steady the pipette tip with the finger of your non-pipetting hand to ensure accurate dispensing. The tip needs to be just inside the well, don’t push it all the way down in the well. Change tips, mix up the next sample with blue dye, and load again. Repeat for all samples, including the marker.

16. Put the lid on the gel box, check that the terminals are connected correctly (negative terminal should be closest to the wells, positive terminal is far from the wells, ie. Run towards Red). Run the gel at ~50 volts up to ~300 V, depending on the buffer system and gel size (see later section for more detail). Check that you have current (non-zero milliamps), and gas bubbles at the electrodes (in clear tanks). If not, check all the wire connections. Running the gels at lower voltages generally gives better resolution of bands (maybe! depends also on other factors).

17. Stop the gel when the fast-running blue dye (bromophenol blue) (= usually the only blue dye) is near the end of the gel (this may take 10-120 min depending on gel size and voltage). You may need to run longer to get good separation of large products (>5 kb). Run for a shorter time for small products (<500 bp), or they may run off gel.

18. If you added GelGreen to the gel, it is now ready to image on the transilluminator. Otherwise, follow post-stain procedure (next section).

**Buffer and staining options for agarose gel electrophoresis**

***"One size fits all" protocol.*** Use 1% Agarose gel made in **TBE** (0.5x). Add 1μL of a dilute GelGreen stock per 10mL of gel while gel is molten (after cooling to pouring temperature ~50°C). Use **TBE** (0.5x)as the running buffer. Run at 150-180V (small tanks) or 200-250V (large tanks).

***"Time is of the essence" protocol.*** Use 1% Agarose gel made in **LAB** (1x). Add 1μL of a dilute GelGreen stock per 10mL of gel while gel is molten (after cooling to ~50°C). Use **LAB** (1x) as the running buffer. Run at 250V. WARNING! this gel will be done in ~15 min.

***"Higher-resolution required" protocol*** (DNA sizes **>**1.5 kb). Use 0.8% Agarose gel made in **TAE** (1x). Add 1μL of dilute GelGreen stock per 10mL of gel while gel is molten (after cooling to pouring temperature ~50°C). Use TAE (1x) as the running buffer. Run at <100V

***"Higher-resolution required" protocol*** (DNA sizes **<**1.5 kb). Use 1.5-2% Agarose gel made in **TBE** (0.5x). Add 1μL of dilute GelGreen stock per 10mL of gel while gel is molten (after cooling to ~50 °C). Use TBE (0.5x) as the running buffer. Run at 150V (small tanks) or 200V (large tanks).

***"Nice gel for publication" protocol.*** Use either of the above high-resolution protocols, but don’t add GelGreen in the gel OR loading buffer. Instead, after the gel has run, add it to 100 ml of post-stain solution. Stain gel on rocking platform or orbital shaker (gentle shaking!) for 30-60 min. This staining solution can be reused a few times. Keep the post-stain solution covered in foil and keep in closed plastic box so it doesn’t evaporate.

Notes on recycling buffers: Its OK to recycle TBE or LAB buffers quite a few times, but its important to use the same \*batch\* of buffer for both gel and tank buffer e.g. don’t use fresh buffer for gel and recycled buffer for the tank. Don’t recycle TAE – the acetate component is lost after the gel is run.

Notes on loading buffers: For all samples, use loading buffer that has GelGreen added to it (1 uL GelGreen per 1 ml loading buffer) – this pre-loading with dye helps to prevent the faster-running DNA bands from ‘sweeping up’ all the GelGreen in the agarose. For restriction digests, use loading buffer that has SDS added (purple lid for NEB buffer). For all other DNA samples, use loading buffer with no SDS (white lid for NEB buffer).

**Reagents**

**TBE:** 20X stock = 1.78 M Tris base, 1.78 M boric acid, 40 mM EDTA (adjust to pH 8)

**LAB:** 25X stock = 250 mM lithium acetate, 250 mM boric acid

**TAE:** 20X stock = 800 mM TRIS base, 400 mM acetate, 20 mM EDTA (adjust to pH 8)

Pre stained No-SDS loading dye: 1µL gel green stock concentrated stock per mL of loading dye (purple capped tube for NEB reagent)

Pre stained SDS loading dye for digest samples: 1µL gel green stock concentrated stock per mL of loading dye (white capped tube for NEB reagent)

Dilute Gel Green stock: 1µL Gel Green in 50µL RO water.

Post Stain Solution: 10 µL of conc. GelGreen dye in 100 mL RO water.

**Protocol for quantitation and restriction digestion of plasmid (gel analysis)**

1. Quantitate your plasmid via running a small amount (e.g. 1 ul and 5 ul) on an agarose gel. Alongside this, run a few lanes with known amounts of DNA ladder (e.g. 2 ul, 5 ul, and 10 ul of a 1/20 dilution of the NEB 1 kb ladder) to enable comparisons. Make sure you run the gel far enough to separate out all the bands in the ladder (till blue dye is right at the end). Using 0.7% agarose instead of 1% agarose and 1xTAE instead of 0.5xTBE can help to resolve large bands in the DNA ladder.

2. Estimate plasmid concentration by finding a band(s) in one of the ladder lanes that corresponds to the band(s) in one of your plasmid dilutions. This is not exact, but is more accurate than using Nanodrop or other UV spectro.– these methods are OK for column-purified PCR products, but overestimate DNA in plasmid preps. The NEB 1 kb ladder is shown (🡪), note the amounts of DNA in each band are given in ng (assumes you load 0.5 µg = 1 µl of undiluted marker)

 3. Choose a restriction enzyme (RE) for the digest that will cut the plasmid 1-3 times. Too many cut sites complicates interpretation. If you use an enzyme that cuts more than once, check that the predicted fragments aren’t close in size. It’s a good idea to choose an enzyme that you are going to use for subsequent cloning steps – this lets you confirm your enzyme is working OK and it cuts the expected number of times. If you are using two enzymes for the cloning, do two separate digests (you won’t usually be able to tell if both of them cut effectively when the RE sites are close together, in the same multiple cloning site)

4. Check the buffer and temperature requirement of the enzyme. Most RE’s work best at 37°C but some (eg SmaI) are better at 25°C. Retrieve the 10x buffer from the freezer and thaw this out thoroughly (e.g. in a 37°C waterbath) – its important that the buffer is fully thawed or the salt conc wont be correct. Give the tube a good mix by vortexing or flicking. Then get out the RE itself – transfer immediately to ice – it is critical that the RE stays cold at all times.

5. Set up the digest with ~250 ng plasmid in a 30 µl volume with 2 µl of RE, as follows. First figure out how much plasmid you need – hopefully this is less than ~ 5 µl (lets call this X µl). If X > 5 µl, the digest may not work due to junk in the plasmid prep (e.g. salts) interfering – in this case, consider further purification and concentration of the plasmid e.g. by ethanol precipitation. Calculate the amount of water (sterile Milli-Q; MQ) to add to the digest; this is (30 – 3 – 2 – X); lets call this Y. Set up the digest in a sterile Eppi tube in this order:

With any molecular biology reaction, you usually add the enzyme **last**. Don’t add enzyme to plain MQ or to 10x buffer. Be careful not to contaminate the enzyme with DNA, and vice-versa ! Change tips for every addition !

- 3 µl of 10x buffer

- Y ul of MQ

- X ul of plasmid

- 2 ul of RE

---------------------------

 total 30 µl

6. Incubate at the preferred temperature for ~1 hr. There is a wide acceptable time range here (10 min – 24 h). 1 hr is a compromise between allowing maximum digestion by the specific RE, and minimising non-specific digestion by traces of other nucleases. Run on agarose gel to check.

**Polymerase chain reaction (PCR)**

**NOTES:** As with all molecular biology and microbiology work, aseptic technique is crucial. Clean your bench, tube racks, and pipettes with 80% ethanol before starting, and work carefully to ensure you don’t contaminate the PCR reagents or mixtures with microbes, DNA, or enzymes from your hands or the bench or the ice bucket etc etc. Ensure pipette tips, Eppi tubes and PCR tubes are sterile.

**Different PCRs behave differently.** Some PCRs are very straightforward, and a trained monkey could make these work, while others are very challenging, and even a highly skilled postdoc might struggle. The factors impacting on the ease / difficulty of PCR include the following:

Type of DNA Template: the easiest template is a plasmid or PCR product containing your sequence of interest. These are like a ‘positive control’– they are very clean, and have a very high ratio of target sequence to non-target sequence. A medium difficulty template would be genomic DNA from a single microbe. The most difficult template is metagenomic DNA from a complex mixture of microbes – this has a very low signal:noise ratio, ie. there are lots of places where the primers can bind incorrectly and only a few places they can bind correctly.

Purity of DNA template: highly purified DNA (e.g. FastPrep or CTAB method) will amplify much better than crude DNA (e.g. boiled cells), which will amplify better than whole cells, which will amplify better than a complex and dirty template (e.g whole soil) (this is unlikely to work at all!). That said, you can certainly do PCR on whole cells of most gram negative bacteria, including E.coli (this is known as ‘colony PCR’.), and this is very useful for rapidly screening clones.

Type of primers: specific primers work well, degenerate primers work less well, and the higher the degeneracy, the worse they perform. Primers that are designed well (no dimers/hairpins or only weak dimers/hairpins) will work better than primers that are poorly designed (strong dimers/hairpins). Primers that have lots of junk at the 5’ end like long non-target sequences or fluorochromes will perform worse than primers that are exactly the same as the template. Primers with mismatches to the template may still work, but the more mismatches present, the worse they will work, and the closer the mismatches are to the 3’ end, the more serious the problems will be.

Type of target gene, and its copy number: If your target gene is on a plasmid, it will amplify more easily than a chromosomal gene (all other things being equal). If your gene has many copies in the template DNA, it will amplify better than a gene which is very rare. If your target gene is 16S rDNA or some other highly-conserved gene, and your primers are ‘universal’ primers, you might expect problems with contamination, since this gene is \*everywhere\*, including on your hands, on the bench, in your pipette etc. (the negative control with no DNA added is critical in this case!). On the other hand, if your target gene is only found in your particular favourite and unusual organism, then contamination will be less of a concern.

Size of PCR product: The smaller the product, the easier the PCR. Product size should not cause problems up to ~ 1 kb, but as you go larger than this, the PCR will become increasingly challenging. This also depends a lot on the type of polymerase used (Phusion is better than Pfu which is better than Taq, in terms of getting large products). Anything larger than ~5 kb is going to be difficult to amplify, even for an experienced user – success here requires using a fancy polymerase, \*excellent\* primers (well-designed, no dimers/hairpins), clean and high-quality template with high signal:noise ratio, fresh reagents, and excellent hands-on technique. In theory, PCR products up to ~20 kb are possible, but these are extremely difficult to obtain in practice.

**Protocol for setup of PCRs**

**NOTES:** The usual PCR is 25 µl volume, this is appropriate for “screening” purposes. However, if you are trying to make a lot of PCR product for cloning, then scale reactions up to 50 µl per tube. A PCR that is working well should give you about 50 ng of product per µl of reaction, thus 2.5 µg per 50 µl. For a cloning experiment, if you prepare 8 x 50 µl PCRs, this should give you plenty of insert DNA (10-20 µg), even if your PCR is not working at high efficiency. This is way in excess of the theoretical requirement, but allows for losses due to subsequent purification steps, and the likelihood that you may need to repeat the experiment a few times :-/

**EXAMPLE REACTION MIX (per 25 µl) FOR 8 REACTIONS\*\* (1 strip of tubes)**

* 10 x buffer: 2.5 µl (🡪 1 x) • 10 x buffer: 22.5 µl
* sterile milliQ water: 20 µl • sterile milliQ water: 182 µl
* dNTPs (10 mM): 0.5 µl (🡪 200 µM) • dNTPs (10 mM): 4.5 µl
* primer #1 (50 µM): 0.25 µl (🡪 0.5 µM) • primer #1 (50 µM): 2.3 µl
* primer #2 (50 µM): 0.25 µl (🡪 0.5 µM) • primer #2 (50 µM): 2.3 µl
* polymerase\* (5 U/µl): 0.25 µl (🡪 0.05 U / µl) • polymerase (5 U/µl): 2.3 µl

 ( DNA template: 1 µl ) ( DNA template: 8 x 1 µl )

\* This means thermostable DNA pol e.g. Taq, Pfu, Phusion, Q5, etc. but NOT Klenow or T4 pol.

\*\* Calculations done as if 9 x 25 µl reactions were being prepared, to ensure mix doesn’t run out.

Template considerations: The correct amount of DNA template depends on the type of sample. If you are using a plasmid, you only need a tiny bit (~1 ng) because the ratio of target:non-target sequences is very high. For genomic DNA of a single microbe, a more usual amount would be 10-20 ng per reaction. For metagenomic DNA, you may need to go even higher (100 ng per reaction), although note that there are diminishing returns here due to the presence of PCR inhibitors in many templates that will interfere with PCR. For complex templates like soil DNA, the optimum concentration of template often needs to be determined experimentally. More is not always better; in some cases, diluting the template (e.g. 1/10) will yield a band when undiluted template gives no band at all.

**SETUP OF MASTER MIX AND REACTIONS**

1. Exact setup of PCR depends on which element is the variable in the reaction. Usually this is the DNA template, but sometimes it is the primer. The ‘Master Mix’ should be made to include everything except the variable being tested. The below protocol and above recipes are written assuming that this variable is the DNA; you need to modify if the same template is being tested with multiple primers.

2. Calculate how many PCRs you need altogether, and thus the total volume of master mix required. Its important to make more than you need (~10% more), as pipetting errors will always change the expected volumes a little bit, e.g. the above recipe for 8 PCRs is calculated as if it were 9 PCRs. Write up the exact recipe that you need in your lab book.

3. Label your strip tubes on the side, at the top, as shown below. If you label the lids or the bottom of the tube, the labels tend to come off during thermocycling. Place the tubes in a tube-rack (a 96-well microtitre plate works well). Leave the lids off the tubes – you are more likely to contaminate the reactions via excessive opening and closing of tubes than from stuff falling in from the air.

4. Retrieve the PCR reagents from the -20°C freezer and thaw them (except polymerase, this remains liquid even at -20°C due to glycerol in buffer). Its important that the 10x buffer, dNTPs, and primers are thoroughly thawed out before use. Give them a brief vortex or flick to mix. These reagents don’t need to be kept on ice during the time needed to set up the PCR, but don’t leave them on the bench longer than needed.

5. Prepare the master mix in a sterile Eppi tube, containing everything except the DNA template. Add ingredients in the order listed in the recipes above. Some folks will insist that this must be done on ice, opinions differ. It is true that you will minimise premature polymerase activity by setting up on ice, but whether this really makes a difference for most PCRs is arguable. Setup on ice is supposedly more important for Pfu and Phusion which contain 3’-5’ exonuclease activity which can ‘eat’ your primers during setup. If you set up on ice, risks of contamination from the ice itself must be managed.

6. Aliquot out the master mix between all of the PCR tubes, putting 25 µl in each tube.

7a. (DNA template in solution) Add DNA template (1 µl) to all the tubes, being very careful to match the sample #’s to the labels on the tubes. You need to concentrate your attention here and really focus in order to avoid missing a tube or putting two templates in the same tube. Be careful also to change tips between every DNA template.

7b. (colony PCR: DNA in cells). Touch a white tip onto a colony or patch of growth, and pick up a \*small\* amount of cells. It should be just enough so that you can see there are cells there, not much more than this. Then dip the tip in and out of the master mix in the first PCR tube approx 5 times, and discard the tip. You don’t need to wipe the whole chunk of growth on the inside of the tube, just the dipping in and out action will dislodge enough cells to give you enough template DNA. Less is more in this case ! Repeat for other colonies/patches.

8. Put lids on tubes, ensure they are snapped on tight, place immediately in thermocycler. Double check your program parameters before starting. See below for detailed thermocycling instructions.

9. Return all reagents to the freezer.

 < thermocycling – see below >

10. After thermocycling is completed, run out your PCRs on an agarose gel to check their size and yield. Typically, we would load 5 µl of PCR mixture. This should give a very strong band if the PCR has been successful. This amount (5 µl) may be hard to quantify if using GelRed in the gel due to smearing; if you are using this staining method, its not a bad idea to run a few different amounts (e.g. 2 µl and 5 µl) to ensure you can accurately size the product.

11. If you are intending to use the PCR product for cloning, check for the presence of non-specific bands (usually fainter, smaller bands than the expected one) – if these are numerous and/or strong, you may need to try a higher annealing temp, or revisit primer design, before the product can be cloned. Alternatively, you can cut out the desired band from the agarose <see other protocol>.

**THERMOCYCLING**

A standard thermocycling protocol is given below (for Taq polymerase)

Initial denaturation: 95°C, 5 min

Denaturation: 95°C, 30 sec

Annealing: **60°C**, 30 sec 30 cycles

Extension: 72°C, **1 min**

Final extension: 72°C, 10 min

Hold: 15°C

The numbers that are underlined and in bold indicate variables that need to be optimised for every individual PCR. Sometimes, other variables may also need to be modified too, as below

Annealing temperature: This is typically set at 5°C lower than the melting temperature (Tm) of the primers. If these Tm’s are different, use the lower one for this calculation. This calculation is very crude, and it is best to optimise annealing temperature experimentally by doing a ‘gradient PCR’, e.g. testing an annealing temp range from 5° lower to 5° higher than the predicted best temp. Aim to make primers with Tm’s 60-65°C, which gives a predicted annealing temp 55-60°C.

Extension time: This is set primarily by the length of the desired PCR product. For Taq polymerase, this is 1 minute of extension per kb of product. However, note that different polymerases require different extension times: Pfu needs 1.5 min per kb, and Phusion needs 0.5 min per kb. If you use a longer extension time than necessary, you risk increasing the yield of non-specific products; if you use a shorter extension time than necessary, you risk a low yield of the desired product.

Denaturation: The optimal denaturation conditions for Phusion pol are different than for Taq or Pfu. Change the initial denaturation to 98°C - 30 sec, and the denaturation in each cycle to 98°C - 10 sec.

Number of cycles: This can be increased to 35 or even 40 cycles to give a higher yield of product, but this risks introducing more mutations into the PCR amplicons, and also gives a higher chance of secondary non-specific products being formed. Conversely, the # of cycles can be reduced to 25, which will minimise mutations and non-specific products, but will also lower yields.

Hold temperature: Its OK to leave your PCRs in the machine overnight. While some protocols recommend fridge temp (4°C) for this ‘Hold’ step, this is actually bad for the machine – it will accumulate condensation on the block, which will degrade the block over time. A good compromise is to set the hold temp to 15°C, which keeps the samples cool(-ish), maintaining them in good condition, but is not cool enough to cause condensation to form.

PCR Enhancer Additions: Two enhancers that might be worth testing for problematic PCRs are Bovine Serum Albumin (BSA) and dimethylsulfoxide (DMSO). BSA is good for binding inhibitors, and is typically added at 0.5 mg/ml final conc. from a 10 mg/ml stock (=1.25 µl per 25 µl reaction). DMSO is good for enabling amplification of GC-rich templates, and is typically added at 1 µl of neat DMSO per 25 µl reaction (=4%).

**PCR primer design**

Designing good primers is crucial to the success of PCR. Time spent on this can save a lot of time wasted later trying to optimise bad primers. For long PCRs or PCRs from complex templates (eg. soil) the need for excellent primers is even more important.

You need 15-20 bases at the 3' end of the primer that are absolutely conserved in the target sequence, but you can add stuff at the 5' end (up to another approx. 30 bases) that may or may not be found in the target sequence, eg restriction sites, mutations, overhangs to join to things etc.

Your forward primer should have the same sequence as the target site on the forward strand of the target DNA, but your reverse primer has the same sequence as the reverse complement of the target site. In both cases, the primer goes in the 5’ 🡪 3’ direction. A simple tool to use for reverse complementing sequences is this: <http://bioinformatics.org/sms/>

If you are adding a restriction site at the 5’ end of the primer, you also need a few extra bases at the 5' end to allow the RE to bind properly. You can look up for each restriction enzyme how many extra bases are needed (see NEB catalog appendix table “Cleavage close to the end of DNA fragments”). If you add 3 extra bases, that is pretty safe for most enzymes. NdeI is annoying and needs > 7 extra bases. You can make the extra bases match the template at that position if you like, but we usually just add AAA or TTT, whichever is least problematic for dimers and hairpins. (see below).

The architecture of a typical FWD primer is shown below:

template FWD strand: 5’ AGTCAGCTCGCATCGAGGGCTCGAGGAC 3’

Fwd primer (adds EcoRI site): 5’ AAAGAATTCAGTGGATCCGCATCGAGGGCTCGAGGAC 3’

 **5’ extra bases EcoRI site Fwd sequence**

The architecture of a typical RVS primer is shown below:

template FWD strand: 5’ ATCGACTATAGGAGCTGAGATATACC 3’

Rvs primer (adds BamHI site): 3’ TAGCTGATATCCTCGACTCTATATGGGGATCCAAA 5’

 **Rev.compl. sequence BamHI site 5’ extra bases**

Aim to have primers with a GC content approx 50-60%, and if possible avoid repeat regions (e.g. AAAAAA). High GC regions are also problematic for primer design, since any dimers or hairpins in these regions will be much stronger than in ‘normal’ DNA region. Aim to end the primer on at least one G or C at the 3’ end – this helps keep the 3’ end of the primer firmly anchored on the template.

Aim to get melting temperatures (Tm) for the primers around 60°C. You want both primers to have very similar melting temps +/- about 3 degrees C, so they work well at the same annealing temp. You can use the IDT website or Snapgene to check melting temps, and the IDT site also gives info on secondary structures (dimers etc).

Note that the predicted primer melting temperature varies depending on the solution chemistry – the values printed on the primer spec sheets are for DNA in 50 mM NaCl. If PCR is performed using Taq, the optimal annealing temperature for the PCR reaction is about 5°C below this Tm in 50 mM NaCl. For newer PCR polymerases, e.g Phusion or Q5, the optimal annealing temperature is higher due to a processivity domain which makes the enzyme bind more tightly to the DNA – in these cases, the optimum annealing temp could be same or even higher than the Tm value.

The real art of primer design is in making primers that don’t make self-dimers, pair-dimers and hairpins - all of these structures will take the primer away from its intended target site in the template. Software eg. PrimerSelect will help you find these. The dimers should have deltaG values higher than approx -5 kcal/mol to be acceptable. eg. a primer where the worst self-dimers are -8 kcal/mol would not work well, but a primer where the worst self-dimer is -3 kcal/mol should be OK. For hairpins, the cutoff is about -2 kcal/mol. You need to check for both self-dimers and pair dimers.

If your primer has bad dimers or hairpins, there are a number of ways to deal with this problem. The first step in all cases is to identify which particular bases are problematic (using the software/websites mentioned above). Then you can try the below:

* slide the primer location up or down a little bit to move it away from those problematic bases. this only works if the problem bases are near the ends of the primer. if the problem bases are in the middle of the primer, you will probably need to choose a new priming location
* if the problem bases are towards the 5’ end, you can sometimes mutate them to another base which doesn’t cause the dimer/hairpin to form. you need to be very careful with this approach though that the mutations you introduce don’t cause problems later.
* you can (at your own peril) ignore the hairpins and dimers and just order the primer and see what happens… sometimes primers that look bad in silico work OK enough in the lab.
* if the problems are arising from the bases in a restriction site, can you change this to a different restriction site?
* if the problems are arising from your extra bases at the 5’ end that are added to facilitate digestion, you can change these to any bases you like (they get cleaved off after digestion)

**Protocol for restriction digestion of plasmid & insert, purification, and ligation**

**NOTES:** First quantify the plasmid (by gel comparison, not nanodrop!), and quantify the insert DNA (usually a column-purified PCR product; you can use nanodrop for this) then set up digests, as below. A typical setup would be 250 ng plasmid and 250 ng insert in digest volume of 100 µl (these numbers used below), but range may be 100-1000 ng of each DNA, in a volume of 30 – 200 µl.

Check which restriction buffer is appropriate. For double digests, it’s OK to use a buffer which gives 100% activity for one enzyme, and 75% activity of the other, but lower than this is not good. Also check the appropriate digest temperature, its usually 37°C, but not always.

<< note – the plasmid and insert digests are described separately and sequentially below for clarity, but these can and should be set up more-or-less at the same time >>

 **DIGEST OF PLASMID**

1. Calculate how much volume of plasmid you need to use to get 250 ng – call this ‘X’, and calculate how much water to add to the digest; this is (100 – 10 – 2 – X) – call this ‘Y’.

3. Retrieve 10 x restriction buffer from freezer, thaw completely, and vortex to mix. The same tube of buffer can be used many times, if you are careful with your aseptic technique.

4. Retrieve the plasmid from the freezer, allow to thaw, (e.g. in 37°C waterbath, or rub in your hands, or on bench etc), then put it on ice when it is thawed. Its not good to leave the plasmid stock at room temp or above for prolonged periods or it may degrade due to traces of nucleases.

5. Retrieve the restriction enzyme(s) from the freezer, put IMMEDIATELY on ice. These are heat-sensitive and you need to look after them. Do not leave them at room temp. Keep on ice while setting up the reaction, then immediately put back in freezer. These don need to be thawed, they are in a glycerol solution which doesn’t freeze at -20°C.

6. Label your tube(s), then set up the digest(s) by adding the ingredients in the following order. Make sure you use excellent aseptic technique, and change tips every time. Its OK to set this up at room temp, the reaction tube doesn’t have to be on ice.

- 10 ul of 10x restriction buffer

- ‘Y’ µl of sterile MQ water

- ‘X’ ul of plasmid DNA

- 2 µl of (each) restriction enzyme

------------------------------------------------

 Total 100 µl

7. Mix by flicking, then tap on bench to get liquid to bottom of tube. Incubate at correct temperature for approx. 2 hours. (1 - 4 hr is OK, but overnight digest is too long; this can lead to ‘raggedy ends’ of the plasmid even if it looks OK on a gel; this is due to non-specific nuclease activity)

NOTE: 100 µl seems like a large volume to use, but this helps dilute any impurities in the plasmid prep. Increasing the total volume of the digest and/or reducing the volume of plasmid added often help to improve the quality of a poor digest. If the digested plasmid is for the purpose of ligation, we can use a large volume, since it will be column-purified & concentrated anyway.

**DIGEST OF INSERT DNA**

1. Calculate how much volume of insert DNA you need to use to get 250 ng – call this A, and calculate how much water to add to the digest; this is (100-10-2-A) – call this B.

3. Label your tube(s). Set up digest by adding ingredients in the following order. Make sure you use excellent aseptic technique, and change tips every time. Its OK to set this up at room temp.

 - 10 µl of 10x restriction buffer

 - ‘B’ µl of sterile MQ water

 - ‘A’ µl of insert DNA

 - 2 µl of (each) restriction enzyme

------------------------------------------------

 Total 100 µl

4. Mix by flicking, then tap on bench to get liquid to bottom of tube. Incubate at correct temperature for approx. 2 hours. (1 - 4 hr is OK, but overnight digest is too long)

5. Dephosphorylate the vector by adding 11 µl of 10x phosphatase buffer and 2 µl of antarctic phosphatase enzyme, incubate 30 min at 37°C. This step prevents the vector religating to itself; it is not required (in theory!) when you are cloning with two different restriction enzymes.

6. Purify both vector and insert DNA using Qiaquick columns (or similar), elute in 15 µl EB.

**LIGATION**

1. Retrieve one 10x ligase buffer aliquot from the freezer. This should be a small amount of buffer (e.g. 10 µl) in a small generic tube, not the large tube from the manufacturer with ~1 ml of buffer Ligase buffer (unlike restriction buffer) cannot be repeatedly frozen and thawed, it starts to ‘die’ after even one freeze/thaw cycle.

2. Retrieve the T4 ligase enzyme from the -20°C freezer and put IMMEDIATELY on ice. This reagent is VERY heat sensitive, and must be handled with care.

3. Put your labelled tube(s) on ice, then set up the ligase reaction in this tube on ice. Be careful not to get ice or melted ice in the tube - this is not sterile! Add reagents in this order, change tips each time :

- 2 µl of 10 x ligase buffer

- 8 µl purified insert DNA

- 8 µl purified plasmid DNA

- 2 µl of T4 DNA ligase enzyme

------------------------------------------------

 Total 20 µl

4. Mix by flicking briefly then incubate either 4°C overnight or room temp for 1 hour. Return the ligase enzyme immediately to -20°C freezer. Throw out any unused thawed ligase buffer.

<<<Note: if things don’t work, see following protocol for details of troubleshooting ligations>>>

**Protocol for troubleshooting ligations and transformations**

**NOTES**: When you first do a particular ligation, you should include all of the controls described below. Once you are ‘up and running’, and just repeating an experiment that has worked well before, you can omit most of these, but you should always do at least the positive control (vector only) and the negative control (no DNA added) in any ligation / transformation experiment.

See the related protocol (above) on setting up ligations for the hands-on details of the basic ligation method. This current protocol just focuses on the different ligation controls, and interpreting the results that arise from them. Note that the use of phosphatase in the procedure, and the inclusion of this control is optional if you are using two different restriction enzymes for cloning, but is essential if you are using a single restriction enzyme for cloning.

 **SUMMARY OF CONTROLS:**

**1. Negative (no plasmid) – are the antibiotic plates OK? (expect no colonies)**

**2. Positive (uncut plasmid) – are plasmid stock + comp cells OK? (expect very many colonies)**

**3. Digest (cut plasmid) – are the restriction enzymes working? (expect very few colonies)**

**4. Ligation (cut, religated plasmid) - is the ligation working? (expect many colonies)**

**5. Dephosphorylation (cut, dephos, religated plasmid) – is phosphatase working? (expect few colonies)**

 **DETAILED EXPLANATIONS:**

1. Transformation negative control: 50 µl of competent cells, no DNA added.

*This control is primarily to check that you have made the antibiotic agar plates correctly, and it should yield no colonies at all*. If you see lots of colonies here, this most commonly means either you forgot to add antibiotic to the plates, or the antibiotic concentration is wrong (too low), or the host bacteria are already resistant to the antibiotic (e.g. TOP10 has chromosomal streptomycin resistance).

Other possibilities could be that the plates were incubated too long (especially with LB-ampicillin), or that there is severe contamination with an antibiotic-resistant bacterium (not E.coli) (this is unlikely!), or that there was a mix up of labelling somewhere e.g. is this really the positive control?

If you see just a few colonies on these plates, this indicates some kind of contamination has occurred during the procedure, e.g. from one of the other samples or the pipette etc. This may not be a ‘deal-breaker’ so long as there are lots more colonies on your experimental test plates.

2. Positive control: 50 µl of competent cells + 1 µl of conc. plasmid

*This control is to check that your stock of the plasmid vector is OK, and that your competent cells are indeed competent; it should yield thousands of colonies on the ‘pellet’ plate*. If you see no growth here, or only a handful of colonies, possibilities are as follows: the cells are not competent, used the wrong antibiotic in the agar (check the sequence of your plasmid to confirm correct resistance), used the wrong concentration of antibiotic (too much), the agar plates are ‘bad’ for some other reason (e.g. added mercuric chloride instead of sodium chloride!), the plasmid stock has gone bad (run a gel to check), there was a mix up of labelling, or there was a pipetting error (look at the pipette tip to ensure you really have 1 µl of plasmid in there!)

3. Digest controls. 50 µl of competent cells + 3 µl of purified digested plasmid

*This control is to check that your restriction enzymes are cutting the plasmid vector effectively. It should yield only a few colonies (approx. < 20 on the ‘pellet’ plate)*. If you see hundreds or thousands of colonies here, either the restriction enzyme has gone bad, the digest was set up incorrectly, your plasmid stock is not sufficiently pure, or you have put too much plasmid into the digest. A separate digest control is needed for each enzyme you are using for cloning – e.g. if you are cloning an EcoRI-XbaI fragment into a vector cut with the same enzymes, you need to test EcoRI digestion and XbaI digestion separately.

Set up digests as described above, as if you were going to ligate the plasmid to an insert (ie. 250 ng plasmid in 100 µl digest, then after incubation, purify on column, and elute in 15 µl EB), but don’t actually set up the ligations, just transform the purified, digested plasmid directly into the cells.

The digest controls should be interpreted alongside an agarose gel run with the remainder of the digested, purified plasmid; the latter should give a single sharp band at the expected total size of the vector plasmid. If you see a lot of smearing in this digest this could indicate non-specific nucleases are contaminating the reaction. If you see additional bands in addition to the band at the expected size, this could indicate incomplete digest (this is quite common) – these extra bands are the supercoiled circular and/or open-circular forms of the plasmid – you need to remove these by re-doing the digest with less DNA and/or more-purified DNA and/or a larger volume digest. The uncut plasmid bands tend to look ‘fuzzier’ than the cut plasmid.

If you have undigested plasmid remaining in the mix, you will get a very high background of vector-only clones, which will make it hard to find your clones of interest. The digest controls will also reveal if your chosen restriction enzymes definitely cut in the expected locations, or if there are other unexpected cut sites in the vector backbone (bad!) – that will give multiple sharp bands, which add up to the expected total plasmid size.

4. Ligation control: 50 µl of competent cells + 3 µl of purified, single-digested, religated plasmid

*This control is to check if the ligation step is working.* *It should yield hundreds of colonies on the ‘pellet’ plate.* If you see only a few colonies or no colonies, this most likely means that either the ligase enzyme is bad or the ligase buffer is bad. Set up digests and ligations as described for the standard ligation procedure, but using a single restriction enzyme only, and no phosphatase step, and no insert DNA. (if you are cloning with two different restriction enzymes, you need to prepare two separate ligation controls, since a double-digest would not be expected to religate in the absence of insert DNA).

This control needs to be interpreted side-by-side with the other controls listed above to ensure that the problem is not poor-quality plasmid DNA or non-competent cells or bad agar plates etc. In addition to bad ligase or bad ligase buffer, this could also be due to bad ligation setup or conditions (e.g. a bad batch of MQ water). Another possibility if the ligation control doesn’t work is that there are non-specific nucleases getting into your restriction digest – this would mess up the ends of the DNA and prevent it religating. (check digests on gel; they will look smeary if you have non-specific nucleases).

5. (optional) Dephosphorylation control. 50 µl of competent cells + 3 µl of purified, single-digested, dephosphorylated, religated plasmid

*This control is to check that the phosphatase enzyme is working. It should yield very few colonies (approx <20 on the pellet plate).* If you see hundreds or thousands of colonies, it means that the phosphatase enzyme or buffer is bad, or you forgot to add phosphatase buffer or enzyme.

Set up the digest, dephosphorylation, and ligation as described for the standard ligation procedure, but using a single restriction enzyme only, and no insert DNA. In this situation, the ligase will attempt to join the cut plasmid backbone to itself, but it should fail to do this since the 5’ phosphate groups have been removed. This control must be interpreted alongside the other controls to rule out e.g. lack of restriction digestion if you see many colonies appearing.

**Cloning/ligation protocol using heat-killed digests**

**Notes:** This protocol is especially useful for ligations involving very small or large fragments which do not get retained very well during column purifications. It’s also worth considering when you don’t have much DNA in your sample and want to minimise losing it. Note that some restriction enzymes are not heat-killable (e.g. PstI-HF and BamHI) so check this first ! (NEB website)

The thermostat on the heat-block isn’t very accurate and usually the block is 5-10°C below the set point. Check the thermometer before you start. Putting the thermometer in an Eppi tube of water in the block will give you the closest idea of what your sample is actually being heated to.

A downside of this protocol is that it doesn’t remove small offcut bits of DNA like column purification does. If for example you are digesting a PCR product that isn’t too small, or a two sites in a plasmid with only a few bases between them, it would be better to column purify these digests to reduce the chance of the small fragment re-ligating back in.

**Protocol**

1. Digest: Set up a restriction digest in a small volume in an Eppi tube. Incubate 37°C for >30 min.

1µL 10x Cutsmart (or other buffer, check first)

1µL Enzyme 1

1µL Enzyme 2

x µL insert DNA

y µL vector DNA

(7-x-y) µL MilliQ-H2O

2. Heat kill. While waiting for digest, turn on the heating block and set to whichever is the highest heat kill temperature of the two restriction enzymes. When digest is complete, place the tube with DNA into the heating block for 20 min. Turn off heat block! Chill DNA on ice for 5 min.

3. Ligation. Give the tube a quick spin to get all the liquid to the bottom. Add 1 µl of 10x T4 DNA ligase buffer and 1µL of T4 DNA ligase. Ligation can be done at room temperature for 30 min on your bench or overnight in the cold room.

**Purification of DNA via spin column (for DNA in solution)**

**GENERAL NOTES**:

This protocol is good for restriction fragments, small digested plasmids, and PCR products. It is NOT good for genomic or chromosomal DNA. Use the FastPrep reagents or CTAB-phenol type prep instead for genomic DNA. Digested plasmids work well up to about 5 kb, but the yield quickly falls off past this point. Generally, plasmids don’t purify as well as PCR products on these columns.

 <<put on gloves + safety glasses. PB contains guanidine which is somewhat toxic >>

1.Figure out what volume of sample you have. If this is less than 100 µl, then make it up to 100 µl with TE. The volume you now have will be called “1 volume”. Add 5 volumes of buffer PB to the DNA solution. e.g. if you have a 100 µl restriction digest, add 500 µl PB.

2. Place a silica-based spin column (e.g. ‘Econospin’) into its 2 ml catch tube (if it isn’t already set up that way). Load up to 750 µl of the DNA-PB mixture onto the column. Spin at ~10,000 g for 30 sec. Discard the flow-through into culture waste.

3. If you still have more DNA-PB mixture left, repeat the previous step until all of the mixture has been put thru the column. The columns will hold a total of ~10 µg DNA, which is a lot!

 <<Can take off glasses and gloves now >>

4. Add 750 μl of buffer PE to the column, spin ~10,000 g for 30 sec, discard flow-through.

5. Repeat step 4.

6.Spin again for 30 sec to remove all traces of PE from the column. Discard both the flow-through and catch tube, and transfer the spin column onto a clean Kimwipe. Leave the column lid open. Transfer Kimwipe to 60°C oven, and allow to dry for 10 min.

7. Transfer spin column to a sterile 1.5 ml Eppi tube, and add 20-50 μl\* of EB buffer to the centre of the spin column – ie on the membrane, not the walls of tube. Allow to sit for 2 min. Spin at ~10,000 g for 1 min, retain Eppi tube with DNA solution in EB, discard spin column.

\* Usually we want high concentration rather than high yield, so use 20 µl. If max. yield is important, or if you have lots of DNA, use 50 µl. Note that you lose approx 5 μl EB during the procedure.

**DNA PURIFICATION SOLUTIONS (SPIN COLUMN) – based on recipes at openwetware.org**

**Buffer PB:** 5 M guanidine HCl, 30% isopropanol. Not sterilised.

Prepare in a sterile bottle or tube containing the correct amount of autoclaved RO water.

**Buffer PE:** 10 mM Tris-HCl, pH 8, 80% ethanol. Not sterilised.

Add appropriate vol. of autoclaved 1 M Tris-HCl to the correct amount of autoclaved RO water in a sterile bottle or tube, then add 100% ethanol to give 80% final conc.

**Buffer EB:** 10 mM Tris-HCl, pH 8. Autoclaved.

 Add appropriate vol. of autoclaved 1 M Tris-HCl to the correct amount of autoclaved RO

 water in a sterile bottle or tube.

**Purification of DNA via spin column (for DNA in a gel slice)**

**GENERAL NOTES**:

This protocol is good for restriction fragments, small digested plasmids, and PCR products. It is NOT good for genomic or chromosomal DNA. Use the FastPrep reagents or CTAB-phenol type prep instead for genomic DNA. Digested plasmids work well up to about 5 kb, but the yield quickly falls off past this point. Generally, plasmids don’t purify as well as PCR products on these columns.

Gel-purification: Don’t expose the DNA to any UV light and don’t use borate-containing buffers in the gel. Even minimal exposure to short-wave UV light will greatly damage DNA, and make it hard to ligate later. Long-wave UV (e.g. hand-held lamp) is less damaging than short-wave UV (transilluminator), but best to avoid UV altogether. Borate can cause problems for later enzyme steps.

**NOTES ON GEL EXTRACTION USING GEL-GREEN (recommended)**

1. If you need to purify DNA from an agarose gel, use TAE-agarose (don’t use TBE!). Put Gel-Green (1 µl) in the gel. Load plenty of DNA so it is easy to visualize.

2. Run gel as usual, then visualize under blue illumination (NOT UV!) in a dark room or box. There is a blue light in the lab for this purpose and orange-tinted glasses also help. Cut out your band(s) of interest with a clean, sharp scalpel blade, and put them into an Eppi tube.

3. Weigh the tube containing gel slice and subtract weight of empty Eppi tube to figure out the weight of gel. Proceed to ‘main protocol’ below.

**NOTES ON GEL EXTRACTION USING GEL-RED or ETHIDIUM (not recommended)**

1. If you need to purify DNA from an agarose gel, use TAE-agarose (don’t use TBE!). For this version of the protocol, you can use GelRed or ethidium bromide, either in-gel or post stain.

****2. Run one lane with a small amount of the sample (e.g. 5 µl; ‘test lane’), then leave a space, then load the rest of the lanes with the remaining sample (e.g. 6 x 50 µl).

3. After the gel has run (and stained if necc), slice off the ‘test lane’ and view on an old-fashioned UV transilluminator where you can cut the agarose while the UV light is on (CAUTION! wear full face shield!).

=== Do not expose the DNA that you want to use for cloning

 to UV light! Only expose the test lane.===

4. Make a notch in the agarose corresponding to your position and width of your band of interest (see pic for an example 🡪 )

5. Line up test gel slice with main part of gel, cut out across the other lanes in line with your notch.

6. Weigh the tube containing gel slice and subtract weight of empty Eppi tube to figure out the weight of gel. Proceed to ‘main protocol’ below.

**MAIN PROTOCOL :**

 <<put on gloves + safety glasses. QG contains guanidine which is somewhat toxic >>

1. If your weight of agarose is less than 100 mg (=100 µl), then make it up to 100 µl with TE. The volume you now have will be called “1 volume”. Then add “3.5 volumes” of QG buffer to your gel slice. eg. if you have 100 mg of agarose, add 350 µl of QG.

2. Slice up or mash the agarose in an Eppi (<300 mg agarose) or a McCartney bottle (>300 mg). This will speed dissolution. Melt the agarose with heating (60C for 5 min is usually enough, mix occasionally), then when it’s all dissolved, allow to cool to room temp.

3.Add “1.5 volumes” of isopropanol to the mixture. e.g. for 100 mg agarose, use 150 µl isopropanol.

4. Place a silica-based spin column (e.g. ‘Econospin’) into its 2 ml catch tube (if it isn’t already set up that way). Load up to 750 µl of the DNA / QG / isopropanol mixture onto the column. Spin at ~10,000 g for 30 sec. Discard the flow-through into culture waste.

5. If you still have more DNA /QG / isopropanol mixture left, repeat the previous step until all of the mixture has been put thru the column. The columns will hold a total of ~10 µg DNA, which is a lot!

 <<Can take off glasses and gloves now >>

6. Add 750 μl of buffer PE to the column, spin ~10,000 g for 30 sec, discard flow-through.

7. Repeat step 6.

8.Spin again for 30 sec to remove all traces of PE from the column. Discard both the flow-through and catch tube, and transfer the spin column onto a clean Kimwipe. Leave the column lid open. Transfer Kimwipe to 60°C oven, and allow to dry for 10 min.

9. Transfer spin column to a sterile 1.5 ml Eppi tube, and add 20-50 μl\* of EB buffer (5 mM Tris, pH 8) to the centre of the spin column – ie on the membrane, not the walls of tube. Allow to sit for 2 min. Spin at ~10,000 g for 1 min, retain Eppi tube with DNA solution in EB, discard spin column.

\* Usually we want high concentration rather than high yield, so use 20 µl. If max. yield is important, or if you have lots of DNA, use 50 µl. Note that you lose approx 5 μl EB during the procedure.

**DNA PURIFICATION SOLUTIONS (SPIN COLUMN) – based on recipes at openwetware.org**

**Buffer QG:** 5.5 M guanidine thiocyanate (GuSCN). 20 mM Tris HCl pH 6.6.

 Prepare by dissolving GuSCN in autoclaved Tris HCl. The GuSCN will take up a large fraction

 of the total volume, so begin with less volume, adjust to final volume with Tris at end.

**Buffer PE:** 10 mM Tris-HCl, pH 8, 80% ethanol. Not sterilised.

Add appropriate vol. of autoclaved 1 M Tris-HCl to the correct amount of autoclaved RO water in a sterile bottle or tube, then add 100% ethanol to give 80% final conc.

**Buffer EB:** 10 mM Tris-HCl, pH 8. Autoclaved.

 Add appropriate vol. of autoclaved 1 M Tris-HCl to the correct amount of autoclaved RO

 water in a sterile bottle or tube.

**Protocol for preparation of chemically competent E.coli cells (rubidium chloride)**

 **NOTES:**

Use excellent aseptic technique at all times. All materials must be sterile.

Protocol can be scaled up or down as required. 100mL of *E. coli* culture produces about 50 x 220 µL aliquots of competent cells. Each individual aliquot of cells is sufficient for 4 transformations (4 x 50µl, with a bit left over as a safety margin).

The competent cells can also be used fresh, without making frozen aliquots, this actually works even better, but it is tedious to do over and over, thus we typically make frozen stocks. These freezer stocks don’t survive multiple freeze/thaw cycles well, so throw out unused cells if there are leftovers after your transformation experiment.

After step 6, work on ice and in the cold room to increase the quality of the final cell prep. Keep RF1 and RF2 solutions in cold room/fridge, so they are always ready to use. At the beginning of the day, place centrifuge bottles in cold room or in the -20°C freezer, ready to use.

 **PROTOCOL:**

1. Streak *E. coli* strain (e.g. JM109, TOP10, DH5α) from glycerol stock or other source onto plain LB agar (no antibiotics). Incubate overnight at 37°C.
2. Check that culture looks pure. Inoculate a 5mL plain LB broth with growth (several colonies) from the LB plate. Incubate overnight (16-24 h) at 37°C, with shaking
3. Aseptically inoculate 100mL plain LB broth (in a 500mL Schott bottle or Erlenmeyer flask) with 3 ml of overnight culture; this should give an initial OD600 of ~0.05. (check this to be sure)
4. Grow cells at 37°C with shaking, until the culture reaches OD600 of ~0.5 (anywhere from 0.3-0.7 is OK, but try to get close to 0.5). This should take 2-3 hours, so check the OD600 every 15 minutes or so after the first hour of incubation.
5. Aseptically transfer (pour) the cells into sterile centrifuge tubes or bottles (one 250 ml Sorvall bottle or 2 x 50 ml Falcon tubes) and balance these, either by transferring culture aseptically between the tubes/bottles or by adding sterile water or LB to the lighter tube/bottle.
6. Centrifuge at 4000 rpm (~3000 g in Centaur/Centurion machine) at 4°C for 10 minutes. Note: centrifuge needs to be cold. Turn on and set temperature beforehand. 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.
7. Working in the cold room with the cells on ice, pour off the supernatant into culture waste (don’t let the centrifuge tube/bottle actually touch the edge of the culture waste bottle). Try to remove as much of the liquid as possible – give it a shake / tap to assist this.
8. Resuspend the pellet gently in 33mL RF1 solution by vortexing and/or shaking the tube/bottle. Its OK to be rough with the cells at this stage in the process, but you shouldn’t need to shake or vortex for more than 10 sec or so.
9. Incubate on ice for 1 hour, then pellet the bacteria again at 4000rpm, 4°C for 10 minutes.
10. Working in the cold room, pour off the supernatant into culture waste. As before, try to remove as much of the residual liquid as possible.
11. Resuspend the pellet in 8mL RF2 solution by vortexing or shaking. At this stage, the cells have become more fragile due to the RF1 treatment, so its important not to shake/vortex any longer than ~10 sec (this shouldn’t be necessary).
12. Incubate on ice for 15 minutes. While the cells are incubating, set up all your Eppi tubes (~50) on ice with the lids open, so they are pre-chilled, and ready to receive cells. Label these tubes on top with the strain name before putting them on ice (labelling becomes difficult with cold and wet tubes!). Be careful to only push the tubes only about 2/3 of the way into the ice. If they are pushed in too far (right up to the lip of the tube), you risk getting ice or melted ice (not sterile!) into your cell aliquots
13. Working quickly (but still carefully!), aliquot 220 µL of cell suspension into the pre-chilled Eppi tubes. Once dispensed, close tube lids tightly, and collect all tubes into a bag/box with a clear and prominent label and store it immediately in the ‑80°C freezer. It’s a good idea to label both the outside of the bag/box AND place a label written on paper inside.
14. Test the transformation efficiency of the freshly-prepared competent cells using a known amount of a plasmid standard (see protocol for heat shock transformation).
15. Streak out a loopful of the cells onto plain LB medium (using either a sample of a frozen aliquot or some residual cells remaining in the large centrifuge tube/bottle), and incubate at 30°C for three days to allow any of the common types of contaminants (e.g. *Staphylococcus*) to grow. This is to check the purity of the cell stock. The streak-plate should look completely uniform, with colonies of only one type (E.coli), and no heterogeneity in the initial patch or the streaklines which would indicate a mixture of bacteria is present.

**COMPETENT CELL SOLUTIONS:**

|  |  |  |
| --- | --- | --- |
| **RF1 solution** |  | **RF2 solution** |
| **Chemical** | **Amount** | **Final conc.** |  | **Chemical** | **Amount** | **Final Conc.** |
| ROW\* | 200 ml\*\* | na |  | ROW\* | 100 ml\*\* | na |
| RbCl | 2.4 g | 100mM |  | MOPS | 0.2 g | 10mM |
| MnCl2 | 2.0 g | 50mM |  | RbCl | 0.1 g | 10mM |
| K Acetate | 0.6 g | 30mM |  | CaCl2 | 1.0 g | 75mM |
| CaCl2 | 0.3 g | 10mM |  | Glycerol | 15 g | 15% (w/v) |
| Glycerol | 30 g | 15% (w/v) |  |  |  |  |

\* ROW = reverse osmosis water

\*\* Add all the ingredients to 100 ml ROW, then make up to final volume in a measuring cylinder

For RF1: adjust pH to 5.8 with conc. acetic acid. pH will change very quickly and only requires ~ 10µL of acetic acid. Sterilize by filtration into an autoclaved media bottle.

For RF2: adjust pH to 6.8 with NaOH or HCl, as appropriate. Sterilize by filtration into an autoclaved media bottle.

**Protocol for heat shock transformation of chemically-competent cells**

1. Remove one or more aliquots (as required) of chemically competent cells of your *E.coli* strain from the -80°C freezer. Thaw the cells e.g. by rubbing them in your hands or put them briefly in a 37°C waterbath, but don’t let them stay warm! As soon as they are thawed, put them onto ice.

2. Divide the cells into the appropriate number of 50 µl aliquots in separate Eppi tubes on ice. Add your DNA samples to each tube; you can use up to ~10 ul of ligation mixture or plasmid here, but note that typically 3 µl of ligation mix or 1 µl of plasmid would be standard.

3. Make sure you include both a positive control and a negative control in the transformation experiment. The positive control should be 1 µl of a plasmid with the correct antibiotic resistance (same resistance as the plasmid used for the ligation), and should also be a plasmid stock that you know is in good condition (based on agarose gel).The negative control is simply no DNA added.

4. Put the cells into a foam ‘floatie’ and put on ice. Ensure at least the bottom half of the tube (approx 2 cm) is embedded in the ice, don’t just rest them on top of the ice. Allow the cell/DNA mixtures to incubate on ice for 15-30 min.

5. Take your esky of ice over to the 42°C waterbath or 42°C heat block. Put the floatie into the waterbath. Allow 45 seconds for heat shock. (Plus or minus 10 seconds, this needs to be exact!). Then transfer the floatie straight back onto ice (embed into ice, as above, don’t just rest on top).

6. Allow transformation mixtures to sit for 2 min on ice, then add 1 ml sterile LB broth to each tube. You can also use more fancy media (e.g. SOC or SOB), but there is not that much difference.

7. Incubate on 37°C shaker for 1 hour. Put the tubes horizontal so they get good shaking action. eg. put the tubes laying flat on the shaker platform and masking-tape into place. Make sure the lids are tight! You can incubate without shaking, and you can incubate for less time (30 min), but it won’t work as well in these cases.

8. Label the LB-antibiotic plates before starting the next bit; you need two plates for each ligation condition or plasmid type, since we will plate out two different cell concentrations of each to ensure we get countable/pickable numbers of colonies. Double check the plates to ensure you are using the correct type of antibiotic(s) for the type of plasmid(s) you are using.

9. Pipette 100 µl of the first cell suspension onto one LB-antibiotic plate (label ‘100 µl’ in addition to other info). Sterilise the glass spreader with ethanol and flame (CAUTION! READ THE **SOP** FIRST!), and spread the cells around the plate with the spreader. Do this by pushing the spreader with a back-and-forth motion, while turning the plate around in a circular motion. Be careful not to touch the spreader on your fingers! Keep spreading for approx 10 seconds.

If the plates are properly dried, you should feel the spreader start to ‘stick’ to the agar, this means the liquid has been drawn into the agar. If this doesn’t happen after ~20 seconds, stop spreading, but next time, dry plates for longer! If the plates are incubated with a lot of liquid still on them, you may not get nice discrete colonies (the cells will swim around in the liquid, making a mess).

10. Spread 100 µl of the remaining samples, each onto a separate, appropriately-labelled plate.

11. Centrifuge all the tubes at ~15,000 rpm for 1 minute in a micro-centrifuge. Pour off most of the supernatant into culture waste (being careful not to touch the tubes on the edge of the culture waste bottle). Leave a little bit of liquid behind (about one or two drops).

12. Vortex the cells in the remaining liquid for about 10 seconds, until they are not sticking to tube anymore, and you have a nice smooth, even, cell suspension.

13. Pipette the cells from the first cell suspension onto the appropriate pre-labelled LB-antibiotic plate (label with ‘pellet’ in addition to other info), spread plate as above. Repeat for the remaining samples

14. Incubate all plates at 37°C overnight. Note that for some plasmids and ligations, it may be beneficial to instead try room temp for 2-3 days – this lowers the copy number of pUC type plasmids, and is useful to allow retrieval of clones that might be toxic to the host.

15. When examining your plates, first check your controls. The positive control should have thousands of colonies, perhaps even a confluent lawn of growth, especially on the ‘pellet’ plate. the negative control should have no colonies at all. If you don’t see these results with the controls, anything you see on your experimental plates is questionable. Common problems and their interpretation are summarised in the Table below. Detailed troubleshooting of the different ligation controls and their results is described in a different protocol.

**“Mix up of labelling” can cause MANY problems – be super careful with your labelling ! (do this BEFORE starting the hands-on bit of the procedure, and double-check everything)**

|  |  |
| --- | --- |
| **Problem** | **Interpretation/solution** |
| Lots of growth of the negative control (thousands of colonies or lawn) | * Forgot to add antibiotic to the plates
* Antibiotic concentration is wrong (too low)
* Host bacteria are already resistant to the antibiotic (e.g. TOP10 has chromosomal streptomycin resistance)
* Plates incubated too long (especially with LB-ampicillin)
* severe contamination with an antibiotic-resistant bacterium (not E.coli) (unlikely!)
* Mix up of labelling somewhere – is this actually the positive control? or one of the experimental tests?
 |
| Some growth on the negative control (a few colonies) | * Contamination during the procedure, e.g. from one of the other samples or the pipette etc. This may not be a ‘deal-breaker’ so long as there are lots more colonies on your experimental test plates
* Mix up of labelling
 |
| No growth or very little growth on the positive control plate | * The cells are not competent
* Used the wrong antibiotic in the agar (check the sequence of your plasmid to confirm correct resistance)
* Used the wrong concentration of antibiotic (too much)
* Agar plates are ‘bad’ for some other reason (e.g. added mercuric chloride instead of sodium chloride!)
* Plasmid stock has gone bad (run a gel to check)
* Mix up of labelling
* Pipetting error (look at the pipette tip to ensure that you really have 1 µl of plasmid in there!)
 |

 **Preparation of electrocompetent cells of *Pseudomonas* or *E.coli***

1. Inoculate a 50 ml LB broth with several colonies from a freshly-grown LB agar plate culture (1-7 days old), grow overnight with shaking at 30°C (for *Pseudomonas*) or 37°C (for *E.coli*)

2. In the morning, add the entire 50 ml culture to 500 ml LB broth in a large Erlenmeyer flask. Measure the optical density (OD600) – this should be ~0.1 - 0.2. Record the value.

3. Incubate the 500 ml culture with shaking at 30°C. Check the OD600 approximately every 30 minutes. When the value reaches 0.4-0.6 (*E.coli*) or 0.8-1.0 (*Pseudomonas*), take it off the shaker, and pour culture aseptically into 2 x 500 ml sterile Nalgene centrifuge bottles which have been chilled on ice. (note that these centrifuge bottles should only be half full so they don’t leak in the centrifuge. Resist the temptation to just use 1 x 500 ml bottle!).

4. Ensure tubes are balanced (weigh them, and top up the lighter one with the appropriate amount of buffer). Centrifuge in large floor centrifuge for 15 minutes at 7500 g (=6700 RPM for Sorvall GS3 or equivalent rotor) at 4°C.

5. Pour off the supernatant liquids into culture waste, and resuspend each pellet in 30 ml of cold electroporation buffer \*. Put caps on tight and shake vigorously to resuspend the cells. (approx 10 seconds), then pour into 2 x 50 ml Falcon tubes.

6. Ensure tubes are balanced (matching volumes by eye is OK this time). Centrifuge at top speed (~4500 rpm, or ~ 3000 g) for 15 minutes in Centaur/Centurion type centrifuge at 4°C.

7. \*Gently\* pour off supernatants into culture waste –the pellets may be quite soft, and are easily lost if you are not gentle! You need to retrieve the tubes immediately from the centrifuge when the spin is finished, and also handle the tubes gently in order to maintain the pellet at the bottom of the tube.

8. Resuspend each pellet in 30 ml of cold electroporation buffer, and spin again.

9. Pour off supernatant, resuspend each pellet in 30 ml cold electroporation buffer, and spin again.

10. Resuspend one pellet in 5 ml of cold electroporation buffer, resuspend cells by vortexing (~10 sec). Pour this cell suspension aseptically into the other tube, and vortex again. Place on ice.

11. Pipette the cell suspension into multiple small aliquots in sterile 1.5 ml Eppendorf tubes. For example, 25 x 200 µl aliquots would be typical. Each of these single 200 µl aliquots has enough cells for four electroporation cuvettes. Label the tubes with the strain name before you start. Although ‘correct practice’ here would involve setting up your 25 Eppi tubes opened, and on ice, I woudn’t do this due to the potential for contamination (melted ice getting in the tubes). But you should aim to do this step quickly, and get the cell aliquots into the -80C freezer ASAP.

12. Put the cell aliquots into a plastic bag or box and freeze at -80 C. Label the bag or box in addition to the individual tubes. Don’t label the bag surface directly with permanent marker – this will come off–better to write with marker in large clear text on a piece of paper, and put this in the bag.

\* Electroporation buffer: For *E.coli* and *Ps.putida*, you can use 10% glycerol, but for *Ps.stutzeri*, you need a more complex buffer containing 10% sucrose, 1 mM HEPES (pH 7), 1 mM MgSO4. This is because the cells are more fragile. Both types of buffer need to be sterilised by autoclaving, and should be chilled on ice before use (at least 30 min on ice; or keep this buffer in the cold room).

**Electroporation of electrocompetent cells of E.coli or Pseudomonas**

1. Retrieve the appropriate number of cell aliquots from the -80°C freezer and allow to thaw. You can do this by putting them directly on ice and waiting a long time (~20 min?) or you can put them in room-temperature water in a floatie, then put on ice as soon as they are fully thawed (~5 min?). In the latter case, don’t leave them too long at room temperature, keep an eye on them ! Occasional mixing by flicking helps to speed the thawing process.

 <<<note ! don’t try to electroporate chemically-competent cells, these will spark! >>>

2. Divide up the cells into multiple 50 µl aliquots in sterile 1.5 ml Eppi tubes, on ice. Also put on ice the same number of sterile electroporation cuvettes (2 mm gap size). These need to chill for at last 10 min before use. Label the cuvettes on the cap before use.

3. Add your plasmid DNA or ligation mixture to the cells. For purified plasmid, 1 µl is heaps ! (and may even be too much). For ligation mixture, it would be typical to use 2-3 µl. In both cases more is not better, because the more DNA you add, the more salt you are also adding, and it doesn’t take very much salt to make the electroporation procedure fail (short circuit = sparks!). Make sure you change pipette tips for each DNA sample.

4. For all electroporation experiments, you should include a negative control (no DNA added) – this will let you know firstly whether your competent cells are OK (not too salty), and secondly whether your aseptic technique is good. If your negative control makes a spark, your cells are no good (need more wash steps!) or the electroporation machine is not set up correctly. If the electroporation procedure works (no spark), but you get colonies on the plates from the negative control, this means you have contamination, either in your competent cells, or getting in somewhere in the electroporation procedure.

5. In the case of electroporation experiments with ligation mixtures, make sure you also include a positive control, which is 1 µl of a purified plasmid of known good quality with the correct antibiotic resistance. This should give you thousands of colonies or a confluent lawn of growth on the appropriate antibiotic agar plate – if it doesn’t, this could mean your cells are not competent, or the plates are bad, or the electroporator is not set up properly, or your ‘good quality’ plasmid stock is no longer good quality.

6. Once all your cell aliquots have the appropriate DNA samples added, transfer each mixture individually to the corresponding electroporation cuvette (again make sure everything is labelled before starting!). Give each cuvette a few taps on the bench to make sure the cell mix is at the bottom of the cuvette.

7. Take your esky, a p1000 pipette, box of blue tips, some sterile LB broth, and some paper towel over to the electroporation machine (You need ~1 ml LB per sample). Turn on the electroporator, and choose “Exponential” protocol. Adjust the settings on the electroporator to 2500 V, 25 µF, and 200 Ω. These settings will work with a wide variety of Gram-negative bacteria, but note that for Gram-positives you need to increase the ohms to 800 Ω (see next protocol). Also note that if you use cuvettes with a different gap size you need to adjust the electroporator settings.

8. Take your first cuvette off ice, and wipe down the outside and underneath briefly with paper towel. Place this firmly into the cuvette holder on the machine, then close the lid.

9. Press the “Pulse” button. After a few seconds, the machine will beep to let you know the pulse has been delivered. Note down the time constant displayed on the screen (ms= milliseconds). This tells you how long the pulse lasted, and a higher number here is better. Retrieve your cuvette and immediately add 1 ml of sterile LB to it. Return to ice.

10. Repeat steps 8 and 9 for all your samples. When finished, turn off electroporation machine, and return to your bench.

11. As the sample becomes more salty with increasing amounts of DNA, the time constant will decrease until eventually you will get a spark (short circuit). If any of your samples make a spark, this means either your competent cells are not prepared properly (the negative control will spark too), or that you have added too much DNA and/or the DNA is of low quality. It is possible to sometimes get transformants from a cuvette which as sparked, but don’t count on it! Its better to repeat the experiment using less DNA (or a dilution of the DNA in sterile MQ water) and/or a different batch of electrocompetent cells.

12. Aseptically pour each transformed cell mixture into a sterile 1.5 ml Eppi tube, as follows. First, label all the Eppi tubes, then open them all, positioned in a rack.(they don’t need to be in ice). Take the cap off the first cuvette and briefly pass the top of the cuvette through the Bunsen flame a few times (if you flame it too much it will melt!), then pour into the first Eppi tube. Tap the cuvette on the tube to ensure you get all the sample out. Repeat for other tubes.

13. Ensure all the Eppi tubes are tightly closed and labelled, and then incubate with shaking for 1 hour at 37°C (E.coli) or 1.5 hours at 30°C (Pseudomonas). Put the tubes lying on their side in a beaker or other container in the shaker, or you can attach them to the shaker with elastic bands or masking tape. Lying horizontal is important for good shaking action in these small tubes.

14. Retrieve tubes from shaker. Prepare 2x the number of appropriate agar plates as you have samples (double check you are using the correct antibiotic(s)!!!), and label all these plates before going any further. In addition to the usual information (antibiotic, plasmid, strain, date), label half of the plates “100 µl” and the other half “pellet”

15. Spread plate 100 µl of each sample onto the appropriate antibiotic agar. Centrifuge the remaining culture (2 min at 10,000 g in Eppi centrifuge), pour off most of supernatant, and resuspend the pellets by vortexing in the drop of liquid that remains. Plate these resuspended cells on a second set of plates of the same antibiotic type. The reason for doing two plates for each sample is to give us the best chance of getting countable and well-isolated colonies on at least one of these plates.

16. Don’t throw out the used electroporation cuvettes. These can be recycled. (repeated rinses with water then 80% ethanol, see elsewhere for this protocol). Don’t let the cuvettes dry out with cell mix still in them, you should give these a couple of rinses with WATER as soon as you finish the experimental part of the work. (discard rinsate into culture waste).

**Preparation of electrocompetent *Mycobacterium smegmatis* & electroporation**

**Protocol for making electrocompetent cells**

1. Inoculate a 5 mL culture of *M. smegmatis* mc2-155 in LB broth containing 0.05% Tween 80 (add after autoclaving from filter-sterile stock) and grow until saturation (~2 days). If there are plasmids in the *M.smeg* cells (e.g. pJV53), add antibiotic too (e.g. Km at 25 µg/ml). You can grow *M.smeg* at either 30°C or 37°C.
2. Subculture into 50 mL of MSM minimal medium in a 250 ml flask (see elsewhere in lab protocols for MSM recipe) containing 1% succinate and 0.05% Tween 80. Start at a final OD600 = 0.02 and grow with shaking overnight. If the culture has plasmids in it like pJV53, it will grow more slowly, and a higher initial OD600 might be helpful (e.g. 0.1).
3. On the following day, when cells are grown up, but not yet in stationary phase (aim for OD600 between 0.6-0.8), transfer the cells to 50 mL Falcon tubes, centrifuge at ~ 4000 g for 10 min at 4ºC, discard the supernatant. For cultures containing pJV53, first add acetamide (0.2% w/v) and grow a further 3 hours to induce the recombination enzymes before centrifuging the cells.
4. Add 20 ml ice cold 10% glycerol + 0.05% Tween solution, centrifuge, discard supernatant. Repeat this step two more times. This removes salt, which is bad for electroporation.
5. Resuspend cells in 2 mL glycerol-Tween solution, divide into 200 µl aliquots, freeze at -80°C.

**Protocol for electroporation**

1. Thaw aliquots of electrocompetent cells (from above) equivalent to half of your number of DNA samples, divide into 100 µl aliquots in Eppi tubes and keep on ice.
2. Add plasmid DNA to the cells. Depending how clean and how concentrated your plasmid is, and what the aim of the experiment is, you may be adding 1-10 µl of plasmid (could be 10-1000 ng, depending on the experiment). If unsure, start with less plasmid and see how you go. Too much plasmid will cause sparking (bad).
3. Allow 10 min on ice for the DNA to adsorb to the cells. While you are waiting, add the electroporation cuvettes to the ice to allow them to chill. We usually use 2 mm gap cuvettes for this. Careful not to get ice inside the cuvettes (ice is not sterile!).
4. Transfer each cell+DNA mix into an electro-cuvette, tap gently to get liquid to bottom. Take samples on ice to the electroporation machine. Set the machine for 2.5 kV, 800 Ω, 25 µF.
5. Wipe down the outside of the cuvette with paper towel, and put it into the holder. Ensure that the metal sides of the cuvette are contacting the electrodes. For some combinations of machine+cuvette, you need to put a spacer in the bottom of the holder to ensure good contact (we use a 5 mm glass bead for this).
6. Hit the button to initiate the electric pulse. Note the time constant – this should be between 15-25 milliseconds. If it sparks, you can try adding 100 µl of 10% glycerol and re-pulsing, but usually this means you need to add less DNA and/or better-quality DNA.
7. Immediately add 1 ml LB-Tween to each cuvette, mix with p1000, transfer to Eppi tube, recover for 2 h with shaking at 30°C or 37°C.
8. Spread-plate 100 µl on the appropriate antibiotic agar plate. Pellet remaining cells, resuspend in a drop of the residual liquid, then spread on a second plate. This gives a mini-dilution series, so hopefully one of the plates will have nice well-separated pickable colonies (this may not matter for some experiments, but does matter for others).

**Screening recombinant clones (patch, PCR, digest, sequence)**

**NOTES:** There are many strategies available for screening recombinant clones. The easiest strategy is when the recombinant has an obviously different phenotype to the vector plasmid alone – e.g. if you are cloning a resistance gene or a fluorescent protein. However, in most cases, we will need to screen the clones by PCR to find the recombinants that we are seeking.

The diagram shows some PCRs that can be used to determine if a plasmid clone is:

A. The desired recombinant

B. Some other kind of recombinant

C. The vector only

The PCR using primers F1-R1 is the best starting point for screening– this is the ‘left junction’ PCR. If you get a product of the expected size in this PCR, this is a good indication that you have the desired recombinants. The PCR using primers F2-R2 (right junction PCR) can then be done on clones that are positive in the F1-R1 PCR. If the clones are also positive in this PCR, this is further evidence that they are the right thing.

The PCR using primers F1-R2 is known as a ‘spanning’ PCR or ‘insert spanning’ PCR – this will amplify the whole insert region. This PCR can be used to determine if the clones have an insert of the expected size, but note that this is not an all or nothing PCR, unlike the junction PCRs. In the spanning PCR, the clones will give a small product if they have no insert, and a large product if they have an insert. This PCR is a bit ambiguous though, since if you have a mixed population of cells in your template DNA, you may see only the small product (this is amplified preferentially), even if you do have some positive clones in there too. The spanning PCR is most useful in preparation for sequencing the insert DNA region, rather than for initial clone screening.

The primers used for amplification of the original DNA insert (not shown in diagram) can also be used for screening PCRs. e.g. these primers used on their own can tell you if the insert DNA is present. (but they wont tell you which way around it has been cloned, unlike the junction PCRs). You can also do a junction PCR using one of the original primers, and a primer in the vector on the other side of the insert DNA, although this may give large products, and a less reliable PCR compared to using dedicated junction screening primers.

The positioning of the screening primers is important for later steps (sequencing). Make sure that the primers F1, R1, F2, R2 are at least 100 bp away from the ligation join – this is because the first ~50 bp of sequence read are usually ‘junk’. Also ensure that these primers are not \*too\* far from the ligation join, otherwise you are amplifying (and later sequencing) stuff that you don’t need to, and it makes the PCR take longer to thermocycle. Placing the primers about 150-200 bp from the ligation join is good, which gives an overall PCR product size of 300-400 bp.

**INITIAL PATCHING AND PCR SCREENING:**

1. Choose 20 well-isolated colonies from your transformation plates. Circle these and number them on the base of the plate.

2. Label three PCR strip tubes with numbers 1- 20, leaving three blanks (one in each strip) as negative controls. (so your first strip should be clones 1-7, the next one should be clones 8-14, and the last one should be clones 15-20)

3. Using an agar plate containing the appropriate antibiotic, divide the plate into patches, using a square grid (5 x 5). Label twenty of the squares with numbers 1 to 20. (see diagram)

2. Prepare a PCR master mix with the appropriate left junction PCR primers, enough for ~30 PCRs, aliquot this into 3 x 8 lots of 25 µl in the labelled PCR strip tubes. Taq polymerase is good for this job (it is very robust to ‘mess’ in the PCR) but other polymerases can also be used.



3. Using a white pipette tip, pick up some growth from the colony of your first clone of interest. Make sure you can see some cells on the tip, but you don’t need a lot (approx 1 mm3 = 1 µl). Dip the tip in and out of the mix in the first PCR tube five times. You don’t need to dislodge the whole chunk of growth, enough cells will fall into the mix to give you a product, if the gene of interest is there. (this is called a ‘colony PCR’)

4. With the same tip, transfer the remaining growth onto the first patch on your agar plate, by scratching the tip across the surface of the agar a few times (e.g. make a set of three closely-spaced parallel lines – see diagram). Stay well clear of the borders of each square, we don’t want the clones to touch each other when they grow up. Then discard the tip.

5. Repeat steps 3 and 4 with the remaining 19 clones, being very careful to keep track of which colony, which tube, and which patch you are up to (labelling before you start is important!)

6. Put the PCRs on the thermocycler and incubate the agar plate at 37°C overnight.

7. Run out the PCRs on an agarose gel, and note which clones give a positive result.

If none of your clones are positive, you can try screening more clones (e.g. another 20), but if you still don’t find your clone of interest after screening ~40 clones, something is wrong – check all your ligation and transformation controls, check all your primer sequences and thermocycling conditions. You may need to repeat the cloning process. It is also possible that the gene you are trying to clone is toxic to the host cells – you may need to try a different vector or different host or different incubation conditions.

**SECONDARY PCR SCREENING**

1. Examine the patch plates prepared above Choose up to seven of the clones that gave a positive PCR in the left junction screen (at least three is recommended).

2. Set up a PCR master mix using the right junction primer set. Remember to make enough master mix so that there is at least 10% more than you need, so you don’t run out.

3. Screen the putative positive clones as described above, by colony PCR. Again, be careful to label everything before you start. Pay close attention to which clone patch is going into which PCR tube.

4. After thermocycling, run out the PCRs on agarose, and note which are positive for the right junction. Choose one of these for a plasmid prep; use this clone to inoculate 100 ml LB-antibiotic broth (see other protocol for details of plasmid prep).

5. Keep the patch plate! (wrap in parafilm, and store in cold room) – you may need to come back to this plate to plasmid prep a different clone if there are problems with the first one.

**VALIDATION OF RECOMBINANT PLASMID BY RESTRICTION DIGESTION**

**NOTES:** When making recombinant clones, we need to have several lines of evidence that the new plasmid has the correct structure. It is not sufficient to rely solely on the junction PCRs. For example, if the same insert has been cloned twice (head to tail) into the vector, of if there is a deletion in the middle of the insert DNA, the junction PCRs will look fine, but the overall structure will be wrong. Doing digests of the new plasmid provides another line of evidence for the correct structure, and also it allows us to quantitate the plasmid accurately, which is usually needed in any case for further work.

1. Using the plasmid prep from your chosen recombinant clone (above), set up a number of restriction digests which will be diagnostic of the correct structure. For example, you can choose an enzyme that cuts in the insert DNA but not in the vector DNA (this is ideal), and/or you can use an enzyme that cuts in the vector only (this is still OK, if the insert is quite large i.e. >500 bp). You want to be generating between 1 and 3 restriction fragments in total, and you want the recombinant clones have a clearly distinct digest pattern to ‘vector only’. You want to be doing two or three different kinds of digests to give maximum confidence in the structure of the plasmid, not just one digest.

2. See other protocol “Protocol for quantitation and restriction digestion of plasmid (gel analysis)” for details of how to set up these plasmid digests.

3. If the chosen plasmid clone looks correct based on both junction PCRs and restriction digests, then you can proceed to sequencing (below).

**VALIDATION OF RECOMBINANT PLASMID BY SANGER SEQUENCING**

**NOTES:** There are two distinct approaches to this, either sequencing the plasmid directly, or amplifying the insert region, and sequencing that. The advantage of sequencing the plasmid directly is that there is no chance of PCR mutations influencing the sequence, but the disadvantage is that we tend to get worse sequencing reads from plasmids compared to PCR products.

In either case, PURITY of the DNA is critical for success of sequencing. A good strategy is to first attempt to sequence the plasmid directly, and if that fails, then amplify the insert region (‘spanning PCR’ above), then sequence that. In the case of PCR products, these must be purified (e.g. Qiaquick kit) \*before\* sequencing.

**SEQUENCING PRIMERS:** The usual primers to use for sequencing would be the F1-R2 primers in the diagram above. However, for large inserts (>1 kb), you will need to design and order additional primers, since the average sequencing read length is only 500-700 bp, depending on template quality. In that case, space additional primers about 400 bp from the F1 and R2 primers, to ensure you get coverage of the entire insert sequence. (and for very large inserts, place further primers again ~400 bp away from the second set of primers). For maximum confidence in the sequence, it is good practice to sequence BOTH STRANDS of the DNA, ie. to ensure you have duplicated sequence data all the way along the insert DNA.

Note that each sequencing reaction only contains ONE PRIMER! It is not a PCR!

See below for detailed instructions on setup of Sanger sequencing reactions for AGRF.

**Preparing and sending samples for Sanger sequencing (AGRF)**

**General notes:** this sequencing method is appropriate for PCR products or plasmids that are expected to be a single sequence. This method is NOT appropriate for diversity analysis (e.g. sequencing 16S PCR products from soil) or other samples where a mix of sequences are expected.

A good Sanger sequencing run should yield at least 800 bp of sequence. If you are sequencing a large construct eg. a plasmid, you should space your sequencing primers about 500 bp apart to ensure overlap of sequence reads, even if the sequence is a bit shorter than usual. The first ~50 bp of sequence is junk, so keep this in mind when thinking about where to design primers.

**Specific instructions:**

Download the pdf “Sanger Sequencing: Sample Preparation Guide” from the AGRF website and read the relevant bits of this document. Especially note the table on page 5. Our samples are almost always the “PD” type (purified DNA).

Set up sequencing mixtures in 1.5 ml tubes with a short informative label on the lid. (e.g. NC1, NC2, NC3…). Wrap top in a little Parafilm to stop it popping open during postage.

Each mixture should contain:

• Column-purified PCR product: typically 1-2 uL (10-75 ng)

• Sequencing primer\*: 2 uL of 5 uM stock solution

• Sterile MilliQ water: Remainder of mixture, to total of 12 uL

\* when sequencing a PCR product, we usually usually one of the initial PCR primers as a sequencing primer too but note that the sequencing primer solution is 10x more dilute. Use sterile MQ to make the dilution. Note that a sequencing reaction only needs ONE primer, not two. It is not a PCR.



Assemble your sequencing mixture on the same day as you plan to send it, ideally just before posting it. Even purified DNA will degrade eventually at room temperature, so we want to minimise this exposure time. Wrap your tubes in bubble wrap or other protective packaging, and add them to an express post envelope (available in G08 store).

Next you need to fill out the online sample submission form on the AGRF website. (www.agrf.org.au) Get the login name and password from Nick.C.

Go to the ‘submit samples’ tab, then choose service type ‘Sanger sequencing’. Region is ‘Sydney’, service is ‘Purified DNA’, sample format is ‘1.5 ml tube’, then choose the correct product type for your samples, then base call method should be ‘N calls for low-quality bases’

Fill out the table at the bottom of the page. It’s very important that the sample names entered in this table are \*exactly\* the same as what is written on your tubes. (You don’t need to worry about the ‘sample name range’ or ‘sample prefix’ boxes.) Don’t tick any of the safety boxes !

Choose purchase by ‘Cost Centre’ then type in your lab account code as the cost centre code (check with Nick.C. for the account code). The delivery method is ‘Post’ then in the next box choose ‘Express Post’. Temperature is ‘Ambient’ Check everything is entered correctly then hit Submit. **You will get a pdf version of the document, which you need to print and add into your package.**

Seal the express post envelope and address to:

Bhawana Nain

AGRF

PO Box 285

Westmead, NSW, 2145

Make sure you sign the hazardous good declaration on the back and peel off the tracking sticker so you can track the package. Post in a yellow express post box. There is one of these on Science Rd (outside the uni post office) and another on King Street (Newtown), near corner of Missenden Rd.

Nick gets an email when your sequencing results are ready (usually 2-3 days after they receive the package), then I will let you know. You can then log in to the AGRF site and retrieve your data. Go to Retrieve Data tab, then Click to connect to AGRF cloud (you may need to enable popups)

The sequence data comes back as several different file types. The .fa and .seq files have basically the same information (i.e. your sequence) ; these can be opened in any text editor (eg. Word or Notepad) and will also open with SnapGene. The .ab1 file has the raw data from the sequencer. If your data is good, you probably wont need the .ab1 file, but if you have N’s in the middle of your data you may need to check the raw data and correct the sequence based on manual inspection of the locations which have given ‘N’s. The ab1 file will open with SnapGene or other dedicated sequence viewer programs. Note that ALL Sanger sequence data will have N’s at the start and at the end, these should be removed before doing any further analysis.

**Protein analysis by SDS-PAGE**

**General notes:** Many of the chemicals involved here are toxic (acrylamide is a neurotoxin, APS is an irritant and TEMED is foul smelling). **Make sure to wear gloves + glasses and remember that anything in the acrylamide area stays in the acrylamide area**. All wastes should go in the acrylamide waste bag for separate disposal.

**Making a gel:**

As a rule whenever you make these gels a few will leak no matter how well you think you’ve sealed it so aim to make twice as many as you need. Any successful gels you don’t need today can be kept for a few days in the cold room wrapped in wet paper towel and gladwrap or you can run a gel at the same time with a different amount of protein per lane in case one comes out nicer than the other.

The trickiest part is assembling the gel former and will take practice to get right. For each gel pick a short plate (the thin, evenly rectangular ones) and a back plate (have raised sides and come in either 1.0 or 0.75mm depths). If they look dirty you can clean them by dipping wet fingertips in Ajax powder and rubbing it into the plates and rinsing it. A few plates have chips – these are a major cause of leaks so either avoid them or make sure the chips aren’t at the bottom of the gel.

Get a green plastic cassette holder from the big plastic beaker (they have folding wings to clamp in on the plates) and slide the plates in. Before you clip them into place make sure the short and back plates are completely flush with each other at the bottom, if they are slightly out you will get a leak and your gel won’t be very nice. Once they’re flush clamp the wings down and check again to make sure they’re still neatly together.

Put a grey foam rubber strip down on the clear plastic gel holders in the groove at the bottom and carefully push your gel down onto it. It matters much more that the plates form a seal with it than that the green part is resting on the bottom or straight. Hold the gel former in place using the plastic clip at the top.

To check that your gel is sealed get a plastic pipette and drip water between the plates to the top and watch closely over a few minutes to see if the level drops. If it does, start again and check for chips or cracks. If it doesn’t you can pick up the whole assembly and tip the water out. Don’t move the glass plates out of the housing at all. The last bit of water can be wicked out with the corner of a paper towel but leaving a little bit doesn’t matter too much.

Get two 50mL falcon tubes and assemble the reagents you’ll need. Acrylamide solutions are in the cold room, TEMED in the flammables cupboard and APS in the common freezer. Use the electric pipetteboy and the glass 10mL tips for the buffers, water, SDS and acrylamide and the acrylamide area pipettes for the other reagents. You can make up everything but the APS and TEMED for both the stacking and resolving gels but only add APS and TEMED right as you’re ready to use the mix.

NOTE 🡪 APS solution must be prepared fresh from powder, immediately before use.

|  |  |
| --- | --- |
| **Resolving gel**  | **Stacking gel** |
| 30% Acrylamide………3.3 ml | 30% Acrylamide………..670 μl  |
| 1.5 M Tris (pH8.8)……2.5 ml | 0.5 M Tris (pH6.8)…….1.25 ml |
| R.O. water…………….4.1 ml | 10% SDS………………..50 μl |
| 10% SDS……………..100 μl  | R.O. water……………….3 ml |
| 10% APS………………100 μl | 10% APS………………...50 μl |
| TEMED……………….10 µl | TEMED…………………..10 μl |

Add the APS and TEMED to the resolving gel mix, and use a plastic ‘squeeze bulb’ pipette to drip the gel into the cassette. Fill to approx. 80% of the glass plate height…leave enough space at the top for the green well former, plus another 5-10 mm. Wait a minute to see if the level drops and if it holds reasonably steady, pipette a layer of MilliQ water over the top to hold it level. Leave a bit of gel in the pipette and/or falcon tube so that you’ll know when it’s set, about 15-30 min later.

Pour off the MilliQ, add the APS and TEMED to the stacking gel mix and pipette that on top of your now set resolving gel and fill it up nearly to the top. Slide in the plastic well comb, making sure to pick the correct size for your gel (0.75 or 1.0mm). Leave some of the stacking gel mix in the pipette and leave it for approx. 15-30 min.

**Preparing samples**

One of the simplest ways to do this is by bead-beating a cell suspension. Prepare beadbeating tubes as follows: Get glass beads plus the scoop and funnel from the chemicals cabinet (under ‘G’ for Glass) and put 2 large beads and two scoops each of the medium and small size beads into each tube. Sterilise by autoclaving (prob. not essential if you are in a hurry or forget, but that would be ‘best practice’). Pellet your cells from broth or scrape off a plate and resuspend in 500µL of TE and 5µL of protease inhibitor cocktail. How much culture to use is tricky to know, more concentrated is usually better, but too many cells and suspension may be gloopy and hard to work with. The pellet from ~ 2 ml of broth culture or a large loopful from a plate is a good starting point.

Take your tubes over to the bead beater and unscrew the tube-holder. Space your tubes around the disc (make sure the caps are on tight!) and replace the cover as tight as you can. Beat for 30 seconds. If results suggest poor lysis, or if you are working with Gram-positive bacteria or yeast etc, you will need to do multiple cycles of beating – in these cases, chill your tubes on ice for 1 min between beatings. You may need 5 x 30 sec beatings to fully lyse tough cells like mycobacteria.

Centrifuge at ~10,000 g for 1 minute (ideally at 4 C) to remove beads and any remaining whole cells. Retain supernatant. Keep on ice. Check the absorbance on the Nanodrop using the protein setting. Ideally we want around 5 mg/ml for nice PAGE gels. (between 2 and 10 mg/mL may be OK) If it’s less than 2 mg/ml, either repeat with more cells or bead beat for more cycles. If it’s more than 10 mg/ml (unlikely!) dilute down to 5 mg/ml.

Keep in mind this Nanodrop reading is only an estimate of the protein concentration, since there is other junk in the cell extract that will interfere. For some applications like enzyme assays, you may need to use a different protein quantitation methods.

**Loading and running the gel**

Calculate how much of each sample to load to get a constant amount of protein in each lane. Its very important to try to match the total protein in each lane so that we can compare e.g. induced vs. uninduced samples. Between 25 and 50µg per lane usually works out well. For example if your sample is 5mg/mL that’s equal to 5µg/µL so for a 40µg lane you would need 8µL of it.

Add an appropriate amount of SDS-PAGE loading buffer (in this case ~3µL of 4x buffer for a total sample volume of 11µL) checking that the buffer has had β-mercaptoethanol added (you’ll know if it has because it will smell sulfurous and awful). Make up 50% more sample + loading buffer than you actually need to prevent loss in the next step. Heat samples at 100C for 3min (heating block) and then allow to cool down before loading on gel. Remember to turn off the heating block!

Take your prepared gel cassette(s) and remove the holders and well combs. Put them into the housing with the short plate sides facing inwards and clip in place. It’s a good idea to mark the location of the wells with a permanent marker at this stage to make it easier to see where the wells are later. Put the assembly into the clear tank and check that the top part makes contact with the terminals. Fill the space between the gels with 1x SDS PAGE running buffer and fill the tank up to the 2 or 4 gel line depending on how many you are running. If you only have one gel, use the “buffer dam” on the second side so that you get a distinct pool of buffer at the top.

Load 5uL of protein standards (these don’t need to be boiled or have buffer added) and the appropriate amounts of each of your samples. Make sure you keep track of which samples are going into which wells. Especially note that the gel may get flipped over later, so ensure the gel is asymmetrical to prevent confusion later, e.g. only put standards on one side).

Run gel at 200V for about an hour or until the dye front reaches the bottom of the gel. Some of the housings leak and this will reduce the amount of current that can flow, if this is happening just check the gel every so often and if it is transfer some buffer from the bottom part of the tank to the space between the gels to top it up. A 50mL syringe is very useful here. When the dye has reached the bottom of the gel, stop and disassemble it. Used buffer can go down the sink.

**Staining and visualising**

Carefully pry open the plates using the green plastic wedge tool and peel out the gel with tweezers. It will be reasonably tough but may still tear or break so be careful. Place it in a plastic dish and pour over enough coomassie blue solution to cover. Put some gladwrap over the top and put on the rocker for half an hour. Pour the stain into the methanol waste bottle and cover with high-destain solution and return, covered, to the rocker for another half an hour. Pour off into the methanol waste and replace with low-destain solution and rock, covered overnight. The high-destain step may be omitted and done the next morning if there is still a lot of background stain. Don’t worry about a bit of background stain, that can be taken out in the image software but less is better as it will enhance the contrast.

To image the gel the best thing to use is the Typhoon scanner on Level 6. The instructions are printed on a piece of paper stuck to the scanner for which software to use. Carefully place the gel on the glass surface (don’t use tweezers, you don’t want to scratch the glass!) being sure to roll it out flat to prevent air bubbles. Something you may want to try is spritzing the gel with water and pressing a clear plastic sheet onto the gel to squeeze out any air and prevent the gel from curling up while you image it.

Start a new protocol and use the preview tool to focus on your gel. Adjust the image settings to the highest resolution if you like and you can also get the software to automatically annotate lanes, bands and even sizes of markers. Press “RUN” to take the image. Adjust the contrast and high/low settings to define the bands more clearly. The software is capable of recolourising the image to look like a very nicely destained coomassie blue gel, which is helpful. It’s worth familiarising yourself with the different imaging and analysis tools as they are quite powerful. If it’s not essential to do all of this you can also use the GelDoc on white light transillumination (there’s a movable tray in the housing at the back for this) for a simple black and white image. In either case save the file and export at maximum quality and save to a USB.

Gels can be stored in the cold room between two sheets of clear plastic sealed together with tape for later reference or mass-spec band excision. You can annotate the plastic directly but be aware the gel can shift in the plastic over time if not kept flat. If you want to discard the gel, this must be treated as contaminated waste, due to possible acrylamide monomer still present…discard into the acrylamide waste bag.