Recombinant DNA technology

It should be noted that the following protocols are for beginners. It is very likely that with just a little experience, any person would be able to give a personal touch to any of the protocols below. For example, one may be able to do preparative digests with much less the 10-20 micrograms once the technique of recovering 90% of the aqueous phase during a phenol extraction is fully under control. Thus, the protocols should be regarded as guidelines only, to get started. But if it works well, then why change a winning horse? A lot of the success in laboratories is based on the principle that basic protocols are carried out routinely with the same protocol by any member of the team, simply because science is difficult enough and nobody can afford to have problems with standard protocols....

Solutions:

LB MEDIUM (1l)
10 g Bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
for solid medium add
15 g/l Bacto Agar
Autoclave!!

TE buffer: (for standard DNA manipulations)
10 mM Tris-HCl pH 8.0
0.1 mM EDTA

TE 50/1 buffer: (For maxipreps only!!)
50mM Tris-HCL pH 8.0
1mM EDTA pH 8.0

TES buffer: (for minipreps)
10 mM Tris-HCl pH 8.0
5 mM EDTA
250 mM sucrose
filter sterilise

10x Restriction buffers:

	RB 0	**RB 50**	**RB 100**	**RB 150**
$MgCl_2$	50mM	50mM	50mM	50mM
Tris pH 8.0	100mM	100mM	100mM	100mM
DTT	10mM	10mM	10mM	10mM
EDTA	1mM	1mM	1mM	1mM
BSA	1mg/ml	1mg/ml	1mg/ml	1mg/ml
NaCl	0	0.5M	1M	1.5M

10x Ligation buffer:
0.5M Tris-HCl (pH 7.6)
100mM $MgCl_2$
100mM dithioltheritol (DTT)
500µg/ml bovine serum albumin (BSA)

This buffer is stored in small aliquots at -20^{o}C.

5xTBE buffer (1l):
54g Tris
27.5g Boric acid
20ml 0.5M EDTA (pH8.0)

4x Loading Dye:(General use)
24% sucrose
0.1% bromophenol blue
40mM EDTA

4x Loading Dye with RNase A:(Miniprep digests)
24% sucrose
0.1% bromophenol blue
40mM EDTA
20µg/ml RNase A

1. Restriction digests of plasmids

1.1. Preparative digest: (plasmid solution in TE that is 1µg/µl and about 3 kb)

Use 10-20 micrograms for a preparative digest. In this particular example, the following could be done:

5 µl of plasmid
5 µl of 10x Restriction buffer
1-2 µl of Restriction enzyme (10-20 u/µl)
bring to 50µl total with TE

(Prepare an agarose gel so that it is ready when you need it. Usually this is the first thing you do in the morning before you start, unless you have a gel with unused slots left from days before. But, never keep a gel for longer than 1 week because the water evaporates and the TBE concentration increases. When you later add some more buffer, you have different salt concentrations in the gel and in the buffer, giving rise to an imbalance in the conductivity.)

Prepare 3 Eppendorf tubes with 5 µl of Loading dye and write 0, 20 and 40 on it.

Then start mixing the reagents, first the TE, then the plasmid, then the Restriction buffer, and then mix thoroughly. Take a sample of 1 µl and mix it with the Loading dye in tube 0. Leave the yellow tip in the tube, it can be used to load the sample on the gel later. Then, and only then, add the enzyme to the reaction mix, and make sure that it is properly mixed. The enzyme is supplied in a 50% glycerol solution and will sink to the bottom if you don't mix it well. Then incubate the reaction at the right temperature (usually 37 °C but always make sure that you are not dealing with an exception) and start your alarm clock (count up). After 20 minutes, take another sample of 1µl and mix it with the blue Loading dye of tube 20. Mix the remainder of the reaction again and incubate further. After 40 minutes since the start of the reaction, repeat the sampling. Then load all 3 samples on the gel with the yellow tips that you have left in the tubes, use a molecular weight marker in an adjacent slot. Incubate the reaction while you wait for the gel (30 minutes). When the gel has run (70 minutes after the reaction was started), check the gel on a transilluminator and decide what to do next.

1) If the reaction has not worked at all, there is no point in waiting longer. If the enzyme has worked on another plasmid before, perhaps re-precipitate the plasmid, or do even a new phenol extraction prior to precipitation and try again. Otherwise, check if the enzyme is still OK by using another plasmid.

2) If the reaction has worked but is not complete after 40 minutes, estimate from the progress at 40 minutes how much longer it will take. You can also double the amount of enzyme at that stage to speed up the reaction. Either way you should be able to stop the reaction at the right time. A new 1µl sample can then be run on a gel (in loading dye) to make sure.

3) If the reaction was complete after 40 minutes or even after 20 minutes, you can stop the reaction immediately and write in your notes that you have an overdigest.

There is no reason for concern at this stage, but if you run into problems, you can take this information into account later.

1.2. Qualitative digest:

This digest is meant as a quality control or to test different recombinants and requires only a small amount of plasmid, to be digested for a standard time (1 hour) with an amount of enzyme that is certainly sufficient, all of which is to be loaded on a gel, none of which is required for further use. There is no need to do a time course as for the preparative digest. 200 ng of plasmid is sufficient and 1 unit of enzyme in a total reaction volume of 10 µl. If the plasmid is not from a relatively clean maxiprep, but a quick miniprep, then triple the amount of enzyme.

For a plasmid with 1µg/µl:

0.2 µl plasmid
1 µl 10x Restriction buffer
8.7 µl TE
0.1 µl enzyme

All of this is mixed with 5 µl of loading dye after 1 hour of reaction, and loaded integrally on the gel.

N.B.
For miniprep plasmid (DNA concentration much lower and usually unknown):

3 µl plasmid
1 µl 10x Restriction buffer
5.7 µl TE
0.3 µl enzyme

Usually, in case of minipreps, you will have 10 or 20 samples to digest. In this case, it is recommended to make a mastermix (n+1) of all the ingredients but the plasmid, and add the mixture to all the tubes. The reaction is started by adding the plasmid. Saves time!!!

Restriction buffer compatibility for commonly used enzymes:

Enzyme	*RB0*	*RB50*	*RB100*	*RB150*	*Enzyme*	*RB0*	*RB50*	*RB100*	*RB150*
BamHI	+	++	+++	+++	PstI	+++	+++	+++	+++
BglII	++	+++	+++	+++	PvuI	+	++	+++	+++
ClaI	+++	+++	+++	++	SacI	+++	+++	++	+
EcoRI		+++	+++	+++	SalI	+	+	++	+++
EcoRV	+	+	+	+++	ScaI	+	+++	+++	++
HindIII	++	+++	+++	++	SmaI	+	+	+	+
KpnI	+++	+	+	+	SnaBI	+++	+++	++	+
NcoI	+	++	+++	+++	SphI	+	+	+++	+++
NdeI	+	+	++	+++	StuI	+++	+++	+++	+++
NheI	+++	+++	+++	++	XbaI	+	+++	+++	+++
NotI	+	+++	+++	+++					

2. Filling up or blunting sticky ends using Klenow

When you want to ligate sticky ends that are not compatible, you can fill up or bite off sticky ends with Klenow so that they become blunt. When you have a 5' overhang, it serves as template and you can fill the site up. When you have a 3' overhang, Klenow will digest off the overhang, which will also result in a blunt end. The latter reaction is much slower than the first, so if you have a 3'overhang, you should prepare yourself for a lower efficiency (which doesn't mean that you will have problems). A typical Klenow reaction is a such:

40 µl of DNA solution
5 µl of RB100 (10x)
5 µl of 1mM dNTPs
1 unit of Klenow (usually 0.2 microlitres), mix and incubate 15 minutes at room temperature (20-25°C)

What can go wrong? The enzyme isn't perfect and contains some nucleases (also true for restriction enzymes). This means, the ends may become shorter than you want, but will still be blunt ends. This can have serious implications when you create fusion proteins or promoter reporter fusions. The ligation may be fine, and the restriction pattern may be perfect, but yet the construct is not working. It is wise to design strategies in which a blunt end ligation results in the reconstitution of a restriction site, then you can check afterwards as you do when you ligate sticky ends.

Often, you can add dNTP and Klenow straight to the restriction digest once you know it is complete. But you have to make sure the buffer is compatible. Suppose you had the following digest:

44 microlitres of DNA solution
5 microlitres of RB100 (10x)
1 microlitre of restriction enzyme (20 units/microlitre)

Incubation at 37°C and samples take at 0, 20, 40 minutes, digest complete after 40 minutes, then you can simply start the Klenow in the following way:

Add 5.5 microlitres 1mM dNTPs
Add 1 unit of Klenow, mix and incubate 15 minutes at room temperature. Then do a phenol extraction (see 'cleaning up').

3. Dephosphorylation of ends

To prevent self-ligation of a vector cut with only one enzyme, the open ends can be dephosphorylated with calf intestine alkaline phosphatase (CIP), this procedure is called "doing a cip" or "cipping". You have to make sure the fragment to be ligated possesses 5'phosphate groups. i.e. PCR products are generally not phosphorylated and must then be treated with kinase (see later).

Procedure:

40 µl of DNA in TE
5 µl of 10xCIP buffer
5 µl of CIP enzyme (1u/µl)
incubate 30 minutes at 37°C

There are other enzymes, for example bacterial alkaline phosphatase, or shrimp alkaline phosphatase, the protocols and in particular the incubation temperatures for those may be different. Follow the manufacturers instructions.

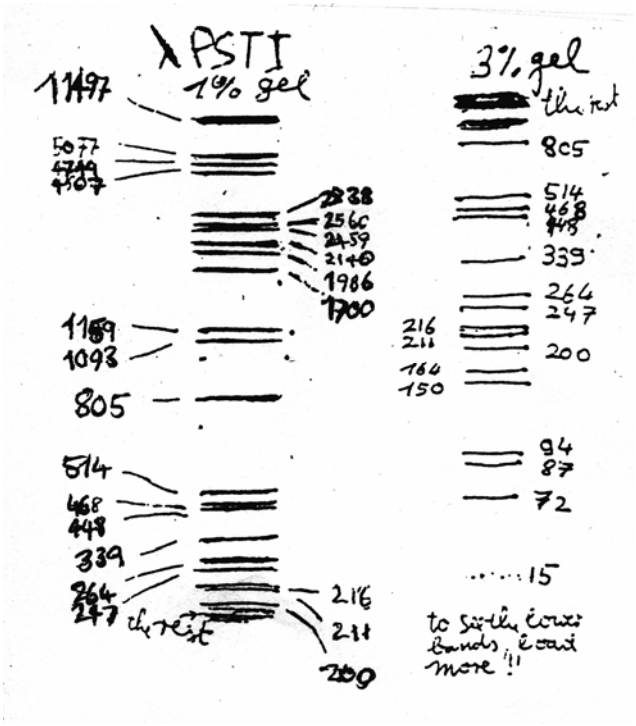
4. Cleaning up

Between different manipulations of plasmids, such as between a restriction digest and a ligation, you have to remove the enzyme and buffer from the previous step so that you can start the next step. This is done by a phenol extraction and is called "clean-up".

50 µl of "dirty" DNA is your starting material.
Add 50 µl of TE
Add 50 µl of equilibrated phenol.
Vortex (should become "milky"). The idea is to get an emulsion with a high surface between the phenol and the water phase. Proteins will migrate to the phenol phase, whereas the DNA stays in solution.
Add 100 µl of chloroform and vortex (now that the DNA and proteins are partitioned, the idea is the opposite, to help separating the two phases).
Centrifuge 5 minutes at maximum speed in a microfuge.
Recover the upper (aqueous) phase (ask for demonstration) and transfer to a new tube containing 100 µl of chloroform.
Vortex and centrifuge again 2 minutes at maximum speed in a microfuge.
Recover the upper (aqueous) phase in a new tube, add 10 µl of 5M $NaClO_4$ and mix.
(these last two steps to recover the aqueous upper-phase are a bit tricky, perhaps the most difficult thing you ever find in recombinant DNA techniques, and it is worthwhile to train it on fake samples (just TE) before you start. Shaky hands are not helpful, so smoke a cigarette before, if you are a smoker...)
Add 110 µl of Isopropanol, vortex and centrifuge 15 minutes at maximum speed in a microfuge.
Discard the supernatant and centrifuge for 1 minute at maximum speed.
Insert a hand made refined pasteur pipette (ask for demonstration) to the opposite side of the pellet (which should be barely visible) and suck out the liquid. Keep sucking even when the liquid has gone, to remove all liquid from the walls of the tube. You should also assemble all droplets that you may see on the walls by moving the pipette and using the capillary forces. It is important to have only a dry pellet left.
Leave the tube open for 1 minute at room temperature and then resuspend it in an appropriate volume of TE. Keep the tube on ice and vortex now and then, it does take a few minutes before all plasmids are dissolved again.
Then you can move to the next manipulation.

5. Agarose gels and Electrophoresis.

Depending on the size of the DNA fragments to be separated agarose gels can be between 0.5-3%. The larger the DNA fragments to be separated, a lower percentage of agarose gel is used. The agarose is added to TBE (0.5x : 45mM Tris-borate 1mM EDTA), the mixture is heated to dissolve and then left to cool till the bottle is not too hot to hold. This can be done quickly by sealing off the blue capped bottle immediately after boiling, which creates a vacuum when you hold the bottle under a stream of cold water. The liquid will start boiling, which gives rise to a much faster heat transfer from the hot solution. 4µl/100ml of EtBr is added before the gel is poured into a gel electrophoresis tray (don't forget to insert the comb!!). You should wait a little while before you use it, even if it appears solid (1 hour). The gel starts to set after 15 minutes, and if you are in a hurry, you may then place the gel in a fridge and wait only a further 15 minutes. To run a sample, the gel is placed in an electrophoresis tank containing buffer so that the gel is just immersed (a few millimetres). A few µl of the digest and loading dye can then be added to each slot by placing the yellow tip carefully over the opening of the slot. The glycerol in the loading dye will make the sample sink to the bottom. A λPstI marker can be run with the samples as a size marker. The gels are run at 10V/cm. Fragments are observed using UV light and a permanent photographic record is made if required. Overloading can cause problems to separate the bands.



6. Ligation of DNA fragment into a plasmid vector.

Ligation reactions are set up along with controls: usually you have two controls, 1) vector alone, 2) vector + ligase, and then the real ligation is vector + fragment + ligase. The ligation mixtures are incubated at room temperature for 2 hours. The amounts of fragments and vector are important and the following golden rules should be followed. Never use more than 10% of the total amount of gel-purified material, otherwise you probably have too much salts and dirt. The amount of vector should be barely visible on gel, so 10-20 nanograms. Fragments should be more abundant, but never more than 10 fold concentrated compared to the vector. A typical reaction is then as follows:

Vector	Fragment	TE	10xLigation buffer	Ligase (1U/µl)
1µl	-	17µl	2µl	-
1µl	-	16µl	2µl	1µl
1µl	2µl	14µl	2µl	1µl

7. The Preparation of Competent Cells, (MC1061 E.coli cells).

Streak out MC1061 E.coli cells on LB. With one colony, inoculate 3ml 2xYT in a new 50ml Falcon tube and incubate 37^{o}C with shaking. At O.D.$_{550}$ = 0.300 (slightly turbid) pour into 200ml of 2xYT that has been prewarmed to 37^{o}C and incubate at 37^{o}C. At O.D.$_{550}$ = 0.480 transfer the culture into four 50ml sterile Falcon tubes. Place on ice for 5 minutes. From now on work in the cold room or keep the tubes on ice while not in the cold room.
Spin down at 3K in a swing-out rotor, 4^{o}C for 20 minutes then discard the supernatent. Resuspend the cells in a total of 80ml of ice cold TFBI and place on ice for 5 minutes. Spin as before and resuspend the cells in 8 ml of TFBII and leave on ice for 15 minutes.
Using pre-chilled pipette tips and working in the cold room, aliquot 100µl into pre-chilled eppendorfs (sitting on ice), frozen in a dry ice/ethanol and stored at -80^{o}C.

To make 1 litre of 2xYT medium:
16g bacto tryptone
10g bacto yeast extract
5g NaCl
Make up to 1 litre and adjust the pH to 7.0 using NaOH and autoclave.

TFBI (200ml):

30mM $KC_2H_3O_2$	0.589g
100 mM RbCl	2.418g
10mM $CaCl_2 \cdot 2H_2O$	0.294g
50mM $MnCl_2 \cdot 4H_2O$	1.979g
15% v/v glycerol	30ml

adjust to pH 5.8 using 0.2 M CH_3COOH, sterilise and filter. Store at + 4^{o}C.

TFBII (200ml):

10mM MOPS	0.419g
10mM RbCl	0.242g
75mM $CaCl_2 \cdot 2H_2O$	2.205g
15% v/v glycerol	30ml

adjust to pH 6.6 using 5 M KOH, sterilise and filter. Store at + 4^{o}C.

8. E.coli transformation

5µl of each ligation mixture is added to 100µl of competent cells, mixed and incubated for 15 minutes on ice (minimum), followed by a heat shock at 37 ^{o}C for 3 minutes during which the E.*coli* cells take up the religated plasmid. 1ml of LB medium is added and the cells are incubated for a further 15 minutes at 37^{o} C. The LB medium with the transformed competent cells is then poured onto Ampicillin containing LB-agar plates, dried and left to incubate overnight at 37^{o}C. The colonies which appear originate from single transformed cells and are resistant to the antibiotic due to the presence of the plasmid. Each colony contains millions of identical copies of the same cell, hence the term clone.

9. Amplification of DNA by the polymerase chain reaction (PCR).

9.1. Pfu Turbo

Reaction Mixture:
5µl Pfu buffer (10X)
0.4µl dNTP's (25mM)
1µl Primer 1 (10pmol/µl)
1µl Primer 2 (10pmol/µl)
1µl Template
5µl Pfu Turbo (10 fold diluted, see below)
dH_2O to give a total volume of 50µl

A master mix, enough for n+2 reactions, is made using only the water, Pfu buffer, and dNTP's. 42µl is placed into a PCR tube and the primers are added along with 1µl of diluted DNA. The remaining master mix is used to make a 10 fold dilution of the enzyme. The tube is heated to 94^{o}C for 2 minutes then 5µl of the diluted enzyme is added to each of the reaction mixtures to start the reaction (hot start). The PCR machine is programmed to do 20-45 cycles of (example).

30 seconds 94^{o}C (denaturation)
1 minute 50^{o}C (annealing)
1 minute 72^{o}C (elongation)

The products are then separated on an Agarose gel to determine the size of the fragment produced and to see therefore if the reaction was successful.

9.2.Taq

Reaction mixture:
5µl Taq buffer (10X) (500mM KCl, 100mM Tris-HCl pH 9.0, and 1% Triton X-100).
5µl $MgCl_2$ (25 mM)
0.4µl dNTP (25 mM)
1µl primer 1 (10 pmol/µl)
1µl primer 2 (10 pmol/µl)
1µl template
5µl Taq polymerase (25 fold diluted, see below)
dH_2O to give a total volume of 50µl.

A master mix, enough for n+2 reactions, is made using only the water, taq buffer, $MgCl_2$ and dNTP. 42µl is placed into a PCR tube and the primers are added along with 1µl of diluted DNA. The remaining master mix is used to make a 25 fold dilution of the enzyme. The tube is heated to 94^{o}C for 2 minutes then 5µl of the diluted enzyme is added to each of the reaction mixtures to start the reaction (hot start).

10. Maxiprep of plasmid DNA

Read the protocol before starting and make sure you have all the stock solutions ready. The lysozyme solution is usually made during the centrifugation period. The rest has to be ready before you start.

All the tubes are re-usable, so don't write on them but mark them with tape and write on the tape!

1) Make a pre-culture of 3 ml LB first thing in the morning starting from a single colony from a fresh plate (ideally streaked the night before).

2) After 3 hours the pre-culture should be slightly turbid, inoculate 800ml LB prewarmed to 37°C (500 fold dilution) and grow overnight (24 hours). Shorter incubations may cause problems with excess proteins and RNA in the cell lysate.

3) Centrifuge the culture using the available infrastructure (for example 60 minutes, 3699rpm, big Mistral Swing-out centrifuge, in 800ml buckets) and remove the supernatant carefully. The supernatant will still contain E.coli so do not tip it straight down the sink.

4) Put the buckets with the cell pellets on ice and resuspend pellets in 8 ml of icecold TE 50/1 (50mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0). Pellets must be completely resuspended by occasional vortexing and placing back on ice, takes some time (5 minutes). Transfer the cell suspension with a 10ml pipette into clean SS34 tubes that are standing on ice already. All further manipulations will be done on ice! At this step, the protocol may be interrupted for a coffee brake...

5) Quickly add 2.5 ml of freshly made lysozyme solution (10mg/ml) to all tubes, close the tubes and turn them quickly upside down and back at least 10 times (preferentially all tubes at once, 4 in one hand 4 in the other). The suspension should become slightly viscous. **Do not shake the tubes, just turn them!** Incubate 5 minutes on ice.

6) Unscrew the bottles and from now on **remember which cap belongs to which tube** (very important because the solution is viscous and a significant portion will stick to the cap). Quickly add 2.0 ml of 0.5M EDTA pH 8.0, close the tube and turn them again as

before. **Again, do not shake them vigorously!** Suspension will become more viscous. Incubate another 5 minutes on ice.

7) Mix 50µl of Ribonuclease A solution (20 mg/ml in distilled water, and stored in 50µl aliquots in -20°C) with 150µl Triton X-100 10% solution and make up to 1ml with TE 50/1. Add this mixture to the tubes. Then close the tubes and turn them as before (no shaking). Suspension will become even more viscous.

8) Incubate 30 minutes on ice, without ever touching them again. The idea is to let the detergent do the rest of the cell lysis in a very gentle way, on ice.

9) Centrifuge at 18,000 rpm in a Sorvall SS34 rotor for 60 minutes. You should get a nice pellet, but sometimes the pellet can be big with very little supernatant. This usually happens when your bacterial culture is too young. In this case, repeat the centrifugation, it will help!

10) Transfer the clear supernatant to a clean Falcon tube. Now it is possible to interrupt the procedure for longer periods by freezing the recovered supernatants at -20°C. It is also possible to have a lunch break and keep the supernatant on ice.

11) Add 20 ml of equilibrated phenol (pH 8.0 with 0.1% 8-hydroxyquinoline) and shake vigorously for 1 minute, spin down at maximum speed in the swing out rotor for 20 minutes.

12) Slowly recover the water phase (upper) and make sure you leave the interphase in the tube. Transfer to a new tube and add 20ml of chloroform, shake again as before and spin as before for 5 minutes.

14) Slowly recover the water phase (this time it is easier to avoid the interphase), transfer to a 30ml Corex tube (between 10 and 15ml, the volumes need to be equalised by the addition of TE 50/1), add 1ml 5M $NaClO_4$ (10% of the water volume), mix and add 8ml isopropanol (80% of the water volume). Seal the tube with parafilm, and turn it around a few times to mix everything. Then spin down in HB4 rotor at 10,000rpm for 15 minutes.

15) Discard the supernatant, dry the pellet, resuspend in 500 µl of TE (10mM Tris-HCl pH 8.0; 0.1 mM EDTA pH 8.0) and transfer to a sterile eppendorf tube. Store at 4°C.

Check the DNA on gel to assess the quantity of RNA, it is usually necessary to carry out a second RNase treatment at this point.

1) Dilute the maxiprep to 5ml with TE in a 50ml Falcon tube.

2) Add 50µl RNase A (20mg/ml) and incubate at room temperature for 30 min

3) Add 5ml phenol, and shake vigorously for 1 minute, spin down at maximum speed in the swing out rotor for 15 minutes.

4) Slowly recover the water phase (upper) and make sure you leave the interphase in the tube. Transfer to a new tube and add 5ml of chloroform, shake again as before and spin as before for 5 minutes.

5) Slowly recover the water phase (this time it is easier to avoid the interphase), transfer to a 15ml Corex tube (approx 5ml), add 500µl 5M $NaClO_4$ (10% of the water volume), mix and add 4ml isopropanol (80% of the water volume). Seal the tube with parafilm, and turn it around a few times to mix everything. Then spin down in HB4 rotor at 10,000rpm for 15 minutes.

6) Discard the supernatant, dry the pellet, resuspend in 500 µl of TE (10mM Tris-HCl pH 8.0; 0.1 mM EDTA pH 8.0) and transfer to a sterile eppendorf tube. Store at 4°C.

Check the DNA on gel to make sure most of the RNA is gone. Determine the DNA concentration spectrophotometrically.

DNA concentration and purity determination:

1) Dilute the DNA as appropriate (usually 1 in 100 for maxipreps)
2) Measure the absorbance at 260nm and 280nm in a quartz cuvette. (for each prep three readings should be made at each wavelength)

3) Calculate the concentration of DNA (A solution containing 50µg/ml of dsDNA has an absorbance of 1 at 260nm)

4) Calculate the ratio of 260nm to 280nm this should be between 1.8 and 2. (if the value is below this then an additional phenol chloroform is required.)

11. Miniprep protocol for liquid cultures

Read the protocol before starting and make sure everything is ready.

This protocol is ideal to analyze individual colonies for cloning purposes. It is a fast and cheap routine procedure that can be carried out without kits and relies entirely on standard chemicals, a waterbath, eppendorf tubes and a microfuge.

1) Start with fresh overnight liquid cultures (with a single colony inoculate 3ml LB medium the evening before, make sure the colonies are well separated), fill approximately 1.5 ml of each culture in an eppendorf tube, don't forget to number the eppendorf tubes according to the numbers written on the culture tubes.

2) Centrifuge 1 minute at maximum speed, put the tubes in the rack and then "throw" the supernatant out. Make sure only minor quantities of LB medium are left with the pellets.

3) Resuspend the pellets in 150 µl TES by pipetting the solution up and down with a yellow tip close to the pellet (systematically going from one side of the pellet to the other until nothing is left sticking to the wall)

4) Quickly add 20µl of Lysozyme solution (10mg/ml in distilled water, usually kept as small stocks frozen. From time to time make a little bit more lysozyme solution while doing a maxiprep and aliquot the excess to maintain your frozen stocks for minipreps) and mix it quickly by pipetting up and down 3 times. Incubate 5 minutes at room temperature.

5) Pipette 300 µl of distilled water quickly to the suspensions, it should mix while the water is sprayed into the tube. Don't shake the tubes afterwards. This should be done as quickly as possible and the tubes are immediately placed in a heat block at 73°C. Incubate there for 15 minutes.

6) Centrifuge at maximum speed for 15 minutes. Sometimes, the pellet is too big (more pellet than supernatant), then just centrifuge for another 15 minutes, be patient, it will pellet down eventually. When the pellet is big, usually the culture was too young (less than 12 hours).

7) Pour the supernatant into another eppendorf tube (don't forget the numbering). Add 5M $NaClO_4$ (approximately 10% of the supernatants volume, usually 10% of 300µl. If

some of your tubes have less supernatant, add some TE to those so that they look like the majority). Shake the tubes.

8) Add 400 µl isopropanol, shake as before and centrifuge 15 minutes maximum speed.

9) "Throw" out the liquid and centrifuge once more for 2 minutes. Remove the last bit of liquid with yellow tips.

10) Dry 15 minutes in the 37°C room (open tubes of course), add 50 µl of TE and put on a shaker for 5 minutes. Ready!

When you do restriction digests, it is recommended to make a master mix of TE, restriction buffer and enzyme, and distribute that over the tubes. Then you just have to add a small amount of your miniprep, and the digest is on. Typically, you will have in each tube 6 µl TE, 1 µl of 10 x restriction buffer, 0.2µl of the restriction enzyme and 3 µl of the miniprep. If you increase/decrease the amount of miniprep, you have to adjust the volume of TE.