Cloning of DNA fragments

General considerations:

1. Before starting an experiment, know the sequence of insert, vector and polylinker. Don't trust predicted restriction sites, as there might be polymorphisms or strain differences. Always check plasmid identity and presence of restriction sites needed by yourself (clones might be wrong). Work out cloning scheme together with an experienced person (what is the aim?). If unsure, always check Maniatis for details.
2. Prepare flow chart (what do I need, how long does it take).
3. DNA quality: Phenol/chloroform- extracted or column-purified DNA
4. Amount of DNA needed: 5- 10 µg of vector (at -20°C, vector can be used for several years), 2-10 µg of insert, depending on size etc..
5. make a note in the GVO chart, and set up a clone chart as soon as a Lb-plate exists
6. control every step of your cloning with the appropriate controls

Calculation of the necessary DNA-amount:

Example: Vector+insert have a size of 5.000bp and the insert size is 500 bp (10 % of total size). To receive 2µg of insert for cloning, you need 20 µg of the plasmid for restriction.

I. Cloning of PCR-Products:

For cloning from plasmid templates, always linearize plasmid outside region of amplification!!!

If product is used for expression of proteins, you must use proof reading polymerase!!!

General procedure:

Linearize a few micrograms of template,

Use 50-100 ng in combination with low cycle number (12-20) to minimize errors.

Depending on cycle number and polymerase, either blunt or sticky ends are created. Choose cloning vector accordingly.

You can always change blunt to TA end by procedure described in appropriate protocol.

Cloning of PCR product should be done instantly after PCR reaction. For analysis of clones, pick 4-12 colonies and analyze by restriction.

Choose 1 or 2 clones for sequencing.

II. Cloning of an insert from vector A to vector B:

1. Retransformation of DNA

1. thaw 20µl XL1 blue comp. cells on ice
2. add 1-10ng DNA and tap lightly
3. 30 min on ice incubation
4. heat-shock for 90s at 42°C water-bath
5. 2 min immediately on ice
6. add 450µl pre-warmed LB
7. incubate 60 min at 37°C in water-bath
8. **plate 50 and 450µl upon plate with the** right antibiotics o/n at 37°C into the incubator
9. next day pick a single colony and do a **mini or midi p**rep

Antibiotics used:
Ampicillin 200µg/ml
Kanamycin 100µg/ml

2. Plasmid preparation see appropriate protocol

3. Restriction of vector and fragment to be cloned

1. Use enough DNA to start with, at least 5µg!
2. pick your restriction enzymes

Set –up:

DNA	x µl	
10X Puffer	5-10 µl	
MilliQ water	x µl	
Enzyme	10-100U	
Total	50-100 µl	(0.2µg DNA/µl reaction)

Remember: 1U enzyme cuts 1µg DNA at 37°C in 1h

incubate at 37°C water-bath for 30-60min, rather use more enzyme and keep the incubation time short. Note: Temperature can vary for different enzymes. Control your restriction with a small aliquot on a mini-gel.

If you are doing a **double digest** which cannot be done in the same buffer, precipitate your DNA as follows:

1. add up to 200µl TE to your sample
2. add 1µl Glycogen
3. add 20µl NaAcetate
4. add 400µl 100% Ethanol
5. centrifuge at max. speed at tabletop centrifuge for 10min
6. take off supernatant

7. add 400µl 70% Ethanol
8. centrifuge at max. speed at tabletop centrifuge for 5min
9. take off supernatant and let pellet air dry, but do not overdry!
10. resuspend pellet in 40µl of TE buffer
11. than set up 2nd digest

DNA	**40 µl**
10X Puffer	**5 µl**
MilliQ	**x µl**
Enzym 10-100U	
Total	**50 µl**

➔ incubate at 37°C waterbath for1h
➔ higher DNA amounts should be cut in a bigger volume!

Dephosphorylation: necessary if vector is cleaved with 1 enzyme or blunt cutters. Prevents relegation. Not necessary if vector is cleaved with 2 different enzymes.
Always do dephosp. reaction after restriction but before gel.
Stop restriction reaction with 0,5M EDTA (10mM end concentration) and precipitate DNA as described above.

Set-up:

DNA	17 µl
10X Puffer	2 µl
SAP	1 µl
Total	20 µl

incubate at 37°C waterbath for1h then stop the reaction at 65°C waterbath for 15min and with additional 0,4µl 0,5M EDTA (10mM end concentration) followed by a gel elution for CIP dephosphorylation see other protocols on M:/!

Procedure: Vectors must be purified on 2 consecutive gels to remove uncut DNA if the bands are too close to each other, to reduce the vector background. The same is done for insert if it runs rather close to the plasmid from which it is cut out. This dramatically reduces false positives. Never use minigels for isolation of fragments but medium-size gels to achieve good separation.

Gel elution is done with a commercial agarose gel elution kit. Follow exact procedure.

Now determine the concentration of your two samples by loading 1µl of each on a gel.
Add 9µl water and 1µl loading dye for easy loading.

You can store restricted vectors for further ligation reactions.

4. Ligation + Transformation

Ligations: 20-50 ng of vector, 5-10 x molar excess of insert (1.000 x for linkers) (as in III). Calculate the amount of fragment and Vector you need, considering the size of vector and fragment, do not use more than 300-500ng of total DNA in one reaction.
Set up a 20µl (max 25µl) reaction with **10µl 2x Rapid Ligation Buffer** and **1µl T4 DNA Ligase**. **Incubate at RT for 1h and transform 10 µl of DNA into competent cells incubate the rest at 16°C over night.**

Note: The 2x Rapid Ligation Buffer contains **ATP**. Thaw the buffer **on ice** and **keep the buffer always on ice**! Store it always at -20°C.

Controls: vector without insert, insert alone. Must always be carried out. If there is no increase on plate with insert, stop cloning and rethink what's gone wrong. The ratio between plates + insert /- insert tells you how many colonies you need to examine.

Transformation: stick to protocol. It is recommended to use recA+ cells (in the lab: TG1, have to be cultured in TB Medium) for routine cloning as they grow fast (colonies after 6-9h). Only if you need DNA for sequencing or transfection into mammalian cells /transgenic mice, retransform miniprep DNA in recA- strain (XL-1 Blue).

1. thaw 50µl comp. cells on ice
2. add 2µl DNA and tap lightly, up to 5µl DNA is ok!
3. 30 min on ice incubation
4. heat-shock for 90s at 42°C water-bath
5. 2 min immediately on ice
6. add 450µl pre-warmed LB
7. incubate 60 min at 37°C in water-bath
8. plate 50 and 450µl upon plate with the right antibiotics
9. o/n at 37°C into the incubator
10. next day pick colonies and set up 10-20 minis and a backup late, see point below
11. as alternative you can do a PCR screening (protocol M:\)

2x Rapid Ligation Buffer:
(Recipe from **Promega**, http//www.promega.com/tbs/9pim822/9pim822.pdf)

2x stock concentration	**Long-term storage of the basic buffer**
60 mM Tris/HCl, pH7.8	6 ml 1M Tris/HCl, pH 7.8
20 mM $MgCl_2$	2 ml 1M $MgCl_2$
20 mM DTT	~~2 ml 1M DTT (added later!)~~
2 mM ATP	~~2 ml 100mM ATP [Fermentas #R0441] (added later!)~~
10% PEG8000	10 g Polyethylene glycol [AppliChem A2204)
H_2O	**Ad 96 ml**

- Prepare 9.6 ml aliquots and freeze at -20°C.

Preparation of Ready-to-use 2x Rapid Ligation Buffer:

- Thaw one **9.6 ml aliquot** of 2x Rapid Ligation Buffer w/o DTT and ATP.
- Add 0.2 ml 1 M DTT and mix.
- Add 0.2 ml 100 mM ATP and mix.
- Prepare 500 and 100 µl aliquots and freeze at -20°C.

5. Inoculation of 3-5 ml liquid cultures for DNA mini-preparations and preparation of a LB-agar master plate

- Prepare a LB-agar back-up (master) plate of your picked clones.
- That is, pick single colonies using a tooth stick.
- "Sting" tooth stick into a defined position on the LB-agar back-up plate.
- "Pitch" tooth stick into 3-ml-LB liquid culture containing the appropriate antibiotics.
- Incubate at 37°C.

6. Mini-preps

Minipreps: Inoculate ~5ml of medium + right antibiotics, prep. from ~**2ml** and keep remainder (up to 1 wk.). Prepare without ph/chl for restriction analysis. Always run out uncut/cut DNA. Always store an aliquot of this DNA as reference. To get more DNA, pick a singl colony from you backup plate and grow a midi culture.

To be sure if your fragment got cloned into your vector check your mini-preps with appropriate restriction digests, don't forget to consider double digests, however 1 digest is sufficient for the initial control to cut out the cloned fragment, further detailed analysis are done with the right candidates.

set-up:

DNA	2 µl	
10X buffer	1 µl	
MilliQ	16 µl	
Enzym	1 µl	
Total	20 µl	incubate at 37°C for 1h

Additionaly set up the uncut control:

DNA	2 µl
MilliQ	18 µl
Total	20 µl

Don't load too much DNA of your restriction on the gel, depending on the size of your fragment (100ng).

III. Adapter ligation:

Before starting check the protocol in the Maniatis. The adapter can be ordered unphosphorylated or phosphorylated, the vector has to be phosphorylated respectively.

For annealing a buffer containing 20mM NaCl and 20mM Tris Buffer is used, however the ligation buffer works nicely as well.

oligo	1 µl	
Annealing Buffer	16 µl	
Total	20µl	5min incubation at 95°C, then slowly cool off in waterbath.

You can control the annealing on a 10% PA gel.

AA (30%)	3,3 ml
TBE 10x	1 ml
MilliQ	5,7 ml
EtBr	10 µl
TEMED	10 µl
APS	100 µl
Total	~10 ml

Ligation with vectors as above. The clone has to be analyzed carefully, as double insertion can happen easily, to prevent this, cut with one enzyme of your adapter, elute it on a gel and re-ligate.