

## PCR-cloning

### 1. PCR with Pfu-Polymerase (has higher accuracy than Taq)

for 100 µl:

A.dest.	85 µl
10x Pfu-Buffer	10 µl
dNTP's (20 mM)	1 µl (ad 200 µM)
Template-DNA	1 µl (50 ng)
Primer forward	1 µl (10 pmol, about 100 ng)
Primer reverse	1 µl (10 pmol, about 100 ng)
Pfu-Polymerase	1 µl (2.5 - 3 units)

94°C	1 min		
94°C	1 min	←	20x
annealing temp.	1 min	—	
75°C	1 min/kb Insert	—	
68°C	5 min		
4°C	unlimited		

Take 10 µl of the PCR-Mix, add DNA-Buffer (2 µl 6x conc.) and run an agarose gel (100 V, 30 min) - the PCR-product should be clearly visible.

### 2. Phenol/chloroform extraction and ethanol precipitation

The aqueous phase of the PCR-Mix (under the paraffin layer) is transferred to a new tube (90µl), an equal volume phenol (or phenol:chloroform:isoamylalcohol=25:24:1) is added (90 µl) and the sample is vortexed for about 10 sec, followed by centrifugation (14000 rpm, Eppendorf Centrifuge, 3 min).

The aqueous phase (upper layer) is carefully taken up with a pipette (using the “screwing mode” for the last few µl). The white material between the two phases should not be taken up. An equal volume chloroform (or chloroform:isoamylalcohol=24:1) is added, followed by vortexing for 10 sec and centrifugation at full speed for 10 sec. The upper phase is again taken up carefully and transferred to a new tube.

The volume of the final DNA-solution should be measured with the pipette and the DNA precipitated by mixing with 1/10 volume 3M Na-acetate and 2.5 volumes absolute ethanol. The solution should be cooled to -70°C for 5 min and the DNA pelleted by centrifugation (14000 rpm, 30 min at 4°C). The supernatant is removed, the pellet is washed with 100 µl 70% EtOH and centrifuged again for 4 min (14000 rpm, 4°C). The washing solution is removed, the pellet is dried for 5 min at r.t. and resolved in 10 µl TE-buffer (by resuspending with a pipette and warming to 58°C for 5 min).

If possible, the amount of DNA is quantified by photometry (1 µl in 300 µl A.dest.).

$A_{260} \times 15 = \mu\text{g}/\mu\text{l}$  (for dilution of 1:300 and dsDNA)

### 3. Restriction digest and electrophoresis

1 - 2 µg of the vector DNA is digested with the restriction enzymes of choice using the appropriate buffer system). Usually a 20 µl solution is prepared (2 µl of 10x buffer, 1 - 2 µl of DNA and A. dest. up to 20 µl). If two enzymes are used, which require different buffers (e.g. L and H buffer), you can start with the low salt buffer and just 1 enzyme (1 µl enzyme, 10 units, 37°C for 1 h), followed by addition of NaCl to obtain the higher salt concentration, addition of the second enzyme and incubation for an additional hour at 37°C.

If you use two different restriction enzymes for cloning (generating incompatible DNA-ends), it is not necessary to dephosphorylate the vector. If you use just one enzyme (or enzymes with compatible ends), you have to dephosphorylate the vector after the restriction digest (by adding 1/10 volume of 10x CIP-buffer and 1 µl of CIP (calf intestine phosphatase) and incubation at 37°C for 15 min. This is necessary to prevent religation of the vector (and a high background in empty vectors after transformation). The phosphatase has to be inactivated afterwards by addition of EDTA (1 µl 0.5 M) and heating to 68°C (for 10 min). Phosphatase treatment usually decreases the efficiency of ligation considerably.

The PCR-product should be subjected to restriction digests as the vector (use an appropriate amount of DNA - e.g. 1 - 2 µg, as well). For the final ligation, you have to consider the molar amount of insert related to vector. Since, the insert is usually much smaller than the vector (e.g. 1 kb compared to 5 kb), 1 µg of insert means a corresponding higher molar amount (5x more than 1 µg of a 5 kb vector).

After restriction digest (and dephosphorylation of the vector) the samples are either extracted with phenol/chloroform and precipitated with ethanol as described above, or subjected to electrophoresis. (Use low melting agarose, if you want to purify the DNA from the cut gel slices by heating and phenol/chloroform extraction, or normal agarose if you want to use GenElute columns from Sigma or similar stuff to obtain the DNA from the gel.).

Electrophoresis is done at 100 V for about 30 min (for normal agarose) or at 80V for 1 h (for low melting agarose).

After electrophoresis, DNA-bands of linearized vectors or PCR-fragments are cut from the gel (as close to the DNA as possible) preventing longer UV-exposure (which damages the DNA). Gel slices can be transferred to GenElute columns (equilibrated with 100 µl TE and centrifuged at full speed for 5 sec), followed by centrifugation at 14000 rpm for 10 min at r.t., which results in the generation of about 40 µl of DNA solution with about 80 - 90% of the original DNA amount. Alternatively, the gel slices are transferred to Eppendorf tubes, centrifuged briefly (to estimate the volume of the gel), covered with an equal amount of TE, heated to 65°C for 5 min, cooled for 30 sec (at r.t.) and extracted with an equal volume of phenol/chloroform as described above.

### 4. Ligation

1 µl 10x ligase-buffer, linearized (dephosphorylated) vector (about 100 - 300 ng), insert (about 50 - 100 ng, depending on length) and A. dest (ad 10 µl) are mixed and ligase is added (e.g. from BioLabs, 0.5 - 1 µl). The molarity of the insert should be between 1 - 2 times the molarity of the vector. The ligation mix is incubated at 16°C over night (or alternatively: 4 h at r.t.). The ligase buffer contains ATP (prevent repeated freeze-thawing; you can aliquot the buffer and store it at -70°C for good results).

**5. Transformation:** Transform 2 µl of the ligation mix (either conventionally or by electro-transformation: 1.9 kV, 25 µF, 200 , 4-5 msec), recover for 1 h at 37°C in 1 ml SOC medium, centrifuge at 3000 rpm for 10 min, decant the supernatant, suspend the pellet in the residual fluid and streak out on appropriate plates.