Blunt-end cloning according to Katri

Preparation of vector

- Check completeness of digestion by agarose gelelectrophoresis

Blunting with Klenow
- also 3'-overhangs can be blunted with Klenow
- final conc. (dNTPs) 50 mM
- same buffer as digestion
- Klenow 1U/pmol DNA-ends
- 15 min RT
- Inactivation 10 min 75°C

Dephosphorylation with CIP
- 1U/pmol DNA-ends
- 1h 37°C (5'-overhangs) or 50°C (3'-overhangs and blunt-ends)
- Inactivation: EDTA to final conc. 5 mM & 10 min 75°C
- Phenol/Chloroform/Isoamylalcohol-extraction 2x
- Chloroform-extraction 2x
- Adjust volume with HPLC-grade H_2O to 2 ml
- Desalt with Centricon C-100 and concentrate to ~100 ng/µl

Preparation of insert

Blunting with Klenow like vector

Isolation from agarose with Qiaex

Ligation

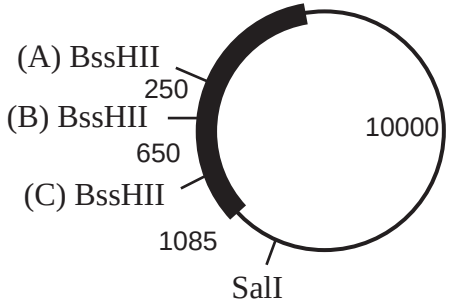
200 ng vector
approx. 2x equimolar amount of insert
1 µl 50% PEG-4000
1 µl ATP 5 mM
1 µl blunt-end-ligation buffer
H_2O to a final volume of 9 µl
1 µl undiluted T4 DNA ligase

Transformation

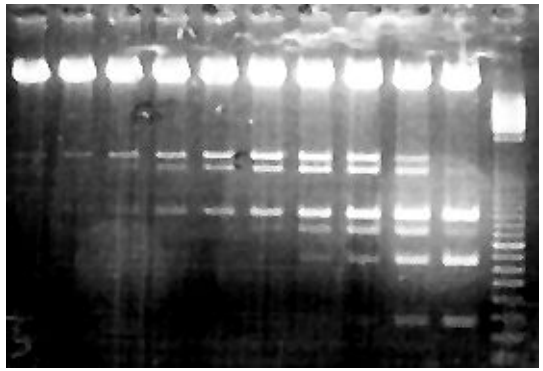
- Ligation mix (between 1 and 5 µl)
- Ligation mix without insert (background control)
- no DNA (contamination control)
- 1-10 ng of supercoiled plasmid (transformation control)

Partial digests are easy!

- Select an enzyme combination that allows isolation of your desired fragment.
- Only do partial digests of linearized plasmids.
- Calculate the exact amount of enzyme needed to completely digest 20-100 µg of plasmid in 2h.
- Start digest and take aliquots at t = 0,1,2,4,8,15,30,60 and 120 min
- Stop reaction adding proteinase K and incubating at 50°C for 10 min
- Heat inactivation works for some enzymes, especially if you want to blunt your fragment thereafter, be aware of the time lag!



BssHII cuts	resulting fragment sizes
nowhere	11985
only at A	10000, **1985**
only at B	10250, 1735
only at C	10900, 1085
at A+B	10000, 1735, 250
at A+C	10000, 1085, 900
at B+C	10250, 1085, 650
all sites	10000, 1085, 650, 250



How do I calculate the exact amount of enzyme needed for complete digestion?

1 unit of a restriction enzyme cuts 1 µg of assay DNA in 1 hour

How many µl of BssHII did I add to 80 µg of the plasmid shown above?

conc. (BssHII) = 20,000 units/ml
assayed on λ (~48 kb)
BssHII cuts λ 6 times
enz. survival: 0.5 units required for digestion of 1 µg assay DNA in 16 h

$$\text{needed units enzyme for complete digest in 1 h} = \frac{(\mu g \text{ target DNA})\,(\text{cuts in target DNA})\,(\text{size of assay-DNA in kb})}{(\text{cuts on assay-DNA})\,(\text{size of target DNA in kb})}$$