Cloning procedure

Overview of a cloning procedure:

1. Digestion of a PCR product (= insert) and a vector
2. Dephosphorylation of the digested vector
3. Ligation of digested insert and vector
4. Transformation of *E. coli*
5. Screening of positive clones by control digestion and/or Colony-PCR
6. Linearization of plasmids for transformation of *B. subtilis*

1. Digestion using restriction enzymes

Calculation of a digestion:

$$\frac{U\,(\text{Enzyme})}{\mu g\,(x)} = \frac{MW\,(R)}{MW\,(x)} \cdot \frac{n\,(x)}{n\,(R)}$$

with	R	Reference-DNA
	x	DNA to digest
	MW	Molecular weight
	n	Amount of cutting sites

- The phage Lambda (λ) serves as reference DNA with a MW of 48.5 kb
- The amount of λ's cutting sites for a specific restriction enzyme is written down in the NEB catalog

EXAMPLE: digestion of 500 ng pXT with *Bam*HI and *Hin*dIII

MW (pXT) = 6.892 kb
MW (λ) = 48.5 kb
n (pXT-*Bam*HI) = 1
n (λ-*Bam*HI) = 5
n (pXT-*Hin*dIII) = 1
n (λ-*Hin*dIII) = 6

*Bam*HI:

➔ $$\frac{U\,(\text{Enzyme})}{\mu g\,(\text{pXT})} = \frac{48.5\ kb}{6.892\ kb} \cdot \frac{1}{5} = 1.4$$

➔ $$\frac{U\,(\text{Enzyme})}{0.5\ \mu g\,(\text{pXT})} = \frac{1.4}{2} = 0.7$$

You need 0.7 U of *Bam*HI to digest 500 ng of pXT

The concentration of the enzyme stock solution is 10 U/µl (for Fermentas enzymes!! → NEB enzymes may have different units per µl)

➔ $\frac{10\,U}{1\,\mu l} = \frac{0.7\,U}{x}$

➔ x = 0.07 µl

To be really sure that almost everything of your DNA will be digested, double the calculated volume of your enzyme:

➔ 0.07 µl · 2 = <u>0.14 µl</u>

You need 0.14 µl of *Bam*HI to digest 500 ng pXT.

*Hin*dIII:

➔ $\frac{\mathrm{U\,(Enzyme)}}{\mu g\,(\mathrm{pXT})} = \frac{48.5\,kb}{6.892\,kb} \cdot \frac{1}{6} = 1.2$

➔ $\frac{\mathrm{U\,(Enzyme)}}{0.5\,\mu g\,(\mathrm{pXT})} = \frac{1.2}{2} = 0.6$

You need 0.6 U of *Hin*dIII to digest 500 ng of pXT

The concentration of the enzyme stock solution is 10 U/µl

➔ $\frac{10\,U}{1\,\mu l} = \frac{0.6\,U}{x}$

➔ x = 0.06 µl

➔ 0.06 µl · 2 = <u>0.12 µl</u>

You need 0.12 µl of *Hin*dIII to digest 500 ng pXT.

Sample preparation:

- 500 ng pXT
- 0.5 µl *Bam*HI
- 0.5 µl *Hin*dIII
- X µl 10×Buffer
- X µl H_2O

Important: DO NOT exceed the volume of the enzymes above 1/10 of the final volume

➔ Incubate for 1.5 to 2 h at 37°C (if you use FastDigest enzymes: incubate for 15 min at 37°C)

2. Dephosphorylation of digested vectors

Important: DO NOT dephosphorylate your inserts!

FastAP (Thermosensitive Alkaline Phosphatase; Fermentas):

- Add 1 µl of Fast AP to the digested vector
- Incubate at 37°C for 15 min

SAP (Shrimp Alkaline Phosphatase; Fermentas):

- Add 1 µl of SAP to the digested vector
- Incubate at 37°C for 30 min
- Add another 1 µl of SAP and incubate further 30 min at 37°C

After dephosphorylation: Purify the digestion samples by using the PCR Purification Kit

3. Ligation

Calculation of a ligation:

$$\frac{\text{ng (Vector)}}{\text{kb (Vector)}} = \frac{ng\ (Insert)}{kb\ (Insert)}$$

EXAMPLE: ligation of 200 ng pXT with a 1000 bp DNA fragment

➔ $\frac{200\,ng}{6.892\,\text{kb}} = \frac{x\,ng}{1\,kb}$

➔ x = 29 ng

Molecular ratio of vector (200 ng pXT) to insert (29 ng of a 1000 bp DNA fragment) = 1:1

In the most cases you will need a 3-times molar excess of insert to vector

➔ 29 ng · 3 = 87 ng

Sample preparation:

- 200 ng pXT
- 87 ng Insert
- 1 µl T4 DNA Ligase (Fermentas)
- X µl 10×Ligase-Buffer (Fermentas)
- X µl dH_2O

➔ Keep the final volume as small as possible!
➔ Incubate for 1-3 h at room temperature

4. Transformation of competent *E. coli* cells ($CaCl_2$)

- Thaw 100 µl of competent cells on ice
- Add ½ volume of your ligation sample to the cells
- Incubate for 20 min on ice
- Heat shock the cells at 42°C for 1.5 min
- Cool down the cells for 2 min on ice
- Add 400 µl LB to the cells
- Shake the cells for 1 h at 37°C
- Plate 50 µl of the cell solution on a LB+antibiotic agar plate
- Centrifuge the remaining cell solution (1 min, 13 krpm, room temperature)
- Discard the medium and resuspend the pellet in the remaining medium drop
- Plate the entire resuspended cells on a LB+antibiotic agar plate
- Incubate the plates at 37°C for overnight

6.1 Control digestion

Sample preparation:

- 200 ng plasmid
- 0.3 µl enzyme 1
- 0.3 µl enzyme 2
- Appropriate buffer
- dH_2O

➔ Incubate for at least 1 h at the dedicated temperature
➔ Mix with DNA loading dye and load the entire sample on an agarose gel

6.2 Colony-PCR → See PCR methods

7. Linearization of integrative plasmids for transformation of *B. subtilis*

Sample preparation: (30 µl final volume)

- 25 µl plasmid (from Mini Prep)
- 1 µl enzyme
- 3 µl buffer
- 1 µl dH_2O

➔ Incubate for 1.5-2 h at 37°C
➔ Use 15 µl of the sample for *B. subtilis* transformation

Examples for linearization enzymes of different vectors: (Check in advance that the indicated enzymes do not cut your insert!)

Vector	**Enzyme for linearization**
pXT	ScaI
pAC5	PstI
pAC6	PstI or ScaI
pAC7	PstI
pSWEET	ScaI
pALFLAGrsiW-1	PvuI
pDG1662	ScaI
pDG1663	NcoI
pDG1731	ScaI

Protocol generously provided by the lab
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