Cloning procedure

(by Karen, 2010)

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:

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

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:

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

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

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