

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:

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

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 *Hind*III

MW (pXT) = 6.892 kb

MW (λ) = 48.5 kb

n (pXT-*Bam*HI) = 1

n (λ -*Bam*HI) = 5

n (pXT-*Hind*III) = 1

n (λ -*Hind*III) = 6

*Bam*HI:

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

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

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

The concentration of the enzyme stock solution is 10 U/ μ l (for Fermentas enzymes!! \rightarrow NEB enzymes may have different units per μ l)

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

$$\rightarrow x = 0.07 \mu l$$

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

$$\rightarrow 0.07 \mu l \cdot 2 = \underline{\underline{0.14 \mu l}}$$

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

*Hind*III:

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

$$\rightarrow \frac{U (\text{Enzyme})}{0.5 \mu g (\text{pXT})} = \frac{1.2}{2} = 0.6$$

You need 0.6 U of *Hind*III to digest 500 ng of pXT

The concentration of the enzyme stock solution is 10 U/ μ l

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

$$\rightarrow x = 0.06 \mu l$$

$$\rightarrow 0.06 \mu l \cdot 2 = \underline{\underline{0.12 \mu l}}$$

You need 0.12 μ l of *Hind*III to digest 500 ng pXT.

Sample preparation:

- 500 ng pXT
- 0.5 μ l *Bam*HI
- 0.5 μ l *Hind*III
- X μ l 10 \times Buffer
- X μ l H₂O

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