Restriction digest, end modification, and ligation of DNA

Recombinant DNA molecules can be generated by breaking and rejoining DNA using restriction enzymes and ligases. The assembly process can be steered by controlling the reactivity of DNA ends. This workflow, restriction-ligation cloning, remains the most reliable and cost-effective way to generate simple or standardized recombinant DNA constructs.
These are modular procedures to generate, modify, and remove DNA overhangs to steer their reactivity, enabling efficient and accurate construction of such DNA products.

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This is a bench card. Full protocol available online.

Procedures

⊞ Optional: *Conversion of DNA mass to moles of ends*

(1.) Some enzymatic activity scales with the concentration of ends of DNA molecules, not mass. Approximate the molar concentration c_i of DNA ends from the mass concentration β_i of n_i kilobase pairs as

$$c_i\,[\mu\mathrm{M}] \approx 3.2{\times}10^{-3} \times \boxed{\beta_i}\,\left[\frac{\mathrm{ng}}{\mu\mathrm{L}}\right] / \boxed{n_i}\,\left[\frac{\mathrm{bp}}{\mathrm{kbp}}\right]$$

A › *Annealing complementary DNA fragments*

- ☐ DNA duplex buffer, pH 7.5

(1.) Complementary DNA oligonucleotides can be annealed to form duplexes with blunt or cohesive ends. To prepare an annealing reaction, mix:

Ingredient	Stock	Final	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			10.0 µL	50.0 µL
DNA duplex buffer	10 ×	1 ×	2.0 µL	10.0 µL
Oligo 1	100 µM	20 µM	4.0 µL	20.0 µL
Oligo 2	100 µM	20 µM	4.0 µL	20.0 µL

> ***Quality assurance:*** To ensure complete duplex formation, confirm the concentration of each oligo by absorbance at 260 nm and calculate the extinction coefficients using the supplier's specification sheets. Equimolar amounts are essential to avoid residual single-stranded material.

(2.) Denature the oligos at 95 °C for 3 min using a thermocycler, heat block, or a beaker with boiling water.

(3.) Allow to cool slowly (1.0–1.5 °C/min) to room temperature. 60 min

(4.) The annealed product is a 10 µM double-stranded DNA fragment with defined ends. Use directly for phosphorylation or ligation.

R › *Restriction digest of DNA*

(1.) Restriction enzymes cleave ("cut") DNA at specific recognition sites to generate DNA fragments with defined ends. To set up a digestion reaction, combine the following:

Ingredient	Stock	Final	Analytical To 10 µL	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			8.5 µL	16.0 µL	78.0 µL
Restriction digest buffer	10 ×	1 ×	1.0 µL	2.0 µL	10.0 µL
DNA	200 ng/µL	10–20 ng/µL	0.5 µL	2.0 µL	10.0 µL
Restriction enzyme (each)	10 U/µL	1 U/µg	0.2 µL	0.4 µL	2.0 µL

> ***Critical:*** Restriction enzymes are sensitive to freeze-thaw cycles and temperature fluctuations. Always transport stocks on ice or in a benchtop cooler immediately after removing them from the −20 °C freezer. Avoid the freezer door for storage.

(2.) Gently flick the tube to mix, briefly spin to collect liquid, and incubate under the recommended conditions, typically 37 °C for 20–60 min. ⏲ 20 min

(3.) *Optional:* Heat-inactivate at 65–80 °C for 20 min. 🕮

(4.) Purify DNA by silica column or agarose gel extraction. 🕮

B › *Selective blunting of 3 or 5 overhangs*

(1.) Blunting can be used to remove undesired restriction sites. Under certain conditions, only one type of DNA overhang is removed while preserving the overhang at the opposite end:

Method	3 overhang	5 overhang	Notes
Mung bean nuclease	Resected	Resected	
T4 DNA polymerase	Resected	Filled	Strong 3→5 exonuclease, use with caution for short DNA
Klenow (+dNTP)	Resected	Filled	Widely used for blunting
Klenow (–dNTP)	Resected	Preserved	
Taq DNA polymerase	Preserved	Filled + Tailed	Adds 1 or 2 untemplated 3 deoxyadenines
RNase T	Resected	Preserved	Inhibited by two or more consecutive 3 (deoxy-)cytidines

> ***Critical:*** 5 single-stranded overhangs cannot be selectively blunted in the presence of 3 overhangs.

Blunting of 3 and 5 overhangs by nuclease digest

(1.) Mung bean nuclease removes 3 or 5 overhangs from double-stranded DNA. Set up on ice: 🐞 🕮

Ingredient	Stock	Final	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			8.0 µL	39.0 µL
Buffer (CS, 2.1, 1.1)	10 ×	1 ×	2.0 µL	10.0 µL
DNA	200 ng/µL	100 ng/µL	10.0 µL	50.0 µL
Mung bean nuclease	10 U/µL	1 U/µg	0.2 µL	1.0 µL

(2.) Incubate at 25 °C for 30 min. ⏲ 30 min

(3.) Inactivate by adding SDS to 0.01% (use a 0.1% SDS stock) or extract with phenol/chloroform.

> ***Critical:*** Do not heat-inactivate! Above 45 °C, DNA may “breathe” and get degraded before the enzyme is fully inactivated.

Blunting of 3 and 5 overhangs by DNA polymerase I (Klenow fragment)

(1.) Klenow fragment removes 3 overhangs via 3→5 exonuclease activity and fills in 5 overhangs by polymerization. Set up on ice: 🐞 🕮

Ingredient	Stock	Final	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			7.0 µL	34.0 µL
Buffer (CS, 3.1, 2.1, 1.1)	10 ×	1 ×	2.0 µL	10.0 µL
DNA	200 ng/µL	100 ng/µL	10.0 µL	50.0 µL
dNTP mix	1 mM	40 µM	0.8 µL	4.0 µL
Klenow fragment, exo–	5 U/µL	1 U/µg	0.4 µL	2.0 µL

(2.) Incubate at 25 °C for 10–15 min. ⏲ 15 min

(3.) Heat-inactivate the enzyme at 75 °C for 10 min.

D › ***Dephosphorylation of DNA and RNA***

(1.) Determine the molar concentration of DNA ends to dephosphorylate and scale volumes accordingly.

(2.) Shrimp alkaline phosphatase (SAP), like other phosphatases, efficiently dephosphorylates the 5-phosphate of single- or double-stranded DNA or RNA. Combine: 🐞 🕮

Ingredient	Stock	Final	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			15.5 µL	112.5 µL
Buffer (CS, 3.1, 2.1, 1.1)	10 ×	1 ×	2.0 µL	10.0 µL
DNA *or* RNA (ends)	300 nM	50 nM	1.5 µL	7.5 µL
SAP (*or* CIP)	1 U/µL	25 U/fmol	1.0 µL	5.0 µL

(3.) Incubate at 37 °C for 30 min. ⏲ 30 min

(4.) *Critical:* Heat-inactivate the phosphatase at 65 °C for 5 min.

P › ***Phosphorylation (or radioactive end labeling) of DNA and RNA***

(1.) Determine the molar concentration of DNA ends to phosphorylate.

(2.) T4 polynucleotide kinase (PNK) transfers the terminal phosphate from nucleotide triphosphates to the 5-hydroxyl group of single- or double-stranded DNA or RNA. Combine the reactants: 🐞 🕮

Ingredient	Stock	Final	Small-scale To 20 µL	Large-scale To 100 µL
Water, reagent-grade			6.0 µL	28.0 µL
T4 PNK reaction buffer	10 ×	1 ×	2.0 µL	10.0 µL
DNA *or* RNA	300 nM	150 nM	10.0 µL	50.0 µL
ATP (*or* NTP, dATP, dTTP)	10 mM	1 mM	2.0 µL	10.0 µL
T4 PNK	10 U/µL	30 U/fmol	0.4 µL	2.0 µL

(3.) Incubate at 37 °C for 30 min. ⏲ 30 min

(4.) Heat-inactivate T4 PNK at 65 °C for 20 min or purify the reaction.

T7 › ***Ligation of cohesive DNA overhangs with T7 DNA ligase***

(1.) Determine the molar concentration of each DNA fragment.

(2.) T7 DNA ligase joins cohesive DNA ends with adjacent 5-phosphate and 3-hydroxy groups: 🐞 🕮

Ingredient	Stock	Final	Small-scale To 5 µL	Large-scale To 20 µL
Water, reagent-grade			1.1 µL	4.2 µL
T7 DNA ligase buffer	2 ×	1 ×	2.5 µL	10.0 µL
Vector DNA	80 nM	1 nM	0.3 µL	1.2 µL
Insert DNA (each)	16 nM	3 nM	0.9 µL	3.6 µL
T7 DNA ligase	3 000 U/µL	150 U/fmol	0.2 µL	1.0 µL

> ***Critical:*** Both 5' ends must be phosphorylated. T7 DNA ligase does not ligate blunt ends. Use T4 DNA ligase in these cases.

(3.) Incubate at 25 °C for 10–15 min. Longer reactions are not typically needed. 10 min

(4.) *Optional:* Heat-inactivate at 65 °C for 10 min.

> ***Critical:*** Do not heat-inactivate if you intend to transform the ligated reaction product and the reaction buffer contained polyethylene glycol (PEG). Denatured PEG-DNA complexes dramatically reduce transformation efficiency.

(5.) *Optional:* Chill on ice and transform 1–5 µL into 10–50 µL competent cells; or store at −20 °C.

T4 › ***Ligation of cohesive or blunt DNA overhangs with T4 DNA ligase***

(1.) Determine the molar concentration of each DNA fragment.

(2.) T4 DNA ligase joins juxtaposed 5-phosphate and 3-hydroxyl termini on double-stranded DNA:

Ingredient	Stock	Final	Small-scale To 5 µL	Large-scale To 20 µL
Water, reagent-grade			3.1 µL	12.2 µL
T4 DNA ligase buffer	10 ×	1 ×	0.5 µL	2.0 µL
Vector DNA	80 nM	1 nM	0.3 µL	1.2 µL
Insert DNA (each)	16 nM	3 nM	0.9 µL	3.6 µL
T4 DNA ligase	400 U/µL	20 U/fmol	0.2 µL	1.0 µL

(3.) Incubate at 25 °C for 10 min (cohesive ends) or 60 min (blunt ends and single-base overhangs).

(4.) *Optional:* Heat-inactivate at 65 °C for 10 min.

(5.) *Optional:* Chill on ice and transform 1–5 µL into 10–50 µL competent cells, or store at −20 °C.

S › ***Self-circularization of DNA***

(1.) Dilute T4 DNA ligase 1 000-fold in reaction buffer.

> ***Critical:*** Do not exceed the recommended ligase concentration. Excess enzyme promotes unwanted intermolecular ligation. At low DNA concentrations, intramolecular end-joining is kinetically favored because the effective local concentration of ends within one molecule exceeds that between molecules.

(2.) To self-circularize linear DNA, prepare the reaction as follows:

Ingredient	Stock	Final	Small-scale	Large-scale
Water, reagent-grade			2.8 µL	11.2 µL
T4 DNA ligase buffer	10 ×	1 ×	0.5 µL	2.0 µL
Vector DNA	3 nM	0.3 nM	0.5 µL	2.0 µL
T4 DNA ligase, 1:1 000 dilution	0.4 U/µL	0.3 U/fmol	1.2 µL	4.8 µL

(3.) Incubate at 25 °C for 10 min (cohesive ends) or 60 min (blunt ends).

(4.) *Optional:* Heat-inactivate at 65 °C for 10 min.

(5.) *Optional:* Chill on ice and transform 1–5 µL into 10–50 µL competent cells, or store at −20 °C.

List of references