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 a reliable and inexpensive 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.

Risk assessment

- Phenol is a REPRODUCTIVE TOXIN and causes serious chemical burns
- ▷ Wear gloves, safety glasses, lab coat



Reviewed: Nov 2, 2026

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

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

> ***Hint:*** For example, 100 ng/µL of a 1 kbp fragment corresponds to 0.32 µM DNA ends, a 2 kbp fragment would be 0.16 µM.

> ***This is why:*** The approximation assumes an average molecular weight of 617.96 g/mol per base pair and neglects terminal modifications. It is sufficiently accurate for DNA larger than 700 bp.

A › ***Annealing complementary DNA fragments***

- ☐ DNA duplex buffer, 10-fold, pH 7.5 ◇ R0016

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

Small	Large	Ingredient		Stock	Final
2 µL	10 µL	DNA duplex buffer	◇ R0016	10 ×	1 ×
4 µL	20 µL	Oligo 1		100 µM	20 µM
4 µL	20 µL	Oligo 2		100 µM	20 µM
10 µL	50 µL	Water, reagent-grade			
To 20 µL	To 100 µL				

> ***This is why:*** This method is often used to generate synthetic inserts, linkers, or adapters compatible with restriction-ligation workflows. Annealed fragments can be directly phosphorylated and ligated into digested vectors.

> ***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 20 µM double-stranded DNA fragment with defined ends. Use directly for phosphorylation or ligation.

R › *Restriction digest of DNA*

- ☐ Restriction enzyme, type IIP (palindromic) or type IIS (asymmetric) recognition
- ☐ Restriction digest buffer

(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:

Analytical	Small	Large	Ingredient	Stock	Final
1 µL	2 µL	10 µL	Restriction digest buffer	10 ×	1 ×
1 µL	2 µL	10 µL	DNA	200 ng/µL	20 ng/µL
0.2 µL	0.4 µL	2 µL	Restriction enzyme (each)	10 U/µL	0.2 U/µL
7.8 µL	15.6 µL	78 µL	Water, reagent-grade		
To 10 µL	To 20 µL	To 100 µL			

This is why: Restriction endonucleases can generate DNA fragments with defined single-stranded overhangs. Fragments with complementary overhangs can be rejoined by ligation since they are 5′-phosphorylated. Recognition sites, cleavage patterns, reaction and inactivation conditions of common restriction endonucleases are provided as Supplementary Resources.

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.

Hint: BamHI, BglII, DraIII, HpaI, KpnI, MluI, NgoMIV, and some other restriction enzymes cannot be heat-inactivated.

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

Hint: If the restriction site is absent from the product and the reaction buffer compatible, purification may be omitted.

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

- ☐ Mung Bean Nuclease

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

Small	Large	Ingredient	Stock	Final
2 µL	10 µL	Buffer (CS, 2.1, 1.1)	10 ×	1 ×
10 µL	50 µL	DNA	200 ng/µL	100 ng/µL
0.2 µL	1 µL	Mung bean nuclease	10 U/µL	1 U/µg
7.8 µL	39 µL	Water, reagent-grade		
To 20 µL	To 100 µL			

This is why: Mung bean nuclease is a single-strand–specific endonuclease. It cleaves unpaired regions both at DNA ends, such as 3′ or 5′ overhangs, and internally, such as in hairpin loops or mismatches, but leaves fully base-paired duplex DNA intact.

> ***Hint:*** Mung bean nuclease is active in many buffers since commercial enzyme stocks provide sufficient zinc even after dilution. Start with 1 U per microgram of DNA and adjust empirically to optimize reaction time and completeness.

(2.) Incubate at 30 °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)

- ☐ DNA Polymerase I, Large (Klenow) Fragment

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

Small	Large	Ingredient	Stock	Final
2 µL	10 µL	Buffer (CS, 3.1, 2.1, 1.1)	10 ×	1 ×
10 µL	50 µL	DNA	200 ng/µL	100 ng/µL
0.66 µL	3.3 µL	dNTP mix	1 mM	33 µM
0.4 µL	2 µL	Klenow fragment	5 U/µL	1 U/µg
6.94 µL	34.6 µL	Water, reagent-grade		
To 20 µL	To 100 µL			

> ***This is why:*** The large fragment of *E. coli* DNA polymerase I (Klenow fragment) can also be used to generate single-stranded DNA probes from random primers, or to synthesize second strands from cDNA. Blunting needs its 3′→5′ exonuclease; the exonuclease-deficient fragment (exo−) cannot remove 3′ overhangs.

> ***Hint:*** The Klenow fragment is active in many restriction enzyme buffers and in T4 DNA ligase buffer. For custom reactions, provide 50 mM Tris-Cl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM DTT, and 20 mg/L BSA.

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

(3.) Stop the reaction by adding EDTA to 10 mM and heating at 75 °C for 20 min.

D › *Dephosphorylation of DNA and RNA*

- ☐ Shrimp Alkaline Phosphatase (rSAP)
  - *or*: Antarctic Phosphatase, New England Biolabs
  - *or*: Quick CIP (calf intestinal alkaline phosphatase), New England Biolabs

(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:

Small	Large	Ingredient	Stock	Final
2 µL	10 µL	Buffer (CS, 3.1, 2.1, 1.1)	10 ×	1 ×
1.5 µL	7.5 µL	DNA *or* RNA (ends)	300 nM	22.5 nM
1 µL	5 µL	SAP (*or* CIP)	1 U/µL	2.2 U/pmol
15.5 µL	77.4 µL	Water, reagent-grade		
To 20 µL	To 100 µL			

> ***This is why:*** Selective dephosphorylation of DNA fragments prevents their self-ligation, since DNA ligases join a 5′-phosphate to an adjacent 3′-hydroxyl. A dephosphorylated vector still accepts a phosphorylated insert; the product carries one nick per junction. Other phosphatases such as the thermolabile antarctic phosphatase (AnP) and calf intestine phosphatase (CIP) can also be used, but may be more difficult to inactivate. All nucleic acid phosphatases act on both 3′- and 5′-terminal phosphates and dephosphorylate dNTPs if present in the reaction.

> ***Hint:*** Shrimp alkaline phosphatase is active in most restriction enzyme buffers and does not require additional cofactors. The enzyme is inhibited by EDTA, high concentrations of DTT or 2-mercaptoethanol, phosphate analogs, and its own reaction product, inorganic phosphate. Reaction volumes should be scaled rather than increasing concentrations.

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

(4.) *Critical:* Heat-inactivate the phosphatase at 65 °C for 5 min (SAP) or at 80 °C for 2 min (Antarctic phosphatase, Quick CIP).

P › *Phosphorylation of DNA and RNA*

☐ T4 Polynucleotide Kinase

(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:

Small	Large	Ingredient	Stock	Final
2 µL	10 µL	T4 PNK reaction buffer	10 ×	1 ×
10 µL	50 µL	DNA *or* RNA	300 nM	150 nM
2 µL	10 µL	ATP (*or* NTP, dATP, dTTP)	10 mM	1 mM
0.4 µL	2 µL	T4 PNK	10 U/µL	1.3 U/pmol
5.6 µL	28 µL	Water, reagent-grade		
To 20 µL	To 100 µL			

This is why: PNK efficiently phosphorylates recessed, blunt, or overhanging 5′-termini but performs poorly on nicks. It also dephosphorylates 3′ ends. Use phosphatase-deficient variants if this is undesired.

Hint: T4 PNK is active in T4 DNA ligase buffer, which includes 1 mM ATP. γ-phosphate-linked substrates are not transferred. The enzyme is inhibited by ammonium ions, EDTA, and high concentrations of salt or inorganic phosphate. Exceeding 6 µM of 5′ ends or 100 ng/µL DNA may reduce enzyme efficiency.

(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*

☐ T7 DNA Ligase

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

(2.) Dilute T7 DNA ligase to 600 U/µL in reaction buffer (1:5 from a 3 000 U/µL stock).

(3.) Dilute Vector DNA to 10 nM (1:8 from a 80 nM stock).

(4.) T7 DNA ligase joins cohesive DNA ends with adjacent 5′-phosphate and 3′-hydroxy groups:

Small	Large	Ingredient	Stock	Final
2.5 µL	10 µL	T7 DNA ligase buffer	2 ×	1 ×
0.5 µL	2 µL	Vector DNA, 1:8 dilution	10 nM	1 nM
0.9 µL	3.6 µL	Insert DNA (each)	16 nM	3 nM
1 µL	4 µL	T7 DNA ligase, 1:5 dilution	600 U/µL	120 U/fmol
0.1 µL	0.4 µL	Water, reagent-grade		
To 5 µL	To 20 µL			

Critical: T7 DNA ligase does not ligate blunt ends under standard conditions; it needs at least 20% PEG to show measurable activity on them, and higher PEG concentrations reduce ligation fidelity. Use T4 DNA ligase for blunt ends.

Hint: For best results, a 3:1 to 5:1 molar insert-to-vector ratio is recommended. Contaminants such as EDTA or phenol inhibit ligation. If substituting buffers, ensure they contain 1 mM ATP and 10 mM $MgCl_2$.

(5.) Incubate at 25 °C for 30 min. 30 min

(6.) *Optional:* Chill on ice and transform at most 0.1 vol of the competent cell volume, such as 5 µL into 50 µL cells; or store at −20 °C.

Critical: Do not heat-inactivate before transformation. The T7 DNA ligase buffer contains polyethylene glycol (PEG), and heat-treated PEG reactions transform poorly.

Hint: To select for correct ligation products with multiple inserts, perform a PCR with primers flanking the cloning site, purify the correct fragment from a gel, and insert into the vector by conventional two-way ligation [AWL10].

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

☐ T4 DNA Ligase

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

(2.) Dilute T4 DNA ligase to 80 U/µL in reaction buffer (1:5 from a 400 U/µL stock).

(3.) Dilute Vector DNA to 5 nM (1:16 from a 80 nM stock).

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

Small	Large	Ingredient	Stock	Final
0.5 µL	2 µL	T4 DNA ligase buffer	10 ×	1 ×
1 µL	4 µL	Vector DNA, 1:16 dilution	5 nM	1 nM
0.9 µL	3.6 µL	Insert DNA (each)	16 nM	3 nM
1 µL	4 µL	T4 DNA ligase, 1:5 dilution	80 U/µL	16 U/fmol
1.6 µL	6.4 µL	Water, reagent-grade		
To 5 µL	To 20 µL			

Hint: One strand may lack a 5′-phosphate, but both strands must not be dephosphorylated. T4 DNA ligase is active in most restriction enzyme buffers and T4 PNK buffer when supplemented with 1 mM ATP. Molar ratios of 1 : 1 to 1 : 10 are optimal for single insertions. For ligations with two or more inserts, double the molar excess of each.

(5.) Incubate at 25 °C for 10 min (cohesive ends) or 2 h (blunt ends and single-base overhangs).

Hint: Use 100 U/fmol to accelerate ligation 5- to 10-fold.

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

(7.) *Optional:* Chill on ice and transform at most 0.1 vol of the competent cell volume, such as 5 µL into 50 µL cells; or store at −20 °C.

S › ***Self-circularization of DNA***

☐ T4 DNA Ligase

(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:

Small	Large	Ingredient	Stock	Final
0.5 µL	2 µL	T4 DNA ligase buffer	10 ×	1 ×
0.5 µL	2 µL	Vector DNA	3 nM	0.3 nM
1.2 µL	4.8 µL	T4 DNA ligase, 1:1 000 dilution	0.4 U/µL	0.3 U/fmol
2.8 µL	11.2 µL	Water, reagent-grade		
To 5 µL	To 20 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 at most 0.1 vol of the competent cell volume, such as 5 µL into 50 µL cells; or store at −20 °C.

Troubleshooting

Annealing complementary DNA fragments

In Step 4:

– Annealing results in incomplete duplex formation

  ◦ Check for potential hairpin or self-dimer formation using an oligo folding tool. Strong intramolecular structures with a predicted $\Delta G < -5\,\text{kcal/mol}$ may outcompete duplex formation. Consider redesigning oligos or modifying the annealing conditions towards slower cooling, and increased oligo concentration.
  ◦ Run a sample on native PAGE to confirm duplex formation. Duplexes migrate differently than hairpins or unpaired oligos.
  ◦ Ensure equimolar concentrations of both strands. Unequal input may result in excess single-stranded or mispaired species.

Restriction digest of DNA

In Step 1:

– No or low restriction activity when starting material has low DNA concentration

  ◦ Concentrate the DNA sample to avoid adding more than 0.25 vol of silica column eluate. Carried-over buffer dilutes the digest and inhibits restriction enzymes.
  ◦ Check that the buffer carries the salt the enzyme needs. BglI, BglII, BsiWI, BsmBI and BstXI reach full activity only in a high-salt buffer and lose most of it in a low-salt one; supplement the salt rather than digesting longer.

– No or low restriction activity when digesting PCR products without prior purification

  ◦ Clean up PCR products before digestion. Most restriction enzymes are active in Taq polymerase buffers, but activity is reduced in high-fidelity polymerase buffers. Residual DNA polymerase activity will blunt overhangs, often blocking further digestion and downstream manipulation.

– No or low restriction activity overall

  ◦ Some restriction sites may be blocked by DNA methylation. If the enzyme is methylation-sensitive such as BsaBI, DpnII, HphI, or XbaI, clone into a methylation-deficient *E. coli* strain.
  ◦ Some restriction enzymes such as FokI, NgoMIV, PaqCI, SacII require two recognition sites for optimal activity. If only a single site is present, supplement with short double-stranded oligonucleotides containing additional sites.
  ◦ How close to a fragment end an enzyme cuts is enzyme-specific. Many cleave efficiently with a single base pair beyond the recognition site, while HindIII, NdeI and SacI-HF reach full activity only from 3 bp. Consult the supplier's table, and allow 6 bp for an enzyme it does not list.

– Non-specific cleavage

  ◦ Do not exceed 0.1 vol enzyme stock in the reaction. Excess enzyme or high glycerol concentrations from storage buffers can promote non-specific cleavage or altered substrate specificity ("star activity").

Blunting of 3′ and 5′ overhangs by nuclease digest

In Step 1:

– Partial digestion or smeared DNA on gel

  ◦ Try reducing the incubation time or lowering the enzyme concentration. Use ice-cold buffer and fresh zinc-containing solution to minimize non-specific activity.
  ◦ Verify the DNA is fully double-stranded. Single-stranded regions from incomplete PCR or overhangs may persist and be unintentionally removed.

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

In Step 1:

– No blunting when used with PCR fragments

  ◦ Clean up the PCR product. Some DNA polymerases tail DNA ends, which interferes with blunt-end reactions. Be sure to add dNTPs back for blunting.
  ◦ If blunting remains incomplete, increase the Klenow amount. Consider re-annealing if overhangs might have reformed.

Dephosphorylation of DNA and RNA

In Step 2:

– Ligation fails after dephosphorylation

  ◦ Residual SAP activity can persist if heat inactivation is incomplete, especially in large volumes or buffers containing stabilizing cations. Purify the DNA using a silica column or magnetic beads before ligation.

– Dephosphorylation is incomplete

- Check for carryover of phosphate-containing buffer from previous steps. Elute DNA in low-salt, phosphate-free buffer before SAP treatment.

Phosphorylation of DNA and RNA

In Step 2:

– Insufficient phosphorylation
  - Prepare fresh ATP.
  - Add fresh DTT to 5 mM from a 1 M stock if needed.
  - Avoid carry-over of phosphate-containing buffers from prior steps. Inorganic phosphate strongly inhibits PNK.

– Short oligonucleotides fail to phosphorylate
  - Verify that 5′-hydroxyl groups are present. Some oligos are synthesized with 5′-phosphate blocking groups or need prior dephosphorylation and rephosphorylation.

Ligation of cohesive DNA overhangs with T7 DNA ligase

In Step 4:

– No ligation product or very low efficiency
  - Verify that overhangs are cohesive and that each junction carries a 5′-phosphate.
  - If DNA was extracted with phenol or contains EDTA, clean up before ligation.

Ligation of cohesive or blunt DNA overhangs with T4 DNA ligase

In Step 4:

– No ligation
  - Thaw T4 DNA ligase buffer on the bench or in the palm of your hand, not at 37 °C, to prevent breakdown of ATP. Once thawed, keep the buffer on ice. Use fresh ATP.
  - Clean up the DNA. High salt, EDTA, or residual phosphatase can inhibit ligation.
  - Lower the DNA concentration. Ligation favors intermolecular reactions at high concentrations and yields linear concatemers rather than circles.
  - Prolong ligation for inserts longer than 2 kbp or perform at 16 °C overnight.
  - Overhangs ending in 5′–`TNA`–3′ or 5′–`TNNA`–3′ (e.g., from PstI, SbfI, XhoI, or SalI digest) ligate with reduced efficiency due to weak terminal base pairing [BPP+22]. Increase insert ratio or ligation time.

– Spurious ligation
  - Dephosphorylate the vector if only one restriction site is used or if gel purification is not feasible.
  - Consider T7 DNA ligase. It offers 89% ligation fidelity compared to 72% for T4 DNA ligase, which may tolerate mismatches at the 5′ overhang, especially G:T or T:G pairs, and single mismatches in GC-rich contexts [BPP+22].

Materials

Annealing complementary DNA fragments

- ☐ **DNA duplex buffer**, 10-fold, pH 7.5 ⟋ R0016

Restriction digest of DNA

- ☐ **Restriction enzyme**, type IIP (palindromic) or type IIS (asymmetric) recognition
  Hint: Select enzyme(s) based on the desired cleavage pattern; see Supplementary Resources for common type IIP and type IIS enzymes with recognition sites, buffer, and inactivation conditions.
- ☐ **Restriction digest buffer**
  Hint: Choose the buffer matching the enzyme(s) being used; see the buffer compatibility table in Supplementary Resources.
  - *e.g.* **CutSmart Buffer**
    New England Biolabs
  - *e.g.* **NEBuffer 1.1**
    New England Biolabs
  - *e.g.* **NEBuffer 2.1**
    New England Biolabs
  - *e.g.* **NEBuffer 3.1**
    New England Biolabs

Blunting of 3′ and 5′ overhangs by nuclease digest

- ☐ **Mung Bean Nuclease**
  New England Biolabs, M0250

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

- ☐ **DNA Polymerase I, Large (Klenow) Fragment**
  New England Biolabs, M0210

Dephosphorylation of DNA and RNA

- ☐ **Alkaline phosphatase**
  - **Shrimp Alkaline Phosphatase (rSAP)**
    New England Biolabs, M0371
  - *or* **Antarctic Phosphatase**
    New England Biolabs, M0289
  - *or* **Quick CIP (calf intestinal alkaline phosphatase)**
    New England Biolabs, M0525

Phosphorylation of DNA and RNA

- ☐ **T4 Polynucleotide Kinase**
  New England Biolabs, M0201

Ligation of cohesive DNA overhangs with T7 DNA ligase

- ☐ **T7 DNA Ligase**
  New England Biolabs, M0318

Ligation of cohesive or blunt DNA overhangs with T4 DNA ligase

- ☐ **T4 DNA Ligase**
  New England Biolabs, M0202

Self-circularization of DNA

- ☐ **T4 DNA Ligase**
  New England Biolabs, M0202

Recipes

DNA duplex buffer, 10-fold, pH 7.5

Amount	Ingredient		Stock	Final
9.0 mL	HEPES, pH 7.5	⟋ R0024	1 M	300 mM
2.94 g	Potassium acetate	[127-08-2]		1 M
To 30 mL	Water, reagent-grade			

DNA duplex buffer

300 mM HEPES, 1 M Potassium acetate, 10-fold, pH 7.5



WARNING

Skin and serious eye irritation

- ☐ Dilute below the discharge limit
- ☐ Flush to drain with excess water

Date: Sign: R0016

Resources

Restriction digest of DNA

Buffer name	Brand	Capacity	System	Salt	Additives
NEBuffer 1.1	NEB	10 mM	Bis-Tris-Propane-Cl, pH 7.0	None	100 µg/mL BSA
NEBuffer 2.1	NEB	10 mM	Tris-Cl, pH 7.9	50 mM Na^+	100 µg/mL BSA
NEBuffer 3.1	NEB	50 mM	Tris-Cl, pH 7.9	100 mM Na^+	100 µg/mL BSA
NEBuffer 4	NEB	20 mM	Tris-acetate, pH 7.9	50 mM K^+	1 mM DTT
CutSmart Buffer	NEB	20 mM	Tris-acetate, pH 7.9	50 mM K^+	100 µg/mL BSA
Tango Buffer	Thermo	33 mM	Tris-acetate, pH 8.2	66 mM K^+	100 µg/mL BSA
T7 DNA Ligase Buffer	NEB	66 mM	Tris-Cl, pH 7.6	None	1 mM DTT, 1.0 mM ATP, 7.5% PEG
T4 DNA Ligase Buffer	NEB	50 mM	Tris-Cl, pH 7.5	None	10 mM DTT, 1.0 mM ATP
	Thermo	40 mM	Tris-Cl, pH 7.8	None	10 mM DTT, 0.5 mM ATP

In Step 1: Common restriction enzyme and ligase buffers. Concentrations refer to the 1 × working solution. All buffers contain 10 mM Mg^{2+} with matched counterion. pH values are at 25 °C.

Group	Enzyme	Recognition site	Organism	Buffer	Temperature	Inactivation
(A)	AgeI	5′–A↓ccggT–3′	*Ruegeria gelatinovora*	CS, 1.1	37 °C	65 °C
(B)	BamHI	5′–G↓gatcC–3′	*Bacillus amyloliquefaciens* H	CS, 1.1	37 °C	No
(3)	BglI	5′–GCCNnnn↓NGGC–3′	*Bacillus globigii*	3.1	37 °C	65 °C
(B)	BglII	5′–A↓gatcT–3′	*Bacillus globigii*	3.1	37 °C	No
None	BsiWI	5′–C↓gtacG–3′	*Bacillus* spp.	CS, 2.1	37 °C	80 °C
(4)	BstXI	5′–CCANnnnn↓NTGG–3′	*Bacillus stearothermophilus* X1	3.1	37 °C	80 °C
(C)	BssHII	5′–G↓cgcgC–3′	*Bacillus stearothermophilus* H3	CS, 3.1, 2.1, 1.1	37 °C	65 °C
(D)	EagI	5′–C↓ggccG–3′	*Enterobacter agglomerans*	CS, 3.1, 2.1	37 °C	65 °C
(E)	EcoRI	5′–G↓aattC–3′	*Escherichia coli* RY13	CS, 2.1	37 °C	65 °C
Blunt	EcoRV	5′–GAT↓ATC–3′	*Escherichia coli* J62 pLG74	CS, 3.1, 2.1	37 °C	65 °C
None	HindIII	5′–A↓agctT–3′	*Haemophilus influenzae* Rd	CS, 2.1	37 °C	80 °C
None	KpnI	5′–Ggtac↓C–3′	*Klebsiella pneumoniae* OK8	CS, 1.1	37 °C	No
(E)	MfeI	5′–C↓aattG–3′	*Mycoplasma fermentans*	CS	37 °C	No
(C)	MluI	5′–A↓cgcgT–3′	*Micrococcus luteus*	CS, 3.1, 2.1	37 °C	No
(F)	NheI	5′–G↓ctagC–3′	*Neisseria mucosa heidelbergensis*	CS, 1.1	37 °C	80 °C
(A)	NgoMIV	5′–G↓ccggC–3′	*Neisseria gonorrhoeae* MS11	CS, 1.1	37 °C	No
(D)	NotI	5′–GC↓ggccGC–3′	*Nocardia otitidis-caviarum*	CS, 2.1	37 °C	65 °C
(G)	NsiI	5′–Atgca↓T–3′	*Neisseria sicca*	3.1	37 °C	65 °C
(G)	PstI	5′–Ctgca↓G–3′	*Providencia stuartii* 164	CS	37 °C	No
None	SacI	5′–Gagct↓C–3′	*Streptomyces achromogenes*	CS	37 °C	65 °C
(H)	SalI	5′–G↓tcgaC–3′	*Streptomyces albus* G	CS, 3.1, 2.1	37 °C	65 °C
(G)	SbfI	5′–CCtgca↓GG–3′	*Streptomyces sp.* Bf-61	CS	37 °C	80 °C
(F)	SpeI	5′–A↓ctagT–3′	*Sphaerotilus* spp.	CS	37 °C	80 °C
Blunt	StuI	5′–AGG↓CCT–3′	*Streptomyces tubercidicus*	CS, 2.1	37 °C	No
(F)	XbaI	5′–T↓ctagA–3′	*Xanthomonas badrii*	CS, 2.1	37 °C	65 °C
(H)	XhoI	5′–C↓tcgaG–3′	*Xanthomonas holcicola*	CS, 3.1, 2.1	37 °C	65 °C
(A)	XmaI	5′–C↓ccggG–3′	*Xanthomonas malvacearum*	CS	37 °C	65 °C
Blunt	XmnI	5′–GAANN↓NNTTC–3′	*Xanthomonas manihotis* 7AS1	CS	37 °C	65 °C

In Step 1: Type IIP restriction enzymes recognize palindromic DNA sequences and cut within or near the recognition site, typically generating cohesive ("sticky") or blunt ends. Enzymes are sorted alphabetically and enzymes that produce compatible overhangs marked with the same group. Type IIP enzymes remain the simplest and most widely used tools for classical restriction-ligation cloning.

Group	Enzyme	Recognition site	Organism	Buffer	Temperature	Inactivation
(4)	BbsI	5′–GAAGACNN↓nnnn–3′	*Bacillus brevis*	CS	37 °C	65 °C
(4)	BsaI	5′–GGTCTCN↓nnnn–3′	*Bacillus stearothermophilus* 6-55	CS, 3.1, 2.1, 1.1	37 °C	80 °C
(4)	BsmBI	5′–CGTCTCN↓nnnn–3′	*Bacillus stearothermophilus* B61	3.1	55 °C	80 °C
(1)	BsmI	5′–GAATGcn↓–3′	*Bacillus stearothermophilus* NUB 36	CS, 2.1	65 °C	80 °C
(3)	BspQI	5′–GCTCTTCN↓nnn–3′	*Bacillus sphaericus*	CS, 3.1, 2.1, 1.1	50 °C	80 °C
(4)	PaqCI	5′–CACCTGCNNNN↓nnnn–3′	*Paucibacter aquatile*	CS, 2.1	37 °C	65 °C

In Step 1: Type IIS restriction enzymes recognize asymmetric DNA sequences and cut outside of their recognition site, generating overhangs of defined but variable sequence. Enzymes are sorted alphabetically and grouped by the number of degenerate bases adjacent to the cleavage site. Type IIS enzymes enable modular DNA assembly strategies such as Golden Gate cloning, allowing scarless ligation of multiple fragments in a single reaction.

Ligation of cohesive or blunt DNA overhangs with T4 DNA ligase

Enzyme	Recognition site	After fill-in/Ligation	Recleaved by
AclI	5′–AA↓cgTT–3′	5′–AA↓cgcgTT–3′	MluI
AgeI	5′–A↓ccggT–3′	5′–ACC↓ggccGGT–3′	EagI
BssHII	5′–G↓cgcgC–3′	5′–G↓cgcgCGCGC–3′	**BssHII**
EcoRI	5′–G↓aattC–3′	5′–GAATT↓AATTC–3′	XmnI
HindIII	5′–A↓agctT–3′	5′–AAG↓ctagCTT–3′	NheI
MluI	5′–A↓cgcgT–3′	5′–ACG↓cgcgCGT–3′	BssHII
NgoMIV	5′–G↓ccggC–3′	5′–GCC↓ggccGGC–3′	EagI, **NgoMIV**
NheI	5′–G↓ctagC–3′	5′–G↓ctagCTAGC–3′	**NheI**

In Step 4: Recleavable sites are generated after fill in of 5′ overhangs of a restriction digest and blunt end ligation or after ligation of certain cohesive ends. Select restriction endonucleases shown only.

List of references

Y. An, W. Wu, and A. Lv, *Anal. Biochem.* **402**(2), 203—205 (2010).

K. Bilotti, V. Potapov, J.M. Pryor, A.T. Duckworth, J.L. Keck, and G.J.S. Lohman, *Nucleic Acids Res.* **50**(8), 4647—4658 (2022).

Change log

2024-07-22 **Benjamin C. Buchmuller** Version 1, 2024. Adaptation as SOP, drawing on NEB product technical resources (neb.com) for restriction, end-modification (CIP, SAP, PNK), DpnI digestion, and T4 ligation procedures.

2026-11-02 **Benjamin C. Buchmuller** Version 2. Reaction conditions change: Klenow with its exonuclease as the blunting enzyme, and new enzyme ratios and ligation times. Credits: Thermo Scientific and IDT alongside NEB; New England Biolabs' buffer activity chart for the salt troubleshooting, where the enzymes named need a high-salt buffer rather than tolerating little salt, and its cleavage-close-to-the-end table, where the enzymes named need more flanking sequence rather than less. Also added the phenol hazard and dropped radioactive end labeling.

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