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.

Risk assessment

▷ Wear gloves, safety glasses, lab coat

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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

$c_i\ [\mu\mathrm{M}] \approx 3.2\times10^{-3} \times \boxed{\beta_i}\ \left[\frac{\mathrm{ng}}{\mu\mathrm{L}}\right] / \boxed{n_i}\ \left[\frac{\mathrm{bp}}{\mathrm{kbp}}\right]$

> ***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, 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**	**Large-scale**
			To 20 µL	**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

> ***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 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**	**Small-scale**	**Large-scale**
			To 10 µL	**To 20 µL**	**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

> ***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, XbaI, 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

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

Ingredient	**Stock**	**Final**	**Small-scale**	**Large-scale**
			To 20 µL	**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

> ***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 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**	**Large-scale**
			To 20 µL	**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

> ***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. For DNA sequencing by chain-termination or blunting 5′ overhangs of DNA prior to ligation, the exonuclease-deficient fragment (exo−) is preferred.

> ***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.) 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**	**Large-scale**
			To 20 µL	**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

> ***This is why:*** Selective dephosphorylation of DNA fragments prevents their ligation as T4 DNA ligase requires at least one phosphorylated strand. T7 DNA ligase requires both strands to be phosphorylated. 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.

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

> ***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. For end labeling with $[\gamma\text{-}^{32}\text{P}]$-ATP, use T4 PNK reaction buffer. 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*

(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.

> ***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 $MgCl_2$.

(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.

> ***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.

> ***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.

[AWL10]

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**	**Large-scale**
			To 5 µL	**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

> ***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.

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

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

(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.

[BPP+22]

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.

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 $\Delta G < -5\,\mathrm{kcal/mol}$ 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. High salt concentrations in column eluates can inhibit restriction enzymes, especially those with low salt tolerance such as BglI, BglII, BsiWI, BsmBI, BstXI.
- 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.
  - Some enzymes such as EcoRI, HindIII, NdeI, NotI, SacI, SpeI, XbaI, XhoI require at least 3 bp distance from DNA fragment ends for efficient cleavage.
- 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 (or radioactive end labeling) of DNA and RNA

In Step 2:

- Insufficient phosphorylation or end labeling
  - 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 label
  - 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 2:

- No ligation product or very low efficiency
  - Verify that overhangs are cohesive and phosphorylated. T7 ligase does not work on blunt ends or unphosphorylated DNA.
  - For cohesive ends shorter than 3 bp, increase PEG concentration to 20–30%. Higher PEG concentrations reduce ligation fidelity.
  - 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 2:

- 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. 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 90% ligation fidelity compared to 70% 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.

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	10% BSA
NEBuffer 2.1	NEB	10 mM	Tris-Cl, pH 7.9	50 mM Na^+	10% BSA
NEBuffer 3.1	NEB	50 mM	Tris-Cl, pH 7.9	100 mM Na^+	10% BSA
NEBuffer 4	NEB	20 mM	Tris-acetate, pH 7.9	50 mM K^+	10% BSA, 1 mM DTT
CutSmart Buffer	NEB	20 mM	Tris-acetate, pH 7.9	50 mM K^+	10% BSA
Tango Buffer	Thermo	33 mM	Tris-acetate, pH 8.2	66 mM K^+	10% 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	A↓ccggT	*Ruegeria gelatinovora*	CS, 1.1	37 °C	65 °C
(B)	BamHI	G↓gatcC	*Bacillus amyloliquefaciens* H	CS, 1.1	37 °C	No
(3)	BglI	GCCNnnn↓NGGC	*Bacillus globigii*	3.1	37 °C	65 °C
(B)	BglII	A↓gatcT	*Bacillus globigii*	3.1	37 °C	No
None	BsiWI	C↓gtacG	*Bacillus* spp.	CS, 2.1	37 °C	80 °C
(4)	BstXI	CCANnnnn↓NTGG	*Bacillus stearothermophilus* X1	3.1	37 °C	80 °C
(C)	BssHII	G↓cgcgC	*Bacillus stearothermophilus* H3	CS, 3.1, 2.1, 1.1	37 °C	65 °C
(D)	EagI	C↓ggccG	*Enterobacter agglomerans*	CS, 3.1, 2.1	37 °C	65 °C
(E)	EcoRI	G↓aattC	*Escherichia coli* RY13	CS, 2.1	37 °C	65 °C
Blunt	EcoRV	GAT↓ATC	*Escherichia coli* J62 pLG74	CS, 3.1, 2.1	37 °C	65 °C
None	HindIII	A↓agctT	*Haemophilus influenzae* Rd	CS, 2.1	37 °C	80 °C
None	KpnI	Ggtac↓C	*Klebsiella pneumoniae* OK8	CS, 1.1	37 °C	No
(E)	MfeI	C↓aattG	*Mycoplasma fermentans*	CS, 1.1	37 °C	No
(C)	MluI	A↓cgcgT	*Micrococcus luteus*	CS, 3.1, 2.1, 1.1	37 °C	No
(F)	NheI	G↓ctagC	*Neisseria mucosa heidelbergensis*	CS, 1.1	37 °C	80 °C
(A)	NgoMIV	G↓ccggC	*Neisseria gonorrhoeae* MS11	CS, 1.1	37 °C	No
(D)	NotI	GC↓ggccGC	*Nocardia otitidis-caviarum*	CS, 2.1	37 °C	65 °C
(G)	NsiI	Atgca↓T	*Neisseria sicca*	3.1	37 °C	65 °C
(G)	PstI	Ctgca↓G	*Providencia stuartii* 164	CS	37 °C	No
None	SacI	Gagct↓C	*Streptomyces achromogenes*	CS	37 °C	65 °C
(H)	SalI	G↓tcgaC	*Streptomyces albus* G	CS, 3.1, 2.1	37 °C	65 °C
(G)	SbfI	CCtgca↓GG	*Streptomyces sp.* Bf-61	CS	37 °C	80 °C
(F)	SpeI	A↓ctagT	*Sphaerotilus* spp.	CS	37 °C	80 °C
Blunt	StuI	AGG↓CCT	*Streptomyces tubercidicus*	CS, 2.1	37 °C	No
(F)	XbaI	T↓ctagA	*Xanthomonas badrii*	CS, 3.1, 2.1	37 °C	65 °C
(H)	XhoI	C↓tcgaG	*Xanthomonas holcicola*	CS, 3.1, 2.1	37 °C	65 °C
(A)	XmaI	C↓ccggG	*Xanthomonas malvacearum*	CS	37 °C	65 °C
Blunt	XmnI	GAANN↓NNTTC	*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	GAAGACNN↓nnnn	*Bacillus brevis*	CS	37 °C	65 °C
(4)	BsaI	GGTCTCN↓nnnn	*Bacillus stearothermophilus* 6-55	CS, 3.1, 2.1, 1.1	37 °C	80 °C
(4)	BsmBI	CGTCTCN↓nnnn	*Bacillus stearothermophilus* B61	3.1	55 °C	80 °C
(1)	BsmI	GAATGcn↓	*Bacillus stearothermophilus* NUB 36	CS, 2.1	65 °C	80 °C
(3)	BspQI	GCTCTTCN↓nnn	*Bacillus sphaericus*	CS, 3.1, 2.1, 1.1	50 °C	80 °C
(4)	PaqCI	CACCTGCNNNN↓nnnn	*Bacillus stearothermophilus* B61	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	`AA↓cgTT`	`AA↓cgcgTT`	MluI
AgeI	`A↓ccggT`	`ACC↓ggccGGT`	EagI
BssHII	`G↓cgcgC`	`G↓cgcgCGCGC`	**BssHII**
EcoRI	`G↓aattC`	`GAATT↓AATTC`	XmnI
HindIII	`A↓agctT`	`AAG↓ctagCTT`	NheI
MluI	`A↓cgcgT`	`ACG↓cgcgCGT`	BssHII
NgoMIV	`G↓ccggC`	`GCC↓ggccGGC`	EagI, **NgoMIV**
NheI	`G↓ctagC`	`G↓ctagCTAGC`	**NheI**

In Step 2: 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

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Change log

2010-09-21	Nick Coleman	Original protocol.
2024-07-22	Benjamin C. Buchmuller	Additions. Adaptation as SOP.