

- PCR: Polymerase Chain Reaction
 - DNA prepared for cloning (copy using PCR)
 - Plasmid vector is prepared in linear form using PCR
 - Plasmid — piece of DNA replicated in the host. Exists separately from host's chromosomal/genomic DNA
- PCR Cloning

Insert Preparation

Steps	PCR Cloning	Seamless Cloning	Ligation Independent Cloning (LIC)
DNA Preparation	PCR 90 mins	PCR 90 mins	PCR 90 mins
DNA end modification	Clean up 15 mins	Clean up 15 mins	Clean up 15 mins
	Phosphorylation (optional) 30 mins	Cohesive-end formation by 5' → 3' exo 30 mins	Cohesive-end formation by 3' → 5' exo 30 mins
Vector & insert joining	Ligation 15 mins	Ligation Same time as previous step	Annealing 30 mins

Vector Preparation

Steps	PCR
DNA Preparation	PCR 2 hrs
	Clean up 15 mins
DNA end modification	T-addition (optional) 1.5 hrs

- NEB PCR Cloning Kit
 - Toxic minigene generated when vector closes on itself → direct cloning from reaction, no purification step

Ligation	Mix insert w/ 1 μ l linearized vector; bring to 5 μ l w/ H ₂ O
	↓
	Add 4 μ l cloning mix 1 & 1 μ l cloning mix 2
	↓ Incubate at room temp for 5-15 mins
	↓ Incubate on ice for 2 mins
Transformation	Add 2 μ l of completed ligation reaction to 50 μ l competent cells
	↓ Incubate on ice for 20 mins
	Heat shock at 42°C for 30 secs
	↓ Incubate on ice for 5 mins
	Add 950 μ l of NEB 10-beta/Stable Outgrowth Medium for outgrowth
	↓ Incubate at 37°C for 1 hr
Plating	Spread 50 μ l and μ l of a 1:10 dilution on ampicillin selection plate; incubate at 37°C overnight

- PCR/Amplification
 - Amplification — often 1st step for PCR cloning, seamless cloning, ligation independent cloning, recombinational cloning
 - Routine PCR with One *Taq*

	Cycles	Temperature	Time
Initial denaturation:	1	94°C	30 secs
Denaturation	30	94°C	15-30 secs
Annealing		45-68°C	15-60 secs
Extension		68°C	1 min per kb
Final Extension:	1	68°C	5 mins
Hold:	1	4-10°C	

- Optimization!!!
 - DNA Template
 - High-quality, purified DNA templates

- Low-complexity templates: use 1pg–10ng of DNA per 50µl reaction
 - Higher DNA concentrations – decrease amplicon specificity, esp. For high # of cycles
 - Primers
 - 20-40 nucleotides in length, 40-60% GC content
 - Primer pairs should have T_m values within 5°C
 - Avoid secondary structure within each primer & potential dimerization between primers
 - Concentrations higher than recommended can decrease specificity
 - Magnesium concentration
 - Most NEB PCR buffers already contain enough levels of Mg⁺⁺ at 1X concentrations
 - Excess Mg⁺⁺ can lead to fake amplification
 - Insufficient Mg⁺⁺ can cause reaction failure
 - Deoxynucleotides
 - Ideal concentration: 200 µM each
 - Starting reactions
 - Assemble all reaction components on ice (unless using hot start enzyme)
 - Add polymerase last
 - Move reactions to a pre-heated (denaturation temp.) thermocycler
 - Pre-heating not necessary when using hot start enzyme
 - Denaturation
 - Avoid longer/higher temp. incubations unless required (high GC content of template)
 - Extension
 - Extension rates are generally from 15-60 s/kb
 - Longer than recommended extension times → higher error rates, spurious banding patterns, reduction of amplicon yields
- Annealing temp
 - Use T_m calculator/NEBaseChanger to determine recommended annealing temp.
 - Non-specific product formation can be avoided by optimizing annealing temp./switching to hot start enzyme
 - Annealing temp. (T_a)
 - Temp. where primers successfully bind to DNA
 - Lower than T_m
- Primer
 - 20-40 nucleotides in length, 40-60% GC content
 - Primer pairs should have T_m values within 5°C
 - Avoid secondary structure within each primer & potential dimerization between primers
 - Concentrations higher than recommended can decrease specificity
 - Melting Temp. (T_m)
 - Temp. where primers fall off from DNA

- Tm & Ta
 - $Ta = 9.3 * Tm \text{ (primer)} + 0.7 * Tm \text{ (product)} - 14.9$

[https://www.researchgate.net/post/What is the relationship between the Tm of primers and annealing temperature#:~:text=Melting%20temperature%20of%20Primer%20\(Tm.identifying%20by%20the%20following%20method.](https://www.researchgate.net/post/What_is_the_relationship_between_the_Tm_of_primers_and_annealing_temperature#:~:text=Melting%20temperature%20of%20Primer%20(Tm.identifying%20by%20the%20following%20method.)