

I. PCR amplification and purification of DNA insert

1. For each plasmid containing the gene to be cloned, set up two reactions on ice:

a. Reaction #1:

10 μ l	5 \times PCR buffer for NEB Pfusion polymerase with 1.5 mM MgSO ₄ (final conc.)
1 μ l	10 mM dNTPs (200 μ M final)
0.3 μ l	50 μ M forward primer
0.3 μ l	50 μ M reverse primer
0.4 μ l	25 ng/ml plasmid template (10 ng; scale volume if concentration is different)
0.5 μ l	Pfusion polymerase (NEB M0530L, add last)
37.5 μ l ddH ₂ O	
50 μ l total volume	

b. Reaction #2:

10 μ l	5 \times PCR buffer for NEB Pfusion polymerase with 1.5 mM MgSO ₄ (final conc.)
20 μ l	5 M betaine (2 M final, Sigma #B0300)
1 μ l	10 mM dNTPs (200 μ M final)
0.3 μ l	50 μ M forward primer
0.3 μ l	50 μ M reverse primer
0.4 μ l	25 ng/ml plasmid template (10 ng; scale volume if concentration is different)
0.5 μ l	Pfusion polymerase (NEB M0530L, add last)
17.5 μ l ddH ₂ O	
50 μ l total volume	

- *Pfusion is a high-fidelity polymerase that is very competitively priced*
- *Betaine relaxes secondary structure in the primers and template and can “rescue” amplifications that fail under Reaction #1 (The 5 \times GC-RICH buffer that comes with the polymerase is probably 5 M betaine). DMSO is also supplied with the enzyme for additional optimization. See www.neb.com/protocols/2012/06/01/guidelines-for-pcr-optimization-with-phusion-high-fidelity-dna-polymerase for further recommendations for optimization.*
- *The specifications for the Pfusion HF polymerase states that this enzyme will handle long range PCR targets, typically up to 20kb but the protocol may be optimized to 30kb. An alternative polymerase for longer amplicons would be Expand High Fidelity polymerase (Roche #11732650001)*

2. Run the PCR reactions on the Roche recommended cycling parameters (“Roche rec” protocol on the MyCyclers):

- 94°C denaturation, 2 min
- 10 cycles of 94°C denaturation (15 sec), 55°C (or 5°C below primer T_m) annealing (30 sec), and 72°C extension (1 min per kb amplicon) note that the extension time must be changed on the protocol
- 20 cycles of 94°C denaturation (15 sec), 55°C (or 5°C below primer T_m) annealing (30 sec), and 72°C extension (1 min per kb amplicon, with 5 sec increase at each cycle) note that the extension time must be changed on the protocol
- Final elongation at 72°C (7 min)
- Indefinite hold at 4°C

3. Electrophorese PCR reactions on an appropriate percentage agarose gel.

4. Excise the amplicon band and purify with the PureLink Quick Gel Extraction Kit (Invitrogen #K2100-25) according to the manufacturer’s recommendation, except use 40 μ l elution buffer at the final elution off the column.

- *Excise the band on the Lucite gel holder with a clean razor blade; do not cut directly on the transilluminator*
- *Be sure to wear a face shield when cutting bands*

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- *Reducing the volume allows the purified amplicon to be digested directly without concentrating it*
5. Add 5 μl 10 \times restriction-enzyme buffer, 2.5 μl of each restriction enzyme, and 0.5 μl of 100 \times BSA and digest at 37 $^{\circ}\text{C}$ for 3 hr.
 - *This amplicon digestion can be performed together with the vector digestion described in Section II Step #1*
 - *Some restriction enzymes have difficulty cutting at the ends of linearized DNA, which is why the longer digest time is preferred*
 - *Directional cloning with two restriction enzymes is preferred over non-directional cloning with one enzyme*
 - *Make sure that the restriction-enzyme buffer is appropriate for the double digest (check the NEB table).*
 - *The new 1.1, 2.1, 3.1, 4.1, and CutSmart buffers that come with NEB restriction enzymes include the BSA. The new buffers can be used with older lots of enzyme.*
 - *Adding more enzyme will increase star activity and hurt the specificity of the digest.*
6. Add 150 μl H₂O, then 200 μl phenol-chloroform in a fume hood. Vortex thoroughly and spin at max speed on a benchtop centrifuge for 1 min.
 - *This step replaces the need to gel purify the insert following PCR amplification.*
7. Transfer 180 μl of the aqueous (top) fraction to a new tube, add 20 μl of 3 M NaOAc (pH 5.2), and 1 μl 20 mg/ml glycogen (Invitrogen #10814-010). Vortex.
8. Add 500 μl ice-cold 100% EtOH, vortex, and incubate at –20 $^{\circ}\text{C}$ for at least 30 min.
 - *Keep EtOH stock at –20 $^{\circ}\text{C}$ to speed the precipitation to completion*
 - *Longer incubations at –20 $^{\circ}\text{C}$ are fine*
9. Spin for 10 min at max speed on a benchtop centrifuge.
10. Carefully aspirate supernatant and wash pellet with 500 μl 70% EtOH at room temperature.
11. Spin for 1 min at max speed on a benchtop centrifuge.
12. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip.
13. Air dry pellets for 5–10 min at room temperature.
 - *The pellet has dried when the color has changed from opaque white to hazy and clear*
 - *Overdrying will make the pellet difficult to dissolve afterwards*
14. Resuspend in 5 μl EB and incubate for 15 min at 37 $^{\circ}\text{C}$ to redissolve.
15. Measure DNA concentration by spectrophotometry on a Nanodrop.

II. Vector digestion and purification

1. Set up two 50 μl restriction digests, each containing 2 μg vector, 5 μl 10 \times restriction-digest buffer, 2.5 μl of each restriction enzyme, and 0.5 μl of 100 \times BSA. Digest at 37 $^{\circ}\text{C}$ for 2.5 hr.
 - *More vector (by mass) is needed for the ligation than the insert, so two digests are needed*
 - *This vector digestion can be performed together with the amplicon digestion described in Section I Step #5*
 - *Directional cloning with two restriction enzymes is preferred over non-directional cloning with one enzyme*
 - *Make sure that the restriction-enzyme buffer is appropriate for the double digest (check the NEB table)*
2. Add 1 μl calf intestinal phosphatase (Invitrogen #18009-027) and dephosphorylate at 37 $^{\circ}\text{C}$ for 30 min.
 - *Dephosphorylation decreases the likelihood of recircularization during ligation*
3. Electrophorese the digested vectors on a 0.8% agarose gel. Include 1 μg of undigested vector in an adjacent lane as a control.
 - *It is important to make sure the linearized vector is clearly separated from all bands visible on the undigested control*
 - *This separation step dramatically increases the number of positive clones after ligation*
4. Excise bands corresponding to the linearized vector and purify with the PureLink Quick Gel Extraction Kit (Invitrogen #K2100-25) according to the manufacturer's recommendation.

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- *Excise the band on the Lucite gel holder with a clean razor blade; do not cut directly on the transilluminator*
 - *Be sure to wear a face shield when cutting bands*
5. Pool the eluate from the two purified bands and add 80 μ l H₂O, 20 μ l of 3 M NaOAc (pH 5.2), and 1 μ l 20 mg/ml glycogen (Invitrogen #10814-010). Vortex.
 6. Add 500 μ l ice-cold 100% EtOH, vortex, and incubate at -20°C for at least 30 min.
 - *Keep EtOH stock at -20°C to speed the precipitation to completion*
 - *Longer incubations at -20°C are fine*
 7. Spin for 10 min at max speed on a benchtop centrifuge.
 8. Carefully aspirate supernatant and wash pellet with 500 μ l 70% EtOH at room temperature.
 9. Spin for 1 min at max speed on a benchtop centrifuge.
 10. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip.
 11. Air dry pellets for 5–10 min at room temperature.
 - *The pellet has dried when the color has changed from opaque white to hazy and clear*
 - *Overdrying will make the pellet difficult to dissolve afterwards*
 12. Resuspend in 5 μ l EB and incubate for 15 min at 37°C to redissolve.
 13. Measure DNA concentration by spectrophotometry on a Nanodrop.

III. Vector-insert ligation

1. Set up a 10 μ l ligation reaction containing 3:1 molar ratio of insert to vector, 1 μ l 10 \times T4 ligation buffer, and 0.5 μ l T4 DNA ligase (New England Biolabs #M0202S).
 - *Be sure that the ligation buffer has thawed completely (no visible precipitates) and is kept at room temperature before use*
 - *0.1 – 1 μ M of 5' termini are recommended by the manufacturer*
 - *Use high concentration enzyme (NEB M0202T HC) for blunt end or difficult ligations.*
 - *Note this reaction volume is half that recommended by the manufacturer*
 - *Be sure to include a control ligation that includes everything but the insert*
 - *Save the leftover digested insert for future ligations into other vectors*
2. Mix by pipetting gently and incubate at room temperature for 1 hr.
3. Add 80 μ l H₂O, 10 μ l of 3 M NaOAc (pH 5.2), and 1 μ l 20 mg/ml glycogen (Invitrogen #10814-010). Vortex.
4. Add 250 μ l ice-cold 100% EtOH, vortex, and incubate at -20°C for at least 30 min.
 - *Keep EtOH stock at -20°C to speed the precipitation to completion*
 - *Longer incubations at -20°C are fine*
5. Spin for 10 min at max speed on a benchtop centrifuge.
6. Carefully aspirate supernatant and wash pellet with 500 μ l 70% EtOH at room temperature.
7. Spin for 1 min at max speed on a benchtop centrifuge.
8. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip.
9. Air dry pellets for 5–10 min at room temperature.
 - *The pellet has dried when the color has changed from opaque white to hazy and clear*
 - *Overdrying will make the pellet difficult to dissolve afterwards*
10. Resuspend in 5 μ l H₂O and incubate for 15 min at 37°C to redissolve.
 - *Do not resuspend in EB at this point, because the purpose of the ethanol precipitation here is to remove salts so that more DNA can be added during the transformation*
11. Proceed to Section IV or store at -20°C .

IV. Transformation into electrocompetent bacteria

1. Thaw homemade electrocompetent bacteria on ice. Label the appropriate number of electroporation cuvettes (Biorad #165-2083) and place on ice.
2. Add 1 μ l of the undiluted ethanol-precipitated ligation to a fresh microcentrifuge tube on ice.

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- *The ethanol precipitation in Section III allows 50+-fold more DNA to be added to the transformation without arcing*
3. Add 20 μ l of the bacteria to the ligation and mix gently by pipetting.
4. Transfer the bacteria to an electroporation cuvette with a gel-loading pipet tip.
 - *Add the bacteria to the very base of the cuvette and then tap the sides so that the bacteria uniformly coat the base of both electroporation plates*
 - *Avoid bubbles when dispensing the bacteria*
5. Electroporate bacteria with the following settings: 1.8 kV, 200 Ω , 25 μ F.
 - *Our electrocompetent bacteria are optimized for these default settings on the Biorad GenePulser*
6. Add 0.5 ml SOC medium immediately after electroporation and transfer bacteria to a 15 ml Falcon tube
7. Incubate tubes in the shaking incubator at 37°C for 1 hr.
 - *The 1 hr incubation is optional for ampicillin resistant plasmids but is absolutely critical for ribosome-targeted antibiotics (e.g., gentamycin)*
8. Streak 200 μ l of each ligation on an LB/Amp, LB/gent, etc. plate and incubate at 37°C overnight.
 - *For difficult ligations, streak more than one plate*
 - *Control ligations only need to be streaked once to check for background colonies*
9. Inspect clones and screen by miniprep, diagnostic digest, and then sequencing.
 - *There should be many colonies on the ligation plate and none on the control; if so, only 2–3 colonies need to be screened by miniprep, because most or all will contain the insert*
 - *High levels of background colonies on the control plate indicates that there is a problem with the vector (not completely cut, self-ligated, etc.); it may still be possible to get positive clones, but 5–10 should be screened by miniprep*