Troubleshooting Guide for Cloning

We strongly recommend running the following controls during transformations. These controls may help troubleshoot which step(s) in the cloning workflow has failed.

1. Transform 100 pg–1ng of uncut vector to check cell viability, calculate transformation efficiency and verify the antibiotic resistance of the plasmid.
2. Transform the cut vector to determine the amount of background due to undigested plasmid. The number of colonies in this control should be <1% of the number of colonies in the uncut plasmid control transformation (from control #1).
3. Transform a vector only ligation reaction. The ends of the vector should not be able to re-ligate because either they are incompatible (e.g., digested with two restriction enzymes that do not generate compatible ends) or the 5´ phosphate group has been removed in a dephosphorylation reaction (e.g., blunt ends treated with rSAP). This control transformation should yield the same number of colonies as control #2.
4. Digest vector DNA with a single restriction enzyme, re-ligate and transform. The ends of the vector DNA should be compatible and easily joined during the ligation reaction, resulting in approximately the same number of colonies as control #1.

The cloning workflow often benefits from an accurate quantitation of the amount of DNAs that are being worked with. We recommend quantification of DNAs whenever possible.

Problem	Cause	Solution
Few or no transformants	Cells are not viable	▪ Transform an uncut plasmid (e.g., pUC19) and calculate the transformation efficiency of the competent cells. If the transformation efficiency is low (<10^4) re-make the competent cells or consider using commercially available high efficiency competent cells.
	Incorrect antibiotic or antibiotic concentration	▪ Confirm antibiotic and antibiotic concentration
	DNA fragment of interest is toxic to the cells	▪ Incubate plates at lower temperature (25 – 30°C). ▪ Transformation may need to be carried out using a strain that exerts tighter transcriptional control over the DNA fragment of interest (e.g., NEB-5-alpha F´ I^q Competent *E. coli* (NEB #C2992))
	If using chemically competent cells, the wrong heat-shock protocol was used	▪ Follow the manufacturer's specific transformation protocol (Note: going above the recommended temperature during the heat shock can result in competent cell death)
	If using electrocompetent cells, PEG is present in the ligation mix	▪ Clean up DNA by drop dialysis prior to transformation ▪ Try NEB's ElectroLigase (NEB #M0369)
	If using electrocompetent cells, arcing was observed or no voltage was registered	▪ Clean up the DNA prior to the ligation step ▪ Tap the cuvette to get rid of any trapped air bubbles ▪ Be sure to follow the manufacturer's specified electroporation parameters
	Construct is too large	▪ Select a competent cell strain that can be transformed efficiently with large DNA constructs (≥10 kb, we recommend trying NEB 10-beta Competent *E. coli* (NEB #C3019)) ▪ For very large constructs (>10 kb), consider using electroporation
	Construct may be susceptible to recombination	▪ Select a Rec A- strain such as NEB 5-alpha (NEB #C2987) or NEB 10-beta Competent *E. coli* (NEB #C3019)
	The insert comes directly from mammalian or plant DNA and contains methylated cytosines, which are degraded by many *E. coli* strains	▪ Use a strain that is deficient in McrA, McrBC and Mrr, such as NEB 10-beta Competent *E. coli* (NEB #C3019)
	Too much ligation mixture was used	▪ Use < 5 µl of the ligation reaction for the transformation

Problem	Cause	Solution
	Inefficient ligation	▪ Make sure that at least one fragment being ligated contains a 5´ phosphate moiety ▪ Vary the molar ratio of vector to insert from 1:1 to 1:10 (1:20 for short adaptors). Use NEBioCalculator to calculate molar ratios. ▪ Purify the DNA to remove contaminants such as salt and EDTA ▪ ATP will degrade after multiple freeze-thaws; repeat the ligation with fresh buffer ▪ Heat inactivate or remove the phosphatase prior to ligation ▪ Ligation of single base-pair overhangs (most difficult) may benefit from being carried out with Blunt/TA Master Mix (NEB #M0367), Quick Ligation Kit (NEB #M2200) or concentrated T4 DNA Ligase (NEB #M0202) ▪ Test the activity of the ligase by carrying out a ligation control with Lambda-HindIII digested DNA (NEB #N3012)
	Inefficient phosphorylation	▪ Purify the DNA prior to phosphorylation. Excess salt, phosphate or ammonium ions may inhibit the kinase. ▪ If the ends are blunt or 5´ recessed, heat the substrate/buffer mixture for 10 minutes at 70°C. Rapidly chill on ice before adding the ATP and enzyme, then incubate at 37°C. ▪ ATP was not added. Supplement the reaction with 1mM ATP, as it is required by T4 Polynucleotide Kinase (NEB #M0201). ▪ Alternatively, use 1X T4 DNA Ligase Reaction Buffer (NEB #B0202) (contains 1 mM ATP) instead of the 1X T4 PNK Buffer
	Inefficient blunting	▪ Heat inactivate or remove the restriction enzymes prior to blunting ▪ Clean up the PCR fragment prior to blunting ▪ Sonicated gDNA should be blunted for at least 30 minutes ▪ Do not use > 1 unit of enzyme/µg of DNA ▪ Do not incubate for > 15 minutes ▪ Do not incubate at temperatures > 12°C (for T4 DNA Polymerase, NEB #M0203) or > 24°C (for Klenow, NEB #M0210) ▪ Make sure to add a sufficient amount of dNTPs to the reaction (33 µM each dNTP for DNA Polymerase I, Large (Klenow) Fragment, NEB #M0210 and 100 µM each dNTP for T4 DNA Polymerase, NEB #M0203). ▪ When using Mung Bean Nuclease (NEB #M0250), incubate the reaction at room temperature. Do not use > 1 unit of enzyme/µg DNA or incubate the reaction > 30 minutes.
	Inefficient A-Tailing	▪ Clean up the PCR prior to A-tailing. High-fidelity enzymes will remove any non-templated nucleotides.
	Restriction enzyme(s) didn't cleave completely	▪ Check the methylation sensitivity of the enzyme(s) to determine if the enzyme is blocked by methylation of the recognition sequence ▪ Use the recommended buffer supplied with the restriction enzyme ▪ Clean up the DNA to remove any contaminants that may inhibit the enzyme ▪ When digesting a PCR fragment, make sure to have at least 6 nucleotides between the recognition site and the end of the DNA molecule
Colonies don't contain a plasmid	Antibiotic level used was too low	▪ Increase the antibiotic level on plates to the recommended amount ▪ Use fresh plates with fresh antibiotics
	Satellite colonies were selected	▪ Choose large, well-established colonies for analysis
Colonies contain the wrong construct	Recombination of the plasmid has occurred	▪ Use a RecA⁻ strain such NEB 5-alpha (NEB #C2987) or NEB 10-beta Competent *E. coli* (NEB #C3019)
	Incorrect PCR amplicon was used during cloning	▪ Optimize the PCR conditions ▪ Gel purify the correct PCR fragment
	Internal recognition site was present	▪ Use NEBcutter® to analyze insert sequence for presence of an internal recognition site
	DNA fragment of interest is toxic to the cells	▪ Incubate plates at lower temperature (25 – 30°C) ▪ Transformation may need to be carried out using a strain that exerts tighter transcriptional control of the DNA fragment of interest (e.g., NEB 5-alpha F´ I^q Competent *E. coli*) (NEB #C2992)
	Mutations are present in the sequence	▪ Use a high-fidelity polymerase (e.g., Q5 High-Fidelity DNA Polymerase, NEB #M0491) ▪ Re-run sequencing reactions

Problem	Cause	Solution
Too much background	Inefficient dephosphorylation	▪ Heat inactivate or remove the restriction enzymes prior to dephosphorylation
	Kinase is present/active	▪ Heat inactivate the kinase after the phosphorylation step. Active kinase will re-phosphorylate the dephosphorylated vector.
	Restriction enzyme(s) didn't cleave completely	▪ Check the methylation sensitivity of the restriction enzyme(s) to be sure it is not inhibited by methylation of the recognition sequence ▪ Use the recommended buffer supplied with the restriction enzyme ▪ Clean up the DNA to remove contaminants. (e.g., too much salt)
	Antibiotic level is too low	▪ Confirm the correct antibiotic concentration
Ran the ligation on a gel and saw no ligated product	Inefficient ligation	▪ Make sure at least one DNA fragment being ligated contains a 5´ phosphate ▪ Vary the molar ratios of vector to insert from 1:1 to 1:10 (1:20 for short adaptors). Use NEBioCalculator to calculate molar ratios. ▪ Purify the DNA to remove contaminants such as salt and EDTA ▪ ATP will degrade after multiple freeze-thaws; repeat the ligation with fresh buffer ▪ Heat inactivate or remove the phosphatase prior to ligation ▪ Ligation of single base-pair overhangs (most difficult) may benefit from being carried out with Blunt/TA Master Mix (NEB #M0367), Quick Ligation Kit (NEB #M2200) or concentrated T4 DNA Ligase (NEB #M0202) ▪ Test the activity of the ligase by carrying out a ligation control with Lambda-HindIII digested DNA (NEB #N3012)
The ligated DNA ran as a smear on an agarose gel	The ligase is bound to the substrate DNA	▪ Treat the ligation reaction with Proteinase K (NEB #P8107) prior to running on a gel
The digested DNA ran as a smear on an agarose gel	The restriction enzyme(s) is bound to the substrate DNA	▪ Lower the number of units ▪ Add SDS (0.1 – 0.5%) to the loading buffer to dissociate the enzyme from the DNA
	Nuclease contamination	▪ Use fresh, clean running buffer ▪ Use a fresh agarose gel ▪ Clean up the DNA
Incomplete restriction enzyme digestion	Cleavage is blocked by methylation	▪ DNA isolated from a bacterial source may be blocked by Dam and Dcm methylation ▪ DNA isolated from eukaryotic source may be blocked by CpG methylation ▪ Check the methylation sensitivity of the enzyme(s) to determine if the enzyme is blocked by methylation of the recognition sequence ▪ If the enzyme is inhibited by Dam or Dcm methylation, grow the plasmid in a *dam-/dcm-* strain (NEB #C2925)
	Salt inhibition	▪ Enzymes that have low activity in salt-containing buffers (NEBuffer 3.1) may be salt sensitive, so clean up the DNA prior to digestion ▪ DNA purification procedures that use spin columns can result in high salt levels, which inhibit enzyme activity. To prevent this, DNA solution should be no more than 25% of total reaction volume.
	Inhibition by PCR components	▪ Clean up the PCR fragment prior to restriction digest
	Using the wrong buffer	▪ Use the recommended buffer supplied with the restriction enzyme
	Too few units of enzyme used	▪ Use at least 3 – 5 units of enzyme per µg of DNA
	Incubation time was too short	▪ Increase the incubation time
	Digesting supercoiled DNA	▪ Some enzymes have a lower activity on supercolied DNA. Increase the number of enzyme units in the reaction.
	Presence of slow sites	▪ Some enzymes can exhibit slower cleavage towards specific sites. Increase the incubation time, 1-2 hours is typically sufficient.
	Two sites required	▪ Some enzymes require the presence of two recognition sites to cut efficiently
	DNA is contaminated with an inhibitor	▪ Assay substrate DNA in the presence of a control DNA. Control DNA will not cleave if there is an inhibitor present. Mini prep DNA is particularly susceptible to contaminants.

Problem	Cause	Solution
		▪ Clean DNA with a spin column, resin or drop dialysis, or increase volume to dilute contaminant
Extra bands in the gel	If larger bands than expected are seen in the gel, this may indicate binding of the enzyme(s) to the substrate	▪ Lower the number of units in the reaction ▪ Add SDS (0.1 – 0.5%) to the loading buffer to dissociate the enzyme from the substrate
	Star activity	▪ Use the recommended buffer supplied with the restriction enzyme ▪ Decrease the number of enzyme units in the reaction ▪ Make sure the amount of enzyme added does not exceed 10% of the total reaction volume. This ensures that the total glycerol concentration does not exceed 5% v/v ▪ Decrease the incubation time. Using the minimum reaction time required for complete digestion will help prevent star activity. ▪ Try using a High-Fidelity (HF) restriction enzyme. HF enzymes have been engineered for reduced star activity.
	Partial restriction enzyme digest	▪ Enzymes that have low activity in salt-containing buffers (e.g., NEBuffer 3.1) may be salt sensitive. Make sure to clean up the DNA prior to digestion. ▪ DNA purification procedures that use spin columns can result in high salt levels, which inhibit enzyme activity. To prevent this, DNA solution should be no more than 25% of total reaction volume ▪ Clean-up the PCR fragment prior to restriction digest ▪ Use the recommended buffer supplied with the restriction enzyme ▪ Use at least 3 – 5 units of enzyme
No PCR fragment amplified	Used the wrong primer sequence	▪ Double check the primer sequence
	Incorrect annealing temperature	▪ Use the NEB Tm calculator to determine the correct annealing temperature
	Incorrect extension temperature	▪ Each polymerase type has a different extension temperature requirement. Follow the manufacturer's recommendations.
	Too few units of polymerase	▪ Use the recommended number of polymerase units based on the reaction volume
	Incorrect primer concentration	▪ Each polymerase has a different primer concentration requirement. Make sure to follow the manufacturer's recommendations.
	Mg^{2+} levels in the reaction are not optimal	▪ Titrate the Mg^{2+} levels to optimize the amplification reaction. Follow the manufacturer's recommendations.
	Difficult template	▪ With difficult templates, try different polymerases and/or buffer combinations
The PCR reaction is a smear on a gel	If bands are larger than expected it may indicate binding of the enzyme(s) to the DNA	▪ Add SDS (0.1 – 0.5%) to the loading buffer to dissociate the enzyme from the DNA
Extra bands in PCR reaction	Annealing temperature is too low	▪ Use the NEB Tm calculator to determine the annealing temperature of the primers
	Mg^{2+} levels in the reaction are not optimal	▪ Titrate the Mg^{2+} levels to optimize the amplification reaction. Follow the manufacturer's recommendations.
	Additional priming sites are present	▪ Double check the primer sequence and confirm it does not bind elsewhere in the DNA template
	Formation of primer dimers	▪ Primer sequence may not be optimal. Additional primers may need to be tested in the reaction.
	Incorrect polymerase choice	▪ Try different polymerases and/or buffer combinations