Ligation of DNA for subcloning

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1. Purify insert and vector. Digest vector and insert DNA to completion. In most cases, use a five to ten-fold excess of enzyme. Since 1 unit is defined as the amount that digests 1 µg in 1 hour, 5 units/µg in two hours is good. Then purify the vector and/or insert as appropriate. It is often helpful to gel-purify the DNA, for example if you have multiple bands in PCR (for the insert) or to eliminate undigested DNA (for vector). It is ESSENTIAL to minimize the amount of time DNA is exposed to UV light, since UV will nick the DNA.

2. Determine DNA concentrations. This can be done using the Nanodrop spectrophotometer, or by agarose-ethidium bromide gel electrophoresis with standards of known concentration. For the latter, prepare a 10 ng/µl stock solution of linear DNA (such as lambda or a digested plasmid) to use as standards. Load 10, 30, and 100 ng as standards on the gel using narrow-tooth combs, plus a minimal amount (about 25 ng) of your DNA; if you purified your DNA using a kit, assume 50% recovery. Run the gel briefly, letting the bromophenol blue dye run <1 cm (if longer, the bands will get diffuse and become hard to see). By comparing the fluorescence of your samples and the standards, you can estimate the DNA concentration with reasonable accuracy (± 50%). Use of the gel method will also confirm that your DNA is not degraded (or lost).

3. Calculate the amount of insert and vector needed. The general idea is to maximize the intermolecular ligation events, while minimizing concatamers (>2 molecules joined to each other). Optimal conditions depend on the size of the DNA, whether the ends are cohesive or blunt, and whether you have used a single enzyme, or two enzymes to force directional ligation. Each vector–insert combination requires unique ligation conditions (determined empirically, here are some general guidelines (see Revie et al., Nucl. Acids Res. (1988) 16: 10301).

Type of ligation	Vector concentration	Insert to vector molar ratio[1]
Vector and insert cut with a single sticky-end enzyme, unphosphatased.	>5 ng/µl (5 to 20 range)	1:1 if insert <1 kb 3:1 if insert > 1 kb
Phosphatased vector with insert	1 ng/µl	3:1 if insert <1 kb, 10:1 if insert >1 kb
Vector and insert cut with two enzymes (forced directional cloning).[2]	<5 ng/µl (1 to 5 range)	1:1 if insert <1 kb 3:1 if insert > 1 kb
Blunt-end ligation	~ 5 ng/µl	1:1 to 6:1

[1] Molar ratio, not DNA concentration ratio. It is the concentration of DNA ends that is important.

[2] A low concentration ensures that slow bimolecular associations between the reactant molecules will be followed by unimolecular cyclization.

4. Choose the optimal temperature. This balances the optimal temperature for T4 DNA catalytic activity (16°C), the higher annealing of sticky ends at low temperatures (4°C), and convenience. When making a library, very high efficiency ligation is desired so 16°C is recommended. For routine subcloning using sticky ends, incubate 12 hours at 12°C, or 18-24 hours at 4°C. For blunt-end cloning, go 2-4 hrs at room temperature or 8-12 hours at 12°C.

5. Set up the ligation. Aim for a minimum volume of 10 µl. Anything much larger is usually just a waste of reagents, unless your competent cells are poor.. The following is a typical recipe:

1 µl	10 X reaction buffer (containing ATP and BSA)
8.5 µl	vector, insert, and water
0.5 µl	T4 DNA ligase (NEB, low conc.; add this last)
10 µl	total volume

Mix gently by flicking with your finger, and spin for 2 seconds in a microcentrifuge to get the liquid down to the bottom of the tube.

When the ligation is finished, freeze just in case DNAses are present. Do not store at 4°C!

6. Other tidbits: For subcloning we use T4 DNA ligase, not RNA ligase and not *E. coli* DNA ligase.

Not all ligases are sold at the same concentration, or use the same unit definition. Be careful!

A common error in ligations is to have too high an overall DNA concentration, which will encourage the formation of long chains of insert, vector, or both.

T4 DNA ligase is impaired at high salt concentrations. That is one reason why it is generally good to purify DNA from restriction digests, which are often 50-100 mM salt. Ligations can be performed in low-salt restriction buffers (such as NEB 1 and 4), or other low-salt buffers such as most Klenow buffers, if 1 mM ATP is added; remember to heat-inactivate the restriction enzyme first!

ATP (not dATP) is provided with most ligation buffers (for example, from NEB). Note that ATP breaks down over time, so 10X buffers should be discarded after ~2 years. For the same reason, thaw the buffers in your hand or at room temperature, not at 37°C, and then put on ice.

ATP concentrations above 0.5 mM inhibit ligation of blunt ends.

Rapid ligation can be achieved by adding PEG6000 to the ligation (5% w/v). This is normally what is added to many "fast ligation" kits on the market. At high DNA concentrations one may get too many oligomers, rather than simple circles.

Beware of "fast ligation" protocols, like in the pGEMT kit; it may say that you can incubate for 30 min, but you will get more colonies after several hours of ligation.

The DNA concentrations described above are for vector-insert ligations, not other applications such as linker addition.

Make sure that at least one of your molecules contains a 5' phosphate! Oligos, for example, lack phosphate at their 5' ends. They will ligate to a digested vector, but not a dephosphorylated (phosphatased) vector.

T4 DNA ligase can be heat inactivated by incubating at 65°C for 15 minutes (don't do this if you have PEG in your ligation buffer).

Single base overhangs are inefficient, and may benefit from doubling the ligase.

For electroporation, you may need to purify the DNA to avoid sparking.