LIGATION PROTOCOL

Introduction

Ligation theory is complicated and confusing. Elaborate calculations can be made to optimize the degree of circle formation versus concatamers (see Maniatis), but in practice we seem to get what we want without going to this trouble, and its worth making the calculations only in special cases, e.g. in ligations for making libraries, etc. where efficiency of the desired vs side reactions is extremely important. For simple constructions as we usually do, the following protocols work well:

Ligations can be done at room temperature for anywhere from 30 minutes to overnight depending on what is most convenient.

Blunt ended and linker ligation

There are two situations where we generally use blunt ended ligations. It can be fairly easily accomplished when you can select for something, e.g. antibiotic resistance conferred by the presence of the fragment you want to blunt in. The other situation in which blunt ligation is used is for the addition of linkers. Here the reaction takes place in the presence of 20 to 100 fold molar excess of linker over vector or fragment, in a small volume, at high enzyme concentration and at room temperature (25C). We add PEG 8000 to a final concentration of approx 10% as it has been demonstrated that this form of macromolecular crowding greatly enhances blunt ended ligation efficiency. Typically the reaction with linkers goes for 3 to 6 hrs.

A common problem is ensuring that the pH of the ligation reaction is approx 7.5 to 8.0 before addition of T4 DNA ligase. Check pH on 6 to 8 pH paper using approx .25 ul droplet of ligation mix, and bring the pH up (or down) as necessary by adding 2 M Tris base (or HCl) in the minutest possible micro-droplets (!). When checking pH this way you have to check it immediately on touching the pH paper as it will fade in seconds, given these small volumes.

A typical linker ligation reaction: (assembled on ice) 30 ul reaction

10ug plasmid DNA (linear gel purified) or (air dried ethanol precipitate)	2uL
CB 10X T4 DNA ligase	3uL
EDTA 2mM (chelates heavy metals0	3uL
PEG 8000 (40% weight/vol)	8uL
linker	1uL
*water	12uL
T4 DNA ligase (high concentration)	1uL

*now check pH using 0.25uL increments of ligation mix and adding the tiniest droplets of Tris

base 2M to adjust.

For a pBR322 sized plasmid linearized, 4-5ug is one picomole, therefore, approx. 2pmoles of DNA to receive linkers is present in the reaction, if the vector has simply been linearized. If more fragments are present, use this ratio to increase the amount of linker and ligation volume correspondingly.

Vortex, spin down and incubate at 25 degrees C (room temp) for 3 to 6 hours or overnight.

Add 3uL of 1M MES pH 6 to your entire ligation reaction (to buffer the pH down so you can transform more than 2.5uL). Then transform 10uL into the appropriate *E.coli* strain (SURE cells are best for standard cloning).

Sticky End Ligation

For sticky-end ligations (eg.: a vector cut with NcoI and PstI and insert with the same ends), the best ligation conditions are those in which there is a 3 to 4 fold molar excess of insert over vector.

For a vector of approx. 3Kb and an insert of approx. 0.5Kb, if you start with 2.5ug of vector DNA and 5ug of insert DNA you will have approx. equimolar recoveries of your vector and insert DNA after gene cleaning.

If you have multiple steps in your cloning, such as phosphatasing or klenow reactions, you may lose some DNA and may need to compensate for this by adding more insert or vector to the ligation reaction (depending on whether it is the vector or insert which has undergone the multiple steps). In this case it is sometimes helpful to start out with a little more DNA (eg. 10ug).

You can then add a 3 to 4 fold molar excess of insert in your ligation reaction.

A sample protocol follows:

Ligation reaction (10 uL)

Vector DNA (gene cleaned	0.5uL
Insert DNA	2.0uL
CB 10XT4 DNA ligase	1.0uL
EDTA 2mM	1.0uL
*water	4.5uL

T4 DNA ligase (0.5units/uL)	1uL

*check pH with 0.5 , add small amount 2 M Tris to adjust to 7.5

After ligation, transform (a) 2.5 ul of ligation reaction directly, (b) 2.5 ul of control ligation of vector alone, and (c) transform an equivalent molar amount of unligated vector to determine the amount of residual (presumably uncut plasmids) transformation in the supposedly totally linearized vector DNA. If you get very few colonies in the third case, very few in the second case and greater than three fold number of colonies in the ligation reaction with insert, you will probably get what you want, although you still have to screen for orientation if the two sticky ends were the same..

If you don't get very many colonies on any of the plates, you can try transforming all of the rest of your ligation mix. To do this, you will have to add 0.75 ul of 1M MES pH 6 to the ligation first to lower the pH so as to not kill the bacteria.