Gibson Cloning, vl.3

Kimble Lab protocol (UW-Madison) via Aaron Kershner -11/2011
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This document is a detailed protocol of the Gibson cloning technique (Gibson DG, Methods Enzymol. 2011. 498:349-61). The published protocol is limited and contains only a few details about how to use Gibson cloning for typical cloning purposes. This document aims to give an account of how to use Gibson cloning to assemble multiple pieces of DNA into any target plasmid.

***1/9/12 UPDATE (vl.l) This version contains an updated protocol for making the reaction buffer. We have been making 2X buffer instead of 5X buffer. For a protocol for making the 5X buffer, see the above citation.

***10/10/13 UPDATE (v1.2) This version clarifies instructions for making the enzyme-reagent master mix.

Overview

Gibson cloning is a technique to assemble overlapping pieces of DNA in vitro. A basic outline of the technique is that, once overlapping DNA fragments are combined in a tube, an enzyme cocktail chews back 5' DNA ends exposing complementary overhangs and then repairs and ligates the DNA fragments. Gibson cloning is rapid (the reaction takes one hour), multiple pieces of DNA can be assembled, and the resulting clones are scarless, i.e. no leftover "junk" sequences remain between your fragments of interest.

Recipes

2X ISO reaction buffer -1.1 ml

Reagent	Stock Concentration	Final Concentration	Volume
PEG 8000	40%	10%	275 ul
Tris, pH 7.5	1M	200mM	220 ul
MgCl2	1M	20mM	22 ul
DTT	1M	20mM	22 ul
dNTP	10 mM (each)	0.4mM	44 ul
NAD*	50mM	2mM	44 ul
Water			473 ul
			Total 1100 ul

*50 mM stocks can be purchased from NEB, cat # B9007S

-Make 133.33 ul aliquots and store at -20. Good for 1 year. Recipe makes 8 aliquots. Scale up or down as you want.

Enzyme-reagent master mix (200 ul, performs 13 reactions)

Reagent	Stock Concentration	Final Concentration	Volume
2X ISO buffer			l33.33 ul
Phusion DNA polymerase *NEB, cat # M0530L*	2 U/ul	0.033 U/ul	3.3 ul
Taq DNA ligase *NEB, cat # M0208L*	40 U/ul	5.3 U/ul	26.6 ul
T5 exonuclease stock* *NEB, cat # M0363L*	5 U/ul*	0.005 U/ul	0.2 ul
Water			36.56 ul
			Total 200 ul

-Store at -20. Good for 2 years.

*TS exonuclease comes from the manufacturer at 10 U/ul. Make 1:2 dilution in IX T5 exonuclease buffer (NE Buffer 4) to create a stock solution before adding it to the enzyme mix.

Protocol

Prepping your vector

1. The first step is to identify the restriction site in your target vector where your assembled DNA will be inserted. Cut the plasmid with your chosen enzyme(s). Uncut vector will increase background, so I recommend digesting until completion.

2. Purify the cut plasmid using gel extraction, and quantify DNA concentration. Your plasmid should be >10 ng/ul.

Prepping PCR fragments for assembly

1. Design PCR primers so that DNA fragments to be fused contain a ≥ 40 bp overlap at the (future site of) junction. For example, if I want to insert one PCR fragment into my target vector, the 5' end of my forward primer would perfectly match the 40 base pairs present on one side of my plasmid cut site, and the 5' end of my reverse primer would perfectly match the 40 base pairs present on the other side of the plasmid cut site. Another example: if I want to put my favorite promoter in front of GFP and assemble that into a vector, the 3' end of the promoter and the 5' end of GFP would overlap; the 5' end of the promoter would overlap with one side of the cut vector; and the 3' end of GFP would overlap with the other side of the cut vector.

2. PCR fragments, purify products using gel extraction, and quantify DNA concentration. It is important that you get a single product, and no secondary bands or primer dimers. Cleanup is optional, but if secondary products are a problem, gel extraction is a good idea. Use high fidelity polymerase (e.g. Phusion, Pfu Ultra II HS, or Platinum Pfx). If your PCR template is plasmid DNA that has the same selectable marker as your target vector, you might want to DpnI treat your PCR products followed by a clean up.

Assembly!

1. Combine DNA in an equimolar ratio in a final volume of 5 ul. A good starting point is 30-50 ng of cut plasmid. Large fragments (e.g. >8 kb) might require more ng of DNA. Use small fragments (≤ 1 kb) in 5-10 fold excess. Include a negative control that contains vector only in 5 ul of water.

2. Add 15 ul of the enzyme-reagent master mix. Flick the tube several times, and centrifuge to collect the sample at the bottom of the tube.

3. Incubate at 50˚C for one hour.

4. Transform DH5α using 10uL reaction products.

5. Sequence clones.