Isothermal assembly (a.k.a. Gibson assembly)

Background

Isothermal Assembly enables you to clone a PCR fragment into a restriction site of choice in a vector at high efficiency without having to actually cut the PCR fragment with a restriction enzyme. It obviates much of the difficulty in subcloning and avoids the intermediate Topo cloning step for moving PCR products into a vector target. One designs 5' and 3' primers with 20-120 bp of homology to the vector (up to the site of insertion) and then 20-30 bp of homology to the sequence to be PCR amplified and inserted. If one wishes to concatenate PCR fragments the 3' primer of one fragment should be homologous to the 5' primer of the next fragment. In this manner the Springer lab as assembled in a single reaction 7 separate PCR amplified cDNAs into a single product! Isothermal Assembly works by combining a cocktail of exonuclease, polymerase, and ligase to fuse dsDNA fragments with sufficiently (20-120 bp) homologous ends. It leaves no "scar" behind, i.e. you can expect your product to contain the EXACT overlap sequence. The reaction may work with shorter ends (e.g. 15 bp), so long as the annealing temperature is higher than 50°C.

Isothermal assembly reactions are stored in the common -20C freezer as 15 ul aliquots.

Isothermal assembly procedure:

1. PCR up your fragments of choice, and gel purify.
2. Not exceeding a total volume of 5 ul, in a PCR tube, combine fragments at equal molecular ratio [e.g. amount fragment1 = 100 ng * (fragment 1 size / backbone size); 100 ng * (fragment 2 size / backbone size); backbone = 100 ng]. If required, bring to 5 ul with ddH2O. I recommend using approx. 100 ng of plasmid backbone (fragment containing antibiotic resistance). Quantitative fusion of two linear PCR fragments is also possible (incubation >30 min will lead to degradation).
3. Add the combined fragments (5 ul) to 1 Isothermal Assembly reaction aliquot (15 ul) and mix by pipetting (20 ul total).
4. Place rxn. at 50°C for 15 min – 1h.
5. (optional for chem. transform.) Purify with Qiagen PCR purification (MinElute) kit. Elute in 20 ul of ddH2O.
6. Transform with 1 ul of assembly rxn.

Recipe to make aliquots:

5x isothermal assembly reaction buffer (assemble on ice):

From the paper:	Actually added:
3 mL 1M Tris-HCl pH 7.5	3 mL 1M Tris-HCl pH 7.5
150 µL 2M MgCl2	300 µL 1M MgCl2
60 µL 100 mM dGTP	600 µL 10 mM each dNTP
60 µL 100 mM dCTP	
60 µL 100 mM dTTP	
60 µL 100 mM dATP	
300 µL 1M DTT	300 µL 1M DTT
1.5 g PEG-8000	1.5 g PEG-8000
300 µL 100 mM NAD	20 mg NAD
ddH2O to 6 mL	ddH2O to 6 mL

* Prepare 320 µL aliquots (18) and freeze all but one. * Label these “5X isotherm buffer”
To the one remaining (320 uL), add:

1.2 µL	T5 Exonuclease
20 µL	Phusion polymerase (NOT HOTSTART)
160 µL	Taq ligase
700 µL	ddH2O

Prepare 15 uL aliquots (~80) on ice in PCR tubes and store at -20°C. These should be good for up to a year.

Shopping list:

Here are some suggestions for where to procure suitable reagents:
Buffer

Item	Vendor	Cat. No.
1M Tris-HCl pH 7.5	Systembio kitchen	none
Magnesium Chloride, 1.00 +/- 0.01M Solution	Affymetrix / USB	78641 10 x 1 ML
Nicotinamide adenine dinucleotide (NAD)	Applichem	A1124,0005
DTT, molecular biology grade	FERMENTAS	R0861
Polyethylene Glycol 8000, Powder	USB / Affymetrix	19966
alternative supplier: PEG8000, Powder	Sigma	89510
dNTP Mix, 10mM each	Fermentas	R0192 1 ml

Enzymes:

T5 Exonuclease	EPICENTRE	T5E4111K
Taq DNA Ligase	NEB	M0208L
Phusion™ High-Fidelity DNA Polymerase	NEB	F-530S

References

Gibson et al (2009) Nature Methods 6(5):343-345.