Gibson Isothermal Assembly (Reck-Peterson Lab Protocol)

Overview

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 50C.
Isothermal assembly reactions are stored in the common -20C freezer as 15 ul aliquots (in a labeled freezer box).

Prepare Isothermal Assembly Aliquots

1. First, prepare 5x isothermal assembly reaction buffer in a 15mL falcon tube (on ice):

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

2. Prepare 320 uL aliquots (18 total) and freeze all but one at -20C. *** Label these "5X isotherm buffer"**
   Expiration note (LS): This buffer will last at least 2 years if frozen & stored properly. I have used 2-year old frozen 5X buffer successfully for recent isothermal assemblies. In general I have more success with fresher isothermal mix, but I think this is more relevant after you add the enzymes (in the next step) versus buffer prep/storage.

3. To the one remaining (320 uL), and still on ice, add:

Volume:	**Reagent:**
1.2 uL	T5 Exonuclease
20 uL	Phusion polymerase (NOT HOTSTART)
160 uL	Taq ligase
700 uL	ddH2O
	(OPTIONAL Note from LS: I have recently been adding **10 uL ET SSB** and only **690 uL ddH2O** after reading the Rabe & Cepko 2020 Biorxiv method; haven't done a robust comparison but I have gotten multiple 5-6 fragment assemblies to work on the first try, so I keep using it since it certainly doesn't harm and is inexpensive!

4. Prepare 15 uL aliquots (~80) on ice in PCR strip tubes and store at -20C. These should be good for up to a year, but tend to perform best when made within the last 1-2 months.

Isothermal Assembly Protocol

1. PCR amplify your fragments of choice and your vector in a 50ul NEB Q5 reaction. Run on an agarose gel to check band size & amplification success. Purify the PCR fragments using one of the following methods. Notes from LS below:
    a. IF PCR TEMPLATE WAS A PLASMID - I do a 1 hour DpnI treatment at 37C to digest remaining plasmid (NEB R0176S). I do not do this for PCR products amplified from genomic DNA or gene blocks.
        i. Add 1 ul DpnI enzyme per 50 ul PCR reaction & close tube
        ii. Incubate in thermalcycler or incubator at 37C for 1 hour
        iii. Proceed with gel purification or column cleanup
    b. Column cleanup - if there was a single crisp band at the desired MW, I use Wizard cleanup kit. Elute in 50ul nuclease free H2O (often I perform the first elution, then reload the eluted sample of 50ul DNA + PCR product to elute a second time for higher yield in the same final volume)
    c. Gel purify - often best way to get high quality PCR fragments with fewer off-target amplicons; however, it is time consuming and often low yield. (I only gel purify if there is a large nonspecific band on the gel)
2. Combine your inserts & vector in a clean PCR tube, not exceeding a total volume of 5 ul.
    a. Vector:insert molar ratio of 2:1. Add all inserts at same molar ratio.
        i. Note by LS: I use 0.025 pmols, or 50 ng, of PCR-amplified pBH vector and 0.050 pmols of each insert.
        ii. Note by AR: For putting a single fragment in a vector, in my hands having a 3- to 5-fold molar excess of the insert over the vector helped increase the fraction of correct products
    b. If required, bring to 5 ul with ddH2O
        i. Note by LS: I have used 6 to 7 ul DNA in our 15 ul isothermal aliquots successfully, so volume does not seem to be super sensitive here. But I always aim for 5 ul anyways!
3. Add combined fragments (5 ul) to one isothermal assembly reaction aliquot (15 ul) and mix gently by pipetting (should have 20 ul final)
4. Incubate reaction at 50C for 15 to 60 minutes; longer for more complicated reactions. Thermalcycler is an excellent place to do this incubation.
5. Transform 1 to 5 ul assembly reaction into competent *E. coli*

Shopping List

Before you buy, first make sure we actually have run out of these reagents. Here are some suggestions for where to procure suitable reagents:

Buffer:

Item	Vendor	Cat. No.
1M Tris-HCl pH 7.5		
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
dNTP Mix, 10mM each	Fermentas	R0192 1 ml

Enzymes:

Item	Vendor	Cat. No.
T5 Exonuclease	NEB	M0363S
Taq DNA Ligase	NEB	M0208L
Phusion™ High-Fidelity DNA Polymerase	NEB	F-530S
(NEW ADDITION SINCE 2020): ET SSB protein	NEB	M2401S

03/08/2018 Note: Tien have been using T5 Exonuclease ([null M0363S]) from NEB instead of Epicenter (T5E4111K) and there has been no problem at all. NEB is easier to procure, so Tien made the permanent change to the protocol.

References:

- Gibson et al (2009) Nature Methods 6(5):343-345; https://doi.org/10.1038/nmeth.1318
- Rabe & Cepko (2020) BioRxiv; https://doi.org/10.1101/2020.06.14.150979