Gibson/Goldengate 2+ Fragment prep

1) Prepare PCR with:
   - Highly accurate polymerase (Q5 or equivalent)
   - Long fragments? GC Enhancer Buffer VERY useful
   - Shorter fragments? <2-3kb? May or may not need GC enhancer -> Lowers accuracy (a bit)
   - LOW template amount (0.1 to 0.5 ng/25 uL PCR reaction) -> Make it easier for the DpnI

2) Thermocycling settings:

Initial Denaturation:
95°C for 90 seconds

30-40 Cycles of:
95°C for 30 seconds
50–67°C for 30 seconds
72°C for 30 seconds/kb

Have a gradient PCR machine?
4-12 reactions from 55-67°C
No gradient PCR?
Start at 59°C and pray

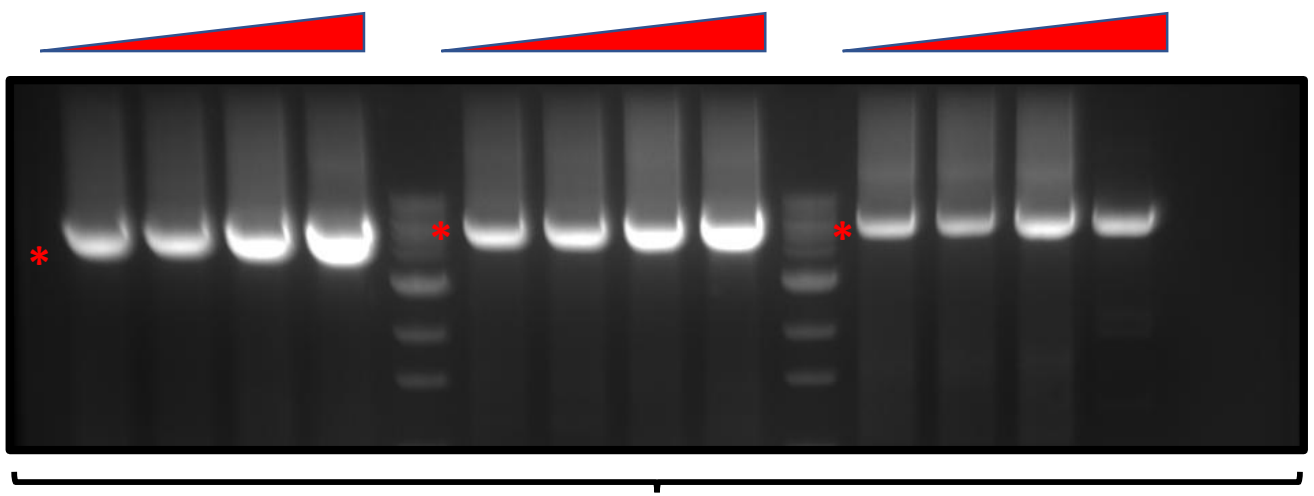
Final Extension:
72°C for 2X Cycling Extension Time
END

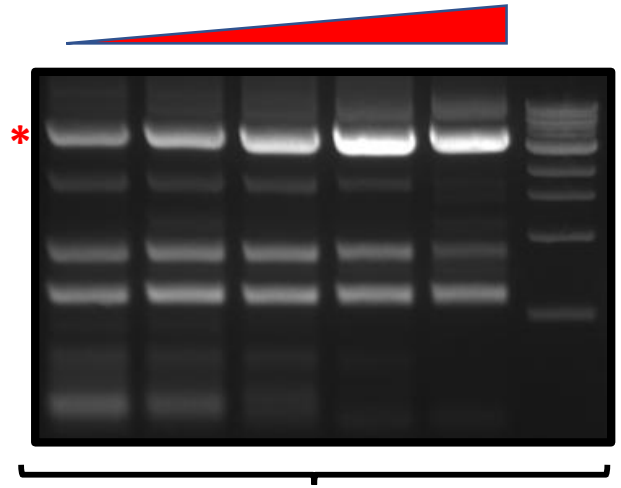
LET THE PCR MACHINE COAST TO ROOM TEMP!
PCR products can survive for DAYS at RT
Your thermocycler is not a refrigerator!

Gibson/Goldengate 2+ Fragment QC

1) Run 5-8uL of PCR product on a 1% agarose
2) Do I have a product of expected size? (*)
3) Is the PCR product pretty clean?

Annealing Gradient





Noice and clean?

DpnI treatment

Optimize PCR
Optimize Primers

Expected product + Nonspecific?

DpnI treatment

Gel Extraction Protocol

Assembly

Less Risk + Slower

Gel Extraction Protocol

PCR Cleanup Column

Riskier + Faster

NO CLEANUP

DO YOU FEEL LUCKY

Assembly

Gibson Assembly

- Determine concentration of all fragments with spectrophotometer
- Calculate fmol/uL for all fragments (NEB Biocalculator -> dsDNA Mass to Moles)
- 1 vector fragment, 1 insert? 1:3 molar ratio
- 1 vector fragment, 1 small insert (100-500bp)? 1:5 to 1:10 ratio
- 1 vector, 2 or more fragments? 1:1:1 etc ratio
- Shoot for 200fmol of all fragments combined, 50fmol is grim but doable, 400+ fmol for 3+ fragment assemblies
- Which mix to use?

1-3 Fragments?

5X TEDA Cloning Mix (1 mL)
500 mM Tris–HCl pH 7.5 (0.5 mL of 1M stock)
50 mM MgCl2 (50 uL of 1M stock)
50 mM dithiothreitol (100 uL of 0.5M stock)
0.25 g of PEG 8000 (May need gentle heat)
1 µl of 10 U/µl T5 exonuclease (NEB)

Combine fragments + mastermix in 10-20uL volume

30C for 40 minutes

1-4 Fragments?

2X Gibson Assembly Master Mix
405µl Isothermal Start Mix
25µl 1M DTT
20µl 25mM dNTPs
50µl NAD+ (NEB Cat. B9007S)
1µl T5 exonuclease (NEB Cat. M0363S)
31.25 µl Phusion High Fidelity DNA Polymerse (NEB Cat. M0530S)
250 µl Taq Ligase (NEB Cat. M0208S)
467.75 µl H2O
Mix by pipetting gently. Make 100 µl aliquots.

Isothermal Start Mix
1.5g PEG8000
3ml 1M Tris-HCl, pH 8.0
150µl 2M MgCl2
Put on tube rotator until PEG is in solution

Recipe/Optimization by Ethan Ford -> Ethanomics blog

Combine fragments + mastermix in 10-20uL volume

50C for 60 minutes

1-4 Fragments?

Commercial Gibson Mix (NEB or Other brands)

Combine fragments + mastermix in 10-20uL volume

50C for 60 minutes

1-5 Fragments?

Commercial Hi-fi Mix (NEB)

Combine fragments + mastermix in 10-20uL volume

50C for 60 minutes

- Mix chosen depends on number of fragments to assemble and budget. Gibson/Hifi mastermix has a notoriously short shelf life! Even at -80C