3G Assembly

Overview

This protocol covers the assembly of circuits using 3G assembly. Transcriptional units will be generated through Golden Gate Assembly, amplified via PCR then combined via Gibson Assembly. Golden Gate Assembly can either be performed with NEB's master mix or with T4 Ligase and BsaI. The instructions are slightly different, and it has been noted that NEB's master mix performs slightly better (~2-3 fold) than using ligase and BsaI.

Materials

- Annealed UNS adapters (50nM stock)
    - Final concentration in Golden Gate is 5nM
- 3G parts (30nM stock)
    - Final concentration in Golden Gate is 3nM
- Either T4 DNA Ligase (**2,000,000 units/mL**), 10x ligase buffer **(small 0.5mL tube aliquot in buffer box)** and BsaI[i] or NEB Golden Gate Assembly Mix and buffer
    - When using ligase buffer, use one of the small aliquots in the buffer box. ATP does not survive freeze thaws well, and so we **aliquot 20µL** tubes.
- Ice
- Q5 2x Hot Start Master Mix
- UNS amplification primers (10µM stock)
- 1% agarose gel(s)
- Qiagen Min-Elute Gel extraction kit
- 2x NEB Hifi DNA Assembly Master Mix
- Linearized destination vector containing UNS1 and UNS10 overlaps.

Procedure

1. Perform a **5µL Golden Gate Assembly** of **each** transcriptional unit. Label a 0.2mL tube for each transcriptional unit that is being assembled.
    - Note: Assemble reaction **on ice**[ii]
2. Add **0.5µL** of the 30nM stock of **each 3G part** (Promoter, UTR, CDS, Terminator)
    - Note that multiple circuits can be assembled in a single pot by preparing an equimolar ratio of parts. (i.e. put in 0.25µL of 30nM promoter #1 and 0.25µL of 30nM promoter #2)
3. Add **0.5µL** of 50nM stock of the **left UNS adapter** (i.e. the **A sticky end**).
4. Add **0.5µL** of 50nM stock of the **right UNS adapter** (i.e. the **E sticky end**)

[i] Note that BsaI-HF does not work. BsaI-HF V2 has not been tested.

[ii] Technically, as long as you are doing the enzyme portions on ice, the DNA stuff can be done at room temperature.

5. When performing more than one Golden Gate, it is easiest to make a Master Mix (**step 6**). Each 5µL Golden Gate reaction uses **either**:
    - 0.5µL of 10x T4 DNA Ligase buffer, 0.25 BsaI, 0.25 T4 DNA Ligase, 1µL NFW: For BsaI and ligase
    - Or 0.5µL Golden Gate Buffer, 0.25µL Golden Gate MM and 1.25µL NFW: For NEB Golden Gate Mastermix
6. Either add NFW, buffer and enzymes (step 5) to the reaction, or assemble a master mix in 0.2mL tube **on ice** (formulas below include 10% upscale).
    - If using BsaI and T4 ligase, for each reaction: Add 0.55µL 10x T4 DNA ligase buffer, 0.275µL BsaI, 0.275µL T4 DNA Ligase, 1.10µL NFW
    - If using NEB Golden Gate MM, for each reaction: Add 0.55µL Golden Gate Buffer, 0.275 Golden Gate MM, 1.375µL NFW
7. Flick and spin to mix
8. Cycle reaction in thermocycler.
    - For **Golden Gate Master Mix,** perform **30 cycles** of incubating at **37C** for **1 minute** then incubating at **16C** for **1 minute**. **After cycles** hold at **37C** for **5 minutes**. Then hold at 4C
    - For **T4 ligase and BsaI**, perform **9 cycles** of incubating at **37C** for **3 minutes** followed by incubating at **16C** for **4 minutes. After cycles** hold at **37C** for 5 minutes Then hold at 4C.
    - Yields of Golden Gate assemblies can be improved by performing more cycles (100 cycles for the Golden Gate Master Mix, 30 cycles for BsaI and T4 ligase). This is often able to fix reactions that fail at the PCR step.
    - Note that Golden Gate product can be stored at 4C overnight
9. Next amplify with a **50µL PCR**.
10. Make a new labeled 0.2mL tube for **each transcriptional unit** and add:
    - **1.5uL** Golden Gate assembly
    - **18.5uL** NFW
    - **2.5uL** 10uM of the appropriate UNS_Forward primer[iii]
    - **2.5uL** 10uM of the appropriate UNS_Reverse primer
    - **25uL** 2x Q5 Hot Start Master Mix
11. Add tubes to thermocycler(s) and incubate reactions using the standard 2x Q5 Hot Start Master Mix protocol. With **27 cycles**, using **30 seconds/kb** and an **annealing temperature of 64C**.
12. Perform a gel extraction on the PCR product (see gel extraction protocol).

[iii] Note that while the original paper uses shorter forward and reverse primers, 3G assembly performance is improved by using primers consisting of the full 40bp of UNS sequence. (~2-3 fold).

13. Perform a **5µL Gibson Assembly** reaction using the purified transcriptional units and vector (see Gibson Assembly protocol). Add **0.015 pmol** of **each transcriptional unit** and **vector**.[iv]
14. Transform Gibson Assembly. Colony PCRs can be performed using UNS1 Fwd and UNS 10 Rev primers.

[iv] Note that it has been found that the inclusion of at least 1µL NFW improves efficiency