Golden Gate Assembly of ϕMINT and ϕMINTO

Purpose

The purpose of this protocol is to detail how we used Golden Gate assembly to build our phagemid ϕMINT and our mobilizable phagemid ϕMINTO. This protocol used around-the-horn (inverse) PCR to amplify the INTEGRATE cassette from pMINT (see pMINT construction) and to introduce BsaI restriction enzyme sites to the ends of this fragment. Using two different forward primers that introduced different overhang sequences after BsaI digestion, we were able to clone these two phagemids in parallel.

We aim to use these phagemids with an M13KO7 helper phage that uses kanamycin resistance, so we replaced the kanamycin resistance with chloramphenicol resistance in these constructs. We also introduced an F1 ori to ϕMINT for phage packaging and an oriT to ϕMINTO for mobilization by the infected host. These gBlocks were acquired from IDT.

Materials

- pMINT around-the-horn template (<1,000 ng)
- Flagged BsaI PCR primers for pMINT (forward and reverse)
- Q5 High-Fidelity 2X Master Mix (New England BioLabs)
- 5X Q5 High GC Enhancer (New England BioLabs)
- Monarch PCR & DNA Cleanup Kit (New England BioLabs)
- F1 ori and chloramphenicol resistance gBlocks (ϕMINT) (IDT)
- F' oriT gBlock (ϕMINTO) (IDT)
- NEB Golden Gate Assembly Kit (BsaI-HFv2)
- 10X T4 DNA Ligase buffer (New England BioLabs)

Protocols

Around-the-horn PCR of pMINT cassette

1. In two, 1.5 mL microfuge tubes, make two master mixes **on ice** with varying forward primers according to Table 1.
   (a) *Add forward primers corresponding to the proper phagemid into their respective master mixes. The two forward primers used contained different overhangs for constructing the two phagemids.
2. **On ice**, dispense 22.5 μL of one master mix into four, 0.2 mL PCR tubes. Repeat for the other master mix.
3. Add 2.5 μL of plasmid to each PCR tube. Tap the tubes gently to mix.
4. Amplify the nucleic acids according to the cycling protocol in Table 2.

Table 1: pMINT around-the-horn PCR reaction table

Component	**Volume for 25 μL reaction**	**Volume for x5 Master Mix**
MilliQ water	2.5 μL	12.5 μL
5X Q5 High GC Enhancer	5 μL	25 μL
Q5 HF 2X Master Mix	12.5 μL	62.5 μL
Forward primer*	1.25 μL	6.25 μL
Reverse primer	1.25 μL	6.25 μL
Purified pMINT miniprep	2.5 μL	OMIT

Table 2: pMINT around-the-horn touchdown PCR cycling protocol

Cycle	**Denaturation**	**Annealing**	**Extension**
1	30 seconds at 98°C	25 seconds at 72°C	480 seconds at 72°C
2–9	20 seconds at 98°C	25 seconds at 72°C (−0.5°C/cycle)	480 seconds at 72°C
10–30	20 seconds at 98°C	25 seconds at 68°C	480 seconds at 72°C
Final	20 seconds at 98°C	25 seconds at 68°C	600 seconds at 72°C
Hold	4°C for ∞		

(a) After running the PCR reaction, visualize the product on a 0.7% agarose gel. The expected size of the product is about 11.1 kb. We recommend running only 2.5 μL of PCR product so as to conserve the amplified material for the assembly.

5. Perform DNA clean up using Monarch PCR clean up kit:

(a) Pool four 25 μL PCR products together into a sterile, 1.5 mL microfuge tube.

(b) Add 200 μL DNA binding buffer to 100 μL sample from step a (2:1 buffer:sample for this amplicon size).

(c) Insert a spin column into a collection tube and load entire sample onto column and close the cap. Spin for 1 minute at 16,000 X G, then discard flow-through.

(d) Re-insert column into collection tube. Add 200 μL DNA Wash Buffer and spin for 1 minute at 16,000 X G. Discarding flow-through is optional.

(e) Repeat wash (Step d).

(f) Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column does not come into contact with the flow-through. If in doubt, re-spin for 1 minute at 16,000 X G to ensure traces of salt and ethanol are not carried over to next step.

(g) Add 20 μL of MilliQ water to the center of the matrix. Wait for 1 minute, then spin for 1 minute at 16,000 X G to elute DNA.

(h) Store and nanodrop the elution, as it contains desired DNA.

Golden Gate assembly of phagemid vectors

1. Rehydrate gBlocks from IDT
   (a) Spin down the dry tubes of DNA to ensure the DNA is at the bottom of the tube
   (b) Add MilliQ water to a final concentration of 10 ng/μL
   (c) Vortex the tubes briefly
   (d) Incubate the tubes at around 50°C for 15–20 minutes to ensure that the DNA has unstuck from the tube.
   (e) Vortex and spin the tubes again
   (f) Verify the expected concentration with a spectrophotometer. Duplicate measurements are recommended here.
2. **On ice**, set up the Golden Gate assembly reactions in 0.2 mL PCR tubes for each phagemid as follows:

Table 3: Golden Gate assembly reaction table: ϕMINT

Component	**Volume for 20 μL reaction**
pMINT PCR product (75ng/μL)	1.2 μL
F1 ori insert	2.7 μL
CmR insert	5.2 μL
10X T4 DNA Ligase buffer	2 μL
NEB Golden Gate assembly Master Mix	1 μL
MilliQ water	7.9 μL

Table 4: Golden Gate assembly reaction table: ϕMINTO

Component	**Volume for 20 μL reaction**
pMINT PCR product (75ng/μL)	1.2 μL
F′ oriT insert	4.4 μL
F1 ori insert	2.7 μL
CmR insert	5.2 μL
10X T4 DNA Ligase buffer	2 μL
NEB Golden Gate assembly Master Mix	1 μL
MilliQ water	3.5 μL

3. In a thermocycler, run the assembly reaction with the cycling protocol for multi-insert assemblies:
   (a) 37°C for 1 minute, then 16°C for 1 minute. Repeat this cycle 30 times.
   (b) 60°C for 5 minutes.
   (c) Hold at 4°C.
4. Transform 10 µL of the assembly reaction into commercial chemicompetent *E. coli*. Recover the transformation for 1–2 hours at 37°C, then plate the entire recovery on chloramphenicol plates (25 µg/mL) for positive selection of successfully assembled constructs. Grow overnight at 37°C.