3A Assembly (Digestion and Ligation)

Aim

To assemble two G-blocks into a new vector.

Procedure

Digestion

1. Prepare the following Master Mixes

Table 1: Master Mixes

Enzyme Master Mix for Plasmid Backbone (20µl total, for 5 runs)	Enzyme Master Mix for Gblock 1 (BioBrick on the 5' end)	Enzyme Master Mix for Gblock 2 (BioBrick on the 3' end)
2 µl Tango Buffer **1.1** µl EcoRI **1.1** µl PstI **16** µl dH2O	**2 µl** Tango buffer **1.1 µl** EcoRI **1.1 µl** KasI **16 µl** dH2O	**2 µl** Tango buffer **1.1 µl** KasI **1.1 µl** PstI **16 µl** dH2O

2. Digest Plasmid Backbone

- Add 4 µl linearized plasmid backbone (25 ng/µl for 100 ng total)
- Add 4 µl of Enzyme Master Mix

3. Digest G-block 1

- Add 5 µl G-block 1 (20 ng/µl for 100 ng total)
- Add 5 µl of Enzyme Master Mix

4. Digest G-block 2

- Add 5 µl G-block 2 (20 ng/µl for 100 ng total)
- Add 5 µl of Enzyme Master Mix

5. Digest all three reactions at 37°C for 60 min, heat kill at 80°C for 20 min

Purification based on the protocol provided by QIAGEN (QIAquick PCR purification kit)

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. (Tips: color → yellow)

2. Place a QIAquick column in a provided 2ml collection tube

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 s. Repeat once or twice.

4. To wash, add 600 uL buffer PE to the QIAquick column and centrifuge for 30-60 s. Discard flow-through and place the QIAquick column back in the same tube.

5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube

7. To elute DNA, add 50 ul buffer EB or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 ul elution buffer to the center of the QIAquick membrane, let the column stand for 1-5 min, centrifuge.

8. If the purified DNA is to be analyzed on a gel add 1 to 5. Mix and Load

9. Measure DNA concentration using Nanodrop

Ligation

Concentration and volumes to be decided on the day.

1. Add 2µl of digested Plasmid Backbone (50 ng)

2. Add equimolar amount of G-block 1 (EcoRI, KasI digested) fragment (< 3 µl)*

3. Add equimolar amount of G-block 2 (KasI, PstI digested fragment) (< 3 µl)*

4. Add 1 µl T4 DNA ligase buffer

5. Add 0.5 µl T4 DNA ligase

6. Add water to 10 µl

7. Ligate at RT for 10 min, heat kill at 80°C for 20 min

8. Transform with 1-2 µl of product

*Easiest to calculate using
http://www.insilico.uni-duesseldorf.de/Lig_Input.html

Start by using a 1:3 vector:insert ratio. If there is enough digested backbone and G-Block left, it is advised to try additional ratios.

NOTE!

We used exceedingly more DNA than iGEM did - DNA volumes of 500-1000 µl.

Recommended Protocol for digestion for KasI (ThermoFisher)

1. Add:
Nuclease-free water 16 µL
10X Buffer Tango 2 µL
DNA (0.5-1 µg/µL) 1 µL and
SspDI 0.5-2 µL

2. Mix gently and spin down for a few seconds.

3. Incubate at 37°C for 1-16 hours.

The digestion reaction may be scaled either up or down.

Sources

This protocol is modified from the 3A Assembly protocol at igem.org:
http://parts.igem.org/Help:Assembly/3A_Assembly

ThermoFisher
https://www.thermofisher.com/order/catalog/product/ER2191