Cloning of software modified sequences into the plasmid

Materials and solutions:

- gBlocks (IDT/TWIST)
- Primers for Gibson assembly reaction and Sanger sequencing/colony-PCR (IDT/TWIST)
- PCR Master Mix) Q5 HF 2X Master Mix, NEB #M0492S, Hy-Fy High Fidelity Mix x2, hylabs EZ-2021)
- DpnI (NEB #RO176S)
- rCutSmart buffer (NEB #6004S)
- DNA electrophoresis gel (see preparation protocol)
- DNA ladder
- DNA loading buffer x6
- PCR cleaning kit (NEB #T1030S or Promega A9281)
- NEBuilder HiFi DNA Assembly Cloning kit+ Competent cells (NEB #E5520S)
- LB-agar plate with 5ug/ml Chloramphenicol
- Ultra-pure water (UPW)

Equipment:

- Thermocycler
- DNA electrophoresis chamber and power supply
- NanoDrop
- Shaking incubator for Eppendorf tubes
- Water-bath
- Ice bucket
- Incubator

Procedure:

Note: in all steps, thaw solutions on ice.

Step #1: Vector linearization and addition of overlap sequences necessary for Gibson assembly, by PCR

Reaction setup:

Component	50 µl Reaction	Final concentration
Q5 HF 2X Mater mix	25 µl	X1
10 µM Forward primer	2.5 µl	0.5 µM
10 µM Reverse primer	2.5 µl	0.5 µM
Template DNA	Variable	10ng
Nuclease-free water	To 50 µl	

Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Transfer PCR tubes to a thermocycler and set the following conditions:

Step	Temperature (°C)	Time (sec)
Initial denaturation	98	30
30 cycles	98 Annealing temp- calc. with NEB Tm calculator 72	30 30 30/kb
Final extension	72	120
Hold	10	

While PCR is running, prepare DNA electrophoresis gel according to the protocol.

Step #2: PCR product evaluation, DpnI digestion, and cleaning:

PCR product evaluation:

When PCR from step #1 is completed, briefly vortex PCR tubes and mix 10 µl with 2 µl DNA loading Dye x6. Load the 12 µl sample into the wells of the DNA electrophoresis gel and set the power supply to 120v. Stop the running until the dye line is approximately 75-80% of the way down the gel and evaluate with a gel imager.

DpnI digestion and cleaning:

To eliminate the original vector from the new PCR product, treat the rest of the PCR product with 1µl DpnI, add rCutSmart to get 10% v/v, and incubate at 37°C for 1hr. Then, clean by centrifugation method with PCR cleaning kit according to the manufacturer's protocol.

Step #3: Gibson assembly and bacterial transformation

- Determine linearized vector and gBlocks (insert) concentrations by using NanoDrop.
- Then, set up the following reaction on ice: (vector∶insert= 1:2)

Component	2-3 Fragment assembly
Vector	0.025/0.05pmols- x µl
Insert	0.05/0.1pmols- y µl
NEBuilder HF DNA assembly Mater mix	10 µl
UPW	10-X-Y µl

It is recommended to calculate pmols with NEBiocalculator tool available online.

- Incubate samples in a thermocycler at 50°C for 1hr. Following incubation, store samples at -20°C or continue for bacterial transformation.
- Transform competent bacteria (provided in the kit) with 2 µl of the chilled assembled product, according to the manufacturer's protocol.
- Incubate LB-agar plates overnight at 37°C.

Step #4: colony PCR and sanger sequencing

- Pick random colonies (4-5) from each plate with a pipette's tips and suspend each in 10µl UPW.
- Set up the following reaction:

Component	20 µl Reaction	Final concentration
HF ready mix x2	10 µl	X1
10 µM Forward primer	1 µl	0.5 µM
10 µM Reverse primer	1 µl	0.5 µM
Bacterial suspension	5 µl	---
Nuclease-free water	To 20 µl	---

- Perform PCR as follows:

Step	Temperature (°C)	Time
Initial denaturation	94	3min
25-35 cycles	94 Annealing temp- calc. with NEB Tm calculator 72	30sec 30 sec 60sec/kb

Final extension	72	10min
Hold	10	

- Run PCR products in DNA electrophoresis gel as described in step #2.
- Meanwhile, streak the rest of bacterial suspension over an LB-agar plate with appropriate antibiotics, and incubate overnight at 37°C.
- Grow insert-positive colonies by picking them into a 4ml LB starter, and incubate overnight at 37°C while rotating at 200rpm.
- Isolate the plasmid with a miniprep kit according to the manufacturer's protocol.
- Measure plasmid concentration with Nanodrop and run it on a gel for evaluation.
- Send the plasmid for Sanger sequencing according to the sequencing unit conditions.

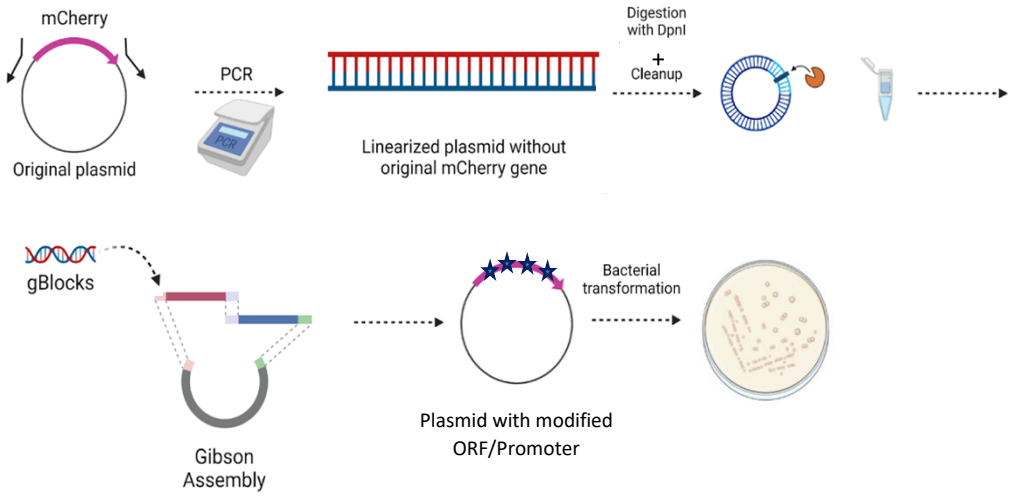

Fig. 1. Illustration of vector construction via Gibson assembly method: original mCherry or P43 promoter were replaced with tested ORFs and promoters (gBlocks) respectively by PCR reaction that excluded mCherry or P43, with a set of primers containing overlapping region to the new ORFs or promoters, necessary for Gibson assembly. Then, a portion of the PCR products was evaluated by DNA electrophoresis gel (not shown), and the rest was treated with DpnI to degrade the remains of the original vector. Next, PCR products were cleaned with a PCR cleaning kit, and the new ORFs or promoters (gBlocks) were cloned into the plasmid via the Gibson method. Then, the modified vector was cloned into competent bacteria.