Insert:

- Use same PCR protocol as original fragment (maybe double check previous optimizations of PCR programs), but use primers called ex. **BB AckA F** (where **BB** stands for **B**io**B**rick). – Use gradient.
- If no unspecific products: use PCR purification kit.
- Measure concentration
- Cut insert with XbaI (**X**) and PstI (**P**) – **60 min** 37 degrees (PstI needs 30 min for PCR products)

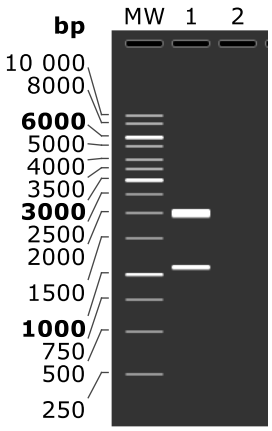
	PCR product
Water, nuclease -free	Up to 20 µL
10X FastDigest Buffer	2 µL
Purified biobrick PCR product	200 ng
XbaI	1 µL
PstI	1 µL
Total	20 µL

- Purify on column (PCR purification kit)
    - Elute with 20 µL elution buffer

Vector BBa_J04450 in pSB1C3:

- Cut vector with XbaI (X) and PstI (P) – **2 hours** 37 degrees

	Plasmid
Water, nuclease -free	Up to 20 µL
10X FastDigest Green Buffer	2 µL
BBa_J04450 (pSB1C3 with RFP)	~1000 ng
XbaI	1 µL
PstI	1 µL
Total	20 µL



- Purify with gel extraction kit (post-stain GelGreen might be preferable). **Don't take picture with the machine before cutting the band out**
    - Band should be 2044 bp (discarded band 1096 bp)
    - Elute with 20 µL elution buffer
- Measure concentration

Ligation:

Linear cut plasmid	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector (use online NEB calculator)
10x T4 DNA Ligase buffer	2 µL
Thermo Scientific T4 DNA Ligase	1 U (1 µL)
Water, nuclease-free	to 20 µL
Total volume	20 µL

Negative control (**no insert)**:

Linear cut plasmid	20-100 ng
10x T4 DNA Ligase buffer	2 µL
Thermo Scientific T4 DNA Ligase	1 U (1 µL)
Water, nuclease-free	to 20 µL
Total volume	20 µL

- Incubate 10 minutes at 22 °C.
- Use up to 5 µL of the mixture for transformation of 50 µL of chemically competent cells
- Negative control: transform 5 µL of the ligation reaction without insert.
- Plate on LB + Chloramphenicol
- 37 degrees' overnight. If no colonies after 16h, put back in the oven again and wait longer (stupid slow iGEM plasmid).

Replate and inoculate colony overnight

- False positive clones are red (still contains BBa_J04450 which has RFP)
- Restreak white colonies on LB+chloramphenicol plates (ex. divide plate into 8 sections to restreak 8 colonies). Dip the green loop in liquid LB+chloramphenicol after you restreaked it. Number tubes and sections on plate.
- Grow liquid culture o/n

Miniprep

Follow protocol. Spin longer with the column empty to remove ethanol. Elute with 50 µL elution buffer.

Restriction verify

	Plasmid
Sterile MQ water	Up to 10 µL
10X FastDigest **Green** Buffer	1 µL
Miniprepped construct	150 ng
XbaI	0.5 µL
PstI	0.5 µL
Total	10 µL

60 min 37 degrees. Post stain with GelRed.
Load 10 µL in gel. Run 85V 1 hour (or maybe longer). **Post-stain with GelRed.**
Bands should be **2044** (vector) + X* (your insert)

Send to sequencing if correct

Gief medals

*Insert lengths:

BB pAQR1: **692**

BB pPCK1: **797**

BB pPYK2: **462**

BB AckA: **1266**