Cloning protocol

DAY 1

1. Resuspend BioBrick
   - Pierce the foil
   - Add 10 µL dH2O and resuspend (pipette up and down several times)
   - Sit for 5-10 minutes
2. Transformation into TOP10 E. coli competent cells → Floor 3, - 80ºC freezer, common competent cells. Remember to split the cells (100µL cells in eppendorfs in freezer).
   - Use 5 µL BioBrick for transforming 50 µL competent cells

Transformation

Components

CaCl2-competent E. coli cells
SOB media or LB
DNA plasmid
Agar plates

Procedure

1. Put LB media in 37°C incubator
2. Turn on a water bath or heating block to 42°C.
3. Thaw competent cells on ice for 15 min.
4. Add 10µL of 5x KCM and 5 µL of ligation reaction mixture (or plasmid) or controls above to 50 µL of competent cells.
5. Incubate for 30 min on ice.
6. Heat shock for 60 s at 42°C.
7. Incubate for 5 min on ice.
8. Add 950 µL of LB media (pre-heated to 37°C).
9. Incubate for 1–1.5 hr at 37°C, with occasional gentle mixing by inversion of the tubes.
10. Plate appropriate antibiotics on plates. 200 µL of 100x CHL (diluted in 95%-70% ethanol) is needed.
11. Spin cells down from remaining 900 µL at 4000 rpm for 5 min.
12. Discard 800 µL of the supernatant and resuspend the pellet in the remaining 100 µL.
13. Spread the remaining suspension bacterial evenly on an agar plate containing the appropriate antibiotic. Continue until all the inoculum has gone into the agar.
14. Put the plates at 37°C overnight.

3. Incubate O/N

DAY 2

4. Preheat LB media
5. (the 6 ml and 6µl can both be 5) Add 6 ml LB media in a sterile erlenmeyer flask and add 6 µl of 1000x of appropriate antibiotic.

6. Pick single colonies from agar plates with 1-10 µl tips. Release tips into media. Pick three single colonies (do triplicates) from each agar plate.

DAY 3

7. Make glycerol stock of O/N culture (600 µl of O/N cell culture + 400 µl 50% glycerol solution, 20 % final glycerol)
8. Purify plasmid with Miniprep kit: Instructions in kit
9. Measure DNA concentration using Nanodrop. You'll get:
    - Concentration (ng/µl)
    - Purity in respect to the RNA concentration (A260/280) = 1.8
    - Purity respect to other contaminants (A260/230)=2.0- 2.2
10. PCR to introduce primer overhangs with restriction sites → if you want to change the vector plasmid for your gene and need other restriction sites

DAY 4

11. Check on gel.
12. Purify the PCR product with a PCR purification kit.
13. Restriction digestion of BioBrick and new plasmid
    - *See Digestion + Ligation protocols belox*
14. Get rid of digestion leftovers
15. PCR purification of digested BioBrick
16. Gel extraction of digested plasmid

DAY 5

17. Ligation of digested BioBrick into digested plasmid.
18. Transformation into E.coli Top10

DAY 6

19. Colony PCR on single colonies
    - KEEP THE COLONY FOR STEP 20
20. Check on gel
21. Send PCR products for sequencing (based on gel) - a few (2 to 3)
22. Grow colonies O/N in liquid media + antibiotics

DAY 7

23. Make glycerol stocks of O/N cultures.
24. Miniprep to extract plasmid.
25. Transformation into E.coli BL21 (host for protein production) with plasmid prep from step 22.

DAY 8

26. Pick single colony and grow O/N in liquid media + antibiotics

DAY 9

27. Make glycerol stocks of O/N culture
28. Start large cultivation for protein production

Transformation

DAY 1

1. Turn on a water bath or heating block to 42°C and put LB medium and agar plates to incubate at 37ºC.
2. Thaw competent cells on ice for 15 min.
3. Gently mix cells and aliquot 50 µL per tube (floor 3 competent cells are 100 µL).
4. Add 10 µL 5x KCM 2ul 5X KCM
5. Incubate cells on ice for 10 min
6. Add 5 µL (2 or 3 µL might also work) of ligation reaction mixture (plasmids from cloning) or controls above to 50 µL of competent cells. 8 µL ligation mixture to 50 µL CC. 8ul water added in place of DNA for negative control. 5 uL of Marits positive control added for positive transformation control. Marit adds KCM to the DNA then incubates 2-5 mins on ice.
   a. Use 10pg from Positive transformation control DNA
7. Incubate for 20-30 min on ice.
8. Heat shock for 60s at 42°C.
9. Incubate for 5 min on ice.
10. Add 950 µL of LB media (pre-heated to 37°C)
11. Incubate for 1–1.5 hr at 37°C, with occasional gentle mixing by inversion of the tubes.
    a. *1h is OK. Inverting the tubes is optional but increases transformation efficiency.*
12. Spin cells down from remaining 900 µL at 4000 rpm for 5 min.
13. Discard 800 µL of the supernatant and resuspend the pellet in the remaining 100µL.

Steps 10-11 are optional but low volumes are easier to plate

14. Spread the remaining suspension evenly out on an agar plate containing the appropriate antibiotic. Spread the bacterial suspension using glass Pasteur pipettes (bend with the fire) until all the inoculum has gone into the agar.
15. Incubate the plates at 37°C overnight.

DAY 2

16. Calculate transformation efficiency (colonies/µg) of the competent cells using the positive control plates. How does it compare with the expected efficiency? In the evening, re-streak appropriate colonies (Protocol 7). You don't need to make a calculation but it's good to analyse the transformation efficiency.
17. Pick single colonies and set overnight cultures.

DAY 3

18. Make glycerol cell stocks (Protocol 2) of strains worth saving and process the rest of the culture according to instructions.

Digestion

1. Make one mix for each plasmid you want to digest (+ negative control) (the negative control is treated the same way as digested plasmids apart from adding restriction enzymes) in 50 µL tubes:
    a. Each tube should contain 1 µg plasmid + nuclease-free water up to 43 µL (total volume)
2. Add 5 µL of 10x reaction buffer for restriction enzymes - CutSmart, stored at -20ºC.
3. Add 1 µL (20 units) of each restriction enzyme (two per tube) to give a final volume of 50 µL.
    a. *Important to design in advance the cloning strategy and decide where to cut with each restriction enzyme*
4. Tap on the tubes to mix. If necessary, centrifuge for a few seconds to spin down the liquid.
5. Incubate at 37°C for 30 min to 1h. (O/N or at several hours during the day)
6. Heat-inactivate the enzymes by incubating at 80°C for 20 min.
    a. *For the last 2 steps there is a thermocycler protocol saved in TC-PLUS (black and orange thermocycler) as iGEM digest.*

At this point, samples may be stored at −20°C.

** Gel analysis of the digests to check digestion efficiency - optional*

- *Run 20 µL (200 ng) of each digestion mixture on a 1% agarose gel. Dissolve 0.50 g agarose in 50ml TBE buffer. Put the 50ml gel mold in the cast and go down to floor 3. Microwave gel for 2 mins until boiling - stir - repeat. Add 5 microliters of cybersafe dye (in small box next to gel station). Quickly pour the gel into the mold. Add the comb (small one max suitable for 10 microliter volume but ideal volume is 6 microliters). Remove bubbles - especially close to the comb.*
- *Use uncut plasmids as negative controls*

How to cut DNA out of gel - using UV imaging in the gel room in floor 3 - remember lab coat, gloves and UV shields!

1. *Make sure you have images your gel*
2. *Weigh your tubes without gel*
3. *Clean cast and scalpel with ethanol*
4. *Put gel on cast*
5. *Put on UV shields + turn the blinds + turn off the lights*
6. *Cut the gel with 4 incisions as close as possible to the DNA*
7. *Image after cutting to see that you have everything*
8. *Weigh your tubes with gel*

Ligation

A) Without kit

1. Add 2 µL (20 ng) of each of the three digestion mixtures to 11 µL of nuclease free water.
   ➢ **Marit uses a 5:1 insert:vector ratio (moles)**
2. Add 2 µL 10x reaction buffer for T4 DNA ligase.
3. Add 1 µL of T4 DNA ligase to give a final volume of 20 µL.
4. Incubate at room temperature (~22°C) for 1h (this was previously 30 min).
5. Heat-inactivate the enzymes by heating at 80°C for 20 min. This is done by using thermocycler deactivate protocol
   ➢ 8 µL ligation mixture + 2µL 5x KCM → transform 50 µL CC

At this point, samples may be stored at −20°C.

B) Using NEBuilder HiFi DNA Assembly Cloning Kit

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2–3 Fragment Assembly*	4–6 Fragment Assembly**	NEBuilder Positive Control†
Recommended DNA Molar Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols** X µl	10 µl
NEBuilder HiFi DNA Assembly Master Mix	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl††	20 µl††	20 µl

* Optimized cloning efficiency is 50–100 ng of vector with 2-fold excess of each insert. Use 5-fold molar excess of any insert(s) less than 200 bp. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%. To achieve optimal assembly efficiency, design 15-20 bp overlap regions between each fragment.

** To achieve optimal assembly efficiency, design 20-30 bp overlap regions between each fragment with equimolarity of all fragments (suggested: 0.05 pmol each).

† Control reagents are provided for 5 experiments.

†† If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional NEBuilder HiFi DNA Assembly Master Mix.

2. Incubate samples in a thermocycler at 50°C for 15 minutes (when 2 or 3 fragments are being assembled) or 60 minutes (when 4–6 fragments are being assembled). Following incubation, store samples on ice or at –20°C for subsequent transformation.
   ➢ *Note: Extended incubation up to 60 minutes may help to improve assembly efficiency in some cases.*
3. Transform NEB 5-alpha or 10-beta Competent E. coli cells (provided in the cloning kit, or from Floor 3) with 2 µl of the chilled assembled product, following the transformation protocol.

At this point, samples may be stored at −20°C.

BioBrick amplification by PCR

1. Prepare the PCR reaction in a 50µL tube:

Component	50µL reaction	Final concentration
5X Phusion HF Buffer	10 µl	1X
Forward primer - VF2 (10µM)	2.5 µl	0.5 µM
Reverse primer - VR (10µM)	2.5 µl	0.5 µM
dNTP mix (10mM)	1 µl	200 µM
template DNA	plasmid (1 ng) genomic DNA (~50-100 ng)	-
Phusion DNA Polymerase (2U/µL)	0.5 µl	0.02U/µL (1U/50µLreaction)
H2O (nuclease-free)	Up to 50 µl	-

⚠️ *Keep tubes on ice.*
⚠️ *Add polymerase last → Phusion DNA Polymerase exhibits 3´→ 5´ exonuclease activity that can degrade primers in the absence of dNTPs.*

Info DNA pol: https://www.thermofisher.com/order/catalog/product/F530S#/F530S

2. Run the following PCR program in the thermocycler:

Initial denaturation → 98ºC 30”

Denaturation → 98ºC 10”
Annealing → 63.4ºC 30”
Elongation → 72ºC 15”

x30 cycles *(25-35 cycles recommended)*

Final elongation → 72ºC 3’
Hold → 4ºC *Forever*

⚠️ *Annealing temperature = 3–5°C lower than the lowest Tm of the primers.*
Tm VF2 (IDT) =55.9ºC *Tm VF2 (thermo) =64.0*
Tm VR (IDT) =55.3ºC *Tm VR (thermo) = 63.4*
→ Annealing temperature = 63.4ºC
www.thermofisher.com/tmcalculator

⚠️ *Elongation time = 15s/kb for extension for low complexity DNA (e.g. plasmid).*
Do not exceed 1 min/kb → protA BB= 235 bp.

3. Purify the amplified BioBrick→ QIAGEN PCR purification kit.
4. Store BioBrick / PCR product at -20°C

Then…

5. Digestion (see Digestion protocol)
6. Use PCR purification kit to get rid of small digestion products
7. Ligation (see Ligation protocol)
8. Transform TOP10 competent cells