Copy of Work Plan - Device 1 using 3A Cloning

WEDNESDAY, 8/11/2021

Objectives:

- Utilize 3A cloning techniques using the linearized plasmid backbones from IGEM 2019 to succesfully clone Device 1 inside Escherichia coli Beta 10 competent cells

Day 0 – Prep Day

A. Prepare Ampicillin, Chloramphenicol and Kanamycin (Source: IGEM Team TU_Darmstadt)

1. **Ampicillin Stock** *(100mg/mL)*
    a. Weigh the 1.0g of ampicillin powder and measure 10mL of ddH2O.
    b. Mix your antibiotic with your ddH2O.
    c. Absorb your solution with the sterile syringe.
    d. Place the syringe filter onto syringe.
    e. Filter your solution into the sterile 15mL centrifuge tube.
    f. Repeat Step e.
    g. Store in 1mL aliquots on -20°C (-4°C can be considered too).
    h. **Always** use 1uL of antibiotic per 1mL of culture media.

2. **Chloramphenicol Stock** *(25mg/mL)*
    a. Weigh the 0.250g of ampicillin powder and measure 10mL of 99.9% pure ethanol.
    b. Mix your antibiotic with your 99.9% pure ethanol.
    c. Store in 1mL aliquots on -20°C (-4°C can be considered too).
    d. **Always** use 1uL of antibiotic per 1mL of culture media.

3. **Kanamycin Stock** *(75mg/mL) light sensitive*
    a. Weigh the 0.750g of ampicillin powder and measure 10mL of ddH2O.
    b. Mix your antibiotic with your ddH2O.
    c. Absorb your solution with the sterile syringe.
    d. Place the syringe filter onto syringe.
    e. Filter your solution into the sterile 15mL centrifuge tube.
    f. Repeat Step e.
    g. Store in 1mL aliquots on -20°C (4°C can be considered too).
    h. **Always** use 1uL of antibiotic per 1mL of culture media.

B. Prepare LB plates with Ampicillin, Chloramphenicol and Kanamycin (Source: IGEM)

1. Prepare three separate bottles with 300mL LB broth each (12 plates per 300mL).
2. Add 4.5g of Agar to each bottle. (15g of agar per 1L, **4.5g agar per 300mL**)
3. Label the bottles, add today's date along with the names of the students who prepared it.
4. Cover the top of the bottle with aluminium foil, add autoclave tape and autoclave.
5. Prepare a sterile area to serve the plates once done autoclaving.
    a. It is recommended you serve them inside a sterile hood that was cleaned beforehand and sterilized with UV light for 15 mins. If unavailable, work near a bunsen burner instead.
6. Once autoclaving is done and the media is at room temperature (but not solid), add...
    a. 300uL of Ampicillin in 300mL broth = final concentration of 100ug/mL
    b. 300uL of Cloramphenicol = final concentration of 25ug/mL
    c. 300uL of Kanamycin to the last bottle = final concentration of 75ug/mL
7. Serve the plates in a sterile area (20-25mL media per plate).
8. Leave to solidify for around 30 to 60 minutes.
9. Make sure to LABEL each and every one of the plates before storing at 4°C.

10. Plates should last around 1-2 months depending on how well stored they are.

Day 1 – Fragment Suspension and Digestion Reactions

!---VERY IMPORTANT, PLEASE READ BEFORE PROCEEDING---!

Step 4 requires the use of "heat block", an apparatus found in the B-266 laboratory. It's a small prop that can heat up tubes to desired temperatures. Make sure to set it up before starting with the suspension protocol. It is a bit slow at heating up and ***you MUST NOT begin the protocol until the thermometer inside the apparatus indicates the temperature has reached 50°C****. Then, you can proceed with the protocol.*

Another important thing is to remove the label around the tube for easier viewing of the inside contents. The tube is a bit weirdly shaped, and the DNA is found around the middle part of the tube because the inside container doesn't go all the way to the bottom. You'll see how it looks once you peel the sticker off. Make sure to be careful when suspending the pellet.

Additionally, remember to set up the Maria Bath ***with anticipation*** *since it does take a while to reach 16°C.*

A. Fragment resuspension (D1F1 and D1F2)

1. Heat up *"bloque seco"* and make sure it reaches a temperature of 50°C.
2. Before opening the tube, spin it down in a microcentrifuge for 3–5 seconds to ensure the DNA is in the bottom of the tube. The pellet can become statically charged and, without this step, can either fly out of the tube or remain in the cap, resulting in loss of yield.
3. Add 20uL nuclease free water, to reach a final concentration of 50 ng/µL. Our experiments have shown that storage concentrations <1 ng/µL result in loss of material due to adherence to the plastic tube in the absence of a carrier such as tRNA.
4. Vortex briefly (15 seconds).
5. Incubate at approximately 50°C for 15–20 min inside the *"bloque seco"*. Heating the tube will ensure the solvent comes in contact with the tiny pellet, even if it is stuck to the side of the tube. Thus, this step will increase the likelihood that the entire pellet will be resuspended.
6. Briefly vortex and centrifuge (15 seconds).
7. Quantify using nanodrop.
8. Store at -20°C once finished.

Materials:

- Microcentrifuge
- Vortex
- 50°C Incubator
- D3Frag3RDX IDT 298bp
- TE Buffer 1X
- Nanodrop
- 20-100uL pipette
- Pipette tips

B. Digestion Reactions (EcoRI and SpeI)

1. Obtain four PCR tubes and name them F1, F2, pC and pK.
2. Add the following reagents inside its respective PCR tube.

Day 1 - Digestion PCR Tube Contents

	A	B	C	D	E
1	Reagents	F1	F2	pC	pK
2	EcoRI	0.5uL	0.5uL	0.5uL	0.5uL
3	SpeI	0.5uL	0.5uL	0.5uL	0.5uL
4	NEBuffer™ r2.1	1.0uL	1.0uL	1.0uL	1.0uL
5	DNA	3uL D1F1	3uL D1F2	4uL pSB1C3	4uL pSB1K3.m1
6	Nuclease Free Water	5uL	5uL	4uL	4uL
7	Total Volume	10uL	10uL	10uL	10uL

3. Incubate at 37°C for two hours.

NOTE:
4uL of plasmid should contain ~100ng of plasmid since the plasmids are at a concentration of 25ng/uL
3uL of insert should contain ~150ng of insert since the concentration of the fragments is 50ng/uL
!--Insert uL are subject to change depending on what our mentors say--!

C. Ligation Reactions - Cohesive End Ligation Protocol using Hi-T4 DNA Ligase (SOURCE: NEB)
***Protocol originally taken from NEB Website, adapted according to CRV's suggestions.**

1. Obtain purified vector/plasmid and suspended insert fragments.
2. Grab two PCR tubes and name them D1F1 and D1F2 respectively.
3. Set up the following reaction in a PCR tube **on ice**. *IMPORTANT* **The T4 DNA Ligase Reaction Buffer should be thawed and resuspended at room temperature. Buffer contains ATP, don't add to solution until it is fully dissolved and no particles can be seen.**

Day 1 - Ligation PCR Tube Contents

	A	B	C
1	Reagents	D1F1	D1F2
2	T4 DNA Ligase Reaction Buffer (10X)	1uL	1uL
3	Vector DNA	1uL (25ng of pSB1C3)	1uL (25ng of pSB1K3.m1)
4	Insert DNA	2uL (100ng of D2F1)	2uL (100ng of D2F2)
5	Nuclease-free water	5.5uL	5.5uL
6	Hi-T4 DNA Ligase (add last)	0.5uL	0.5uL
7	Total Volume	10uL	10uL

***Important note regarding the Ligase, it contains 400 units/uL. Might want to consider diluting 1uL in 4uL of water to make it 100u/uL?**

3. Gently mix the reaction by pipetting up and down and microfuge briefly.
4. For cohesive (sticky) ends (1X T4 DNA Ligase Buffer), **incubate between 25-50°C for 4 hours minimum or overnight**.

5. Heat inactivate at 65°C for 10 minutes.
6. Once the ligation is done, the sample tube will be stored at -20°C.

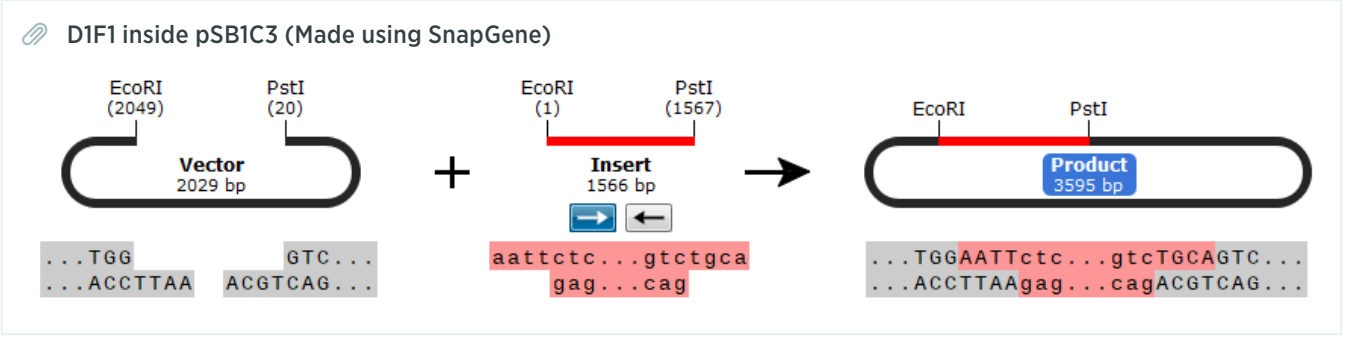
Materials:

- Microcentrifuge
- Ice Box
- Maria Bath at 16°C
- D3Frag3RDX IDT 298bp
- Purified vector (pUC19)
- Hi-T4 DNA Ligase (add last)
- StickTogether DNA Ligase Buffer (2X)
- Nuclease-free water
- One microcentrifuge tube
- 2-20uL pipette
- 0.2-2uL pipette
- Pipette tips

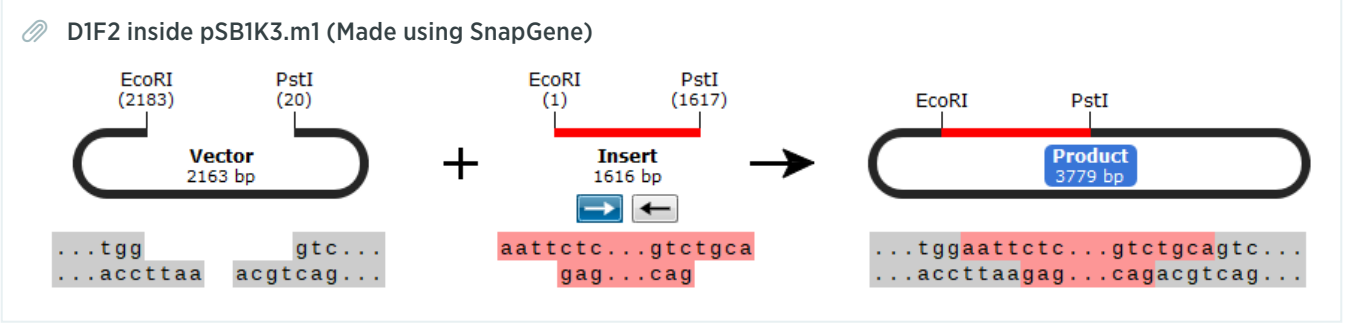
Tips from the CRV Workshop (Friday June 11th):

- For every 1uL of vector add 3uL of insert (or rather, in terms of nanograms)
- Run ligation reaction at the heat indicated by the Ligase's instructions
- Use Maria Bath for heat conditions, leave incubating overnight
- If you have to incubate in a rush, leave it running for 4 hours minimum
- Buffer contains ATP, don't add to solution until it is fully dissolved and no particles can be seen

Expected Results:



Visualization of Device 1 Fragment 1 inside plasmid pSB1C3.



Visualization of Device 1 Fragment 2 inside plasmid pSB1K3.m1.

!---VERY IMPORTANT, PLEASE READ BEFORE PROCEEDING---!

Set up maria bath at **exactly 42°C** before proceding with the transformation protocol.

D. Bacterial Transformation

1. Thaw a tube of NEB 10-beta Competent *E. coli* cells on ice for 10 minutes. Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will decrease the transformation efficiency.
2. Add 1-5 µl containing 1 pg-**100 ng** of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex**.
3. Place the mixture on ice for 30 minutes. **Do not mix.** For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes this step is shortened.
4. Heat shock at **exactly 42°C** for exactly 30 seconds. **Do not mix.** Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 30 seconds at 42°C is optimal.
5. Place on ice for 5 minutes. **Do not mix.**
6. Pipette 950 µl of room temperature NEB 10-beta/Stable Outgrowth Medium into the mixture.
    a. Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes this step is shortened. NEB 10-beta/Stable Outgrowth Medium gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.
7. Place at **37°C** for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C while the 60 minutes pass.
    a. Selection plates can be used warm or cold, wet or dry without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in NEB 10-beta/Stable Outgrowth Medium.
10. Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

Day 2 - Colony Screening

A. Colony Screening

1. Verify if there are any colonies present in the experimental plates. If so, proceed to inoculate said colonies in 3mL of LB broth and 3uL of antibiotic (Cloramphenicol or Kanamycin)
2. Incubate at 37°C shaker.

Day 3 - Miniprep and Transformation Validation

A. Run a MiniPrep with the transformed colonies. (pCF1 and pKF2) (SOURCE: QIA MiniPrep Handbook / Video Tutorial **)**

1. Name the samples pCF1 and pKF2.
2. All protocol steps should be carried out at room temperature.
3. Add 3mL of culture media to microtube. Centrifuge 8,000 rpm x 3 minutes until a pellet forms.
4. Discard supernatant.
5. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. No cell clumps should be visible after resuspension of the pellet. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain
6. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, because this will result in shearing of genomic DNA and contamination of plasmid. If continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
7. Add 350 µl Buffer N3. Mix immediately and thoroughly by inverting the tube 4–6 times. The solution should become cloudy.

8. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
9. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 Spin Column by pipetting.
10. Centrifuge for 30–60 s. Discard the flow through.
11. **Recommended:** Wash the QIAprep 2.0 Spin Column by adding 500uL Buffer PB and centrifuging for 30–60 s. Discard the flow through. This step is necessary to remove trace nuclease activity when using endA+ strains, such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains, such as XL-1 Blue and DH5α, do not require this additional wash step.
12. Wash QIAprep 2.0 Spin Column by adding 750uL ml Buffer PE and centrifuging for 30–60 s.
13. Discard the flow through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer. **Important:** Residual wash buffer will not be completely removed unless the flow through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
14. Place the QIAprep 2.0 Spin Column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add **15 µl** Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of each QIAprep 2.0 Spin Column, let stand for 1 min, and centrifuge for 1 min.
15. Flowthrough has desired DNA. Discard column.
16. Store DNA at -20°C
    - For long periods of time, store at -80C

B. Double Digestion (XbaI and PstI)

1. Obtain two PCR tubes and name them pCF1 and pKF2.
2. Add the following reagents inside its respective PCR tube.

DAY 2 - Digestion Tube Contents

	A	B	C
1	Reagents	pCF1	pKF2
2	XbaI	0.5uL	0.5uL
3	PstI	0.5uL	0.5uL
4	NEBuffer™ r2.1	1.0uL	1.0uL
5	DNA	4uL pCF1	4uL pKF2
6	Nuclease Free Water	4uL	4uL
7	Total Volume	10uL	10uL

3. Incubate at 37°C for two hours.

C. Agarose Gel Electrophoresis with Digestion Samples (SOURCE: Plasmid Electrophoresis / Normal Electrophoresis)

1. Add 30 ml 1X TAE buffer to a conical flask. (If there is none, dilute the 50X TAE buffer by 50 times.)
2. Then, add 0.3 g agarose (1%) to the conical flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. Pour the agarose into a gel tray with the well comb in place.
6. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.
7. Once solidified, place the agarose gel into the gel box (electrophoresis unit).

8. Orient the gel with wells (comb removed) facing the BLACK negative electrode. Check if the gel is covered by TAE buffer in the tank.
9. Fill gel box with 1x TAE until the gel is covered.
10. Add 1/6 of 6X loading dye to the DNA to a total volume of <25 µl (depended on the well) before adding to the wells. Mix loading dye to DNA to make the solution colored.
    I. Example, if total sample is 5uL add 1uL of loading dye.

DAY 2 - Agarose Gel Electrophoresis Samples

	A	B	C	D
1	Reagents	Well 1	Well 2	Well 3
2	DNA	5uL 1kb Ladder	3uL pCF1	3uL pKF2
3	Loading Dye 6X		0.6uL	0.6uL

DNA SUBJECT TO CHANGE ACCORDING TO THE CONC. OBTAINED BY THE MINIPREP...

11. Load the sample to the wells (<25 µl/ well)
12. Connect the electrodes to the power supply with the correct color, black to black, red to red. Apply power supply with 120 V. Check if there are bubbles on the negative electrodes.
13. Run the gel at 90 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 80 minutes, depending on the gel concentration and voltage. (The time is variable based on the gel concentration and the size of interested DNA. Be aware the samples run into the gel by checking if the blue band stays on the gel.)
    - **Remember that the voltage depends of the sample.**
14. After electrophoresis for 30-90 min, turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
15. If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 10 mins.
16. Take the gel to imager, and turn UV on to observe bands. *REMINDER: Never run a gel with >200V, as the heat so generated can melt the gel and also easier to cause electric leakage. Range from 80 – 160 V is acceptable. Usually it needs at least 100 ng DNA for a band to be seen and visualized on the UV trans-illuminator.

C. Using GELDOC

1. Open GELDOC tray and clean with alcohol and kimwipes.
2. Place gel in the tray, with the bottom of the gel close to the crack on the GELDOC glass.
3. Turn on GELDOC (back - left side of GELDOC)
4. Press EPIWHITE.
5. Open program in the computer.
6. Press FILE and GELDOC XR.
7. Press Trans UV on GELDOC.
8. Verify that the computer program is set to "UV" and press autoexpose.
9. Once you see the gel and bands, press "Freeze" once you see the best image resolution.
10. Press "Save" and save on IGEM RUM 2021 folder in the Raw format.
11. Click FILE and "Export as .jpeg", set to 100 and save on IGEM RUM 2021 file.
12. Send the image through email (to yourself) for you to add to Lab Notebook.
13. **Change the GELDOC setting back to EPIWHITE before opening the tray,** discard gel on Biohazard, and clean tray with alcohol and kimwipes.
14. Turn off GELDOC.

Day 4 – Double Digestion Reactions

!---VERY IMPORTANT, PLEASE READ BEFORE PROCEEDING---!

*Remember to set up the Maria Bath **with anticipation** since it does take a while to reach 42°C.*

A. Digestion Reactions (EcoRI and PstI)

1. Obtain four PCR tubes and name them F1A, F2A, pA.
2. Add the following reagents inside its respective PCR tube.

DAY 4 - PCR Digestion Tube Contents

	A	B	C	D	E	F
1		F1A		F2A		pA
2	EcoRI	0.5uL	XbaI	0.5uL	EcoRI	0.5uL
3	SpeI	0.5uL	PstI	0.5uL	PstI	0.5uL
4	NEBuffer™ r2.1	1.0uL	NEBuffer™ r2.1	1.0uL	NEBuffer™ r2.1	1.0uL
5	DNA	3uL D2F1	DNA	3uL D2F2	DNA	4uL pSB1A3
6	Nuclease Free Water	5uL	Nuclease Free Water	5uL	Nuclease Free Water	4uL
7	Total Volume	10uL	Total Volume	10uL	Total Volume	10uL

3. Incubate at 37°C for two hours.

NOTE:
4uL of plasmid should contain ~100ng of plasmid since the plasmids are at a concentration of 25ng/uL
3uL of insert should contain ~150ng of insert since the concentration of the fragments is 50ng/uL
!--Insert uL are subject to change depending on what our mentors say--!

C. Ligation Reactions - Cohesive End Ligation Protocol using Hi-T4 DNA Ligase (SOURCE: NEB)
***Protocol originally taken from NEB Website, adapted according to CRV's suggestions.**

1. Obtain purified vector/plasmid and suspended insert fragments.
2. Grab one PCR tube and name it D1A.
3. Set up the following reaction in a PCR tube **on ice**. *IMPORTANT* **The T4 DNA Ligase Reaction Buffer should be thawed and resuspended at room temperature. Buffer contains ATP, don't add to solution until it is fully dissolved and no particles can be seen.**

Table2

	A	B
1	Reagents	D1A
2	T4 DNA Ligase Reaction Buffer (10X)	1uL
3	Vector DNA	2uL (50ng of pSB1A3)
4	Insert #1 DNA	2uL (100ng of D2F1)
5	Insert #2 DNA	2uL (100ng of D2F2)
6	Nuclease-free water	2.5uL
7	Hi-T4 DNA Ligase (add last)	0.5uL
8	Total Volume	10uL

***Important note regarding the Ligase, it contains 400 units/uL. Might want to consider diluting 1uL in 4uL of water to make it 100u/uL?**

3. Gently mix the reaction by pipetting up and down and microfuge briefly.
4. For cohesive (sticky) ends (1X T4 DNA Ligase Buffer), **incubate between 25-50°C for 4 hours minimum or overnight.**
5. Heat inactivate at 65°C for 10 minutes.
6. Once the ligation is done, the sample tube will be stored at -20°C.

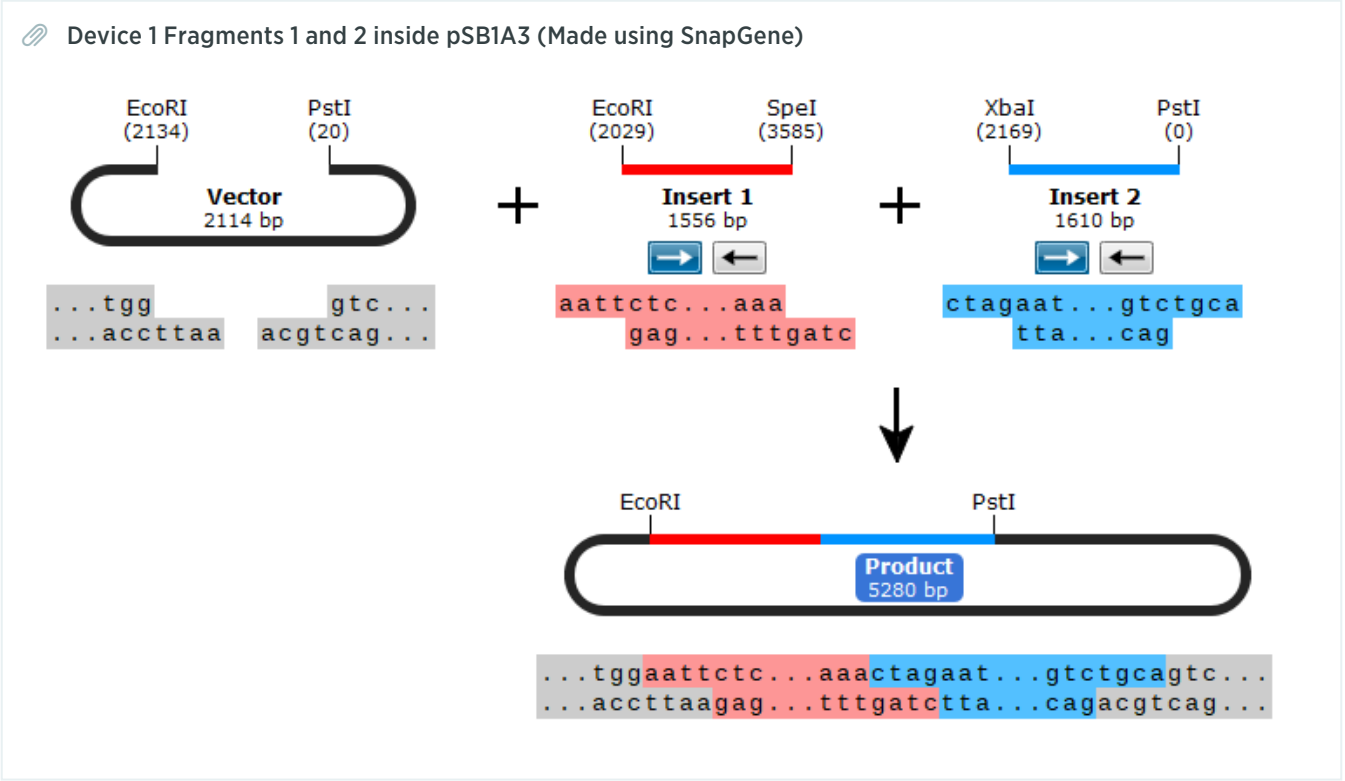
Materials:

- Microcentrifuge
- Ice Box
- Maria Bath at 16°C
- D3Frag3RDX IDT 298bp
- Purified vector (pUC19)
- Hi-T4 DNA Ligase (add last)
- StickTogether DNA Ligase Buffer (2X)
- Nuclease-free water
- One microcentrifuge tube
- 2-20uL pipette[
- 0.2-2uL pipette
- Pipette tips

Tips from the CRV Workshop (Friday June 11th):

- For every 1uL of vector add 3uL of insert (or rather, in terms of nanograms)
- Run ligation reaction at the heat indicated by the Ligase's instructions
- Use Maria Bath for heat conditions, leave incubating overnight
- If you have to incubate in a rush, leave it running for 4 hours minimum
- Buffer contains ATP, don't add to solution until it is fully dissolved and no particles can be seen

Expected Results:



Visualization of Device 1 (Complete) inside plasmid pSB1A3.

!---VERY IMPORTANT, PLEASE READ BEFORE PROCEEDING---!

Set up maria bath at **exactly 42°C** before proceding with the transformation protocol.

D. Bacterial Transformation

1. Thaw a tube of NEB 10-beta Competent *E. coli* cells on ice for 10 minutes. Cells are best thawed on ice and DNA added as soon as the last bit of ice in the tube disappears. Cells can also be thawed by hand, but warming above 0°C will decrease the transformation efficiency.
2. Add 1-5 µl containing 1 pg-**100 ng** of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **Do not vortex**.
3. Place the mixture on ice for 30 minutes. **Do not mix.** For maximum transformation efficiency, cells and DNA should be incubated together on ice for 30 minutes. Expect a 2-fold loss in transformation efficiency for every 10 minutes this step is shortened.
4. Heat shock at **exactly 42°C** for exactly 30 seconds. **Do not mix.** Both the temperature and the timing of the heat shock step are important and specific to the transformation volume and vessel. Using the transformation tube provided, 30 seconds at 42°C is optimal.
5. Place on ice for 5 minutes. **Do not mix.**
6. Pipette 950 µl of room temperature NEB 10-beta/Stable Outgrowth Medium into the mixture.
    a. Outgrowth at 37°C for 1 hour is best for cell recovery and for expression of antibiotic resistance. Expect a 2-fold loss in transformation efficiency for every 15 minutes this step is shortened. NEB 10-beta/Stable Outgrowth Medium gives 2-fold higher transformation efficiency than LB medium; and incubation with shaking or rotating the tube gives 2-fold higher transformation efficiency than incubation without shaking.
7. Place at **37°C** for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C while the 60 minutes pass.
    a. Selection plates can be used warm or cold, wet or dry without significantly affecting the transformation efficiency. However, warm, dry plates are easier to spread and allow for the most rapid colony formation.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in NEB 10-beta/Stable Outgrowth Medium.

10. Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

Day 5 - Colony Screening

A. Colony Screening

1. Verify if there are any colonies present in the experimental plates. If so, proceed to inoculate said colonies in 3mL of LB broth and 3uL of antibiotic (Ampicillin)
2. Incubate at 37°C shaker.

Day 6 - Miniprep and Transformation Validation

A. Run a MiniPrep with the transformed colonies. (pAD1) (SOURCE: QIA MiniPrep Handbook / Video Tutorial)

1. Name the samples pAD1.
2. **All protocol steps should be carried out at room temperature.**
3. Add 3mL of culture media to microtube. Centrifuge 8,000 rpm x 3 minutes until a pellet forms.
4. Discard supernatant.
5. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. No cell clumps should be visible after resuspension of the pellet. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain
6. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, because this will result in shearing of genomic DNA and contamination of plasmid. If continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
7. Add 350 µl Buffer N3. Mix immediately and thoroughly by inverting the tube 4–6 times. The solution should become cloudy.
8. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.
9. Apply 800 µl of the supernatant from step 4 to the QIAprep 2.0 Spin Column by pipetting.
10. Centrifuge for 30–60 s. Discard the flow through.
11. **Recommended:** Wash the QIAprep 2.0 Spin Column by adding 500uL Buffer PB and centrifuging for 30–60 s. Discard the flow through. This step is necessary to remove trace nuclease activity when using endA+ strains, such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains, such as XL-1 Blue and DH5α, do not require this additional wash step.
12. Wash QIAprep 2.0 Spin Column by adding 750uL ml Buffer PE and centrifuging for 30–60 s.
13. Discard the flow through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer. **Important:** Residual wash buffer will not be completely removed unless the flow through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
14. Place the QIAprep 2.0 Spin Column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add **15 µl** Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of each QIAprep 2.0 Spin Column, let stand for 1 min, and centrifuge for 1 min.
15. Flowthrough has desired DNA. Discard column.
16. Store DNA at -20°C
    - For long periods of time, store at -80C

B. Double Digestion (EcoRI and PstI)

1. Obtain one PCR tube and name it pAD1.
2. Add the following reagents inside its respective PCR tube.

Table1

	A	B
1	**Reagents**	**pAD1**
2	**EcoRI**	0.5uL
3	**PstI**	0.5uL
4	**NEBuffer™ r2.1**	1.0uL
5	**DNA**	4uL pAD2
6	**Nuclease Free Water**	4uL
7	**Total Volume**	10uL

3. Incubate at 37°C for two hours.

C. Agarose Gel Electrophoresis with Digestion Samples (SOURCE: Plasmid Electrophoresis / Normal Electrophoresis)

1. Add 30 ml 1X TAE buffer to a conical flask. (If there is none, dilute the 50X TAE buffer by 50 times.)
2. Then, add 0.3 g agarose (1%) to the conical flask.
3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).
4. Let agarose solution cool down to about 50 °C (about when you can comfortably keep your hand on the flask), about 5 mins.
5. Pour the agarose into a gel tray with the well comb in place.
6. Place newly poured gel at 4 °C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.
7. Once solidified, place the agarose gel into the gel box (electrophoresis unit).
8. Orient the gel with wells (comb removed) facing the BLACK negative electrode. Check if the gel is covered by TAE buffer in the tank.
9. Fill gel box with 1x TAE until the gel is covered.
10. Add 1/6 of 6X loading dye to the DNA to a total volume of <25 µl (depended on the well) before adding to the wells. Mix loading dye to DNA to make the solution colored.
    I. Example, if total sample is 5uL add 1uL of loading dye.

Table3

	A	B	C
1	Reagents	Well 1	Well 2
2	DNA	5uL 1kb Ladder	3uL pAD1
3	Loading Dye 6X		0.6uL

DNA SUBJECT TO CHANGE ACCORDING TO THE CONC. OBTAINED BY THE MINIPREP...

11. Load the sample to the wells (<25 µl/ well)
12. Connect the electrodes to the power supply with the correct color, black to black, red to red. Apply power supply with 120 V. Check if there are bubbles on the negative electrodes.

13. Run the gel at 90 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 80 minutes, depending on the gel concentration and voltage. (The time is variable based on the gel concentration and the size of interested DNA. Be aware the samples run into the gel by checking if the blue band stays on the gel.)
    - **Remember that the voltage depends of the sample.**

14. After electrophoresis for 30-90 min, turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
15. If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 10 mins.
16. Take the gel to imager, and turn UV on to observe bands. *REMINDER: Never run a gel with >200V, as the heat so generated can melt the gel and also easier to cause electric leakage. Range from 80 – 160 V is acceptable. Usually it needs at least 100 ng DNA for a band to be seen and visualized on the UV trans-illuminator.

C. Using GELDOC

1. Open GELDOC tray and clean with alcohol and kimwipes.
2. Place gel in the tray, with the bottom of the gel close to the crack on the GELDOC glass.
3. Turn on GELDOC (back - left side of GELDOC)
4. Press EPIWHITE.
5. Open program in the computer.
6. Press FILE and GELDOC XR.
7. Press Trans UV on GELDOC.
8. Verify that the computer program is set to "UV" and press autoexpose.
9. Once you see the gel and bands, press "Freeze" once you see the best image resolution.
10. Press "Save" and save on IGEM RUM 2021 folder in the Raw format.
11. Click FILE and "Export as .jpeg", set to 100 and save on IGEM RUM 2021 file.
12. Send the image through email (to yourself) for you to add to Lab Notebook.
13. **Change the GELDOC setting back to EPIWHITE before opening the tray,** discard gel on Biohazard, and clean tray with alcohol and kimwipes.
14. Turn off GELDOC.