<u>Instructions: Write the date, your name, and what you did in the lab on that day. Make sure to keep this continuously updated so we don't mess up.</u>

<u>4/24/17 (Christina)</u>

We followed a digest protocol.
Digest: One or more restriction enzymes are used to digest the DNA resulting in either non-directional or directional insertion into the compatible plasmid.

We had 4 tubes including 2 controls, which included Puc 19 and 2 digests. The following parts were digested with the enzymes written adjacent to the part.

1. Part 1 - RllClpXP with E and S enzymes
2. Part 2 - CI with X and P enzymes
3. Control 1 - Puc19 E and S enzymes
4. Control 2- Puc19 X and P enzymes

We utilized Cutsmart Buffer.

RllClpXP
4 ul (E) + 2 ul (S) + 34 uL (DNA) = 40 uL reaction

CI
4 uL (Buffer) + 2 uL (X) + 2 uL (P) + 22 uL (DNA) = 40 uL reaction

DNA → Spin → Pipet

We inserted the microcentrifuge tubes inside the Edvo Cycler
37C for 60 min
80C for 20 min

We inserted the tubes in this order.

Puc 19	Puc19XP
RllClpXP	CI

4/25/2017 (Gaurav)

Results from the gel indicate that the CI part was not present and the R0011ClpXP had impurities in it (Gel was stained with FastBlast). We have stopped this workflow, and are starting over using stock solutions from GA Tech's Lab.

Transformation-

List of Samples: ClpXPCI (3K5), ClpXP (AK3), CI Bricking R (IC3), R11ClpXP (Unknown Backbone)

Transformation Solution- 2uL of sample, 50uL of competent cells, 950uL of SOC Outgrowth Medium

Protocol- Followed New England Biolabs Protocol for Transformation

Plates (Total of 7 plates)-

- ClpXPCI- Kan
- ClpXP- Carb, Kan
- CI Bricking R- Carb
- R11ClpXP- Tet, Kan, Carb

***Carbenicillin** can be used in place of **ampicillin**. **Carbenicillin** is more stable, so it is potentially more effective at selecting only bacteria containing the plasmids of interest (for example, fewer satellite colonies will grow). However, it is also more expensive.

Stored in incubator at 37.3 C at 9:25 AM.

4/26/2017 (Gaurav)-

Checked plates for growth. ClpXP (Carb), ClpXP (Kan), and ClpXPCI (Kan) had growth, while R0011ClpXPCI and CI Bricking R (Carb) had no growth. We are now running a colony PCR on the two plates with growth.

i) Diluted 1uL of DNA from each colony in 40 uL of mqH20.
ii) Master Mix (In order)- 63 uL mqH20, 20uL Q5 Reaction Buffer, 5uL VF2 Primer, 5uL VR Primer, 2uL dNTP, 1uL Q5 Polymerase. Total Master Mix is 96uL for 24uL to be distributed to each PCR Tube. Following aliquot into each tube, 1uL (2) of colony dilution was added to each appropriate tube.

iii) Run in thermocycler with program 1.

ClpXPCI (Kan)	ClpXP (Kan)
ClpXP (Carb)	puc19

Plates with successful transformations are stored in fridge, unsuccessful transformations are in incubator to see whether any growth occurs. Colony dilutions are also stored in fridge.

4/27/17 (Christina Lee)

We ran a gel electrophoresis to visualize our parts and their presence.

<u>How to Make a Gel</u>

Materials
50 mL of 1X TBE
.50g agar

Protocol

1. Bring buffer to 100 celsius add stir bar (Microwave ~ 2 min 30 sec)
2. Add agar and stir vigorously on stir/hot plate
3. Mix until agar dissolves completely
4. Add 10 uL of gel dye (found in cabinet)
5. Cool to 60 celsius and pour into molds
6. Add combs and let solidify (~20 minutes)
7. Remove from mold and store in TAE buffer

Microwave Instructions

1. Time
2. Power
3. 5 (50% power)
4. Start

Gel Order

1. Empty
2. Ladder (100uL)
3. Sample Puc19
4. ClpXP carb
5. Ladder (500 bp)

6. Ladder (100 bp)
7. Sample ClpXP (kana)
8. ClpXP CI (kana)
9. 500 bp ladder

Dilution

- **100 bp ladder**
  - 4 uL of water
  - 2 uL DNA ladder
- **500 bp ladder**
  - 2 uL of water
  - 4 uL DNA ladder

Running a gel

120 Voltage
45 Minutes
200 MA

4/27/2017 (Gaurav)-

Calculations for potential ligation-

1.1.1. 20uL total reaction = 1uL Vector + uL Part 1 + uL Part 2 + 2uL Ligase Buffer + 1uL Ligase + uL dH_2O

Parts	Length of Insert	Length of Vector	Total	~[DNA] = 1/total length in kilobases (kb)	uL
Vector IC3	1000	2200	3200	0.31	1 uL
Part 1 ClpXP AK3	1899	3189	5088	0.197	5.08 uL
Part 2 ClpXPCI 3K5	2674	2936	5610	0.178	1.74 uL

If buffer precipitate forms, vortex to dissolve the precipitate. Heat the buffer with hands first since increasing temperature increases solubility.

DON'T VORTEX ENZYMES

4/27 Error Analysis (Christina)

Only our ladders showed up in our gel.
Our control (Puc19) did not show up.
We used a different program (3), which is programmed for digest. We should have ran it in program 1.

4/27 Finishing Up PCR (Kevin)
Use 2 uL of colonies instead of 1
Spun down PCR tubes
Use Program 001 in PCR machine

PCR Layout

	ClpXP Carb		ClpXP Kan	
	ClpXP CI		Puc19	

4/27/2017 (Gaurav)-

Inoculated liquid cultures from colony dilution

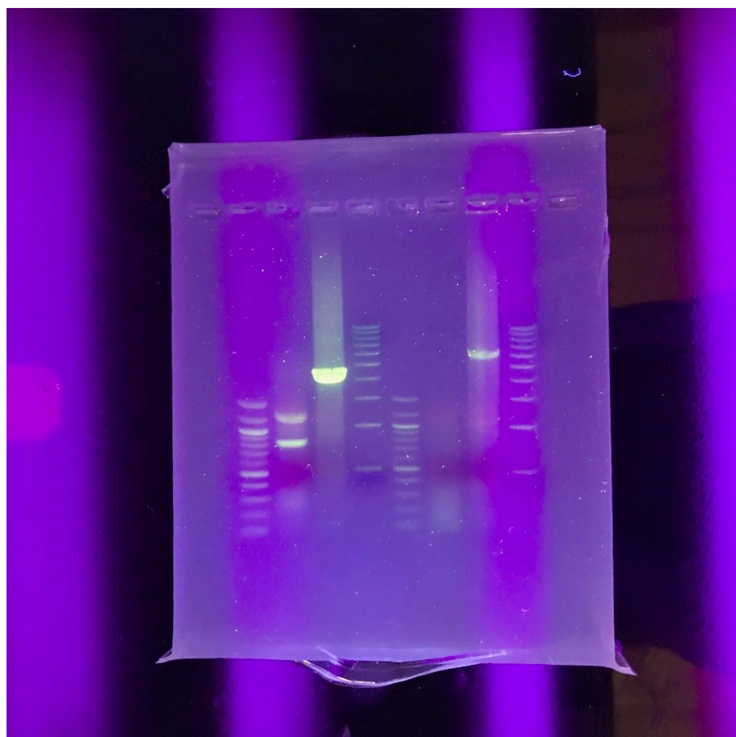
Liquid Culture: 39uL colony dilution, 5mL LB, 5uL Antibiotic Resistance
ClpXP Carb contains 6uL of kan resistance instead of 5uL.
Placed in incubator

Gel has solidified. Left in enclosed environment under box for use tomorrow.

Thermocycler reaction completed. PCR products stored in PCR box in refrigerator. Placed in same arrangement as table above.

4/28/2017 (Christina)-
Ran a gel (2nd time after our 1st one failed)

Gel Results
Non-specific binding: Nothing (ClpXP `kana)
ClpXP Carb and ClpXP CI



MiniPrep

1. Grow 1-5 mL culture overnight in a 10-20 mL culture tube.
2. Centrifuge at 10,000 x g for (depends on centrifuge min). We ran for 10 minutes.
3. Add 250 uL Solution I mixed with RNase A. Vortex to mix thoroughly. Transfer suspension into a new 1.5 mL microcentrifuge tube.
4. Add 250 uL Solution II. Invert and gently rotate (2-3 times) the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary. Avoid vigorous mixing and do not exceed a 5 minute incubation.
5. Add 350 uL Solution III. Immediately invert several times until a flocculent white precipitate forms. Centrifuge at maximum speed (17,900 rep. 10 minutes) A compact white pellet will form. Promptly proceed the next step.
6. Insert a HiBind DNA Mini Column into a 2 mL Collection Tube.
7. Transfer the cleared supernatant from Step 6 by CAREFULLY aspirating it into the HIBind DNA Mini Column. Centrifuge at maximum speed for 60 seconds
8. Add 500 uL HBC Buffer diluted with isopropanol → centrifuge (60 seconds)
9. Centrifuge at maximum speed (17,500) for 60 seconds. Discard the filtrate and reuse the collection tube.
10. Add 750 uL DNA Wash Buffer diluted with ethanol. Centrifuge at maximum speed for 60 seconds. Discard the filtrate and reuse the collection tube.
11. ****Make sure to do this step even though it is crossed out. Repeat step 10 for a second DNA Wash Buffer wash step.
12. Centrifuge the empty HiBind DNA Mini Column at maximum speed for 2 minutes to dry the column. This step is critical for removal of trace ethanol that may interfere with downstream applications.
13. Transfer the HiBind DNA Mini Column into a nuclease-free 1.5 mL microcentrifuge tube.
14. Add 30 uL Elution Buffer. Let sit at room for 5 minutes. Centrifuge at maximum speed for 60 sec.
15. Store eluted DNA at -20 C

Calculating Concentration
-Blank with elution buffer
-1 uL of each

ClpXP (Carb) = A260/A280: 1.85
72.5 ng/uL

ClpXP CI = *Low copy* 3K5 = A260/A280 = 1.91
23.5 ng/uL

Making Buffer
20 mL
50X (V) = 1000 (1X)

V = 20 mL of buffer

980 mL of DI water

*Tip: Make sure you burp before you put the stock back

5/1/17 (Christina)

Digest

40 uL reaction

Parts:
PlambdaLacI (E & S)
PlambdaLacI

TsPurple (X & P)
AmilCP

BB-IC3 (E & P)

PlambdaLacI (77.5)
12.9 uL DNA
30.1 uL MQ Water
5 uL Buffer
1 uL E
1 uL S

PlambdaLacI (97.7)
10.3 uL DNA
32.7 uL MQ Water
5 uL Buffer
1 uL E

1 uL S

Amil CP
5.5 uL DNA
32.7 uL Water
5 uL Buffer
1 uL X
1 uL P

tsPurple
2.8 uL DNA
40.2 Water
5 uL Buffer
1 uL X
1 uL P

IC3 (BB)
2 uL E
1 Pst
23 uL Water

99.7 PlambdaLacI	77.5 PlambdaLacI

	Backbone in Middle (IC3)
Amil	TsPurple

Program 3 - Digest

EX _____SP

E plambdaLacITspurpleP

5/2/17

New Gel
7.5 DNA
1.5 Loading DNA

500 bp ladder

1. Empty
2. 100 bp Ladder (5)
3. AMil CP
4. Tspurple
5. IC3
6. 100 bp ladder
7. 500 bp adder
8. Plambda 77.5 (12)
9. 100 bp Ladder

5/3/17 (Ellie)
Made another gel for pLambdaRIacI
Materials
50 mL of 1X TBE
.50g agar

8. Bring buffer to 100 celsius add stir bar (Microwave ~ 2 min 30 sec)
9. Add agar and stir vigorously on stir/hot plate
10. Mix until agar dissolves completely
11. Add 10 uL of gel dye (found in cabinet)
12. Cool to 60 celsius and pour into molds
13. Add combs and let solidify (~20 minutes)

14. Remove from mold and store in TAE buffer

Shrimp Alkaline Phosphatase (rSAP)

1. Prepare a 20 ul reaction (1 pmol of DNA ends is about 1 ug of a 3 kb plasmid)
    a. DNA: .69 ul
    b. Cutsmart Buffer (10x): 2 ul
    c. rSAP: 1 ul
    d. mQ H_2O: 16.31
2. Incubate at 37 C for 30 minutes
    a. Program 4

Gel Wells

Well 5: p(Lambda)RLacI - 30 uL
Well 9: 500 bp Ladder - 5 uL

5/3/17 (Kevin)

Standy decided we needed more copies of the P(Lambda)RLacI so we decided to run a PCR of it to meet our needs.
PCRed original DNA stock (77.7 and 37.7 concentration)
Created a 48 uL Master Mix. Instead of using 1 uL of diluted DNA, we used 1ul of DNA from miniprep

PCR Setup:

77.7 ng/uL		
		37.7 ng/uL

May 4th (Christina Lee)

PCR Purification Kit

PlambdaR 37.7
455.6 ng/uL
A260/A280: 1.79

PlambdaR 77.5
A260/A280:1.77
441.2 ng/uL

*Different than original protocol since we ran a 20 uL rxn instead of a 50 uL rxn

Step 1:
100 uL Buffer and 20 uL sample into each microcentrifuge tube

Step 2:
Add solution (120 uL) into QIAquick column in a provided 2 mL collection tube.

Step 3:
Centrifuge for 60 seconds

Step 4:
300 uL PE Buffer (Instead of 750 uL) into each tube

Step 5:
Centrifuge for 60 seconds to dry

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

***Multiplying everything by .4 (Calculations)

Digest

2ul E, 1uL S, 7.8uL DNA, 9.2uL mqH20

May 5, 2017
Ligations (Christina)

Parts	Length of Insert	Length of Vector	Total	~[DNA] = 1/total length in kilobases (kb)	uL
Vector (1C3)	805	2070	3875	(1 / 3.875) = .258	1
RlambdaL acI	1115	3252	4367	(1 / 4.367) = .229	3.381
TsPurple	690	2070	2760	(1 / 2.76)	2.139

20 uL Total Reaction: (PlambdaLacITsPurple)
1uL Vector
+ 3.381 uL PlambdaLacI
+ 2.139 uL TsPurple
+ 2 uL Ligase Buffer
+ 1 uL Ligase + 10.48 uL dH_20

20 uL Total Reaction: (PlambdaLacIAmilCp)
1 uL Vector
+ 3.381 uL PlambdaLacI
+ 2.13 uL AmilCp
+ 2 uL Ligase Buffer
+ 1 uL Ligase
+ 10.489 uL dH_20

Put in incubator at 8:45 (60 minutes)
Take out at 9:45

Re-labeled: 100 uL new centrifuge tube (T4 ligase buffer) **Buffer degrades because ATP
DNA does not degrade

5/5/17

Followed NEB Transformation Protocol to transform ligation mixtures into E Coli.

1. C3019H: Thaw a tube of NEB 10-beta Competent *E. coli* cells on ice for 10 minutes.
2. C3019I: Thaw a tube of NEB 10-beta Competent *E. coli* cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
3. 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**.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
6. Place on ice for 5 minutes. Do not mix.
7. Pipette 950 µl of room temperature NEB™ 10-beta/Stable Outgrowth Medium into the mixture.
8. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in NEB™ 10-beta/Stable Outgrowth Medium.
11. 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.

12.

Plated onto Antibiotic Plates. AmilCP and tsPurple plates had no growth. Puc19 had successful growth as well as R11ClpXPCI.

5/9/17 (Christina Lee)

Colony PCR
50 uL reaction (We will continuously run 50 uL reactions)
Protocol: divide everything by 2

Materials

Materials
31.5 uL of dH_20 → PCR tube 40 uL of d_2H0 → Microcentrifuge
10 uL of Buffer (Q5 Reaction Buffer)
2.5 uL VF_2
2.5 uL VR
1 uL dNTP
.5 uL Q5 Polymerase
2 uL Colony DNA (diluted in microcentrifuge tube → PCR tube)

Program 1 = Colony PCR

5/12/17

Liquid Culture
5 uL of antibiotics (Kanamycin) 1k3
Placed in incubator, set to shake

5/15/17 (MiniPrep)

Followed mini prep protocol

30 uL of elution buffer instead of 50 uL

Nanodrop

- Using elution buffer (1 uL) for blanking
- Concentrations
    - R0llClpXPCI:

  A260 (10 mm): 0.876
  A260/A280: 1.87
  43.8 ng/ul
  or
  A260 (10 mm): 1.264
  A260/A280: 1.66
  63.2 ng/ul

Transformations
May 16 2017

- 50 uL of Competent cells
- 2 uL of plasmid DNA
- Skip serial dilutions
- 4 transformations: TsPurple (1C3), B0015 6A1, RFP 1AT3, Plambda RFP 3T5,
- Incubator at 9:15
- Take out at 10:15
- 150 uL of each dilution → selection plate

Competent Cell Day- 5/17/17

- We put in keio wild at 7:45 am into the LB
- DH10 at 7:50

- Started to spin at 8:09

Transformation of Competent Cells - 5/18/17
Flowing transformation protocol but using ClpX and ClpP K.O.

8/1/17 (Gaurav)

I digested the stock backbones IC3, IA3, IT3, and IK3 using the below protocol.

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)
  - 5 ul NEB Buffer 2
  - 0.5 ul BSA
  - 0.5 ul EcoRI-HF
  - 0.5 ul PstI
  - 0.5 ul DpnI (Used to digest any template DNA from production)
  - 18 ul dH20
- Digest Plasmid Backbone
  - Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
  - Add 4 ul of Enzyme Master Mix
  - Digest 37C/30 min, heat kill 80C/20 min

We also ran a gel on the tsPurple Digests from the previous day using 5uL amounts, but there were no results on the gel. We are rerunning the gel with 15uL amounts to see a banding pattern.