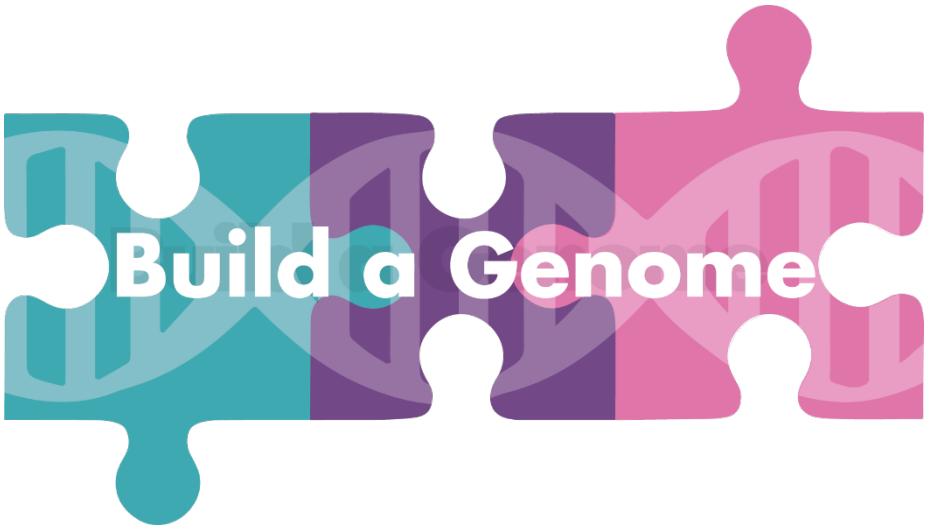

2023 Workshop

August 4-6, 2023
Hartwick College
Oneonta, NY

HARTWICK COLLEGE
est. 1797

NSF

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Build-a-Genome Workshop 2023 Logistics

Timing

You should plan to arrive here in Oneonta some time on Thursday evening, August 3rd to check into the housing (more details below), and we will wrap things up by about 1 pm on Sunday, August 6th. Also, there are no activities scheduled for Thursday Aug 3rd, so you will be on your own for dinner that night (but please save any receipts). But we will start the workshop with breakfast at 8 am on Friday Aug 4th in the lobby of the Johnstone Science Center.

Travel

By car: See the "Driving directions" section below. Please note your starting and ending mileage so we can reimburse your travel.

By bus: The bus will leave you at the bus station in the downtown area on Market St. You can either call/text Eric Cooper (585-746-7035) to pick you up at the bus station (and I am happy to do so!), or call ABC taxi (607) 437-5267 to drive you to the apartments. Please save all receipts, and we will provide you with paperwork at the end of the workshop to get reimbursed for travel.

Lodging

Most of you will stay in one of Hartwick's on-campus "apartments," but a couple of you will be in a "townhouse" (which is nearby). I should mention that both the apartments and townhouses are used as dorms during the school year, but they were built with the purpose of renting them out during the summer, so they are nicer than any dorm I stayed in during college. Here is some information about each:

Apartments: Each individual will have their own multi-bedroom apartment (either a 2- or 4-bedroom). Each apartment also has a kitchenette, its own bathroom(s) and is climate-controlled. Bedding and towels are provided, but (just so you know) the bedrooms only have twin beds.

Townhouses: Each individual will have their own two-story townhouse. The second floor has 3 bedrooms -- one "master bedroom" with a queen-size bed and its own bathroom. The other two bedrooms are singles (with twin beds) and there is a second bathroom in the townhouse. Downstairs has a kitchen and living room. Bedding and towels are provided, and the unit is climate-controlled. You can look at an apartment and townhouse using the links below:

Link to townhouse:
https://www.tours.vividmediany.com/3d-model/hartwick-townhouse-3bedroom-apartment/fullscreen/

Link to apartment:

https://www.tours.vividmediany.com/3d-model/hartwick-apartment-community-4bedroom/fullscreen/

Checking into your housing (beginning 1pm Thurs, August 3rd)

Please go to the front desk inside the main entrance to the apartment building (directions below) to obtain a key for either your apartment or townhouse.

If you arrive between 1pm – 4pm, you will be greeted at the front desk inside the main entrance to the apartment building by James Cummings, who has generously helped with coordinating the housing for the workshop. He will provide your key, which will open both the main door to the building and your apartment unit. He can also, of course, answer any questions you may have.

If you arrive between 4pm-7pm, either Eric Cooper or Chelsea Kotey (who is an amazing Hartwick student) will greet you at the front desk inside the main entrance to the apartment building. If, for some reason, no one is at the front desk when you arrive, then please do not hesitate to call or text Eric Cooper at 585-746-7035, so he can arrange to get you checked in.

If you arrive after 7pm, you can let yourself into the apartment building. The front door will be propped open, and please leave it open for anyone else who arrives after you. The room keys will be in small manila envelopes on the front desk and these will have your name and room number written on them. If you are staying in a townhouse, then your key will still be on the front desk of the apartments, but then you will need to proceed to the townhouses, of course. Please make yourself at home! But, if there are any problems/concerns/questions, do not hesitate to call/text Eric Cooper at 585-746-7035. Or, if there is something wrong (or missing from) your apartment/townhouse, you can also call Hartwick Accommodations (and James Cummings should answer) at (607) 431-4519.

Here is a link to a webpage with more details about the accommodations:
https://hartwick.presence.io/organization/hartwick-accommodations

In case of emergency

If you require any emergency assistance during your stay, please contact Campus Safety at (607) 431-4111.

Checking out of your apartment (by 12 pm Sunday, August 6th)

You might want to bring your luggage with you to the Johnstone Science Center on Sunday morning, since check-out is at 12:00 p.m. Before you leave, please place your keys in the black key box at the front desk. We would also be eternally grateful if you could throw out any trash/recycling in the dumpsters behind the apartment building and strip the linens off of the beds. Thank you so much!

Meals

Please save receipts for reimbursement for any meals during your travel to and from the workshop. We will provide all meals starting Friday morning when the workshop begins at 8 am. Please let us know if you have any dietary restrictions or requests. If you are looking for

something to eat in Oneonta on Thursday evening, there are a variety of restaurants on/near Main St. You would probably want to drive down there from the apartments (well, you *can* walk there in about 20 minutes, but there is a VERY steep hill to get back up to the campus)! Here are a few suggestions:

Latte Lounge: great coffee shop but closes at 5pm
Yellow Deli: good sandwiches; cozy and welcoming place to dine in
Wise Guy Sammy's: more good sandwiches, but is take-out only
Tino's, Sal's, or Nina's: these are three of the many pizza places in our college town (and there is much discussion around here about which is best!)
Simply Thai: really good Thai food; dine in or take-out
Star Mix Grill: excellent gyros, shawarma, etc. A couple of tables in there, but great for take-out.
Sloans's New York Grill: Steaks, burgers, etc.
Brooks BBQ: This is a regionally famous BBQ spot, and is currently just for take-out. Be sure to order the BBQ chicken!
Andre's Blue Ribbon BBQ: Here is a tip from a local – Brooks is fantastic and more well-known, but Andre's is excellent, too! It is more of a mom-and-pop operation and is a little farther out of town, but great for take-out.
B-Side Ballroom: Nice bar with cocktails, really good food, and occasional live music.
Nag's Bar and Grill: Bar food (tacos, bowls, burgers) and has nice booths to sit in.
The Depot: Bar food; has locally famous chicken wings (called "garbage wings")

Good place for a beer: Roots Brewing Company on Main St.

Parking at Hartwick College

If in an apartment:
Parking spaces are right in front of the on-campus apartments. Detailed directions are below.

If in a townhouse:
You can park temporarily in front of the townhouses to load/unload your vehicle, but please minimize the time that your vehicle remains there, as they are emergency lanes. Dedicated parking is in the lot directly in front of the apartment building (for townhouse guests, you may access the parking lot by walking up the wooden stairs next to the laundry building). Handicap-accessible parking for the townhouse quad is available in the parking lot next to van Ess Hall (please refer to the campus maps below).

Meeting Location

We'll be meeting in the Johnstone Science Center. Directions are below:

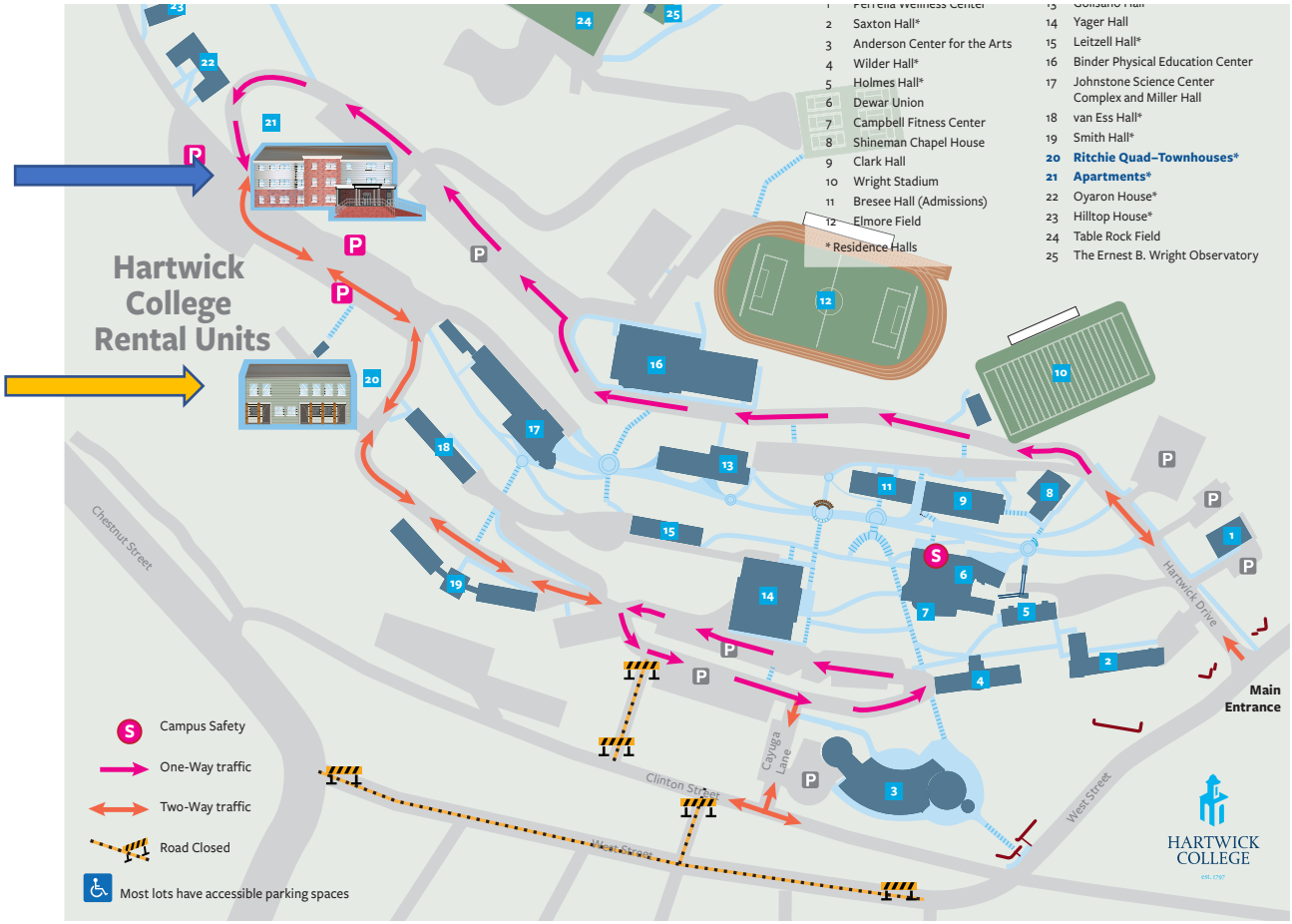
When walking from the apartments: Turn left out of the apartment building and walk through the parking lot. The greenhouse you will see in front of you is attached to the Johnstone Science Center. Proceed to the right of the greenhouse and the door to the building will be on your left, as you pass the greenhouse. Proceed down the hallway to the main lobby, where breakfast will be

available. For talks, we will meet in Room 215, (and when you enter the building on the "main floor," you are actually on the second floor), and we will move to room 412 for lab activities.

When walking from the townhouses: Walk up the stairs next to the laundry building (in the middle of the row of townhouses) to the parking lot. Turn right and you will see the greenhouse in front of you, which is attached to the Johnstone Science Center. Proceed to the right of the greenhouse and the door to the building will be on your left, as you pass the greenhouse. Proceed down the hallway to the main lobby, where breakfast will be available. For talks, we will meet in Room 215, (and, just to confuse you, when you enter the building on the "main floor," you are actually on the second floor) and we will move to room 412 for lab activities

Driving directions to Hartwick College apartments from the "main entrance" at the corner of West St and Hartwick Drive

There is a rather confusing system of one-way roads on the Hartwick college campus. It might be easiest to set Google maps to bring you to 86 West St, Oneonta, NY, which is the address of a residence right across the street from the main entrance to our campus. The route to follow is indicated with red arrows along the top of the map below. The apartments are indicated with the blue block arrow, and the townhouses are indicated with the orange/yellow block arrow.

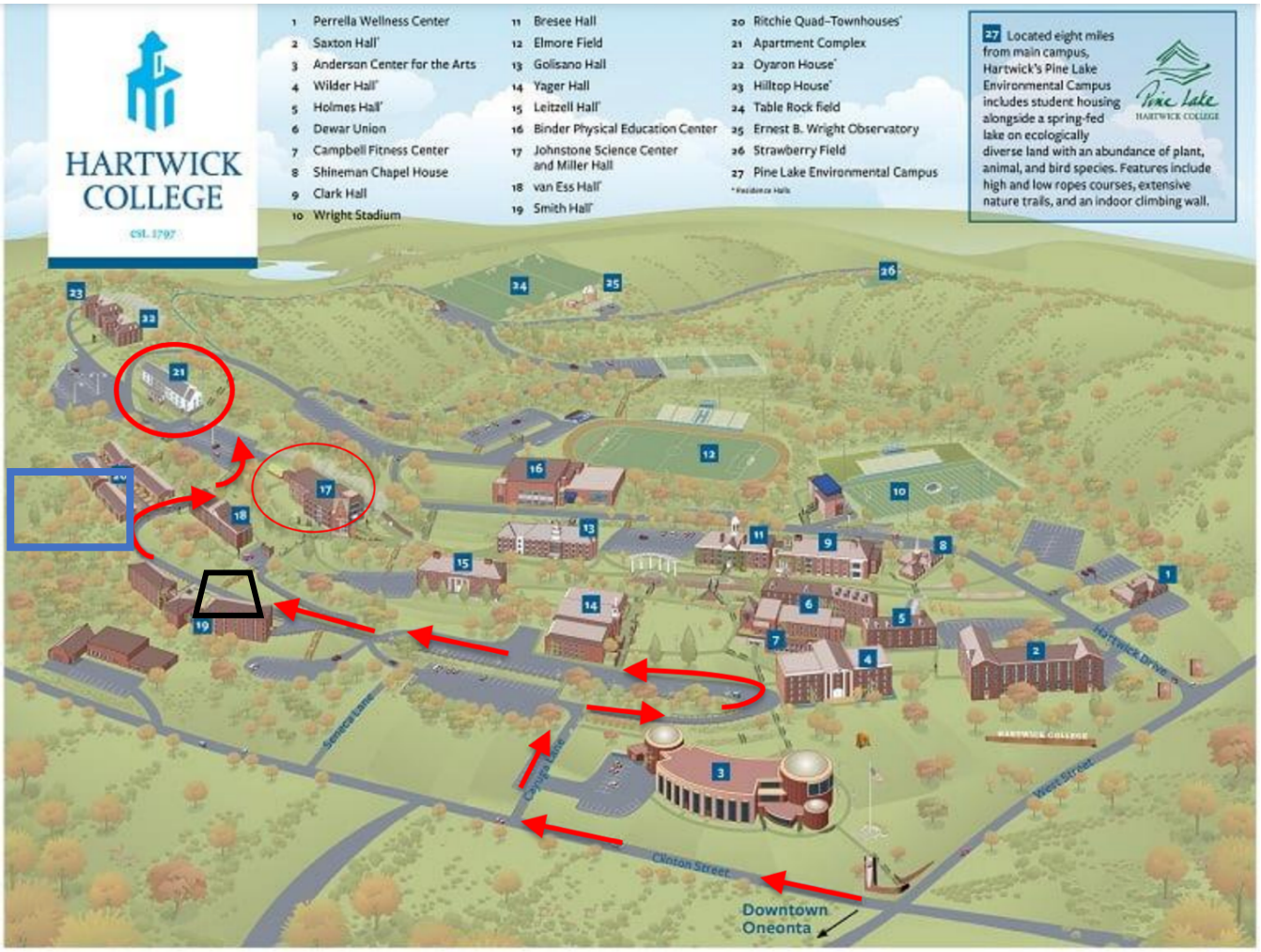


• You will be on West St. when you get to our campus. If you are driving UPHILL on West St with the campus on your left, you will pass a large brick sign that says "Hartwick College" -- the main entrance on Hartwick Drive is just past it on the left (and across the street from the residence at 86 West St.).

• As you enter the campus, you will drive up a very steep hill. After passing a couple of parking lots, turn RIGHT at the fork, immediately after Shineman Chapel (which will be on your left) and toward the playing fields. There may be a sign that says “Hartwick Rental Units” for you to follow. You will continue up another hill on one-way road that will take you across campus.

• As you pass the Binder Physical Education Center (#16) on your right, the road turns right and up another very steep hill. At the crest of the hill, turn left and proceed through another parking lot. At the end of the parking lot, the road then (finally!) goes down a steep hill and curves left. At the bottom of this hill, turn left at the stop sign. You will see the apartment building (#21) on your left and you can park in font. Here is a picture of the apartment building:

• To get to the townhouses from the apartments, drive toward the greenhouse, turn right at the end of the parking lot down a steep hill, and then make the first right. You can park temporarily in front of a townhouse to load/unload your car, but this is an emergency lane, so you will need to park elsewhere (either back up the hill near the greenhouse or by van Ess Hall). See map above or the one I pasted on the next page (in case it is easier to decipher).



In the map above:

Thick red circle = Apartments

Thin red circle = Johnstone Science Center

Blue rectangle = Townhouses

Black trapezoid = van Ess Hall parking lot (one option if you are staying in a townhouse)

AGENDA FOR BAG WORKSHOP AUGUST 2023 v1.0

Friday August 4th

*_For early risers_: the Latte Lounge on Main St. is a great place for coffee!

8:00-8:30 am	**Breakfast** - Johnstone Science Center (JSC) lobby
8:30-8:45 am	**Welcome, introductions and overview** – JSC 215 Eric Cooper/Lisa Scheifele
8:45 -9:15 am	**Introduction to Build-a-Genome** Eric Cooper, Hartwick College
9:15-9:45 am	**Introduction to three BAG Workflows** Lisa Scheifele, Loyola University Maryland
9:45-10:30 am	**Lab: Gene assembly from oligos I: T-PCR reactions**
10:30-11:00 am	**Break** - Johnstone Science Center (JSC) lobby
11:00-12:00 pm	**Neochromosomes and synthesis of large DNAs** - JSC 215 Dr. Leslie Mitchell, CEO, Neochromosome, Inc.
12:15-1:15 pm	**Lunch** - Johnstone Science Center (JSC) lobby
1:15-2:00 pm	**Lab: Gene assembly from oligos II: F-PCR reactions** – JSC 412
2:00-3:15 pm	**Lab: Neochromosome assembly via Golden Gate cloning -** JSC 412
3:15-3:30 pm	**Break** - Johnstone Science Center (JSC) lobby
3:30-4:30 pm	**Ethical issues in synthetic biology** Dr. Todd Kuiken, Policy Analyst - Science & Emerging Technology, Congressional Research Service
4:30-6:15 pm	**Lab: Run gels and transform neochromosome assemblies**
6:30-8:30 pm	**Dinner** in Chesbro Room, Dewar Hall (~5-minute walk from Johnstone)

Saturday August 5th

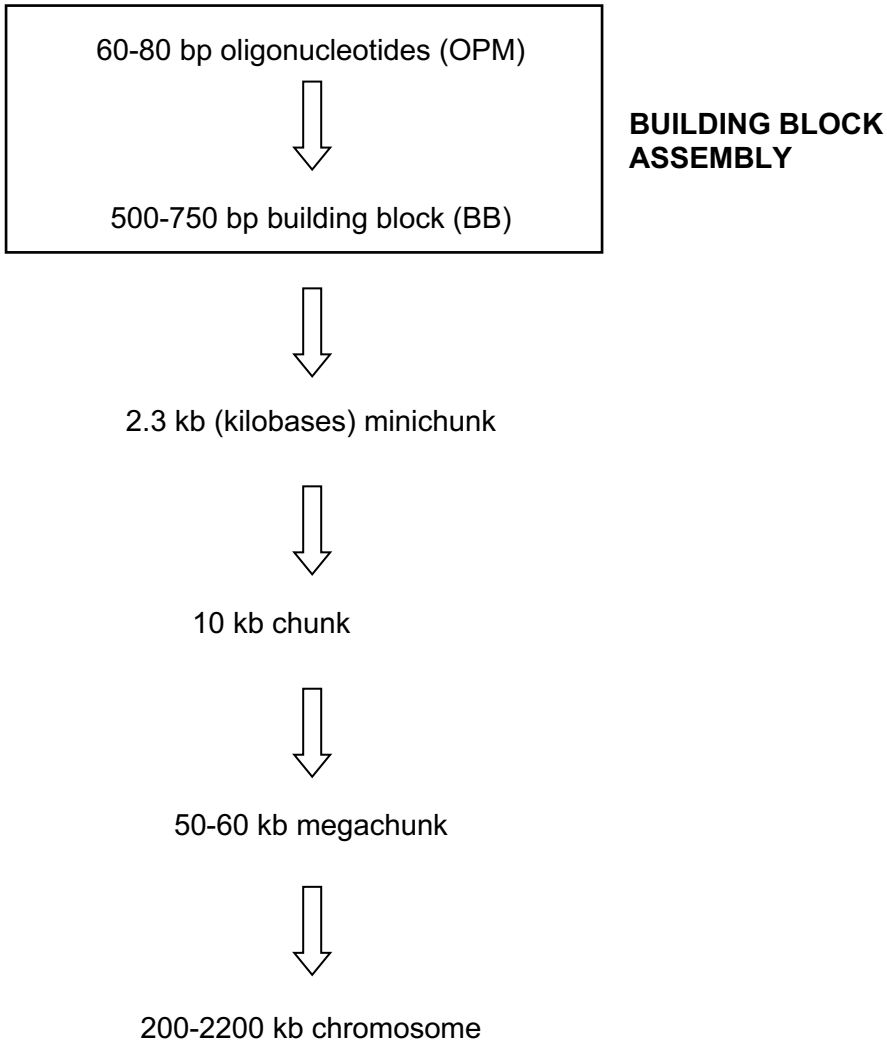
8:15-9:00am	**Breakfast** - JSC lobby

9:00-10:00 am	**The Synthetic yeast project and its applications** - JSC 215 Dr. Yu (Jeremy) Zhao, Postdoctoral fellow in Boeke lab, New York University Institute for Systems Genetics
10:00-10:30 am	**Lab: Analyze previous day's work** – JSC 412
10:30-10:45 am	**Break** - Johnstone Science Center (JSC) lobby
10:45-12:00 pm	**Lab: Yeast culture, transformation, and Scramble** – JSC 412
12:15-1:00 pm	**Lunch** - Johnstone Science Center (JSC) lobby
1:00-2:00 pm	**Case studies in Synthetic Biology** Dr. Fran Sandmeier, University of Colorado-Pueblo
2:00-3:00 pm	**Working session I: Models for incorporating BAG into courses and research projects/Incorporating BAG into syllabi** – JSC 215
3:00-3:15pm	**Break** - Johnstone Science Center (JSC) lobby
3:15-4:30pm	**Lab: Phage electroporation and culture** – JSC 412
4:30-5:30 pm	**Hike** – Table Rock Trail near the apartments
6:00 pm	**Dinner** – Autumn Café, 244 Main St. Oneonta, NY

Sunday August 6th

8:15-9:00 am	**Breakfast** – JSC lobby
9:00-9:30 am	**GeneDesign and bioinformatics**
9:30-10:30 am	**Working session II: Incorporating BAG into syllabi or research projects/ designing your own genes and/or "neochromosomes"** – JSC 215
10:30-11:00 am	**Break**
11:00 am-12:00 pm	**Introduction to QUBEShub and Post-Workshop Activities** – JSC 215
12:00-1:00 pm	**Lunch** - Johnstone Science Center (JSC) lobby
1:00 pm	**Departure**

OVERVIEW OF GENOME ASSEMBLY STEPS



OVERVIEW OF BUILDING BLOCK ASSEMBLY STEPS

Step 1: ***Templateless*** *PCR*

Templateless PCR is performed to form a larger construct (the building block, or BB) from mixtures of oligonucleotides.

Step 2: Finish PCR and Gel electrophoresis

Finish PCR is performed to amplify the full-length building block that is produced in small quantities in the templateless PCR. The success of the finish PCR is gauged by running a portion of the finish PCR reaction on a gel.

Step 3: Ligation and Bacterial Transformation

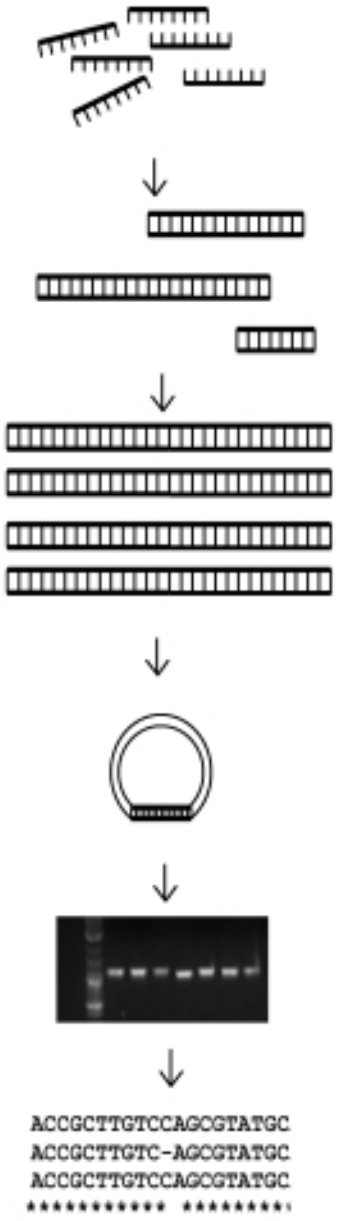
Building blocks that are of the correct size are cloned into a genetic cloning vector and used to transform bacteria. Bacteria are transformed with the cloned construct to create a population of clones for sequencing and future assembly into larger synthetic yeast constructs.

Step 4: Troubleshooting PCR

Since many BBs will not assemble correctly the first time, we change the templateless PCR and finish PCR reaction conditions to enable assembly of each building block.

Step 5: DNA Sequence Analysis

Bacterial clones containing building blocks that are the correct size are sequenced to make sure that there are no sequence errors. Once the DNA has been sequenced, we compare the DNA sequence of each clone to the desired sequence to identify those without sequence errors (we call these “perfect clones”). This completes BB synthesis.



Oligo Mixes for TPCR

Typically, 12-18 oligos are used to synthesize each building block (BB). These oligos are ordered individually and are delivered to us in 96 well plates. Each 96-well plate can contain oligos to make up to 4-5 complete building blocks.

For each building block that you are assigned, you will need to combine these 12-18 oligos together to create a templateless primer mix (TPM). This TPM will be used in a templateless PCR reaction (TPCR), which will anneal the oligos together and extend them to make longer DNA sequences, including only a small amount of the full-length building block sequence. Next week, you will use the outer primer mix (OPM) to amplify the full-length BB in a finish PCR reaction (FPCR) so that the predominant DNA following FPCR is your desired full-length BB.

Templateless primer mix (TPM)

The templateless primer mix (TPM) contains the 12-18 oligos that comprise the building block. All primers must be present at a concentration of 300nM in the TPM (a dilution of 1/20). These dilutions should be done in a 1.7ml microcentrifuge tube, and primer mixes should be stored at -20°C when not in use.

1. Label your tube with: the BB name, the type of oligo mix (TPM), and the primer concentration (300 nM)
2. To the tube, add 10 ul of each of the TPM oligos
3. Add H2O to get a final volume of 200ul.
4. Mix thoroughly and keep the tube on ice.

In some cases, there may be more than 20 oligos that comprise a building block. Mix 10 ul of each oligo and add no water. Even though the concentration will be slightly more dilute than 300 nM this does not seem to have any effect on reaction efficiency.

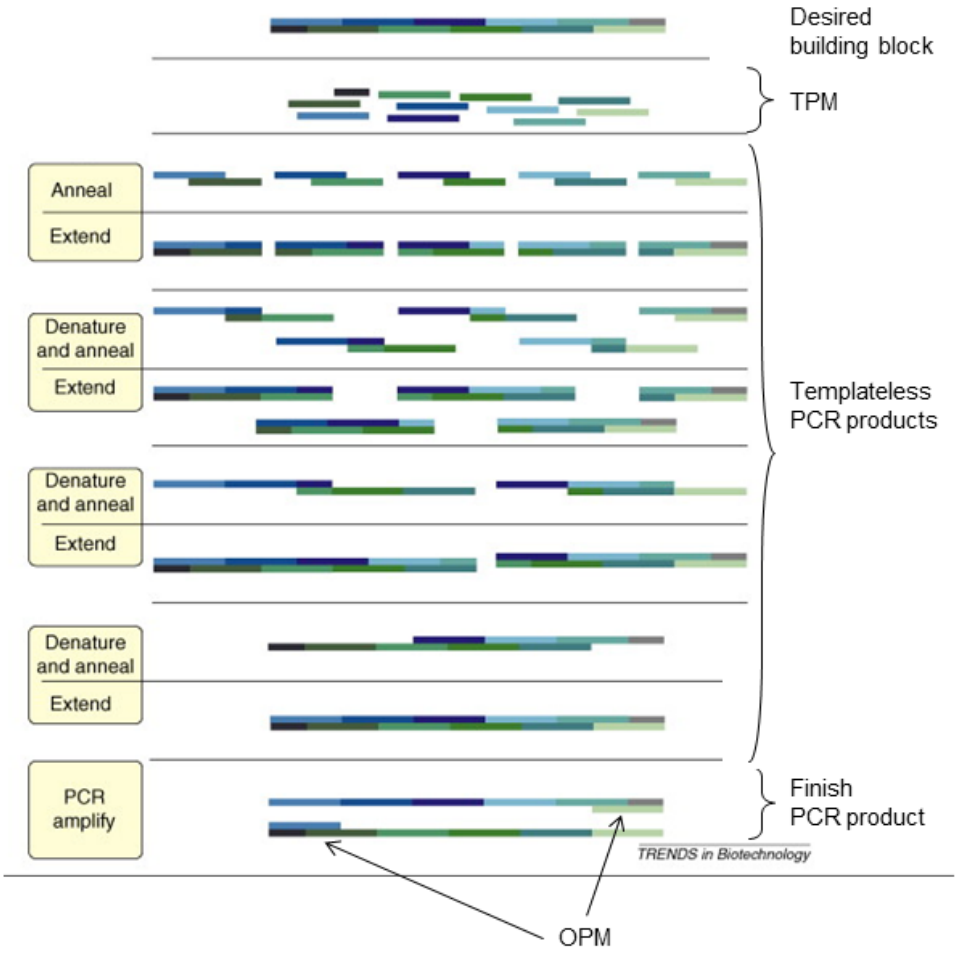
Outer primer mix (OPM)

For each building block, the outer primer mix (OPM) contains **only** the first and last oligos. The outer primers must be present at a concentration of 3uM in the OPM (the oligos must both be diluted by 1/2). Again, these dilutions should be done in a 1.7ml microcentrifuge tube, and primer mixes should be stored at -20°C when not in use.

1. Label your tube with: the BB name, the type of oligo mix (OPM), and the primer concentration (3 uM)
2. To the tube, add 10 ul of each of the OPM oligos
3. Mix thoroughly
4. Put this primer mix into the box marked “OPM mixes” to be stored until next week

Templateless PCR

The goal of templateless PCR is to combine a mixture of short single-stranded DNA oligonucleotides (the TPM) and assemble them into a longer double-stranded DNA sequence, which we term a building block (BB). This building block DNA may have a sequence that is completely new and may not have ever previously existed in nature.
The oligos are annealed together during the templateless PCR reaction and are then extended through multiple rounds of PCR until finally a small amount of the full-length BB (approximately 500-750 bp) is synthesized.



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Volume 27, Issue 2, p63–72, February 2009

Reaction Setup

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either "BB", "PC" or "NC".

1. You will be performing 3 PCR reactions (1 building block (BB) plus one positive control reaction (PC) and one negative control reaction (NC).

2. Combine all reagents listed below into three different PCR tubes (these are the very small tubes).

Reagent	Vol per 25ul reaction
Water	10 ul
2X Phusion Master Mix	12.5 ul
Total	22.5 ul

3. To the first tube, add 2.5 ul of the TPM.

4. To the second tube, add 2.5 ul from the tube marked PC. This will be your positive control reaction.

5. To the third tube, add 2.5 ul of water. This will be your negative control reaction.

6. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate.

7. Place your tubes in the PCR machine in the position for which you signed up. Make sure that you have recorded which sample (BB, PC, or NC) is in each position in the PCR machine.

Reaction Conditions:
1 cycle:
94°C, 3 minutes
55°C, 30 seconds
72°C, 1 minute
5 cycles:
94°C, 30 seconds
69°C, 30 seconds
72°C, 1 minute
5 cycles:
94°C, 30 seconds
65°C, 30 seconds
72°C, 1 minute
20 cycles:
94°C, 30 seconds
61°C, 30 seconds
72°C, 1 minute
1 cycle:
72°C, 3 minutes

Finish PCR

We just used templateless PCR to assemble a small amount of the full-length building block (BB) from oligonucleotides. However, templateless PCR produces very little of the full-length BB. Because templateless PCR is so inefficient at synthesizing the full-length BB, it also creates many smaller DNA fragments that are intermediates in building block synthesis. Therefore, the templateless PCR that we performed last week resulted in a mixture of DNA products: a small amount of the full-length BB is present among DNA products of varying sizes.

The goal of finish PCR is to exponentially amplify the building block sequence so that most of the DNA present in the mixture following finish PCR will be the full-length BB. This is accomplished by using the outer primer mix (OPM), which contains two oligos to amplify only full-length DNA. The OPM primers bind to either end of the building block (which now serves as a template), enabling only BBs that are full-length (and therefore contain both ends of the BB sequence) to be amplified by PCR (see figure on page 11).

Reaction Setup

- Before you set up reactions, make sure you reserve a spot in the PCR machine. You will need to sign up for 3 spaces in the PCR machine-label each with your initials and either “BB”, “PC” or “NC”.

1. Obtain your templateless PCR reactions from the PCR machine.

2. You will no longer need your PC and NC reactions today. To store them, transfer each to a separate tube by pipetting the liquid into a 1.7 ml tube. Label each tube with:
   - Your initials
   - ”TPCR”
   - Either “PC” or “NC”

Put these tubes away to be frozen for next week, when we will run it on a gel-if the PC reaction shows a band of DNA and the NC reaction does not, it will indicate that you properly set up your TPCR reactions.

3. For your BB reaction, add 175 ul of water, cap the tube and invert to mix. We will use this TPCR BB reaction in step 4 below.

You will be performing 3 PCR reactions (one building block (BB) plus one **NEW** positive control reaction (PC) and one **NEW** negative control reaction (NC).

4. Combine all reagents listed below into 3 different PCR tubes (the very small tubes).

Reagent	Vol/25ul reaction
Water	8 ul
2X Phusion Master Mix	12.5 ul
OPM	2 ul
Total	22.5 ul

4. To the first tube, add 2.5 ul of the TPCR BB reaction from step 2 above. Check with you instructor if you should keep or discard the remainder of the TPCR BB reaction.

5. To the second tube (PC), add 2.5 ul from the tube labeled PC.

6. To the third tube (NC), add 2.5 ul of water.

7. Cap your tubes and **make sure that they are sealed tightly** so that the liquid will not evaporate. Place your tubes in the PCR machine in the position for which you signed up. Make sure that you have recorded which sample (BB, PC or NC) is in each position in the PCR machine.

Reaction Conditions:

1 cycle:
94°C, 3 minutes

25 cycles:
94°C, 30 seconds
55°C, 30 seconds
72°C, 1 minute

1 cycle:
72°C, 3 minutes

Gel Electrophoresis

So far, we have performed two steps of synthetic gene assembly to create a building block: (1) tempateless PCR to assemble oligos into a small amount of full-length building block, and (2) finish PCR to amplify the full-length BB so that it becomes the most abundant DNA following PCR.

Now, we need to check how well each of these steps worked by running our PCR products (and controls!) on an agarose gel to verify whether we have assembled a significant amount of the full-length BB. Agarose gel electrophoresis will separate DNAs based upon their size. Therefore, if assembly of our BBs has been successful, we expect to see one DNA band whose size should approximate that of our desired BB. You can determine the size of each of your DNAs by comparing the size of your band to the size of the DNA marker (the 2-Log DNA ladder), which you will run on the gel with your DNAs.

Remember, we also have 4 control reactions (the PC and NC controls from the templateless PCR and the PC and NC controls from the finish PCR).

Pouring a Gel:

1. Weigh out 0.5 g of agarose on a piece of weigh paper. Transfer to an Erlenmeyer flask. Add 50 ml of 1x TAE.

2. Place the flask on a hot plate and heat until the agarose is completely transparent and colorless. Using a rubber heat mitt, periodically swirl the flask to ensure that the agarose is melting evenly. If there is any cloudiness or particulates, then place the agarose back on the hot plate for additional time until clear (check frequently).

3. Remove the flask of clear agarose and allow it to cool. This will take about 10 min. If you can hold your hand on the flask for 20 sec, then the agarose is cool enough to pour. (Warning: if poured while too hot, the agarose will leak and warp the plastic gel tray.)

4. While the agarose is cooling, place the gel tray into the gel box and add the black blockers and a comb.

5. When the agarose is cool, bring it to the instructor to add 1 ul of ethidium bromide to the melted agarose (**CAUTION: ethidium bromide is a mutagen. Wear gloves at all times when handling gels and gel equipment!**).

6. Swirl the agarose to incorporate the ethidium bromide and pour the agarose into the gel tray –your gel should be about 1 cm thick.

7. Allow at least 20 minutes for the gel to solidify. Once solid, carefully remove the comb and the black blockers, and place the solidified gel (still on the tray) into the gel box so that the wells are oriented on the same side as the black electrode.

8. Add enough 1x TAE buffer to completely cover the gel by about 1 cm.

Preparing your samples:

1. Transfer each of your PCR products from the small PCR tubes to a new larger tube. Label each tube with either “BB”, “FPCR PC”, or “FPCR NC”.

2. Obtain your TPCR PC and TPCR NC tubes that were stored in the freezer from last week. You should have 5 total samples now.

3. On a piece of parafilm, spot out 2 ul of 5x DNA loading dye with a P20 pipet. You should have 5 spots (one for each of your PCR products). Wait until your gel has started to solidify before beginning this step since the spots will dry out after ~10 minutes.

4. Add 3 ul of water to each spot of dye.

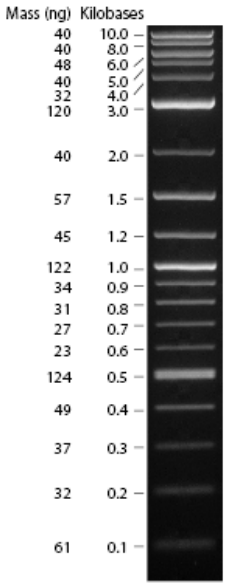
5. Add 5 ul of each PCR product to a separate spot of dye:

    1. TPCR PC reaction
    2. TPCR NC reaction
    3. FPCR PC reaction
    4. FPCR NC reaction
    5. Building block (BB)

Running a Gel:

1. Into the first lane of the gel load 5 ul of the 2-Log DNA ladder (mixed with water and dye).

2. Into lanes 2-5, load 9 ul of each of your PCR products (mixed with water and dye).



3. Place gel lid with electrodes on gel box. REMEMBER DNA RUNS FROM NEGATIVE (black) TO POSITIVE (red). CHECK YOUR LEADS: BLACK SHOULD BE AT THE TOP, RED SHOULD BE AT THE BOTTOM!

5. Set voltage to 100V. You should see bubbles coming up from the wire electrode.

6. After a few minutes, check to make sure everything is going smoothly. Run gel approximately 30 minutes or until the dye is 2/3 of the way down the gel.

7. Take a picture of your gel. Use the DNA ladder (a picture is on the right) to approximate the size of each of your building blocks. Make sure you check that your building blocks are the correct size - do not assume that because you have a band, it is the correct product!

9. You may discard all of your positive and negative control reactions once you have run your gel, but **be sure to keep your building block DNA in a tube that is clearly labeled with your initials and the name of the DNA building block.**

PCR assignment

1. (6 pts) Attach a printout of your gel. Label each lane to indicate what sample was run in each lane.

2. (12 pts) Complete the table below.

PCR product	Are you expecting a PCR product?	If so, what is the expected size (bp)?	Actual size (bp; estimate from the gel picture)
TPCR PC			
TPCR NC			
FPCR PC			
FPCR NC			
BB			

3. (3 pts) The positive control (PC) consists of a plasmid and two primers that have been successfully amplified in the past. Why do you include this control when running a templateless or finish PCR reaction?

4. (3 pts) The negative control (NC) contains only DNA primers and water. Why do you include this control when running a templateless or finish PCR reaction?

5. (6 pts) The only PCR reaction that we did not run on a gel is the BB PCR reaction after templateless PCR but before finish PCR. What would you expect to see if you ran this PCR reaction on a gel? Therefore, why is there little point in running the templateless PCR BB product on a gel?

6. (6 pts) After DNA fragments have been separated by gel electrophoresis, how does the dye enable us to visualize the DNA (which is normally clear and colorless)? Why do we need to use both loading dye and also ethidium bromide?

7. (6 pts) When we set up the templateless PCR, we add all of the oligonucleotides to the reaction (the TPM), but when we set up the finish PCR we only add two of the primers. Why? Is finish PCR a template-dependent or template-independent PCR and what is the difference between the two in terms of what we are trying to accomplish with each reaction?

8. (6 pts) Look at the reaction conditions for the templateless PCR. Notice that the reaction begins at an annealing temperature of 69 degrees and then uses annealing temperatures of 65 and 61 degrees. In contrast, the finish PCR uses only one annealing temperature that is much lower (55 degrees). Why do the annealing temperatures differ and how does this enable the goals of each PCR reaction?

9. (6 pts) If you run your building block DNA on a gel after finish PCR and you get no PCR product, what are two things that you could change (in terms of the reaction conditions) to improve your results to get a PCR product?

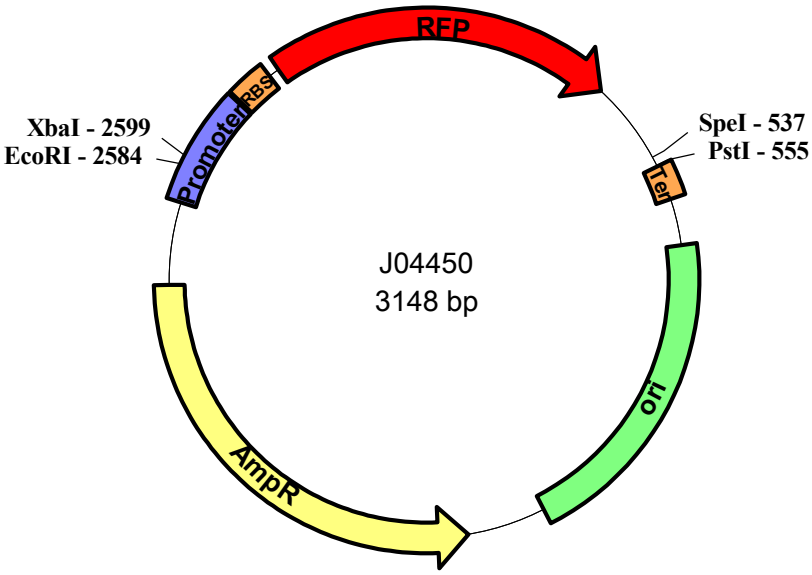
10. (6 pts) When performing PCR, you want to maximize the yield of specific product and minimize the amount of nonspecific product that can result from primers binding to non-identical regions of DNA. If your PCR is not specific enough and you get more than one PCR product, what are two things that you could change (in terms of reaction conditions) to improve your results to get only one PCR product?

Gibson Assembly and Bacterial Transformation

Following the production of our DNA building block (which codes for the Red Fluorescent Protein-RFP) by templateless and finish PCR and verification that most of this DNA is the correct size by gel electrophoresis, we now must clone this building block into a plasmid vector. This cloning step is important for two reasons. First, it allows us to separate the population of DNA molecules into individual DNA molecules, thereby enabling us to screen the individual molecules to identify those that have no errors in the DNA sequence. Second, cloning allows us to create a permanent frozen bacterial stock of each plasmid so that we may store each building block.

Our building block PCR product (the RFP gene) will be cloned into a plasmid called J04450 which has the following features:

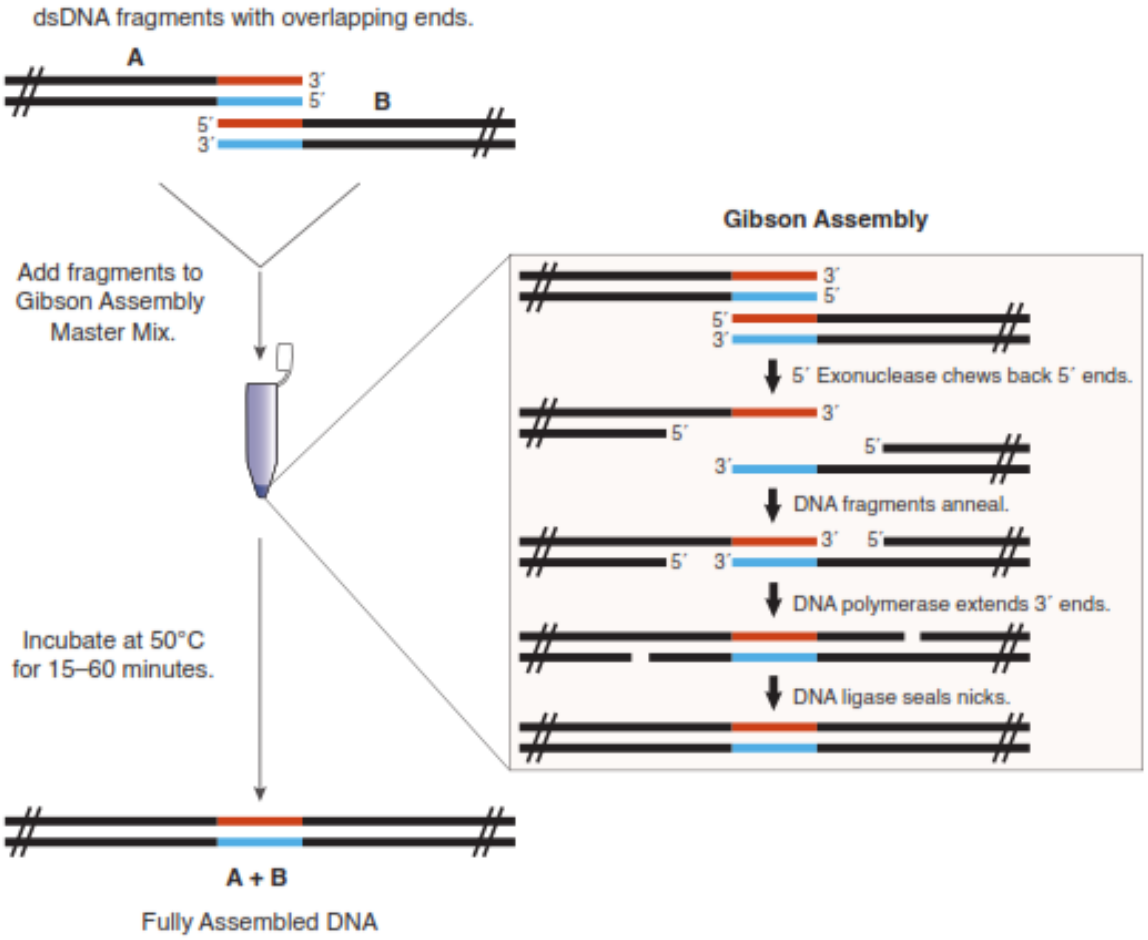
- Bacterial origin of replication (ori)
- Multiple cloning site (MCS)
- Selectable marker (ampicillin resistance gene)
- Bacterial promoter
- Ribosome binding site (RBS)
- Transcription terminator (Ter)



Traditionally, we would clone the PCR product into the vector by using restriction enzymes to cut both the vector and DNA insert and then we would join them together with the enzyme DNA ligase. Instead of the traditional method, we will use a newer method called Gibson assembly. Gibson Assembly was developed by Dr. Daniel Gibson and his colleagues at the J. Craig Venter Institute. It allows for successful assembly of multiple DNA fragments, regardless of fragment length or end compatibility (the presence of compatible restriction sites). It has been rapidly adopted by the synthetic biology community due to its ease-of-use, flexibility and suitability for large DNA constructs.

Gibson assembly involves combining the vector (fragment A below), the DNA insert (fragment B below) and the Gibson Assembly Master Mix. The Gibson Assembly Master Mix includes three different enzymes:

- The exonuclease creates single-stranded 3´overhangs in each of the DNA fragments. Note that the two fragments, A and B, have the same sequence at their ends (overlapping ends). The 3' overhangs therefore allow the fragments, which have complementary sequences, to anneal together.
- The DNA polymerase synthesizes DNA to fill in gaps within each annealed fragment.
- The DNA ligase seals each of the two DNA strands.



Gibson Assembly

Remember that the Gibson Assembly Master Mix contains enzymes and therefore **should be kept on ice at all times!**

1. Label a PCR tube (the tiny tubes) with your initials.

2. In that tube (**on ice)**, combine:

Vector	2.5 ul
Building block insert	2.5 ul
Gibson Assembly Master Mix	5 ul

2. Place tube in the PCR machine and incubate at 50C for 15 minutes.

3. Remove tube from PCR machine and place on ice.

Bacterial Transformation

Once the vector and DNA insert are joined together with the Gibson Assembly Master Mix, the resulting recombinant DNA molecule is transformed into bacteria. Only a small number of bacterial cells in the population will become transformed. We can detect transformed cells because the plasmid DNA contains an antibiotic resistance gene; bacterial cells that become transformed will become resistant to the antibiotic (in this case, ampicillin). Ampicillin acts as a selective agent, allowing only those cells that contain a plasmid to grow.

E. coli that efficiently take up foreign DNA are said to be *competent*, and the method that they use to take up DNA is called *transformation*. Bacterial cells can be made competent by growing them at log phase, harvesting them from the growth media and then treating with calcium chloride. (It has been suggested that the Ca^{2+} neutralizes the negative charge of the phosphate backbone of the DNA. This neutralization prevents the DNA from being repulsed by the negatively charged phospholipids on the cell membrane, allowing the DNA to more efficiently enter the cell.) These competent bacteria can be stored at -80°C and subsequently used for transformation.

Competent bacteria take up DNA when the membrane is permeabilized, typically by heating quickly to 42°C. It is a tricky process because bacteria must be kept cold right until the heat shock step. **Taking the bacterial cells off of ice even briefly can lead to a dramatic decrease in transformation efficiency.**

It is important to use sterile technique throughout the transformation procedure. Keep the lids on your Petri dishes as much as possible and keep the foil top on the beaker of microcentrifuge tubes. Remove the cap as briefly as possible when pipetting the media. **Please remember to wash your hands with soap before leaving the lab!**

This is the easiest step to "mess up" in the entire workflow. Cells must be kept cold at all times! As soon as they thaw, they start dying. The more time passes (up to the point where LB is added) the more they die and the fewer transformants you'll get. Chill labeled tubes, ligation mixes, etc., on ice prior to mixing of cells and DNA (ligation mix).

1. Get three LB/Amp plates and label them “BB”, “PC”, and “NC”. Put the plates in the 37˚C incubator to warm up.

2. Label three microcentrifuge tubes (the larger size tubes) “BB”, “PC”, and “NC”. Close the caps and place these empty tubes on ice.

3. Add 2.0ul of the Gibson Assembly reaction to the “BB” tube. Place tube back on ice.

4. Add 2.0ul of the positive control DNA to the “PC” tube. Place tube back on ice.

5. Bring your tubes (**on ice**) up to the front to have 25ul of thawed competent cells transferred into each of your microcentrifuge tubes.

6. Incubate all tubes on ice for 30 minutes.

7. **Bring your ice bucket and a timer to the water bath**, and heat shock your tubes by placing at 42^{o}C for 30 seconds. **Timing is critical here!**

8. Immediately (ie, **bring your ice bucket to the water bath**) place heat shocked cells back on ice for 2 minutes.

9. Add 350 ul room temperature SOC medium to each transformation reaction and then put in a microcentrifuge rack at room temperature.

10. Put the rack with tubes in the 37˚C incubator for 1 hr.

11. Pipette 100 ul from each tube onto corresponding LB/Amp plates (only remove the tops from the plates long enough to add the cells). Spread bacteria by adding 5-6 sterile glass beads, shaking the plates with beads and then pouring beads into the waste.

12. Allow the liquid to soak into the plates. Once the plates appear dry, incubate the plates overnight at 37^{o}C (When you are incubating or storing plates, they should be stored upside down to prevent condensation from dripping onto them).

INTRODUCTION TO NEOCHROMOSOME ASSEMBLY ACTIVITY:

One strategy for constructing yeast "neochromosomes" involves yeast Golden Gate (yGG) cloning, as described by Neta Agmon and Leslie Mitchell (Agm*on, et al. ACS Synth. Biol.* 2015, 4, 853−859). This method efficiently and rapidly assembles multiple DNA fragments in a precise order for generating synthetic genes (which can then be assembled into multi-gene pathways using further iterations of Golden Gate assembly). Several advantages of this system are that: 1) it is standardized, so genetic parts can be mixed-and-matched, shared, and easily incorporated into other projects, 2) screening is color-based, which facilitates identifying positive clones, 3) it uses a single restriction enzyme, making it simple to simultaneously set up different experiments (e.g. generating a panel of mutants).

Today, we will work on several activities related to neochromosome assembly via Golden Gate cloning:

1. *Set up Golden Gate cloning reactions*: To see how this works, we will set up a Golden Gate cloning experiment to assemble two fragments that will effectively turn bacteria from red to green.

2. *Deep dive into how the Golden Gate cloning reaction works:* While things are incubating, let's discuss the nuts and bolts of how the experiment works and then "act it out".

3. *Transform products of Golden Gate assembly into bacteria.*

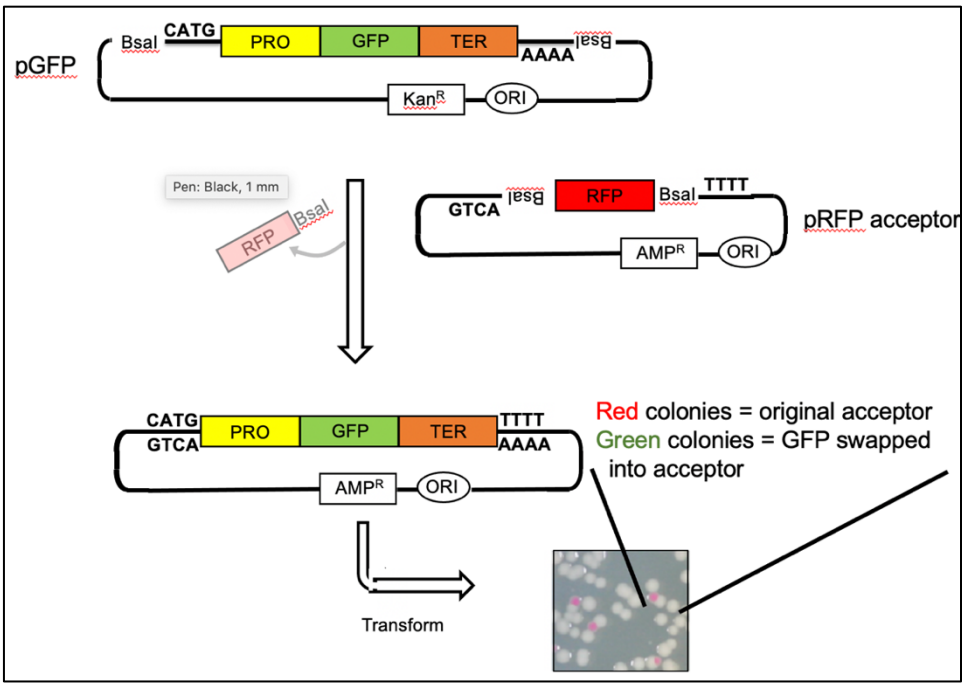
4. *If time allows*, *design a strategy to construct a synthetic gene comprised of a promoter, coding sequence and terminator*. As an example, we will design a reporter gene in which the yeast *GAL1* promoter is fused to the gene encoding GFP and a transcriptional terminator.

ACTVITY #1: SET UP GOLDEN GATE CLONING REACTIONS

• *Let's perform a "proof-of-principle experiment" to see how Golden Gate cloning reaction works.* We will start with the following two plasmids:

1) **pGFP**: This plasmid contains a gene encoding Green Fluorescent protein (GFP) and has features (e.g. promoter and ribosome-binding site) that enable the gene to be transcribed and translated in bacteria (so the bacteria appear green). This plasmid also contains a gene conferring resistance to the antibiotic kanamycin.

2) **pRFP acceptor plasmid**: This plasmid contains a gene encoding Red Fluorescent Protein (RFP) and an ampicillin-resistance gene. The plasmid contains features that enable the RFP gene to be transcribed and translated in bacteria (so the bacteria appear red).



Using Golden Gate cloning, we will swap out the RFP gene from the acceptor plasmid and replace it with the GFP gene (from the pGFP plasmid). This will effectively enable us to turn bacteria from red to green.

• ***Procedure:***

1. Each individual should obtain TWO PCR tubes – one "larger" tube and one "tiny" one.

2. Label larger tube "+50 and your initials." Label tiny tube "-50 and your initials."

3. Add the following ingredients to EACH tube in the order written from top to bottom. In other words, each tube will get the ***same*** stuff – the two reactions only differ in whether or not the program has a final 50°C incubation step. We are setting up two reactions today, so prepare the "Master mix for 3 rxns," which will provide more than enough material.

Ingredient	**Volume for 1 rxn**	**Master mix for 3 rxns**
H2O	4.5 µL	13.5 µL
pGFP plasmid*	1.5 µL	4.5 µL
pRFP acceptor plasmid	1.5 µL	4.5 µL
2x GG assembly mix**	7.5 µL	22.5 µL
TOTAL VOLUME	15 µL	45 µL

* ***Note****: In my hands, using 1.5 µL each of the pGFP and pRFP plasmid minipreps works just fine. This is very convenient in a course lab, since students can purify their own plasmids in a previous week (and it is not necessary to determine the DNA concentrations, which would be awkward since we just have one spectrophotometer). That said, we do perform restriction digests with some of the purified plasmids, so I get a sense of the yield of plasmid. If one student had a very low (or no) plasmid, then I supplement from a previously made prep (or their lab partner's).*

** ***Note***: *2x GG assembly mix contains DNA ligase, BsaI-HFv.2 restriction enzyme, a buffer, and ATP. I prepare the homemade mix shown below, but a pre-made version is also available from NEB. Below is the recipe for one reaction, and you can scale up accordingly.*

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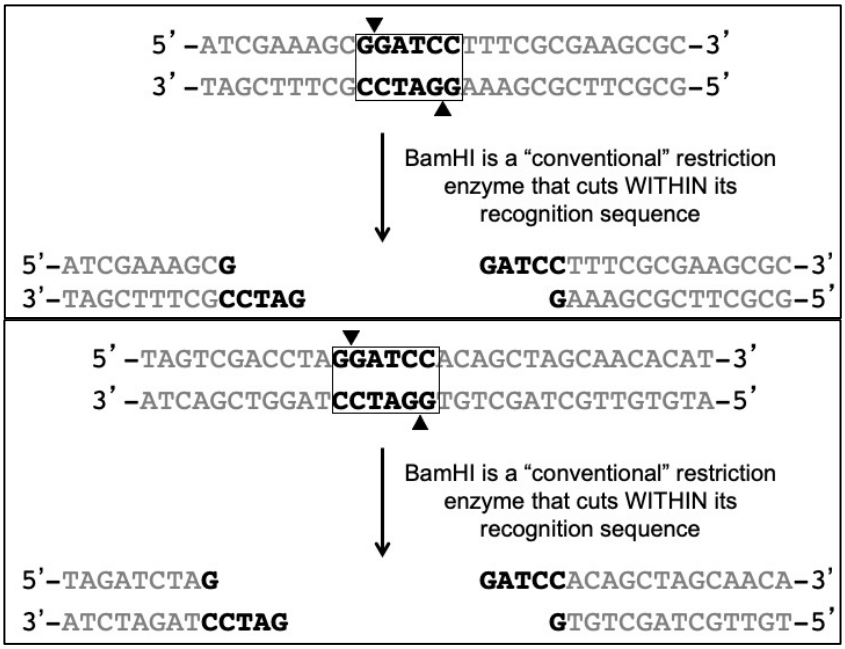
Ingredients for 2x yGG assembly mix	Volume for 1 reaction
1 mg/mL Bovine serum albumin (BSA)	1.5 µL
T4 DNA ligase (400,000 units/ml)	0.3 µL
BsaI-HFv2 (20,000 units/ml)	0.5 µL
10x T4 ligase buffer	1.5 µL
H2O	3.7 µL
Total volume	7.5 µL

4. Place your "+50" and "-50" tubes into the appropriate thermocyclers. Here are the reaction conditions:
   Step 1: 37°C for 3 min
   Step 2: 16°C for 4 min
   Step 3: Go to Step 1 for 4 more cycles
   Step 4 (for the +50 reaction only): 50°C for 5 min***
   Step 5: 80°C for 5 min

***<u>NOTE</u>: *For this example reaction, we are using a program with only 5 cycles. This will take about 45 minutes, should give a nice mixture of red and green colonies, and will fit nicely within a standard 3-hour lab period. But a typical Golden Gate reaction with more DNA parts would run for more like 25 cycles to maximize the yield of desired product (and this takes about 3 hours).*



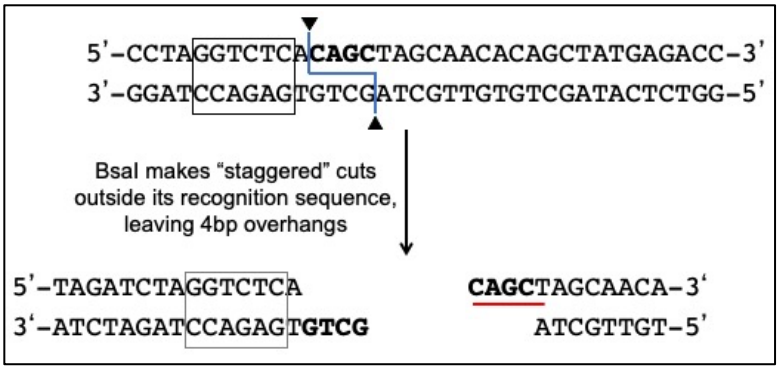
ACTIVITY #2: DEEP DIVE INTO HOW GOLDEN GATE CLONING WORKS

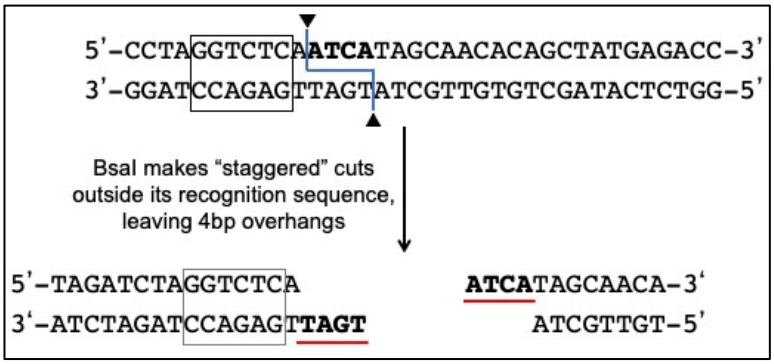
Golden Gate cloning utilizes a class of enzymes called Type IIS restriction endonucleases that cut DNA *outside* their recognition sequences and leave unique single-stranded overhangs. Let's quickly compare Type IIS and "conventional" restriction enzymes:

Conventional restriction enzymes cut WITHIN their recognition sites, so **they always generate the SAME overhangs** (no matter what sequence lies adjacent to the recognition site). For instance, the two panels above contain two different DNA fragments, each containing the 6-base sequence 5'-GGATCC'3' recognized by the restriction enzyme BamHI.

BamHI cuts *within* its recognition sequence between the two adjacent "G" nucleotides on each strand (indicated by arrowheads), and always generates the same single-stranded overhangs, even if the sequences flanking the recognition site vary (compare top and bottom panels).

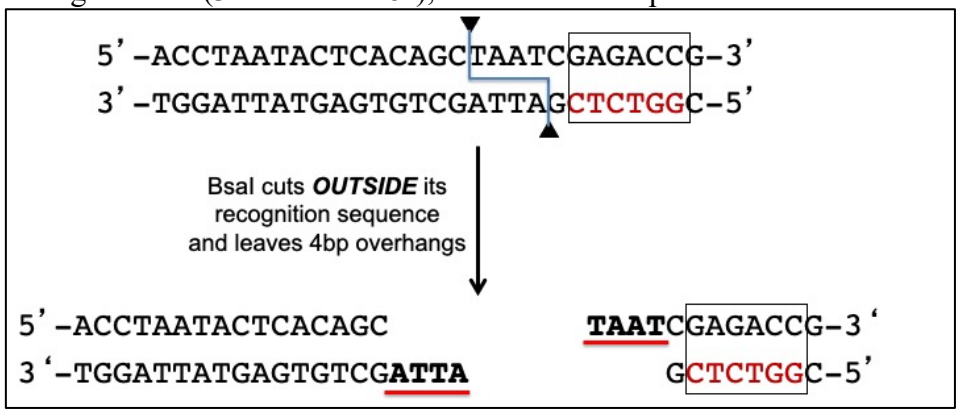
However, Type IIS restriction enzymes cut ***OUTSIDE*** their recognition sequences, enabling them to leave ***different*** overhangs (if the sequences adjacent to the recognition sites vary). The two panels below illustrate the activity of a Type IIS restriction enzyme called BsaI. It recognizes the 6-base sequence 5'-GGTCTC-3' (shown in rectangles), and then cuts ***outside*** this sequence -- one nucleotide away on the top strand and five nucleotides away on the bottom strand (indicated by the arrowheads), thus leaving 4-base overhangs. When the sequences adjacent to the recognition site vary (as they do below), BsaI generates ***different*** 4-base overhangs (compare the two panels).





Golden Gate assembly takes advantage of these different 4-base overhangs! By incorporating specific nucleotide sequences *adjacent to* BsaI recognition sites, one can generate DNA fragments with complementary base pairs that will "fit" together like puzzle pieces in a precise configuration.

There is one more important thing to mention about Type IIS restriction enzymes -- the orientation of the recognition sequence is important! In the above panels, the BsaI recognition sequence is on the top strand and oriented left to right (5'-GGTCTC-3'). When oriented this way, BsaI cuts one nucleotide away on the top strand and five nucleotides away on the bottom strand. But the BsaI recognition sequence ***can also lie on the bottom strand*** and be oriented right to left (3'-CTCTGG-5'), as shown in the panel below.



The easiest way to appreciate why the orientation of the BsaI sites matters is to "act out" the RFP → GFP Golden Gate reaction we are setting up! Please grab the plasmid handouts and your scissors!

ACTING OUT THE GOLDEN GATE CLONING REACTION

Let's "act out" what is happening in the Golden Gate cloning reaction – grab your scissors! To set things up, first cut out your circular plasmids from the papers provided.

Here is the program we are using:

Step 1: 37°C for 3 min
Step 2: 16°C for 4 min
Step 3: Go to Step 1 for 4 more cycles
Step 4 (for the +50 reaction only): 50°C for 5 min
Step 5: 80°C for 5 min

The reaction program consists of 5 cycles of the following…

- 37°C cutting step = the **restriction enzyme BsaI** is most active at this temperature and cuts the DNA

- 16°C ligating step = this temperature is optimal for ligating DNA fragments to one another (using the enzyme **DNA ligase**)

…and then the reaction includes the following:

- 50°C final cutting step = the BsaI enzyme is *still* active at this temperature, but the ligase is *not.* This ensures that the reaction ends on a cutting step, which will be important for reasons you will see later.

- 80°C heat inactivation step = this denatures both the BsaI enzyme and the ligase, thereby terminating the reaction.

As we do this, keep in mind that the goal is to replace the RFP in the ampicillin-resistant plasmid with GFP from the other plasmid.

1. Note the orientations of the BsaI restriction sites flanking the GFP and RFP genes in the two starting plasmids. These are key to this experiment! Are the BsaI sites (5'-GGTCTC-3') located on the ***top or bottom*** strands? Are they oriented ***toward or away*** from the GFP/RFP genes?

Let's perform a virtual 37°C incubation! Pretend you are the restriction enzyme BsaI and cut both plasmids along the dashed lines.

2. First focus on the GFP gene fragment:

a. Can the GFP fragment "fit" via complementary base pairing into the "space" opened in the ***Ampicillin***-resistant plasmid (that previously housed RFP)?

b. If, during the 16°C step, GFP *IS* ligated into the ***ampicillin***-resistant plasmid, will the plasmid still possess BsaI restriction sites? A related question -- could GFP be cut out of this ***ampicillin***-resistant plasmid by BsaI during the next 37°C incubation step (i.e. in the next "cycle"), OR is GFP "permanently stuck" in there?

c. After GFP is cut from its original plasmid, can it "fit" via complementary base pairing *back* into the "space" opened in the original ***kanamycin***-resistant plasmid that it came from (i.e. is there a chance of just regenerating the original GFP-containing/kanamycin-resistant plasmid instead of making the desired ampicillin-resistant product)?

d. If, during the 16°C step, the GFP fragment is ligated *BACK* into the ***kanamycin***-resistant plasmid that it originally came from (to regenerate the starting plasmid), will the plasmid still possess BsaI restriction sites? Could GFP be cut out of this plasmid by BsaI during the next 37°C incubation step (i.e. in the next "cycle"), OR is GFP permanently "stuck" in there?

3. *Now let's focus on the RFP gene fragment:*

a. Can the RFP fragment "fit" via complementary base pairing into the "space" opened in the ***kanamycin***-resistant plasmid (that previously housed GFP)?

b. If, during the 16°C step, RFP *IS* ligated into the ***kanamycin*** -resistant plasmid, will the plasmid still possess BsaI restriction sites? A related question -- could RFP be cut out of this ***kanamycin*** -resistant plasmid by BsaI during the next 37°C incubation step (i.e. in the next "cycle"), OR is RFP "permanently stuck" in there?

c. After RFP is cut from its original plasmid, can it "fit" via complementary base pairing back into the "space" opened in the ***ampicillin***-resistant plasmid that it originally came from (i.e. is there a chance of just regenerating the original RFP-containing/ampicillin-resistant plasmid instead of making the desired GFP/ampicillin-resistant product)?

d. If, during the 16°C step, the RFP fragment is ligated *BACK* into the ***ampicillin***-resistant plasmid that it originally came from (to regenerate the starting plasmid), will the plasmid still possess BsaI restriction sites? Could RFP be cut out of this plasmid by BsaI during the next 37°C incubation step (i.e. in the next "cycle"), OR is RFP permanently "stuck" in there?

Summarize the above by answering the following:

4. Why do you think it is necessary to perform multiple cycles of 37°C cutting and 16°C ligating? In your answer, be sure to mention the importance of the orientations of the BsaI sites.

5. Draw simple diagrams representing all the possible plasmids you could end up with at the end of this Golden Gate cloning reaction (<u>*Hint*</u>: there are four possible plasmids, including the two original ones ☺). Include the following features in your diagrams: GFP or RFP, Kan^R or Amp^R, and BsaI sites (if applicable)

* * *

If four possible plasmids are present at the end of the reaction, how do we identify the desired one (i.e. an ampicillin-resistant plasmid with the GFP gene ligated into it)? Let's consider how to do so!

6. We will "transform" our ligation products into bacteria and plate them on medium containing ampicillin. Which of the four potential plasmids will enable transformed bacteria to survive on this ampicillin-containing medium? Does this help to "narrow" down the search for the desired plasmid?

7. Most likely, you will obtain a mixture of green and red bacterial colonies on the ampicillin-containing transformation plate. Which plasmids must be present in the green vs. red bacteria? Does this help to "narrow" down the search for the desired plasmid?

8. *Challenge question*: Recall that we actually set up TWO Golden Gate assembly reactions – one that included a 50°C incubation step near the end and one that did not. Although the components in each reaction were identical, you most likely obtained different ratios of red to green bacterial colonies. How does including the 50°C incubation step (at which BsaI is still active, but ligase is not) alter the red to green ratio? (Hints: 1) can linear DNA be replicated in bacteria, and 2) do all the plasmids contain BsaI sites (i.e. can they all be cut during this final 50°C step)?

ACTIVITY #3: TRANSFORMING GOLDEN GATE ASSEMBLIES INTO BACTERIA

1. Obtain bucket of ice from the machine at end of the hall on the 4th floor.

2. Obtain TWO tubes of "competent" bacteria (I will give these to you) – one for each of your two Golden Gate assemblies. ***For optimum results, keep cells ice-cold as much as possible!***

3. Thaw bacteria on ice – you can monitor this by gently flicking the tube to see whether cells are still frozen. Label one tube "+50 and your initials" and the other ""-50 and your initials"

4. Transfer 5 µL of your Golden Gate assembly to bacteria in the appropriate tube. Mix by gently flicking the tube.

5. Incubate on ice for ~10 minutes.

6. Obtain two LB + AMP plates. Label one plate "+50 and your initials plus the date" and the other "-50 and your initials plus the dates"

7. **Heat shock**: bring ice bucket containing tube to the 42°C water bath. Place tube in floating rack in 42°C water bath for 45 seconds. Put tube back on ice and carry back to your table.

8. Using sterile transfer pipet, add ~500 µL of LB to each tube. Place in the 37°C incubator for ~30 minutes.

9. Spin cells 10 seconds in microfuge (set to max speed although it may not get to max speed during this short spin).

10. You should see a small pellet. Using a pipetman, remove 400 µL of the supernatant and discard it in beaker at your space.

11. Resuspend cells in remaining liquid by pipetting up and down. Pipet all cells into the center of appropriate plate.

12. Spread cells evenly over surface of medium with sterile spreader.

13. Put plates upside down in the 37°C incubator.

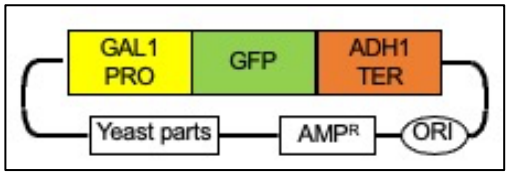
ACTIVITY #4: DESIGN A 3-PART TRANSCRIPTION UNIT VIA yGG CLONING

The trick is to Golden Gate assembly is to generate DNA parts with the following features:

1) DNA parts must be flanked by BsaI sites in proper orientation

2) DNA parts possess appropriate 4-base overhangs (that will be revealed after BsaI cuts the DNA)

Both of the above features can be easily added to each DNA part using PCR, if you include the necessary sequences in the primers (i.e. since PCR amplifies everything in between ***and including*** the primers, the complete primer sequences end up in the products). Let's see how to do this!



Your mission: Design PCR primers to generate a "reporter" construct in which the yeast *GAL1* promoter is fused to the GFP coding sequence and yeast *ADH1* transcriptional terminator, as indicated in the diagram at left. The DNA sequences you will need are located in the "Appendix." The standard primer design system below will help you add the necessary sequences onto your primers (i.e. the BsaI site and appropriate 4-base overhangs).

STANDARD PRIMER DESIGN

Primer set to amplify any promoter (PRO):

Forward PRO primer:
5'-ctaGGTCTCa**CAGT**…(insert forward PRO-specific priming sequence)…

Reverse PRO primer:
5'-agcGGTCTCa**CATT**…(insert reverse PRO-specific priming sequence)…

Primer set to amplify any coding sequence (CDS)

Forward CDS primer:
5'-ctaGGTCTCa**AATG**…(insert forward CDS-specific priming sequence starting with the ***second*** codon, since the ATG start codon is built into the 4-base overhang shown in bold)…

Reverse CDS primer:
5'-agcGGTCTCa**CTCA**…(insert reverse CDS-specific priming sequence starting with the ***last amino acid-specifying*** codon, since a stop codon is built into the 4-base overhang shown in bold (although the sequence is the reverse complement)…

Primer set to amplify any terminator (TER):

Forward TER primer:
5'-ctaGGTCTCa**TGAG**…(insert forward TER-specific priming sequence)…

Reverse TER primer:
5'-agcGGTCTCa**AAAA**…(insert reverse TER-specific priming sequence)

Below is a guide to the features included in the above PCR primers:

1. Red letters indicate the BsaI recognition sequence

2. The blue "a" is a single nucleotide "spacer" needed because BsaI cuts the top DNA strand *one nucleotide away* from the 3' end of the recognition sequence.

3. The bold, underlined, capital letters indicate the 4-base overhang that will be generated after digesting the PCR product with BsaI

4. Lower-case black letters are three random nucleotides added to the 5' end of each primer to enable BsaI to efficiently cut the DNA close to the end if you plan to directly use a PCR product in the Golden Gate reaction, instead of cloning the PCR product into a plasmid first. (and these extra 3 nucleotides are not necessary if you clone it into a plasmid).

APPENDIX

• Below are DNA sequences you will need for designing your synthetic gene:

PRO = Full-length yeast GAL1 promoter
CGGATTAGAAGCCGCCGAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTG
CGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGATGTGCCTCGCGCCGCACTG
CTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATT
GGCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAG
GATGATAATGCGATTAGTTTTTTAGCCTTATTTCTGGGGTAATTAATCAGCGAAGCG
ATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAACCACTTTAAC
TAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATC
AACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGAG

CDS = GFP (a BsaI-less version of superfolder from Dueber lab MoClo collection)
ATGCGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGAT
GGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAAC
TAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTTCCTTG
GCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGA
CCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGA
ACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAAT
TTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAG
GACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTAC
ATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCACAA
CGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCG
GTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGT
CTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCA
GCGGGCATCACGCATGGTATGGATGAACTGTACAAA

TER = Yeast ADH1 terminator
GCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTG
TATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAA
CTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGCATGAGGTCGCTCTTATTGAC
CACACCTCTACCGG

• If you are not used to designing PCR primers, here is one way to do it:

I think it is easiest to visualize what is happening by pasting the template sequences above individually into SnapGene Viewer, which is a free DNA analysis software package. SnapGene will make the DNA sequence double-stranded and also has tools to assist with the design. You can download SnapGene Viewer from this link.: https://www.snapgene.com/snapgene-viewer/

1. Open SnapGene Viewer and choose "New DNA or RNA file." Paste a template DNA sequence (e.g. the GAL1 promoter sequence) into the box and press "OK."

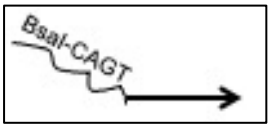
2. SnapGene detects commonly used sequences (like the *GAL1* promoter). If SnapGene asks you to "Add features," then choose OK. If it does not detect a sequence, then just go to the next step.

3. By default, you will likely be looking at a linear "map" representation, but you can switch to the sequence view by pressing the "Sequence" tab at the lower left of the window.

4. Easy way to design your Golden Gate cloning PCR primers:

a. Your primers will contain two "parts:"

1) The 5' end of each primer will contain the BsaI site, a 4-base overhang, and a few additional nucleotides. In the diagram at right, the 5' end with these "bells and whistles" is represented by the squiggle.



2) The rest of the primer will consist of the sequence that actually anneals to your original template. It is represented by the solid arrow in the diagram at right.

b. Use the sequences written in the "Standard primer design" section above to obtain the 5' ends of each primer (i.e. the squiggly parts). You can actually just copy and paste those parts.

c. Here's an easy way to design the template-binding portion of the FORWARD primer (i.e. the "solid arrow" part, do the following) using SnapGene Viewer:

1) In SnapGene, start highlighting from the beginning of the sequence you want to amplify and drag along the top strand. For the FORWARD primer, pay attention to the TOP strand and drag from LEFT TO RIGHT.

2) As you drag past the 8th nucleotide, SnapGene begins calculating the melting temperature (Tm) of your primer in a purple window directly above the sequence.

3) Keep dragging until the Tm is about 59°-60°C, which is a good "all-purpose" melting temperature for a primer AND the last nucleotide is a G or C (if possible). Copy this sequence – it is the one you need! Then, paste it onto the 3' end of the of the "standardized beginning" of your primer (i.e. the standard "squiggly" part) to complete the primer design.

4) Most primers will be 18-24 nucleotides in length, but A-T rich primers will need to be longer since there are only 2 hydrogen bonds in an A-T base pair (as opposed to three in a G-C base pair).

d. To design the template-binding portion of the REVERSE primer (i.e. the "solid arrow" part, do the following):

1) In SnapGene, start highlighting from the beginning of the sequence you want to amplify, and drag along the BOTTOM strand. For the REVERSE primer, pay attention to the BOTTOM strand and drag from RIGHT TO LEFT (i.e. this is the 5' to 3' direction along the bottom strand).

2) Again, keep dragging until the Tm is about 60°C. Copy this sequence by doing the following: Go to Edit → Copy Bottom strand bases → 5' → 3'

3) You can then paste this sequence onto the 3' end of the of the beginning of your primer (i.e. the standard "squiggly" part) to complete the design

*The melting temperature provides an indication of how "strong" the primer binds to its complementary "target" in the template. It represents the temperature at which half the primer dissociates from its target. A primer with a Tm that is too low is not strong enough (i.e. cannot form enough hydrogen bonds) to bind to its target.

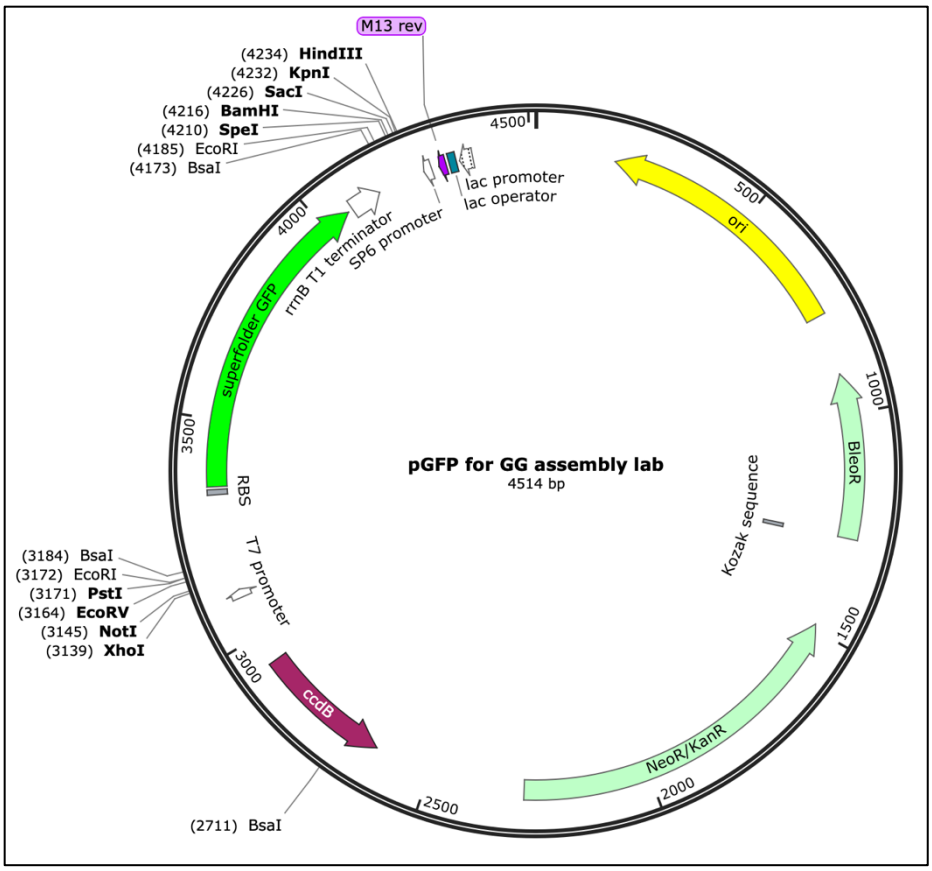
ADDITIONAL NOTES AND INFORMATION:

• Information about the plasmids used for the above experiment.

- **pRFP plasmid.** I called this "pRFP" to make it easy for students to identify, but its "official" name is pAV116. This plasmid was generated in the Boeke lab and is described in Neta Agmon and Leslie Mitchell's paper at the following link: https://pubmed.ncbi.nlm.nih.gov/25756291/. The plasmid is available from AddGene, and you can information about is here: https://www.addgene.org/63183/

- **pGFP plasmid.** This can be obtained from Eric Cooper (and it is also called EMCB81). The plasmid contains superfolder GFP cloned into the pCRBlunt plasmid from ThermoFisher. To construct this plasmid, superfolder GFP was amplified from plasmid pYTK047 (from John Dueber's lab and described here: https://pubs.acs.org/doi/10.1021/sb500366v) using the following primers that added BsaI restriction sites and appropriate 4-base sequences flanking the GFP gene:

  Forward primer: 5'-GGTCTCa**CAGT**gtgaaacgtgatttcatgcgtc-3'
  Reverse primer: 5'-GGTCTCa**AAAA**gaaggtgagccagtgtgactc-3'



In the above primers, the BsaI sites are capitalized but *NOT* in bold. The 4-base overhangs left after BsaI digestion are capitalized *AND* in bold. The PCR product was then ligated into pCRBlunt from ThermoFisher.

A map of the resulting plasmid is shown at left (and note that this plasmid contains a gene conferring resistance to **kanamycin)**.

- **One disadvantage of golden gate cloning – internal BsaI sites!**

What if your desired DNA "part" contains an internal BsaI site? Unfortunately, this is one disadvantage of this type of cloning, but here are a several options:

1. Find a source of BsaI-free (i.e. "domesticated") DNA fragments, and many are out there! Addgene sells "MoClo" kits used for bacterial and yeast gene assembly (and for other organisms as well), and the DNA parts included in these will be BsaI-free. Also, any lab using Golden Gate cloning will undoubtedly have some BsaI-free parts they might be willing to share.

2. Choose a different Type IIS restriction enzyme (i.e. other than BsaI) such as BsmBI or BbsI. However, this may affect the ability to use this part with other ones generated with BsaI.

3. Perform site-directed mutagenesis to eliminate the BsaI site(s), which may be a good side project for an interested student! Here is another paper from Leslie Mitchell that outlines a strategy for rapidly eliminating multiple BsaI sites from a plasmid using a single "round" of mutagenesis:

   Multichange isothermal mutagenesis: a new strategy for multiple site-directed mutations in plasmid DNA. Mitchell, LA, et al. *ACS Synth Biol* 2013 Aug 16;2(8):473-7.

Yeast Transformation

The plasmid DNA which induces the Scramble system will be transformed into competent yeast cells. This plasmid will be selected using a selectable (auxotophic) marker on the plasmid. Yeast cells containing the selectable marker will be able to grow on media lacking an amino acid. [Note to instructors: We can provide you with the yeast strains containing this plasmid already if you want to skip the yeast transformation step].

1. Obtain one tube of yeast cells (10_8 cells). Spin these down in the microcentrifuge for 1 min at full speed.

2. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of sterile water. Spin cells down in the microcentrifuge for 1 min at full speed.

3. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate). Spin cells down in the microcentrifuge for 1 min at full speed.

4. Remove the liquid (you can toss it with the pipet tip into the biohazard trash. Resuspend the cells in 1 ml of 0.1M LiAc (lithium acetate).

5. Obtain four sterile microcentrifuge tubes (one for the assembly transformation, one for the positive control, and one for the negative control). Add 100 ul of yeast competent cells from step 4 to each tube. Label these tubes “CRE”, “NO CRE”, and “NC”.

6. Centrifuge the yeast cells at full speed for 1 minute. Remove the liquid (called the supernatant; you can toss it with the pipet tip into the biohazard trash.

7. Prepare a yeast transformation master mix. You have a tube containing 960 ul of 50% polyethylene glycol (PEG). To this tube add 144 ul of 1.0M lithium acetate (LiAc) and 40 ul of single-stranded herring sperm DNA. Mix very well by vortexing for 10 seconds.

8. Aliquot (distribute) 286 uL of yeast transformation mix into each of the three microcentrifuge tubes containing the yeast cell pellets from step 6. Pipet up and down very well to mix the yeast cells.

9. To the “NC” tube, add 25 uL of sterile water.

10. To the “CRE” tube, add 25 ul of provided plasmid DNA (plasmid pLM161; 500 ng).

11. To the “NO CRE” tube, add 25 ul of provided plasmid DNA (plasmid pRS416; 500 ng).

12. Vortex the tubes for 10 seconds to thoroughly mix the DNA with the transformation mix and yeast cells.

13. Incubate in a 42°C water bath for 20 minutes. During this time, obtain 3 SC-Ura dropout plates. Label one “CRE”, one “NO CRE”, and one “NC”.

14. After the heat shock, centrifuge the tubes at top speed for 30 seconds.

15. Remove the supernatant with a P1000 pipette set to 1000 ul.

16. Add 300 µL of sterile water to each tube. Gently pipette to resuspend the pellet.

17. From each tube, transfer 250 ul of the transformation product onto your appropriately labeled SC-Ura dropout plates. Add ~5-7 sterile large glass beads to each plate, shake the plates and toss the glass beads into the waste container.

18. Once the plates are no longer wet, incubate the plates at 30°C for 2 days (remember to turn the plates upside down).

19. Pick 2 new SC-Ura plates, label them “CRE” and “NO CRE” and with your initials. Parafilm and leave in the incubator with your transformation plates

20. After 2 days, select a single colony from the CRE plate and streak it on the new SC-Ura plate to isolate a single colony and remove any background (non-transformed) cells. Repeat for the NO CRE sample, streaking a single colony onto a new SC-Ura plate

Inducing the Scramble system

Your instructor has inoculated a liquid culture (in 200 ml SC-Ura media) of your yeast strain containing the Cre plasmid (pRS413-CreEBD). The plasmid contains the Cre gene (remember that Cre induces the Scramble system) under the control of the estradiol promoter. In this experiment, you will add estradiol to the cells to induce the expression of Cre and therefore the Scramble system.

1. Measure the optical density of the yeast culture, which gives you an estimate of the cell density.

    a. Transfer 1 ml of media to a cuvette. Place in the spectrophotometer and blank the instrument (this only needs to be done once for the class).

    b. Transfer 1 ml of your yeast culture to a cuvette. Place in spectrophotometer and record the optical density. If the density is greater than OD=1, dilute the cells and repeat the measurement. Remember to account for the dilution when entering the OD into the formula below

2. Use the formula $C_1V_1=C_2V_2$ to determine the volume of cells equal to an OD of

    0.1 (Concentration (OD as measured on spec)(Volume to be

    determined) = (0.1) (25 ml) Volume to be determined =_ml

3. Into a sterile flask, add:
    a. The volume of cells that you calculated above
    b. 25 ml of SC-Ura liquid media
    c. 5 ul of beta-estradiol (1 uM final concentration).

4. Incubate the culture with shaking for 2-6 hours. (The cells are Scrambling now!)

5. Transfer 100 ul of the cell culture to a microcentrifuge tube.

6. Spin down the cells for 1 min at full speed. Remove the supernatant. Resuspend the culture in 1 ml sterile water.

7. Repeat step 7 two more times, ending with the cells resuspended in 1 ml sterile water.

8. Onto two selective plates, add 100 ul water. Into this drop, add 10 ul of cells from step 7. Spread the cells on the plates with glass beads.

9. Discard beads and when the liquid has absorbed into the plates, incubate the plates upside down at 30C for 2 days.

Scramble Assignment

The Scramble system requires two steps: (1) introduction of the Scramble plasmids into yeast cells and selection of those plasmids using genetic markers, and then (2) induction of the Scramble system and selection of yeast cells with interesting properties. The questions below will address your understanding of those two steps. To answer these questions, use:

1. Use your class notes
2. The websites listed in the questions themselves
3. The Synthetic Yeast design paper (Reading 6 Synthetic Eukaryotes on Moodle)
4. The Chapter 4: Working with Yeast article that was shared with you, and
5. The yeast transformation video: https://www.youtube.com/watch?v=izquCFtH5Ps

1. The yeast strain we are using has the genotype his3delta1 leu2delta0 met15delta0 ura3delta0. Go to: http://wiki.yeastgenome.org/index.php/Commonly_used_auxotrophic_markers and look up each of the four alleles and then complete the table below (you need to scroll all the way to the bottom of the page for the last 3 markers)

Allele	Is this marker a complete deletion of the gene?	If not, what portion of the gene is missing?
his3delta1		
leu2delta		
trp1delta		
ura3delta		

2. Each allele contains a hyperlink to the Saccharomyces Genome Database. Each of these alleles is an auxotrophic marker, meaning that yeast containing the allele requires a particular nutrient. Under "Name Description", determine what nutrient yeast containing each of the alleles requires.

Allele	Name of gene	Nutrient Required
his3delta1		
leu2delta		
trp1delta		
ura3delta		

3. For the Scramble experiment, the Cre gene was cloned into a plasmid containing the URA3 gene as its selectable marker, transformed into yeast, and selected on SC-Ura medium. Based on your results above, what three other genes (selectable markers) could have been used instead of *URA3* and what medium would you use for selection?

4. The article, Chapter 4: Working with Yeast, describes different types of yeast media. If you were to make one of the types of ***selective*** media described above (SC-Ura, for example), would this be rich media or defined media? How do you know?

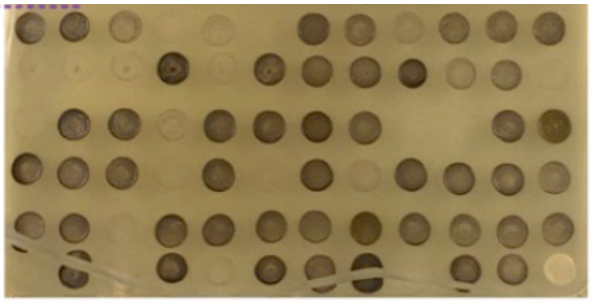
5. Why is it harder to introduce DNA into yeast cells, causing the heat shock step of transformation take 20 minutes for yeast cells but only 45 seconds for *E. coli* bacteria?

6. Where are the LoxP sites located in the yeast genome? What function do LoxP sites confer to the genome (in the presence of the Cre protein)?

7. Watch the Transformation of Yeast video. What percent of genes that are the same between humans and yeast have an important role in human disease? This includes genes that are involved in cancer-what cellular process are many of these genes involved in (study of this process in yeast won the Nobel Prize in 2001)? Why are yeast also being used to screen new cancer drugs?

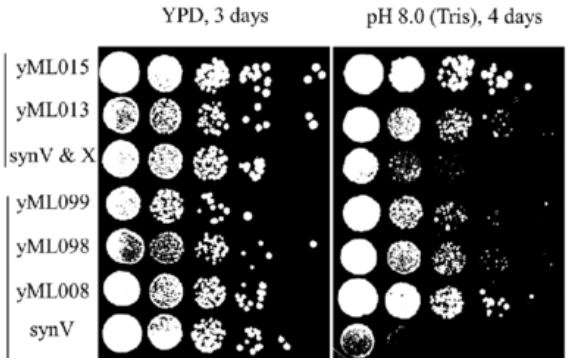
8. From the article, Chapter 4: Working with Yeast, describe 4 methods that are used to maintain sterility when working with yeast.

9. The figure below is the result of a Scramble experiment. The cells all express the pigment violacein (purple-black) and they all started off making the same amount of pigment. The Scramble experiment was performed multiple times and each spot represents the result of an independent Scramble experiment.



Even though the experimental procedure was the same each time, why do the resulting cells produce different levels of pigment?

10. The figure below represents the results of a Scramble experiment. The strains labeled synV and syn V&X are the starting strains (not Scrambled) and they contain either synthetic chromosome 5 only or synthetic chromosomes 5 and 10. The strains are serially diluted and spotted onto rich media plates (YPD, left) and plates with alkaline media (pH 8.0, right).
    Strains yML015, yML013, yML099, yML098, and yML008 are five strains resulting from five independent Scramble experiments. They were diluted and plated in a similar manner.
    a) Which of the strains show the greatest improvement in its tolerance for alkaline conditions following the Scramble experiment? Describe how you can tell.



    b) When performing spot dilutions like in the experiment above, why is it important to plate the cells on both the desired condition (alkaline conditions, pH8.0) and also on rich media (YPD)? How would you interpret the results if the Scrambled strain grew significantly better on both YPD and on alkaline conditions?

11. The yeast genome was selected as the first synthetic genome because of the simplicity of the yeast genome. Think about the differences between the yeast and human genomes. What features or aspects of the human genome would make it much more complicated to implement the Scramble system and the process of Scrambling chromosomes?

Measuring growth rates by serial dilution and plating

Up to now we have Scrambled the synthetic yeast chromosomes and selected for our phenotype of interest. We selected this phenotype based on the size of colonies on the initial selection plates (high temperature, ethanol, or caffeine). Now we need to confirm that these larger colonies really do grow better on the selective conditions. We will do this by:

1. Selecting 11 colonies that you believe show increased growth in the selective conditions
2. Transferring an equal number of cells from each of the 11 colonies to one well of a cell culture dish. You also transfer cells that have not been Scrambled into one well of the cell culture dish.
3. Performing serial dilutions of those cells. This means that in each subsequent well of the cell culture dish, you will have progressively fewer cells
4. Transfer the diluted cells from each well onto a Petri dish containing media with the selective condition
5. Incubate the plates for 2 days and then assess the growth of the diluted cells (both the size and the number of colonies) relative to the non-Scrambled strains.

An example of serial dilutions of yeast cells to compare growth rate on selective conditions (size and number of cells)

Procedure

1. Obtain your yeast cells on the selective plate from last week. Also obtain a plate with non-scrambled cells.

2. Obtain a new 96-well plate. When working with the plate, keep the lid on as much as possible; remove the lid to add or pipette the yeast cells, but put the lid right back on when you're done.

3. Transfer 200 ul of sterile water to each of the wells in the first row of a 96-well plate.

4. Using a sterile toothpick, select a colony from the non-scrambled cells. Swirl the toothpick into the water in the first well of water in the 96-well plate to disperse all the yeast cells into the liquid.

5. Using a sterile toothpick, select one of the largest colonies from the scrambled cells on the selective plate. Swirl the toothpick into the water in the first well of water in the 96-well plate to disperse all the yeast cells into the liquid.

6. Repeat step 5 for 10 more colonies, resuspending each in a new well.

7. Determine the OD of the cells in each well by taking a reading on the plate reader at an absorbance of 600 nm.

8. For each sample, calculate the volume of cells needed to prepare 200 ul of culture at an OD of 0.5 by using the formula C1V1=C2V2. Use the formula below and then complete the table below it.

   (0.5 OD) (200 ul) = (OD measured from plate reader)(Volume of cells needed).

Well	1	2	3	4	5	6	7	8	9	10	11	12
Volume of cells needed (calculated above)												
Volume of water needed to achieve a total volume of 200 ul												

9. Obtain a new 96-well plate. Into the first row, transfer the volume of cells calculated above for each well (ie, you are transferring the calculate volume from well A1 on plate 1 to well A1 on plate 2, etc.)

10. To these cells, add the volume of water calculated above to make a total volume of 200 ul.

11. Into each of the other wells in the dish, add 200 ul of water.

12. Using a P200 pipette set to 40 ul, pipette up and down 3-4X in well A1 and then transfer 40 ul of cells to the row beneath it (well B1). Pipette up and down 3-4X in well B1 and transfer 40 ul to well C1. Repeat all the way down to row H1.

13. Repeat step 12 for each column of cells (ie, transferring from A2 to B2 to C2…..)

14. Obtain 2 rectangular petri dishes with media. One should have rich media (YPD) and the other should have your media with your selective condition. Label the back of these plates with your initials and dates.

15. Obtain two paper templates containing the layouts of each plate. These will show you where on the smooth surface of the agar plate to place your cells. Place these under each of your dishes.

16. You should now have: your 96-well plate with diluted cells, your YPD plate, and your selective plate. Take 2 ul from the first well (A1) of diluted cells and pipette the liquid onto the

corresponding area of the YPD plate (A1). Take another 2 ul from the first well (A1) of diluted cells and pipette the liquid onto the corresponding area of the selective plate (A1).

17. Repeat step 16 for all wells of the 96-well plate.

18. Incubate the cells at 30C (or 40C if that is your selective condition) for 2 days.

Extraction of Yeast DNA

Now that we've Scrambled the yeast DNA and selected our phenotype of interest, we need to see which gene or region of the chromosome is responsible for our phenotype. To do this, we must extract the DNA from the yeast and amplify the PCR Tags. This will allow us to determine, for each section of the chromosome, whether the wild-type or synthetic DNA is present. Today we will extract the DNA, and next week, we will amplify the PCR tags. The following week, we will run our PCR reactions on agarose gels.

1. Transfer 1ml of yeast cell culture into a 1.5ml microcentrifuge tube.

2. Centrifuge at 13,000–16,000 *× g* for 2 minutes to pellet the cells. Remove the supernatant.

3. Resuspend the cells thoroughly in 293µl of 50mM EDTA.

4. Add 7.5µl of Zymolyase enzyme, and gently pipet 4 times to mix.

5. Incubate the sample at 37°C for 60 minutes. During this time, the Zymolyase will digest the yeast cell wall. Cool to room temperature.

6. Centrifuge the sample at 13,000–16,000 *× g* for 2 minutes and then remove the supernatant.

7. Add 300µl of Nuclei Lysis Solution to the cell pellet and gently pipet to mix.

8. Add 100µl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds.

9. Let the sample sit on ice for 5 minutes.

10. Centrifuge at 13,000–16,000 *× g* for 3 minutes.

11. Transfer the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube.

    Note: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

12. Add 300µl of room temperature isopropanol and gently mix by inversion. You may see thread-like strands of DNA forming a visible mass.

13. Centrifuge at 13,000–16,000 *× g* for 2 minutes.

14. Carefully remove the supernatant with a P1000 pipet. Add 300µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.

15. Centrifuge at 13,000–16,000 *× g* for 2 minutes. Carefully remove the supernatant with a P1000 pipet.

16. Centrifuge at 13,000–16,000 *× g* for 30 sec. Carefully remove the supernatant with a P200 pipet. Allow the pellet to air-dry for 10–15 minutes by leaving the tube sitting open in a rack.

17. Add 50µl of DNA Rehydration Solution.

18. Add 1.5µl of RNase Solution to the purified DNA sample. Vortex the sample for 1 second. Centrifuge briefly in a microcentrifuge for 5 seconds to collect the liquid and incubate at 37°C for 15 minutes.

19. Nanodrop the samples to determine the concentration of DNA. Turn on the machine, use DNA Rehydration Solution to "blank" the reading, then add 1 ul of your DNA solution to the machine and collect the reading.

Concentration:_____ng/ul 260/280 : _____

Amplification of PCR Tags

For each strain of yeast, we will now verify that the strain contains both one copy of the wild-type chromosome and one copy of the synthetic chromosome, as expected. In amplifying these regions, you will also be able to determine whether there are any structural changes in these regions of the chromosomes that are the result of the Scramble process; these will be visible as larger, smaller, or missing DNA bands.

For this procedure, you will amplify three strains of DNA:
1. Wild-type (non-synthetic strain)
2. Synthetic strain prior to Scrambling
3. Your synthetic Scrambled strain.

Each strain of DNA will be amplified with the following primer sets:

1. YEL071W gene, wild-type primers
2. YEL071W gene, synthetic primers
3. YER188W gene, wild-type primers
4. YER188W gene, synthetic primers

Therefore, your reactions will be:

	Template DNA	Primer 1	Primer 2
	Wild-type	YEL071W_F_WT	YEL071W_R_WT
	Wild-type	YEL071W_F_SYN	YEL071W_R_SYN
	Wild-type	YER188W_F_WT	YER188W_R_WT
	Wild-type	YER188W_F_SYN	YER188W_F_SYN
	Synthetic, non-Scrambled	YEL071W_F_WT	YEL071W_R_WT
	Synthetic, non-Scrambled	YEL071W_F_SYN	YEL071W_R_SYN
	Synthetic, non-Scrambled	YER188W_F_WT	YER188W_R_WT
	Synthetic, non-Scrambled	YER188W_F_SYN	YER188W_F_SYN
	Synthetic, Scrambled	YEL071W_F_WT	YEL071W_R_WT
	Synthetic, Scrambled	YEL071W_F_SYN	YEL071W_R_SYN
	Synthetic, Scrambled	YER188W_F_WT	YER188W_R_WT
	Synthetic, Scrambled	YER188W_F_SYN	YER188W_F_SYN

Protocol:

1. Dilute your DNA to a concentration of 20 ng/ul by using the formula C1V1=C2V2.

2. Obtain one set of 12-tube PCR strip tubes. Distribute 12.5 ul of the 2X PCR master mix to each of your 12 PCR tubes.

3. To each tube, add 5 ul of the corresponding primer (1 uM concentration) according to the table above. It is highly recommended that you make a check mark next to each primer as you add it to the tube to keep yourself organized and to remember what was added.

4. To each tube, add 2.5 ul of template DNA according to the table above.

5. Cap your tubes (make sure they are on tightly!) and place them in the PCR thermocycler.

6. Make sure that the machine is set to the following program:

    94 deg, 30 sec
    30 cycles of 94 deg for 30 sec, 64 deg for 30 sec, 68 deg for 1 min
    68 deg, 5 min

Name:

Genome Databases Assignment

Step 1: DNA alignment: What portion of the chromosome are we amplifying?

The first thing that we would like to do is to determine what sequence in the yeast genome our DNA primers (PCR Tags) correspond to. This can be accomplished by performing an alignment. The sequences of our PCR tags are (remember there are two primers for each PCR reaction):

>PCR Tag 1
GTCAGGGTCCAATAAGAGACAC
>PCR Tag 2
CCACCTTGTGGTACTACTGCCA
>PCR Tag 3
ATGTCGGATCCAAGTTCTATTAAC
>PCR Tag 4
TTCTTCCTTCAACTTATATAGGTT

Align each of the PCR tags to the reference genome using BLAST and complete the table below. **Table 1.**

	PCR Tag 1	PCR Tag 2	PCR Tag 3	PCR Tag 4
How many hits does your query retrieve?				
What is the score (bits) for your top hit?				
For your top hit, what is the probability the alignment would occur by random chance (the e-value)?				
Scroll down to look at the aligned sequences. What is the percent sequence identity between the search result and your query sequence?				
What chromosome is your sequence on?				
What are the chromosome coordinates of the sequence?				
For one of your PCR tags, go back and shorten your query sequence by three nucleotides. What does this do to the e-value of your BLAST hit(s) and why?				

Step 2: Annotation: What is the location of the sequence in the genome?

From your top alignment, click on "Genome Browser". This will give you a visual representation of the chromosome, including the sequence that your primer corresponds to. The yellow vertical line corresponds to the boundaries of your query sequence. Genes (open reading frames or ORFs) are colored red. There are many other genomic features that are indicated as well. Click on the name of the gene that corresponds to your DNA sequence. This will give you information about this gene.

Table 2.

	PCR tags 1 and 2	PCR tags 3 and 4
What is the name of this gene?		
What is the length of this gene?		
What is the position on the chromosome of this gene (starting and ending coordinates)?		
Is this gene encoded on the positive or negative strand of the DNA double helix?		

What is the systematic name (ID) of this ORF?		
What is the function of this gene?		

Step 3: Annotation: Learn more about your gene:

Click on the name of the gene, which will take you to the gene page where there is even more information, including functional information, links to information on gene and protein interactions, and perhaps a summary paragraph about the gene. Complete the table below for both of your DNA sequences.

Table 3.

	PCR Tags 1 and 2	PCR Tags 3 and 4
What is the closest gene just upstream of your gene?		
What is the closest gene just downstream of your gene?		
Does this gene make a protein? If so, what is its length in amino acids? About how many copies of this protein exist in the cell?		
Is this gene essential in the yeast cell? (You can tell this by looking in the Phenotype section; next to “null”, it should say either that the null mutation (deletion) is viable or inviable).		
Does this gene have a human homolog, and if so, what is it? (You can tell this by looking in the Phenotype section).		
Is the human homolog associated with any disease, and if so, which one (you can tell this by looking in the Disease section).		

Step 4: Gene Ontology

Watch the gene ontology video: https://www.youtube.com/watch?v=KzbX7tKeb5o&t=5s

Table 4.

	PCR Tags 1 and 2	PCR Tags 3 and 4
What is the molecular function(s) of your gene?		
What is the biological process that your gene is involved in?		
Where in the cell does your gene function?		

Synthetic DNA purification

We need to combine the phage building block with the rest of the phage genome. Before we can join these DNAs together, we must remove the old buffers and proteins from the PCR reaction using a purification kit. (This protocol uses Promega's Wizard SV PCR purification kit, but any equivalent kit can be substituted). We will purify the phage gene that you just amplified (you can throw out your PC and NC reactions).

1. Add 80 ul of water to your PH gene and mix by pipetting.
2. Add 100 ul of Membrane Binding Solution and pipet up and down several times to mix.
3. Obtain a spin column/collection tube and label with your initials. Add the liquid from step 2 to the top of the column. Let the tube sit for 1 min.
4. Spin in centrifuge for 1 min (be sure the centrifuge is balanced with another tube on the opposite side).
5. Remove the spin column and pour out the liquid from the bottom collection tube. Put the spin column back in the collection tube.
6. Add 700 ul of Membrane Wash Solution to the top of the column.
7. Spin in centrifuge for 1 min (be sure the centrifuge is balanced with another tube on the opposite side).
8. Remove the spin column and pour out the liquid from the bottom collection tube
9. Add 500 ul of Membrane Wash Solution to the top of the column.
10. Spin in centrifuge for 1 min (be sure the centrifuge is balanced).
11. Remove the spin column and pour out the liquid from the bottom collection tube
12. Without adding any additional liquid, spin the empty tube in centrifuge for 1 min (be sure the centrifuge is balanced).
13. Obtain a 1.7 ml microcentifuge tube and label with your initials and “PH”.
14. Discard the collection tube and transfer the column to the new microcentrifuge tube.
15. Add 30 ul of nuclease-free water to the column. The microcentrifuge tube cap will not close over the spin column, just leave the tube open.
16. Wait 1 min and then spin the tube and column in centrifuge for 1 min.
17. Throw out column. The liquid in the bottom of the microcentrifuge tube is your purified DNA.

Electroporation of Bacteria with Synthetic Gene & Phage DNA

Note for instructors: These protocols are based on those from Graham Hatfull's lab. The originals are available here:
http://phagesdb.org/media/workflow/protocols/pdfs/BRED_Main_Protocol_2.2013.pdf
and here:
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003957

Now that we have created synthetic genes, we need to combine that synthetic gene with the rest of the phage genome. We will do this by combining the native phage genome with the synthetic gene inside the host cell (the bacteria *Mycobacterium smegmatis*). Within the bacterial cell, the native phage genome and the synthetic gene will undergo the process of recombination, which will join them into a semi-synthetic phage genome.

If it is infectious, the semi-synthetic phage genome will burst the initial host bacterial cell, causing it to rupture and release phages which will then infect neighboring bacterial cells. These neighboring cells will then be ruptured, and the process will repeat, resulting in the formation of a plaque, a region where all of the bacterial cells have been burst.

Today we will introduce the native phage genome and synthetic gene into the bacterial cells and then plate those cells onto a petri dish. If the semi-synthetic phage that is formed is infectious, we will see plaques on the petri dishes next week. The method that we will use to introduce the phage genome and synthetic gene into bacteria is called electroporation; rather than using heat to get the DNA into the bacteria (as we did a few weeks ago), we will use a short pulse of electricity.

Preparation of Electro-competent Cells:

1. Measure the optical density of the M. smegmatis overnight culture in the spectrophotometer. The value should be between 0.4 and 0.8
2. Transfer 1.5 ml of the culture into a sterile microcentrifuge tube (use the P1000 pipet set to 750 ul twice). Incubate on ice for 30 min.
3. Spin the cells down at 5000 rpm for 10 min in the cold room.
4. Remove the liquid with a pipet tip and resuspend the cells in 800 ul 10% sterile ice cold glycerol. Pipet up and down until no clumps remain.
5. Repeat step 4.
6. Repeat step 4, but resuspend in 400 ul 10% sterile ice cold glycerol.
7. Repeat step 4, but resuspend in 200 ul 10% sterile ice cold glycerol.

Electroporation:

1. Label two tubes (+DNA and -DNA) and place them on ice so that they become cold.
2. Transfer 100 ul of cells to each of the two empty tubes.

3. To the first tube (+DNA), add 1 ul of the phage DNA and 300 ng of the synthetic gene building block (the PH that you purified last week). To the second tube (-DNA), add 4 ul of water. Incubate on ice for 10 min.

5. During the 10 min incubation:

- Obtain 2 cuvettes and place on ice so that they become cold.
- Obtain one tube of 7H9 media with ADC
- Obtain two Pasteur pipettes

6. After the 10 min incubation:

- Transfer the DNA and cells from the first tube into the first cuvette (on ice!)
- Wipe the cuvette with a Kimwipe to remove any wetness
- Transfer the cuvette into the black cuvette holder and insert into electroporation machine
- Press the PULSE button twice to deliver the pulse of electricity
- Pick up the Pasteur pipette with liquid. As soon as the machine beeps, pull out the cuvette holder and immediately add the 7H9 media with ADC on top of the cells

7. Transfer the mixture of 7H9 media without ADC and cells to a glass test tube and place in 37 degree incubator for 1 hour

8. 15 minutes before the incubation is done, prepare the top agar. Melt the MBTA (Middlebrook top agar) on a hot plate until just melted and allow to cool for 10 min.

9. Once the one hour incubation time is done, add the following to a new tube tube (add in the order listed):

10 ul of cells from the glass test tube shaking
at 37C 300 ul of *Mycobacteria smegmatis*
cells
1.5 ml 7H9 media without ADC
2.5 ml MBTA

Vortex gently and pour the mixture into a petri dish. Swirl gently to distribute the mixture evenly in the plate. Allow to cool for ~10 minutes and then incubate at 37 degrees.

Electroporation of Bacteria with Phage DNA Assignment

1. What were the results of your TPCR and FPCR assembly of the phage gene? How could you change the conditions to improve the results (if necessary)?

2. Fill in the chart with the results of your electroporation below:

Sample	Number of plaques
+DNA	
-DNA	

3. Before we used our DNA for electroporation, we were careful to purify the DNA using a column purification kit. One of the reasons was to remove excess salt that was present in the PCR buffer. Why is it crucial to remove all the salt when using electroporation to introduce DNA into cells?

4. Look back at the previous bacterial transformation lab protocol that we performed earlier in the semester. What are two ways in which that protocol is similar to this electroporation protocol? What are two ways in which they are different?

5. How does a plaque assay allow you to determine the number of infectious phages present?

6. Last time we performed transformation, we plated cells directly on to the top of the petri dish. This time we used top agar to plate the cells. Top agar has a lower agar concentration than the regular agar in petri dishes. When working with phages and performing plaque assays, why would this be advantageous?

7. Our FPCR products (our synthetic genes) contain codon optimized versions of the synthetic gene. What do we mean by codon optimizing a gene? Why might we use different codons depending on which species our gene will be expressed in?

8. What is the reason that synthetic biologists might codon optimize a gene that they are working with in the lab?

9. What is one possible negative unforeseen consequence of codon optimizing a gene?

10. Our next step will be to screen our semi-synthetic phage using PCR and then to dilute our phages so that we are sure that each plaque is generated from a single phage particle? Why is this step necessary if during electroporation we were able to successfully create the semi-synthetic phage?

Picking Plaques

When phages successfully propagate among bacteria growing in a lawn, plaques are formed. These are areas of clearing where phages have infected and lysed cells. Plaques can range in size, turbidity, and morphology. Often, it is necessary to collect a sample of the phages that have made a particular plaque (or putative plaque) to perform further experiments with them (such as testing their relative infectivity). This protocol describes how to "pick" the plaque and obtain a liquid sample of phage.

Reaction setup:

1. Distribute 100 ul of phage buffer into 6 different microcentrifuge tubes. Label these tubes 1-6.

2. Place a new sterile pipet tip on the end of your pipet. Pick one plaque on the plate and touch the center of the plaque once with the tip. The idea is to penetrate the top agar layer, but not go all the way through to the bottom agar. The angle of the pipette tip is not critical, although with small plaques, approaching the plaque perpendicularly seems to work best.

3. Move the tip to the prepared tube of Phage Buffer, lower the point under the level of the liquid, and shake moderately from side to side to release phages into the buffer. It's also a good idea to pipet up and down several times to "wash" any phages from inside the tip into the buffer.

4. Repeat for tubes 2-6, picking a fresh plaque for each tube. Allow to sit at room temperature for 30 minutes

5. Parafilm your bacterial plates from last week and store in the fridge until we analyze the PCR reactions.

PCR Screening of Phage Plaques

Now that we have selected several plaques and isolated the phages, we need to check whether these phages have the synthetic gene or the native gene. The synthetic gene contains two watermark sequences within the synthetic gene which mark the DNA sequence as being synthetic. We will therefore perform PCR with primers that bind to these watermark sequences. If the synthetic sequence is present, the primers will bind and we will get a DNA product. If the primers do not bind because the synthetic sequence is absent then we will not get a DNA product amplified. When screening, we want to start with several plaques to maximize the likelihood that we have at least one that is correct (contains the synthetic gene). We will therefore be setting up 8 PCR reactions (6 different plaques plus positive and negative controls).

1. In a large microcentrifuge tube **on ice**, combine the following components:

- 100 ul of 2X master mix (2X MM)
- 10 ul of primer WM1 (watermark 1)
- 10 ul of primer WM2 (watermark 2)
- 74 ul of sterile water
- 6 ul of DMSO

2. Pipet up and down 10X to completely mix the contents of the tube.

3. Aliquot (distribute) 20 ul per tube of the mix into 8 PCR tubes (the very small ones).

4. Into PCR tube 1, add 1 ul from phage tube 1.

5. Repeat step #4 for phage tubes #2-6.

6. Into PCR tube #7, add 1 ul from tube PC.

7. Into PCR tube #8, add 1 ul of sterile water.

8. Put your 8 PCR tubes into one column of the PCR machine and begin the PCR reaction.

Reaction Conditions:

1 cycle:
98°C, 30 seconds

35 cycles:
98◦C, 10 seconds
55◦C, 30 seconds
72◦C, 30 seconds

1cycle:

72◦C, 10 minutes

9. Run PCR products on a 1% gel in TAE buffer.

If the PCR reactions indicate that we have successfully introduced the synthetic gene into the rest of the phage genome, we now have a stock of the phage sample (the plaque that you picked into phage buffer). If none are positive, we can pick more plaques and screen more phages for positives.

Creation and Screening of semi-synthetic phages

4. Results of your electroporation and plaque assay. Describe and interpret your results and describe any alterations to the protocol that you would make next time.

5. Results of your PCR screening. Fill out the table below, **attach a picture of your gel with each lane labeled**.

Sample	PC	NC	Plaque 1	Plaque 2	Plaque 3	Plaque 4	Plaque 5	Plaque 6
Expected size of PCR product								
Actual size of PCR product								

6. Interpret the results of your PCR screening. Describe any alterations to the protocol that you would make next time.

Genome Databases

Once a genome is sequenced, that data must be deposited in a databank and then annotated. Annotation provides functional information about the location of genes and regulatory elements. This information is cataloged along with data relating to phenotypes and interactions between genes.

7. Sequence Retrieval

The first thing that we must do is to retrieve the sequence of your gene from the database. Go to the NCBI Nucleotide database at http://www.ncbi.nlm.nih.gov/nucleotide . Enter the name of our organism: Mycobacterium phage Giles. For this genome, what is the:

Accession number: _____ Genome length: _____

You will see that there are several different submissions for this genome data. For the original submission, what is the:

Molecule type (mol_type): _____

Isolation source: _____

Country and location: _____

Collection date: _____

Collected and identified by: _____

How many genes are in this genome (you may need to scroll down): _____

8. Characterization of your gene

Scroll down and find your gene. Click on the Gene ID number to open a new page. What is the lineage of your gene: _____

What is the length of your gene? _____
What are its sequence coordinates in the phage genome? _____

9. DNA alignment

Under “NCBI Reference Sequences (RefSeq)”, click on FASTA to get the nucleotide sequence of your gene. On the right toolbar, click Run BLAST to look for alignments in the database. We are using the BLAST algorithm (Basic Local Alignment Search Tool) to quickly find the sequence(s) that closely match our query sequence (your building block DNA). You should get a colored graph representing the quality of alignment generated by BLAST which ranges from dark blue (poor alignment) to red (very good alignment). BLAST provides statistics to help determine how good your alignment is. The e-value (the expect value) represents the probability that the alignment you produced could occur by random chance.

Gene	
How many hits does your query retrieve? What are they?	
For each hit, what is the probability the alignment would occur by random chance?	
What is the percent sequence identity between the search result and your query sequence?	

10. Go back and switch the Program Selection at the bottom of the BLAST page from “Highly similar sequences (megablast)” to “Somewhat similar sequences (blastn)”. What does this do to the number of hits that you retrieve and their e-values? Why?

Dilution of semi-synthetic phage to determine titer

1. Obtain your phage stock tube from last week. Select one of the phage stocks that was positive by PCR.

2. Obtain 5 sterile microcentrifuge tubes and label them 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Add 90 ul of phage buffer into each tube (keep tubes closed at all times!)

3. Take 10 ul from your phage stock tube (pipet up and down 3 times before removing the 10 ul) and transfer to the 10^{-1} tube. Invert the tube 10 times to mix well.

4. Take 10 ul from your 10^{-1} tube (pipet up and down 3 times before removing the 10 ul) and transfer to the tube10^{-2}. Invert the tube 10 times to mix well.

5. Repeat step 4, transferring from the 10^{-2} tube to the 10^{-3} tube, etc. until you have all dilutions completed through 10^{-5}.

6. You are done with the 10^{-1} and 10^{-2} tubes, so you can discard them in the biohazard trash now.

7. Obtain 3 new microcentrifuge tubes. Label them 10^{-3}, 10^{-4}, and 10^{-5}. Transfer 10 ul of the diluted phages to these new tubes (10 ul from the old 10^{-3} tube to the new 10^{-3} tube, etc.). Keep the new tubes with 10 ul and discard the old set of tubes in the biohazard trash.

8. To each tube, add 300 µl of fresh *M. smegmatis* cells.

9. Allow tubes to sit at room temp for 30 minutes.

10. Obtain 3 7H10 plates and label them with your initials and 10^{-3}, 10^{-4}, and 10^{-5}.

11. After 25 minutes, melt your MBTA in the microwave, mixing frequently to keep it from boiling over.

12. Obtain 3 15 ml conical tubes. To each, add 2.5 ml of MBTA and 2.5 ml of 7H9.

13. One at a time, take one of your phage/ *M. smegmatis* cell tubes, transfer the entire volume of liquid to the MBTA/7H9 solution. Mix by vortexing gently and pour onto the surface of a 7H10 plate (make sure the label corresponds to the dilution that you are using). Swirl the plate gently to distribute.

14. Allow plates to harden for ~10 minutes on the bench and then incubate at 37C overnight. Be sure to invert your plates.

BUILD-A-PHAGE SUPPLY LIST

PCR purification and Electroporation of Bacteria with Phage DNA:

- Promega Wizard SV Gel and PCR Cleanup kit (A9281; 50 preps)
- Electroporation apparatus
- Electroporation cuvettes (VWR #89047-206)
- Sterile 7H9 media WITH ADC and sterile 7H9 media WITHOUT ADC
- Bottles containing 40 ml sterile MBTA (recipe below)
- Sterile 5 ml pipets and pipet aids
- Microwave
- 7H10 plates
- Sterile glass test tubes with caps
- Wild-type phage DNA (we use Mycobacteriophage Giles)
- *Mycoplasma smegmatis* (or other bacterial host) cells containing pJV53 plasmid; grown overnight in 7H9 media with 10%ADC (see these references for details on the strain and plasmid: http://phagesdb.org/media/workflow/protocols/pdfs/BRED_Main_Protocol_2.2013.pdf http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0003957)

Picking plaques and PCR screening:

- Phage buffer (recipe below)
- Sterile microcentrifuge tubes and racks
- PCR positive control (combination of any plasmid template and two primers that are known to work well)
- NEB OneTaq Hot Start 2X Master Mix
- Watermark primers

Diluting phages:

- Sterile 1.7 ml microcentrifuge tubes
- Sterile 7H9 liquid media
- 15 ml conical tubes or sterile test tubes with lids
- Sterile 5 ml pipets and pipet aids
- Bottles containing 40 ml sterile MBTA
- Microwave
- 7H10 plates
- Phage buffer
- Incubator set to 37C

Media recipes:

Phage buffer:
Recipe available at: http://phagesdb.org/media/workflow/protocols/pdfs/PHProtocol_PhageBuffer.pdf
Note that you must add CaCl2 to 1 mM before using phage buffer.

7H10 plates:

Combine 19g 7H10 agar, 12.5 ml 40% glycerol, and 890 ml distilled water. Autoclave and cool to 55 degrees. Add 100 ml ADC, 10 ml of 0.1M CaCl2, 1 ml of 50 mg/ml carbenicillin, and 10 mg/ml cycloheximide. Mix well and pour plates (makes ~40 plates).

MBTA:

Combine 4.7g 7H9 powder, 7g Bacto agar, and 900 ml distilled water. Autoclave, mix well, and distribute ~40 ml into each sterile glass bottle. Allow to cool and harden (be careful not to close the cap tightly or it will be difficult to reopen).

7H9 media:

Combine 2.4 g 7H9 powder, 2.5 ml of 40% glycerol, and 450 ml distilled water. Autoclave and allow to cool.
For 7H9 without ADC, add 15 ml of 0.1M CaCl2.
For 7H9 with ADC, add 15 ml of 0.1M CaCl2 and 50 ml ADC.

Should gene drives be considered a conservation strategy in New Zealand?

Figure 1. Many species of plants and animals found in New Zealand are endemic, or completely unique to the country. All these species have declined greatly and are some of New Zealand's iconic avifauna (a) kakapo, a ground-dwelling forest parrot, (b) kea, the only alpine species of parrots, (c) North Island brown kiwi, and (d) tui.

Overview

One of the best detailed proposals to use gene drives for conservation purposes suggests using this technology to exterminate non-native, European rats (*Rattus rattus* and *Rattus norvegicus*) from New Zealand. New Zealand is a very unique land mass, as it has no native species of mammals except for bats. Therefore, many species - including plants, reptiles, insects, and bird - have declined dramatically due to herbivory and predation by introduced rats, stoats, and possums. In particular, New Zealand has a very rich avifauna - including such birds as the forest-dwelling parrot (kakapo), kiwi, and many others (Fig. 1) - some of which have only been saved from the brink of extinction due to captive breeding and release on mammal-free, offshore islands. While stoats and possums also pose a significant threat, these are less acceptable targets of genetic manipulation due to more limited genetic sequencing and their importance to other ecosystems. For example, the species of possum that is invasive in New Zealand is protected in Australia, and accidental escape of genetically modified

individuals to this nearby continent could be devastating. In contrast, rats are commonly used as a laboratory species and are invasive in many parts of the world.

The purpose of this case study is to discuss the benefits and costs of using this technology to restore the altered ecosystem of New Zealand. One of the reasons we focus on New Zealand is due to a large initiative called “Predator-Free 2050” which was initially started as a lobby group by Les Kelly. Kelly lived abroad for many years before returning to New Zealand. He was struck by the huge decline of birds within the span of his own lifetime. As the idea won support from conservation biologists, the government, and the public, it has caught on as a goal to restoring some of the former ecology of the country by the year 2050.

Proposed use of gene drives to eliminate rats

Two types of gene drives have been proposed to eventually eliminate populations of rats. One is a gene drive to turn all offspring into females (all gametes will possess and X chromosome), which should lead to a skewed sex ratio and eventual local extinction of populations. Another proposed mechanism is to reduce fertility in homozygous female offspring (gene drive depicted in the presentation), which again would cause populations to decrease. These mechanisms first spread the gene drives and then causes reduced population growth within generations (both are “daisy-chain gene drives”, which are explained in more detail below).

Risk: If local release becomes global invasion:

1. What happens if these genetically modified rats escape to different parts of the globe?

2. Rats have not been studied as species of conservation-concern. But what do you think ecological effects of complete extermination would be in areas where they are native?

3. In sexually-reproducing species, how are the effects of gene drives different from the introduction of a genetically-modified animal without the use of gene drives?

4. Given many of these risks, why might New Zealand be a good place for the release of gene-drive engineered rats?

Proposed safe-guards:

Currently, New Zealand has closely-monitored safe-guards against importation of invasive species. These safe-guards were not in place in the past, when many of these mammals were intentionally or accidentally introduced. The IUCN (International Union for the Conservation of Nature) report on this project suggests that these safe-guards can also be used to limit exportation of potentially invasive species as well. However, two of the experts of gene drive technology in this example also note that there are economic incentives for smuggling engineered rats to different parts of the world for rodent control, as these same two species are introduced/seen as pests in large parts of the world.

A second safe-guard has been proposed by Esvelt, which are called “daisy-chain gene drives” and are self-limiting gene drives. For example, a gene C would drive (or perpetuate, via CRISPR/CAS) a gene B, which drives a gene A. Gene A produces the phenotype, but does not have its own gene drive. After multiple generations, these gene drives should die out. This happens because gene C does not drive, or perpetuate itself - and is eventually lost. As C is lost, gene B will also be reduced and eventually lost in the population, with the same thing happening to A as B is lost.

1. How do you think mutations might affect the gene-drives and daisy-chain gene drives?

2. Many models of spread of gene drives include the idea of panmixis (i.e. complete mixing of a population). Can you think of reasons why a rat population might not be in panmixis, as barriers to gene flow or mate-selection?

3. If there are barriers to gene flow, do you think the gene drives would spread more quickly or die out more quickly?

4. If gene drives die out too quickly, can you think of an argument against their use in eradicating a species?

Reducing widespread application of rodenticide

Currently, rats and other invasive mammals are controlled by various means, but include the widespread application of toxic chemicals. These are also toxic to pets and livestock.

1. How might gene drives be considered more humane than poisoning live animals?

2. Do you think the risks (stated so far) outweigh the benefits of potentially saving species from extinction and reducing pollution?

Communities (including indigenous communities) rightfully feel skeptical about new technology being used for conservation

Humans - especially from industrialized nations - have a bad track record in biocontrol and causing more harm than good in the name of economic or environmental benefits. Examples include the introduction of cane toads to Australia to limit pests in sugar fields. Cane toads outcompete some native species, are toxic to native predators, and spread disease. In fact, stoats and possums were intentionally introduced to New Zealand to limit introduced rabbit populations and as part of the fur industry, respectively. Finally, the use of genetically modified crops has been criticized in some regions as disenfranchising local communities, leading to an increase in monocultures, poor local nutrition, and widespread application of herbicides.

In New Zealand, the Maori (native population of Polynesian descent) have lived in New Zealand for roughly 800 years and have a strong ethic for preservation of the land. Luckily, open forums about the clear problem of invasive mammals and the risks of genetic modification have led to thoughtful debates, and some community support, mixed with concern and a cautionary view towards conservation. Esvelt and Gemmel (2017) specifically address the importance of open conversation and only using this technology with approval of the local communities in New Zealand.

1. As part of the local community, what might some of your concerns be about the release of gene-drive rats?

2. The kiore rat (*Rattus exulans*) was brought to New Zealand by Maori people and is culturally important. While it might still be considered a threat to some native species, this species of rat is relatively localized in the southern parts of the South Island, and causes much less damage than the two more common species. Do you think hybridization with the other species and spread of gene drives could negatively affect these small populations?

3. One worry is that this new technology will be seen as a “cure” to species extinctions and more traditional conservation strategies, such as preserving habitat and land stewardship, will no longer be emphasized. Do you agree or disagree with this sentiment?

What is our ethical obligation?

Finally, two other questions emerge from this debate. These are entirely based on your opinion/world view:

1. Is it ethical to fundamentally change an organism?

2. If we have the means to save species that we, as humans, have driven to the brink of extinction, is it not ethical to use technology and science to reverse those extinctions?

Regulations

While the IUCN reviewed the potential use of gene drives for conservation, they proposed a cautionary look at this new technology, the need for new regulations (beyond those now in place for agricultural genetically modified organisms), the need for international as well as national agreements and laws, and the review of proposals on a case-by-case basis. In short, all biological and ecological effects cannot be predicted, but the hope is that scientists can use lessons learned in the past from both failed and successful manipulation of the ecosystem to use this technology only with great care and full community support.

Read more here (click on links or ask your instructor for these resources):

Websites (Predator Free 2050):

https://predatorfreenz.org/big-picture/pf-2050-vision/

https://predatorfreenz.org/research/public-attitudes-worldview-gene-drive-technologies/

(2020 survey of New Zealanders)

https://www.doc.govt.nz/nature/pests-and-threats/predator-free-2050/

Popular science article:

Young, E. 2017. New Zealand's war on rats could change the world. Atlantic Daily. https//www.theatlantic.com/science/archive/2017/new-zealand-predator-free-2050-rats-gene-drive-ruh-roh/546011

Peer-reviewed publication:

Esvelt, KM, NJ. Gemmell. 2017. Conservation demands safe gene drive. PLOS Biol. 15:e2003850.

IUCN Report:

Genetic frontiers for conservation: an assessment of synthetic biology and biodiversity conservation. IUCN Task Force on Synthetic Biology and Biodiversity Conservation. 2019. doi: 10.2305/IUCN.CH.2019.04.en; available at https://portals.iucn.org/library/node/48408

Should gene drives be considered a conservation strategy in New Zealand?

For instructors: *The goal of this case study is for students to evaluate both the biology and ethics behind the use of synthetic genetics and gene drives to potentially solve a conservation problem. Currently, this is the most realistic proposal that involves genetic manipulation of a vertebrate animal. Some questions do not have a single correct answer, but logical suggestions are detailed below (in italics). The IUCN concluded that each proposal will have its own benefits and risks, and the technology should not be reviewed as one technique, but as individual applications. Hopefully, the students will reach a similar conclusion and discuss the pros and cons of this approach. (The presentation should be delivered prior to handing out the case study and be made available for reference: https://qubeshub.org/publications/2026/1).*

Figure 1. Many species of plants and animals found in New Zealand are endemic, or completely unique to the country. All these species have declined greatly and are some of New Zealand's iconic avifauna (a) kakapo, a ground-dwelling forest parrot, (b) kea, the only alpine species of parrots, (c) North Island brown kiwi, and (d) tui.

Overview

One of the best detailed proposals to use gene drives for conservation purposes suggests using this technology to exterminate non-native, European rats (*Rattus rattus* and *Rattus norvegicus*) from New Zealand. New Zealand is a very unique land mass, as it has no native species of mammals except for bats. Therefore,

many species - including plants, reptiles, insects, and bird - have declined dramatically due to herbivory and predation by introduced rats, stoats, and possums. In particular, New Zealand has a very rich avifauna - including such birds as the forest-dwelling parrot (kakapo), kiwi, and many others (Fig. 1) - some of which have only been saved from the brink of extinction due to captive breeding and release on mammal-free, offshore islands. While stoats and possums also pose a significant threat, these are less acceptable targets of genetic manipulation due to more limited genetic sequencing and their importance to other ecosystems. For example, the species of possum that is invasive in New Zealand is protected in Australia, and accidental escape of genetically modified individuals to this nearby continent could be devastating. In contrast, rats are commonly used as a laboratory species and are invasive in many parts of the world.

The purpose of this case study is to discuss the benefits and costs of using this technology to restore the altered ecosystem of New Zealand. One of the reasons we focus on New Zealand is due to a large initiative called “Predator-Free 2050” which was initially started as a lobby group by Les Kelly. Kelly lived abroad for many years before returning to New Zealand. He was struck by the huge decline of birds within the span of his own lifetime. As the idea won support from conservation biologists, the government, and the public, it has caught on as a goal to restoring some of the former ecology of the country by the year 2050.

Proposed use of gene drives to eliminate rats

Two types of gene drives have been proposed to eventually eliminate populations of rats. One is a gene drive to turn all offspring into females (all gametes will possess an X chromosome), which should lead to a skewed sex ratio and eventual local extinction of populations. Another proposed mechanism is to reduce fertility in homozygous female offspring (gene drive depicted in the presentation), which again would cause populations to decrease. These mechanisms first spread the gene drives and then causes reduced population growth within generations (both are “daisy-chain gene drives”, which are explained in more detail below).

Risk: If local release becomes global invasion:

1. What happens if these genetically modified rats escape to different parts of the globe?

One threat is the extirpation of native populations in other countries, in which they play an important role in the ecosystem. Other human communities have not approved this technology and may not be in favor of it - which could be seen as an infringement of rights.

2. Rats have not been studied as species of conservation-concern. But what do you think ecological effects of complete extermination would be in areas where they are native?

Extirpation could cause ecosystem-level effects. Rats may provide food sources to other mammals. Rodents have been shown to be important as seed dispersers. Alternatively, the impacts may not be severe, if other rodents in those areas perform similar ecological roles.

3. In sexually-reproducing species, how are the effects of gene drives different from the introduction of a genetically-modified animal without the use of gene drives?

Gene drives are self-perpetuating and the gene of interest is passed on to all the offspring. If a genetically-modified animal (without the use of gene drives) mates with a normal animal, only half of the modified genes would be expected to be passed down to the offspring.

4. Given many of these risks, why might New Zealand be a good place for the release of gene-drive engineered rats?

New Zealand is an island, so it is easier to contain a genetically-modified organism.

Proposed safe-guards:

Currently, New Zealand has closely-monitored safe-guards against importation of invasive species. These safe-guards were not in place in the past, when many of these mammals were intentionally or accidentally introduced. The IUCN (International Union for the Conservation of Nature) report on this project suggests that these safe-guards can also be used to limit exportation of potentially invasive species as well. However, two of the experts of gene drive technology in this example also note that there are economic incentives for smuggling engineered rats to different parts of the world for rodent control, as these same two species are introduced/seen as pests in large parts of the world.

A second safe-guard has been proposed by Esvelt, which are called “daisy-chain gene drives” and are self-limiting gene drives. For example, a gene C would drive (or perpetuate, via CRISPR/CAS) a gene B, which drives a gene A. Gene A produces the phenotype, but does not have its own gene drive. After multiple generations, these gene drives should die out. This happens because gene C does not drive, or perpetuate itself - and is eventually lost. As C is lost, gene B will also be reduced and eventually lost in the population, with the same thing happening to A as B is lost.

1. How do you think mutations might affect the gene-drives and daisy-chain gene drives?

Mutations in general, might cause gene drives to not work properly. For example, loss- of-function mutations in any one of the three genes would not lead to the expected phenotype spreading throughout the population. Other

possible answers: Mutations could cause unexpected changes to be driven into the population. Other genes might become linked to the drives and also become fixed in the population.

2. Many models of spread of gene drives include the idea of panmixis (i.e. complete mixing of a population). Can you think of reasons why a rat population might not be in panmixis, as barriers to gene flow or mate-selection?

Rats are small and geographic barriers (rivers, mountains, etc.) would be obstructions to gene flow. Roads with high traffic may limit gene flow. Rats may also inbreed within family group, also reducing gene flow. Some rats favor human establishments, so gene flow may actually be limited across large expanses of natural areas.

3. If there are barriers to gene flow, do you think the gene drives would spread more quickly or die out more quickly?

They would spread more slowly. Multiple introduction of manipulated rats might be needed for population sizes to shrink.

4. If gene drives die out too quickly, can you think of an argument against their use in eradicating a species?

This technology might be more expensive than other means of control. Public opinion might change if gene drives do not work as well as promised.

Reducing widespread application of rodenticide

Currently, rats and other invasive mammals are controlled by various means, but include the widespread application of toxic chemicals. These are also toxic to pets and livestock.

1. How might gene drives be considered more humane than poisoning live animals?

Gene drives reduce fertility, but do not kill adult animals. Gene drives would also reduce accidental poisoning of non-target mammals.

2. Do you think the risks (stated so far) outweigh the benefits of potentially saving species from extinction and reducing pollution?

Yes: Gene drives seem humane and clean. We have an ethical obligation to reverse risks of extinction that humans have caused.

No: Gene drives are too risky. Humans should not change the genomic signature of a species. We cannot risk the chance of causing the complete extinction of one species that happen to be invasive in some parts of the world.

Communities (including indigenous communities) rightfully feel skeptical about new technology being used for conservation

Humans - especially from industrialized nations - have a bad track record in biocontrol and causing more harm than good in the name of economic or environmental benefits. Examples include the introduction of cane toads to Australia to limit pests in sugar fields. Cane toads outcompete some native species, are toxic to native predators, and spread disease. In fact, stoats and possums were intentionally introduced to New Zealand to limit introduced rabbit populations and as part of the fur industry, respectively. Finally, the use of genetically modified crops has been criticized in some regions as disenfranchising local communities, leading to an increase in monocultures, poor local nutrition, and widespread application of herbicides.

In New Zealand, the Maori (native population of Polynesian descent) have lived in New Zealand for roughly 800 years and have a strong ethic for preservation of the land. Luckily, open forums about the clear problem of invasive mammals and the risks of genetic modification have led to thoughtful debates, and some community support, mixed with concern and a cautionary view towards conservation. Esvelt and Gemmel (2017) specifically address the importance of open conversation and only using this technology with approval of the local communities in New Zealand.

1. As part of the local community, what might some of your concerns be about the release of gene-drive rats?

This is just another release of a non-native species. Unpredictable ecological ramifications may occur - such as a disruption to predator-prey relationships, increased populations of stoats, etc.

2. The kiore rat (*Rattus exulans*) was brought to New Zealand by Maori people and is culturally important. While it might still be considered a threat to some native species, this species of rat is relatively localized in the southern parts of the South Island and causes much less damage than the two more common species. Do you think hybridization with the other species and spread of gene drives could negatively affect these small populations?

Hybrids of most species of mammals are not fertile, and therefore would not spread the gene drive. Studies should be done on interbreeding among all three species, to rule out a risk to the kiore. Gene drives may actually benefit the kiore, if less rodenticide poisons are spread on the landscape.

3. One worry is that this new technology will be seen as a “cure” to species extinctions and more traditional conservation strategies, such as preserving habitat and land stewardship, will no longer be emphasized. Do you agree or disagree with this sentiment?

Agree: usually there is limited money for conservation. It might make more sense to continue with trapping rodents and also fund other types of land conservation. Public sentiment might also stop favoring more traditional conservation.
Disagree: New Zealand has a good track record for caring about conservation and Maori culture also favors land stewardship. I do not think the additional use of genetic manipulation will change that sentiment. New Zealand also has strong tourism industry, due to natural features and unique species: there is also economic pressure to preserve the ecosystem.

Pro/Con: What is our ethical obligation?

Finally, two other questions emerge from this debate. These are entirely based on your opinion/world view:
1. Is it ethical to fundamentally change an organism?

Various answers.

2. If we have the means to save species that we, as humans, have driven to the brink of extinction, is it not ethical to use technology and science to reverse those extinctions?

Various answers.

Regulations

While the IUCN reviewed the potential use of gene drives for conservation, they proposed a cautionary look at this new technology, the need for new regulations (beyond those now in place for agricultural genetically modified organisms), the need for international as well as national agreements and laws, and the review of proposals on a case-by-case basis. In short, all biological and ecological effects cannot be predicted, but the hope is that scientists can use lessons learned in the past from both failed and successful manipulation of the ecosystem to use this technology only with great care and full community support.

Read more here (click on links or ask your instructor for these resources):
Websites (Predator Free 2050):
https://predatorfreenz.org/big-picture/pf-2050-vision/
https://www.doc.govt.nz/nature/pests-and-threats/predator-free-2050/

Popular science article:

Young, E. 2017. New Zealand's war on rats could change the world. Atlantic Daily. https//www.theatlantic.com/science/archive/2017/new-zealand-predator-free-2050-rats-gene-drive-ruh-roh/546011

Peer-reviewed publication:

Esvelt, KM, NJ. Gemmell. 2017. Conservation demands safe gene drive. PLOS Biol. 15:e2003850.

IUCN Report:

Genetic frontiers for conservation: an assessment of synthetic biology and biodiversity conservation. IUCN Task Force on Synthetic Biology and Biodiversity Conservation. 2019. doi: 10.2305/IUCN.CH.2019.04.en; available at https://portals.iucn.org/library/node/48408

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Evaluation form: https://qubeshub.org/publications/2026/1

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