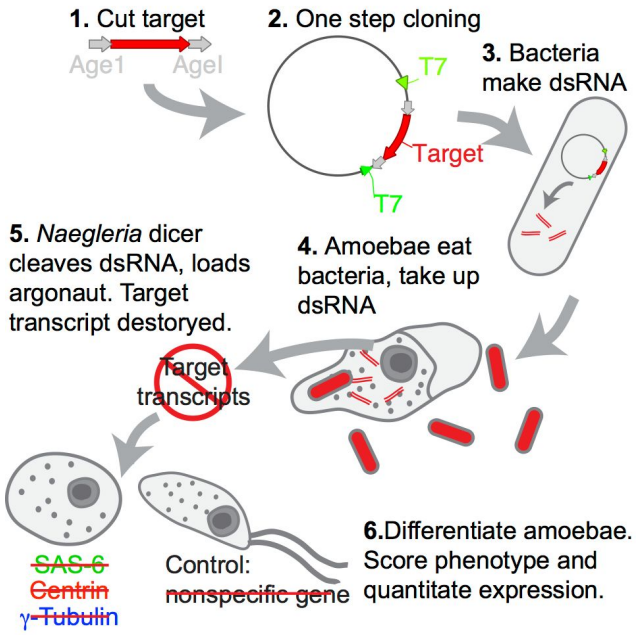
Molecular Cloning bootcamp 1

Diagram of overall plan for *Naegleria* module as we discussed in class. This diagram may help you design a flow chart to represent your experiment. Note: this diagram does not include every step of today's work (that's what you will be drawing in your flow chart), but is intended to give you a visual picture of the entire *Naegleria* module. I have already done step 1 for you. Today we are doing Step 2.



Today's mission: build dsRNA expression plasmids by inserting coding sequence of a Naegleria gene between two T7 promoters that can be activated by a special sugar (IPTG). This is represented in Step 2 of the above figure.

We will ligate together two pieces of DNA: the first has the gene we are targeting for RNAi flanked by Age1 restriction enzyme cut sites (I will have already digested this for you and purified the target gene away from the starting vector), the other is a plasmid that has two T7 promoters with an Age1 site between them (this will be the vector of the final ligation product). This plasmid also has a gene for Ampicillin antibiotic resistance for selection. We will ligate these two pieces together, and transform them in to DH5alpha bacteria to select for plasmids with our insert. On the second day of the cloning bootcamp, we will perform analytical digests to confirm our products.

Before beginning your flowchart, participate in the forum on Moodle which includes useful videos.

Flow Charts:
Before you begin, you must draw a diagram representing the experiment we are doing today. Avoid words, but instead use symbols or pictures (labels are OK). Feel free to ask questions of other students and/or instructors. The point of this process is to make sure everyone understand why they are doing each of these steps, and how each contributes to the experiments potential for success.

When you are done, find another student to pair up to give feedback. Trade flow charts. Look over each others and discuss. Are all the questions listed below answered by the flow chart? **Write feedback on the charts**

and add your name (hint, using another color will help). Useful feedback on someone else's chart is part of your grade, so be thoughtful. Again, as questions arise, ask! Be sure to turn in your flow charts before leaving.

Questions to be sure to address in your flow chart:
Why do we dephosphorylate the target vector digest reaction?
Why heat inactivate? What would happen if we didn't?
Explain the purpose of the two ligation controls. How will you evaluate these controls? (Or, what does success look like? Failure?)
Draw a diagram of the two pieces of starting DNA. Include all features discussed above.
What are all of the possible products of the ligation reaction? Draw them. Which will allow growth on ampicillin plates?

Experimental Steps:

1.) Digest the target vector with Age1 restriction enzyme. Dephosporylate target vector.
2.) Column purify vector
3.) Ligate (remember controls: no insert, no vector)
4.) Transform DH5alpha bacterial cells.
5.) Plate transformations on ampicillin plates

We purchased the enzymes you will be using from New England Biolabs. In case you have more questions, you can find out lots of information about these and other common cloning reagents on their website (including detailed protocols and tips):

AgeI: https://www.neb.com/products/r0552-agei
rSAP: https://www.neb.com/products/m0371-shrimp-alkaline-phosphatase-rsap
Ligase: https://www.neb.com/products/m2200-quick-ligation-kit

Step 1: Digest + dephosphorylate vector (10ul of starting insert vector = 300 ng)
Mix: plasmid, 10X buffer, and water by pipetting up and down. Add enzymes. Pipette up and down again gently to mix. Centrifuge briefly to make sure all contents are at the bottom of the tube. Incubate at 37C for 30 minutes.

Reaction contents:

10ul vector
2ul Digest Buffer (NEB 1.1)
1ul (2 units) Age1
1ul rSAP
6ul water

Step 2: Column purify your DNA
To purify your vector away from the restriction enzyme, follow the step-by-step instructions provided at each lab station.

Principle: This column purification system uses a simple bind-wash-elute procedure. Binding buffer is added directly to the PCR sample or other enzymatic reaction, and the mixture is applied to the QIAquick spin column. Nucleic acids adsorb to the silica membrane in the high-salt conditions provided by the buffer. Impurities are washed away and pure DNA is eluted with a small volume of low-salt buffer provided or water, ready to use in all subsequent applications.

Note: although this text was copied from the Qiagen PCR Purification Handbook (https://www.qiagen.com/us/shop/sample-technologies/dna/dna-clean-up/qiaquick-pcr-purification-kit/#product details), there are many brands of columns that work in the same way.

Step 3: Ligation

First, fill out the grid below with volumes to use for your controls. This is a thought exercise. Have someone check your work! When assembling your ligation reaction, add DNA, and Rxn buffer first, pipette up and down to mix. Then add ligase buffer and mix. Then add ligase and mix by pipetting up and down gently. Incubate reaction at room temperature for 5 min.

Component	Ligation	Insert only control	Vector only control
Water	0 ul		
Digested vector	5 ul *		
Digested insert	5 ul *		
Ligase Buffer	10 ul		
Ligase	1 ul		

*Note: These volumes are based on the fact that we want to add insert to vector at a 3:1 Molar ratio, and a total DNA content of 150 ng, this works out to be 75ng of each.

Step 4: Transformation

Keep your cells cold! Any warming of competent cells is bad for the efficiency of the transformation. (Except for the heat shock step. Obviously.) Each group gets one tube of competent cells.

1. Pre-chill tubes for transformation, one tube for each ligation.
2. Aliquot 100ul of competent cells into each tube.
3. Add 5ul of each reaction to an aliquot of competent cells. Mix by flicking. DO NOT LET THEM WARM UP. Put your tube back on ice.

Heat shock cells by putting them into 42C heat block for 30 s. Immediately return cells to ice. Incubate on ice for 2 min. Add 200ul of LB media, and plate your transformations by pipetting onto selection media (LB+Amp plates), adding a dozen or so sterile glass beads, and shaking to spread (instructors will demonstrate). Dump out used beads in dirty bead jar.

The next morning: move plates from 37 to 4C. (Lil will do this for you).