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Instructor Handbook

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Yeast ORFans CURE

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ABSTRACT

This document describes the theory behind the Yeast ORFans CURE and addresses operational details. Actual instructor protocols (for setting up the student labs) are in the same workspace as this document.

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ABSTRACT

This document describes the theory behind the Yeast ORFans CURE and addresses operational details. Actual instructor protocols (for setting up the student labs) are in the same workspace as this document.

Introduction

As the accompanying publication explains, the goal of this CURE is to gather data about genes of unknown function in brewer's yeast *Saccharomyces cerevisiae*. The approach we take is called "reverse genetics" -- we disable a gene, then screen the resulting knockout strain for phenotypes. I usually tell the following story to illustrate:

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"Imagine you are an alien who, after watching a bunch of Earth television from orbit, has a pretty good idea of how a car works. To learn more, you steal a car from a parking lot and are amazed to find that it is a hugely complicated machine with lots of smaller parts. How can you figure out what each part does? Well, one approach is to remove the part and see how that changes the car's function. For example, you could remove the wheels -- and you'd discover that the wheels are pretty important to the function of the car! Or you could remove just the hubcaps -- and you'd find that not much had changed.

What happens, though, if you remove the windshield wipers? If you were driving the car on a sunny day, it wouldn't have much impact. However, if you drove on a dark and stormy night, you would find that they were very important indeed -- but only under particular conditions."

We take a similar approach to discovering the function of a gene in *S. cerevisiae*. First we disable it -- "knock it out" in genetics parlance -- and then we see what has changed. It's unlikely that the knockout will keep the cell from growing entirely, because most of those genes have already been discovered. However, a huge number of genes have so-called conditional phenotypes -- like the wipers in the storm, they only have an effect on cell growth under particular conditions. And *which conditions show us a phenotype tell us something about the gene!* For example, if we knock a gene out and those cells are subsequently more sensitive to being poisoned by hydrogen peroxide, then that's a pretty good clue that that gene (and the protein that it encodes) is involved in the cell's response to oxidative stress.

What follows is an overview of the molecular biology we use to create the knockouts. For actual protocols, see the accompanying documents elsewhere in this workspace.

For a more student-focused introduction, have a look at the student guide, which accompanies this document.

Experimental Overview

The knockout approach we're using is based on a molecular genetics phenomenon called homology-directed repair, or HDR. Basically, when DNA is damaged, the cell will repair the damaged site with DNA that has sequence homology -- either the homologous gene (in a diploid cell) or the sister chromatid (in a cell that is in the S or G2 phase of the cell cycle.)

For years, this approach has been used to generate knockout strains in yeast and in other organisms -- see

Hu Y, Jia Y, Zhao X, Yang Z, Hao Z, Dong J, Zeng F (2019). A new strategy for seamless gene editing and marker recycling in Saccharomyces cerevisiae using lethal effect of Cwp1.. MicrobiologyOpen.
https://doi.org/10.1002/mbo3.750

The approach is straightforward:

1. Choose the gene to knock out and collect its genome sequence from an online database.
2. Design PCR primers to amplify a selection marker and attach homologous sequences from upstream and downstream of the target gene to the amplicon.
3. Perform the PCR, purify the PCR product and transform it into the cells. Select for transformants using the

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selection marker.

Enough random DNA damage happens in a culture that, with a bit of luck, you'll get transformants where your gene of interest has been damaged and the cell has "repaired" it using the PCR product, disrupting the gene. (You can verify this with a subsequent PCR.) Unfortunately, this process is inefficient enough that it requires an extremely good yeast transformation.

Enter CRISPR technology. Cas9 is a "programmable" endonuclease -- it's sequence specific, but the sequence at which it cuts is determined by a guide RNA that it complexes with. The "usual" Cas9 protein, from *Streptococcus pyogenes*, can't cut just anywhere -- the cut site needs to be downstream of a "PAM" (protospacer-adjacent motif), which is 3'-NGG-5'. However, Cas9 can serve as an "amplifier" of PCR-based knockouts, because it can cause a double-stranded break in the genome. If we co-transform the PCR product from above with a plasmid that expresses Cas9 and a guide RNA targeting the gene, this stimulates homology-directed repair at that locus and greatly increases the efficiency of editing. Per a colleague, something like 80% of the colonies that are transformed with the plasmid also had a correctly-edited gene.

This, then, is the plan:

- Choose a gene of unknown function (henceforth called "YFG", for "your favorite gene"). Use bioinformatics software to choose a cut site that Cas9 can target in that gene.
- Create a plasmid that expresses Cas9 and a guide RNA for the selected cut site.
- Create a PCR product with a selection marker flanked by homologous sequences upstream and downstream from the cut site.
- Co-transform yeast with BOTH the Cas9-expressing plasmid and the PCR product. Select for the selection marker on the PCR product -- but use the plasmid marker as a transformation control.
- Do a quick-and-dirty genomic DNA extraction and PCR YFG back out of the genome. Make sure that length of the PCR amplicon matches the expected length if the gene was knocked out.

The rest of this handbook outlines the equipment and reagents required, walks through the semester, and ends with some general tips. Most of the individual student protocols have instructor protocols detailing the setup required, as well as protocol-specific tips and common student errors to watch out for.

Major Equipment

- Autoclave
- -80 freezer
- Incubator
- Incubating shaker
- Swinging-bucket or high-speed refrigerated centrifuge
- Thermocycler
- Gel imager
- Nanodrop (or equivalent for measuring DNA concentration and purity)
- Cuvette spectrophotometer (for measuring culture density)
- Cold room (not strictly required but highly recommended)
- 96-well plate reader with incubation (for growth curve experiments)

Minor equipment

- Micropipettors - P-1000, P-200, P-20 and P-10 or P-2.
- Microcentrifuges
- Mini-centrifuges with a 200-ul PCR tube rotor
- Microwave oven
- Submerged gel tanks and power supplies for agarose gel electrophoresis
- Water bath

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- Dry bath (with microfuge tube block)
- Mr. Frosty (3) (or a large styrofoam container)

Materials

- 1.7 ml microcentrifuge tubes
- 200 ul PCR tubes
- 15 ml conical centrifuge tubes
- 50 ml conical centrifuge tubes
- Test tubes for microbial cultures -- either disposable or reusable
- Petri dishes (disposable polystyrene)

Reagents

Many of the kits and enzymes from NEB are available free to educational institutions! See https://www.neb.com/promoting-science-education/course-support-and-reagent-donation

Recombinant DNA Kits

☒Monarch® Plasmid Miniprep Kit **New England**

- **Biolabs Catalog #T1010**

☒Monarch® PCR & DNA Cleanup Kit (5 µg) **New England**

- **Biolabs Catalog #T1030**

Enzymes and enzyme buffers

☒Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns **New England**

- **Biolabs Catalog #M0494S**

☒Esp3I **New England**

- **Biolabs Catalog # R0734S**

☒PvuII-HF - 5,000 units **New England**

- **Biolabs Catalog #R3151S**

☒T4 DNA Ligase - 20,000 units **New England**

- **Biolabs Catalog #M0202S**

☒CutSmart® Buffer **New England**

- **Biolabs Catalog #B7204S**
- ☒10X NEB T4 DNA ligase buffer **New England Biolabs**

☒Diluent A - 5.0 ml **New England**

- **Biolabs Catalog #B8001S**

☒Diluent B - 5.0 ml **New England**

- **Biolabs Catalog #B8002S**

Other recombinant DNA reagents

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☒Agarose **Contributed by**

- **users Catalog #A5304**

☒Quick-Load Purple 1 kb Plus DNA Ladder - 250 gel lanes **New England**

- **Biolabs Catalog #N0550S**

☒Gel Loading Dye Purple (6X) - 4.0 ml **New England**

- **Biolabs Catalog #B7024S**

☒SYBR SAFE DNA stain **Invitrogen - Thermo**

- **Fisher Catalog #S33102**
-

☒Parafilm™ M Laboratory Wrapping Film, 4 in. W x 125 ft. L; (10cm x 38m) **Thermo Fisher Catalog #1337410**

☒Lithium Acetate Dihydrate **Sigma**

- **Aldrich Catalog #L4158**

☒Boric acid **Fisher**

- **Scientific Catalog #BP1681**
- ☒Nuclease free water **Contributed by users**
- ☒TE Buffer **Contributed by users**

Microbiological media

☒Tryptone **Fisher**

- **Scientific Catalog #BP1421-500**

☒Bacto™ Peptone **Thermo Fisher**

- **Scientific Catalog #211677**

☒BD Bacto™ Yeast Extract **BD**

- **Biosciences Catalog #212750**

☒α-D-Glucose **Sigma**

- **Aldrich Catalog #158968**

☒Agar, bacteriological

- grade **Amresco Catalog # J637**

☒Yeast Nitrogen Base (YNB) **Sunrise**

- **Science Catalog #1500-100**

☒CSM-Leu-Ura Powder **Sunrise**

- **Science Catalog #1038-010**

☒L-Leucine Yeast Culture Grade **Sunrise**

- **Science Catalog #1980-010**

☒Uracil Yeast Culture Grade **Sunrise**

- **Science Catalog #1906-010**

☒Ammonium sulfate **Sigma**

- **Aldrich Catalog #A4418**

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Miscellaneous (microbiological)

- ☒Mix & Go! E.coli Transformation Buffer Set **Zymo**
- **Research Catalog #T3002**
- ☒Kanamycin **Research Products International**
- **(rpi) Catalog #K22000-25.0**
- ☒Sodium Chloride **Fisher**
- **Scientific Catalog #S271**
- ☒Potassium chloride **Sigma**
- **Aldrich Catalog #P9333**
- ☒Magnesium chloride hexahydrate **Sigma**
- **Aldrich Catalog #M2670**
- ☒Magnesium sulfate heptahydrate **Sigma**
- **Aldrich Catalog #M2773**
- ☒Glycerol **Contributed by**
- **users Catalog #G5516**
- ☒DMSO (dimethyl sulfoxide) **Sigma**
- **Aldrich Catalog #D8418**
- ☒Sodium dodecyl sulfate **Sigma**
- **Aldrich Catalog #436143-25G**
- ☒Polyethylene Glycol (PEG) 3350 **Electron Microscopy**
- **Sciences Catalog #19760**
- ☒Salmon Sperm DNA **Research Products International**
- **Corp Catalog #D52150**
- ☒Ethanol (100%, Molecular Biology Grade) **Fisher**
- **Scientific Catalog #BP2818500**
- ☒10 mL syringes **BD**
- **Biosciences Catalog #BD 309695**
- ☒0.2 µm syringe
- filter **Corning Catalog #CLS431212**
- ☒Glass beads 5 mm **VWR**
- **Scientific Catalog #26396-596**

Key Reagents

Beyond the "usual" molecular biology reagents for recombinant DNA work, there are a few specialized reagents that are required for this CURE. (Contact me for samples!)

Plasmid L2-01

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L2-01 is the plasmid that expresses Cas9 and a guide RNA in yeast. It is also a shuttle plasmid -- it can be maintained in both E. coli and yeast. Its construction is described in the Supplemental Information section (along with a plasmid map and a GenBank file with its sequence and annotations), below, but briefly, it has four major groups of functional sequences:

- The bits required for E.coli cloning -- a ColE1 origin of replication and the KanR resistance marker (which makes transformants resistant to the antibiotic kanamycin.)
- The bits required yeast cloning -- a CEN6/ARS4 (centromeric / low copy number) origin of replication and a LEU2 auxotrophic marker (which allows it to complement leucine auxotrophy in LEU2 knockout strains of yeast)
- The bits needed to express the Cas9 protein: a strong constitutive promoter, the Cas9 open reading frame, and a terminator.
- The bits needed to construct a custom single guide RNA (sgRNA) and express it. As described in the Lee et al paper linked from the Supplement, the sgRNA is based on a phenylalanine tRNA, an HDV ribozyme, a 20 bp targeting sequence, the sgRNA, and an SNR52 terminator. The targeting sequence is the variable region that needs to be changed to re-target the Cas9 protein -- in this vector, it is replaced by a GFP cassette flanked by BsmBI cut sites.

To customize L2-01 to target a specific gene requires the following steps:
- Digest the L2-01 plasmid with BsmBI (or Esp3I, which is an isoschizomer.)
- Optional: gel-purify the backbone.

> Improves ligation efficiency (decreases background), but not strictly required if your digestion is efficient and you use a 10:1 molar excess of annealed oligo to backbone in the ligation (below)

- Synthesize two oligonucleotides containing the 20 bp target sequence. If we write the target sequence as (TS) and its reverse complement as (TSrc), then the two oligonucleotides are:

  5' GACTTT-(TS) 3'

  and

  5' AAAC-(TSrc)-AA 3'

- Anneal the two nucleotides to produce a short double-stranded piece of DNA
- Ligate the annealed oligos into the digested backbone
- Proceed with the "usual" recombinant DNA workflow: transform into E. coli, pick (white!) colonies, grow up a couple of 5 ml cultures, miniprep to purify the plasmid DNA, and verify with a restriction digest.

Plasmids YTK74 and YTK96

These plasmids are from the Dueber lab's yeast toolkit. YTK74 is used as the template for the URA3 amplification, and YTK96 is used for both yeast and E. coli transformation controls (it has both KanR and URA3 markers -- you could also use undigested L2-01.) Plasmid maps and Genbank sequences are below.

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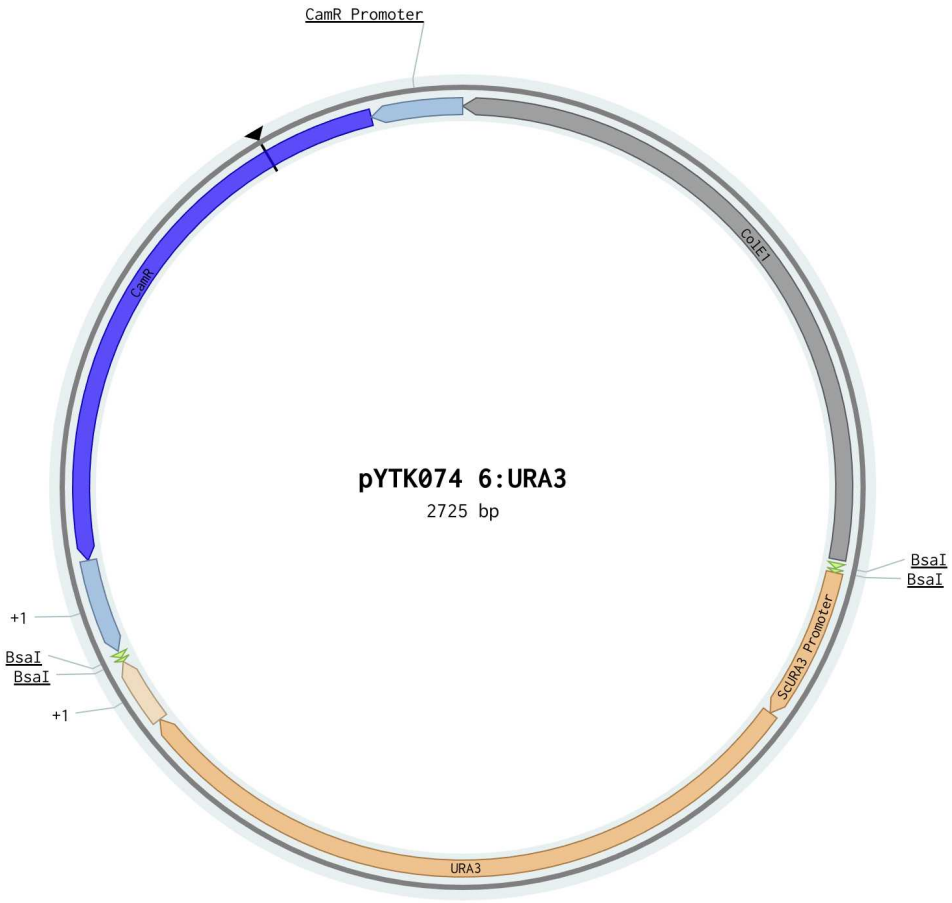

pYTK074 6-URA3 (seq_S6e0lmcS).gb

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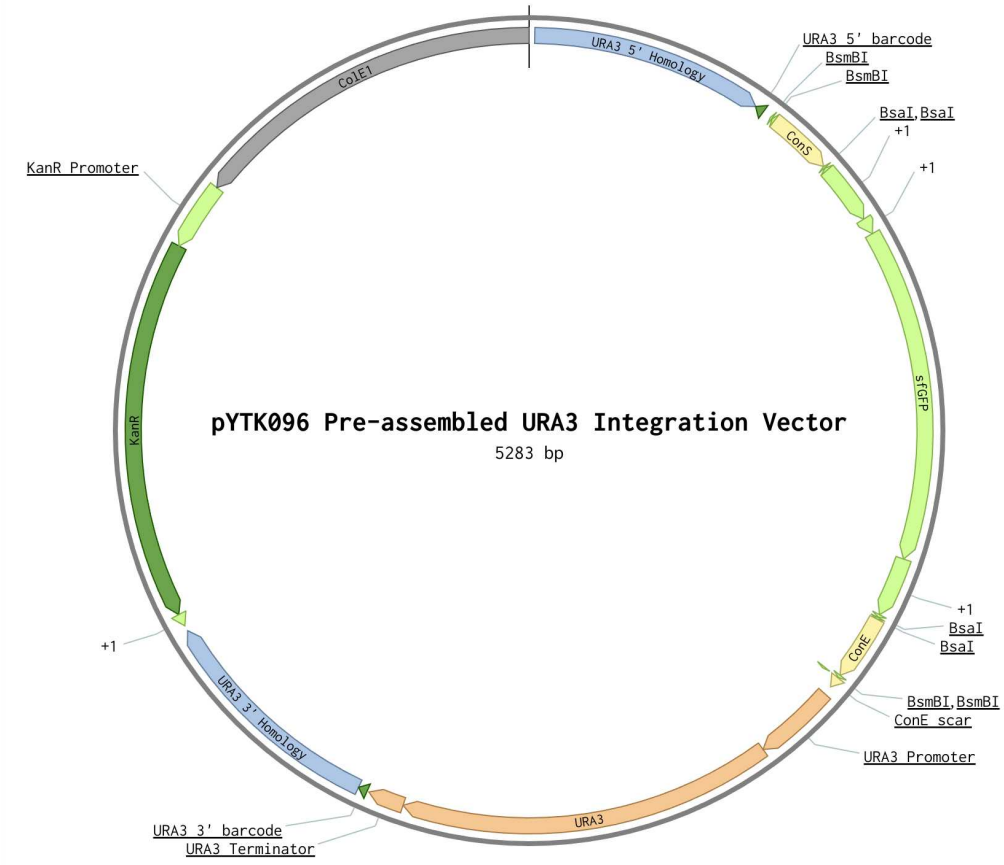

pYTK096 Pre-assembled URA3 Integration Vector (seq_f3kyxzGc).gb

Auxotrophic yeast strain and media

Transforming microbes -- either E. coli or S. cerevisiae -- is highly inefficient, which necessitates *selection* to recover successfully transformed clones. In E. coli, the traditional approach is *antibiotic selection*. You make media containing an antibiotic such as ampicillin or kanamycin; and on the plasmid is a gene that makes the E. coli resistant to the antibiotic. When you transform and grow on the selective media, only cells that transformed successfully will grow. (These cells are clonal -- hence the word "cloning", of course.)

This approach can also be used to select transformants in yeast, but it is less common. Instead, one often uses *auxotrophy,* which depends on a yeast strain that has been genetically modified so it cannot produce a necessary metabolite such as uracil or leucine. On the plasmid is a gene that complements the disabled metabolic gene, and now the selective media is synthetic defined (SD) media without the auxotrophic metabolite. Untransformed cells cannot make the metabolite and cannot get it from the media, while transformed cells can make it and proliferate.

There are several common auxotrophic markers: URA3 (uracil), LEU2 (leucine), and HIS3 (histidine) are among the most common. Laboratory strains often have multiple auxotrophies, which allow for multiple genetic modifications simultaneously. The strain that we've used is BY4735, which is derived from S288C. Its genotype is MATalpha ade2Δ::hisG his3Δ200 leu2Δ0 met15Δ63 ura3Δ0, and it's described in this publication:

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Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications.. Yeast (Chichester, England).

The auxotrophies we use are uracil and leucine; any haploid ura3Δ / leu2Δ strain should be fine.

Semester Walkthrough

This is the structure of a "usual" semester, in greater detail than is laid out in the accompanying manuscript. Our semesters are 14 weeks long, and my course meets twice weekly for two hours each time.

Week 1

Protocol 0 - Pipetting Practice

Most students have never used a micropipettor before, and subsequent protocols are MUCH more likely to be successful if we've done some intentional practice before. This is also an opportunity to practice making dilutions -- many students can recite "c1 * v1 = c2 * v2" but have never actually needed to use it to make a dilution! In particular, the idea that they need to compute the amount of water (or other diluent) is often a challenge.

The protocol is adapted from **Dilution and Pipetting Lesson Using Food Dyes**, by James Burnette, Lisa Kanizay, Nikki Chester, Susan R Wessler on CourseSource here:

https://qubeshub.org/community/groups/coursesource/publications?id=2556&tab_active=about&v=1

Protocol 1 - Design oligos

This protocol walks students through choosing a gene to target and designing the three pairs of oligonucleotides they'll need to knock out their gene and verify the knockout. My lab meets Tuesday and Thursday -- if we can get these done, double-checked and ordered from IDT on Thursday, they are here and ready to use by the following Tuesday.

Week 2

Protocol 2 - Annealing Oligonucleotides

Students resuspend and mix together oligos to be annealed. I am deliberately pretty hands-off about the dilution math etc, and this is students first "real" molecular biology protocol -- finding reagents, still getting used to the micropipettors, record keeping, etc. As such, a protocol that would take me 10 minutes usually takes students an entire two-hour lab period.

Protocol 3 - Ligation

Students dilute their annealed oligos and ligate them into the plasmid backbone that expresses Cas9 and the

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guide RNA. The ligation incubates on the benchtop for an hour, so I usually request that students complete Protocol 4 (URA3 PCR, below) on the same day.

Protocol 4 - URA3 PCR

Students PCR the URA3 cassette off of the YTK74 template, adding 40 bp homology arms on each end. PCR is often quite finicky, especially for beginners. Here, we're using a purified plasmid template, which helps immensely. Also, I like to use

Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0494S** -- it's robust and causes less primer degredation when students have assembled their reactions but are waiting to load in to the thermocycler. We see substantially better results with this than the

OneTaq 2X Master Mix with Standard Buffer - 100 rxns **New England Biolabs Catalog #M0482S** that we used to use.

Week 3

Protocol 5 - Transformation

Students transform their ligation into chemically competent E. coli and plate on LB-agar + kanamycin plates. There is a lot of waiting in this one -- I always suggest students begin brainstorming their knockout experiments.

Protocol 6 - Agarose gel electrophoresis (of the URA3 PCR)

Students analyze their PCR with gel electrophoresis. They also interpret their transformations and pick colonies to miniprep (if there are some white colonies on the plate.)

Week 4-6

By this point, most groups will have failed at least once -- no colonies on the transformation plate, or all-green colonies, or no PCR band. It's a good point to take a moment and normalize failure! And remind students that this is real research -- if they want to knock out their genes, they need to get their transformation and PCR to work! Prioritize the transformation; as soon as there are white colonies, students can move on to:

Protocol 7 - Miniprep

The instructor picks colonies that the students have indicated to grow up 5 ml cultures (two colonies per group), and students use a miniprep kit to purify their plasmids back out of the E. coli. DNA concentration and purity is checked with a Nanodrop or equivalent (Qbit, DyNAquant, etc).

Protocol 8 - Diagnostic restriction digest

Students digest part of their miniprep with PvuII. Because PvuII cuts inside the GFP dropout as well, it discriminates between a "correct" plasmid and a common failure mode.

Protocol 6 - Agarose gel electrophoresis (of the diagnostic restriction digest)

This agarose gel is just like the last one, with one important difference -- the students need to load the ENTIRE digest in order to get bright enough bands to interpret.

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When a group's PCR works, they can do:

Protocol 9 - PCR Purification

Students purify the PCR amplicon DNA away from the buffer & protein in the PCR reaction. DNA concentration & purity are checked with the Nanodrop or equivalent.

Groups that are at the front of the success curve, I encourage to get to work planning their knockout experiments. I start waving the Hampsey paper, below, in their face and suggesting that they NOT reinvent the wheel. If you have the bandwidth, they can also move forward with the yeast transformation steps, below

Hampsey M (1997). A review of phenotypes in Saccharomyces cerevisiae.. Yeast (Chichester, England).

If, by the beginning of Week 6, groups don't have their plasmid and PCR ready to go, then I'll spend a couple of afternoons making the plasmid and PCR products for them. (I generally don't do the PCR purification.) This balances "normalize failure" and "do real research" with meeting the CURE's learning goals.

Week 7 - Yeast transformation

Let's knock out some yeast genes!

Protocol 10 - Yeast transformation

I always double-check groups' work before I let them do a yeast transformation -- I want to see two high Nanodrop concentrations (and good A230/A260 and A280/A260 numbers) for their plasmid and purified URA3 amplicon, and two correct gels (the diagnostic restriction digest and the PCR). Then students can transform their yeast.

If students do the yeast transformation on a Tuesday, their plates won't be grown up enough to move forward on Thursday. This is the week that I request students settle on a stressor for their phenotype study and communicate it to the rest of the class, so that we don't have multiple groups studying the same phenotype.

Weeks 8-10 - Yeast transformation retries & verification

If the yeast transformation didn't work, then they need to be tried again! If there are colonies on the -Ura plate, students can proceed to

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Protocol 11 - Yeast DNA extraction

Students do a "quick and dirty" genomic DNA extraction. There's no phenol chloroform and no zymolase -- just a quick lithium acetate / SDS lysis followed by an ethanol precipitation. Produces genomic DNA good enough for PCR (but not much else.)

Protocol 12 - Knockout verification PCR

Students use primers that flank the insertion site to determine if the insertion mutagenesis worked or not. They analyze their PCR using agarose gel electrophoresis, as usual.

When students have a succesfully verified knockout, the yeast strain is archived (both on a rich media plate, for the refrigerator, and in a glycerol stock at **-80 °C**)

Weeks 11-14 - Phenotype experiments and write-up

Phenotype experiments usually take 4 weeks or so. This is enough time to prepare reagents (usually takes one day), run a pilot experiment and analyze the data (two days), and run the actual phenotype experiment (several replicates over several days.) If there are several groups in a section, and each group has (a) generated a knockout strain and (b) designed a phenotype experiment, then we take an all-tests-all approach. That is to say, each group does not just test THEIR yeast strain; instead, they test EVERYBODY'S strains. This makes the likelihood of identifying a real phenotype much higher.

Finally, students write up their results and post them to the Saccharomyces Genome Database wiki.

Notes and Tips for Success

Each instructor protocol has a tips section. In addition, here are some overall tips for a successful implementation:

Make the lab reflect your teaching philosophy. My goal in developing this CURE was to create an *authentic research experience* for my students, and this informs how I deliver the lab too. I am notably hands-off -- happy to consult when students have trouble and to double-check answers, but I'm not going to hand a student a result that they should be working for (and learning to develop) themselves. For me, the best lab is one that requires an understanding of the molecular principles at work for success. As a colleague likes to remark, "if you're not uncomfortable, you're not learning."

Focus on transferable skills. This flows from the first tip, but I think it merits closer examination. How many of these students will need to know how to transform E. coli later in their education or their career? Not many. Instead, these labs are an opportunity to learn, and refine, some really important transferable skills:

- *Record keeping* - My students are using Benchling for sequence manipulation already, so I ask them to keep their notebooks there too (using a template I provide.) How are you going to have your students record their work? Demonstrate their thinking about the work they're doing in the lab?
- *Sample tracking* - closely related to record keeping. As the CURE progresses, student groups accumulate many tubes containing small volumes of clear liquids. How are they labeled and recorded and tracked?
- *Data analysis* - knowing whether a result is "right" or "wrong" depends on a molecular understanding of what's happening. Is the band on this gel in the right place? Do I pick the green or the white colony?
- *Experimental design* - especially towards the end of the semester, we focus on designing a good experiment. What controls are necessary? How do we balance doing an interesting experiment with feasibility and safety concerns?
- *Communication and collaboration* - If you see a puzzling result on a gel or a graph, who are you going to

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puzzle over it with? And publishing the experimental results "for real" really makes students focus on the question "what does a reader who's not familiar with my work need to know?"
- *Identity as a scientist* - an important part of authenticity is autonomy and ownership. The answers aren't going to be handed to you -- but the flip side is, you get to make decisions that affect the outcome of the experiment.

Normalize failure and repetition. An experienced scientist could complete all of these steps in just a few afternoons. But students are novices and they're still learning -- and that's okay! Sometimes "real" science doesn't work, but we don't just shrug and move on. Instead, we ask what might have led to the failure we observed, and then we try again -- optimally, modifying something to make the failure less likely. (Commonly misattributed to Albert Einstein, "insanity is defined as doing the same thing and expecting a different result.") And there's nothing like watching a student's face when the PCR finally works.

Adopt and adapt as necessary. I've described this CURE as I run it currently - but it may not work for you as written! In particular, meeting twice a week for two hours is a huge luxury. If we met once per week, for example, I might have multiple groups working on the same gene to increase the chances of success. Or I might do more of the cloning -- for example, make a few plasmids myself, and have students do the PCR and the yeast transformation. Decide what is important and what is expendable in your situation, what supports the things you want your students to get out of the CURE.

Get help. This CURE can require a LOT of student support, especially in the beginning. I find it incredibly useful to have a good student TA to help, preferably someone who has completed the CURE before.

Get in touch. If you're implementing this CURE, I'd love to know about it! I'm happy to send samples of the plasmids, consult on technical issues, etc.

Supplemental Information

Assembly of plasmid L2-01.

Student-created variants of L2-01 express Cas9 and a guide RNA that is specific to the gene they're trying to knock out (YFG). I assembled L2-01 from parts in the Dueber lab's yeast toolkit, described here:

Lee ME, DeLoache WC, Cervantes B, Dueber JE (2015). A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly.. ACS synthetic biology.
https://doi.org/10.1021/sb500366v

The Dueber lab toolkit uses GoldenGate cloning, first described here:

Engler C, Kandzia R, Marillonnet S (2008). A one pot, one step, precision cloning method with high throughput capability.. PloS one.
https://doi.org/10.1371/journal.pone.0003647

The following description assumes basic familiarity with GoldenGate cloning.

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Two L1 plasmids were assembled.

L1-01 was assembled with a BsaI GoldenGate reaction from the following yeast toolkit plasmids:

- YTK002 ConLS
- YTK011 pPGK1
- YTK036 Cas9
- YTK054 tPGK1
- YTK067 ConR1
- YTK095 AmpR-ColE1 backbone

L1-02 was assembled with a BsaI GoldenGat reaction from the following yeast toolkit plasmids:

- YTK003 ConL1
- YTK050 sgRNA Dropout
- YTK072 ConRE
- YTK095 AmpR-ColE1 backbone

Both L1 plasmids were verified with a diagnostic restriction digestion. Then, a backbone plasmid named BB-01 was created using a BsaI GoldenGate Reaction from the following yeast toolkit plasmids:

- YTK008 ConLS'
- YTK047 GFP Dropout
- YTK073 ConRE'
- YTK075 LEU2 yeast selection marker
- YTK081 CEN6/ARS4 yeast origin of replication
- YTK084 KanR-ColE1 backbone

Finally, L1-01, L1-02 and BB-01 were assembled into an L2 named L2-01 using an Esp3I GoldenGate reaction. (Esp3I is an isoschizomer of BsmBI.)

The plasmid map is below, and the GenBank file follows.

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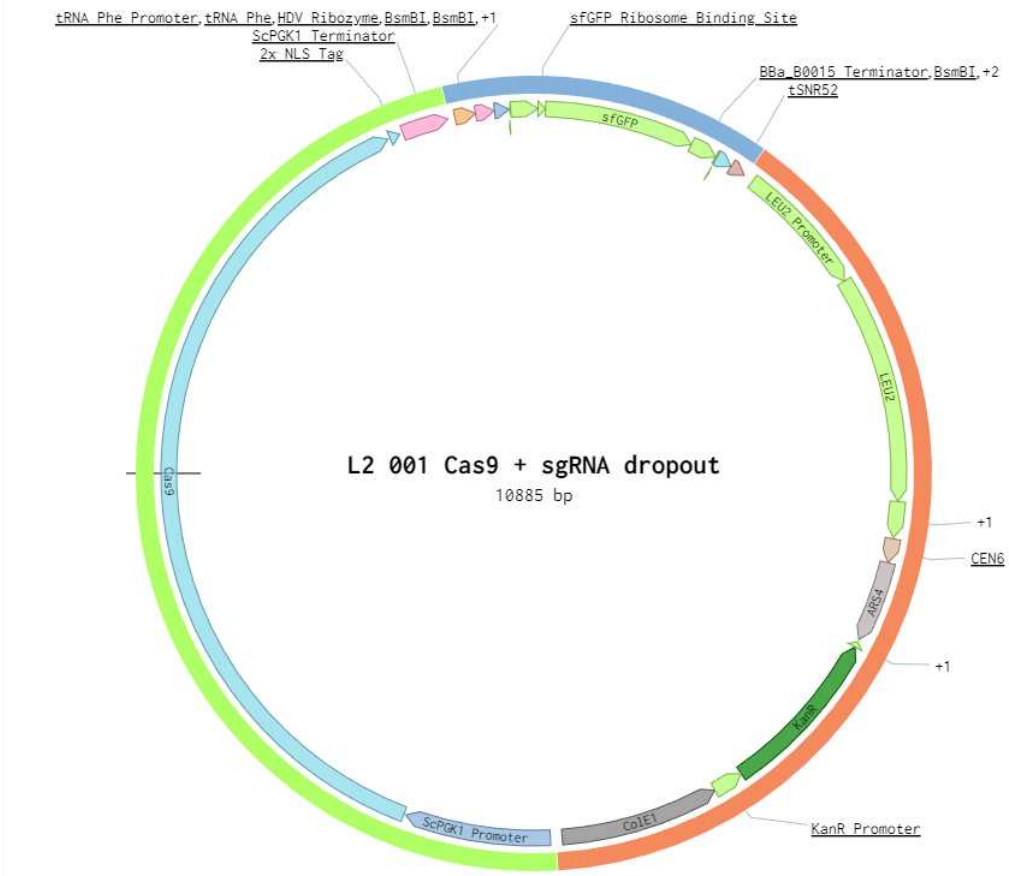

L2-01.gb

After cutting L2-01 with BsmBI (or Esp3I), the GFP dropout can be replaced with annealed oligos as per the supplement to the Lee paper, linked above. The map and annotated sequence of the resulting plasmid follow -- the targeting sequence is replaced with Nx20:

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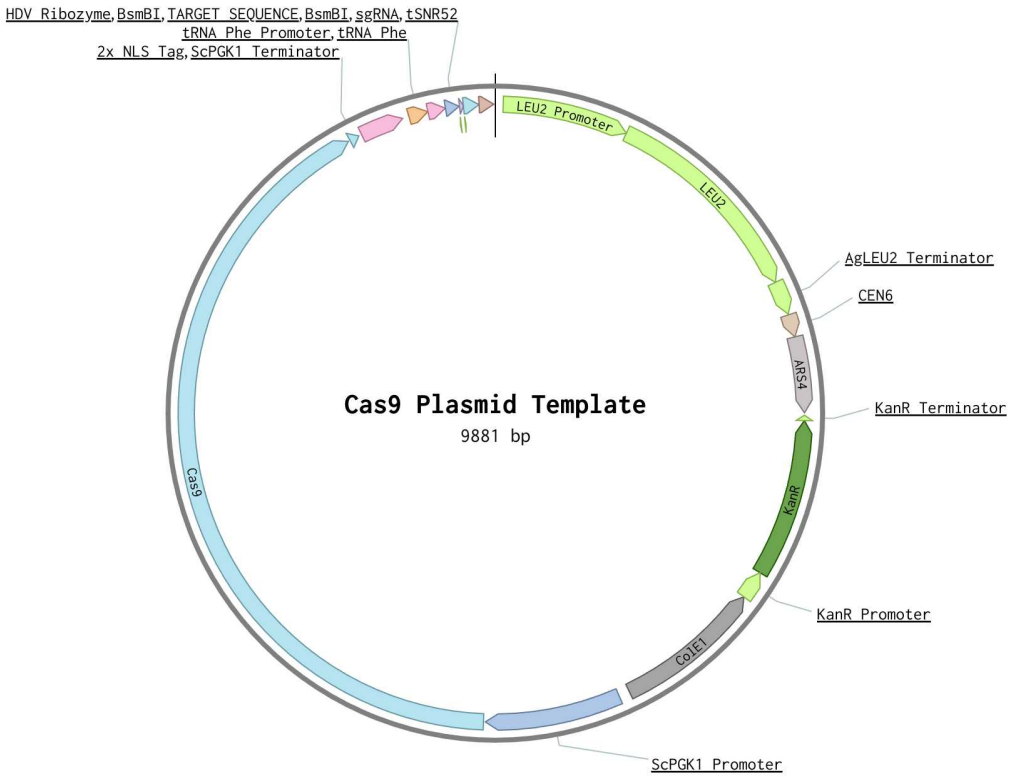

Cas9 Plasmid Template.gb

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