Hangover Free beer workshop - Introduction to CRISPR

End of July to October workshop dates

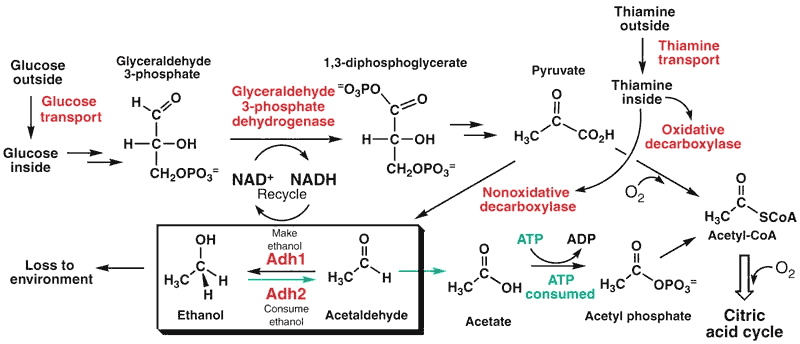
1. Why is it useful
2. Talk about pros and cons of this and some
   1. Talk about josiah
   2. Don't worry about stepping on toes
3. First group older, the team
4. Second group younger
   1. Potentially run 2 segments

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# Overview

[Acetaldehyde](https://health.howstuffworks.com/wellness/drugs-alcohol/hangover4.htm) is identified to be a strong contributor to hangovers. While the human body also creates Acetaldehyde from breaking down ethanol after a long night of drinking, it is [often found naturally in beer](https://www.ncbi.nlm.nih.gov/pubmed/19335652) puting us at an unwelcome risk to cancers. Acetaldehyde arises in beer as yeast can convert glucose into ethanol, but when glucose is in low supply, it can also metabolize ethanol into acetaldehyde for energy. The expression of ADH2 is under the control of a [glucose regulated promoter](https://www.ncbi.nlm.nih.gov/pubmed/15849781).



The goal of the workshop is get workshop participants to knock out the ADH2 gene in brewers yeast to prevent the reverse metabolism of Ethanol into acetaldehyde when it is used to brew. Thereby, reducing levels of acetaldehyde in the beer.

Part of the workshop should engage workshop participants in theorizing the next steps in bringing beers closer to hangover free. Include a question and answer series of what other modifications we could make to yeast to get it to make completely hangover free beer. Provide information in the form of handouts OR web references.

1. <https://www.wired.com/2014/05/hangover-cure/>
   1. Alternate ethanols - Known isomers that have antidotes
   2. Opunita ficus indica extract - reduces inflamation
   3. Unique flavanoids
2. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3929114/>
   1. Alda 1 - ALDH2 agonist, if you wanted a beer that could clear the acetaldehyde from your body due to the breakdown of alcohol, getting the yeast to produce this in your beer would be on another level!
   2. <https://www.deltanutra.com/blogs/news/an-aldh2-deficiency-drug-mystery-aldea-pharmaceutical-s-alda-1>

Workshop participants will be given various CRISPR gRNA sets to use in their own experiments. These will target various areas within the ADH2 gene for double stranded breaks (DSB) by the CAS9 protein. Some of these breaks may induce errors due to non-homologous end joining (NHEJ) and result in a variety of effects from loss of function, impaired function, to no change. Following each users transformation, beer will be brewed using their resulting mutants and screened for their acetaldehyde content using a purchased assay kit and compared against a control strain of yeast.

# Considerations

Feedback from various professionals in industry and academia and general to do’s

1. How can we get more than 10 colonies less than 100 (need a minimum of 10 to be sure that we have 10 colonies) - this can depend on the amount of DNA and the strain that we are using, and the size of the plasmid we are using. Aim for between 4,000 - 10,000 bp
2. How much overhang (10bp) restriction site , gRNA, restriction site (10bp)  
   Check using Oligo calc to check for hairpins.
3. Select plasmid
4. Create a list of gRNA sets for participants to use
5. Transform open plasmid into yeast with gRNA, this ensures that only yeast that have taken up and gone through a homologous recombination event has worked.

# Experimental Design

Since the goal requires beer to be brewed from the resulting mutants, it’s important that mutants be able to be used in this capacity. This makes auxotrophic selection markers unsuitable for selecting positive transformants of the CRISPR plasmid. Instead, it is better to have a selection method such as

Literature recommends that a variety of gRNA’s be used if possible to target genes for knockout to ensure the success of knockouts.

## Flow

1. Order in plasmids
   1. AQIS permits (non standard biological product)
2. Grow culture of bacteria on selective plates/selective broth
3. Mini prep
   1. Store vials at -20
4. Glycerol stocks
5. Construction of Plasmid
   1. Order in gRNA with protospacer
   2. PCR amplification with long range high fidelity polymerase and gRNA primers
   3. Gel clean up kit to get proper 10.4kbp product
   4. [T5 exonuclease/T4 DNA ligase assembly](https://openwetware.org/wiki/Gibson_Assembly) - recipie

## Plasmid selection

Factors to be aware of when choosing plasmids; Better if they are under 10kbp, successful transformants must be able to brew unhindered (can’t use auxotrophic mutants).

Candidates

1. [pRCC-N](https://www.addgene.org/81192/)- 10kbp, ROx3 promoter, Natmx6 marker, SNP52p promoter for gRNA, BamHI/NotI for gRNA insert.
2. [pML 104-KanMx4](https://www.addgene.org/83476/) - 11kbp, Bcll-Swal cloning sites for gRNA
3. [bRA89](https://www.addgene.org/100950/) - 11kbp, PGK1 promoter, HPH marker, Bpll cloning sites for gRNA
4. [bRA66](https://www.addgene.org/100952/) - 11kbp, GAl1 promoter for cas9, HPH marker, Bpll cloning sites for gRNA with SNR52 promoter for gRNA, AmpR for bacterial selection

## Primers for Yeast ID and ADH2

ITS1F- TCCGTAGGTGAACCTGCGG - **primarily for ensuring the right strain of yeast**  
Primers for ADH2 sequence (covers PAM sites) - **Was able to amplify target PCR product on Sach previously in 2017**  
Fwd - CAGTTCCAAAGCCAAAGCCC   
Rev - TTCGATAGCGGCTTCGGAAA   
  
 gRNA targeting sets (show picture of what this is targeting)

>gRNA\_for\_Sc:13:873,519  
GAGGAGCACTTTGCACCGGC  
>gRNA\_for\_Sc:13:873,453  
CCATTGTCGGCTCTTACGTG

## gRNA

<https://www.dna20.com/eCommerce/cas9/delivery>

[gRNA synthesis](https://media.addgene.org/cms/files/hCRISPR_gRNA_Synthesis.pdf)

ADH2 - regulation/operon look at pathway

## Testing

Acetaldehyde assay kit  
<https://secure.megazyme.com/Acetaldehyde-Assay-Kit>

<http://www.yeastgenome.org/locus/S000004918/overview>

<https://www.dna20.com/products/crispr#4>

## General Steps

1. Select Suitable plasmid
   1. Neuro (Natmx6)
      1. <https://www.addgene.org/74215/>
      2. <https://www.addgene.org/81192/>
   2. Kanamycin
      1. <https://www.addgene.org/78231/>
      2. <https://www.addgene.org/83946/>
2. Design gRNA’s

## Plasmid Construction

## 

## Transformation

Options

Going with the basic method of yeast transformation (LIAC/PEG/SSDNA)

Yeast colonies should be grown up on YAPD overnight to induce log phase

### Solutions

1. Lithium acetate (1.0 M). Add 5.1 g of lithium acetate dihydrate (Sigma Chemical  
   Co. Ltd., St. Louis, MO, cat. no. L-6883) to 50 mL of water in a 100-mL Pyrex  
   medium bottle, stir until dissolved, autoclave for 15 min, and store at room temperature.

Table 4

|  |  |
| --- | --- |
| Table 4  Transformation Mix  PEG 3500 (50% [w/v])  LiAc 1.0 M  SS carrier DNA (2.0 mg/mL)a  Plasmid DNA (100 ng) plus water (distilled/deionized)  Total volume (excluding cells) | -  Component Volume  240 μL  36 μL  50 μL  34 μL  360 μL |

*“aVortex mix the carrier DNA before pipetting it.”*

2. PEG MW 3350 (50% [w/v]). Add 50 g of PEG 3350 (Sigma Chemical Co. Ltd.,  
cat. no. P-3640) to 30 mL of distilled/deionized water in a 150-mL beaker. Dissolve  
on a stirring hot plate with medium heat and then cool to room temperature.  
Make the volume up to 100 mL in a 100-mL measuring cylinder, cap the cylinder  
with Parafilm’, and mix by inversion. Transfer the solution to a glass storage  
bottle and autoclave for 15 min. The polyethylene glycol (PEG) can be stored at  
room temperature. The bottle must be securely capped bottle to prevent evaporation,  
which will increase the concentration of PEG in the transformation reaction  
and severely reduce the yield of transformants.

3. Single-stranded carrier DNA (2.0 mg/mL). Dissolve 200 mg of salmon sperm

DNA (Sigma Chemical Co. Ltd., cat. no. D-1626) in 100 mL of TE (10 mM Tris-

HCl, 1 mM Na2 EDTA, pH 8.0) on a stir plate overnight at 4°C. Dispense 20

samples of 1.0 mL into 1.5-mL microcentrifuge tubes and the remainder in 5 mL

samples in 15-mL screw-capped plastic centrifuge tubes and store at –20°C.

Denature the carrier DNA in a boiling water bath for 5 min and chill immediately

in an ice/water bath before use. Denatured carrier DNA can be boiled three or

four times without loss of activity.

4. Transformation Mix All three transformation protocols use the same basic Transformation

Mix (T Mix). The recipe below is for the transformation of 1 × 108

cells; the volumes can be amended as appropriate for larger and smaller numbers

of cells. T Mix can be made up in bulk and kept in ice/water until required. The

highest transformation efficiencies (transformants/μg plasmid DNA/108 cells) are

obtained with 100 ng plasmid DNA but the yield (number of transformants) will

be increased if more plasmid is used.

### Method

1. Inoculate a 2 cm2 patch of the yeast strain onto YPAD agar and incubate overnight  
   at 30°C (see Note 1).
2. Boil a tube of carrier DNA in a boiling water bath for 5 min and chill immediately  
   in ice/water. We suggest that you do this first, otherwise you will have to  
   put subsequent steps on hold for 10 to 15 min.
3. Scrape a 50 μL blob of yeast from the YPAD plate and suspend the cells in 1.0  
   mL of sterile water in a 1.5-mL microcentrifuge tube. The suspension will contain  
   about 5 × 108 cells.
4. Pellet the cells at top speed in a microcentrifuge for 30 s and discard the supernatant.
5. Add 360 mL of T Mix to the cell pellet. Resuspend the cell pellet by vortex  
   mixing briskly. For a single transformation, the ingredients should be added in  
   the order listed and mix vigorously.
6. Incubate the tube in a water bath at 42°C for 20–180 min (see Notes 2 and 3).
7. Microcentrifuge the transformation tube at top speed for 30 s and remove the T  
   Mix with a micropipettor.
8. Pipet 1.0 mL of sterile water into the transformation tube. Stir the pellet with a  
   sterile micropipet tip to resuspend the cells and then vortex mix vigorously.
9. Pipet 10 and 100 μL samples of the cell suspension onto plates of appropriate SC  
   selection medium. The 10 μL samples should be pipetted into 100 μL puddles of  
   sterile water. Transformants can be isolated after incubation at 30°C for 3 or 4 d.

### NOTES 1. This protocol can be used to transform cells that have been stored in a refrigerator

or at room temperature. The yield will be reduced but there will generally be

sufficient transformants of the desired genotype.

2. Incubation at 42°C for 20 min will result in several thousand transformants per

tube. With many yeast strains, extending the duration of this incubation can increase

the yield of transformants significantly. We have obtained 1 × 105

transformants/μg plasmid after 60 min incubation and >1 × 106/μg plasmid DNA

after 180 min.

3. The addition of dimethylsulfoxide (DMSO) to the T Mix increases the yield of

transformants with some strains. For example, when strain Y190 was transformed

by the Rapid Protocol the yield of transformants increased over 10-fold when 5%

DMSO was added to the T Mix and incubation at 42°C was extended to 180 min.

Zymogen kit allows easy transformation  
 1. Zymogen easy transformation of plasmids into yeast cells (no carrier DNA required) $210 AUD  
 a. <http://www.integratedsci.com.au/product/frozen-ez-yeast-transformation-ii-kit.html>

2. Protocol  
 a. <http://2014.igem.org/wiki/images/5/51/Forzen_EZ_yeast_transformation_protocol.pdf>

3. For figuring out the gs to RPM needed for the cells to make them competent  
 a. <https://tools.thermofisher.com/content/sfs/brochures/TR0040-Centrifuge-speed.pdf>

Transformation  
Yeast transformation Yeast cells were transformed by PEG/LiAc method according to Gietz and Woods (2006). The heat shock time was prolonged to 90 min for strain CBS7960 in order to get higher transformation efficiency  
  
Cloning  
E.coli  
  
  
Regarding using salmon sperm for yeast transformation  
  
*Hello Felicia,  
Thanks for the great question!  
As a former yeast biologist, I have transformed yeast dozens of times without really giving a lot of thought to the role that carrier DNA plays. In my lab, we used calf thymus DNA in lieu of salmon sperm DNA, but their purpose is roughly equivalent. Simply stated, scientists use carrier DNA because it increases transformation efficiencies by 10- to 100-fold. There are two prevailing theories as to for how this happens:  
 1. There are a multitude of nucleases in the yeast cell, and by adding DNA bulk, the plasmid of interest is more likely to make it to the nucleus without being degraded.  
 2. The yeast cell wall, because of its chemical composition, binds DNA; adding more extraneous DNA allows the wall to bind that DNA, allowing some of the plasmid to make it into the yeast cell.  
Unfortunately, this is as much information as I was able to find about the purpose of carrier DNA. I hope this answers your question!  
Sources:   
Burgers, PMJ and Percival LJ. Transformation of yeast spheroplasts without cell fusion. Analytical Biochemistry. 163. 391-397. 1987.   
http://www.ncbi.nlm.nih.gov/pubmed/3310730  
Gietz RD, Schiestl RH, Willems AR, and Woods RA. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast. 11. 355-360. 1995*  
From <<http://www.madsci.org/posts/archives/2012-04/1334787478.Cb.r.html>>

Obtain pure yeast strain  
  
Sanger sequence to ID yeast strain  
  
Sanger sequence to locate targeted PAM site  
  
  
 4. Use BmtI and EcoR1  
 a. <https://www.thermofisher.com/order/catalog/product/ER2041?ICID=search-product>

b. <https://www.thermofisher.com/order/catalog/product/FD0274?ICID=search-product>

Anneal grna to form over hanging sticky ends  
  
<https://www.sigmaaldrich.com/technical-documents/protocols/biology/annealing-oligos.html>

Clone CRISPR plasmid with gRNA in E. Coli  
Grow up E. Coli   
Purify Plasmid  
  
OR using DNA ligase to ligate gRNA in vitro   
  
Yeast transfection  
  
Screen yeast colonies

## Selection

<https://www.goldbio.com/product/1679/nourseothricin-sulfate-streptothricin-sulfate>

Growth on acitemide <https://www.sigmaaldrich.com/catalog/product/sial/a0500?lang=en&region=AU>

Kanamycin sulfate

<https://www.sigmaaldrich.com/catalog/product/sigma/60615?lang=en&region=AU>

DNA sequencing to check for gene deletion.  
  
Brew first batch of GMO 1% hangover beer using modified yeast  
*Recombinant industrial brewing yeast strainswith ADH2 interruption using self-cloning GSH11CUP1cassette - 2008*  
<https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1567-1364.2009.00502.x>

Costing

1. Plasmid - $60
2. Transformation in E.coli
   1. DH5a
3. Oligo synthesis $40
4. Plasmid mini prep
5. PCR
   1. gRNA primers
   2. Long range PCR 10kbp plus
   3. Ladder 1kbp
   4. Agarose
   5. PCR clean up
6. Transformation in yeast
   1. PEG/Liac
7. Plating
   1. Ypd media
   2. Nourseothricin Sulfate
8. Wort
9. Sanger Seq

# Resources

* [CRISPR/Cas system for yeast genome engineering: advances and applications-2017](https://academic.oup.com/femsyr/article-pdf/17/5/fox030/23892752/fox030.pdf)
  + General how to for CRISPR in yeast
* [Simplified Simplified CRISPR-Cas genome editing for Saccharomyces cerevisiae. - 2016](https://www.sciencedirect.com/science/article/pii/S016770121630149X?via%3Dihub)
  + Paper citing the use of the plasmid we will likely use
* <https://www.sigmaaldrich.com/technical-documents/protocols/biology/annealing-oligos.html>

# Appendices

ADH2 in Yeast  
  
ATGTCTATTCCAGAAACTCAAAAAGCCATTATCTTCTACGAATCCAACGGCAAGTTGGAGCATAAGGATATCCCAGTTCCAAAGCCAAAGCCCAACGAATTGTTAATCAACGTCAAGTACTCTGGTGTCTGCCACACCGATTTGCACGCTTGGCATGGTGACTGGCCATTGCCAACTAAGTTACCATTAGTTGGTGGTCACGAAGGTGCCGGTGTCGTTGTCGGCATGGGTGAAAACGTTAAGGGCTGGAAGATCGGTGACTACGCCGGTATCAAATGGTTGAACGGTTCTTGTATGGCCTGTGAATACTGTGAATTGGGTAACGAATCCAACTGTCCTCACGCTGACTTGTCTGGTTACACCCACGACGGTTCTTTCCAAGAATACGCTACCGCTGACGCTGTTCAAGCCGCTCACATTCCTCAAGGTACTGACTTGGCTGAAGTCGCGCCAATCTTGTGTGCTGGTATCACCGTATACAAGGCTTTGAAGTCTGCCAACTTGAGAGCAGGCCACTGGGCGGCCATTTCTGGTGCTGCTGGTGGTCTAGGTTCTTTGGCTGTTCAATATGCTAAGGCGATGGGTTACAGAGTCTTAGGTATTGATGGTGGTCCAGGAAAGGAAGAATTGTTTACCTCGCTCGGTGGTGAAGTATTCATCGACTTCACCAAAGAGAAGGACATTGTTAGCGCAGTCGTTAAGGCTACCAACGGCGGTGCCCACGGTATCATCAATGTTTCCGTTTCCGAAGCCGCTATCGAAGCTTCTACCAGATACTGTAGGGCGAACGGTACTGTTGTCTTGGTTGGTTTGCCAGCCGGTGCAAAGTGCTCCTCTGATGTCTTCAACCACGTTGTCAAGTCTATCTCCATTGTCGGCTCTTACGTGGGGAACAGAGCTGATACCAGAGAAGCCTTAGATTTCTTTGCCAGAGGTCTAGTCAAGTCTCCAATAAAGGTAGTTGGCTTATCCAGTTTACCAGAAATTTACGAAAAGATGGAGAAGGGCCAAATTGCTGGTAGATACGTTGTTGACACTTCTAAATAA

## OLD NOTES

Transform Yeast  
Use IDT for primers  
Test via brewing  
2 for double stranded  
Use zang lab MIT  
Design CRISPR website for yeast  
When designing guides need to design 3 guides, FW, REV, ssDNA (never know which will work)  
Clone in bac first, yeast 2nd (need stuff?)  
DNA mini prep  
GET DNA sequencer primer for CRISPR to check guide RNA is in plasmid (no empty vector)  
Cut plasmid with 2 RE's  
Put in bac to transform  
Choose 3 after you do transformation using control and ligase  
Mini prep one of these then do a diagnostic digest, cut vs not cut, try to design in a single RE site to remain  
Sequence  
  
[Genome editing with the CRISPR-Cas9 system](https://www.youtube.com/watch?v=h18HmFtybnQ)    
  
