Protocols

His-Tag Protein Purification

Part I: Cell Extract Preparation

1. Grow 200 ml cultures (LC) of desired E.coli strains overnight
2. Centrifuge sample at 5,000 g, 10 mins., and 4 degrees Celsius to harvest cells
3. Completely resuspend pellet in 20 mL xTractor lysis buffer
4. Incubate 10 minutes at room temperature with gentle shaking
5. Centrifuge sample at 13,300 rpm, 20 mins, 4 degrees Celsius to spin down insoluble debris
6. Transfer supernatant to a new tube
7. Filter cell extract through a 0.45 um syringe filter

Part II: His-tagged protein purification:

1. Remove pre-packed, pre-charged His GraviTrap column cap (GE Health Cat. No. 11-0033-99) and pour out buffer as clean as possible
2. Add 10 ml of washing buffer, cut open the tip of the column to start the flow
3. Allow washing buffer to flow through
4. Load cell extract and let it flow through
5. Add 10 mL washing buffer and let it flow through
6. Add 3.0 mL eluting buffer and let it flow through
7. Assay purified protein

*Buffers obtained by mixing different buffers from purchased kit by mixing as described in given protocol

ALDH2 Functional Test

***For candy prototype functional test, first dissolve candy and proceed directly to PART 1 STEP 3 (population has already been standardized during candy production).

PART I: Cell Lysate Preparation (if using purified enzymes, skip directly to PART II)

1. Grow liquid cultures (LC) of desired E.coli strains overnight

2. *E. coli* population standardization
    a. Take 3mL from each LC and transfer them into the cuvettes
    b. Prepare a blank (3mL of LB w/ antibiotic)
    c. Change the mode to time based on the spectrophotometer
    d. Set the wavelength to 600 nm
    e. Calibrate / warm-up the spectrophotometer with the blank solution
    f. Take absorbance of the samples
    g. Convert the absorbance value (@OD600) to bacterial cell count (Cells/mL)
        i. Bacteria Cell Count = Measured Absorbance Value x 8 x 10^8
        ii. Conversion calculator: http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp
    h. Determine dilution recipe in order to obtain 2.93 x 10^9 cells/mL (a number that can be used in

modeling): http://www.celeromics.com/en/Support/cell-dilution-calculator.php

3. Preparing Sample
    a. Centrifuge diluted LC at 5000 rpm, 10 minutes, 4 degrees C
    b. Decant LB
    c. Add 600 ul xTractor lysis buffer and pipet up and down to completely re-suspend pellet
    d. Incubate mixture with gentle shaking for 10 minutes at room temperature
    e. Centrifuge 13300 rpm (max speed) for 20 mins. at 4 degrees Celsius
    f. *if using boiled cell extract as blank, take cell extract out of centrifuge and boil it at 95 degrees Celsius for 10 minutes, then centrifuge one minute at 13300 rpm to remove insoluble debris
    g. Use supernatant to conduct functional testing

PART II: [NADH] quantification

4. Obtain quartz cuvettes and stoppers/caps

5. Turn on CT-400 Spectrophotometer
    a. Set to 340 nm
    b. The spectrophotometry automatically blanks everything to air, so there is no need to blank

6. Add the following recipe into the cuvettes:

Recipe	
Distilled water / Artificial Saliva	2000 µl
Cell extract / Purified Enzymes / Negative Control Buffer	500 µl
Acetaldehyde stock solution (0.45 M)	50 µl
Megazyme Buffer 1 (pH buffer)	200 µl
Megazyme Buffer 2 (NAD^+)	200 µl
Total Volume	**2.95 mL**

*Recipe volume can be diluted by 10 if using a 300 µl quartz cuvette

7. Immediately invert cuvettes and put into spectrophotometer

8. Put into incubator without shaking if controlling temperature

9. Take data points every 5 minutes

Ethanol Promoter Test

1. Prepare LC's overnight for desired bacterial strains
    a. LB (- control)
    b. AlcR only
    c. PalcA + GFP only
    d. GFP only (+ control)
    e. AlcR + PalcA + GFP (+1g ethanol)
    f. AlcR + PalcA + GFP (+0.1g ethanol)

    g. AlcR + PalcA + GFP (+0.01g ethanol)
    h. AlcR + PalcA + GFP (+0.001g ethanol)
2. Add ethanol based on the protocol above and dilute the rest with water
3. Take fluorescence reading for T0
4. Screw cap on and let shake for 24 hours
5. Take fluorescence at the end of 24 hours for T1
6. 3 replicates, each 100ul, into the well

7. Go take fluorescence using the Interlab conditions OR clicking on past files and opened up the file we used last time
    a. Take a time 0 fluorescence
    b. Send the data to me and Tim
8. Take the plate back to lab and throw away
9. ADDING ETHANOL TO LIQUID CULTURES
    a. Add 1270ul of ethanol into the liquid cultures called (1g)
        i. No water needs to be added
    b. Add 127ul of ethanol into the liquid culture called (0.1g)
        i. Add 1143ul of ddH2O into liquid culture to maintain equal volume
    c. Add 12.7ul of ethanol into the liquid culture called (0.01g)
        i. Add 1257.3ul of ddH2O into liquid culture to maintain equal volume
    d. Add 1.27ul of ethanol into the liquid culture called (0.001g)
        i. Add 1268.73ul of ddH2O into liquid culture to maintain equal volume
10. Place these cultures into the shaking incubator and shake at 60 RPM

Protein Purfication/ Western Blot

Useful links to learn about western blotting:

- https://www.abcam.com/ps/pdf/protocols/Western_blot_diagram.pdf
- https://www.jove.com/science-education/5065/the-western-blot

Protein Sample Prep (same as SDS)

1. Prepare bacterial liquid culture overnight
2. Set hot plate to 95C
3. Centrifuge 1 ml LQ at 12000 rpm for 1 minute at 4C
4. Discard supernatant, thoroughly decant by pipetting excess liquid
5. Add 100 ul 1x SB (with B-Mercapotoethanol)
6. Carefully pipette to re-suspend bacteria pellet, avoid causing bubbles
7. Boil suspension at 95C for 10 mins
8. Centrifuge at 13300 rpm (max speed) for 10 mins for 4C

Making SDS Page Gel

Preparing SDS-PAGE gels WARNING: Unpolymerized acrylamide is a neurotoxin!

(1) **Clean the plates and combs.** For each gel, you will need one short plate, one spacer plate, and one comb. These

are usually found on the gray rack by the sink. Spray a little bit of 70% ethanol on the plates, and wipe dry with Kimwipes. Wash the combs thoroughly with tap water. It is critical to remove all dust and small particles, especially any bits of left-over polyacrylamide. **WIPE THE SIDES**

(2) **Set-up the plates on the rack.** Layer the short plate on the spacer plate, with the spacers in between and slide the two plates into the green holder. Make sure that the bottom edges of the two plates are flush to avoid leakage. Lock the plates in, and place the holder on the rack, with the bottom edges of the plates pushed into the gray foam gasket to make a water-tight seal. Test the seal by pipetting or squirting a small volume of water between the plates and making sure there is no leakage. Blot dry with filter paper.

(3) **Pour the separating gel.** For each minigel (1 mm thick) you will need slightly more than 5 mL of reagent mix. (3mL) Use the table below as a guide to calculate the total volumes you will need. Pipette solutions in order. Avoid introducing bubbles, which will inhibit polymerization. Swirl the solution gently to mix thoroughly after addition of each component.

	7.5% gel	10% gel	12.5% gel	15% gel	18% gel
ddH_2O	2.81 mL	2.50 mL	2.19 mL	1.88 mL	1.50 mL
40% acrylamide/bis stock	0.94 mL	1.25 mL	1.56 mL	1.88 mL	2.25 mL
1.5 M Tris, pH 8.8	1.25 mL	1.25 mL	1.25 mL	1.25 mL	1.25 mL
10% ammonium persulfate	50 µL	50 µL	50 µL	50 µL	50 µL
TEMED	5 µL	5 µL	5 µL	5 µL	5 µL
TOTAL VOLUME	~5 mL	~5 mL	~5 mL	~5 mL	~5 mL

Once TEMED is added, the gel will begin to polymerize, so you need to work fast (but carefully). Pipette the gel mix between the plates, making sure you leave enough space at the top for the stacking gel and comb. Carefully layer water on top of the gel solution. Once the gel has polymerized (about 10-15 mins), wash off the top of the gel with water. Carefully blot off excess water with a filter paper. Take care not to disturb or damage the top of the gel.

(4) **Pour the stacking gel.** For each gel you will need about 1.2 mL of reagent mix. Again, pipette the solutions carefully and swirl to mix after addition of each component. Pipette the gel mix between the plates up to just below the edge of the short plate. Carefully place in the comb.

ddH_2O	3.13 mL
40% acrylamide/bis stock	0.62 mL
1.5 M Tris, pH 6.8	1.25 mL
10% ammonium persulfate	50 µL
TEMED	5 µL
TOTAL VOLUME	~5 mL

Once the gel has polymerized, slowly remove the comb under running water. Wash the wells carefully to avoid distorting them.

(5) Gel storage. Put back the comb, and sandwich the gel between two wet pieces of paper towels. The gel can be stored horizontally at 4 °C for up to 1 week.

Making E. coli Competent

E. coli Calcium Chloride competent cell protocol

1. Inoculate a single colony into 5mL Lb in 50mL falcon tube. Grow O/N @ 37°C.
2. Use 1mL to inoculate 100mL of LB in 250mL bottle the next morning.
3. Shake @ 37°C for 1.5-3hrs.

Or

1. Inoculate a single colony into 25mL LB in a 250 mL bottle in the morning.
2. Shake @ 37°C for 4-6 hrs.

Then....

1. Put the cells on ice for 10 mins (keep cold form now on).
2. Collect the cells by centrifugation in the big centrifugue for 3 mins @6krpm
3. Decant supernatant and gently resuspend on 10 mL cold 0.1M CaCl (cells are susceptible to mechanical disruption, so treat them nicely).
4. Incubate on ice x 20 mins
5. Centrifuge as in 2
6. Discard supernatant and gently resuspend on 5mL cold 0.1MCaCl/15%Glycerol
7. Dispense in microtubes (300µL/tube). Freeze in -80°C.

Transformation of Ca++ competent cells

1. Put 1µL of circular plasmid or all of a ligation reaction of plasmid DNA in a microtube. Gently add ~100µL of competent cells. Do NO DNA control tube with cells and no DNA.
2. Incubate for 30 mins on ice.
3. Heat shock for 2 mins @ 42°C. Put back on ice.
4. Add 900 µL of LB to tubes. Incubate @ 37°C for 30 mins.
5. Plate 100-1000 µL of the cells in LBAmp or LBCarb (100µg/ml) plates. Plate 100 µL of the NO DNA control in a blood plate (to check for quality of cells). Grow O/N. U can save the rest in the cold room or freeze with 15% of Gly in case u get no colonies (v. unlikely).
6. If you need a lot of colonies or the ligation is of low efficiency, centrifuge the transformation for 1 min @ 8krpm, discard 900 µL of supernatant, resuspend on the 100 µL left and plate the whole lot.

Lactobacillus/Lactococcus glycerol perservation

1. Grow LC (10mL) for the lacto strains
2. Centrifuge bacteria culture
    a. Take 1.5mL of LC into each of 5 microcentrifuge tube
    b. Repeat step for all strains of lactobacillus
    c. Spin down centrifuge tubes at 12000rpm for 1 minute
    d. Discard supernatant
3. Resuspend pellet with 20% glycerol + 80% broth
    a. Add 300uL to each tube
    b. Freeze at –80C
4. Retrieval
    a. Thaw the cells on ice
    b. Centrifuge and discard supernatant
    c. Take 0.1mL of thawed cells and add to 5mL of MRS broth
    d. Shake overnight

Lactobacillus/Lactococcus Electroporation

Prepping cell culture

1. Grow LC
    a. 2mL original LC + 8mL MRS (PEG)

b. 2mL original LC + 8mL MRS (sucrose + MRS)
c. 2mL original LC + 8mL MRS with 1.0% glycine
d. 2mL original LC + 8mL MRS with 0.9M NaCl
e. 2mL original LC + 8mL MRS (for growth)

2. Place on stirrer overnight
3. Measure OD at 600nm
    a. Take 800uL from each LC and transferred them into the micro-volume cuvettes
    b. Prepare a blank (800mL of MRS, MRS + 1% glycine, or MRS + 0.9 NaCl)
    c. Change the mode to time base on the spectrophotometer
    d. Set the wavelength to 600 nm
    e. Calibrate/ warm-up the spectrophotometer with the blank solution
    f. Take absorbance of the samples
    g. Make sure the value lands around 0.6~1.2

Pretreatment #1 (WITH WATER)

1. Thaw -80 degree cells on ice
2. Centrifuge until supernatant is clear
3. Resuspend pellet in ~500uL of 30% PEG to make 600uL cell suspension (or match to the 0.6mL line on 15mL tube)
4. Add 900uL of cold sterile water
    a. Pipet or stir to mix
5. Let sit on ice for 30 minutes
6. Spin culture down at 12000rpm for 2~3 minutes
    a. Discard supernatant
7. Wash in 1mL of cold sterile 0.5M sucrose (or cold sterile 30% PEG solution)
8. Spin culture down at 12000rpm for 2~3 minutes
    a. Discard supernatant
9. Add () of cold sterile 0.5M sucrose (or cold sterile 30% PEG solution)

Pretreatment #2 (WITH LITHUM ACETATE)

1. **Take out solutions from –20 C**
2. Spin culture down in 15mL tubes at 4500rpm for ~7mins until the supernatant is clear
    a. (in hood) Pipet out supernatant until it reaches the 600uL mark
    b. Resuspend cell pellet
3. Add 750 uL of lithium acetate solution (200mM lithium acetate, 1.2M sucrose, 20mM Tris, pH 7.5) and 150uL of 100mM DTT solutions
    a. Solution has to be cold and filter sterilized
4. Transfer to micro centrifuge tubes
5. Let sit on ice for 30 minutes
6. Spin culture down at 12000rpm for 2~3 minutes
    a. Discard supernatant
7. Wash in 1mL of cold sterile 30% PEG solution
8. Spin culture down at 12000rpm for 2~3 minutes
    a. Discard supernatant
9. Add 300 uL of cold sterile 30% PEG Solution

Electroporation

1. Calculate the volume of plasmid DNA needed
    a. 200ng needed per transformation

i. Maybe increase?
ii.

b. 6/13
i. 2 uL for 133 ng/uL

2. Mix with 300 uL of cell suspension
3. Voltage setting to 2000 V
4. After electroporation, IMMEDIATELY add 900 uL recovery medium (0.5 M Sucrose MRS broth)
5. Incubate at 37 C for 4 hr
6. Preheat MRS plates 1 hr before plating
7. Then plate on MRS plate
   a. 2 plate depending on assituation

Transformation

- Centrifuge tubes
- Carefully discard supernatant with pipet
- Wash with MRS + cm broth
- Centrifuge & discard
- Add MRS + cm broth to yield around 300uL cell suspension
- Resuspend
- Take 2 x 100uL to plate two plates
- Take the rest 100uL and add 10mL MRS + cm broth
- Shake overnight

Transformation Protocol **Protocol No. 4308 915.519 – 12/2001**

Microorganism	*Lactococcus lactis* MG1363
Cell type	Bacteria, gram positive
Molecules injected	Plasmid DNA (pGK12)
Growth medium	Complex medium with 1% glycine
Washing solution	0.5 M sucrose, 10% glycerol
Electroporation solution	0.5 M sucrose, 10% glycerol
Outgrowth medium	Ice-cold complex medium with 0.5 M sucrose, 20 mM $MgCl_2$, 2 mM $CaCl_2$
Cuvette	1 mm gap width
Reference	Dr. Horst Neve • Bundesanstalt für Milchforschung • Institut für Mikrobiologie Hermann-Weigmann Str. 1 • D-24103 Kiel • Phone +49 431 6091 • Fax +49 431 609222

Making electrocompetent cells:

1. Grow cells overnight at 30 °C to an $O.D._{620}$ of 0.7.
2. Wash twice with ice-cold washing solution.
3. Resuspend cells in 1/100 volume of electroporation solution. Keep on ice.

Electroporation of cells:

1. Add 0.25 µg plasmid DNA (in water) to 100 µl of electrocompetent cells. Homogenize by gently mixing with pipette several times. Transfer mixture into cuvette.
2. Wipe moisture from the cuvette and insert the cuvette into the device.
3. Electroporation:

Mode	Prokaryotes "O"
Voltage (V)	2,000 V
Time constant (τ)	5 ms

4. Add 1 ml ice-cold complex medium, incubate 2 hours at 30 °C.
5. Plate diluted cells on selective chloramphenicol plates. Incubate 2 days at 30 °C.

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5. Plate diluted cells on selective chloramphenicol plates. Incubate 2 days at 30 °C.

Expected results:
Transformation efficiency up to 1.4 x 10^6 transformants/µg of DNA.

Source: Eppendorf Eporator

Testing temperature threshold of *E. coli* Nissle 1917

1. Grow 20mL LC of Nissle and GFP overnight
2. Spin down the cultures
3. Resuspend and gently pipet in 20 mL of water
4. Spin down and decant
5. Repeat step 3 and 4 to wash twice
6. Resuspend in 10mL water
7. Pour the mixtures into two small beakers and place on hot plate
8. Place a stirrer in the beakers
9. Set the temperature to 150 degree
10. Place a thermometer in the beaker
11. When the thermometer reaches 25 degree, take 10uL of culture out of the beaker and place one of the 12-wheels
12. Repeat step 11 for the following temperatures: 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140

MRS plate

1. Suspend 23.6 g of MRS broth into 500 mL of distilled water
2. Add 9 grams of agar powder
3. Add 0.5 ml polysorbate 80 (TWEEN 80)
4. Boil to dissolve the medium completely and mix thoroughly
5. Autoclave at 15 lbs pressure (121°C) for 15 minutes

Making Acetaldehyde

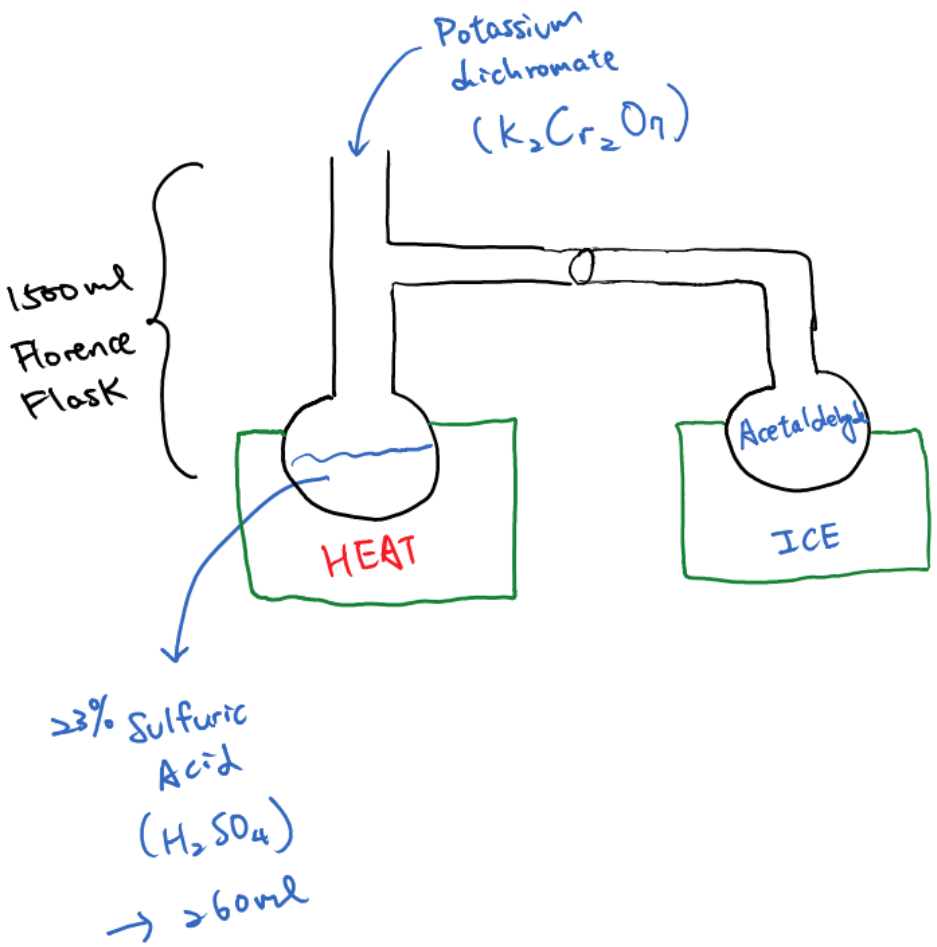
Procedure #1:

1. Place 260 mL of 23% sulfuric acid in a 1500-mL Florence flask.
2. Connect an addition funnel to the flask, and set it up for vacuum distillation with a 500-mL receiving flask.
3. The receiving flask should be immersed in a salt-ice bath to cool the distillate when it comes over. Although a setup for vacuum distillation is used, it is not necessary to apply a vacuum; the vacuum adapter merely provides a means of equalizing pressure.
4. Prepare a solution of 100 g of sodium dichromate in 200 mL of water and 127 mL of ethyl alcohol. Factor in the amount of water that is already in the ethyl alcohol when making the solution, i.e. 100 mL of 95% alcohol has 5 mL of water in it already.
5. Place this solution in the addition funnel, position the stem of the funnel so that it is about 3 cm above the surface of the acid. Heat the acid until it just begins to boil, then add the mixture in the funnel in a slow steady stream to the acid.
6. It will not be necessary to heat the flask during the addition because it will generate its own heat. The heat will be sufficient to distill over the acetaldehyde along some alcohol and waste acetal. If acetaldehyde vapors begin to escape from the flask, regulate the distillation by decreasing the amount of dichromate solution being added. If the reaction flask does not boil on its own, gently heat it for a short time until boiling

begins.

(https://erowid.org/archive/rhodium/chemistry/acetaldehyde.html)

Setup Diagram: (**Not yet Complete**)



Procedure #2:
Preparation of acetaldehyde from paraldehyde

1. For the preparation of acetaldehyde a simple fractional distillation apparatus is assembled fitted with a long all-glass Dufton column, or Hempel column.
2. A mixture of 50 ml of paraldehyde and 0.5 ml. of concentrated sulfuric acid (as the depolymerizing agent) and a few small fragments of porous porcelain are placed in the distillation flask (the sulfuric acid may be replaced by 1-2 g of sulphamic acid (NH2SO3H) or by p-toluenesulfonic acid).
3. The receiver is cooled in crushed ice, meanwhile, the flask is gently heated at 50-60° C, by not allowing the temperature at the head of the column to rise above 30-32° C.
4. The distillation must be conducted very slowly in order that the fractionation may be efficient, since acetaldehyde and paraldehyde form a constant boiling point mixture, b.p. 42° C (53.4% and 46.6% respectively).
5. Most of the acetaldehyde distils at 21-25° C and the distillation is stopped when 10 ml of liquid remain in the flask.
6. The obtained acetaldehyde, produced in excellent yield, is sufficiently pure for many purposes, however, in order to obtain pure acetaldehyde, the product must be redistilled. Pure acetaldehyde boils at 21° C.

(http://www.prepchem.com/synthesis-of-acetaldehyde/)

Ball Drop (Viscosity) Experiment

1. Mass and add arbitrary amount of Biotene gel into 50 mL of water
2. Heat and stir gel on hot plate to completely dissolve gel
3. Pour 50 mL of saliva model into a long test tube
4. Pour 50 mL of water to another long test tube
5. Drop balls down the test tube and record time it takes for ball to drop down a prescribed length
   *we used Logger Pro video analysis to obtain drop times
6. Use collected data to calculate viscosity of artificial saliva
7. Keep trying different amounts of gel until the viscosity is similar to the viscosity of actual saliva

Our calculations

*Viscosity equation and procedures were given in a published paper (Tang, 2016).

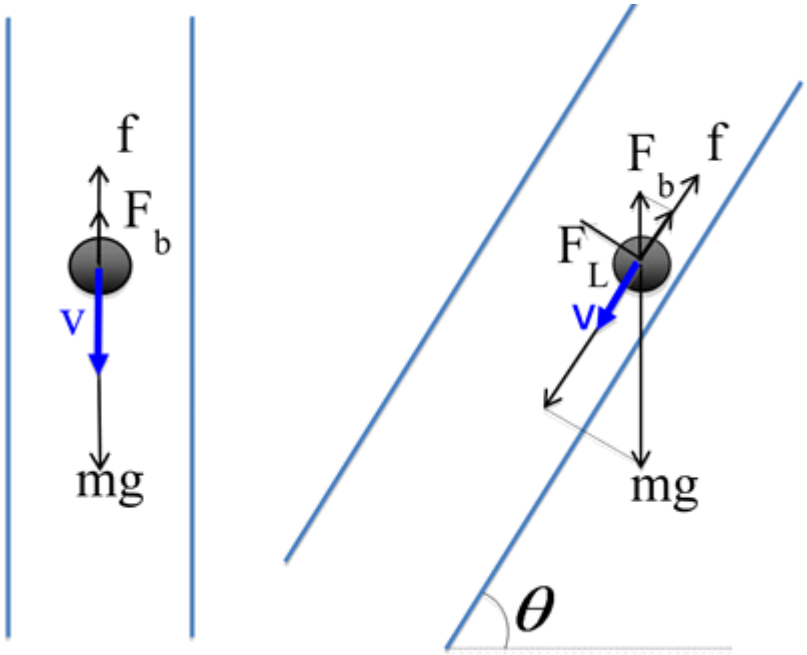


Figure 1: Free body diagram of ball drop

When all forces are in equilibrium, the following equation can be derived:

$$v = \frac{d^2(p_b - p_l)g \sin\theta}{18L}$$

Viscosity is given by the equation above, where:

Variable	Representation
$v(cP)$	viscosity
d(mm)	diameter of ball
p_b(g/mL)	density of ball
p_l(g/mL)	density of liquid
g(m/sec^2)	gravitational acceleration
L (cm)	distance of ball drop
T(seconds)	time of ball drop

Plugging in the values for our ball drop in water, we get:

$$v = \frac{d^2(p_b - p_l)g \sin\theta}{18L}\mathsf{T}$$

$$v_w = \frac{0.003^2(6.38 - 1.006)10^3 * 9.81 * \sin 90}{18(0.3)}0.498$$

$$v = 0.0428$$

Plugging in the values for our ball drop in our artificial saliva, we get:

$$v = \frac{d^2(p_b - p_l)g \sin\theta}{18L}\mathsf{T}$$

$$v_{as} = \frac{0.003^2(6.38 - 1.028)10^3 * 9.81 * \sin 90}{18(0.3)}1.08$$

$$v = 0.0646$$

Finding the relative viscosities between water and artificial saliva:

$$\frac{v_{as}}{v_w} = \frac{0.094}{0.0428} = 2.196$$

Finding viscosity of artificial saliva based on relative viscosity to water:

$$2.196 * 0.95\ cP = 2.09cP$$

Reference:

Tang JX. (2016). Measurements of fluid viscosity using a miniature ball drop device. Rev Sci Instrum. 87(5):054301.

Artificial Saliva

Based on the experiment above, dissolving 6.5 grams of Biotene gel into 50 mL of water will closely mimic the viscosity of saliva. (artificial saliva is 2.09 cP; mean saliva viscosity is 2.1 cP)

Cloning Cycle

Miniprep (Geneaid Mini Plasmid Kit)**: Isolating plasmids from bacterial cells**

- Prepare 3 mL LB + 3 uL (1000x) antibiotic liquid cultures.
  - Shake at 37 °C overnight (225 rpm)
- Transfer the cultures to 1.5 mL microcentrifuge tubes (centrifuge at 12,000 rpm for 1 min)
  - Discard supernatant
- Repeat to pellet the rest of the bacterial culture
  - Remove supernatant (discard, then use micropipette to remove rest of liquid)
- Add 200 uL **PD1 Buffer (+RNase A)** and mix by vortexing or pipetting
- Add 200 uL **PD2 Buffer**. DO NOT VORTEX. Invert tubes 5-10 times to mix
  - Let sit for 2 min (DO NOT EXCEED 5 MINUTES)
- Add 300 uL **PD3 Buffer**. DO NOT VORTEX.
  - Invert tubes 5-10 times to mix
  - Centrifuge at 12,000 rpm for 5 min at room temperature
- Transfer supernatant to PD Column (with Collection Tube attached)
  - Centrifuge at 12,000 rpm for 30 sec
  - Discard flow-through and put Collection Tube back on
- Add 600 uL **Wash Buffer (with Ethanol added already)** into the PD Column
  - Centrifuge at 12,000 rpm for 30 sec
  - Discard flow-through
  - Centrifuge again at 12,000 rpm for 2 min
  - Transfer the PD Column to a new 1.5 mL microcentrifuge tube
- Add 30 uL **Elution Buffer** into the center of the column matrix
  - Let stand for at least 2 min
  - Centrifuge at 12,000 rpm for 2 min

Digestion: Vector and Insert

- Prepare 2 reactions as follows in 1.5 mL microcentrifuge tubes
  - One tube to cut vector
  - One tube to cut insert
  -

Volume	Component
1 ug	DNA
1 uL	Restriction enzyme
1 uL	Restriction enzyme
2 uL	10X NEBuffer 2.1
to 20 uL	Water

- Incubate the reactions at 37 °C for (at least) 1 h

Gel Extraction: Using gel electrophoresis to isolate digested DNA fragments

Volume	Component
20 uL	Digestion mix
4 uL	6x DNA dye
--	Water
24 uL	TOTAL

- Prepare separate samples of cut vector and cut insert to run **DNA agarose gel**
  - Load **all sample (in a single well)** and run gel
  - **Cut out** desired DNA bands and transfer to clean microcentrifuge tubes
  - Run **Gel Extraction Kit (Geneaid Purification Kit)** to isolate digested DNA

Ligation: (New England BioLabs) "Connect" DNA after complementary sticky ends anneal

___ uL	Digested vector
___ uL	Digested insert
2 uL	10x T4 DNA ligase buffer
1 uL	T4 DNA ligase
to 20___ uL	Water
20 uL	**TOTAL**

- Calculate how much vector and insert you need to
  - Have a 3 Insert : 1 Vector molar ratio
    - Use the calculator from **http://2011.igem.org/Team:UT_Dallas/ligation**
  - Have a total of 50-100 ng DNA in the reaction tube.

- Incubate at room temperature for at least 1 hour.
- Use 10 uL of the ligation mixture for transformation in 100 uL competent cells.

(**Geneaid Elite Competent E.coli Cells**): Inserting DNA into Bacteria

Three tubes: Ligated DNA + cells, Ligated vector control + cells

Perform steps with open samples by the flame or in the hood

- Thaw competent cells on ice (~ 50 uL in each tube)
- Add 5 uL of Ligation reaction to competent cells
  - Mix by gently swirling. DO NOT PIPETTE OR VORTEX
  - Leave on ice ~5 min

- Heat shock at 42 °C for 30 sec
- Put tubes on ice for 2 min
- Add 1 ml LB [no antibiotics] and shake at 37 °C for ~45 min
- Centrifuge at 12,000 rpm for 1 min.
    - Gently pour out supernatant
    - Re-suspend pellet in remaining supernatant
- Plate ALL on LB + [antibiotic] agar plates
    - Incubate at 37 °C overnight
- Check for colonies the next day

Basic Housekeeping

LB

For 250 mL

1. ***Dissolve 6.25g LB Broth Powder in 250 mL dH_2O***
2. Wrap aluminum foil on the cap
3. Loosen the cap
4. Stick the autoclave sticker with dates at the top of the aluminum foil
5. Autoclave at Liquid Setting
6. Close the cap tightly (prevent any contamination)
7. Cool and dry in the hood (make sure ventilation is on)
8. Store at room temperature at anywhere in the lab

- For 500mL LB
    1. ***Dissolve 12.5g LB Broth Powder in 500 mL dH_2O***
    2. Wrap aluminum foil on the cap
    3. Loosen the cap
    4. Stick the autoclave sticker with dates at the top of the aluminum foil
    5. Autoclave at Liquid Setting
    6. Close the cap tightly (prevent any contamination)
    7. Cool and dry in the hood (make sure ventilation is on)
    8. Store at room temperature at anywhere in the lab

Note: *Volume of Antibiotic: Volume of LB = 1 : 1000 = **1µL : 1mL*

1x TAE

- For 500 mL 1x TAE
    1. Mix 10mL 50x TAE with 490mL dH_2O
- For 1L 1x TAE
    1. Mix 20mL 50x TAE with 980mL dH_2O

DNA GEL

1. 1g of agarose powder per 100 mL 1x TAE
2. Microwave 30 seconds, stop, stir, and **repeat** until the agarose is completely dissolved

*NOTE: **DO NOT over boil the solution**

1. Let agarose solution cool down
2. Add 1µl of Seeingsafe per 20mL of solution
3. Pour agarose in the gel tray with the well comb in place

*Note: Pour slowly to avoid bubbles/ use a pipette tip to push the bubble away to the side

1. Cover the gel tray with a box
2. Wait for the gel to solidify (~1 hour)
3. After the gel is solidified, remove the well comb carefully
4. Transfer the gels to the gel box and store them at 4°c (Keep them in the front part of the fridge, or else they'll freeze!)
5. Throw away gel remnants from the gel tray and table into the GARBAGE CAN !!!

*Note: DO NOT WASH THEM DIRECTLY IN THE SINK !!!

1. Wash everything & let them dry on paper towels

AGAR PLATE

1. Take a 500mL bottle from the syn bio cabinet
2. Mix 7.5 g of agar with 500 mL of LB
3. Autoclave
4. Cool it down with running water
5. Add 500µ of antibiotics
6. Pour the solution into the plates in the hood

*Tip: Fill to ½ height of the plate only

*Note: Remove any bubbles

1. Label the plates with the initial of the antibiotics on side
2. Let them solidify in the hood

CLEANING

Making 75% Ethanol (from *95% ethanol*)

Total Volume (mL)	Volume of 95% Ethanol (mL)	Volume of Distilled Water (mL)
500	395	105
1000	798	202
1500	1184	316
2000	1579	421
2500	1974	526
3000	2368	632

Making 10% Bleach

- 100% Bleach : Tap water = 1:10
- Located in the cabinet under the sink

Dish Washing

1. Load everything in upside down (heavy and larger objects on the bottom tray; lighter objects on the top tray; spatulas/stirring rods on the right side of the top tray)
2. Add dish soap in a drawer on the door of the dishwasher
3. Press start
4. When it's done washing, take them out and put the objects back to where they belong to

Throwing away UNWANTED plates

1. Take the plate to the Chemistry Storeroom's hood
2. Cover the agar completely with 10% bleach
3. Let the plate sit for 5 minutes
4. Dump the bleach into Liquid Waste bottle in the hood
5. Stack the plate in a plastic bag

Dumping Liquid Waste

1. When the liquid waste is 70% full, take the liquid waste to the Chemistry Storeroom's hood
2. Fill about 1/10 of the total volume of the liquid waste with 10% bleach
3. Let the bottle (with cap on) sit in hood overnight

(DO the following steps right before the lab closes)

1. Let the tap water run through the sink continuously while dumping the liquid waste in the sink
2. Add soap into the bottle to make it smell better
3. Rinse the bottle in and out with water until it doesn't smell that disgusting
   *Note: Do not brush
4. Let the bottles (with cap on) dry on paper towels (let the outside of the bottle dry)
5. Put the dried bottle back to its original place
6. Spray 75% ethanol in the bottle
7. Remember to put the lid back on

Throwing Out Waste Tips/ Cleaning the Waste Bin

1. Take a super big plastic bag (with sticker, located on the top shelf in the syn bio cabinet)
2. Dump all the waste tips into the bag
3. Spray the empty bin with A LOT of alcohol
4. If the bag is too full, try to squeeze everything together and seal the bag with extra tapes
5. Stick an autoclave tape on the bag
6. Autoclave at ***trash*** setting
7. After autoclaving, throw the whole bag into the garbage can

Cleaning Beads

1. Accumulate glass beads in a beaker (if not already in "waste beads" beaker)
2. Add tap water until beads are all submerged.
3. Stir and shake up and down violently to make sure all beads are cleaned.
4. Drain off water.
5. Add tap water and a little bit of soap until beads are all submerged.
6. Stir and shake up and down violently to make sure all beads are cleaned.
7. Drain off water.
8. Add 10% bleach until beads are all submerged.
9. Stir and shake up and down violently to make sure all beads are cleaned.
10. Drain off 10% bleach.
11. Add tap water until beads are all submerged
12. Stir while tap water is running continuously into the beaker (constant overflow) for 1 minute.
13. Use a sieve (metal wire net) to catch all the beads and drain off water.
14. Add 75% ethanol to about half way the height of beaker
15. Stir and shake up and down violently to make sure all beads come into contact with ethanol.
16. Use a sieve (metal wire net) to catch all the beads and drain off ethanol.
17. Let beads dry in the sieve.
18. Pour beads back into an Erlenmeyer flask.
19. Put aluminum/tin foil over the opening to make a cap.
20. Put autoclave tape over cap.
21. Autoclave at "Dry" settings.

22. Once autoclaved, DON'T open the cap unless the flask is in the hood (ALWAYS REMEMBER TO CLOSE THE CAP)

Filling Pipette tips

1. Spray the table with ethanol
2. Pour the tips out of the bag onto a paper towel
3. Spray your hands with ethanol
4. Fill them into the box until full
5. Write the date on the autoclave tape
6. Stick autoclave tape over the opening of pipette box
7. Autoclave (DO NOT put too much stuff in at once)
8. Check if the autoclave tape showed black stripes
9. Dry it in the hood next to the autoclave (make sure ventilation is on)

Cleaning the Autoclave

- Weekly
    1. Wash the tray and tray rack
    2. Completely drain the water from the reservoir and replenish with fresh ***distilled water***.
- Monthly
    1. Use the ***autoclave cleaner*** (located in the first drawer under the autoclave), follow the cleaner's instructions on the back of the package

Jello candy with Nissle

1. Grow 10mL of Nissle overnight
2. Spin down Nissle, resuspend in water, and decant
3. Place a beaker/pan on a hot plate set at around 70 degrees
4. Add 120 mL water, 30mL gelatin, 30mL sugar
5. Heat and stir until solutes melt
6. Remove from hot plate
7. Use a temperature probe to measure temperature
8. When the temperature cools down to 55~60 degree, mix with Nissle (resuspended in small amount of water)
9. Aliquot 3mL of solution into each dent of cooling tray
10. Let sit overnight