LAB MANUAL: SYNTHETIC BIOLOGY PROTOCOLS



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Lab Manual: Synthetic Biology Protocols

Nathan Reyna, Ruth Plymale, & Kristine Johnson

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2.3.1.1: MAKING MEDIA (LB BROTH AND AGAR)

LB Agar:

Making 1 L of LB Agar:

- 5 g Yeast extract
- 10 g Peptone
- 10 g NaCl
- 12 g Agar

1. Mix this with 1 L of Distilled H2O
2. Autoclave at 121 °C for 30 minutes (If there is a setting, set it to slow/liquid)
3. Let cool before pouring into plates (approximately 25 mL of agar in each plate)

This recipe makes about 40 plates and can be adjusted as needed!

LB Broth:

Making 1 L of LB Broth:

- 25 g pre-mixed broth powder

This powder contains 10 g tryptone, 5 g yeast extract and 10 g NaCl

1. Mix this with 1 L of Distilled H2O
2. Autoclave at 121 °C for 30 minutes (If there is a setting, set it to slow/liquid)
3. Let cool before pouring into **STERILE** test tubes with lids (approximately 5 mL of broth per test tube)

This recipe makes about 200 tubes of broth and can be adjusted as needed!

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2.3.1.2: ADDING ANTIBIOTICS

Color coding LB-agar plates is recommended to simplify which plate contains which antibiotic

Addition of antibiotics to media is done after autoclaving, when the media has cooled to 50-55oC

**Ampicillin (Amp/A): ***Working concentration 100 ug/mL freezer stock 100 mg/mL (in water)

- Add 1 mL of 100 mg/mL ampicillin (dissolved in water) into 1 L of LB broth or agar

Chloramphenicol (Chlor/C): *Working concentration 35 ug/mL freezer stock 35 mg/mL (in EtOH)

- Add 1 mL of 35 mg/mL chloramphenicol (dissolved in ethanol) into 1 L of LB broth or agar

Kanamycin (Kan/K): *Working Concentration 50 ug/mL freezer stock 50 mg/mL (in water)

- Add 1 mL of 50 mg/mL kanamycin (dissolved in water) into 1 L of LB broth or agar

**Tetracycline (Tet/T): ***Working concentration 10 ug/mL freezer stock 10 mg/mL (in water)

- Add 1 mL of 10 mg/mL tetracycline (dissolved in water) into 1 L LB broth or agar

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2.3.1.3: MAKING LINEARIZED BACKBONE PCR PROTOCOL

PCR Protocol for Linearized Backbones

All procedures copied or modified from iGEM's linear backbone protocol and Q5® Hot Start High-Fidelity 2X Master Mix Protocol

1. Grow up part BBA_J04450 from the iGem plasmid. Grow 1 part for each different backbone (**psB1C3**, **psB1K3**, **psB1T3**, **psB1A3**)
2. Isolate the DNA from culture and obtain DNA concentration (via DeNovix/Nanodrop/Qubit)
3. Make PCR reaction mixture for plasmid amplification using the following table:

**Keep everything on ice and label PCR tubes before starting. Always add the DNA polymerase last as it will sink to the bottom. Use the 50 µL reaction for linear backbones. Usually, 1µL of DNA is sufficient. **

Component	25 uL Reaction	50 uL Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5 uL	25 uL	1X
10 uM Forward Primer (SB-prep-3P-1)	1.25 uL	2.5 uL	0.5 uM
10 uM Reverse Primer (SB-prep-2Ea)	1.25 uL	2.5 uL	0.5 uM
Template DNA (psB1C3, A3, K3, T3)	Variable	10 ng	< 1,000 ng
Distilled Water	up to 25 uL	up to 50 uL	

Primers for linear backbone:

SB-prep-3P-1 gccgctgcagtccggcaaaaaa,

SB-prep-2Ea atgaattccagaaatcatccttagcg

4. Place tube in the thermocycler, making sure there are **no bubbles** and the lids are closed tight
5. Set the thermocycler to the following settings for plasmid amplification:

Step	Temperature	Time
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C	10 seconds
	66°C	30 seconds
	72°C	3 minutes
Final Extension	72°C	1.5 minutes
Hold	4°C	

6. Run 1 uL of the **unpurified** PCR product on a gel to verify correct band size and concentration

a. **psB1C3**= 2077 bp
b. **psB1A3**= 2155 bp
c. **psB1K3**= 2204 bp
d. **psB1T3**= 2461 bp

7. Use a PCR cleanup kit to purify the samples (Qiagen or ZymoPure recommended)
8. Test concentration of purified PCR Product (via DeNovix/Nanodrop/Qubit) **Expected yield should be 40 ng/uL or higher*
9. Run a digestion with the purified PCR product

1. **Digestion:**
    1. **4 uL 10X NEB Buffer 2.1**
    2. **1 uL EcoRI-HF**
    3. **1 uL PstI**
    4. **1 uL DpnI *(Used to digest any template DNA from production)***
    5. **25 uL PCR Linearized plasmid product (*use total amount from purified PCR product)***
    6. **Digest at 37°C for 3 hours**
    7. **Heat kill at 80°C for 20 minutes**

10. PCR purify using the same kit as before, quantify DNA concentration, and adjust final concentration of the plasmid to 25 ng/uL using distilled water

11. Ligate each purified backbone to another plasmid part. Also set-up one control tube for each backbone. *(This tube will not have ANY insert added and will just have water added in place. This tests how often the backbone just closes on itself instead of ligating the insert inside).*

Choose a part with a relatively large insert that you know is correct and you have plenty to spare. Digest 300 ng of the plasmid with EcoRI and PstI according to the Double Digestion instruction. Heat inactivate the sample

1. **Ligation:**
    1. 1 uL purified E/P digested plasmid backbone (25 ng)
    2. Equimolar amount of E/P digested fragment (2 uL)
    3. 1 uL T4 DNA Ligase Buffer
    4. 0.5 uL T4 DNA Ligase
    5. Distilled water up to 10 uL
    6. **Ligate at 16°C for 30 minutes**
    7. **Heat kill at 80°C for 20 minutes**

12. Transform 25 uL of bacteria (JM109 recommended) with 1-2 uL of ligation product

See "Cell Transformation: Zippy Transformation of Z-competent Cells" protocol above for information on how to perform a transformation

13. Plate on the appropriate antibiotic plate according to backbone resistance gene

Any colonies on the backbone-only control plates represent background to the three antibiotic assembly process. ***If there are too many, this could present a problem during the student projects.***
Many colonies on the backbone+insert plates indicate that the backbones are amplified and cut effectively to be ligated to E/P cut inserts.

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2.3.1.4: DNA EXTRACTION SUPPLIES

DNA Extraction:

While many vendors offer mini-prep kits that will work for this step, Zymo provides an excellent kit (Cat#: D4210) with great results at a reasonable price.

Materials	Amount Needed (Per Reaction)
ZymoPURE™ P1 (Red)	250 uL
ZymoPURE™ P2 (Green)	250 uL
ZymoPURE™ P3 (Yellow)	250 uL
ZymoPURE™ Binding Buffer	275 uL
ZymoPURE™ Wash 1	800 uL
ZymoPURE™ Wash 2	1000 uL
ZymoPURE™ Elution Buffer	25 uL
Zymo-Spin™ II-P Columns **(must have purple ring)**	1
Collection Tubes	1
Serological Pipets	2 (at least 2 mL)
Microcentrifuge Tubes	3
Centrifuge	1 (used by class)
Micropipettes and Tips	100-1000 uL, 10-100 uL, 1-10 uL
Ice Bucket	1
UV Spectrophotometer	1 (used by class)

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2.3.1.5: DOUBLE DIGESTION SUPPLIES

Double Digestion:

Enzymes and buffers are indicated from New England Biolabs.

Materials	**Amount Needed (Per Reaction)**
Distilled Water	25 uL-(Sum of DNA+NEB+Enzyme1+Enzyme2)
DNA Sample	Determined by spectrophotometer (300ng/DNA concentration)
10x NEBuffer 2.1 (Cat#: B7202S)	2.5 uL
Restriction Enzyme 1 (EcoRI [Cat#: R0101S]) or SpeI [Cat#: R0133S])	1 uL
Restriction Enzyme 2 (XbaI [Cat#: R0145S] or PstI [Cat#: R0140S])	1 uL
Water/Bead Bath (37C)	1 (used by class)
Heat Block (80C)	1 (used by class)
Microcentrifuge Tube	1
Micropipette and Tips	1-10uL, 2-20uL
Microcentrifuge	1 (used by class)

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2.3.1.6: GEL LOADING/RUNNING SUPPLIES

Gel Loading and Running:

Materials	**Amount Needed (Per Reaction)**
Agarose	Varies depending on gel percentage
1x TBE	Approximately 165 mL (to make gel and for gel rig)
SybrGreen, SybrSafe, or GelRed (Cat#: S33102)	2-3 uL
Digested DNA	5 uL
DNA Ladder (Quick-Load Ladder Cat#: N0550S)	5 uL
6x DNA Loading Dye (Cat#: B7024S)	1 uL
Microwave	1 (used by class)
Flask	1 (at least 50 mL in size)
Micropipette and Tips	1-10 uL
Gel Rig (MiniOne; Cat#s: M1000, M1002, M1010, M1012)	1 (with all necessary parts included with the rig)
Weigh Tray	1
Scale	1 (used by class)

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2.3.1.7: DNA LIGATION SUPPLIES

DNA Ligation:

Materials	Amount Needed (Per Reaction)
Distilled Water	5 uL (up to 10 uL)
10X DNA Ligation Buffer (Cat#: B0202S)	1 uL
Linearized Plasmid Backbone (Amp,Chlor,Kan,Tet)	1 uL
Double Digested DNA insert fragments	1 uL
T4 DNA ligase (Cat#: M0202S)	1 uL
Ice Bucket	1
Microcentrifuge Tube	1
Micropipette and Tips	1-10 uL
Heat Block (80C)	1 (used by class)

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2.3.1.8: TRANSFORMATION SUPPLIES

Transformation:

Materials	**Amount Needed (Per Reaction)**
Competent Bacteria Cells (**JM109=** Cat#: T3003)	25-50 uL
Ligation Mixture	1-5 uL
LB Agar with Antibiotics (either Kan, Chlor, Amp, or Tet)	1 plate (depending on backbone)
SOC Media (Cat#: 15544034)	200 uL
Sterile Glass Beads	Approximately 4-8 beads
Ice Bucket	1
Microcentrifuge Tube	1
Shaking Incubator (37C)	1 (used by class)
Plate Warmer (37C) (Can be the same as the Shaking Incubator)	1 (used by class)
Incubator	1 (used by class)

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2.3.1.9: INOCULATION SUPPLIES

Inoculation:

Materials	Amount Needed (Per Reaction)
Sterile Culture Tube (with cap)	1
Sterile Toothpick	1
LB Broth	5 mL
Lab Hood	1 (used by class)
Shaking Incubator	1 (used by class)
Antibiotics (Amp, Chlor, Kan, or Tet)	Optional (see Appendix I or Instructor's Notes)

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2.3.1.10: GLYCEROL STOCK SUPPLIES

Glycerol Stock:

Materials	Amount Needed (Per Reaction)
80% Glycerol	500 uL
Cryovial (Cat#: 5000-0020)	1
Vortex	1 (used by class)
Micropipette and Tips	100-1000 uL

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2.3.1.11: LINEARIZING BACKBONE SUPPLIES

Linearizing Backbones:

Materials	Amount Needed: (Per Backbone Reaction)
Q5 High-Fidelity 2X Master Mix (Cat #: M0492S)	Up to 25 uL
10 uM Forward Primer (SB-prep-3P-1)	Up to 2.5 uL
10 uM Reverse Primer (SB-prep-2Ea)	Up to 2.5 uL
Template DNA (psB1C3, A3, K3, T3)	10 ng
Distilled Water	Up to 60 uL
Thermocycler	1
UV Spectrophotometer	1
Micropipette and Tips	1-10 uL, 0.2-1 uL, 20-200 uL
Heat Block (80C)	1
Gel Rig	1
PCR Clean-up Kit (Cat #: D4033)	1
10X NEB Buffer 2.1	4 uL
Digestion Enzymes (EcoRI, PstI, DpnI)	1 uL of each
Competent Bacteria Cells (**JM109**= Cat#: T3003)	25 uL
LB Agar Plate with Antibiotic (Chlor, Amp, Tet, Kan)	1 plate (depending on backbone)
Sterile Glass Beads	Approximately 4-8 beads
SOC Media (Cat#: 15544034)	200 uL
Shaking Incubator (37C)	1
T4 DNA ligase (Cat#: M0202S)	0.5 uL
T4 DNA Ligase Buffer(Cat#: B0202S)	1 uL

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2.3.1.12: TIPS FOR ORGANIZING THE LAB FOR LARGE CLASSES

Preparing for a Class

Example of a Lab Room

Pre-established Freezer Boxes:

It can be helpful to have some already stocked freezer boxes ready for your students before they get into the lab. This can work both for individual students as well as teams. These freezer boxes can be marked via color and assigned to students when the lab has started. The boxes can be pre-stocked with the various reagents your students will need so they will be able to have ready access to them.

This CryoSafe Cooler Box (Cat# 03-410-497) should consist of the following reagents:

- Restriction enzymes (EcoRI, SpeI, XbaI, PstI)
- 10x NEBuffer 2.1
- 10x DNA Ligation Buffer
- T4 DNA Ligase

It is helpful to have another color coded cardboard freezer box that is empty alongside the reagent box. This way your students will ideally have no problem storing their parts as they move through the protocol as well as ensuring fewer mixups between the various student parts as they are stored.

Pre-established Student/Team Work Stations:

Much like the freezer boxes, pre-stocked work stations allow for you to be able to provide your students with access to tools/materials such as micropipettes and microcentrifuge tubes. It is helpful to color coordinate a student/team work station with a freezer box.

This work station should consist of:

- A set of micropipettes (ranging from 1-10 uL to 100-1000 uL) per student
- At least one tube rack
- A container of microcentrifuge tubes
- A Zymo Mini-Prep kit (this can be the kit itself or pre-aliquoted amounts of the reagents)
- Sharpie
- Pipet tips (a box per type of micropipette)
- Any other room temperature reagents you would want easily accessible

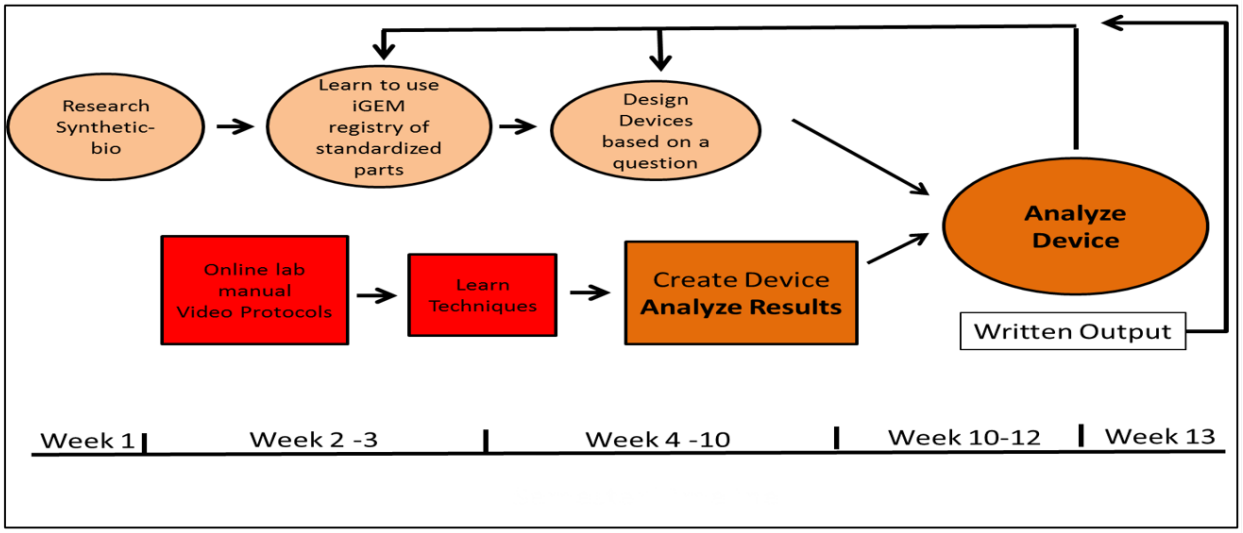
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2.3.1.13: BEFORE YOU BEGIN A SEMESTER OVERVIEW

The protocols included in this lab manual are part of a Synthetic Biology undergraduate research course (CURE) designed by the authors. The lab presented in this manual is designed for a Genetics or Biotechnology class and has been used at both two-year and four-year institutions. The workflow for the semester long course is shown below. The lab is based off the iGEM genetic engineering competition (iGEM.org). Students are tasked with designing and then engineering a biological device in a bacterium (*E.coli*) using standardized DNA parts called Bio-Bricks from the iGEM registry. The process of engineering the device requires iteration of standard molecular biology cloning techniques which are outlined in this lab manual, some including video protocols. Additionally, provided are appendices with recipes for reagents, supply/resources list, and instructor recommendations. **Instructors are encouraged to watch a series of implementation videos created for the Arkansas-CURE Projects Synthetic Biology faculty training workshop.**



Support for this manual was provided by:

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SECTION OVERVIEW

2.3.2.1: VISUAL REPRESENTATION OF THE PROTOCOL



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2.3.2.2: PLASMID DNA EXTRACTION (MINI-PREP)

ZYMO-PURE PLASMID DNA EXTRACTION (MICRO-CENTRIFUGE METHOD)

LEARNING OBJECTIVE

- Isolation of Plasmid DNA from overnight cultures in LB.

This method is spin column-based and purifies up to 100 μg of ultra-pure endotoxin-free plasmid DNA in less than 15 minutes. The result is plasmid DNA suitable for transfection, restriction endonuclease digestion, bacterial transformation, PCR amplification, and DNA sequencing. (ZymoPURE Plasmid Miniprep Kit)

NOTE

Watch the Youtube video for questions about the Miniprep protocol



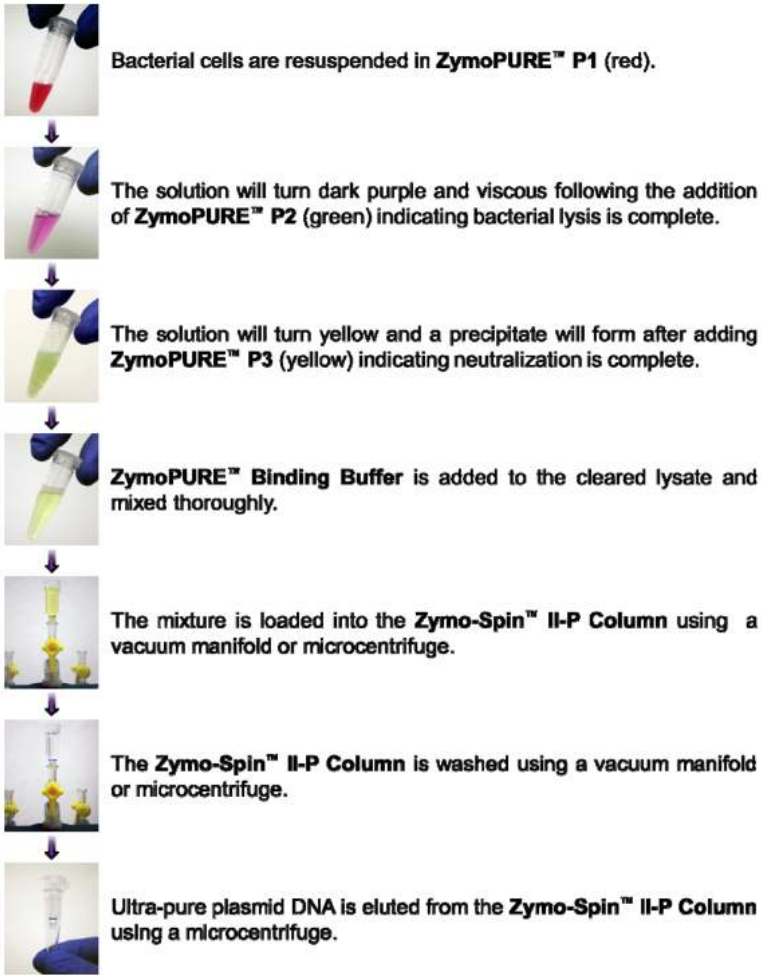
ZymoPURE Plasmid Miniprep video

NOTE

The P1 reagent is temperature sensitive due to RNase being present, and should be kept in the fridge or on ice at all times. All other reagents will be stored at room temperature.

Reagent name and Code	Storage Temperature
ZymoPURE™ P1 (Red)	4°C
ZymoPURE™ P2 (Green)	Room Temp.
ZymoPURE™ P3 (Yellow)	Room Temp.
ZymoPURE™ Binding Buffer	Room Temp.
ZymoPURE™ Wash 1	Room Temp.
ZymoPURE™ Wash 2	Room Temp.
ZymoPURE™ Elution Buffer	Room Temp.
Zymo-Spin™ II-P Columns **(must have purple ring)**	Room Temp.
Collection Tubes	Room Temp.

Visual Procedure of Zymo Plasmid Mini-Prep



TIPS BEFORE STARTING

The first couple of times you do the Mini-Prep, on the protocol indicate/LABEL **WHERE your DNA is** for each of the steps! (e.g., DNA is in the supernatant/liquid OR DNA is in the pellet). Each step below has a "circle one" option asking where the DNA is! (pellet or supernatant).

NOTE

If you *understand* exactly where your DNA is in each one of the steps, you won't LOSE your DNA!!

PROTOCOL: (MAKE SURE YOU HAVE ALL REAGENTS BEFORE YOU BEGIN… GET ICE!!!)

1. Using a serological pipet, pipet out **1.0 to 3 mL** (preferably 3 ml) of bacterial culture into a 1.5 ml microcentrifuge tube. Centrifuge this microcentrifuge tube at **full speed** for **20 seconds**. *You will see a small pellet form at the bottom of the tube.* (You must **repeat** steps 1-2 to get more than 1 ml worth of cells

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

2. Remove as much media as possible by pouring it off into the Biohazard bag. **Do not disrupt the cell pellet**! (**Repeat** steps 1-2 to increase cell concentration: However, 1-2ml of culture should be enough plasmid.)

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

3. Add **250 µL** of **ZymoPURE™ P1 (Red)** to the bacterial cell pellet and vortex or pipet the pellet to resuspend it completely. (**P1 is stored in the fridge at 4°C. Keep on ice when not in use.)** *This step involves RNase to purify the DNA by destroying any RNA in the sample.*

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

4. Add **250 µL** of **ZymoPURE™ P2 (Green)** and **immediately mix** by gently inverting the tube **6-8 times**. *Do not vortex!* Let sit at room temperature for **2-3 minutes**. Do not let sit for longer than 3 minutes. (*Cells are completely lysed when the solution appears clear, purple, and viscous.)*

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

5. Add **250 µL** of ice cold **ZymoPURE™ P3 (Yellow)** and mix thoroughly by inversion. Do not vortex! Invert the tube an **additional 3-4 times** after the sample turns completely yellow. (*The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.)*

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

6. Incubate the neutralized lysate in the microcentrifuge tube on ice for **5 minutes**

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

- Centrifuge the neutralized lysate for **5 minutes** at 16,000 *xg (*or **Max speed***)*. (Do not disrupt the white/pelleted cell debris).

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

8. Transfer **600 µL** of supernatant from step 7 into a clean 1.5 ml microcentrifuge tube. *Be careful not to disturb the yellow pellet and avoid transferring any white cellular debris to the new tube.*

Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

9. Add **275 µL** of **ZymoPURE™ Binding Buffer** to the cleared lysate from step 8 and mix thoroughly by inverting the capped tube **8 times**.
10. Place a **Zymo-Spin™ II-P Column** (**Purple ring**) in a Collection Tube and transfer the entire mixture from step 9 by pouring or pipetting it into the Zymo-Spin™ II-P Column.
11. Incubate the **Zymo-Spin™ II-P/Collection Tube** assembly at room temperature for **2 minutes** and then centrifuge at 5,000 *xg* for **1 min**. Discard the flow through in the Biohazard bag.
12. Add **800 µL** of **ZymoPURE™ Wash 1** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
    (The collection tube will hold 900ul of liquid. It is important that the flowthrough does not touch the bottom of the column!)
13. Add **800 µL** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
14. Add **200 µL** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
15. Centrifuge the Zymo-Spin™ II-P Column at ≥ 10,000 *xg* (**full speed**) for **1 min** in order to remove any residual wash buffer.
16. Transfer the Zymo-Spin™ II-P Column into a clean 1.5 ml microcentrifuge tube and add **25 µL** of (warmed 50°C- optional) **ZymoPURE™ Elution Buffer** directly to the column matrix. Incubate at room temperature for **2 minutes**, and then centrifuge at ≥ 10,000 xg (**full speed**) for **1 minute** in a microcentrifuge. (*warmed buffer and a 5 min incubation may increase plasmid concentrations*)
17. Determine the concentration of your sample using a spectrophotometer (E.g. Denovix, NanoDrop, Qubit). (*See appendices II, III, IV on how to use one of the aforementioned machines.)*

    DNA Purity: Eluted DNA is ultrapure, endotoxin-free, and well suited for transfection, transformation, sequencing, restriction endonuclease digestion, *in vitro* transcription, and other sensitive applications.
    Typical: Abs 260/280 ≥ 1.8 and Abs 260/230 ≥ 2.0
18. Clearly label each plasmid tube; store it at -20 degrees and record it in the lab notebook!

What should be on your label?

TOP LABEL:

Part # (Bba_J0176)

SIDE LABEL:

Team Name/Initials (SV/JH Team)

Part Name (RBS: GFP)
Concentration (ng/µL) 260/280 ratio
Plasmid Backbone (Antibiotic Resistance) / psB1C3 (Chlor)
(Date) 6/14/2021

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2.3.2.3: DNA DOUBLE DIGESTION

DNA DOUBLE DIGESTION PROTOCOL

Two restriction enzymes are used simultaneously to digest DNA in a single reaction.

Reagent name	Storage Temperature
Distilled Water	Room Temp
DNA Sample	4°C
10x NEBuffer 2.1	-20°C
Restriction Enzyme 1 (EcoRI or XbaI)	-20°C
Restriction Enzyme 2 (SpeI or PstI)	-20°C

TIPS BEFORE STARTING:

- As you add each reagent to a 1.5 ml microfuge tube, stir it in well with the pipet tip and always change your tip in between steps - **NEVER double dip your tip!**
- ***VERY IMPORTANT!*** Do not dispense the liquids onto the sides of the tube. Always dispense the volumes into the very bottom of the tube!
- Enzymes are stored in glycerol and will never freeze and are ready to be used immediately. They never should be out of the table freezer block or an ice bucket. However, **the buffer must be completely thawed before use**. (Gently flick the tube and spin the contents to the bottom). You can store the buffers at 4°C but they will only last two weeks. *You must keep up with how long your buffer has been at this temperature!*

PROCEDURE: (TAKE YOUR TIME! MAKE SURE YOU'RE DOING EACH STEP CORRECTLY!)

1. Pipette the following into a 1.5 ml microfuge tube: **Reagents must be added in the specified order (water is always the first reagent to add!)**.

25uL final volume reaction system

If your DNA concentration is too low you can increase the reaction volume to 40-50 ul.

X μL of water to get **final reaction volume of 25 μL**
X is equal to *25* - *(NEB+DNA+Enzyme 1+Enzyme 2)*
2.5 μL NEB buffer 2.1
Y μL DNA
(usually ~300 ng-Use spectrophotometer to determine concentration and calculate volume needed).
Y is equal to 300 ng/DNA Concentration(ng/uL)

1 μL BioBricks enzyme 1 (either EcoRI or SpeI)
1 μL BioBricks enzyme 2 (either XbaI or PstI)

2. Mix well by pipetting slowly up and down approximately **5x**. **Be gentle, and do not vortex**. Spin the samples for **5 seconds** in a balanced microcentrifuge, or flick them to collect the mixture at the bottom of the tube.

3. Incubate at 37 degrees for at least 1 hour. Samples can be stored at -20 degrees at this point, but DO NOT forget about step 4 before ligation.

4**.** After digestion, **incubate your samples for 80°C for 20 minutes (in heat block)**. For 3A assembly it is important you heat inactivate your samples after digestion. **THIS MUST BE DONE or NOTHING ELSE WILL WORK!**

5. Run 5 μL of your digested sample in an agarose gel in order to check that your digestion worked. (*See Section 1.4 for making/pouring of agarose gel as well as running a gel electrophoresis).* Make sure you run the proper controls with your samples on the gel: (1) a small volume of uncut DNA for each plasmid digested. (2) **Also, always run a lane with DNA Ladder**!

Be sure to clearly label and freeze the remainder of your digestion. This will be used for the ligation step.

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2.3.2.4: GEL LOADING AND RUNNING

GEL LOADING AND RUNNING USING MINIONE INSTRUCTIONS

Using agarose gel electrophoresis for the visualization of digested DNA.

Video example of pouring and running a gel

Reagent name	Storage Temperature
Agarose	Room Temp
1x TBE	Room Temp
SybrGreen/SybrSafe/GelRed	Room Temp (Keep in dark place)
Digested DNA	$4°C$
DNA Ladder	Room Temp
Loading Dye	Room Temp

TIPS BEFORE STARTING:

Start by making a bottle of 500 ml stock of 1x TBE (*C1V1=C2V2*) for your team's running buffer and to make agarose gels. (You can store this for future use- label the bottle clearly!) You will utilize either 10X, 25X or 50X TBE stock buffer– Pay attention to what stock solution you have been provided with!

Procedure:

1. You will make 30ml of agarose that is 1X TBE and usually 0.75-1.5% agarose. (**See Appendix V for suggestions on what percentage to use.**)

a. Measure ___ g of agarose (0.75-1.5%)

b. Add ___ mL of 1x TBE buffer.

1. Heat gel for **1+ min** in microwave – (**Use plastic flasks!**). Swirl to make sure all crystals are in solution!
2. Let the gel cool for 1 minute.
3. Add **2-3** μL of SybrGreen, SybrSafe or GelRed *(ask your instructor where this is stored; keep tube in the dark at all times)*
4. Let the gel cool for another minute - Do not pour HOT!!!!!
5. Split warm agarose between the two gel containers (you will only need one). Do not forget the combs.
6. Load **5** μL of your DNA ladder per gel lane. (*Your DNA ladder should preferably be in the first lane of your gel).*
7. Load your samples into the wells of the gel, and mark down in your notebook the order in which you loaded the lanes. a. Loading your samples: **5** μL of DNA mixed with **1** μL of 6x loading dye. Load this directly in the well.
8. Run gel for **20 min** in the Gel rig (set up according to diagrams below). While the gel is running, simulate the digest(s) in your lab (physical/digital) notebook, adding the *Quick-Load Purple 1 kb Plus DNA Ladder* as your ladder and paste the image of the expected gel in your notes. Take a picture of your actual gel results and paste them into your lab notebook.

The gel can be visualized in the gel rig (blue light) or for more sensitivity on the UV light box. WARNING: UV light is dangerous. Do not use unless you have talked to your professor!



See Appendix V for any questions you might have about the agarose gel or the electrophoresis protocol

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2.3.2.5: LIGATION OF PRODUCTS

LIGATION OF PRODUCTS

Joining of 2 DNA molecules catalyzed by DNA ligase

This process forms a phosphodiester bond between the 3' hydroxyl and 5' phosphate of adjacent DNA strands, creating recombinant DNA. (NEB)

Video example of performing a ligation

Materials	Storage Temperature
Water	Room Temp
10X T4 DNA Ligation Buffer	-20°C
Linearized Plasmid Backbone (Amp,Chlor,Kan,Tet)	-20°C
Double Digested DNA insert fragments	-20°C
T4 DNA Ligase	-20°C

TIPS BEFORE STARTING:

An equal (1:1) molar quantity of each insert is best. However, 1 ***µL*** of each digested insert is commonly used for the ligation and gets consistent results. **If your parts are drastically different in size, you may want to adjust for molar quantity. (***If you are not getting good results/colonies upon transformation, start looking at changing the molar ratio of your inserts. Use NEBioCalculator to calculate molar ratios.)*

PROCEDURE

1. Thaw 10X T4 DNA **Ligation Buffer** at room temperature and then store it on ice until needed. Leave **DNA ligase (enzyme)** in the freezer block until immediately before it is needed; always keep cold!

2. **Always add water and buffer first**. Buffer must be fully thawed and mixed before use:

5 ***µL*** of Water *(water up to 10ul, if insert volumes are adjusted)*
1 ***µL*** of **10X DNA ligation buffer**
1 ***µL*** (25ng) **linearized plasmid (destination) backbone**
1 ***µL*** of **insert A** *(digested with appropriate enzymes / denatured)*
1 ***µL*** of **insert B** *(digested with appropriate enzymes / denatured)*
1 ***µL*** of **T4 DNA ligase** *(actual enzyme)*

3. Incubate at room temperature for **1hr to 2hr.** *Ligations can go overnight or longer if stored at 4 °C (refrigerated). This works the best.*

4. Heat inactivate the reaction at **65°C** (or 80°C) for **10 minutes**. *(Optional but company recommended.)*

5. Samples can be stored at -20°C or used immediately in the Zippy transformation protocol. It is best if you immediately transform the **5 *µL*** of your reaction into a tube of zippy cells. (*Ligation buffer can reduce transformation efficiency. Using more than 5ul may actual decrease transformation efficiency)*

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2.3.2.6: CELL TRANSFORMATION

CELL TRANSFORMATION: ZIPPY TRANSFORMATION OF Z-COMPETENT CELLS

Introducing foreign DNA into E. coli bacteria

Bacterial transformation is when cells take up foreign DNA from the environment. Cells may express the genetic information they received from this foreign DNA. (ThermoFisher)

TIPS BEFORE STARTING:

- Depending on the situation, particularly when you get to the analysis phase and you request a different type of bacterial chassis, you will use a variety of competent cells. Be sure you know what cells you are using and if there is a specific protocol for the type of cell. (The competent bacteria described in this protocol are Zippy Competent JM109, ZymoResearch)
- Most plasmids carry a marker gene for a specific antibiotic resistance. By supplementing the growth medium with the antibiotic of choice, only cells containing the plasmid of interest will propagate. **You must know the antibiotic resistance associated with your plasmid backbone!! (See Appendix I)**

All competent cells are stored in the -80 freezer. Ask the instructor for help when you are ready to get the competent cells. You must have ICE, first!

Materials	Storage Temperature
Zippy Competent Bacteria Cells	-80°C
Ligation Mixture	fresh / -20°C
LB Agar plate w/Antibiotic	Room Temp / 37°C
SOC	4°C / Room Temp
Sterile Glass Beads	Room Temp

Procedure: Read every step before you begin!

1) Make sure you have a warm plate. Pre-warm by placing it in the **37°C** incubator or by taking it out at room temperature before starting the procedure. *See color coding (Appendix I) to ensure you have appropriate LB+antibiotic plate type!*

2) Get ice, then request cells / Let the cells thaw for **1-2 minutes on ice**. – The 50 μL of cells can be split into **2 tubes of 25 μL** each for two different transformations if used immediately to transform different plasmids. *(If you don't have two plasmids to transform, consider sharing with another team.)*

3) **Before** the cells are completely thawed add **1-5 μL** of ligation (plasmid) mixture directly to the cells (do not add to the side of the tube). For best results add the plasmid ligation to the cells while they are still slushy.

4) Incubate on ice for **5 minutes**.

5) Add **200 μL** of **SOC media** with no antibiotic to the cell-ligation mix.
(SOC amount = ~4X volume of cell + plasmid mix; thus only 120ul needed for 25ul transformation)

6). Incubate with gentle shaking (200-300 RPM) for **1-2 hrs at 37°C**. *(1 hr will work well but you get a higher transformation efficiency with 2 hr out-growths*). **Skip this step if using Ampicillin resistance and proceed directly to step 7.**

7) Spread transformed cells using glass beads onto a **LABELED** warmed (22-37°C) plate containing antibiotic. Allow plates to dry (37°C) before flipping plates upside down. *(You do not want media/bacteria dripping onto the lid. You can flip plates early the next day, if needed.)*

8) Incubate overnight (18-20 hrs) at **37°C**. The next day, remove plates from the incubator, seal the lids with parafilm and store at 4°C.

The next day: All colonies should be about the same size (diameter). It is not uncommon with 3A assembly to see only 1 to 10 colonies.

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2.3.2.7: INOCULATION

INOCULATION OF OVERNIGHT CULTURES

Small sample of bacteria is taken from a plate and placed in LB broth to be used in ZymoPure miniprep or for long term storage.

Materials	Storage Temperature
Sterile Toothpicks	Room Temp
LB Broth	Room Temp
Isolated Colony of Transformed Bacteria	Room Temp

Procedure:

Using sterile technique, pick an isolated colony from a fresh plate (less than seven days old) and inoculate LB-Miller medium.

1. You will use a **sterile toothpick** to pick the colony, and you can simply drop the entire toothpick into the liquid culture tube.
2. **Sterile 5 mL** of **LB** broth will be provided for you for this step. You may add the required antibiotic to the LB prior to inoculating with your colony (**optional**).
3. Incubate with shaking for **8–16 hours at 37°C** before harvesting. This results in maximum yields of a high-copy-number plasmid.

(Typically, after overnight incubation, the absorbance of a tenfold dilution of the culture at a wavelength of 600nm (A600) with a 1cm path length should range from 0.10–0.35.)

These cultures can now be used in the Mini-prep procedure or stored long term in glycerol. (See Appendix VI for how to make your own glycerol stock.)

Growth of Overnight Cultures – Helpful Hints if you are having difficulty.

Different culture media will also have a profound effect on the growth of different bacterial strains. Most plasmid DNA purification systems are appropriate for bacterial cultures grown in 1X Luria-Bertani (LB) medium. However, use of a LB-Miller medium containing more NaCl will produce significantly greater yields and is highly recommended. Richer media such as 2X YT, CIRCLEGROW® or Terrific Broth may be used to increase plasmid yields by increasing the biomass for a given volume of culture. Keep the biomass in a range acceptable for the plasmid isolation system used, as overloading may result in poor purity and yield of the plasmid DNA

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SECTION OVERVIEW

2.3.3: APPENDICES- EQUIPMENT USE

2.3.3.1: APPENDIX I- ANTIBIOTICS AND COLOR CODING

2.3.3.2: APPENDIX II- USING THE DENOVIX

2.3.3.3: APPENDIX III- USING THE NANODROP

2.3.3.4: APPENDIX IV- USING THE QUBIT

2.3.3.5: APPENDIX V- COMMON AGAROSE GEL QUESTIONS AND TIPS

2.3.3.6: APPENDIX IV- PREPARING GLYCEROL STOCKS

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2.3.3.1: APPENDIX I- ANTIBIOTICS AND COLOR CODING

LB Plates plus antibiotics (color coding)

***Green* = LB + Ampicillin^{100*}**

***Black* = LB + Chloramphenicol^{35**}**

***Blue* = LB + Kanamycin^{50***}**

***Red* = LB + Tetracycline^{10****}**

Antibiotic Selection

Ampicillin (Amp/A) A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis. The resistance gene (*bla*) specifies a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.

*Working concentration 100µg/mL; freezer stock 100mg/mL (in water)

Chloramphenicol (Chlor/C) A bacteriostatic agent that binds to the 50S subunit of the ribosome and blocking peptidyl transferase and inhibiting peptide bond formation. The resistance gene, chloramphenicol acetyltransferase (*cat*), specifies an enzyme that renders the antibiotic incapable of binding to the ribosome.

**Working concentration 35µg/mL; freezer stock 35mg/mL (in EtOH)

Kanamycin (Kan/K) A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA. The resistance gene (*kan*) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.

***Working concentration 50µg/mL; freezer stock 50mg/mL (in water)

Tetracycline (Tet/T) A bacteriostatic agent that binds to the 30S ribosome and disrupts codon and anticodon interactions. The resistance gene (*tet*) specifies a multimeric transporter protein that participates in the rapid efflux of tetracycline from the bacterial cell rendering it incapable of accessing the ribosomes.

****Working concentration 10µg/mL; freezer stock 10mg/mL (in water)

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2.3.3.2: APPENDIX II- USING THE DENOVIX

How to Use the Denovix



Watch the YouTube videos regarding questions for the DeNovix.

(https://www.youtube.com/user/denovixvideos/videos)

The Denovix allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5μL to 2μL** for accurate measurements. It also has the ability to measure fluorescently labeled molecules.

Startup Denovix by touching the screen. Set up parameters for measuring **nucleic acid/double stranded DNA** (dsDNA).

1. **Clean Denovix**. Pipette **5-10 μL** of water on the pedestal (metal part that was under the arm with a hole in the middle), close arm, and then wipe off water with a **Kim-wipe(no paper towels)**.
2. You will now need to **measure a blank**. *(This should be the same liquid your sample is dissolved in)*. Use the **elution buffer** you use in your Zymopure miniprep. Lift metal arm and pipette **2μL** of your blank liquid onto the pedestal. (*Avoid introducing bubbles*.)

4. **Gently** lower arm and click on the **blank button** on the screen**.**

5. When complete, lift the arm and simply wipe clean with a Kim-wipe (**do not** use a paper towel!).
You are now ready to measure multiple samples.
(*It is recommended you re-blank the instrument every 30 min.)*

6. Add **1.5** or **2μL** of your DNA sample and click **measure** on the screen.

*Note: You do **not** have to clean with water between samples. Simply wipe the pedestal dry when you are ready for the next sample. Repeat for all samples.*

7. Record DNA concentration (ng/μL), 260/280 and 260/230 ratios for all samples.
260/280 = a ratio of ~1.7- 1.80 is considered pure DNA (**most important value**)
260/230 ratios are commonly in the range of 2.0-2.2 (can vary a lot)

8. **Clean Denovix**. Pipette **10 μL of water** on the pedestal, close arm, and then wipe off water with a
Kim-wipe….then close out the program. (Press home button.)

What do my values mean?

Link to DeNovix Website to learn more: https://www.denovix.com/tn-130-purity-ratios-explained/

- 260/280 Nucleic Acid Purity Ratios

"260/280 ratios are routinely used to determine the purity of nucleic acid measurements. This ratio is most commonly used to determine the presence of protein and or phenol in the isolated nucleic acid sample. Table 1 describes a general acceptable range for these ratios; however, they are not a guarantee of sample purity."

- 260/230 Nucleic Acid Purity Ratios

"The 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL and guanidine thiocyanate. Generally acceptable 260/230 ratios are in the range of 2.0 – 2.2. Values higher than this may indicate contamination with the aforementioned compounds."

*A 260/280 ratio below 1.5 **does not** render the DNA unsuitable for any application, but lower ratios indicate more protein contaminants are present which may interfere with downstream steps. 260/230 measure residual contaminants from the purification process. Low ratios may indicate impure DNA that will be less successful in downstream steps.*

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2.3.3.3: APPENDIX III- USING THE NANODROP

How to Use the NanoDrop



Watch the YouTube videos regarding questions for the NanoDrop.

(https://www.youtube.com/watch?v=y3ICiycWeCA&t)

The NanoDrop allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5μL to 2μL** for accurate measurements. Some models have the ability to measure fluorescently labeled molecules.

Procedure:

1. Wash the NanoDrop pedestal. Lift the upper arm of the NanoDrop and remove the wipe.
   Add 4-5 μL of purified water to the lower pedestal, then lower the arm.
   Wait 30-60 seconds.
   Lift the upper arm and use the wipe to vigorously scrub both the upper and lower pedestals.
2. Open the NanoDrop software on the computer by double-clicking the “ND-1000” icon that looks a bit like an hourglass
3. Initialize the NanoDrop:
   Click on the “Nucleic Acid” button in the NanoDrop software. This brings up q dialog box; **DO NOT click “okay” until you have added water.**
   Add 2 μL of purified water to the lower pedestal, then lower the upper arm.
   Click “okay” on the computer and wait about 20 seconds while the NanoDrop initializes
   When it is done, lift the upper arm and dry it off with a wipe
4. Blank the NanoDrop:
   Add 2 μL of the buffer your sample is in (most likely elution buffer).
   Lower the upper arm of the NanoDrop and click the “Blank” button on the software.
   Wait about 20 seconds for the blank measurement to be made.
   Lift the upper arm and dry the pedestal with a wipe
5. Measure your Sample:
   Add 2 μL of your sample to the lower pedestal, then lower the upper arm.
   In the “Sample ID” box, type the name of your sample.
   Click the “Measure” button on the software and wait about 20 seconds for the measurement to be complete.
   Lift the upper arm and dry the pedestal **OR** lift the upper arm and carefully pipet up as much of the sample as you can to retain for further use.
6. Collecting Data:
   Write down any measurements you are interested in.
   Click the “Print Screen” button to print the complete spectrum if desired.
   When finished making all measurements, click “Print Report” to get a table of all data.
7. Cleaning the Pedestal:
   Add 4-5 μL of purified water to the lower pedestal, then lower the arm.
   Wait 30-60 seconds.
   Lift the upper arm and use a wipe to vigorously scrub both the upper and lower pedestals.
   Place a new folded lab wipe on the lower pedestal and close the upper arm.
8. Shut down and close the NanoDrop computer.

More information on what these NanoDrop values mean:

https://tools.thermofisher.com/content/sfs/brochures/T123-NanoDrop-Lite-Interpretation-of-Nucleic-Acid-260-280-Ratios.pdf

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2.3.3.4: APPENDIX IV- USING THE QUBIT

How to Use the Qubit Fluorometer

Watch the YouTube video regarding questions for the Qubit Fluorometer.

(https://www.youtube.com/watch?v=z5g6pAFAWEc)

The Qubit allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5 μL to 2μL** for accurate measurements. Some models have the ability to measure fluorescently labeled molecules.

Procedure:

1. Calibration:
    1. On the "Home Screen," choose the type of assay for which you want to run the standards. Standards Screen is automatically displayed.
    2. The screen will prompt you to choose between reading new standards and using the previous calibration. Press "Yes" to read new standards.
    3.
    4. Insert Standard #2, and press "Read." Ensure that you are using the Standard #2 appropriate for the assay you are performing.
    5. Calibration is complete. The new standards graph with data points for standards connected by a line appears on the screen.
2. Reading Samples:
    1. Choose "Sample" to go on the Sample Screen
    2. Insert a sample into the Sample Chamber and press "Read." The measurement takes approximately 3 seconds. Upon the completion of the measurement, the result is displayed on the screen. The number displayed is the concentration of the nucleic acid in the assay tube.
    3. To read the next sample, remove the sample from the Sample Chamber, insert the next sample, and press "Read Next Sample."
    4. Repeat until all samples are read.

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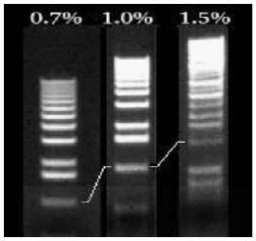
2.3.3.5: APPENDIX V- COMMON AGAROSE GEL QUESTIONS AND TIPS

Common Agarose Gel Related Questions and Tips

(http://www.lifetechnologies.com/us/en/home/life-science/pcr/elevate-pcr-research/agarose-content-with-tips-and-tricks.html#2)

1. What Percent Agarose is best?

The standard percentage of agarose used to run a DNA gel is usually around 1.0%. A higher agarose percentage enhances the resolution of smaller bands; conversely, a lower agarose percentage gives better resolution and separation of higher-molecular-weight bands. If the wrong percentage is used, it can be difficult to visualize the DNA bands reliably



1KB ladder run in different % agarose gels. Arrow indicates 1kb DNA fragment. Notice clarity of different size bands on each gel.

% Gel	Optimum Resolution/ Linear DNA (kb)
0.5	30 to 1.0
0.75	**12 to 0.8**
1.0	**10 to 0.5**
1.2	7 to 0.4
1.5	3 to 0.2

*****For very small parts, 2% gel may be an option. Talk to your instructor about it!**

2. How does gel casting affect the band resolution?

The recommended thickness for agarose gel is **3–4 mm**; a gel thicker than 5mm will result in fuzzy bands and higher staining background. Similarly, the amount of running buffer to cover over the gel in an electrophoresis apparatus is 3–5 mm. Too much buffer will decrease DNA mobility and cause band distortion. The thickness of the comb is also important and significantly affects resolution. A thin comb (1 mm) gives very well-defined bands, while a thick comb gives thick bands leading to reduced resolution.

3. How much DNA to run (Less is best)

The minimal quantity of DNA that may be detected is dependent on the stains used. The maximum quantity of DNA in a band that is still clear and well-defined is approximately 100 ng.

4. What is the right buffer for your agarose gel electrophoresis?

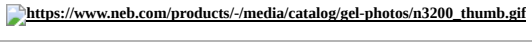
The two most popular types of buffers for running agarose gels are Tris-acetate with EDTA (TAE) and Tris-borate with EDTA (TBE). **For small DNA (<1000 bp), and if there is no plan to extract the DNA, then 1x TBE buffer is recommended.** TBE buffer has a high ionic strength and buffering capacity. TAE buffer, together with low field strength (1–2 V/cm), is preferred for separating large DNA (12–15 kb). TAE buffer interacts with agarose, resulting in lower electroendosmosis, larger apparent pore size, and lower field strength compared to agarose gels in TBE buffer. **Buffer must be added to the GEL.**

5. Why do I see so many bands?

Uncut DNA will typically appear to run faster than fully linearized plasmids. Isolated, uncut plasmid should be in the supercoiled form. The use of restriction enzymes will linearize our plasmids.
(http://bitesizebio.com/13524/how-to-identify-supercoils-nicks-and-circles-in-plasmid-preps/)

How to identify supercoils, nicks and circles in plasmid preps

DNA Ladder (*Quick-Load Purple 1kb Plus DNA Ladder, New England Biolabs)*



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2.3.3.6: APPENDIX IV- PREPARING GLYCEROL STOCKS

Making a Glycerol Stock

ONLY if you have confirmed that your NEW composite part *(newly built plasmid)* is **CORRECT.** This means that after conducting a Zymo Miniprep and restriction digest, the new plasmid now contains the parts that you have ligated together.

How to make a glycerol stock?

1. Using sterile technique, aliquot **500µL** of 80% glycerol into a cryovial (This may or may not have been done already, ask your instructor)
2. Label the cryovial clearly on top or on the side with the part number, the antibiotic resistance (amp, chlor, tet, kan), as well as any other labeling standard your lab uses ***(LABELING IS VERY IMPORTANT!!)***
3. Vortex your culture
4. Carefully add about **500uL** (0.5mL) of your culture to the cryovial. Vortex and place the vial on ice.

Store the vial in a -80 freezer that has been designated for long term storage.

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