Opentrons
for Education

Prep Sheet

Lab Module 4: DNA Normalization

Dr. Cătălin Voiniciuc, Dr. Moni Qiande, and Abigail Lin

University of Florida

Getting Started

This lesson plan uses values from an Excel template to customize the DNA Sample Normalization protocol. Directions are included in this lesson plan to create and use the Excel template. Values from the template are copied and pasted into labeled sections of the Python protocol in a code editing program. Questions about using the Excel template for protocol customization can be directed to cvoiniciuc@ufl.edu.

This course does not require previous coding experience. For guidance working with Python code in this Opentrons protocol, you can refer to the following resources:

- ☐ Python Protocol API Tutorial
- ☐ Python Protocol API- Labware
- ☐ Python Protocol API- Temperature Module
- ☐ Python Protocol API- Loading Labware on Adapters

Additional Support and Resources

OT-2 Manual
Temperature Module video
Running a protocol on the OT-2

For technical support, please check our Opentrons Help Center for relevant articles. If you need further support, please contact support@opentrons.com. Inform them that you are a part of the Opentrons for Education program and provide the date of your next laboratory class.

If you have questions related to the lesson plan, please reach out to Dr. Cătălin Voiniciuc at cvoiniciuc@ufl.edu.

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Educator Guide
Lab Module 4: DNA Normalization

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Contents

The educator guide includes the following sections:

- Purpose
- Background Knowledge
- Supplies
- Experimental Duration
- Basic Troubleshooting and Tips
- Procedure Guide
- Discussion Questions

This lesson plan includes suggestions to allow for instructor flexibility in student learning outcomes. Cloning in this course incorporates selection markers into a set of suggested plasmid backbones; however, instructors can create their own workflow to include more or less genetic components, or specific genes of interest.

Purpose

Students will use the OT-2 to normalize previously isolated plasmid DNA to prepare for modular cloning (MoClo) assembly. They'll calculate DNA dilution volumes using NanoDrop measurements of DNA concentration. In addition, students will use different methods (as appropriate) to visualize the selection markers in their randomized *Agrobacterium* reporter strains.

During this lab class, students will learn about and gain experience with:

- Basic principles of cloning using plasmid vectors
- Automating pipetting tasks
- Customization of protocols for the OT-2
- Common reporters and markers

Core Competencies

Laboratory Skills

- Basics of cloning experiments, including preparation for a cloning experiment
- Common reporters and markers

Automation Skills

- Automation of pipetting tasks
- Python protocol customization

Background Knowledge

Students should begin this lab with an understanding of the cloning workflow used in this course from the previous week. Instructors may wish to review this workflow with students before class. An included pre-lab reading builds on the concepts of plasmids as used in cloning introduced in the previous week. *No coding experience is required for this lab,* but students and/or instructors will need to edit a Python protocol file.

Supplies

Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ Temperature Module GEN2 in deck slot 3
- ☐ OT-2 P300 Single-Channel GEN2 Pipette in left mount

Opentrons Protocol

- ☐ DNA Sample Normalization protocol

Non-Opentrons Equipment

- ☐ Plate reader and/or epifluorescence microscope (depending on markers used in *Agrobacterium* reporter strains)

Labware

- ☐ Opentrons OT-2 Filter Tips, 300µL in deck slot 1
- ☐ Opentrons 24 Well Aluminum Block with NEST 1.5 mL Snapcap tubes in deck slot 2
- ☐ Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 uL tubes (in deck slot 3, on the Temperature Module)

- [ ] Opentrons 15 Tube Rack with Falcon 15 mL Conical tubes in deck slot 5

Reagents and Other Materials

- [ ] 70% ethanol for cleaning
- [ ] Student plasmid DNA samples isolated in Lab Module 3 in snapcap tubes (in deck slot 2)
- [ ] Molecular biology grade water, such as HyClone Water, in a Falcon 15 mL conical tube
- [ ] Plasmid maps or sequence information for *Agrobacterium* reporter strains

Experimental Duration

Required Class Sessions

1

Lab Run Time

This lesson plan was prepared for a traditional laboratory class time of 80–90 minutes. Students continue work from the previous week with viewing and assignment of *Agrobacterium* reporter strains, and measurement of their plasmid DNA concentration on the NanoDrop.

Basic Troubleshooting and Tips

- We recommend completing a trial run of the protocol used for this lesson plan prior to class. On the OT-2 robot, this trial run can be completed with or without tips.

- Issues with tips striking plates are almost always due to using alternate labware or robot calibration issues. If you experience this issue, first confirm that the correct labware specified in the protocol is in use; then, re-calibrate the robot. A Labware Position Check is also recommended after importing a protocol and before you

run it to confirm the combination of deck slot and labware definition on the OT-2.

- Demonstrate proper techniques when working with DNA for students, including establishing and working in a DNase- and RNase-free environment, and using sterile equipment and labware.

- Please note that, in this class, students normalize their plasmid DNA to a concentration of 20 ng/uL. This is a typical normalization concentration used in MoClo (modular cloning, a Golden Gate cloning method) tested by Dr. Voiniciuc and his lab. Student DNA yields can be up to 300 ng/uL, resulting in a large amount of diluted plasmid DNA. Digest reactions in the following lab are scaled down to 10 µL to allow effective cleavage by restriction enzymes.

- The labware definition for Opentrons 96 Aluminum Block with Generic PCR Strip 200 uL assumes “generic” PCR strip tubes loaded into the aluminum block. Instructors should confirm labware dimensions of your chosen PCR strip tubes with the labware definition, and complete the recommended labware position check prior to running this protocol. If labware is changed in your protocol, labware API names must be updated to match the template name (for example, ‘strip_tubes’ =

opentrons_96_aluminumblock_generic_pcr_strip_200ul) in the protocol's labware dictionary, beginning around line 80 of code. A labware position check should be completed prior to running your protocol with updated labware.

- When editing your protocol using your Excel template, please note that Source_Labware and Destination_Labware names are *not* API names for labware, but rather are common names entered into your Excel template. They are defined for the OT-2 in the labware_dict section of the protocol. If your chosen labware has a definition (from the Labware Library or a custom definition) and is compatible with the module it will be loaded into, labware can be replaced to further customize the protocol.

Procedure Guide

Before Class

1. Turn on any required equipment (microscopes, plate reader) ahead of time.
2. Pre-chill a 24-well aluminum block at -20 °C.

Lab Introduction

1. Instructors should review the course cloning workflow with students.

2. Students should understand why DNA normalization is important for a molecular cloning workflow.

Quantification of Plasmid DNA on the NanoDrop

1. Students will remove their sample from storage and bring samples (on ice) to the NanoDrop.
2. Open the program on the computer and demonstrate use of the NanoDrop to students.
3. Direct students to complete control measurements of a blank (molecular grade water) first.
4. As students take measurements of samples on the NanoDrop; ensure they write down the following numbers from the program:
    - concentration of plasmid DNA (in ng/µL or µg/µL)
    - the 260/280 ratio and the 260/230 ratio

DNA Normalization on the OT-2

1. Direct students to calculate the amount of molecular biology grade water which will need to be added to their plasmid DNA for a final concentration of 20 ng/uL. Instructors may want to create a table, like the example shown below. Students can fill in a smaller example table to check their understanding in the **Student Guide**.

Tube Label	Plasmid Name	DNA concentration (ng/µL)	µL needed for 20 ng/µL	Water volume	Total volume

C1	pAGM1263	71.4	56	144	200
C2	pICH41264	114.1	35	165	200
C3	pICH47742	201.3	20	180	200
C4	pICH47822	130.0	31	169	200
C5	pICH41744	285.4	14	186	200
C6	pICH49277	230.9	17	183	200
C7	pICH83999	160.6	25	175	200
C8	pAGM8079	197.3	20	180	200
C9	pICH50927	187.1	21	179	200
C10	pICH75388	231.9	17	183	200
C11	pICH54011	109.8	36	164	200
C12	pICH82113	140.0	29	171	200

2. Next, create an Excel file as a class. The file will be used as a template throughout the course to customize protocols for your unique number of samples, volumes, and other factors.
3. First, create a “Step 1” table for initial volumes of liquids.

Step 1: Put columns B-F into "csv_volume_data_raw" This is how you tell the robot how much liquid is initially in the wells.	Labware	Initial_Wells	Initial_Volume	Liquid_Name	Description	Color
	DNA_tubes	A1	0.4	Plasmid DNA	Plasmid DNA sample to	#00FF00
	tube_rack	A2	3	Water	Molecular grade water	#FFC0CB

   The table can include common labware names; in other words, descriptive names for your labware that are separate from the API names. Here, we use “DNA_tubes” to describe a single plasmid DNA sample (see “liquid name” and “description” columns) in a NEST 1.5 mL snap cap tube, and “tube_rack” to describe the Falcon 15 mL conical tube containing molecular grade water. Initial volumes are entered in mL. *Note: this example template includes only one tube with one DNA sample; however, you can add as many as 24 individual DNA samples to customize the Python protocol for your experiment.*

4. Next, create a “Step 2” table for your volume transfers. The table should define transfer steps from source

labware (and source wells) to wells in the destination labware.

Step 2: Put columns H-L into "csv_transfer_data_raw". Volume is in uL. This is the table for transferring liquid.

Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volum	Pick_Up_Tip
DNA_tubes	A1	strip_tubes	A1	56	TRUE
DNA_tubes	A1	strip_tubes	A2	35	TRUE
DNA_tubes	A1	strip_tubes	A3	20	TRUE
DNA_tubes	A1	strip_tubes	A4	31	TRUE
DNA_tubes	A1	strip_tubes	A5	14	TRUE
DNA_tubes	A1	strip_tubes	A6	17	TRUE
tube_rack	A2	strip_tubes	A1	144	TRUE
tube_rack	A2	strip_tubes	A2	165	FALSE
tube_rack	A2	strip_tubes	A3	180	FALSE
tube_rack	A2	strip_tubes	A4	169	FALSE
tube_rack	A2	strip_tubes	A5	186	FALSE
tube_rack	A2	strip_tubes	A6	183	FALSE

This example table demonstrates transfer of plasmid DNA samples (from DNA_tubes) into the destination labware, “strip_tubes” (PCR strip tubes placed in the aluminum block on the Temperature Module). Transfer volumes are entered in µL. The Pick_Up_Tip column specifies when the OT-2 will select a new tip in the protocol timeline (before each DNA transfer, and before the first water transfer).

5. As a class, download and open the DNA Sample Normalization protocol in a code editing program.
6. Starting at line 8, follow the directions to modify your code by copying and pasting in data from your tables.
    a. First, copy and paste your “Step 1” table under csv_volume_data_raw. Be sure to copy and paste your data to replace the current values. This includes the column titles (Labware, Initial_Wells, etc.) and all values.

    b. Next, copy and paste your “Step 2” table under csv_transfer_data_raw in the same method.
7. As needed, update the starting tip for the protocol (the first available tip the OT-2 should pick up in the tip box; around line 120).
8. Save your customized protocol and import into the Opentrons App. *Note:* remember to save your Excel template for later use in the course!
9. Remove the pre-chilled, 24-well aluminum block from storage at -20 °C.
10. Set up your labware and liquids as follows. Liquid well locations are specified by the values in your data tables.
    a. Opentrons OT-2 96 Filter Tip Rack 300 µL in deck slot 1
    b. Opentrons 24 Well Aluminum Block (can be pre-chilled) with plasmid DNA samples in NEST 1.5 Snapcap tubes (in deck slot 2)
    c. Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 L (in deck slot 3, on the Temperature Module)
    d. Opentrons 15 Tube Rack with molecular grade water in a Falcon 15 mL Conical (in deck slot 5)
11. Run the protocol.
12. The OT-2 will begin by setting the Temperature Module to 4 °C.

13. The OT-2 will transfer concentrated DNA from 1.5 mL snapcap tubes to 8-well PCR strip tubes (volume dependent on table values).
14. The OT-2 will add molecular grade water to a final volume of 20 ng/uL.
15. Diluted plasmid DNA samples should be stored at -20 °C.

Identification of *Agrobacterium* Reporter Strains

1. Students should take photos and use a plate reader or microscope to quantify the absorbance and/or fluorescence spectra of *Agrobacterium* strains.
2. Students will then "assign" the plasmid sequence belonging to each of the strains growing in different wells from the anonymized pre-cultures.

Discussion Questions

Direct students to discuss the lab activities with one another. Example prompts might include:

- What are some of the different techniques you saw today for selection? How can you be sure you are using the right plasmid?
- What are the next steps in the cloning process? What will happen next with the plasmid DNA you normalized today?
- Why did we need to normalize the plasmid DNA to a standard concentration?

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Student Guide

Lab Module 4: DNA Normalization

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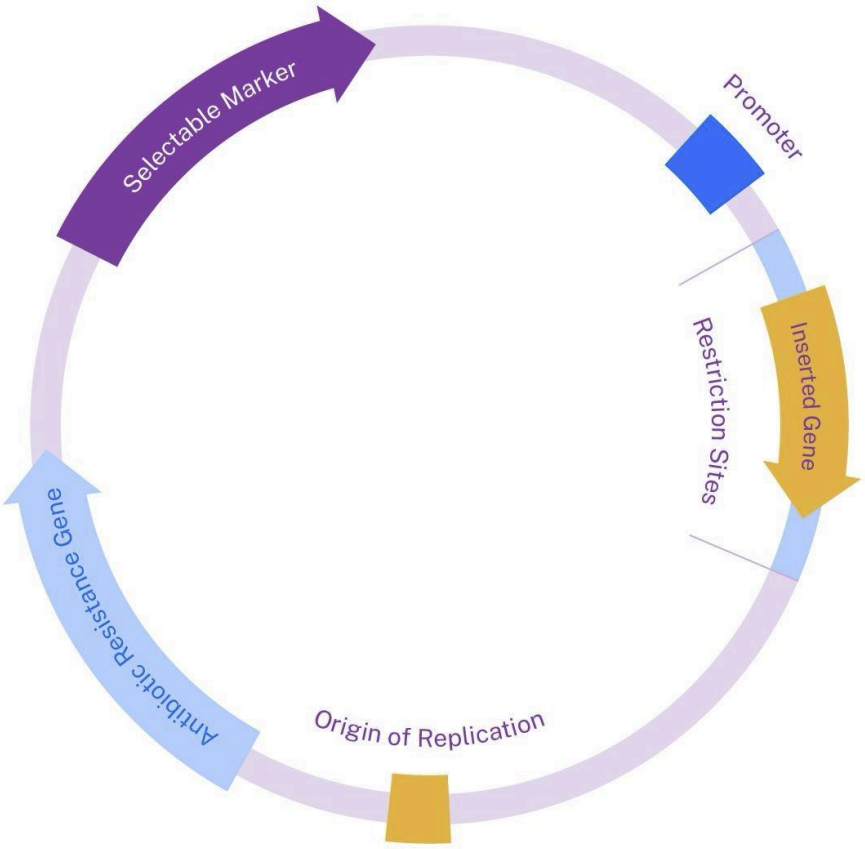
Pre-Lab Reading

Today in lab, you'll prepare your plasmid DNA for use in a *cloning* experiment. In *molecular cloning,* scientists use plasmids to insert a new gene of interest.

Let's say you have a gene you want to work with, maybe another antibiotic resistance or selection marker gene, or even a specifically altered gene that you'd like to study the effects of. Cloning, at its most basic, is inserting that gene you are interested in into a *vector*, which is often a plasmid. That vector can be used to deliver your gene of interest, or even several genes, like selection markers *and* your gene of interest, into your research organism, like other cells in culture, or even *Arabidopsis* plants. To understand how this works, let's revisit the plasmid "map" we looked at last week:



There are four plasmid components, shown here, that we should consider in two groups:

1. *Antibiotic resistance gene* and *selectable marker*
2. *Restriction sites* and *inserted gene*

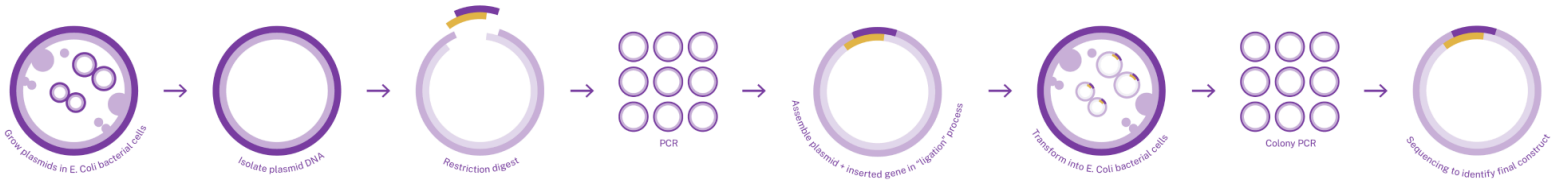
Today, let's discuss the second important group of plasmid components: the restriction sites and inserted gene. This plasmid already contains selectable (or selection) markers, and antibiotic resistance genes, so here, the inserted gene will be a gene of interest you'd like to use in your research. You'll notice

that the inserted gene can be found between two *restriction sites.* Restriction sites are like a roadmap for *restriction enzymes.* A restriction enzyme is like a pair of molecular scissors. It will cut DNA at a very specific site, usually at a given sequence of less than 10 base pairs (you guessed it, the restriction site). In a plasmid like this, the restriction sites tell us where the enzyme will cut DNA, and where we can insert our gene of interest, as well as *which* enzyme we can use to do the cutting. Specific enzymes cut at specific sites, or small sequences of DNA.

Other plasmid components that we can see in the diagram are the *origin of replication* and the *promoter.* An origin of replication (sometimes referred to as an ORI) is the site where DNA replication, to make more DNA, begins. The promoter is a region in the DNA where transcription factors and polymerases will bind so that our gene of interest can be transcribed. Remember that one of the reasons scientists work with plasmids as vectors is that, using bacterial culture, it is easy (and relatively inexpensive) to grow large quantities of bacteria, and therefore large quantities of plasmid DNA. We need these important elements so that DNA reproduction can occur, and the plasmid can reproduce itself.

Now, before we start the cloning experiment, we need to normalize the plasmid DNA to a certain concentration. Why is

this important? Let's take a look at our basic cloning workflow for this course:



So far, the plasmid *backbones*, or the basic plasmid that we want to add genetic components to, have been grown in bacterial culture. You isolated those plasmids from *E. coli,* which we now have in different concentrations. Next week, we'll complete a *restriction digest*: the cutting of the plasmid DNA with restriction enzymes. That means we'll be adding your plasmid DNA to restriction enzymes, and adding anything else (like other DNA fragments) you want the enzyme to cut. Restriction enzymes, like other enzymes, have a limited amount of substrate (in this case, our plasmid DNA) they can bind to and work on. We could add more enzyme for a higher concentration of DNA, but DNA concentration will be important for later reactions, like PCR and *ligation*, assembling the genetic components we want into the plasmid. It's easiest, and the best practice, to normalize the DNA now to prepare for what's next.

To learn more about the cloning workflow we'll use in this class, read this article: Modular Cloning (MoClo) Guide.

Purpose

In this lab, you will use the OT-2 to normalize your plasmid DNA to prepare for modular cloning (MoClo) assembly. In addition, we'll revisit and visualize selection markers in your randomized *Agrobacterium* reporter strains from last class. You should be able to identify which plasmid is which by comparing the visual markers and the plasmid maps or sequences your instructor will provide.

Learning Outcomes

- Understand the use of plasmids in cloning, and the cloning workflow we'll use in this course
- Be able to use data from a CSV file in your automated protocol to complete a pipetting task
- Understand the use of common reporters and markers in plasmids

Supplies

Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ Temperature Module GEN2 in deck slot 3
- ☐ OT-2 P300 Single-Channel GEN2 Pipette in left mount

Opentrons Protocol

- ☐ DNA Sample Normalization protocol

Non-Opentrons Equipment

- ☐ Plate reader and/or epifluorescence microscope (depending on markers used in *Agrobacterium* reporter strains)

Labware

- ☐ Opentrons OT-2 Filter Tips, 300µL in deck slot 1
- ☐ Opentrons 24 Well Aluminum Block with NEST 1.5 mL Snapcap tubes in deck slot 2
- ☐ Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 uL tubes (in deck slot 3, on the Temperature Module)
- ☐ Opentrons 15 Tube Rack with Falcon 15 mL Conical tubes in deck slot 5

Reagents and Other Materials

- ☐ 70% ethanol for cleaning

- ☐ Student plasmid DNA samples isolated in Lab Module 3 in snapcap tubes (in deck slot 2)
- ☐ Molecular biology grade water, such as HyClone Water, in a Falcon 15 mL conical tube
- ☐ Plasmid maps or sequence information for *Agrobacterium* reporter strains

Procedure Guide

Before Class

1. Complete the pre-lab reading.

Quantification of Plasmid DNA on the NanoDrop

1. Bring your sample (on ice) to the NanoDrop and open the program on the computer.
2. You need to take a measurement of a *blank* sample first. This gives the NanoDrop a negative control to compare your sample against. Molecular grade water is often used.
3. Take a measurement of your sample on the NanoDrop. Make sure to write down the following numbers from the program:
    - concentration of your plasmid DNA (in ng/µL or µg/µL)
    - the 260/280 ratio and the 260/230 ratio (these numbers give you an estimate of how pure your sample is)

DNA Normalization on the OT-2

1. You'll need to calculate the amount of molecular grade water to be added to your plasmid DNA to reach a final concentration of 20 ng/uL. A table, like the example shown below, can be helpful:

Tube Label	Plasmid Name	DNA concentration (ng/µL)	µL needed for 20 ng/µL	Water volume	Total volume
C1	pAGM1263	71.4	56	144	200
C2	pICH41264	114.1	35	165	200
C3	pICH47742	201.3		180	200
C4	pICH47822	130.0	31		200
C5	pICH41744	285.4			200
C6	pICH49277	230.9			200

Fill in the table with the correct amounts to check your understanding.

2. Next, create an Excel file as a class. The file will be used as a template throughout the course to customize protocols for your unique number of samples, volumes, and other factors.

3. First, create a “Step 1” table for initial volumes of liquids.

Step 1: Put columns B-F into "csv_volume_data_raw" This is how you tell the robot how much liquid is initially in the wells.	Labware	Initial_Wells	Initial_Volume	Liquid_Name	Description	Color
	DNA_tubes	A1	0.4	Plasmid DNA	Plasmid DNA sample to	#00FF00
	tube_rack	A2	3	Water	Molecular grade water	#FFC0CB

The table can include common labware names; in other words, descriptive names for your labware that are separate from the API names. Here, we use “DNA_tubes” to describe a single plasmid DNA sample (see “liquid name” and “description” columns) in a NEST 1.5 mL snap cap tube, and “tube_rack” to describe the Falcon 15 mL conical tube containing molecular grade water. Initial volumes are entered in mL. *Note: this example template includes only one tube with one DNA sample; however, you can add as many as 24 individual DNA samples to customize the Python protocol for your experiment.*

4. Next, create a “Step 2” table for your volume transfers. The table should define transfer steps from source labware (and source wells) to wells in the destination labware.

Step 2: Put columns H-L into "csv_transfer_data_raw". Volume is in uL. This is the table for transferring liquid.

Source_Labware	Source_Well	Destination_Labware	Destination_Well	Transfer_Volum	Pick_Up_Tip
DNA_tubes	A1	strip_tubes	A1	56	TRUE
DNA_tubes	A1	strip_tubes	A2	35	TRUE
DNA_tubes	A1	strip_tubes	A3	20	TRUE
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DNA_tubes	A1	strip_tubes	A5	14	TRUE
DNA_tubes	A1	strip_tubes	A6	17	TRUE
tube_rack	A2	strip_tubes	A1	144	TRUE
tube_rack	A2	strip_tubes	A2	165	FALSE
tube_rack	A2	strip_tubes	A3	180	FALSE
tube_rack	A2	strip_tubes	A4	169	FALSE
tube_rack	A2	strip_tubes	A5	186	FALSE
tube_rack	A2	strip_tubes	A6	183	FALSE

This example table demonstrates transfer of plasmid DNA samples (from DNA_tubes) into the destination labware, “strip_tubes” (PCR strip tubes placed in the aluminum block on the Temperature Module). Transfer volumes are entered in µL. The Pick_Up_Tip column specifies when the OT-2 will select a new tip in the protocol timeline (before each DNA transfer, and before the first water transfer).

5. As a class, download and open the DNA Sample Normalization protocol in a code editing program.
6. Starting at line 8, follow the directions to modify your code by copying and pasting in data from your tables.
    a. First, copy and paste your “Step 1” table under csv_volume_data_raw. Be sure to copy and paste your data to replace the current values. This includes the column titles (Labware, Initial_Wells, etc.) and all values.
    b. Next, copy and paste your “Step 2” table under csv_transfer_data_raw in the same method.

7. As needed, update the starting tip for the protocol (the first available tip the OT-2 should pick up in the tip box; around line 120).
8. Import your saved version of the protocol into the Opentrons App.
9. Remove the pre-chilled, 24-well aluminum block from storage at -20 °C.
10. Set up your labware and liquids as follows. Liquid well locations are specified by the values in your data tables.
    a. Opentrons OT-2 96 Filter Tip Rack 300 µL in deck slot 1
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    d. Opentrons 15 Tube Rack with molecular grade water in a Falcon 15 mL Conical (in deck slot 5)
11. Run the protocol.
12. The OT-2 will begin by setting the Temperature Module to 4 °C.
13. The OT-2 will transfer concentrated DNA from 1.5 mL snapcap tubes to 8-well PCR strip tubes (volume dependent on table values).
14. The OT-2 will add molecular grade water to a final volume of 20 ng/uL.

15. Diluted plasmid DNA samples should be stored at -20 °C.

Which *Agrobacterium* strain is which?

1. Use an epifluorescence microscope and/or a plate reader to quantify the absorbance or fluorescence spectra of the *Agrobacterium* strains.
2. Now, you should be able to assign the plasmid sequence belonging to each of the *Agrobacterium* strains growing in different wells. Which is which?

Discussion Questions

Discuss the lab activities with a neighbor:

- What are some of the different techniques you saw today for selection? How can you be sure you are using the right plasmid?
- What are the next steps in the cloning process? What will happen next with the plasmid DNA you normalized today?
- Why did we need to normalize the plasmid DNA to a standard concentration?