Opentrons
for Education

Prep Sheet

# Lab Module 3: Assembling Your Molecular Toolkit

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

University of Florida

## Getting Started

Review the following resources prior to class.

- ☐ Running a protocol on the OT-2
- ☐ Opentrons HEPA Module

## Additional Support and Resources

### OT-2 Manual

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.

Opentrons
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Educator Guide

# Lab Module 3: Assembling Your Molecular Toolkit

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## Contents

This educator guide includes the following sections:

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

This lesson plan includes both full protocols and suggestions to allow for instructor flexibility in both the classroom (available equipment and time) and in student learning outcomes. Students can use buffers and solutions prepared before class, or follow a protocol from a commercially available kit. Suggestions are included for plasmids to be used in this course's cloning workflow; however, instructors can create their own workflow to include use of more or less genetic components, or specific genes of interest. Questions can be directed to cvoiniciuc@ufl.edu.

Prior to class, instructors will need to begin culture of selected plasmid backbones in *E. coli*. In addition, instructors will need to introduce visible markers, such as rainbow plasmids (Rainbow Chromoprotein Plasmid Set or others), RUBY, or fluorescent markers into competent *Agrobacterium* strains, or

use their own *Agrobacterium* reporter strains. Introduction of these reporters can also be done by students in either of the first two labs.

## Purpose

Students will perform a spin column purification to manually extract plasmid DNA from bacterial cells. In addition, students will use the OT-2 to perform sterile transfer and begin culture of randomized *Agrobacterium* reporter strains. In the following lab class, students will use visible markers in the reporter strains to identify their randomized plasmids, and quantify extracted plasmid DNA concentrations using a NanoDrop.

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

- Comparison of manual and automated pipetting tasks
- Working with a protocol to accomplish a quantifiable outcome
- Basic principles of  bacterial culture

### Core Competencies

**Laboratory Skills:**

- Follow a molecular biology protocol
- Perform accurate manual pipetting
- Basics of bacterial culture and common reporter strains

**Automation Skills:**

- Automation of pipetting tasks for sterile transfer

## Background Knowledge

Students should begin this lab with an understanding of the use of plasmids for cloning in molecular biology, as well as the use of fluorescent or colorimetric markers for identification and selection in plants and other organisms. An included pre-lab reading introduces working with  plasmids and selection markers. *No coding experience is required for this lab.*

## Supplies

### Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ OT-2 P1000 Single-Channel GEN2 Pipette (loaded in left mount)
- ☐ Heater-Shaker Module GEN1 in deck slot 10
- ☐ OT-2 HEPA Module

### Opentrons Protocol

- ☐ LB Distribution protocol

### Non-Opentrons Equipment

- ☐ NanoDrop spectrophotometer
- ☐ Bacterial culture equipment (inoculating loops, culture tubes and flasks, 37 °C shaking incubator)
- ☐ Tabletop microcentrifuge

### Labware

- ☐ Corning 24-Well Deep Well Plate (10,000 µL volume) from Sigma in deck slot 1 (labware definition can be downloaded with the LB Distribution protocol)

- ☐ Opentrons 10 Tube Rack with Falcon 4x50 mL, 6x15 mL Conical in deck slot 2
- ☐ Opentrons OT-2 Filter Tip Rack 1000 uL in deck slot 6
- ☐ Universal Flat Adapter on the Heater-Shaker Module in deck slot 10

### Reagents and Other Materials

- ☐ Empty vector plasmid backbones from Addgene (see table containing suggested plasmids in the **Before Class** section of the educator's **Procedure Guide**)
- ☐ *Agrobacterium* competent cells from GoldBio (see the **Before Class** section of the **Procedure Guide** for recommendations)
- ☐ visible markers (colorimetric markers as in Rainbow Chromoprotein Plasmid Set or similar, RUBY, or fluorescent markers) for introduction into *Agrobacterium* competent cells
- ☐ 70% ethanol for cleaning
- ☐ DNAse- and RNAse-free 1.5 mL snapcap tubes appropriate for classroom microcentrifuge
- ☐ Sterile LB media
- ☐ Kanamycin, spectinomycin, ampicillin, and/or other appropriate antibiotics with 500X stock (powdered or liquid stocks)
- ☐ 96-100% ethanol for use in plasmid purification

- ☐ QIAprep Spin Miniprep Kit OR Syd Labs Spin Columns for Plasmid Miniprep and reagents sufficient to make buffers for miniprep (see “Before Class” in the educator’s Procedure Guide).
- ☐ Molecular grade water, like HyClone water
- ☐ Tape for plate lids

## Experimental Duration

### Required Class Sessions

1

### Lab Run Time

This lesson plan was prepared for a traditional laboratory class time of 80-90 minutes, with planned continuation into the next laboratory class (for viewing and assignment of *Agrobacterium* reporter strains). Students arrive to class with 2 types of overnight bacterial cultures prepared: for 1) harvest and plasmid purification, and 2) sterile transfer of randomized *Agrobacterium* strains. NanoDrop measurements of isolated DNA concentration may be taken in this class or the following class. In this lesson plan, students are directed to store both their isolated DNA sample and an aliquot for NanoDrop measurements at -80 °C for the following class.

## Basic Troubleshooting and Tips

- We recommend completing a trial run of a simple protocol from our Protocol Library 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.

- Growth of bacterial cultures for longer than 16 hours can cause cell lysis and reduce plasmid DNA yields. Tubes or flasks with a volume at least 4 times that of the culture volume should be used.

- The spin column protocol used in this lab to isolate plasmid DNA has been adapted based on lab protocols by Dr. Cătălin Voiniciuc and the Qiagen QIAprep Spin Miniprep kit to provide options for educators. If using the

Qiagen kit, please also refer to the included handbook and manufacturer instructions.

- The LB Distribution protocol uses a Corning 24-Well Deep Well Plate (10,000 µL volume) from Sigma to incubate reporter strains and LB broth on the Heater-Shaker Module. Follow the link above to download the labware definition (.json file) with the protocol from the Opentrons Protocol Library.

- Instructors should direct students to complete the lab activities in an order that works for your class (and available time and reagents). Several options are included in the lesson plan to accommodate this flexibility:
    - Based on desired learning outcomes, students can assist in the preparation of LB broth, *Agrobacterium* reporter strain transformation, or *E. coli* culture prior to class.
    - Buffers can be prepared by instructors before class, or a Qiagen kit may be purchased and used.
    - Quantification of students' plasmid DNA concentrations using a NanoDrop may be completed in this class or the following class. Instructions for NanoDrop measurements are included in the **Student Guide** of Lab Module 4.

## Procedure Guide

### Before Class

1. Prepare *Agrobacterium* cultures for the sterile transfer activity. Colorimetric or fluorescent markers can be introduced into *Agrobacterium* reporter strains and cultured prior to class, either by instructors or students. See the table below for *Agrobacterium* competent cell recommendations:

| Supplier | Catalog # | Description | Link |
|---|---|---|---|
| GoldBio | CC-105-5x50 | GV3101 Agrobacterium Chemically Competent Cells | GV3101 Agrobacterium \| GoldBio |
| GoldBio | CC-240-5x50 | C58C1 Agrobacterium ElectroCompetent Cells | C58C1 Agrobacterium \| GoldBio |
| GoldBio | CC-220-5x50 | LBA4404 Agrobacterium ElectroCompe | LBA4404 Agrobacterium \| GoldBio |

| | | | |
|---|---|---|---|
| | | tent Cells | |
| Lifeasible | ACC-108 | EHA101 Chemically Competent Cell | EHA101 - Lifeasible |
| LifeScience Market | STR3008 | GV2260 Agrobacterium Strain | GV2260 Agrobacterium Strain |

Competent *Agrobacterium* cells should be chosen based on available equipment and desired student learning outcomes. Once visible markers are introduced, assign an anonymous label to each pre-culture.

2. Prepare plasmid backbone bacterial cultures. Below are suggested Addgene plasmids (empty backbone vectors) for use in this course.

| Plasmid ID | Addgene Catalog # | Description | Selectable Markers | Antibiotic Resistance | Link |
|---|---|---|---|---|---|
| pAGM1263 | 47985 | Level 0 cloning vector; empty backbone | LacZ alpha | Spectinomycin, 50 ug/mL | Addgene: pAGM1263 |
| pICH41264 | 47993 | Level 0 cloning vector; empty backbone | LacZ alpha | Spectinomycin, 50 ug/mL | Addgene: pICH41264 |
| pICH47742 | 48001 | Level 1 cloning vector; empty backbone | LacZ alpha | Ampicillin, 100 ug/mL | Addgene: pICH47742 |
| pICH47822 | 48009 | Level 1 cloning vector; empty backbone | LacZ alpha | Ampicillin, 100 ug/mL | Addgene: pICH47822 |
| pICH41744 | 48017 | Level 2 end linker; empty backbone | - | Spectinomycin, 50 ug/mL | Addgene: pICH41744 |
| pICH49277 | 48025 | Level 2 end linker; empty backbone | LacZ alpha | Ampicillin, 100 ug/mL | Addgene: pICH49 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 277 |
| pICH83999 | 48033 | Level 2 end linker; empty backbone | Violacein synthetic operon | Ampicillin, 100 ug/mL | Addgene: pICH83999 |
| pAGM8079 | 48041 | Level M cloning vector; empty backbone | LacZ alpha | Spectinomycin, 50 ug/mL | Addgene: pAGM8079 |
| pICH50927 | 48049 | Level M end linker; empty backbone | - | Ampicillin, 100 ug/mL | Addgene: pICH50927 |
| pICH75388 | 48057 | Level P cloning vector; empty backbone | LacZ alpha | Kanamycin, 50 ug/mL | Addgene: pICH75388 |
| pICH54011 | 48065 | Level 2 dummy fragment; empty backbone | - | Ampicillin, 100 ug/mL | Addgene: pICH54011 |
| pICH82113 | 48073 | Empty backbone | LacZ alpha | Kanamycin, 50 ug/mL | Addgene: pICH82113 |

These plasmids are part of the Weber et al., 2011 collection. A full list of plasmids in this collection can be found at Addgene. Sequence maps, depositor comments, and additional information are available at individual plasmid links. Instructors may wish to select plasmids to ensure variety, yet some overlap between students. In Lab Module 5, students will complete a restriction digestion using isolated plasmid DNA.

    a. Pick a single colony of each plasmid from a freshly streaked selective plate and inoculate a culture of 1–5 mL sterile LB medium with kanamycin, spectinomycin, ampicillin, or other appropriate antibiotic.
    b. Incubate overnight (for 12–16 hours) at 37 °C with vigorous shaking.
2. To save time during class, buffers for plasmid extraction can be prepared ahead of time. Instructors can reach out to Syd Labs for detailed instructions on use of their spin columns.
3. Import the LB Distribution protocol into the Opentrons App.

### Lab Introduction

1. Instructors should introduce a workflow for the class, ensuring that students understand both (separate) activities and the steps involved.

2. Instructors may want to provide a brief overview of cloning, including uses of plasmids and selection markers.

### Harvest Bacterial Cultures

1. Students will harvest bacterial cells by centrifugation at >8,000 RPM (6,800 *x g*) in a tabletop microcentrifuge for 3 minutes at room temperature. Instructors should demonstrate proper use and balance of the microcentrifuge for students, and check their balance before use.
2. Students will remove supernatant from bacterial pellet.

### Spin Column Plasmid Purification

1. Students will resuspend pelleted bacterial cells in 250 µL of P1 buffer, and transfer to a microcentrifuge tube.
2. Students will add 250 µL of P2 buffer and mix thoroughly by inverting the tube 4-6 times. Remind students *not* to vortex the sample, as this will cause shearing of plasmid DNA. The sample should become viscous, and students should move onto the next step after fewer than 5 minutes of mixing.
3. Students will add 350 µL of N3 buffer, and mix immediately and thoroughly (inverting the tube 4-6 times). The solution should become cloudy.
4. Students will centrifuge the sample for 10 minutes at 13,000 RPM. A compact white pellet should be visible.

5. Students will apply 800 µL of supernatant from the previous step for the spin column with a pipette.
6. Students will centrifuge the spin column for 30-60 seconds and discard flow through.
7. Students should wash the spin column by adding 0.5 mL of PB (binding) buffer and centrifuge spin columns again for 30-60 seconds. Flow through should be discarded.
8. Students should wash the spin column again by adding 0.75 mL of PE (wash) buffer and centrifuge again for 30-60 seconds.
9. Students can discard flow through and centrifuge spin columns again at full speed for an additional 1 minute to remove any remaining PE (wash) buffer.
10. Students can place spin columns into clean, 1.5 mL microcentrifuge tubes, and add 50 µL of molecular grade water to the center of each spin column to elute DNA.
11. Students should let the spin column (with molecular grade water added) stand for 1 minute, followed by centrifugation for 1 minute, to fully elute DNA.
12. Students should aliquot a small amount of their isolated plasmid DNA to bring to the NanoDrop for quantification (ex. 5 µL). Store both the aliquot and full sample at -80 °C.

### Sterile Transfer of *Agrobacterium* Strains

1. Turn on the OT-2 HEPA Module for at least 30 minutes before liquid transfer.

    a. The introduction to the OT-2 HEPA Module (OT-2 HEPA Module) includes a demonstration of cleaning the robot interior with 70% ethanol. One student can do this prior to sterile transfer.
2. Students should prepare a sufficient volume of LB broth. Using antibiotic stock (powdered or liquid antibiotics), direct students to prepare a 1000X stock.
3. Students should dilute their stock to 500X.
4. Open the LB Distribution protocol in the Opentrons App.
5. Set up your labware:
    a. Opentrons OT-2 96 Filter Tip Rack (1000 µL) in deck slot 6
    b. Opentrons 10 Tube Rack with 4x50 mL Falcon tubes in deck slot 2
    c. Corning 24-well deep well plate in deck slot 1
6. Set up your liquids:
    a. Sterile 500X LB media + antibiotics for selected *Agrobacterium* strains (in a 50 mL Falcon tube in well A3 of the Opentrons tube rack)
        i. At least 50 mL of appropriate antibiotic in well A3
7. Run the LB Distribution protocol.
8. The OT-2 will transfer 2000 µL of sterile LB media to each well of the 24-well plate, using the same tip.
9. The OT-2 will open the latch of the Heater-Shaker Module and pause the protocol.

10. Students should select their randomized *Agrobacterium* colony using a sterile tip and inoculate a well of the 24-well plate. Remind students to take notes on their well location within the plate.
11. Manually cover the plate with a lid and seal with tape.
12. Move the plate manually to the Heater-Shaker Module.
13. The Heater-Shaker Module will incubate the plate for 16 hours at 37 °C, shaking at 1700 RPM. In the next class, students will take photos and use a plate reader or microscope, as needed, to identify their randomized *Agrobacterium* strains.

## Discussion Questions

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

- Turn to a neighbor and discuss- what will happen with the plasmids you isolated today? What are the next steps in the cloning process?
- Why is a sterile work environment important when working with bacteria?

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Student Guide

# Lab Module 3: Assembling Your Molecular Toolkit

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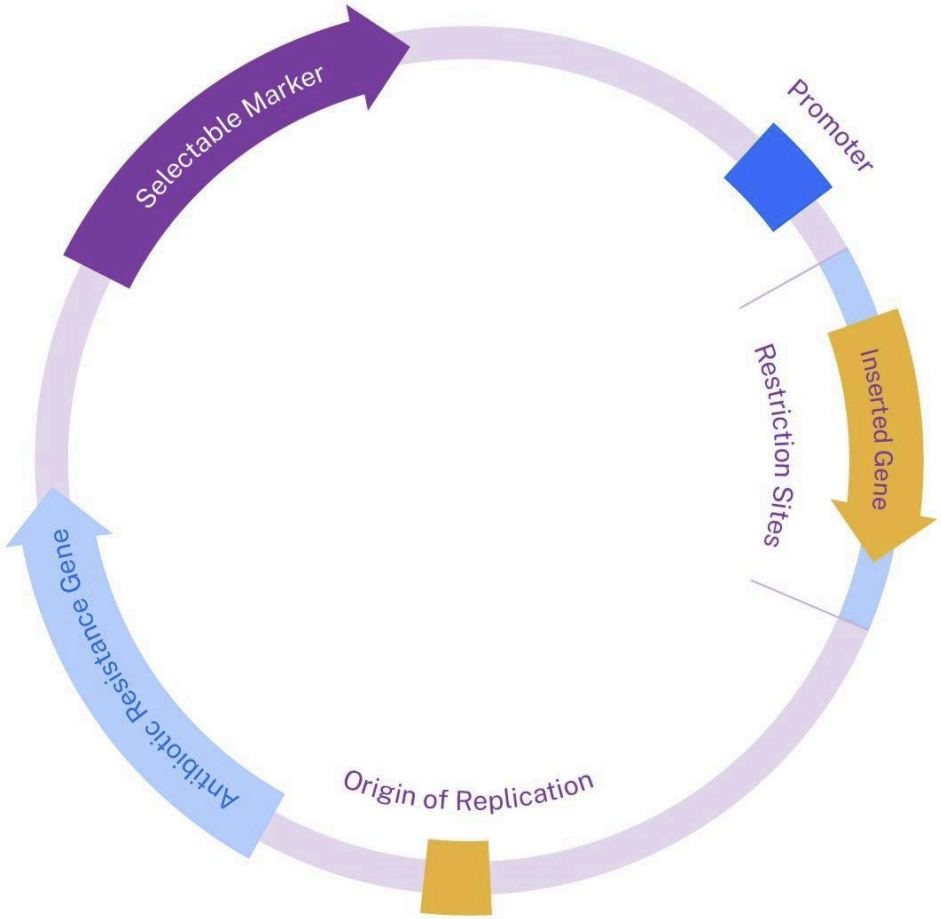
## Pre-Lab Reading

Today in lab, you'll be working with *plasmids.* Plasmids are small, circular pieces of DNA that are found in unicellular organisms, like bacteria. For decades, plasmids have been an absolutely essential tool in molecular biology- but why?

DNA can be a challenge for scientists to work with. Different organisms have different amounts of DNA. For example, the human genome contains 3 billion base pairs, meaning that each strand has 3 billion nucleotides! Bacterial plasmids, like those we'll work with today, generally range in size from 1 to >200 kilobase pairs (1 kilobase = 1,000 nucleotides). This makes plasmids much smaller pieces of DNA, and therefore easier to work with. When working with DNA, RNA, or proteins, it's also nice to have a lot of material to work with. Each of these molecules is targeted by naturally occurring DNases, RNases, and proteases, or enzymes that can degrade the molecules. Remember that plasmids are found in bacteria, which grow quickly and are not expensive to maintain. In this way, scientists can obtain a lot of plasmid DNA from bacteria, which you'll do today!

Plasmids are also incredibly useful because of their structure. Let's take a look:




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*

Antibiotic resistance genes and selectable markers are used by scientists to confirm that they are choosing and using the correct piece of DNA. Bacteria grow in *media*, also sometimes called broth, that contains nutrients needed for their growth.

Let's say that this plasmid contains an antibiotic resistance gene that allows them to survive exposure to the antibiotic kanamycin, and a selectable marker that produces green fluorescent protein (GFP). Now, if your media contains kanamycin, bacteria that do *not* contain the antibiotic resistance gene will die, but bacteria that contain your plasmid of interest will survive. You can check to make sure you chose the correct bacteria, again, containing your plasmid of interest, by using fluorescence to see the bacteria glow green with GFP.

To learn more about plasmids, check out this video from Addgene: What is a Plasmid? - Plasmids 101

## Purpose

In this lab, you'll learn techniques for working with plasmids and selection markers in plant molecular biology. First, you'll perform a protocol known as a *spin column purification* to isolate plasmid DNA from bacterial cell cultures. Afterwards, a *NanoDrop* can quantify how much plasmid DNA you have. Next, you'll use the OT-2 to perform sterile transfer and begin culture of *Agrobacterium* reporter strains. These are bacteria that contain different kinds of selection markers, but it'll be up to you to figure out just which ones!

### Learning Outcomes

- Be able to work with a protocol to isolate plasmid DNA

- Understand common reporters and markers used to identify plasmids
- Understand basic principles of bacterial culture used for molecular biology

## Supplies

### Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ OT-2 P1000 Single-Channel GEN2 Pipette (loaded in left mount)
- ☐ Heater-Shaker Module GEN1 in deck slot 10
- ☐ OT-2 HEPA Module

### Opentrons Protocol

- ☐ LB Distribution protocol

### Non-Opentrons Equipment

- ☐ NanoDrop spectrophotometer
- ☐ Bacterial culture equipment (inoculating loops, culture tubes and flasks, 37 °C shaking incubator)
- ☐ Tabletop microcentrifuge

## Labware

- ☐ Corning 24-Well Deep Well Plate (10,000 µL volume) from Sigma in deck slot 1 (labware definition can be downloaded with the LB Distribution protocol)
- ☐ Opentrons 10 Tube Rack with Falcon 4x50 mL, 6x15 mL Conical in deck slot 2
- ☐ Opentrons OT-2 Filter Tip Rack 1000 uL in deck slot 6
- ☐ Universal Flat Adapter on the Heater-Shaker Module in deck slot 10

## Reagents and Other Materials

- ☐ Empty vector plasmid backbones from Addgene
- ☐ Randomized *Agrobacterium* cells with visible markers
- ☐ 70% ethanol for cleaning
- ☐ DNAse- and RNAse-free 1.5 mL snapcap tubes appropriate for classroom microcentrifuge
- ☐ Sterile LB media
- ☐ Kanamycin, spectinomycin, ampicillin, and/or other appropriate antibiotics with 500X stock (powdered or liquid stocks)
- ☐ 96-100% ethanol for use in plasmid purification
- ☐ QIAprep Spin Miniprep Kit OR Syd Labs Spin Columns for Plasmid Miniprep and buffers for miniprep

- ☐ Molecular grade water, like HyClone water
- ☐ Tape for plate lids

## Procedure Guide

### Before Class

1. Complete the pre-lab reading.

### Harvest Bacterial Cultures

1. Harvest bacterial cells from overnight cultures by centrifugation at >8,000 RPM (or 6,800 *x g*) in a table-top microcentrifuge for 3 minutes at room temperature. Have the instructors check the balance of your microcentrifuge before use.
2. Carefully remove the *supernatant* (upper liquid in the tube) from the bacterial *pellet* (bacterial cells clumped at the bottom of the tube).

### Spin Column Plasmid Purification

1. Resuspend the pelleted bacterial cells in 250 µL of P1 buffer, and transfer to a microcentrifuge tube.
1. Add 250 µL of P2 buffer and mix thoroughly by inverting the tube 4-6 times. Do *not* use a vortex to mix the sample- this will cause shearing of your plasmid DNA and destroy the sample. You should see the sample in your tube begin

to become viscous. Do not mix for longer than 4-5 minutes.

2. Add 350 µL of N3 buffer, and mix immediately and thoroughly (inverting the tube 4-6 times). The solution should become cloudy.
3. Centrifuge the sample for 10 minutes at 13,000 RPM. You should be able to see a compact white pellet at the bottom of the tube.
4. Using a pipette, apply 800 µL of supernatant from the previous step to a spin column.
5. Centrifuge the spin column for 30-60 seconds. The *flow through* (liquid that has come out of the spin column; at the bottom of the tube) can be discarded.
6. Wash the spin column by adding 0.5 mL of PB (binding) buffer. Centrifuge the spin column again for 30-60 seconds. Again, flow through should be discarded.
7. Wash the spin column again by adding 0.75 mL of PE (wash) buffer. Centrifuge the spin column again for 30-60 seconds.
8. Discard flow through. Centrifuge spin columns again, this time at full speed, for an additional 1 minute to remove any remaining PE (wash) buffer.
9. Place the spin column into a clean, 1.5 mL microcentrifuge tube. Add 50 µL of molecular grade water to the center of each spin column to elute DNA.
10. Students should let the spin column (with molecular grade water added) stand for 1 minute, followed by

centrifugation for 1 minute, to fully *elute* (remove bound DNA from the column) DNA.

11. Remove a small *aliquot* (a small sample, for example, 2-5 µL) of your isolated plasmid DNA to bring to the NanoDrop for quantification. Store this aliquot and the rest of your plasmid DNA at -80 °C.

## Sterile Transfer of *Agrobacterium* Strains

1. At least thirty minutes before beginning, your instructor will turn on the OT-2 HEPA Module and clean the OT-2 interior with 70% ethanol.
2. Follow directions from your instructor to prepare a sufficient volume of LB broth. You will use antibiotic stocks (powdered, or liquid antibiotics) to prepare a 1000X stock.
3. Dilute your 1000X stock to a 500X stock.
4. Open LM3_LB_Distribution.py in the Opentrons App.
5. Set up your labware:
    a. Opentrons OT-2 96 Filter Tip Rack (1000 µL) in deck slot 6
    b. Opentrons 10 Tube Rack with 4x50 mL Falcon tubes in deck slot 2
    c. Corning 24-well deep well plate in deck slot 1
6. Set up your liquids:

    a. Sterile 500X LB media + antibiotics for selected *Agrobacterium* strains (in a 50 mL Falcon tube in well A3 of the Opentrons tube rack)
        i. At least 50 mL of appropriate antibiotic in well A3
7. Run the protocol.
8. The OT-2 will transfer 2000 µL of sterile LB media from a Falcon tube in the Opentrons 10 Tube Rack to each well of the 24-well plate, using the same tip.
9. The OT-2 will open the latch of the Heater-Shaker Module and pause the protocol.
10. Select your randomized *Agrobacterium* colony using a sterile tip and inoculate a well of the 24-well plate. Remember to take notes on the well location of your randomized strain within the plate.
11. Manually cover the plate with a lid and seal with tape.
12. Move the plate manually to the Heater-Shaker Module.
13. The Heater-Shaker Module will incubate the plate for 16 hours at 37 °C, shaking at 1700 RPM. In the next class, you will analyze the bacterial colonies to determine the identity of your randomized *Agrobacterium* strains.

## Discussion Questions

Discuss the lab activities with your labmates.

- Turn to a neighbor and discuss- what will happen with the plasmids you isolated today? What are the next steps in the cloning process?

- Why is a sterile work environment important when working with bacteria?
- What are some of the different techniques you saw today for selection? How can you make sure you are using the right plasmid?