Prep Sheet

Lab Module 11: Genotyping Bacteria with Colony PCR

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

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Getting Started

This lesson plan uses values from an Excel template to customize the Colony PCR Preparation 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.

Educator Guide

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Contents

Included in this document are the following sections:

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

Purpose

Students will prepare for colony PCR using the OT-2. The OT-2 performs a simple transfer of master mix and desired colonies will be added manually. Primer design for this lab comes from section **B** of the previous Primer and PCR Design worksheet, building on concepts from previous labs to begin to identify students' experimental results.

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

- Experimental design and completion of colony PCR
- Troubleshooting and analyzing experimental results
- Laboratory automation of pipetting tasks

Core Competencies

Laboratory Skills:

- Design and completion of colony PCR experiments
- Understanding experimental analysis and *how* to properly identify an assembled plasmid

Automation Skills:

- Automation of pipetting tasks
- Protocol customization

Background Knowledge

Students should begin this lab with a thorough understanding of the cloning workflow used in this course. The included pre-lab reading introduces colony PCR and reviews the workflow steps used in this lab and the next to properly identify the students' assembled plasmids. *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
- ☐ OT-2 P20 Single-Channel pipette (in left mount)
- ☐ Temperature Module GEN2 (in deck slot 3)

Opentrons Protocol

- ☐ Colony PCR Preparation protocol

Non-Opentrons Equipment

- ☐ Benchtop thermocycler
- ☐ Gel electrophoresis equipment:
  - ☐ casting tray
  - ☐ well combs
  - ☐ voltage source
  - ☐ gel box
  - ☐ Microwave
  - ☐ Gel imager

Labware

- ☐ Opentrons OT-2 96 Filter Tip Rack 20 µL 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 µL in the Temperature Module in deck slot 3

Reagents and Other Materials

- ☐ Assembled plasmid products in *E. coli* bacterial culture from the previous class
- ☐ REDTaq 2x Master Mix with 1.5 mM $MgCl_2$
- ☐ Appropriate primers (forward and reverse, 10 µM working dilution stocks)
- ☐ Molecular grade water, such as HyClone Water
- ☐ 50X TAE buffer stock (to make 1X TAE)
- ☐ Loading buffer for samples
- ☐ Gel stain such as GelRed® Nucleic Acid Gel Stain - Biotium
- ☐ Appropriate molecular weight ladder for gel electrophoresis, such as GeneRuler 1 kb DNA Ladder

Experimental Duration

Required Class Sessions

1

Lab Run Time

This lesson plan was prepared for a traditional laboratory class time of 80-90 minutes. While the PCR reaction runs to completion in a benchtop thermocycler, students should prepare and pour agarose gels to analyze their results. Instructors may need to complete the run and image gels after class.

Basic Troubleshooting and Tips

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

- 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.

- Remind students to be aware of the potential for false positive results in a colony PCR experiment. A PCR product of the expected size run on a gel does not ensure that the insert is ligated correctly, or that there are no mutations in the insert.

Procedure Guide

Before Class

1. Check growth on plates with recovered *E. coli* cells a day before class.

2. *Optional:* Agarose gels can be prepared and poured before class begins to save time in the lab.

Lab Introduction

1. Instructors should review the differences between colony PCR (or *genotyping PCR*) with the other uses of PCR students have seen in the course.

PCR Preparation

1. Students should manually assemble a master mix with the following components (volumes shown are for a single reaction, with a total master mix volume of 10 uL). Store the master mix on ice.
    a. **RedTaq 2x master mix** (with 1.5 mM $MgCl_2$); 5 µL
    b. **Forward primer** (10 µM working stock dilution); 0.5 µL
    c. **Reverse primer** (10 µM working stock dilution); 0.5 µL
    d. **Hyclone water**; 4 µL
2. Open your Excel template used for protocol customization. First, edit your “Step 1” table to input initial volumes of liquid in your labware.

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
	snap_caps	A1	1.5	Master Mix	Manually assembled master mix	#00FF00
	snap_caps	A2	1.5	Master Mix	Manually assembled master mix	#00FF00

The example table shown here includes snap_caps (NEST 1.5 mL snapcap tubes in a 24 well aluminum block). We recommend using a larger initial volume in your protocol (10-20 µL).

3. Next, edit your "Step 2" table for liquid transfer steps the OT-2 should perform.

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_Volume	Pick_Up_Tip
	snap_caps	A1	PCR_tubes	A1	10	TRUE
	snap_caps	A1	PCR_tubes	A2	10	FALSE
	snap_caps	A1	PCR_tubes	A3	10	FALSE
	snap_caps	A2	PCR_tubes	A4	10	FALSE
	snap_caps	A2	PCR_tubes	A5	10	FALSE
	snap_caps	A2	PCR_tubes	A6	10	FALSE

The example table shown here includes transfer steps of master mix (from snap_caps tubes) to PCR_tubes (generic PCR strip tubes in the aluminum block on the Temperature Module).

4. Download and open the [Colony PCR Preparation]() protocol in a code editing program. Follow the directions to copy and paste your values into the protocol.

a. 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).

5. Save a version of the protocol file with your changes and import into the Opentrons App.
    a. Check setup instructions in the app to confirm hardware, labware, and liquid used in your protocol.
6. Set up your labware and liquids:
    a. Opentrons OT-2 96 Filter Tip Rack 20 uL; in deck slot 1
    b. Opentrons 24 Well Aluminum Block with NEST 1.5 mL Snapcap tubes in deck slot 1; contains the manually assembled master mix
    c. Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 uL; on the Temperature Module in deck slot 3
7. Run your modified protocol.
8. The OT-2 will set the Temperature Module to 4 °C.
9. The OT-2 will dispense 10 μL of master mix into each specified PCR strip tube.
10. The OT-2 will deactivate the temperature module and end the protocol.
11. Manually remove the PCR plate from the OT-2.
12. Students will use a sterile P2 tip to touch the desired colony (already numbered on the plate), re-streak it on a new plate (if not already done), and transfer their desired

colonies to individual wells of the PCR plate using a sterile tip.

13. Seal tubes and flick to mix.

Colony PCR

1. Load the PCR plate into a benchtop thermocycler.
2. Set the thermocycler with the following experimental conditions:

Thermocycler Conditions	**°C**	**min:sec**	**Cycles**
1. Initial Denaturation	95	3:00	
2. Denaturation	95	0:20	20x
3. Annealing	**57**	0:30	
4. Extension	72	**1:30**	
5. Final Extension	72	5:00	
6. Storage	12	∞	

Note: no lysis step is required prior to beginning the reaction; cells will be lysed in the first step of PCR.

Gel Electrophoresis- Gel Preparation, Electrophoresis, and Analysis

1. Students can prepare and pour gels while the PCR reaction runs to completion. For this experiment, we will need a 0.7% agarose TAE gel.
    a. Prepare 1X TAE from a 50X TAE stock.
    b. Measure 0.7 grams of agarose powder.
    c. Mix the agarose powder with 100 mL 1X TAE in a microwaveable flask.
    d. Microwave 1-3 minutes to dissolve agarose. *Reminder:* do *not* overboil the mixture. Microwaving for 30-45 seconds, stopping, and swirling the flask can help.
    e. Let the agarose solution cool to around 50 °C (or for about 5 minutes).
    f. Pour agarose mixture into a gel tray with a well comb in place. Pour slowly to avoid bubbles.
    g. Let gel set at room temperature for 20-30 minutes until completely solid.
    h. Students (or instructors) can place the solidified gel into the gel box.
2. When the PCR reaction is complete, students should add an appropriate amount of loading buffer to their samples.
3. Students should fill the gel box with 1X TAE until the gel is covered.

4. Students (or instructor) should load 1 μL of an appropriate GeneRuler ladder in the first ladder of the gel.
5. Students will load 5 μL of each sample into the other lanes of the gel. Remind students to take notes on which lanes samples are loaded into.
6. The gel should be run for 45 minutes at 100 volts (time and voltage adjusted as needed). Note that the gel may need to be run, and images taken, outside of class time.

Discussion Questions

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

- Why did you use Taq polymerase in this PCR reaction, instead of the Phusion polymerase we used in Lab Module 8 (DNA amplification ahead of cloning)?
- Why is colony PCR not the *final step* of this process? In other words, what can happen in your results from colony PCR and why do we need a final step of sequencing, like Sanger sequencing?

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Student Guide

Lab Module 11: Genotyping Bacteria with Colony PCR

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Pre-Lab Reading

In the previous class, you *transformed* your assembled plasmids into competent *E. coli* cells. As we have seen previously in this course, *E. coli* culture is an extremely useful method for growth, storage, and maintenance of plasmids. At the beginning of today's class, you should now have *colonies*- groups of *E. coli* cells- in your culture, which contain many copies of your assembled plasmid product. Today, you will take the first step to test your experimental results, and determine the identity of your assembled plasmid, with *colony PCR.*

Colony PCR, also sometimes referred to as *genotyping PCR*, is known as a *high-throughput method*- that is, a method you could use to test many different colonies at one time. By using a 96-well PCR plate, you can determine the presence or absence of a DNA insert in 96 different plasmid constructs in a single thermocycler reaction! Colony PCR includes the same reagents you are by now familiar with in a PCR reaction, with a few key differences:

- **A DNA template**, in this case, your assembled plasmid products in *E. coli* colonies
- **Master mix** containing a Taq DNA polymerase
- **Forward and reverse primer pairs** to amplify <u>both</u> plasmid and gene insert

- **Molecular grade water**, such as HyClone Water

Here, the primer pairs used must be able to amplify both the plasmid and the DNA insert, or gene of interest, that was assembled into the plasmid during the ligation reaction. Open your Primer and PCR Design worksheet from Lab Module 7, and find Section **B**. Here, you will see that you've already completed the primer design for this experiment to *genotype* your constructs using Taq DNA polymerase. In addition, you've already considered the primer pairs needed to run the PCR reaction in a single thermocycler, which we'll do today in our colony PCR reaction!

Today, you'll carefully transfer cells from an *E. coli* colony to wells of a PCR plate. The OT-2 will then automate pipetting of master mix into each well to prepare for PCR. The actual colony PCR reaction itself will be carried out in a benchtop thermocycler with the following steps:

1. **Initial denaturation-** this initial denaturation stage is important to *lyse* the bacterial cell walls, so the DNA can be accessed by the primers and polymerase.
2. **Denaturation, annealing, and extension-** the typical PCR steps are carried out here for 25 cycles to amplify our assembled plasmid sequence.

3. **Final extension and storage-** the reaction ends with storage at 12°C until the plate is removed.

When the PCR reaction is complete, you'll run your PCR products on a gel. This gel electrophoresis experiment will allow you to visualize your assembled plasmid products, especially the *size.* You already know the size (number of base pairs) you should expect based on the size of your plasmid backbone and DNA insert. Although colony PCR and gel electrophoresis will confirm whether or not your DNA insert is present, the experiment can give a false positive result- confirming that your PCR product is the expected size is a great start, but this doesn't necessarily mean that your product was assembled correctly, or that there are no mutations in the insert. To fully confirm the identity of your assembled plasmid, you will prepare for Sanger sequencing in the next class.

You can learn more about colony PCR in this Addgene article.

Purpose

In today's lab, you will prepare for colony PCR using the OT-2. Desired colonies will be added manually to a plate prefilled with water, with the OT-2 adding the rest, including Taq polymerase and gene and vector-specific primer pairs. Primer design for this lab comes from section **B** of the previous

Primer and PCR Design worksheet. Gel electrophoresis will then be used to analyze your PCR product (amplified assembled plasmid products).

Learning Outcomes

- Understand experimental design and completion of colony PCR
- Be able to use gel electrophoresis to analyze and troubleshoot your experimental results
- Planning to automate your experiment, including protocol customization for pipetting tasks

Supplies

Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ OT-2 P20 Single-Channel pipette (in left mount)
- ☐ Temperature Module GEN2 (in deck slot 3)

Opentrons Protocol

- ☐ Colony PCR Preparation protocol

Non-Opentrons Equipment

- ☐ Benchtop thermocycler
- ☐ Gel electrophoresis equipment:
  - ☐ casting tray
  - ☐ well combs
  - ☐ voltage source
  - ☐ gel box
  - ☐ Microwave
  - ☐ Gel imager

Labware

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Reagents and Other Materials

- ☐ Assembled plasmid products in *E. coli* bacterial culture from the previous class
- ☐ REDTaq 2x Master Mix with 1.5 mM $MgCl_2$

- ☐ Appropriate primers (forward and reverse, 10 µM working dilution stocks)
- ☐ Molecular grade water, such as HyClone Water
- ☐ 50X TAE buffer stock (to make 1X TAE)
- ☐ Loading buffer for samples
- ☐ Gel stain such as GelRed® Nucleic Acid Gel Stain - Biotium
- ☐ Appropriate molecular weight ladder for gel electrophoresis, such as GeneRuler 1 kb DNA Ladder

Procedure Guide

Before Class

1. Complete the pre-lab reading.
1. Revisit your Primer and PCR Design Worksheet (section **B**) from Lab Module 7 before class.

PCR Preparation

1. First, you will manually assemble your master mix according to section **B** of your Primer and PCR Design worksheet (from Lab Module 7). Example volumes are shown here for a single reaction, with a total master mix volume of 10 uL. Store the master mix on ice.
    a. **RedTaq 2x master mix** (with 1.5 mM $MgCl_2$); 5 µL
    b. **Forward primer** (10 µM working stock dilution); 0.5 µL

c. **Reverse primer** (10 µM working stock dilution); 0.5 µL
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The example table shown here includes transfer steps of master mix (from snap_caps tubes) to PCR_tubes (generic PCR

strip tubes in the aluminum block on the Temperature Module).

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13. Seal tubes and flick to mix.

Colony PCR

1. Load the PCR plate into a benchtop thermocycler.
2. Set the thermocycler with the experimental conditions from section **B** of your Primer and PCR Design worksheet (from Lab Module 7). An example table is shown below.

Thermocycler Conditions	**°C**	**min:sec**	**Cycles**
1. Initial Denaturation	95	3:00	
2. Denaturation	95	0:20	20x
3. Annealing	**57**	0:30	
4. Extension	72	**1:30**	
5. Final Extension	72	5:00	

6. Storage	12	∞	

Note: no lysis step is required prior to beginning the reaction; cells will be lysed in the first step of PCR.

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    b. Measure 0.7 grams of agarose powder.
    c. Mix the agarose powder with 100 mL 1X TAE in a microwaveable flask.
    d. Microwave 1-3 minutes to dissolve agarose. *Reminder:* do *not* overboil the mixture. Microwaving for 30-45 seconds, stopping, and swirling the flask can help.
    e. Let the agarose solution cool to around 50 °C (or for about 5 minutes).
    f. Pour agarose mixture into a gel tray with a well comb in place. Pour slowly to avoid bubbles.
    g. Let gel set at room temperature for 20-30 minutes until completely solid.
    h. You (or your instructor) can place the solidified gel into the gel box.

2. When the PCR reaction is complete, add an appropriate amount of loading buffer to your PCR samples.
3. Fill the gel box with 1X TAE until the gel is covered.
4. You (or your instructor) should load 1 µL of an appropriate GeneRuler ladder in the first ladder of the gel.
5. Load 5 µL of your PCR sample into another lane of the gel. Remember to take notes on which lane your samples are loaded into.
6. The gel should be run for 45 minutes at 100 volts (time and voltage adjusted as needed). Note that the gel may need to be run, and images taken, outside of class time.

Discussion Questions

Discuss the lab activities with a labmate.

- Why did you use Taq polymerase in this PCR reaction, instead of the Phusion polymerase we used in Lab Module 8 (DNA amplification ahead of cloning)?
- Why is colony PCR not the *final step* of this process? In other words, what can happen in your results from colony PCR and why do we need a final step of sequencing, like Sanger sequencing?