Opentrons
for Education

Prep Sheet

Lab Module 6: Restriction Digest Analysis

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

University of Florida

Getting Started

Review the following resources prior to class.

- ☐ Opentrons Protocol Designer Instruction Manual

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.

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Educator Guide
Lab Module 6: Restriction Digest Analysis

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Contents

Included in this document are the following sections:

Purpose

Students will analyze the results of the previous lab's restriction digest using gel electrophoresis. Here, the amount of time required for running the gel allows for an artistic use of the OT-2! Using "rainbow" plasmid pre-cultures expressed in *E. coli* cells, students can create a drawing of their choice. Unlike the "drawing" completed in the first lab, students have an opportunity to participate more actively, both in the automation and culture of their drawing.

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

- Analysis and troubleshooting of restriction digests in cloning workflows
- Gel electrophoresis as a laboratory tool
- Automating (fun) pipetting tasks while developing bacterial culture skills

Core Competencies

Laboratory Skills:

- Use of modern biology tools in cloning workflows
- Experimental troubleshooting and bacterial culture

Automation Skills:

- Designing automation of sterile pipetting tasks on the OT-2

Background Knowledge

Students should begin this lab with an understanding of the cloning workflow used in this course from previous weeks, as well as a basic understanding of gel electrophoresis. An included pre-lab reading introduces gel electrophoresis and its uses for downstream applications. *No coding experience is required for this lab.*

Supplies

Opentrons Equipment

- [ ] OT-2 automated liquid handling robot
- [ ] OT-2 Single-Channel Pipette (P20, P50, P300, or P1000 GEN2)
- [ ] OT-2 HEPA Module

Opentrons Protocol Tools

- [ ] Opentrons Protocol Designer

Non-Opentrons Equipment

- [ ] Gel electrophoresis equipment:
  - [ ] casting tray
  - [ ] well combs
  - [ ] voltage source, such as the Mini-Sub Cell GT System for the Classroom
  - [ ] gel box
  - [ ] Microwave
  - [ ] gel imager
- [ ] Bacterial culture equipment (inoculating loops, culture tubes and flasks, 37°C shaking incubator)

Labware

- [ ] Single well plate or reservoir, such as an Axygen 1 Well Reservoir 90 mL (see **Basic Troubleshooting and Tips** for more details)
- [ ] NEST 12 Well Reservoir 15 mL (or similar labware, for multiple plasmid colors)

Reagents

- [ ] 50X TAE buffer stock (to make 1X TAE buffer)
- [ ] Agarose powder
- [ ] Loading buffer
- [ ] Gel stain such as GelRed® Nucleic Acid Gel Stain - Biotium
- [ ] Appropriate molecular weight ladder for gel electrophoresis, such as GeneRuler 1 kb DNA Ladder
- [ ] LB media (to make LB agar plates)
- [ ] Kanamycin (at 100 mg/L)
- [ ] Rainbow Chromoprotein Plasmid Set or other constitutively expressing, colorimetric plasmids
- [ ] Microwaveable flasks

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 running their restriction digest results on a gel, students can use the Opentrons Protocol Designer to create a “drawing.” A set of “rainbow” colorimetric plasmids in *E. coli* (we recommend starting cultures either in the prior lab class or outside of class time) are used as colors, pipetted by the OT-2 into LB agar on a plate to “draw.” To save time in class, gels can be poured beforehand.

Basic Troubleshooting and Tips

- We recommend completing a labware position check on the OT-2 before running a protocol, especially when using new labware. We also recommend a dry run, or running the protocol without tips or liquid on the OT-2, to help prepare for using any new protocol.

- Restriction digestion results can be difficult to interpret and challenging to understand. Additional resources, such as this article and video from Addgene, may be helpful.

- This lesson plan uses a gel stain from Biotium as a safer alternative to the traditional use of ethidium bromide. The provided Biotium Tech Tips may be helpful during first use. The gel stain reagent is microwaveable and can be added during gel preparation.

- Students will use the Opentrons Protocol Designer to create a simple protocol for their "drawing," using multiple transfers of "rainbow" plasmids from a reservoir into an LB agar plate. The OT-2 always requires directions for each part of a transfer step, meaning that wells must be specified in both source and destination labware.

  In this activity, students will choose wells in Protocol Designer to create their drawing, but ultimately add different colors of plasmid culture into a single-well plate or reservoir filled with LB agar. We recommend adding a 96 well plate, like the NEST 96 Well Plate 200 µL Flat in Protocol Designer. Use the plate to specify wells in transfer steps in Protocol Designer. However, we recommend actually placing a single-well plate or

reservoir, like the Axygen 1 Well Reservoir 90 mL , on the deck.

- In Protocol Designer, students will add transfer steps to dispense culture of a given color into their LB agar plate. When creating a transfer step, you'll use a two-part menu to select source and destination information and advanced settings. In the second menu, click **Dispense** and **Dispense tip position**. Here, you can adjust the position in the "well" the OT-2 will dispense in. Enter values for the Z position to change the depth the OT-2 will dispense into the LB agar at. We suggest using recommended labware and carefully checking labware dimensions (well depth) in the Labware Library. You can read more about transfer steps and advanced settings here in our Protocol Designer manual.

- If using different labware, we recommend choosing two pieces of labware with dimensions as similar as possible. Selecting a well plate with a shorter well depth (in mm) than your reservoir or single-well plate is important to avoid labware crashes. Follow the included links to view dimensions for the NEST 96 Well Plate 200 µL Flat and Axygen 1 Well Reservoir 90 mL. Instructors can also create custom labware definitions to use in Python protocols and Protocol Designer.

- Students can review the basic guidelines for using Protocol Designer in the Lab Module 1 lesson plan. The Protocol Designer Instruction Manual includes additional guidelines for creating and editing Protocol Designer protocols.

Procedure Guide

Before Class

1. Students or instructor(s) should begin overnight cultures with “rainbow” plasmid pre-culture strains.
2. Streak out each “color” of plasmid onto an LB agar plate (with 100 mg/L kanamycin). Grow overnight at 37 °C.
3. Pick a single colony of each plasmid from the agar plate and inoculate a culture of sterile LB broth (with 100 mg/L kanamycin). Incubate overnight for 12-16 hours at 37 °C with vigorous shaking. Total volume of broth required depends on the number of colors used and number of students in class.
4. To save time in the lab, gels can be poured before class. Instructions are included as the first step of both the educator and student guides to allow for flexibility.
5. LB agar plates (with 100 mg/L kanamycin) should be prepared for use in the OT-2 “drawings.”

Gel Electrophoresis- Gel Preparation, Electrophoresis, and Analysis

1. Gels may be prepared as the first step of class, or poured prior to class. For this experiment, prepare a 0.7% agarose TAE gel.
    a. Prepare 1X TAE from a 50X TAE stock.
    b. Add 2.5 µL of GelRed Nucleic Acid Gel Stain per 50 mL of 1X TAE buffer.
    c. Measure 0.7 grams of agarose powder.
    d. Mix the agarose powder with 100 mL 1X TAE in a microwaveable flask.
    e. 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.
    f. Let the agarose solution cool to around 50 °C (or for about 5 minutes).
    g. Pour agarose mixture into a gel tray with a well comb in place. Pour slowly to avoid bubbles.
    h. Let gel set at room temperature for 20-30 minutes until completely solid.
2. Students (or instructors) can place the solidified agarose gel into the gel box.
3. Students will remove restriction digest samples from 12 °C storage and allow samples to thaw, then add an appropriate amount of loading buffer.

4. Students should fill the gel box with 1X TAE until the gel is covered.
5. Students (or instructor) can load the molecular weight ladder into the first lane of the gel.
6. Students will load samples (10 µL each) into the other lanes of the gel. Remind students to take notes on which lanes samples are loaded into.
7. Gel should be run for 60 minutes at 120 volts (time and voltage adjusted as needed).

Rainbow "Drawing" on the OT-2

1. While the gel(s) run for 60 minutes, students can begin using Opentrons Protocol Designer to design their drawing.
    a. Students should use a well plate, like the NEST 96 Well Plate 200 µL Flat, in Protocol Designer to create transfer steps for their "drawing."
    b. The OT-2 should pipette liquid bacterial culture from a reservoir (we recommend a single reservoir with multiple wells, like the NEST 12 Well Reservoir 15 mL).
    c. Liquid bacterial culture can be dispensed into a single-well reservoir or plate (we recommend the Axygen 1 Well Reservoir 90 mL, filled with LB agar with 100 mg/L kanamycin) in the desired pattern to create a "drawing."

        i. Click **Dispense** and **Dispense tip position** in the second menu when adding a transfer step. Here, you can change the Z position to make sure the OT-2 dispenses into the LB agar. Have your instructor check your work.
2. Direct students to export their Protocol Designer "drawing" protocol(s) and import into the Opentrons App.
    a. Check the setup instructions to confirm hardware, labware, and liquid, including initial volumes.
    b. Students can use the run preview in the app to confirm the actions the OT-2 will perform.
3. Run the protocol.
4. Incubate the LB agar plates upside down at 37 °C for 24-48 hours.

Restriction Digest Analysis

1. Direct students to visualize their bands with a gel imager.

Discussion Questions

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

- Did your restriction digest results match the virtual digest results? How many bands did you see?
- If you saw different results than you expected, what are some reasons this might have occurred? Discuss with your labmates and your instructor as needed.

- What is the next step in the cloning workflow? In your own words, describe the step you will need to complete next.

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Student Guide

Lab Module 6: Restriction Digest Analysis

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Pre-Lab Reading

Today in lab, you will revisit the restriction digestion experiment you performed in the last class. Let's take a look at the steps again:

A restriction digest generally consists of several steps:

1. Planning your experiment- *how can I insert my DNA fragment, or gene of interest? Which restriction enzyme(s) do I need?*
2. Assembling your reagents- often combining restriction enzymes and buffer into a *master mix* and keeping DNA separate
3. Restriction digestion- incubating DNA and master mix at the required temperature for optimal enzymatic activity
4. Inactivation- inactivating restriction enzymes at a specific temperature, so they can no longer cut the DNA
5. Analysis- gel electrophoresis to determine band size and number. *Did the digest work?*

Today, you'll focus on step 5- using *gel electrophoresis* to determine whether or not the digest worked as expected. You'll be able to compare the bands (size and number) to the results of your virtual digest. How does this work?

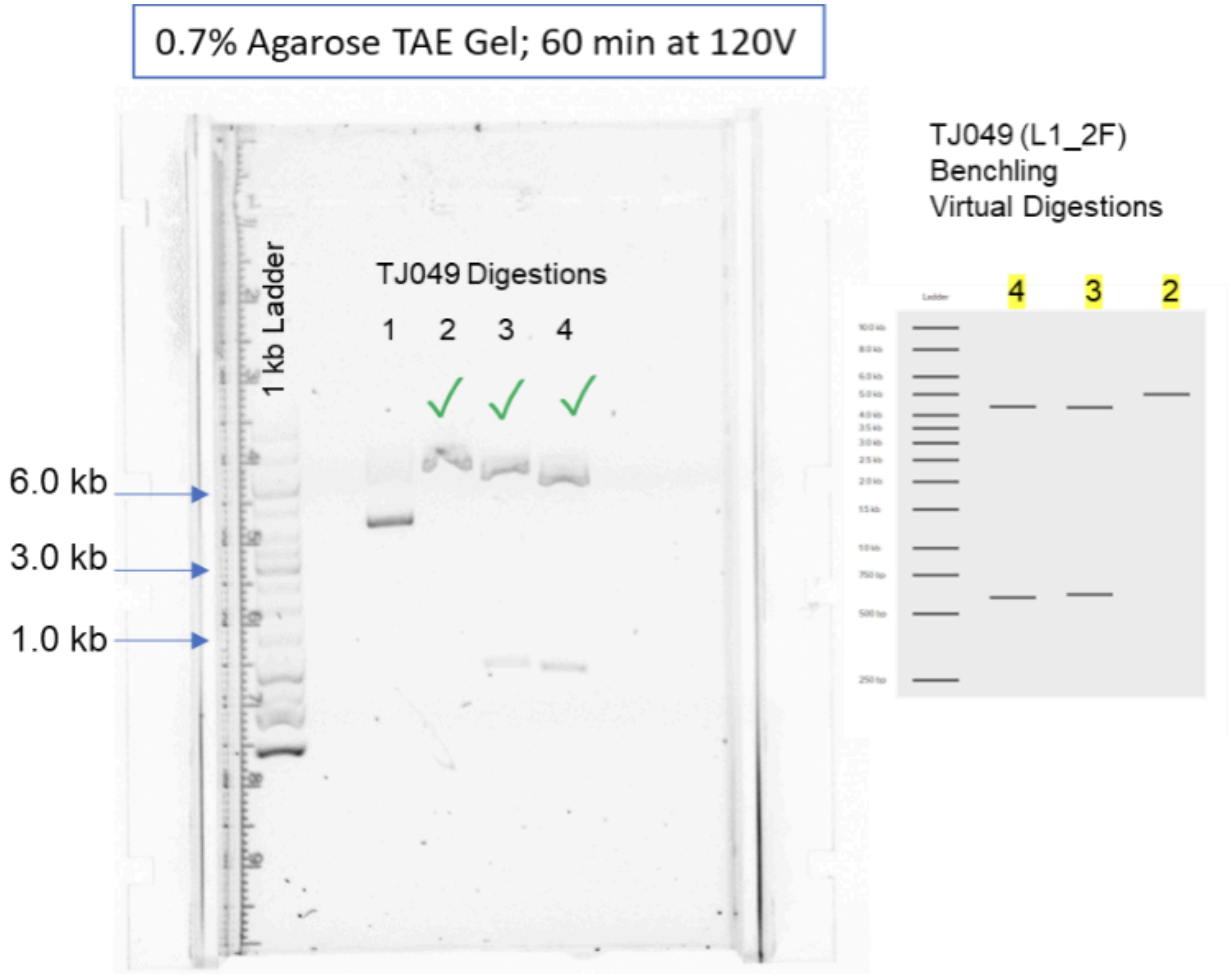
We can think of gel electrophoresis in terms of its two words: 1) gel and 2) electrophoresis. In this process, we use an

agarose gel. Agarose, which is used in a powder and mixed into a solution to form a gel, is just like the agar used in plates for bacterial cultures. In the gel (or the plate), the agarose forms a *matrix* with the buffer. You can think of the matrix like a mesh, with holes, or pores, that molecules like DNA can pass through. Changing the amount of agarose in the gel can change the size of those pores. Today, we'll use a 0.7% agarose gel, but it's common to use up to 2% agarose in gels for electrophoresis. In a gel with a higher percentage of agarose (for example, 2% agarose), the pores are smaller, and smaller DNA fragments will have better separation- they can fit through those small pores, while larger DNA fragments can't. A gel with a lower percentage of agarose (like 0.7%) is better to use with larger DNA fragments- the pores are larger, and those large fragments can fit through.

Now, let's consider the second word: *electrophoresis.* We use this word because an electrical field is applied to the gel and buffer using *electrodes*. Why? DNA is a negatively charged molecule. In electrophoresis, scientists take advantage of its negative charge by applying an electrical field to move the negatively charged DNA towards a positive electrode.

So, an applied electrical field can move negatively charged DNA molecules through the gel. But the real purpose of gel electrophoresis is to separate DNA by size and length. How does this work? Because the gel is a matrix, smaller pieces of

DNA will fit through (and move through) the gel (via the “holes” in the matrix) more quickly. Adding a molecular weight ladder, or a standard group of DNA molecules of known sizes to compare your samples to, will allow you to determine the size of your DNA pieces, in *bands* on the gel.



You'll be able to compare the gel you run today with the results of your virtual digestion, confirming the number of bands (the number of pieces of DNA after the restriction enzyme(s) cut your DNA) and the size of each band. Because of

this, gel electrophoresis is an important step in any cloning workflow, allowing researchers to check that their digest occurred as planned.

It does take time to separate DNA molecules in a gel, which leaves time for other lab activities, such as pipetting a "drawing" using the OT-2! Unlike the last drawing you made with colored liquid, this drawing uses bacteria (*E. coli*) expressing different colorimetric markers- they are different colors to the naked eye! Because we'll use bacteria, you'll design your drawing, let the OT-2 pipette, and wait for it to "appear" as the bacterial colonies grow. Remember that the OT-2 always requires directions for each part of a transfer step, both aspirating from the source and dispensing into the destination labware. In this lab, you will choose wells in Protocol Designer to create a "drawing," but ultimately add different colors of plasmid culture into a single-well plate or reservoir filled with LB agar. In order to do this, a 96-well plate should be included in the transfer steps in your protocol, but *not* placed on the deck.

Purpose

In this lab, you will analyze the results of the previous lab's restriction digest using gel electrophoresis. Here, the amount of time required for running the gel allows for an artistic use of the OT-2! You will use "rainbow" plasmid pre-cultures expressed in *E. coli* cells to create a drawing.

Learning Outcomes

- Understand the uses of gel electrophoresis in a molecular biology lab
- Analyze and troubleshoot your restriction digest using gel electrophoresis
- Understand principles of bacterial culture, used here to "draw"

Supplies

Opentrons Equipment

- ☐ OT-2 automated liquid handling robot
- ☐ OT-2 Single-Channel Pipette (P20, P50, P300, or P1000 GEN2)
- ☐ OT-2 HEPA Module

Opentrons Protocol Tools

- ☐ Opentrons Protocol Designer

Non-Opentrons Equipment

- ☐ Gel electrophoresis equipment:
  - ☐ casting tray
  - ☐ well combs
  - ☐ voltage source, such as the Mini-Sub Cell GT System for the Classroom
  - ☐ gel box
  - ☐ Microwave
  - ☐ gel imager
- ☐ Bacterial culture equipment (inoculating loops, culture tubes and flasks, 37°C shaking incubator)

Labware

- ☐ Single well plate or reservoir, such as an Axygen 1 Well Reservoir 90 mL (see **Basic Troubleshooting and Tips** for more details)
- ☐ NEST 12 Well Reservoir 15 mL (or similar labware, for multiple plasmid colors)

Reagents

- ☐ 50X TAE buffer stock (to make 1X TAE buffer)
- ☐ Agarose powder
- ☐ Loading buffer
- ☐ Gel stain such as GelRed® Nucleic Acid Gel Stain - Biotium
- ☐ Appropriate molecular weight ladder for gel electrophoresis, such as GeneRuler 1 kb DNA Ladder
- ☐ LB media (to make LB agar plates)
- ☐ Kanamycin (at 100 mg/L)
- ☐ Rainbow Chromoprotein Plasmid Set or other constitutively expressing, colorimetric plasmids
- ☐ Microwaveable flasks

Procedure

Before Class

1. Complete the pre-lab reading.
2. You or your instructor(s) should begin cultures for “rainbow” plasmid pre-cultures.
    a. Streak out each “color” of plasmid onto an LB agar plate (with 100 mg/L kanamycin). Grow overnight at 37 °C.
    b. Pick a single colony of each plasmid from the agar plate and inoculate a culture of sterile LB broth (with

100 mg/L kanamycin). Incubate overnight for 12-16 hours at 37°C with vigorous shaking. Total volume of broth needed depends on the number of colors used and number of students.

c. LB agar plates (with 100 mg/L kanamycin) should also be prepared for use in the OT-2 “drawings.”

3. If directed by your instructor, follow the instructions in Step 1 of **Gel Electrophoresis- Gel Preparation, Electrophoresis, and Analysis** to prepare and pour your gel.

Gel Electrophoresis- Gel Preparation, Electrophoresis, and Analysis

1. Gels may be prepared as the first step of class, or poured prior to class. For this experiment, we will need a 0.7% agarose TAE gel.
    a. Prepare 1X TAE from a 50X TAE stock.
    b. Add 2.5 µL of GelRed Nucleic Acid GEl Stain per 50 mL of 1X TAE buffer.
    c. Measure 0.7 grams of agarose powder using a balance scale.
    d. Mix the agarose powder with 100 mL 1X TAE in a microwaveable flask.
    e. Microwave 1-3 minutes to dissolve agarose. It’s important to not allow the flask to overboil. Try microwaving for 30-45 seconds at a time, and stop

and swirl the flask between each interval. Use caution and proper protection; the flask will be hot.

f. Let the agarose solution cool to around 50 °C (or for about 5 minutes).
g. Pour agarose mixture into a gel tray with a well comb in place. Pour slowly to avoid bubbles.
h. Let gel set at room temperature for 20-30 minutes until completely solid.

2. You (or your instructor) should place the solidified agarose gel into the gel box.
3. Remove your restriction digest samples from 12 °C storage.
4. After allowing samples to thaw, add an appropriate amount of loading buffer to each DNA sample. Check manufacturer instructions for your chosen loading buffer.
5. Fill the gel box with 1X TAE until the gel is covered.
6. You (or your instructor) should load the molecular weight ladder into the first lane of the gel by pipetting.
7. Load 10 µL of your restriction digest samples into another lane of the gel. Remember to take notes on which lanes samples are loaded into.
8. Gel should be run for 60 minutes at 120 volts (time and voltage adjusted as needed).

Rainbow “Drawing” on the OT-2

1. While your gel runs, you should use Opentrons Protocol Designer to design a drawing you’d like the OT-2 to pipette.
    a. You’ll use a well plate, like the NEST 96 Well Plate 200 µL Flat, in Protocol Designer to create transfer steps for your “drawing.”
    b. The OT-2 should pipette liquid bacterial culture from a reservoir (we recommend a single reservoir with multiple wells, like the NEST 12 Well Reservoir 15 mL).
    c. Liquid bacterial culture can be dispensed into a single-well reservoir or plate (we recommend the Axygen 1 Well Reservoir 90 mL, filled with LB agar with 100 mg/L kanamycin) in the desired pattern to create a “drawing.”
        i. Click **Dispense** and **Dispense tip position** in the second menu when adding a transfer step in Protocol Designer. Here, you can change the Z position to make sure the OT-2 dispenses into the LB agar. You can read more about changing the tip dispense height here.
        ii. Have your instructor check your work.
    d. Be sure to check your gel periodically- it is possible for your DNA bands to “run off” the end of the gel!
2. Export your Protocol Designer “drawing” protocol and import into the Opentrons App.

3. Check the setup instructions and run preview to confirm the actions the OT-2 will perform.
4. Run your protocol.
5. After, incubate your LB agar plates upside down at 37°C for 24-48 hours.

Restriction Digest Analysis

1. Follow directions from your instructor to visualize bands in your gel with a gel imager.

Discussion Questions

Discuss the lab activities with a neighbor.

- Did your restriction digest results match the virtual digest results? How many bands did you see?
- If you saw different results than you expected, what are some reasons this might have occurred? Discuss with your labmates and your instructor as needed.
- What is the next step in the cloning workflow? In your own words, describe the step you will need to complete next.