Opentrons
for Education

Prep Sheet

# Lab Module 5: Restriction Enzyme Digest

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 Restriction Enzyme Digest 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- Heater-Shaker Module
- ☐ Python Protocol API- Temperature Module
- ☐ Python Protocol API- Loading Labware on Adapters

## Additional Support and Resources

OT-2 Manual
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.

Opentrons
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Educator Guide

# Lab Module 5: Restriction Enzyme Digest

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

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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 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. This educator guide contains suggestions for digestion components and the appropriate restriction enzyme.

## Purpose

Students will perform a restriction digest to “test” the digestion of their modular cloning (MoClo) components with restriction enzymes. This lab module is designed to emphasize student understanding of cloning workflows, including experiment planning and troubleshooting. An attached plasmid digestion worksheet and virtual digestion activity on Benchling introduce these concepts. Students use the OT-2 to distribute master mix to prepare for restriction digests for a number of samples.

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

- Principles of modern cloning, including restriction digestion
- Experimental planning and troubleshooting
- Protocol customization to automate pipetting tasks

### Core Competencies

**Laboratory Skills:**

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

**Automation Skills:**

- Automation of a pipetting task
- Protocol customization

## 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 restriction digestion, including the use of restriction enzymes. Instructors may want to cover real-world restriction digest examples, using specific enzymes, prior to class. An included pre-lab reading introduces the restriction digestion-specific workflow. *No coding experience is required for this lab,* but students and/or instructors will need to edit a Python protocol file.  To save time in the lab, students can sign up for a free Benchling account before class.

## Supplies

### Opentrons Equipment

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

### Opentrons Protocol

- ☐ Restriction Enzyme Digest protocol

### Non-Opentrons Equipment

- ☐ Benchtop thermocycler

### Labware

- ☐ Opentrons OT-2 Filter Tips, 20µL in deck slot 1
- ☐ Opentrons 24 Well Aluminum Block with NEST 2 mL Screwcap tubes (on the Temperature Module in deck slot 3)
- ☐ Opentrons 96 Well Aluminum Block with NEST Well Plate 100 uL (2; in deck slots 2 and 4)

## Reagents

- ☐ 70% ethanol for cleaning
- ☐ Isolated plasmid backbone DNA from Lab Module 3
- ☐ DNA fragments of choice, intended for insertion in cloning workflow (see the **Before Class** section for additional information)
- ☐ Plasmid map and/or sequence information in appropriate files for loading in Benchling (from Genbank, FASTA, Snapgene, or other resources)
- ☐ Benchling accounts for instructors and students
- ☐ Digestion buffer to support activity of chosen restriction enzymes, such as ThermoFisher's FastDigest Green Buffer (10X)
- ☐ Appropriate restriction enzymes, such as FastDigest Eco31I (IIs class) (BsaI)
- ☐ Molecular grade water, such as HyClone water

## Experimental Duration

### Required Class Sessions

1

### Lab Run Time

This lesson plan was prepared for a traditional laboratory class time of 80-90 minutes. After completing the restriction digest, samples can be frozen at 4 °C until the following class, in which students will run gel electrophoresis to analyze their digest results.

## Basic Troubleshooting

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

- Information from Benchling on their "Simulate a digest " feature and other recommended articles may be useful.

- For beginners, the pPICZ B vector is a good basis with which to educate students about classical cloning. This vector is used as an example in this lesson's virtual digest

activity. Students can also read the instruction manual for the EasySelect™ Pichia Expression Kit, which uses the pPICZ B vector.

- Demonstrate proper techniques for enzyme work with students. Discuss methods to limit “star activity,” or the cutting at sequences that are similar to a restriction enzyme’s recognition site, with students. Store enzymes at the manufacturer-recommended temperature before and after use. Remind students to work quickly with enzymes.

- Check requirements for buffers and enzymes used in your class, including temperature guidelines. Temperatures for the restriction digest in this lesson plan (incubation and inactivation) are based on the activity of BsaI. The manufacturers may also recommend adding bovine serum albumin (BSA) during a restriction digest.

- Two restriction enzymes may be required to cut both the plasmid backbone and insert (fragment, gene or marker of interest) DNA. This online NEB Restriction Enzyme Single/Double Digestion tool can be helpful to find an enzyme that may cut both sequences.

- When customizing the 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 you can enter into your spreadsheet. 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.

## Procedure Guide

### Before Class

1. Obtain chosen selection markers or genes of interest for insertion into plasmid vector backbones. Gene inserts can be custom-built for compatibility with plasmid backbones.
2. Download (and upload in Benchling) an example plasmid map and sequence data file (.dna file from Snapgene, or other resource) for use in the virtual plasmid digestion activity.
3. Import the Restriction Enzyme Digest protocol into the Opentrons App.

4. *Optional:* to save lab time, direct students to sign up for Benchling accounts prior to class.

## Lab Introduction

1. Review cloning workflow with students. Instructors may want to cover real-world restriction digest examples using specific enzymes.

### Virtual Restriction Digest on Benchling

1. Demonstrate a virtual digest for students using Benchling with a simple plasmid map, such as the pPICZ B Sequence and Map. pPICZ B is an empty vector with several unique restriction enzyme cut sites.
    a. Download the sequence and map of the chosen plasmid from Snapgene, as above, or another resource.
    b. Upload the plasmid file into Benchling by using the + button on the left toolbar and choosing “create DNA/RNA sequence.”
    c. Show students the available plasmid tools in Benchling, such as the linear map and plasmid map features. Students should be familiar with several key features of the plasmid map, including the ORI (origin of replication) site and labeled restriction enzyme cut sites.

d. Click the scissors icon on the right toolbar (hover over to view “Digests” label). Here, select the enzyme type (single vs. double cutters, etc.) and an appropriate restriction enzyme. Choose “run digest.”
e. In the virtual digest tab, show students the example gel and expected number of bands.

2. Direct students to run their own virtual digest. Section **A** of the attached Plasmid Digestion Preparation worksheet (in the **Student Guide**) includes space for multiple test digestions, and questions to test student understanding.
    a. Students should run their virtual digest with the tools they will actually use during class, and can try multiple digests to test their predictions. For example, a first virtual digest with their plasmid backbone and restriction enzyme, and a second virtual digest with their plasmid backbone, a DNA fragment for insertion, and restriction enzyme.

### Restriction Digest Preparation

1. After the virtual digestion, students can use section **B** of the attached plasmid digestion preparation worksheet (in the Student Guide) to prepare for their restriction digests. The worksheet considers DNA concentration, plasmid size, and reaction size with required reagents amounts, such as master mix.
2. Students will prepare enzyme master mix manually for the number of samples required. Note that more than

one master mix may be required to accommodate different DNA concentrations or restriction enzymes.

3. Open the Restriction Enzyme Digest protocol in a code editing program.
4. Using the Excel template previously created in Lab Module 4, edit your tables as follows to customize for your experiment:
    a. First, edit your “Step 1” table to include both liquids and their initial volumes.

| **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 |
|---|---|---|---|---|---|---|
| | screw_caps | A1 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | screw_caps | A2 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | screw_caps | A3 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | pcr_plate | A1 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A2 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A3 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A4 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A5 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A6 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | | | | | | |

The example table shown here includes both manually assembled master mix (in screw cap tubes in the Temperature Module) and plasmid DNA to be cut (in a 96-well plate on the deck).

    b. Next, edit your “Step 2” table to include transfer steps, specifying source and destination labware, transfer volumes, and when the OT-2 should pick up a new tip.

| **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 |
|---|---|---|---|---|---|---|
| | screw_caps | A1 | pcr_plate | A1 | 0.0125 | TRUE |
| | screw_caps | A1 | pcr_plate | A2 | 0.0125 | FALSE |
| | screw_caps | A2 | pcr_plate | A3 | 0.0125 | FALSE |
| | screw_caps | A2 | pcr_plate | A4 | 0.0125 | FALSE |
| | screw_caps | A3 | pcr_plate | A5 | 0.0125 | FALSE |
| | screw_caps | A3 | pcr_plate | A6 | 0.0125 | FALSE |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

Remember when editing your protocol that Source_Labware and Destination_Labware names are NOT API names for labware, but rather are common names you can type into your spreadsheet. They are defined for the OT-2 in the labware_dict section of the protocol. As long as your labware has a Opentrons-supported 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.

   c. 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 your version of the protocol and import into the Opentrons App.
6. Set up your labware and liquids:
   a. Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1
   b. Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 2 (contains plasmid DNA to be cut in the specified wells)

c. Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 4 (empty at the beginning of the protocol)
d. Opentrons Temperature Module in deck slot 3
e. Opentrons 24 Well Aluminum Block with NEST 2 mL Screwcap tubes in deck slot 3, on the Temperature Module (screwcap tubes contain the manually assembled master mix).

### Restriction Digest

1. Run the Restriction Enzyme Digest protocol.
2. The OT-2 will set the Temperature Module to 4 °C.
3. The OT-2 adds cooled master mix from the NEST 2 mL screwcap tubes in the Temperature Module to the empty NEST 100 µL well plate in deck slot 4. A “blow out” setting makes sure no master mix is left in the pipette tip.
4. The OT-2 adds plasmid DNA to be cut to each well of the NEST plate that now contains master mix. A “blow out” setting makes sure no DNA sample is left in the pipette tip.
5. The protocol will pause. Manually remove the Opentrons 24 Well Aluminum Block with NEST 2 mL screwcap tubes (now empty) from the Temperature Module.
6. Manually move the NEST Well Plate 100 µL from deck slot 4 to the Temperature Module in deck slot 3.

7. The OT-2 will set the Temperature Module to the required incubation temperature. At each step, the OT-2 will wait for the module to reach the specified temperature.
    a. First, the OT-2 incubates samples at 37 °C on the Temperature Module for 10 minutes.
    b. Next, the OT-2 incubates samples at 65 °C for 5 minutes to inactivate the restriction enzyme(s).
    c. The OT-2 sets the Temperature Module at 4 °C and ends the protocol.
8. Manually remove the NEST well plate from the Temperature Module.
9. Store samples at 4 °C until the following class. Students will use gel electrophoresis to analyze their restriction digest results. Samples should be stored at -20 °C if more than one week is needed before gel electrophoresis.

## Discussion Questions

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

- Which restriction enzyme did you choose to use in your virtual restriction digest? How many sites did it cut at? How many bands were on the example gel in the virtual digest?
- How did you know if your coding sequence was “MoClo ready?” (section **A** of the plasmid digestion preparation worksheet)

- Why is there a section of the OT-2 protocol to inactivate the restriction enzymes?

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Student Guide

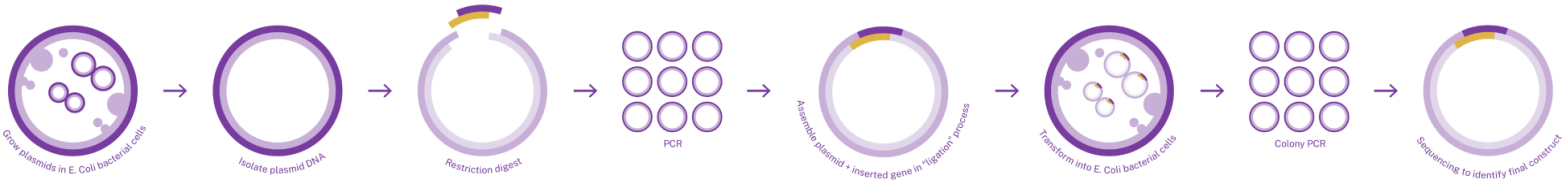
# Lab Module 5: Restriction Enzyme Digest

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

University of Florida

## Pre-Lab Reading

Today in the lab, you'll complete a *restriction digest.* In our cloning workflow, you can see that a restriction digest takes place after the isolation of your plasmid DNA, and a few steps before assembly, or ligation, of your DNA components.




A restriction digest has two purposes: first, to cut the DNA and prepare it for ligation and assembly with other pieces of DNA (in workflow step #5), and second, to confirm that the restriction enzymes, and the cut they will make, work as expected. If the restriction enzymes do not cut the DNA, or cut it incorrectly, ligation and assembly won't work either.

To complete a restriction digest, you need the following reagents:

- your **plasmid DNA backbone** (isolated in Lab Module 3), normalized to 20 ng/µL
- **DNA fragments**, or pieces of DNA you eventually want to insert into your plasmid backbone
- **Restriction enzyme(s**) that will cut the DNA at specific locations, or *restriction sites*

- A **digestion buffer** that supports the activity of your restriction enzyme(s)

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 that they can no longer cut the DNA. Inactivated enzymes also minimize the possibility of off-target cuts.
5. **Analysis**- gel electrophoresis to determine band size and number. *Did the digest work?*

As you can see, a restriction digest, like many biological reactions, has many moving parts that require special considerations. You can use the attached Plasmid Digestion Preparation Worksheet to help you understand how to plan your experiment. You can also use a virtual tool on a molecular biology platform called Benchling to “practice” your digestion virtually before beginning. This virtual digestion can show you what results you can expect, from cuts made with

different restriction enzymes to the number of bands that might show up on your gel.

Once you have an experimental plan, it's time to assemble reagents. A restriction digest involves lots of careful calculations and pipetting. You'll first assemble a *master mix*, a premixed solution of reagents for your experiment. To save time and ensure accuracy, the OT-2 will handle other steps, such as combining the DNA and master mix, and incubation at the proper temperature and time.

Before class, you can create a free Benchling account by following the link.

You can read the following paper to learn more about the modular cloning workflow we will use in this course (and to help you answer a bonus question during class!): **A Golden Gate Modular Cloning Toolbox for Plants | ACS Synthetic Biology**.

## Purpose

In this lab, you will complete a restriction digest in our cloning workflow. You'll begin by using an attached plasmid digestion worksheet and virtual digest activity to test your understanding of restriction digests in cloning and to prepare your experiment. After manually preparing the master mix,

the OT-2 will automate restriction digests for a number of samples.

### Learning Outcomes

- Understand the use of a restriction digest, including choosing the right restriction enzymes, the components required, and experimental steps
- Customize a Python protocol to automate pipetting tasks

## Supplies

### Opentrons Equipment

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

### Opentrons Protocol

- ☐ Restriction Enzyme Digest protocol

### Non-Opentrons Equipment

- ☐ Benchtop thermocycler

## Labware

- ☐ Opentrons OT-2 Filter Tips, 20µL in deck slot 1
- ☐ Opentrons 24 Well Aluminum Block with NEST 2 mL Screwcap tubes (on the Temperature Module in deck slot 3)
- ☐ Opentrons 96 Well Aluminum Block with NEST Well Plate 100 uL (2; in deck slots 2 and 4)

## Reagents and Other Materials

- ☐ 70% ethanol for cleaning
- ☐ Isolated plasmid backbone DNA from Lab Module 3
- ☐ DNA fragments of choice, intended for insertion in cloning workflow
- ☐ Plasmid map and/or sequence information in appropriate files for loading in Benchling (from Genbank, FASTA, Snapgene, or other resources)
- ☐ Digestion buffer to support activity of chosen restriction enzymes, such as ThermoFisher's FastDigest Green Buffer (10X)
- ☐ Appropriate restriction enzymes, such as FastDigest Eco31I (IIs class) (BsaI)
- ☐ Molecular grade water, such as HyClone water

## Experimental Procedure

### Before Class

1. Complete the pre-lab reading.
2. Create your Benchling account and explore. Can you add a DNA or RNA sequence? What kinds of files can you upload with sequence information?
3. Read the **Golden Gate Modular Cloning Toolbox for Plants** paper to help you understand the cloning workflow (and to help answer a bonus question during lab).

### Virtual Restriction Digest on Benchling

1. Your instructor will demonstrate a virtual restriction digest using Benchling with a simple plasmid map, such as the pPICZ B Sequence and Map. pPICZ B is an empty vector with several unique restriction enzyme cut sites. You can follow the instructions below to complete your own virtual digest:
    a. Download the sequence and map of the chosen plasmid from Snapgene, as above, or another resource.

a. Upload the plasmid file into Benchling by using the + button on the left toolbar and choosing “create DNA/RNA sequence.”
b. You can now view the linear and plasmid map of the sequence you uploaded. You should also be able to see several important features of your plasmid, such as the ORI (origin of replication) site and labeled restriction enzyme cut sites.
c. Click the scissors icon on the right toolbar (hover over to view “Digests” label). Here, select the enzyme type (single vs. double cutters- for one or two cuts) and a restriction enzyme. Choose “run digest.”
d. You can now see the results you would expect- an example gel and expected number of bands- in the virtual digest tab.

2. Now, try running your own virtual digest. You can follow section **A** of the attached plasmid digestion preparation worksheet.
    a. Try out multiple digests with different components- what happens? For example, try a digest with the plasmid backbone and restriction enzyme you’ll use today. Then, try a second digest, this time adding a DNA fragment for insertion. What happens differently?

## Restriction Digest Preparation

1. After the virtual digestion, follow section **B** of the attached plasmid digestion preparation worksheet to prepare for your restriction digest. The worksheet considers DNA concentration, plasmid size, and reaction size with required reagents amounts, such as master mix.
2. After completing the worksheet, prepare your enzyme master mix for restriction digestion manually. The table below is an example of the ratio of buffer, enzyme, and water to add for A) a given amount of DNA and B) your chosen number of reactions. The master mix consists of the following:
    a. Digestion buffer to support activity of chosen restriction enzymes, such as ThermoFisher's FastDigest Green Buffer (10X)
    b. Appropriate restriction enzymes, such as FastDigest Eco31I (IIs class) (BsaI)
    c. Molecular grade water, such as HyClone water
3. Mix by pipetting.

| **Reactions (# samples)** | **µL DNA** | **10x FD Green Buffer** | **FD Eco31I (BsaI)** | **HyClone water** | **Total µL** |
|---|---|---|---|---|---|
| **1** | 1 | 1 | 0.2 | 7.8 | 10 |
| **15** | | 15 | 3 | 117 | |

4. As a class, open the Restriction Enzyme Digest protocol file in a code editing program.
5. Using the Excel template previously created in Lab Module 4, edit your tables as follows to customize for your experiment:
    a. First, edit your “Step 1” table to include both liquids and their initial volumes.

| **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 |
|---|---|---|---|---|---|---|
| | screw_caps | A1 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | screw_caps | A2 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | screw_caps | A3 | 1.5 | Master Mix | Manually assembled master mix | #00FF00 |
| | pcr_plate | A1 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A2 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A3 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A4 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A5 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | pcr_plate | A6 | 0.001 | Plasmid DNA | DNA to be cut | #FF0CB |
| | | | | | | |

The example table shown here includes both manually assembled master mix (in screw cap tubes on the Temperature Module) and plasmid DNA to be cut (in a 96-well plate on the deck).

    b. Next, edit your “Step 2” table to include transfer steps, specifying source and destination labware, transfer volumes, and when the OT-2 should pick up a new tip.

| **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 |
|---|---|---|---|---|---|---|
| | screw_caps | A1 | pcr_plate | A1 | 0.0125 | TRUE |
| | screw_caps | A1 | pcr_plate | A2 | 0.0125 | FALSE |
| | screw_caps | A2 | pcr_plate | A3 | 0.0125 | FALSE |
| | screw_caps | A2 | pcr_plate | A4 | 0.0125 | FALSE |
| | screw_caps | A3 | pcr_plate | A5 | 0.0125 | FALSE |
| | screw_caps | A3 | pcr_plate | A6 | 0.0125 | FALSE |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

Remember when editing your protocol that Source_Labware and Destination_Labware names are NOT API names for labware, but rather are common names you can type into your spreadsheet. They are defined for the OT-2 in the labware_dict section of the protocol. As long as your labware has a Opentrons-supported 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.

   c. 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).

6. Save your version of the protocol and import into the Opentrons App.
7. Set up your labware and liquids:
   a. Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1
   b. Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 2 (contains plasmid DNA to be cut in the specified wells)

c. Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 4 (empty at the beginning of the protocol)
d. Opentrons Temperature Module in deck slot 3
e. Opentrons 24 Well Aluminum Block with NEST 2 mL Screwcap tubes in deck slot 3, on the Temperature Module (screwcap tubes contain the manually assembled master mix).

### Restriction Digest

1. Run the Restriction Enzyme Digest protocol.
2. The OT-2 will set the Temperature Module to 4 °C.
3. The OT-2 adds cooled master mix from the NEST 2 mL screwcap tubes in the Temperature Module to the empty NEST 100 µL well plate in deck slot 4. A “blow out” setting makes sure no master mix is left in the pipette tip.
4. The OT-2 adds plasmid DNA to be cut to each well of the NEST plate that now contains master mix. A “blow out” setting makes sure no DNA sample is left in the pipette tip.
5. The protocol will pause. Manually remove the Opentrons 24 Well Aluminum Block with NEST 2 mL screwcap tubes (now empty) from the Temperature Module.
6. Manually move the NEST Well Plate 100 µL from deck slot 4 to the Temperature Module in deck slot 3.

7. The OT-2 will set the Temperature Module to the required incubation temperature. At each step, the OT-2 will wait for the module to reach the specified temperature.
    a. First, the OT-2 incubates samples at 37 °C on the Temperature Module for 10 minutes.
    b. Next, the OT-2 incubates samples at 65 °C for 5 minutes to inactivate the restriction enzyme(s).
    c. The OT-2 sets the Temperature Module at 4 °C and ends the protocol.
8. Manually remove the NEST well plate from the Temperature Module.
9. Store samples at 4 °C until the following class, where you will use gel electrophoresis to analyze their restriction digest results. Samples should be stored at -20 °C if more than one week is needed before gel electrophoresis.

## Discussion Questions

Discuss today's lab activities with a labmate.

- Which restriction enzyme did you choose to use in your virtual restriction digest? How many sites did it cut at? How many bands were on the example gel in the virtual digest?
- How did you know if your coding sequence was "MoClo ready?" (section **A** of the plasmid digestion preparation worksheet)
- Why is there a section of the OT-2 protocol to inactivate the restriction enzymes?

## Plasmid Digestion Preparation Worksheet

This worksheet will guide you through a virtual digestion and help you prepare for your first restriction digestion.

### Section A: Virtual Restriction Digestions

1. Open Benchling.
2. Download a plasmid sequence and map, such as the pPICZ B Sequence and Map. You can find other plasmids to try at sites such as GenBank and Snapgene.
3. Upload the plasmid file into Benchling by using the + button on the left toolbar and choosing “create DNA/RNA sequence.”

1. Explore the linear and plasmid map of the sequence you uploaded. You should also be able to see several important features of your plasmid, such as the ORI (origin of replication) site and labeled restriction enzyme cut sites.
2. Click the scissors icon on the right toolbar (hover over to view “Digests” label). Here, select the enzyme type (single vs. double cutters- for one or two cuts) and a restriction enzyme. Choose “run digest.”
3. You can now see the results you would expect- an example gel and expected number of bands- in the virtual digest tab.
4. Now, try running your own virtual digests using the table below. Start with a simple digest of a plasmid backbone

(such as pPICZ B). Which restriction enzymes can you use for digestion? For a second digest, repeat the above, but add in a gene (Gene 1, a DNA fragment for insertion). What happens differently?

a. You can use the following paper to help you answer the bonus question: **A Golden Gate Modular Cloning Toolbox for Plants**
b. Continue to fill in the remaining rows with your own test digestions.

| **Plasmid to Digest** | **How was the gene cloned?** | **Enzymes for test digestion** | **Bonus question: is the CDS MoClo ready?** |
|---|---|---|---|
| pPICZ B | | | |
| pPICZB + Gene 1 | | | |
| | | | |
| | | | |
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## Section B: Plasmid Restriction Digest

Use the following examples and table to consider each of your components: DNA concentration, plasmid size, and reaction size with required reagents amounts, such as master mix.

Your restriction digest should look like this:

100-300 ng of DNA to be digested (volume in µL will vary, depending on your DNA concentration)
0.2 µL of restriction enzyme (BsaI)
1 µL of buffer (10x FD Green Buffer)
7.8 µL molecular grade water

First, decide how much DNA you'll need (based on your normalized concentration of DNA). Then, calculate how much molecular grade water you'll need for a final reaction volume of 10 uL. The first row of the table below is filled out for you as an example, using DNA at a concentration of 200 ng/uL. Continue to fill out the table for 1 reaction, and 15 reactions, of your restriction digest. The final two rows of the table can be used to fill out your reaction conditions for a plasmid backbone and a gene insert (your gene of interest).

| Description | Reactions (# of samples) | µL of DNA (for 500 ng | 10X buffer | Restriction Enzyme | Molecular grade water | Total volume |
|---|---|---|---|---|---|---|

| | | DNA) | | | | (µL) |
|---|---|---|---|---|---|---|
| Plasmid backbone | 1 | 1 | 1 | 0.2 | 7.8 | 10 |
| Plasmid backbone | 1 | | | | | |
| Plasmid backbone | 15 | | 15 | 3 | 117 | |
| Plasmid + insert | 1 | | | | | |
| Plasmid + insert | 15 | | | | | |

Now, use the above table to assemble your master mix. Manually pipette to combine the 10X FD Green Buffer, restriction enzyme, and molecular grade water.

**A few helpful reminders:**

- Looking up some basic information about enzymes can help you decide how much to use. Let's consider BsaI-HF®v2 from New England Biolabs. "One unit (of enzyme) is defined as the amount of enzyme required to digest (cut) 1 ug of DNA in 1 hour at 37°C in a total reaction volume of 50 uL." Enzymes are sold in units/mL; for example, a concentration might be 20,000 units/mL. You can use this ratio to calculate the amount of enzyme

you need to use. It's common to use ~0.2-0.5 µL of restriction enzyme, because it's difficult to accurately pipette less volume than this.

- Loading an undigested control in your gel with your experimental, restriction digest sample can help to troubleshoot your results.