Opentrons
for Education

Prep Sheet

Lab Module 9: DNA Assembly Methods

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University of Florida

Getting Started

This lesson plan uses values from an Excel template to customize the MoClo Assembly 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
Running a protocol on the OT-2

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

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

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Educator Guide
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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

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

Purpose

Students will use the OT-2 in MoClo assembly (cloning new genes, or their DNA insert, into a standardized plasmid backbone). Here, MoClo assembly methods allow for a “one-pot” digestion and ligation, building on concepts learned in previous classes. Students will use the OT-2 to assemble their reaction components. Thermocycler reactions will be carried out in a benchtop thermocycler.

During this lab class, students gain experience with:

- Experimental design for MoClo assembly
- Laboratory automation of pipetting tasks

Core Competencies

Laboratory Skills:

- Design and completion of MoClo assembly experiments
- Understanding of Golden Gate cloning methods

Automation Skills:

- Automation of pipetting tasks
- Protocol customization

Background Knowledge

Students should begin this lab with a thorough understanding of the cloning workflow and MoClo assembly concepts used in this course. The included pre-lab reading reviews the steps of a one-pot digestion and ligation in MoClo assembly, and introduces the identification methods that follow in upcoming lab classes. *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 GEN2 pipette (in left mount)
- [ ] Temperature Module (in deck slot 3)

Opentrons Protocol

- [ ] MoClo Assembly Preparation protocol

Non-Opentrons Equipment

- [ ] Benchtop thermocycler

Labware

- [ ] Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1
- [ ] Opentrons 24 Tube Rack with NEST 1.5 mL Snapcap tubes (in deck slot 5)
- [ ] Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 µL tubes (on the Temperature Module in deck slot 3)

Reagents and Other Materials

- ☐ PCR products (amplified plasmid backbone, DNA insert) from previous classes
- ☐ BsaI restriction enzyme
- ☐ DNA ligase, such as Promega T4 ligase enzyme
- ☐ Ligase buffer, such as 10X FD Buffer
- ☐ 10 mM ATP
- ☐ 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. Assembled DNA products can be stored at -20 °C until the following class.

Basic Troubleshooting and Tips

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

2. 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.

3. 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.

4. Storage of assembled products is permissible at 4 °C for up to 2 days before the next class; otherwise, storage at -20 °C is recommended.

Procedure Guide

Lab Introduction

1. Instructors may wish to review the basic cloning workflow for the course with students, paying special attention to any new or complex concepts (Golden Gate cloning, MoClo assembly).
2. Students should understand the multiple, different uses of thermocycler reactions- PCR for amplification ahead of digestion compared to *what* takes place in the thermocycler reaction steps in this lab class.

Reaction Component Assembly

1. Students should manually prepare their master mix with the following components:
    a. **10x FD Buffer**; 1 µL
    b. FD enzyme (such as BsaI); 0.5 µL
    c. T4 DNA ligase; 0.5 µL
    d. 10 mM ATP; 1 µL
    e. Hyclone water; 5 µL
2. Open the Excel template used in previous classes for protocol customization.
    a. First, modify your “Step 1” table.

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_Wel	Initial_Volume	Liquid_Name	Description	Color
	PCR_tubes	A1	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	PCR_tubes	A2	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	PCR_tubes	A3	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	PCR_tubes	A4	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	PCR_tubes	A5	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	PCR_tubes	A6	0.002	DNA sample	This is DNA sample(s) for assembly	#00FF00
	snapcap_tubes	A1	0.048	Master mix	This is master mix	#FFC0CB

Here, our example table assumes that 6 different DNA samples are being used, with 2 µL of each loaded into an individual PCR tube. We also have one master mix aliquot that will be used for each of our samples.

    b. Next, edit your “Step 2” table.

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
	snapcap_tubes	A1	PCR_tubes	A1	8	TRUE
	snapcap_tubes	A1	PCR_tubes	A2	8	FALSE
	snapcap_tubes	A1	PCR_tubes	A3	8	FALSE
	snapcap_tubes	A1	PCR_tubes	A4	8	FALSE
	snapcap_tubes	A1	PCR_tubes	A5	8	FALSE
	snapcap_tubes	A1	PCR_tubes	A6	8	FALSE

The example table shown here demonstrates a transfer of 8 µL of master mix (in “snapcap_tubes”) into each specified well of “PCR_tubes” containing DNA samples.

3. Download and open the MoClo Assembly Preparation protocol in a code editing program.
4. Follow the directions to copy and paste in your data and customize 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 your customized 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 24 Tube Rack with NEST 1.5 mL Snapcap tubes in deck slot 5; contains master mix in the specified tubes

c. Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 µL tubes on the Temperature Module in deck slot 3; contains DNA to be assembled in the specified wells

7. Run the protocol.
8. The OT-2 will set the Temperature Module to 4 °C.
9. The OT-2 will transfer 8 µL of master mix into each PCR tube containing DNA to be assembled.
10. When the protocol is complete, manually remove the PCR strip tubes from the Temperature Module.

"One-Pot" Digestion and Ligation

1. Gently mix the PCR strip tubes by flicking.
2. Place PCR strip tubes in the benchtop thermocycler and set the reaction to run as follows:

Thermocycler Method		**Description**
37°C	5 minutes	5 cycles
22°C	10 minutes	
37°C	5 minutes	Final digest
75°C	10 minutes	inactivation
12°C	∞	storage

3. Assembled plasmid products should be stored at 4 °C (or -20 °C for longer storage).

Discussion Questions

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

- Describe in your own words what took place in the thermocycler reaction you ran in class today. What happens during digestion? Ligation? What are your final products?
- What are the different reactions we use the thermocycler for in this class? Name 3. What are the differences between them?

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Student Guide

Lab Module 9: DNA Assembly Methods

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Pre-Lab Reading

Today in class, you will complete a “one-pot” digestion and ligation. Last week, you used a PCR reaction to amplify your plasmid backbone and DNA insert (gene of interest). The PCR reaction (and primer design) can also be used to add restriction sites, so restriction enzymes will be able to cut your DNA components.

Today, in a single tube and in a single thermocycler reaction, you will complete two steps in a MoClo assembly:

- *Digestion*: cutting of your DNA pieces with a single restriction enzyme. Remember that both your plasmid backbone, or *destination vector,* and gene of interest, or *insert fragment,* both need to contain compatible restriction sites for a Type IIS restriction enzyme in Golden Gate assembly methods.
- *Ligation:* sealing together your cut pieces of DNA into a single assembled plasmid

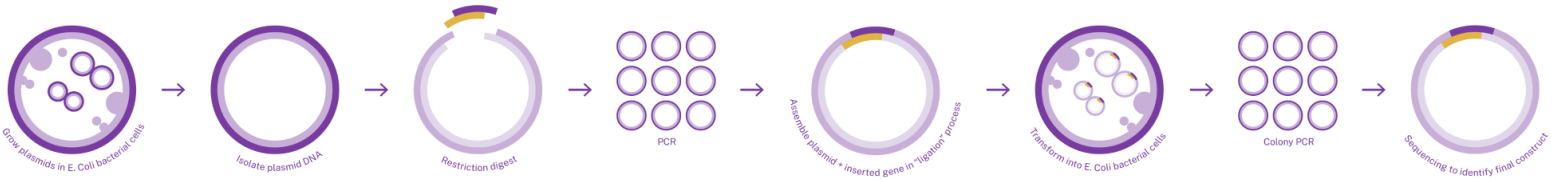
As a reminder, MoClo (or *modular cloning*) is a Golden Gate method of assembling standardized DNA parts. You can revisit the pre-lab reading for Lab Module 8 (and the linked resources) to learn more. To begin, we will use the OT-2 to combine the following parts into a single tube:

1. **PCR products:** your plasmid backbone and gene of interest, amplified and, if required, restriction sites added using the previous PCR reaction
2. **Restriction enzyme:** such as BsaI, to cut your DNA components.
3. **BSA (bovine serum albumin)**: optionally added to stabilize enzymes in your reaction
4. **DNA ligase:** an enzyme to *ligate*, or seal, your DNA components together into a new assembled product
5. **DNA ligase buffer:** optimizes the activity of the DNA ligase enzyme
6. **10 mM ATP:** improves performance of T4 DNA ligase and buffer
7. **Molecular grade water:** add up to your final reaction volume

These reaction tubes will be placed into a benchtop thermocycler to carry out the single, “one-pot” reaction. The thermocycler profile should include three steps:

- *Incubation:* incubating the reaction at a specific temperature to allow enzymatic activity
- *Inactivation:* brings the reaction to a specific temperature to deactivate both restriction enzymes and the DNA ligase
- *Holding:* holds assembled products at a safe temperature for storage, often 4°C

When the reaction is complete, congratulations! You should have successfully completed a cloning experiment, and your tube should contain assembled plasmid products containing your gene of interest. Now, *how* would you check your results to ensure your experiment worked? Let's revisit the cloning workflow:



As you can see, the steps following digestion and ligation include transformation into *E. coli*, colony PCR, and Sanger sequencing. Let's break these down.

You'll begin by transforming your assembled products 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. Here, you will transform a small amount of your assembled plasmid product into *E. coli*, allow the cells to grow, and will obtain more of your assembled product than you began with.

Growth in *E. coli* is helpful for another reason, too. Plasmid backbones generally contain an antibiotic resistance gene. By growing *E. coli* containing your assembled products in LB broth or agar supplemented with a specific antibiotic, you can select

for your plasmid of choice. Any bacteria without the antibiotic resistance will die, but your plasmid should be able to thrive in the presence of the antibiotic it is resistant to. This is the first step to making sure that your reaction worked; however, it will not provide a definitive result, because your plasmid backbone *without* the DNA insert (gene of interest) would also be able to grow in the presence of the antibiotic.

Therefore, we will use other methods to test your experimental results- including *colony PCR* and *Sanger sequencing*. These experiments, done in upcoming classes, will allow you to determine whether or not your DNA insert is present in your plasmid, and whether or not it was inserted correctly.

You can learn more about the assembly methods used in today's class at Snapgene.

Purpose

In today's class, you will use the OT-2 in MoClo assembly of a plasmid backbone and DNA insert (gene of interest). Here, MoClo assembly methods allow for a "one-pot" digestion and ligation. First, the OT-2 will be used to pipette reaction components into a single tube, and a single thermocycler reaction will be carried out in a benchtop thermocycler.

Learning Outcomes

- Understand experimental design for MoClo assembly
- Customize your protocol to pipette required volumes

Supplies

Opentrons Equipment

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

Opentrons Protocol

- ☐ MoClo Assembly Preparation protocol

Non-Opentrons Equipment

- ☐ Benchtop thermocycler

Labware

- ☐ Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1

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Reagents and Other Materials

- ☐ PCR products (amplified plasmid backbone, DNA insert) from previous classes
- ☐ BsaI restriction enzyme
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- ☐ Ligase buffer, such as 10X FD Buffer
- ☐ 10 mM ATP
- ☐ Molecular grade water, such as HyClone Water

Experimental Procedure

Before Class

1. Complete the pre-lab reading.

Reaction Component Assembly

1. Start by manually assembling your master mix with the following components:
    a. **10x FD Buffer**; 1 µL
    b. FD enzyme (such as BsaI); 0.5 µL
    c. T4 DNA ligase; 0.5 µL
    d. 10 mM ATP; 1 µL
    e. Hyclone water; 5 µL
2. As a class, open the Excel template used to customize your protocols for previous classes.
3. Download and open the MoClo Assembly Preparation protocol in a code editing program.
    a. First, modify your “Step 1” table.

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.

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6. Set up your labware and liquids:
    a. Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1
    b. Opentrons 24 Tube Rack with Eppendorf 1.5 mL Safe-Lock Snapcap tubes in deck slot 5; contains master mix in the specified wells
    c. Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 µL tubes on the Temperature Module in

deck slot 3; contains DNA to be assembled in the specified wells

7. Run the protocol.
8. The OT-2 will set the Temperature Module to 4 °C.
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10. When the protocol is complete, manually remove the PCR strip tubes from the Temperature Module.

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