Opentrons
for Education

Prep Sheet

Lab Module 12: Sanger Sequencing and Multiple Sequence Alignment

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 Sanger Sequencing 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.

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Educator Guide

Lab Module 12: Sanger Sequencing and Multiple Sequence Alignment

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Contents

Included in this document are the following sections:

Purpose

Students will use the OT-2 to prepare plasmids and/or PCR fragments for Sanger sequencing at Genewiz. This lab builds on concepts from previous labs, including *how* scientists confirm results of their cloning experiments. Final Sanger sequencing data can be used as the basis for student final lab reports.

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

- Use of automation to prepare samples and use of common molecular biology services
- Principles of Sanger sequencing
- Analysis, interpretation, and troubleshooting of experimental results

Core Competencies

Laboratory Skills:

- Sample preparation ahead of Sanger sequencing
- Understanding and interpreting sequencing results

Automation Skills:

- Automation of pipetting tasks to prepare molecular samples
- Python protocol customization

Background Knowledge

Students should begin this lab with an understanding of the results that Sanger sequencing will produce- and *why* sequencing results are more definitive than results of their previous colony PCR experiment. An included pre-lab reading introduces the principles of Sanger sequencing. *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 GEN2 (in deck slot 3)

Opentrons Protocol

- ☐ Sanger Sequencing Preparation protocol

Non-Opentrons Equipment

- ☐ Tabletop microcentrifuge

Labware

- ☐ Opentrons OT-2 96 Filter Tip Rack 20 µL (in deck slot 1)
- ☐ Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 2
- ☐ Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 µL tubes on the Temperature Module in deck slot 3

- ☐ Opentrons 24 Well Aluminum Block with NEST 1.5 mL Screwcap tubes in deck slot 6

Reagents and Other Materials

- ☐ Assembled plasmid products in *E. coli* culture
- ☐ DNAse- and RNAse-free 1.5 mL snapcap tubes appropriate for classroom microcentrifuge
- ☐ Primers (10 µM working stock dilution)
- ☐ Molecular grade water, such as HyClone Water
- ☐ 96-100% ethanol for use in plasmid purification
- ☐ QIAprep Spin Miniprep Kit OR Syd Labs Spin Columns for Plasmid Miniprep and reagents sufficient to make buffers for miniprep (see **Before Class** in the educator's **Procedure Guide**).

Experimental Duration

Required Class Sessions

1

Lab Run Time

This lab class was prepared for a traditional laboratory class time of 80-90 minutes. Samples will be sent out for Sanger sequencing by Genewiz, and sequencing results can be included in students' final lab reports.

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.

- This lesson plan includes a mini-prep for plasmid isolation; however, bacterial samples are accepted for sequencing by Genewiz and other commercial services. Please reference the sample submission guidelines from Genewiz or your provider of choice.

- 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.

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

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

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

Procedure Guide

Before Class

1. Review Genewiz's Sanger Sequencing product offerings and Sanger Sequencing Sample Submission Guidelines. Sequencing data should be reported 1 business day after samples are received.
2. To save time during class, buffers for plasmid extraction can be prepared ahead of time. Instructors can reach out to Syd Labs for detailed instructions on their spin columns.

Lab Introduction

1. Instructors should review the differences between results obtained from colony PCR and the results students can expect from Sanger sequencing.

Harvest Bacterial Cultures

1. Students will harvest bacterial cells by centrifugation at >8,000 RPM (6,800 *x g*) in a tabletop microcentrifuge for 3

minutes at room temperature. Instructors should demonstrate proper use and balance of the microcentrifuge for students, and check their balance before use.
2. Students will remove supernatant from bacterial pellet.

Spin Column Plasmid Purification

1. Students will resuspend pelleted bacterial cells in 250 µL of P1 buffer, and transfer to a microcentrifuge tube.
2. Students will add 250 µL of P2 buffer and mix thoroughly by inverting the tube 4-6 times. Remind students *not* to vortex the sample, as this will cause shearing of plasmid DNA. The sample should become viscous, and students should move onto the next step after fewer than 5 minutes of mixing.
3. Students will add 350 µL of N3 buffer, and mix immediately and thoroughly (inverting the tube 4-6 times). The solution should become cloudy.
4. Students will centrifuge the sample for 10 minutes at 13,000 RPM. A compact white pellet should be visible.
5. Students will apply 800 µL of supernatant from the previous step for the spin column with a pipette.
6. Students will centrifuge the spin column for 30-60 seconds and discard flow through.

7. Students should wash the spin column by adding 0.5 mL of PB (binding) buffer and centrifuge spin columns again for 30-60 seconds. Flow through should be discarded.
8. Students should wash the spin column again by adding 0.75 mL of PE (wash) buffer and centrifuge again for 30-60 seconds.
9. Students can discard flow through and centrifuge spin columns again at full speed for an additional 1 minute to remove any remaining PE (wash) buffer.
10. Students can place spin columns into clean, 1.5 mL microcentrifuge tubes, and add 50 µL of molecular grade water to the center of each spin column to elute DNA.
11. Students should let the spin column (with molecular grade water added) stand for 1 minute, followed by centrifugation for 1 minute, to fully elute DNA.

Sanger Sequencing Sample Preparation

1. Students should add ~500 ng of assembled plasmid DNA (our example assumes 5 µL of plasmid DNA sample) to the PCR strip tubes that will be placed in the 96-well aluminum block on the Temperature Module.
2. Open your Excel template used for protocol customization. Start by editing your “Step 1” table to enter 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
	PCR_tubes	A1	0.005	DNA template	Assembled plasmid products	#00FF00
	PCR_tubes	A2	0.005	DNA template	Assembled plasmid products	#00FF00
	PCR_tubes	A3	0.005	DNA template	Assembled plasmid products	#00FF00
	screw_caps	A1	0.1	Primer	10 uM primer working dilution	#FFD966
	screw_caps	A2	0.1	Primer	10 uM primer working dilution	#FFD966
	screw_caps	A3	0.1	Primer	10 uM primer working dilution	#FFD966
	water_tube	A4	5	Molecular water	Water to add to final volume	#FDF1BC

The example table shown here includes assembled plasmid products in the PCR_tubes (generic PCR strip tubes in the aluminum block on the Temperature Module), primer stocks in screw_caps tubes (NEST 1.5 mL screwcap tubes in a 24-well aluminum block), and a water_tube (Falcon conical tube in an Opentrons 10 tube rack).

3. Next, edit your “Step 2” table to describe the transfer steps.

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_tubes	A1	2.5	TRUE
	screw_caps	A2	PCR_tubes	A2	2.5	TRUE
	screw_caps	A3	PCR_tubes	A3	2.5	TRUE
	water_tube	A4	PCR_tubes	A1	7.5	TRUE
	water_tube	A4	PCR_tubes	A2	7.5	FALSE
	water_tube	A4	PCR_tubes	A3	7.5	FALSE

The example table shown here includes transfer steps of primer (from screw_caps tubes to PCR_tubes) and molecular grade water (from water_tube to PCR_tubes).

4. Download and open the Sanger Sequencing Preparation protocol in a code editing program. Follow the directions

to copy and paste in your values to customize the protocol for your experiment.
    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 protocol and import into the Opentrons App.
    a. Check setup instructions in the app to confirm hardware, labware, and liquids used in your protocol.
6. Load your labware and liquids:
    a. Opentrons OT-2 96 Filter Tip Rack 20 µL in deck slot 1
    b. Opentrons 24 Well Aluminum Block with NEST 1.5 mL screwcap tubes in deck slot 6 (containing molecular grade water and primer working dilution stocks)
    c. Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 2 (contains plasmid DNA templates)
    d. Opentrons 96-well aluminum block with generic PCR strip tubes, 200 µL; in deck slot 3 (on the Temperature Module; all wells are empty on the protocol starting deck)
7. Run your modified protocol.
8. The OT-2 will set the Temperature Module to 4 °C.
9. The OT-2 will transfer 10 µL of molecular grade water from the NEST 1.5 mL screwcap tubes to each required

well of the generic PCR strip tubes in the Temperature Module.

10. The OT-2 will transfer 2.5 µL of primer from the NEST 1.5 mL screwcap tubes into each required well of the generic PCR strip tubes in the Temperature Module, combining primers and molecular grade water.
11. The OT-2 will transfer 2.5 uL of plasmid DNA template from the NEST 100 µL well plate to generic PCR strip tubes in the Temperature Module (to a final reaction volume of 15 µL).
12. The OT-2 will deactivate the Temperature Module and end the protocol.

GeneWiz Submission

1. Remove the PCR strip tubes from the Temperature Module. Carefully label the tubes and complete the online submission form.
2. Gently mix the contents of the tubes.
3. Place in a plastic bag labeled GeneWiz and drop off.

Discussion Questions

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

- Describe in your own words the Sanger sequencing reaction that Genewiz will complete. What happens to your primer? The DNA template?
- What information will these results provide you that colony PCR *cannot?*

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Student Guide

Lab Module 12: Sanger Sequencing and Multiple Sequence Alignment

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Pre-Lab Reading

Today in lab, you will prepare samples for *Sanger sequencing*. Sanger sequencing, as the name suggests, will provide the exact sequence of your plasmid and DNA insert. This will allow you to confirm whether or not your cloning experiment was successful, and will reveal any mutations or insertion issues in the sequence. Sanger sequencing, the first ever method of DNA sequencing, was developed by Fred Sanger in 1975. This method won Fred Sanger a Nobel Prize, was instrumental for the sequencing of our DNA in the Human Genome Project, and still is the *most accurate* method of sequencing available today!

So, how does Sanger sequencing work? The basis of this method is a reaction you are very familiar with by now- the polymerase chain reaction (PCR). In a normal PCR reaction, dNTPs are added to the mixture as the nucleotide building blocks that DNA copies will be assembled from. A Sanger sequencing reaction uses both dNTPs (deoxynucleotide triphosphates) and ddNTPs (dideoxynucleotide triphosphates). What is the difference? Either dNTPs or ddNTPs are incorporated, one at a time, into the DNA strand being assembled; however, ddNTPs are *chain-terminating* nucleotides. When a ddNTP is added to the strand, no further nucleotides can be added. This means that the dNTP/ddNTP mix results in strands (strings of nucleotides) of each possible

length being produced, since a ddNTP can be randomly incorporated and terminate the chain at any time. In addition, ddNTPs each contain a fluorescent label: one for each possible base in DNA (adenine, cytosine, guanine, and thymine). By analyzing each of these chains- their size, and the color of the fluorescent label on the ddNTP at the end- researchers can identify the sequence of their DNA strand. For these reasons, Sanger sequencing is sometimes also referred to as the *chain-termination* method. A complete Sanger sequencing reaction looks like this:

1. **Cycle sequencing:** combines fluorescently labeled ddNTPs, dNTPs, a single sequencing primer, DNA polymerase, and the DNA of interest (often purified PCR products or a plasmid) to generate chain-terminated, fluorescently-labeled fragments
2. **Cycle sequencing clean up:** clean-up method, like a column, to bind and remove unincorporated, or “loose” ddNTPs from the reaction mixture
3. **Capillary electrophoresis:** electrophoresis to separate DNA fragments by size; each sequencing reaction is carried out in a single capillary in special equipment, such as a genetic analyzer
4. **Laser excitation:** a laser excites the fluorescent label at the end of each fragment to determine nucleotide identity

In the end, you can think of Sanger sequencing results as placing DNA fragments side by side- the shortest fragment (for example, AAGC) would be near the beginning of the sequence, and a longer fragment (such as AAGCTACGTA) would be further down the sequence. Each fragment increases in length by one nucleotide- a ddNTP with a fluorescent label. A laser excites that fluorescent label and special equipment, such as a genetic analyzer, can complete steps 3 and 4 to generate a file with a DNA sequence! Although genetic analyzers and similar equipment can be used in the lab, today it is more common to outsource Sanger sequencing to companies that specialize in this and other molecular biology services. Today in lab, you will use the OT-2 to prepare your samples for Sanger sequencing before sending them in the mail.

You can read more about Sanger sequencing in the following articles:

- Sanger sequencing — National Genetics Education Programme
- How Does Sanger Sequencing Work? - ThermoFisher Behind the Bench

Purpose

Today in lab, you will use the OT-2 to prepare plasmids and/or PCR fragments for Sanger sequencing at Genewiz. This experiment allows you to confirm results of your cloning experiment, and final Sanger sequencing data can be used in your final lab report.

Learning Outcomes:

- Be able to prepare samples and use a common molecular biology service
- Understand the principles of Sanger sequencing
- Analyze, interpret, and troubleshoot experimental results

Supplies

Opentrons Equipment

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

Opentrons Protocol

- ☐ Sanger Sequencing Preparation protocol

Non-Opentrons Equipment

- [ ] Tabletop microcentrifuge

Labware

- [ ] Opentrons OT-2 96 Filter Tip Rack 20 µL (in deck slot 1)
- [ ] Opentrons 96 Well Aluminum Block with NEST Well Plate 100 µL in deck slot 2
- [ ] Opentrons 96 Well Aluminum Block with Generic PCR Strip 200 µL tubes on the Temperature Module in deck slot 3
- [ ] Opentrons 24 Well Aluminum Block with NEST 1.5 mL Screwcap tubes in deck slot 6

Reagents and Other Materials

- [ ] Assembled plasmid products in *E. coli* culture
- [ ] DNAse- and RNAse-free 1.5 mL snapcap tubes appropriate for classroom microcentrifuge
- [ ] Primers (10 µM working stock dilution)
- [ ] Molecular grade water, such as HyClone Water
- [ ] 96-100% ethanol for use in plasmid purification

- ☐ QIAprep Spin Miniprep Kit OR Syd Labs Spin Columns for Plasmid Miniprep and reagents sufficient to make buffers for miniprep (see **Before Class** in the educator's **Procedure Guide**).

Procedure Guide

Before Class

1. Complete the pre-lab reading.

Harvest Bacterial Cultures

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

Spin Column Plasmid Purification

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

the sample. You should see the sample in your tube begin to become viscous. Do not mix for longer than 4-5 minutes.

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

10. Students should let the spin column (with molecular grade water added) stand for 1 minute, followed by centrifugation for 1 minute, to fully *elute* (remove bound DNA from the column) DNA.

Sanger Sequencing Sample Preparation

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7. Run your modified protocol.
8. The OT-2 will set the Temperature Module to 4 °C.
9. The OT-2 will transfer 10 µL of molecular grade water from the NEST 1.5 mL screwcap tubes to each required well of the generic PCR strip tubes in the Temperature Module.
10. The OT-2 will transfer 2.5 µL of primer from the NEST 1.5 mL screwcap tubes into each required well of the generic PCR strip tubes in the Temperature Module, combining primers and molecular grade water.
11. The OT-2 will transfer 2.5 uL of plasmid DNA template from the NEST 100 µL well plate to generic PCR strip tubes in the Temperature Module (to a final reaction volume of 15 µL).
12. The OT-2 will deactivate the Temperature Module and end the protocol.

GeneWiz Submission

1. Remove the PCR strip tubes from the Temperature Module. Carefully label the tubes and complete the online submission form.
2. Gently mix the contents of the tubes.
3. Place in a plastic bag labeled GeneWiz and drop off.

Discussion Questions

Discuss the lab activities with a neighbor.

- Describe in your own words the Sanger sequencing reaction that Genewiz will complete. What happens to your primer? The DNA template?
- What information will these results provide you that colony PCR *cannot?*