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Prep Sheet

16S PCR Setups

Authored by Carlos Goller, Ph.D.

Getting Started

Before teaching the lesson plan, complete the following steps prior to class.

- ☐ Unbox the OT-2
- ☐ Set up the Opentrons app
- ☐ Attach pipettes
- ☐ Calibrate the deck
- ☐ Calibrate tip length & pipette offset
- ☐ Import any related protocols to the app
- ☐ Test run the protocol on the OT-2

Need Additional Support?

For technical support, please check our Opentrons Help Center for relevant articles. If you need further support, please contact support@opentrons.com.

If you have questions related to the lesson plan, please reach out to the author, Carlos C. Goller, at ccgoller@ncsu.edu.

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Educator Guide

16S PCR Setup

Authored by Carlos Goller, Ph.D.

Contents

Included in this document are the following sections:

Purpose

Students will amplify the bacterial 16S rRNA gene using an automated liquid handling robot (OT-2) for PCR setup, gaining experience with:

- Basic practice with reagent preparations, manual pipetting, and centrifuge use
- Sanger sequencing technology
- Chromatogram analysis
- Running a robotics protocol to efficiently scale gene sequencing

Student Audience

This lab was modified from the course, Biotechnology and Sustainability (BIT 295), at North Carolina State University.

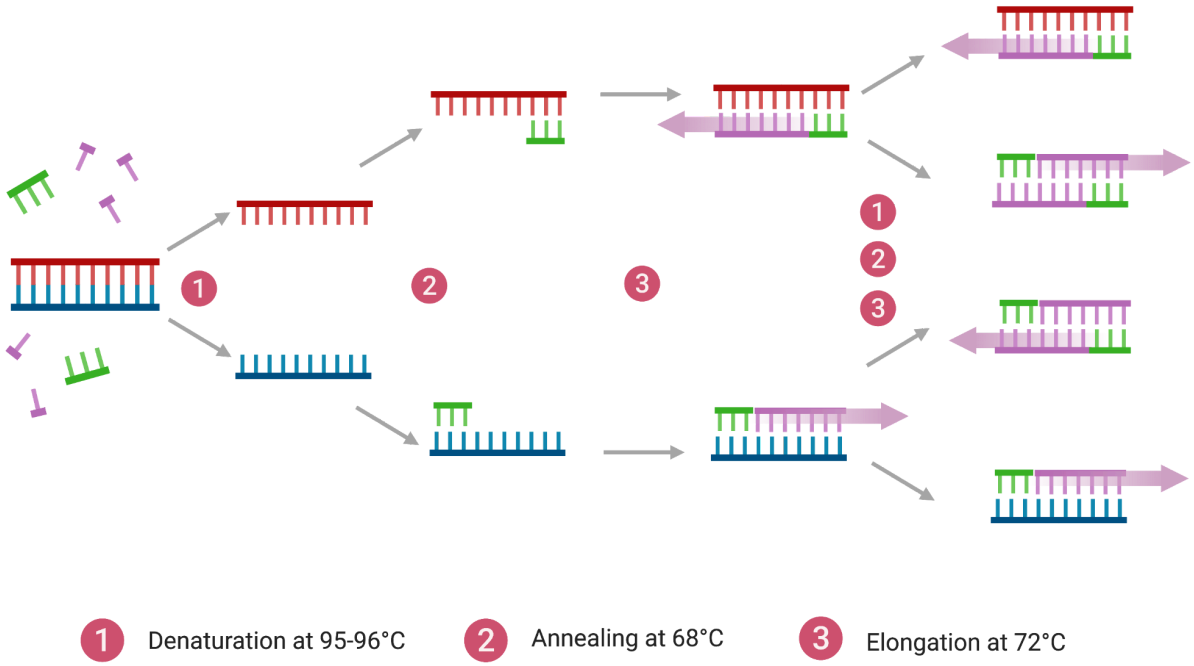
It is adapted for use in entry-to-mid-level undergraduate biology courses. It can accommodate class sizes of 12 working in groups and adapted for larger lab sections.

Background Knowledge

Students should start this lab with a conceptual understanding of pipetting, centrifuging, and interpreting agarose gel electrophoresis. An introduction to bacterial diversity and how the 16S rRNA gene can be used to classify organisms is helpful. We encourage educators to share information about how sequencing variable regions of the 16S rRNA gene can be utilized to classify organisms. The following script below may be useful:

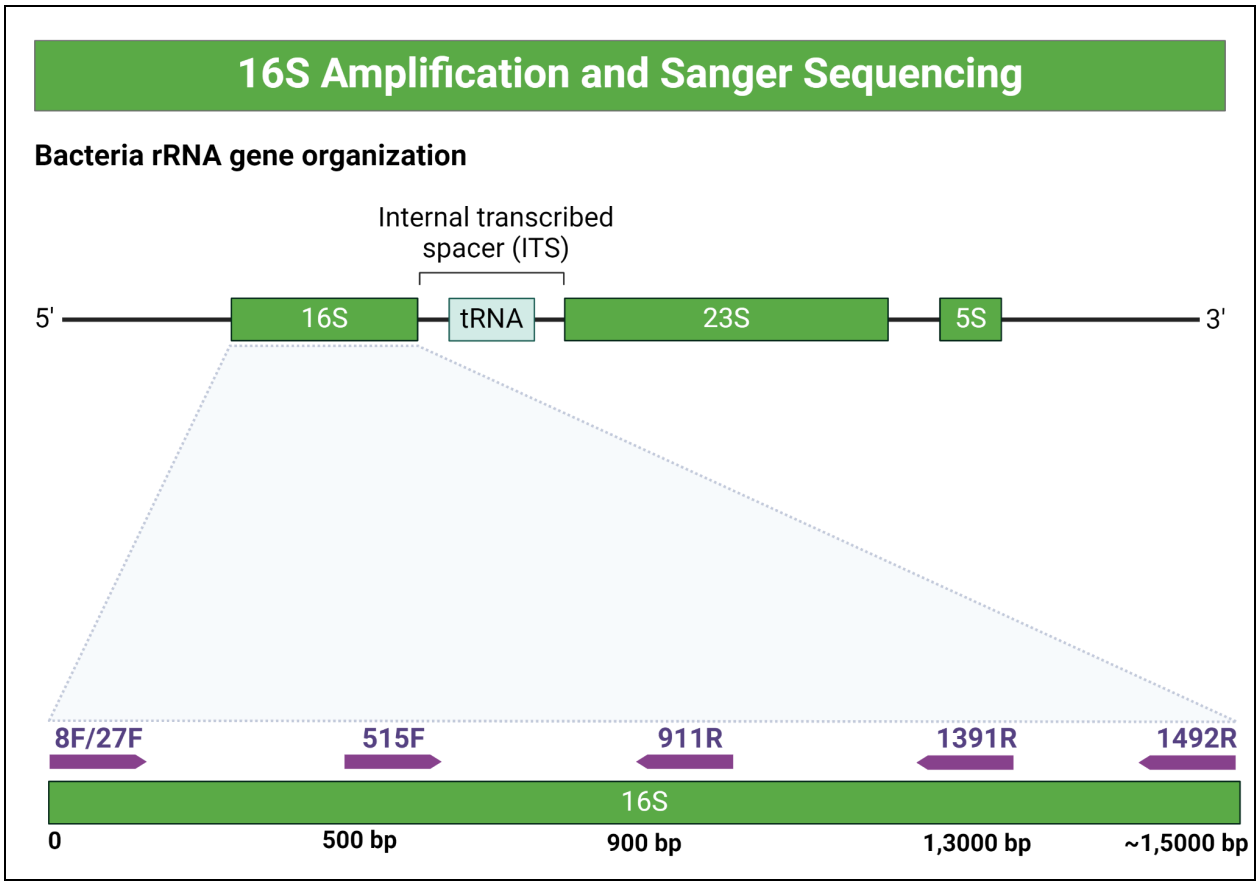
> *We will amplify the bacterial 16S gene, which encodes a subunit of ribosomes. Biologists use this gene to compare evolutionary relationships by sequence similarities and differences to organize biological taxonomies. Polymerase*

Chain Reaction (PCR) enables scientists to amplify many copies of a gene, and we will target 16S for amplification.



PCR Cycles Diagram indicating the three main steps: denaturation at 95-96°C, annealing at 68°C, and elongation at 72°C. Created with BioRender.com

You can learn about this reaction by watching the video "Polymerase Chain Reaction."



Bacterial 16S Gene Schematic for Amplification and Sanger Sequencing. The structure of the 16S rRNA gene is presented along with the primers and their annealing sites. Created with BioRender.com

Temperature (°C)	Time	Repetitions
98°C	30 seconds	1X
98°C	10 seconds	35 X
63°C	30 seconds	
72°C	45 seconds	
72°C	2 minutes	1X
4°C	Hold	Hold

Thermocycler protocol table.

Core Competencies

Laboratory skills

Manual pipetting, reagent preparation and enzyme handling, centrifuging, PCR and gel electrophoresis procedures, and the use of laboratory automation equipment**Critical thinking**

Interpretation of data, troubleshooting, chromatogram analysis, and drawing reasonable conclusions

Supplies

Opentrons Protocol

- ☐ Download protocol from https://library.opentrons.com/p/pcr_amplification

Opentrons Equipment

- ☐ Opentrons OT-2 automated liquid handling robot
- ☐ Opentrons 20uL tip rack
- ☐ Opentrons 300uL tip rack
- ☐ Opentrons 24 tube rack using Eppendorf 1.5mL Snap Cap Tubes

- [ ] Opentrons P20 Single Channel Pipettes, Gen 2
- [ ] Opentrons P300 Single Channel Pipettes, Gen 2

Non-Opentrons Equipment

- [ ] BioRad CFX 96 Thermocycler
- [ ] Electrophoresis materials
- [ ] PCR cleanup reagents (for example, QIAGEN spin columns or NEB PCR Clean-up kit)

Labware

- [ ] Microfuge tubes (Eppendorf DNA LoBind 1.5 ml)
- [ ] PCR plate or strips (Eppendorf or BioRad)

Reagents

- [ ] NEB Q5 Hot Start High-Fidelity 2X Master Mix, 25 µL per reaction (M0494S). DNA polymerase master mix (contains proof-reading) DNA polymerase, dNTPs, buffer, Mg2+), 25 µL per student

- [ ] 10 µMForward Primer, 2.5 µL per student

  27fwd 5′ `AGA GTT TGA pTCM TGG CTC AG` 3′
- [ ] 10 µMReverse Primer, 2.5 µL per student′

  1492rev 5′ `CGG TTA CCT TGT TAC GAC TT` 3′
- [ ] Nuclease-free water, 17.5 µL per student
- [ ] gDNA (at least 5 ng/µl ng/µL), 2.5 µL per student

Adapted from: [Identification of unknown bacterial isolates using Sanger sequencing of the 16S rRNA gene | CHMI services]

Experimental Duration

Required Class Sessions

2

Lab Run Time

Total time: This lesson plan was prepared for a course with limited lab time, 1:15 per session. Thus, students arrive to class with the OT-2 set up and ready to take their genomic DNA and prepare 16S PCR reactions.

Basic Troubleshooting

1. Do a trial run before class; this way any unexpected occurrences can be resolved before students arrive.
2. Issues with tips striking plates are almost always due to using alternate labware or robot calibration. If you experience this and have confirmed the correct labware, try re-calibrating the robot.
3. If you need to reach out to Opentrons Support, please inform them that you are part of our Opentrons for Education program and the date of your next lab class.

Required Pre-Lab Activities

Prior to starting this lab, students should have the following technical abilities and theoretical knowledge:

- Pipetting basics, including loading tips and making volume adjustments for manual pipettes
- Theoretical knowledge of PCRs and gel electrophoresis

Procedure Guide

Session One

1. Lab Introduction ~ 10 minutes

Prior to the lab, students should review the lab protocol and be ready to begin. The OT-2 should be set up and basic troubleshooting should be conducted to ensure class time is used effectively.

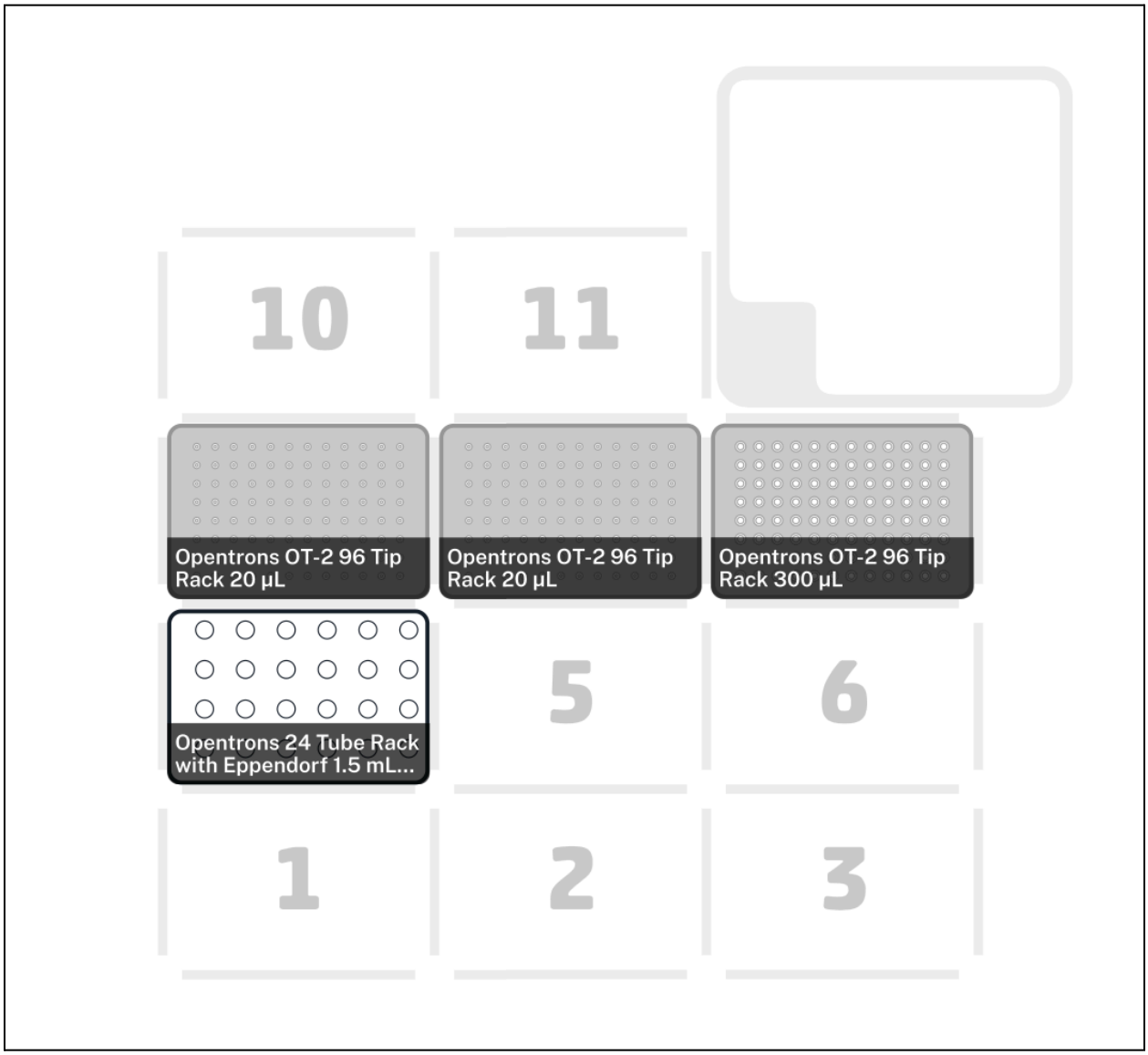
During the lab introduction, plan to briefly review the lab protocol and answer student questions.

2. 16S PCR Setup (Part A) ~ 45 minutes

During this portion of the lab, students will be working with the OT-2 and Thermocycler. You may wish to help students one-on-one as they operate the OT-2.

Students will be using the following protocol:

1. Please label your genomic DNA tubes with all the details.
2. The OT-2 deck should be laid out as follows (see image on following page):
    a. Load p20 single pipette on the right.
    b. Load p300 single pipette on the left.
    c. Load Opentrons 20uL Tip Racks on deck slots 7 & 8.
    d. Load Opentrons 300uL Tip Rack on deck slot 9.
    e. Load Opentrons Tube Rack on deck slot 4.



3. Run the OT-2 automated liquid handler protocol script to prepare reagents for the PCR reaction.
    a. You will set up two mastermixes, aliquot them, and make a dilution using nuclease-free water to obtain at least 20 µl of 5 ng/µl genomic DNA.
4. For each gDNA sample, set up two reactions:

a. One LONG reaction with primers 27fwd + 1492rev
b. One SHORT reaction with 515fwd + 1492rev

Primer name	Primer sequence 5'→3'
27fwd	AGA GTT TGA TCM TGG CTC AG
515fwd	GTG CCA GCM GCC GCG GTA A
1492rev	CGG TTA CCT TGT TAC GAC TT

5. Use the following thermocycler conditions:
    a. 98°C, 30 seconds
    b. 98°C, 10 seconds
    c. 63°C, 30 seconds
    d. 72°C, 45 seconds
    e. Repeat steps b-d 35 times
    f. 72°C, 2 minutes
    g. 4°C, HOLD

3. PCR Clean Up (Part B) ~ 15 minutes

In Part B, students will clean up amplified samples and properly store them for lab session two.

1. Add all 50 µL of the PCR reaction to five volumes (250 µL) PB buffer.
2. Mix and transfer to the pink spin column.

3. Centrifuge at 10,000 rpm for 30 sec.
4. Remove flow through.
5. Add 750µL PE to wash the column. Incubate at RT for 1 minute.
6. Centrifuge at 10,000 rpm for 30 sec.
7. Remove spin column from collection tube and add to a new wash tube.
8. Centrifuge at 10,000 rpm for 30 sec to remove all ethanol wash.
9. Remove spin column from collection tube and add to a new collection tube.
10. Add 50 µL EB to elute. Incubate at RT for 1 minute.
11. Centrifuge at 10,000 rpm for 30 sec.
12. Remove spin column and throw away.
13. Quantify 2 µl using the NanoDrop that has been blanked with the elution buffer (EB).
14. DNA should be stored at -20°C until the following session.

Session Two

1. Lab Introduction ~ 10 minutes

Review session one, review theoretical knowledge of Sanger Sequencing, and leave a few moments for questions.

2. **Sanger Sequencing Sample Preparations (Part C) ~ 15 minutes**

During Part C, students will prep their samples for Sanger Sequencing:

1. Start by sending out 20 µL of ~100 ng/µL DNA of your SHORT amplicon for two sequencing reactions with the three primers 27fwd, 515 fwd, and 1492rev.
2. Prepare 10 µL a 1:10 dilution of your 100 µM primers.

3. **Analyze Chromatograms (Part D) ~ 45 Minutes**

Lastly, students will BLAST 16S sequences to identify bacterial species or related species.

4. **Review Lab Report ~ 5 Minutes**

Briefly prepare students for their lab reports, leaving a few moments for questions.

Lab Report

Instructions

Assign students to prepare a comprehensive lab report. Provide guidelines for report structure and data presentation. Some ideas are included below:

- Why might lab automation be helpful for sample preparation? Give at least two reasons.

- Why do you think we use two forward primers and one reverse primer for this protocol?

- Why are stage b-d of the thermocycler conditions repeated 35 times? Would more or fewer repetitions change the results? If so, how?

- What is Sanger DNA sequencing, and why would it require “clean” DNA?

- Why might we want overlapping sequences between our primers?

- What applications could this lab have in “real life?” Give two examples.

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Student Guide

16S PCR Setup

Authored by Carlos Goller, Ph.D.

Purpose

Background: The bacterial gene 16S encodes a subunit of ribosomes. This gene is used by biologists to compare evolutionary relationships by sequence similarities and differences to organize biological taxonomies. Polymerase Chain Reaction (PCR) enables scientists to amplify many copies of a gene.

In this lab, you will amplify the bacterial 16S gene using automated liquid handling robotics (OT-2), gaining experience with:

- Basic practice with reagent preparations, manual pipetting, and centrifuge use
- Nanopore and Sanger sequencing technology
- Chromatogram analysis
- Running robotics protocol to efficiently scale gene sequencing

Required Equipment

Opentrons Protocol

- ☐ Download protocol from https://library.opentrons.com/p/pcr_amplification

Opentrons Equipment

- ☐ Opentrons OT-2 automated liquid handling robot
- ☐ Opentrons 20uL tip rack
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- ☐ Opentrons 24 tube rack using Eppendorf 1.5mL Snap Cap Tubes
- ☐ Opentrons P20 Single Channel Pipettes, Gen 2
- ☐ Opentrons P300 Single Channel Pipettes, Gen 2

Non-Opentrons Equipment

- ☐ BioRad CFX 96 Thermocycler
- ☐ Electrophoresis materials
- ☐ PCR cleanup reagents (for example, QIAGEN spin columns or NEB PCR Clean-up kit)

Labware

- ☐ Microfuge tubes (Eppendorf DNA LoBind 1.5 ml)

- [ ] PCR plate or strips (Eppendorf or BioRad)

Reagents

- [ ] NEB Q5 Hot Start High-Fidelity 2X Master Mix, 25 µL per reaction (M0494S). DNA polymerase master mix (contains proof-reading) DNA polymerase, dNTPs, buffer, Mg2+), 25 µL per student
- [ ] 10 µMForward Primer, 2.5 µL per student

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- [ ] gDNA (at least 5 ng/µl ng/µL), 2.5 µL per student

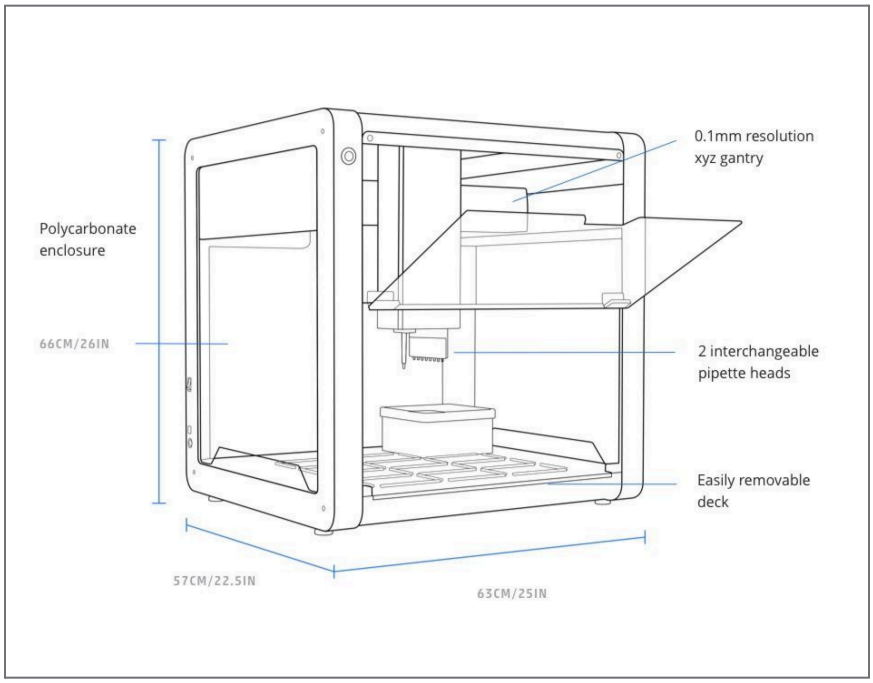
Adapted from: [Identification of unknown bacterial isolates using Sanger sequencing of the 16S rRNA gene | CHMI services]

Experimental Procedure

Session One

Part A: 16S PCR Setup

During Part A, you will be using the OT-2 automated liquid handling robot. Here is a diagram that will help you understand the OT-2 anatomy you should be aware of:



Overview of OT-2

OT-2 deck layout at start of protocol

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2. The OT-2 deck should be laid out as follows (see diagram on following page):
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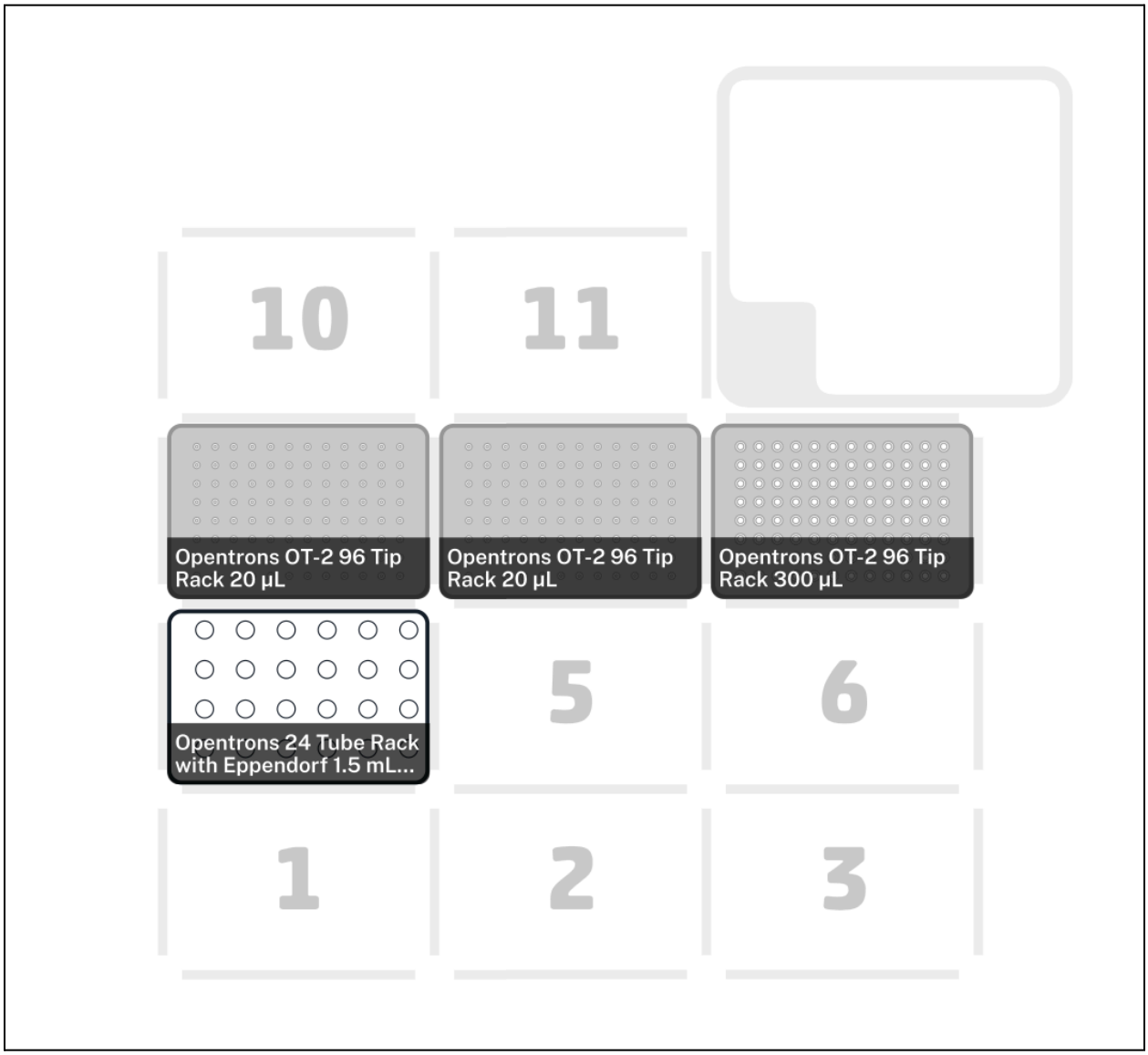


3. Run the OT-2 automated liquid handler protocol script to prepare reagents for the PCR reaction.

a. You will set up two mastermixes, aliquot them, and make a dilution using nuclease-free water to obtain at least 20 µl of 5 ng/µl genomic DNA.

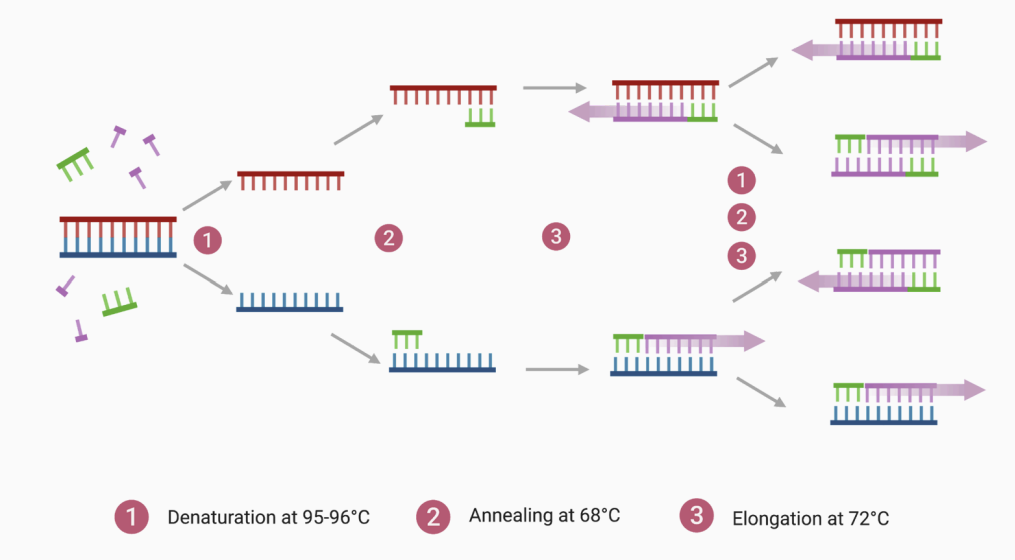
4. For each gDNA sample, set up two reactions:
    a. One LONG reaction with primers 27fwd + 1492rev
    b. One SHORT reaction with 515fwd + 1492rev

Primer name	**Primer sequence 5'→3'**
27fwd	AGA GTT TGA TCM TGG CTC AG
515fwd	GTG CCA GCM GCC GCG GTA A
1492rev	CGG TTA CCT TGT TAC GAC TT

5. Use the following thermocycler conditions:
    a. 98°C, 30 seconds
    b. 98°C, 10 seconds
    c. 63°C, 30 seconds
    d. 72°C, 45 seconds
    e. Repeat steps b-d 35 times
    f. 72°C, 2 minutes
    g. 4°C, HOLD

Why do we do this? This diagram (created with BioRender) indicates the temperatures at which denaturing, annealing,

and elongation occur:



Created with BioRender.com

Part B: PCR Clean Up

In order to remove the reagents from the PCR and isolate pure DNA for Sanger sequencing, we need to "clean up" our amplified DNA samples. To create this lab, we use reagents from QIAGEN DNA Clean Up Kit.

1. Add all 50 µL of the PCR reaction to five volumes (250 µL) PB buffer.
2. Mix and transfer to the pink spin column.
3. Centrifuge at 10,000 rpm for 30 sec.
4. Remove flow through.

5. Add 750µL PE to wash the column. Incubate at RT for 1 minute.
6. Centrifuge at 10,000 rpm for 30 sec.
7. Remove spin column from collection tube and add to a new wash tube.
8. Centrifuge at 10,000 rpm for 30 sec to remove all ethanol wash.
9. Remove spin column from collection tube and add to a new collection tube.
10. Add 50 µL EB to elute. Incubate at RT for 1 minute.
11. Centrifuge at 10,000 rpm for 30 sec.
12. Remove spin column and throw away.
13. Quantify 2 µl using the NanoDrop that has been blanked with the elution buffer (EB).
14. DNA can be stored at 4°C or on ice while being used and should be stored long term at-20°C.

Session Two

Part C: Sanger Sequencing Sample Preparations

1. Start by sending out 20 µL of ~100 ng/µL DNA of your SHORT amplicon for two sequencing reactions with the three primers 27fwd, 515 fwd, and 1492rev.

2. Prepare 10 µL a 1:10 dilution of your 100 µM primers.
    a. Note: If you do not get clear chromatograms to interpret the sequence, next send out your SHORT amplicon for Sanger sequencing with only primers 515fwd and 1992rev. Then prep 10 µL a 1:10 dilution of your 100 µM primers.

Unlike Nanopore sequencing (whole genome sequencing), Sanger sequencing can be used to amplify one gene. These methods are contrasted below:

Nanopore Sequencing	**Sanger Sequencing**
High throughput (many genes at once)	Low throughput (one gene at a time)
Sequences gDNA directly	Sequences PCR amplified DNA
Ion current base calling	Fluorophore base calling

Part D: Analyze Chromatograms

BLAST 16S sequence to identify bacterial species or related species.