

Identifying Unknown Bacteria Using Biochemical and Molecular Methods

Credits:

This lab was created by Robert Kranz, Kathleen Weston-Hafer, and Eric Richards. The lab was developed and written by Kathleen Weston-Hafer. Specific protocols were optimized by Kathleen Weston-Hafer and Wilhelm Cruz. This document was written and assembled by April Bednarski.

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Correspondence:

April Bednarski: aprilb@biology2.wustl.edu

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Beginning of Instructor Pages

Purpose

The purpose of this lab is to introduce a variety of lab techniques to students working on the common problem of identifying an unknown bacterium. This lab helps students develop an understanding of the biochemical and molecular differences in bacteria and introduces the concept of identifying species based on characteristic gene sequences. Students work through two types of identification procedures, one classical and one involving DNA sequencing, then compare the results of the two methods.

Educational Context

The lab was created to accompany lecture topics in bacterial genetics and biochemistry. The main topics covered in lecture that relate to this lab are prokaryotic **replication**, **transcription**, and **translation**, **enzyme function**, and **cellular respiration**. This lab was tailored for second semester freshmen who are in their first semester of a three-semester introductory biology course. The first semester focuses on molecular biology, bacterial genetics, and introductory biochemistry. This lab was designed for 500 students split into lab sections of 20. However, this curriculum is easily adaptable to accommodate any number of students.

In this lab, students identify an unknown bacteria using a biochemical method and a molecular method. For the biochemical method, students use a combination of differential growth tests and enzyme tests developed for clinical use. For the molecular method, students PCR amplify and sequence the 16S rRNA gene from their bacteria, then use BLAST to search the bacterial database and identify the species that most closely matches their sequence results for this gene.

Summary

This section contains a brief summary of the exercises contained in this lab. More thorough discussion of the materials follow in the General Materials section. The detailed protocol for each exercise is in the Student Section.

Unknown bacteria are first collected by swabbing surfaces around and near the lab, then streaked on sterile LB agar plates and grown overnight in an incubator. A different “unknown number” is given to each place bacteria are collected. Students then use these plates to make their own “unknown plate” by streaking for single colonies.

In the first step in the biochemical identification, students use a single colony to streak an EMB-lactose agar plate to determine if their unknown is gram positive or gram negative. The EMB dye will enter the gram positive bacteria and inhibit growth, but gram negative bacteria are protected by their enhanced cell wall and will be able to grow on these plates. If any students are working with a gram positive unknown, they pair up with a student with a gram negative unknown since the following methods in this lab were developed for gram

negative bacteria only. In the next step, students determine if their bacteria are positive for cytochrome c oxidase. In this test, the students are using dry oxidase test slides and pipet a small amount of their unknown from a liquid culture. If the bacteria contain the enzyme, then a substrate of this enzyme on the slide will be converted to a purple product and a spot will appear. The results of this oxidase test determine if students use an Enterotube or an Oxi/Ferm tube in the next step. These tubes were developed for clinical use to identify bacteria. They contain thirteen compartments, each with a different type of media, which will test for the presence of a different enzyme or set of enzymes in the unknown bacteria. Students inoculate the compartments with their unknown bacteria and place the tubes in the 37°C incubator. After overnight incubation, students examine each compartment to determine the color of the media and look for gas production. Students compare the color of the compartments with a reference guide to determine if the color indicates a positive or negative result for the presence of that particular enzyme(s). Each positive result is used in generating a five-digit number. This five-digit number, or “biocode,” can then be looked up in either the Enterotube or Oxi/Ferm tube code book, as appropriate; the number will correspond to a species of bacteria that produces that particular combination of enzymes. Students will usually successfully identify their unknown bacteria on completion of this test.

The molecular identification protocol introduces students to PCR and cycle sequencing. Students first follow a simple protocol to isolate genomic DNA from their unknown bacteria. This protocol involves breaking the cells open with a series of freeze/thaw cycles, then centrifuging to remove cellular debris. Students then set up a PCR reaction to amplify a region of the 16S rRNA gene. The PCR product is cleaned up using an ExoSAP-IT kit, which cleaves excess primers and inactivates free nucleotides. The cleaned PCR product is then used as the template for a sequencing reaction. Students set up the sequencing reaction using BigDye reagents and the reactions are run in a thermocycler (PCR machine). The completed samples are then sent to a core facility to obtain the sequence. In the final exercise, students view the electropherograms from their sequencing reaction, then use the sequence in a BLAST search limited to a bacterial data base. Students identify their unknown bacteria by examining the top-scoring sequences from the BLAST search results.

Additional background information for the biochemical tests described here is best obtained from the product information guides from the manufacturers. Background and animations of PCR and DNA sequencing are available on the following Websites:

PCR:

<http://www.dnalc.org/ddnalc/resources/pcr.html>

Cycle sequencing:

<http://www.dnalc.org/ddnalc/resources/cycseq.html>

Sanger sequencing:

<http://www.dnalc.org/ddnalc/resources/sangerseq.html>

www.nslc.wustl.edu/elgin/genomics/

Select "Genome Sequencing Center Video Tour" in the first paragraph. This 30 min video provides a tour of the Washington University Genome Sequencing Center with explanations and animations of each step of the sequencing process, which includes PCR and cycle sequencing.

Time Table

The table below provides a general outline for student lab time to perform the experiments. The table does not include the time it may take in lab for students to view and discuss their results or to complete their lab reports.

Part 1	Activities	Unknown Preparation	Student Lab Time	Incubation/Reaction Time
Exercise 1	Streaking for Single Colonies	Lawn plate	10 min	Overnight, 37°C*
Exercise 2	EMB Analysis	Single colony	10 min	Overnight, 37°C*
Exercise 3	Oxidase Test	Liquid culture	10 min	20 seconds, room temperature
Exercise 4	Oxi/Ferm or Enterotube Test	Single colony	10 min	2 Days, 37°C*
Part 2				
Exercise 1	PCR	Liquid culture	90 min	3 hours, thermocycler*
Exercise 2	DNA Sequencing		45 min	Time varies**

* Can remove plates from incubator and store at 4°C until students can view results in lab

** Sample preparation, time required to obtain results, and retrieval guidelines will vary depending on what facility generates the sequencing results. Refer to the sequencing core facility you choose to use for more information.

Note: The lab is presented here with students performing one exercise during each lab period. However, if desired, students could view their EMB results and perform Exercises 3 and 4 from Part 1 and Exercise 1 from Part 2 on the same day.

Collection and Sample Preparation of Unknown Bacteria***

Use a sterile swab to collect bacteria from a commonly touched area in or near the lab. Some examples include elevator buttons, drinking fountains, toilet flushers, faucets in the bathroom, and doorknobs. Rub the swab onto a sterile LB/agar plate. Use a new sterile swab and plate for each location and write the location on the LB/agar plate. Place the plates in a 37°C incubator overnight. Students will use these plates to streak for single colonies in the first exercise.

***As written, this step is performed by the lab instructor, but if the class is small, this could also be performed by the students.

In order to prevent culturing possibly harmful bacteria, strains can be ordered from the American Type Culture Collection (ATCC), a nonprofit organization which provides strains at a reasonable cost for educational purposes. See www.atcc.org for more information. A list of strains used previously used in this lab along with their experimental results is provided in the following table.

Strain Table and Results

Bacteria	Gram	Oxidase	Biocode
<i>Enterobacter cloacae</i>	NEGATIVE	NEGATIVE	32163
<i>Serratia liquifaciens</i>	NEGATIVE	NEGATIVE	26061
<i>Escherichia coli</i>	NEGATIVE	NEGATIVE	26170
<i>Pseudomonas aeruginosa</i>	NEGATIVE	POSITIVE	30303
<i>Enterobacter agglomerans</i>	NEGATIVE	NEGATIVE	20100
<i>Alcaligenes faecalis</i>	NEGATIVE	POSITIVE	10001
<i>Klebsiella pneumoniae</i>	NEGATIVE	NEGATIVE	24373
<i>Enterobacter aerogenes</i>	NEGATIVE	NEGATIVE	36361

General Materials and Equipment:

micropipettors - 0-20 μL , 20-200 μL , and 100-1000 μL
sterile tips for micropipettors
markers for labeling
vortexer
microcentrifuge, 14,000rpm max speed, rotor holds 1.5 mL tubes
2 water baths
thermocycler
37 °C incubator with shaker
benchtop biohazard waste containers (or jars with 10% bleach)
wet and dry ice
ice buckets
sterile loops
microcentrifuge tubes (1.5 mL and 0.2 mL) and racks

Materials Preparation Directions (for 5 students or student groups – adjust as needed)

LB agar plates

10 g tryptone
5 g yeast extract
5 g sodium chloride
15 g agar
Dilute to 1 L. Autoclave, cool 5 min, then and pour into 15 mm petri dishes (~ 25 mL per plate) before completely cooled.

EMB + lactose plates

0.4 g Eosin Y
0.065 g Methylene Blue
5 g lactose
13.5 g agar
10 g pancreatic digest of casein
5 g sucrose
2 g K_2HPO_4
Dilute to 1 L. Autoclave, cool 5 min, then and pour into 15 mm petri dishes (25 mL per plate) before completely cooled.

LB sterile media

10 g tryptone
5 g yeast extract
5 g sodium chloride
Dilute to 1 L. Autoclave and store sterile at room temperature.

PCR mix

Component	Stock	Final	5 rxn
dH ₂ O			195.25 μ L
Taq buffer	10x	1x	25 μ L
MgCl ₂	50 mM	1.5 mM	7.5 μ L
dNTP's	10 mM	0.25 mM	6.25 μ L
Forward primer	20 μ M	0.4 μ M	5 μ L
Reverse primer	20 μ M	0.4 μ M	5 μ L
Taq polymerase	5 U/ μ L	0.02 U/ μ L	1 μ L

Student directions state to use 49 μ L of the above mix and 1 μ L of their prepared template DNA per PCR reaction.

Sequencing mix

Make a 1.6 μ M stock solution of the forward primer.
For each reaction, add 2 μ L of primer, 8 μ L of BigDye

Student directions state to add 10 μ L of their PCR products (after Exo-SAP IT protocol) to the Big Dye/primer mix.

Ordering Information (for 5 students or student groups – adjust as needed)

General chemicals not listed here can be purchased from Sigma-Aldrich
(www.sigmaaldrich.com)

General materials not listed here can be purchased from Fisher Scientific
(www.fishersci.com)

Oxidase test slides (need 5 slides)

Becton, Dickinson, and Company (www.bd.com)

Catalog number 231746

Enterotubes II (need 5 or less)

Becton, Dickinson, and Company (www.bd.com)

Catalog number 211832

Oxi/Ferm tubes II (need 5 or less)

Becton, Dickinson, and Company (www.bd.com)

Catalog number 212116

Enterotube Interpretation Guide (need 1)

Becton, Dickinson, and Company (www.bd.com)

Catalog number 243383

Oxi/Ferm Interpretation Guide (need 1)

Becton, Dickinson, and Company (www.bd.com)

Catalog number 243235

Forward and reverse primers for 16S rRNA gene (in *E. coli* K12) to give a 481 bp product

Forward sequence: CGG CCC AGA CTC CTA CGG GAG GCA GCA G

Reverse sequence: GCG TGG ACT ACC AGG GTA TCT AAT CC

Invitrogen Life Sciences Custom Primers (www.invitrogen.com/oligos)

(Order 40 nmoles of forward primer and 20 nmoles of reverse primer)

Taq polymerase and buffer (5 units)

Invitrogen (www.invitrogen.com)

Catalog number 18038-018

PCR-grade dH₂O (500 mL)

Invitrogen (www.invitrogen.com)

Catalog number 10977-015

Big Dye[®] Terminator v1.1 Cycle Sequencing Kit (1 kit)

Applied Biosystems (www.appliedbiosystems.com)

Product number 4336774

dNTP mix (10 mM) (10 µL)

Invitrogen (www.invitrogen.com)

Catalog number 18427-013

ExoSAP-IT (5 reactions)

GE Healthcare (Amersham Biosciences –

www.amershambiosciences.com)

Catalog number US78200

Instructor Preparation (for 5 students or student groups – adjust as needed)

Method 1

Exercise 1:

5 types of unknown bacteria streaked to create a lawn on sterile LB agar plates (prepare one unknown per student or student group)

5 LB/agar plates

15 sterile loops for streaking (each group uses 3)

Exercise 2:

Plates containing single colonies of unknown bacteria (prepared by students in Exercise 1)

5 EMB + lactose agar plates

5 LB agar plates

10 sterile loops

Exercise 3:

Prepare 4 mL sterile LB broth in culture tubes (one tube for each unknown). Label each tube with the unknown number, then inoculate with a colony from each unknown plate the day before the students meet again. Grow the samples overnight in a shaker at 37°C to an approximate OD of 2. These samples will be used for the oxidase test slide. Students only need 20 µL per test, so each 4 mL liquid culture can be re-used many times if needed. If desired, also prepare a liquid culture of a known oxidase positive strain and a known oxidase negative strain to use as controls in the oxidase test.

5 Oxidase test slides

Exercise 4:

Plates containing single colonies of the unknown bacteria (prepared by students in Exercise 1)

5 (or less) Enterotubes II and Oxi/Ferm Tubes II

5 (or less) Enterotubes and Oxi/Ferm Interpretation guides will be needed, but tubes must first incubate at 37°C overnight. When interpreting these results, it is useful for students to have a copy of the interpretation table that accompanies each kind of tube. This table contains columns labelled “reaction name,” “negative,” “positive,” “remarks,” and has a row corresponding to each

compartment. The remarks column contains information about the specific reaction for that compartment and how the color change or gas was produced. Using this table, students can learn more about the specific biochemical test in each compartment and get information about how to best interpret unclear results. For more information about how these tests were developed, see the references below:

Enterotubes:

MacFaddin, J.F. *Biochemical Tests for Identification of Medical Bacteria*, 2nd ed., Baltimore: Williams and Wilkins, 1980.

Farmer, J.J. et al., "New groups of Enterobacteriaceae," *Journal of Clinical Microbiology*, Vol.21, pp.46-76, 1985.

Oxi/Ferm tubes:

Gilardi, G.L.: *Nonfermentative Gram-Negative Rods. Laboratory Identification and Clinical Aspects*. Marcel Dekker Inc., 1985.

Lennette, E.H., Balows, A., Hausler, W.J., Jr., Shadomy, H.J. (ed.): *Manual of Clinical Microbiology* 4th ed. Washington, D.C.: American Society for Microbiology, 1985.

Method 2

Exercise 1:

Prepare liquid cultures of unknown bacteria as described in Part 1 Exercise 3 above. Students will use 500 μ L per PCR reaction.

245 μ L PCR mix – If preparing in advance, prepare as directed under General Materials, omitting Taq polymerase, and store at -20°C until ready to use. Add 1 μ L Taq polymerase to the mix immediately prior to use.

Taq polymerase
5, 0.2 mL microcentrifuge tubes
1.25 mL PCR-grade dH₂O
Dry ice
Water bath at 70°C

Exercise 2:

PCR samples from Part 2 Exercise 1

5, 0.2 mL microcentrifuge tubes, each with 2 μ L ExoSAP-IT
35 μ L dH₂O

Water baths at 37°C and 80°C
5, 0.2 mL microcentrifuge tubes, each with 10 µL Sequencing mix
Companies for DNA sequencing:

<http://www.genegateway.com/>

<http://www.genomex.com/>

Sample Data and Results

Unknown bacteria collected from drinking fountain handle:

Method 1:

EMB-lactose plate – see growth

Oxidase test slide – negative

Enterotube II – 24373

Method 2:

16S rRNA sequence –

```
CCGTGGTTGTTTGTGAGCTGGGGCTTGTGCGTGATGCAGCATGG
GGGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGG
AGGAAGGCGATAAGGTTAATAACCTTGTGCGATTGACGTTACCCGCAG
AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC
GGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACT
GCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAG
CGTGCGGGAGCAAACCGGATTAGATACCCTGGTAGTCCCACGC
```

Unknown is *Klebsiella pneumoniae*.

Unknown bacteria collected from elevator button:

Method 1:

EMB-lactose plate – see growth

Oxidase test slide – positive

OxiFerm tube - 00001

Method 2:

16S rRNA sequence –

```
TGGGAATTTTGGAAATGGGGGAAACCCTGATCAGCCTCCCGCGTGTAT
GATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGG
TATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAATAAGCAC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCCGAAA
GAAAGATGTGAAATCCCAGGGCTCAACCTTGGAAGTGCATTTTTAACT
GCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCA
GTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCC
CCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGCCACGCA
```

Unknown is *Alcaligenes faecalis*

Unknown bacteria collected from faucet in bathroom:

Method 1:

EMB-lactose plate – see growth

Oxidase test slide – positive

Oxi/Ferm tube – 30303

Method 2:

16S rRNA sequence –

```
GTACGCCTTCTTCGGATTGTTAGCCCCTTTTGTGGGTTACTGCTGTA
GTTATTTCCCTTGCTGTTTTGACGTTACCATCAGTATTAGCACCGGCTA
TCTTCGTGCCAGCAGCCGCGGTATTACGATGGGTGCAAGCGTTAATC
GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGA
TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAACTACTGA
GCTAGAGTACGGTAGAGGGTGGTGGAAATTTCTGTGTAGCGGTGAA
ATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTG
GACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTATGCAACGCAATTGGGGTCTCGTTTTTAGAAGG
GGGTTTCGTTAAAACTAAAGCGTTTGACGCTTTT
```

Unknown is *Pseudomonas aeruginosa*.

Some common mistakes:

Oxidase test:

It is common for students to obtain a false positive on the oxidase test slide if the slides are old and/or if students wait longer than about 2 min for the color to develop. It's useful to do this test in duplicate and to have a positive control for comparison. If students interpret this test incorrectly, they will get uninterpretable results from the Oxi/Ferm tube.

Identifying Unknown Bacteria Using Biochemical and Molecular Methods

Beginning of Student Pages

Method 1: Biochemical Identification of Unknown Bacteria

Background:

In this lab, you will begin the experiment by preparing an "unknown" bacterial sample for biochemical identification. There are many methods for identifying bacteria. Traditionally an observational and biochemical approach has been used. Simply looking at (and even smelling) a bacterial colony growing on an agar plate can give an experienced researcher clues to a bacterium's identity. Bacteria are categorized as "Gram Positive" or "Gram Negative" according to whether or not they are stained by a chemical dye, a common biochemical technique. (The basis for the differential Gram Stain is a difference in cell wall construction.) Which sugars bacteria ferment, which antibiotics they have resistance to and which enzymes they produce are all important identifying characteristics that can be reasonably easily tested.

Currently, molecular methods of identification are often used in addition to or instead of biochemical techniques. Molecular methods involve examining the DNA of the bacterium in question, either by using a technique to map certain important characteristics of an organism's genome, or by sequencing a portion of the organisms DNA. Results are then compared to a database of known bacteria, hopefully resulting in a "match" that allows identification. DNA sequencing has become so standard and straightforward that it is now often easier and quicker than traditional biochemical methods.

We will start at the beginning of the semester with samples of unknown bacteria that have been collected from various places. We have intentionally avoided collecting from places likely to harbor human pathogens (ie human bodies). We will first learn techniques for growing and studying bacteria in the laboratory. Next we will work on a biochemically based identification of the bacteria, testing growth characteristics, enzyme production and sugar fermentation. Last, we will amplify and sequence a specific gene from each unknown sample (the 16s rRNA gene). By comparing our sequence results to a database of known sequences, we will try to identify the unknown bacteria.

Exercise 1: Streaking plates for isolation of single colonies

For many experiments involving bacteria, it is imperative to use a bacterial culture in which all cells are genetically identical. Because all cells in a colony develop from a single cell, colonies are excellent sources of genetically pure bacterial stocks. Single isolated colonies can be obtained by streaking small volumes of a liquid culture onto a Petri plate containing nutrient media. During the procedure, single bacterial cells are spatially isolated from each other on the surface of the media. These cells then develop into isolated colonies that can be picked off the plate using a wire loop or any sterile instrument.

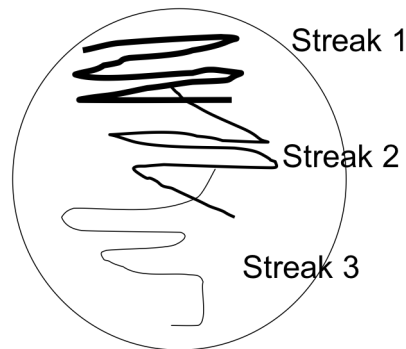
Streaking for isolation of single colonies is traditionally performed using a wire inoculating loop that is sterilized before and during the streaking procedure. Instead of the wire loop and flame, we will be using presterilized plastic loops. Your instructor will diagram the technique for you in the lab.

1. Label the bottom of an agar plate with your name, section letter and date.

2. Choose an "unknown" bacterial sample. Record the unknown bacteria number in **LINE 1** of your lab report and in the box below:

MY UNKNOWN BACTERIA NUMBER IS:

3. Open the petri dish of your unknown, without placing the top of the dish down on the bench top. Using the loop gently swipe across an area of bacteria to transfer the bacteria from the dish to your loop.
4. Gently streak the bacteria onto the agar surface of your labeled fresh plate as demonstrated by your lab instructor. You should make three successive streaks of bacteria. Dispose of the loop in the benchtop biohazard waste container and get a new one **before each new streak**. See the diagram below for an example:



5. You will examine your plate in lab next week. Things you should look for are: Can you see a progressive dilution? Did you isolate single colonies?

Exercise 2: EMB Analysis of Unknown Bacteria

For this experiment, you will transfer some of the bacteria you isolated last week in lab to a different kind of solid media (agar plate), EMB + lactose media. With this media we can determine which bacteria are Gram-negative and which are Gram-positive, because **only Gram-negative bacteria grow on this special media**. The enhanced cell wall of Gram-negative bacteria protect these bacteria from the dye in the EMB plates. The dye is able to enter the cells of Gram-positive bacteria and kill them.

1. Obtain the LB plate of your unknown bacteria that you prepared last week and an EMB+lactose plate. Label the new EMB+lactose plate with your unknown bacteria number.
2. On **LINE 2** of your lab report, illustrate the results of your plate from last week. Recall that you were streaking the bacteria onto the plate to isolate individual colonies. Answer **LINE 3** of your lab report.

3. Using a sterile loop, carefully select a bacterial colony and streak it on EMB + lactose plate.

Exercise 3: EMB + Lactose Results and Performing an Oxidase Test

1. Obtain your EMB + lactose plate that you prepared last week.
2. Observe the results on the EMB + lactose plate. Record your observations/results in **LINE 4 (a & b)** of your lab report.

After you have obtained the EMB + lactose results and concluded whether your bacteria is Gram-positive or Gram-negative, you will pair up with a lab partner to work together to further identify your unknown bacteria. However, for the remainder of the semester, your group will be working with a **Gram-negative** unknown bacteria strain. (The rest of the test we will do were developed specifically for Gram-negative bacteria.) So, if you concluded that your starting bacteria is Gram-positive, you must pair up with another student that has a Gram-negative bacteria. Record the unknown number of the Gram-negative bacteria number in **LINE 5** of your lab report and in the box below:

My Gram-negative, unknown bacteria number is



3. Obtain a liquid culture stock of your Gram-negative unknown bacteria.
4. Obtain a dry oxidase test slide. On each slide, there are four test areas. Therefore, up to four samples can be tested on one slide.
5. Transfer 20 µl of your liquid culture stock onto the center of the 2 test areas, noting which of the four test areas contains your sample. If any control samples are available, pipet them into the remaining test areas.
6. Incubate the test slide on your bench at room temperature for 20 seconds and no longer than 2 minutes.
7. Record the results of the oxidase test on **LINE 6** of your lab report. A blue/purple color indicates that the bacteria is oxidase positive while no color change indicates oxidase negative.

Exercise 4: Performing the Oxi/Ferm or Enterotube Test

1. Based on the results from the dry oxidase test slide last week, select either an Oxi/Ferm tube or an Enterotube to inoculate. If your unknown was oxidase positive, use the Oxi/Ferm tube. If your unknown was oxidase negative, use the Enterotube. Record which tube you used on **LINE 7, page 38**.
2. Inoculate your Enterotube or Oxi/Ferm tube as demonstrated in lab using an isolated colony from the LB plate of your unknown bacteria.

Exercise 5: Analyzing the Results of the Oxi/Ferm or Enterotube Test

Follow the directions given by your lab instructor for analyzing the results of your Enterotube or Oxi/Ferm tube. You will obtain a code number based on your results and then a bacterial strain name based on your code number. Record your results on **LINE 8 & 9** of your lab report.

NAME: _____

Lab Report: Biochemical Identification of Unknown Bacteria

1. Record your unknown bacteria number from week 1 here : _____
2. Diagram the results of your streaking for isolation below:

3. Did you isolate single colonies? _____ If not, is there anything you could do differently next time to increase your chances of isolating single colonies?

4. (a) Record the results of your replica plating. Did your bacteria grow on the EMB + lactose plate? _____

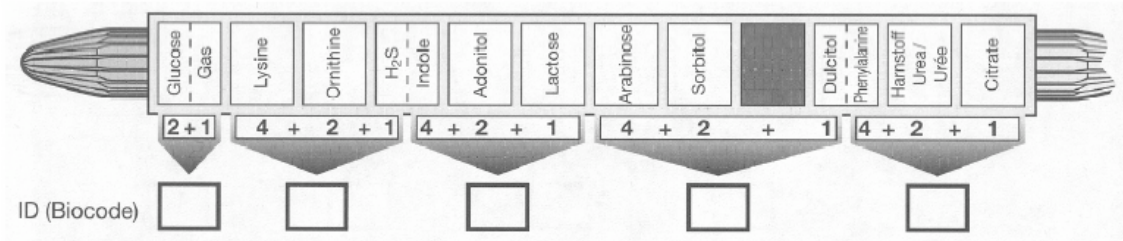
(b) What is the Gram classification of your unknown bacteria?

After you have obtained the EMB+lactose results and concluded whether your bacteria is Gram positive or Gram-negative, you will pair up with a lab partner to work together to further identify your unknown bacteria. However, for the remainder of the semester, your group will be working with a **Gram-negative** unknown bacteria strain. So, if you concluded that your starting bacteria is Gram-positive, you must pair up with another student that has a Gram-negative bacteria.

5. Record your unknown **Gram-negative** bacterial strain number here:

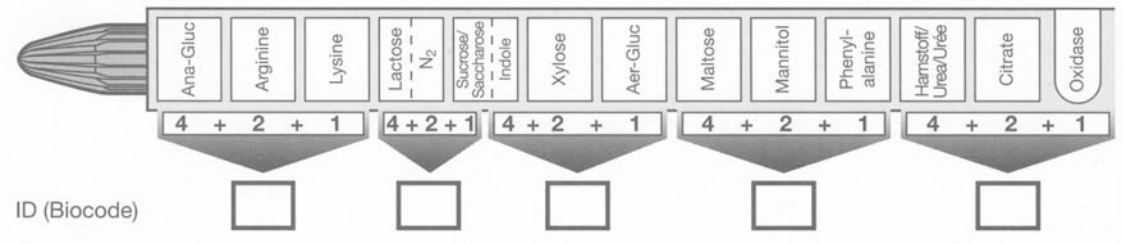
6. Record the results of your oxidase test here. Was your bacterial strain oxidase positive or oxidase negative? _____
7. Did you inoculate your unknown bacterial strain into an enterotube or an oxi/ferm tube? _____
8. What code number did you get from your enterotube or oxi/ferm tube?

ENTEROTUBE: Use this form if your bacteria is OXIDASE NEGATIVE



ID (Biocode)

OXI/FERM: Use this form if your bacteria is OXIDASE POSITIVE



ID (Biocode)

9. To which bacterial species does your code number correspond?

Method 2: Molecular Identification of Unknown Bacteria

Background:

This week you will set up a PCR (**P**olymerase **C**hain **R**eaction) amplification of the 16s rRNA gene of your unknown bacterial strain. We will be determining the DNA sequence of the 16s rRNA genes; by comparing the generated sequence to a database of known sequences, we will determine a "molecular identification" of the unknown bacteria.

Since its development in the 1980's, PCR has become a tool used almost universally by geneticists. PCR is used to quickly **amplify** specific regions of a particular segment of a DNA strand (creating millions of copies). PCR allows researchers to study very small amounts of DNA without resorting to laborious cloning procedures. The technique has had an impact in many areas of biology and has greatly facilitated the field of forensics.

PCR is a very simple reaction in both concept and practice. First, template DNA that contains some region of interest is isolated. The template does not need to be purified, and one can start with a very tiny amount of template. In this experiment, our source of template DNA will be the genome of your unknown bacterial strain. The experimenter does not need to have some prior sequence information about the segment to be amplified, because primers (for DNA replication) need to be used that are complementary to sequence on both sides of the segment to be amplified. In this experiment, we will be using "universal" 16s rRNA gene primers. Because we are hoping to amplify genes that are similar but not identical from the different bacteria, primers need to be designed to anneal to the parts of the DNA sequence that are most similar in all bacteria. The actual production of DNA is carried out by a DNA polymerase enzyme, which starts at the primers and uses the template to produce copies of the template sequence. Many copies of the template are created by repeating the reaction many times (many cycles). Your textbook should have a diagram of the PCR reaction. Additionally, a great animation is available at: www.dnalc.org/ddnalc/resources/pcr.html

After creating many copies of the 16S rRNA genes of your unknown, we will determine the sequence of the DNA using a modified "dideoxy" sequencing reaction. Once the DNA sequence is determined, we will compare the sequence to known sequences in a computer database. The 16S rRNA genes of many bacterial species have already been sequenced, so that it is likely that the sequence of the 16S rRNA gene of your unknown is in the database.

Exercise 1: PCR Amplification of the 16s rRNA gene (work in pairs)

A. Isolation of Template DNA

1. Pipet 500 μ l of overnight culture of your unknown bacteria into a 1.5 ml microcentrifuge tube.
2. Centrifuge at 13,000 rpm for 1 minute.

3. Remove the liquid supernatant from the tube and discard in the biohazard waste container on your bench.
4. Add 250 μ l of water to the cell pellet in the tube. Vortex well to resuspend the cells in the water.
5. Place the tube of cells in the dry ice bath for 3 minutes.
6. Transfer the tube to the 90°C water bath for 3 minutes.
7. Repeat steps 5 and 6 twice. (The freeze/thaw cycle lyses the cells and releases the template DNA into solution).
8. Centrifuge at 13,000 rpm for 1 minute.

B. PCR Amplification

1. Label the top of a sterile 0.2 mL microcentrifuge tube with your initials.

Pipet 49 μ l of the "PCR mix" in your ice bucket into the 0.2 mL microcentrifuge tube. The PCR mix contains the forward and reverse primers, dNTPs, Taq polymerase, MgCl₂ and PCR reaction buffer.

2. To the PCR mix add 1 μ L of the cell solution from step 7 above. (Note how little of the template solution we are adding.)
3. Your sample is now ready for amplification. Your instructor or TA will collect your reaction tube for amplification with the rest of the section. The reaction is conducted in a PCR machine (also known as a thermal cycler). The reaction will proceed as follows:

1 cycle	94°C for 3 minutes (Denature)
followed by 40 cycles	94°C for 1 minute (Denature)
	50°C for 1 minutes (Anneal)
	72°C for 1 minutes (Elongation)

Exercise 2: DNA Sequencing

In the last exercise, you used PCR to amplify a specific region of the 16s rRNA gene of your unknown bacteria. In this exercise, you will set up the reaction to generate the DNA sequence of the amplified DNA. The amplified fragment will be used as template in a DNA sequencing reaction. The actual DNA sequencing gel will be run and read at the Washington University Biology Department DNA sequencing facility.

The DNA sequencing reaction you will be using is actually quite similar to a PCR reaction. The DNA of interest will be used as a template, and DNA polymerase will copy the template to produce a new strand of DNA. The main difference will be the inclusion in

the DNA replication reaction of special nucleotides called 2', 3'-dideoxynucleotides. As the name implies, these nucleotides do not have an -OH group on either the 2' or 3' carbon. Whenever they are incorporated into a growing DNA strand, they are the last nucleotide incorporated, because there is no 3' -OH available for the next phosphodiester bond. If one has a way of identifying the different dideoxynucleotides (A or G or T or C), one can know the last base incorporated in any newly replicated strand of DNA. In our sequencing reactions, we will use dideoxynucleotides labeled with different colored fluorescent tags. Also, in DNA sequencing only one primer is used, so only one of the two strands is used as a template in the sequencing reaction.

Once the results of the DNA sequencing are known (next week), we will be able to search the database of known sequences for a match to your sequence.

A. Removal of free primer and nucleotides from the PCR reaction

1. Recall your PCR sample code number and obtain your PCR sample tube that you set up last week.
2. Obtain an "ExoSAP-IT" tube which is a small (0.2 ml), PCR reaction tube that has a red mark (dot) on the lid and is kept on ice.

This tube contains 2 μ l of ExoSAP-IT. ExoSAP-IT is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that removes left-over primers and free nucleotides from the PCR reaction. Having "left-over" components from the PCR reaction will complicate the sequencing reaction.

3. Transfer 5 μ l of your PCR product into the ExoSAP-IT tube.
4. Label this tube so that you can differentiate it from other tubes in the class.
5. Incubate your tube at 37 °C for 15 minutes to allow the degradation of primers and free nucleotides.
6. Transfer your tube to the 80 °C water bath and incubate for 15 minutes to inactivate the ExoSAP-IT enzymes.
7. Add 7 μ l of water to the tube. You are now ready to prepare your DNA sequencing reaction.

B. Cycle-Sequencing

1. Obtain a "Big Dye Terminator" tube from your lab instructor. This tube (which is kept on ice) is a small (0.2 ml), unmarked PCR reaction tube that contains 10 μ l of a pinkish solution. This pinkish solution consists of 2 μ l of primer and 8 μ l of BigDye Terminator Reagent.

2. Transfer 10 µl of your "clean" PCR product (from step 7 above) to the Big Dye Terminator tube. Flick the tube with your finger to mix the contents.
3. Label the Big Dye Terminator tube with your PCR sample code number and return this tube your lab instructor.
4. The samples will be run through a cycling protocol similar to the PCR protocol, purified to remove excess dyes, and sent to a sequencing facility to be run on a gel and read by the automated reader. We will have the sequence data back next week.

Exercise 3: BLAST Analysis of 16S rRNA Gene Sequence

BLAST (Basic Local Alignment Search Tool) is a web-based program that is able to align your search sequence to thousands of different sequences in a database (that you choose) and show you a list of the top matches. This program can search through a database of thousands of entries in under a minute. (The time will be longer if there are many users using the search program at the same time.) For this lab, you will use a database that contains all the bacterial sequences that have been published. BLAST performs its alignment by matching up each position of your search sequence to each position of the sequences in the database. For each position, BLAST gives a positive score if the nucleotides match. BLAST can also insert gaps when performing the alignment. Each gap inserted has a negative effect on the alignment score, but if enough nucleotides align as a result of the gap, this negative effect is overcome and the gap is accepted in the alignment. These scores are then used to calculate the alignment score in "bits" which is converted to the statistical E-value. A high bit score correlates with a low E-value. The lower the E-value, the more similar the sequence found in the data base is to your query sequence. When the results are given, the most similar sequence is the first result listed.

This program can be accessed at:

<http://www.ncbi.nlm.nih.gov/BLAST>

Reference: Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410.

NAME: _____

Lab Report: PCR Amplification and DNA Sequencing

In order to provide you with a thorough understanding of PCR, the following exercise leads you through three rounds of PCR from a hypothetical DNA template. Read carefully to ensure that you answer all of the questions. One strand of the double helix of DNA will be designated Original-1 (O1). While our starting template DNA is actually very long, we will show only a short sequence for convenience.

- Write the nucleotide sequence of the complementary strand designated Original-2 (O2).

(O1) Original-1:	5'-	C	G	T	A	A	T	G	T	A	T	C	A	T	G	G	C	T	T	C	A	G	C	C	T	A	-3'
(O2) Original-2:	3'-																										-5'

A chemically synthesized piece of DNA known as the primer is made that has a nucleotide sequence complementary to the bases adjacent to the segment of interest on the 3' end of Original-1. Another primer is made that has a nucleotide sequence complementary to the bases adjacent to the segment of interest on the 3' end of Original-2. The primer sequences are shown in bold. **(Note: In experiments primers are typically about 20 nucleotides long, not as short as shown here. This is important since a 5 bp sequence is much less likely than a 20 bp sequence to be unique to the DNA of interest.)**

In cycle 1, and in all subsequent cycles of the PCR reaction, a copy of each of the two original strands will be made beginning at the 3' end of the primer and continuing to the 5' end of the original strand.

- Write the sequence of the copies that are made from the strands of (O1) and (O2) in the blanks below.

(O1) Original-1:	5'-	C	G	T	A	A	T	G	T	A	T	C	A	T	G	G	C	T	T	C	A	G	C	C	T	A	-3'
(C1) Copy of O1:	3'-																					G	T	C	G	G	-5'

(O2) Original-2:	3'-	G	C	A	T	T	A	C	A	T	A	G	T	A	C	C	G	A	A	G	T	C	G	G	A	T	-5'
(C2) Copy of O2:	5'-			T	A	A	T	G																			-3'

During the second cycle of PCR, a copy is made of each of the strands of Original-1, Copy-1 (C1), Original-2, and Copy-2 (C2) obtained in cycle 1.

3a. In the appropriate blanks below, write the sequence of C1 formed during the replication of O1 in cycle 2.

(O1) Original-1:	5'	C	G	T	A	A	T	G	T	A	T	C	A	T	G	G	C	T	T	C	A	G	C	C	T	A	-3'
(C1) Copy of O1:	3'																										-5'

3b. Does the sequence differ from that of C1 made in the first cycle? _____

3c. In the appropriate blanks below, write the sequence of C2 formed during the replication of O2 in the second cycle.

(O2) Original-2:	3'	G	C	A	T	T	A	C	A	T	A	G	T	A	C	C	G	A	A	G	T	C	G	G	A	T	-5'
(C2) Copy of O2:	5'																										-3'

3d. Does the sequence differ from that of C2 made in the first cycle? _____

To make a copy of the C1 strand, a primer attaches to appropriate sequences on the strand. Note that only one of the two primers will be appropriate. The other primer will anneal to the C2 strand.

3e. Complete the sequence of (CC1) and (CC2) below:

Note: in subsequent steps all strands can be categorized as O, C or CC (1 or 2). Think about the size differences among these three choices.

(C1) Copy of O1:	3'	A	T	T	A	C	A	T	A	G	T	A	C	C	G	A	A	G	T	C	G	G	A	T	-5'
(CC1) Copy of C1	5'	T	A	A	T	G																			-3'

(C2) Copy of O2:	5'	C	G	T	A	A	T	G	T	A	T	C	A	T	G	G	C	T	T	C	A	G	C	C	-3'
(CC2) Copy of C2	3'																								-5'

4. How many strands of each of the following are present after the second cycle?

(O1)_____ (O2)_____

(C1)_____ (C2)_____

(CC1)_____ (CC2)_____

5a. For the third cycle of PCR, each of the eight strands present after the completion of Cycle 2 will act as a template and be replicated. Using CC1 and CC2 as your template, complete the sequences below *and* label the new strand that is produced in the blank space provided to the left of each sequence:

(CC1) Copy of C1:	5'	T	A	A	T	G	T	A	T	C	A	T	G	G	C	T	T	C	A	G	C	C	-3'	
	3'																		G	T	C	G	G	-5'

(CC2) Copy of C2:	3'	A	T	T	A	C	A	T	A	G	T	A	C	C	G	A	A	G	T	C	G	G	-5'	
	5'	T	A	A	T	G																		-3'

5b. In the blanks below, write the symbol of the strand produced by replication of O1, O2, C1, C2, CC1, and CC2. *Note that O, C, and CC are the only choices. CCC is not an acceptable choice. Think about the sequence and length of the strand produced.*

Replication of (O1) produces _____ Replication of (O2) produces _____

Replication of (C1) produces _____ Replication of (C2) produces _____

Replication of (CC1) produces _____ Replication of (CC2) produces _____

6. How many strands of each of the following types are present after the third cycle?

Total number _____

(O1) _____ (O2) _____

(C1) _____ (C2) _____

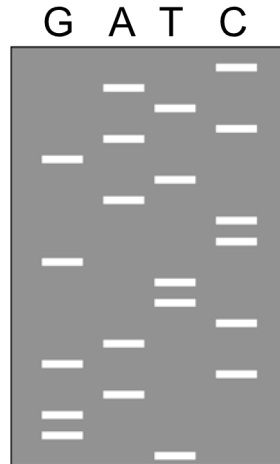
(CC1) _____ (CC2) _____

7. Review the numbers you have compiled so far, and deduce the patterns. Use this information to fill in the table below.

Cycle	Number of Strands						
	Total	O1	O2	C1	C2	CC1	CC2
0							
1							
2							
3							
4							
5							
10							
20							

8. Go to the website below to read about "traditional" Sanger dideoxy sequencing:
<http://www.dnalc.org/ddnalc/resources/sangerseq.html>

The following diagram represents what would be seen on an autoradiogram (film) at the end of the gel electrophoresis. Starting at the bottom of the gel, "read" the DNA sequence shown and record it below.



9. Think about the way DNA sequencing works. Does the sequence you wrote above represent the sequence of the template DNA or the newly synthesized strands? Provide a sketch showing the template, primer, and labeled product.
10. In lab we used a modified version of Sanger "dideoxy" sequencing called cycle sequencing. Use the website below to learn about cycle sequencing. Give two important similarities and two important differences between traditional dideoxy and cycle sequencing.

Cycle sequencing:

<http://www.dnalc.org/ddnalc/resources/cycseq.html>

NAME: _____

Lab Report: Molecular Identification of Bacterial Unknown

1. Examine the sequence from the 16s rRNA gene of your unknown bacteria. You can look at either the graphic (color peaks) or text version of the sequence. About how many bases of quality sequence did you get in your experiment?
2. Submit your sequence to the NCBI database using these directions and answer the following questions:

Go to the NCBI website (www.ncbi.nlm.nih.gov/BLAST)

Select the nucleotide-nucleotide BLAST (blastn)

Copy your sequence from the file then past it in the search box on the blastn website.

Click "BLAST," then on the next page, click "Format"

You will be asked to wait a few seconds while the program runs.

Your search results page should open. It will contain a graphic with several red horizontal lines representing homologous sequences.

Scroll to the text list of sequences below this graphic.

a) How many matches ("hits") did you obtain from the search?

b) What is the name of the organism with the best match to your sequence?

c) How good is the match between your sequence and the top match (score)?

- d) Give the name of one other organism that has a match to your sequence.
- e) Is sequencing the 16S rRNA gene a useful way to discriminate among bacteria?
3. What result did you get several weeks ago from the biochemical identification of your unknown bacteria? Did your sequencing results match the biochemical identification? Discuss, in general, why the results may not match and which technique you think is the best for identifying an unknown bacterial strain.