BI302 Clinical Microbiology Lab Manual

BI302 Clinical Microbiology Lab Manual

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Contents

1. Lab 1: Aseptic Technique

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to inoculate a broth, deep, plate and slant.
- Explain how aseptic technique prevents contamination, maintains culture purity, and why this is a key concept of microbiology.
- Apply microbial terminology to describe growth patterns on each media type.
- Determine when each media type should be used to cultivate bacteria.

Introduction

Aseptic Technique was developed in the early to mid-1800s through the efforts of such scientists as Robert Koch, Louis Pasteur, Ignaz Semmelweis, and Joseph Lister. As with many standardized methods in microbiology, Aseptic Technique has been modified over time as more knowledge was gained within the field of microbiology and culturing methodologies advanced. However, throughout its development, Aseptic Technique has maintained three essential goals:

- Prevent contamination (unwanted microbial growth) of specimen or culture
- Prevent contamination of self
- Prevent contamination of work area (the “lab space”)

In this lab, you will learn the methods we use in the microbiology

laboratory to maintain a clean environment and prevent contamination. These methods will include:

- Utilizing a good hand washing technique
- Disinfecting work surfaces before and after working with microbes
- Using a Bunsen burner or Bacti-cinerator to sterilize inoculating loops/ needles and test tubes or flasks
- Disposing of used or contaminated equipment and specimens properly

Media comes in four basic types – broths, deeps, plates, and slants. Plates (also called Petri plates), deeps and slants are all solid forms of media that rely on agar isolated from seaweed to provide a solid growth surface while broths are a liquid type of growth medium. It is important to note that bacterial cells will have different characteristics when grown on solid versus liquid media.

Colonies are formed only on solid media where cells can form visible masses on top of agar surfaces. Slants and plates differ in their surface area – slants will have a smaller surface area and a smaller exposure to the environment when the test tube is opened compared to the much larger surface area of plates. Because plates are the easiest form of media to contaminate, it is important to hold a plate in such as way as to minimize air exposure. Whereas Petri plates can be used to obtain isolated colonies, slants lack sufficient surface area to achieve isolation in most cases. Deeps, while a solid form of media, lack the ability to provide isolate colonies because growth only occurs along a narrow inoculation band termed the stab line or slightly away from the stab line in motile species. It should be noted that deeps will contain an oxygen gradient where oxygen is plentiful at the top at the deep but limited at the bottom of the deep due to poor oxygen diffusion across thick agars.

Growth in broths in indicated by a general cloudiness called **turbidity**. Cells may form clumps termed flocculent that are

suspended within the turbid broth. Oxygen-loving cells may cluster at the top of an inoculated broth and form a film on the broth surface termed a pellicle. Cells grown to saturation – that is cells that fall out of solution and settle in a precipitate at the bottom of tube – are referred to sediment. Sediment can usually be resuspended by lightly thumping the tube to remix the culture.

Overall, each form of media is used for:

- **Broths** – determining growth characteristics in a liquid culture and growing a large number of cells in a small space.
- **Deeps** – determining motility and oxygen usage of a culture. It should be noted that the mechanism of motility cannot be determined only that an organism is motile.
- **Plate** – isolation of colonies.
- **Slant** – long-term storage (several weeks – a few months) of cultures.

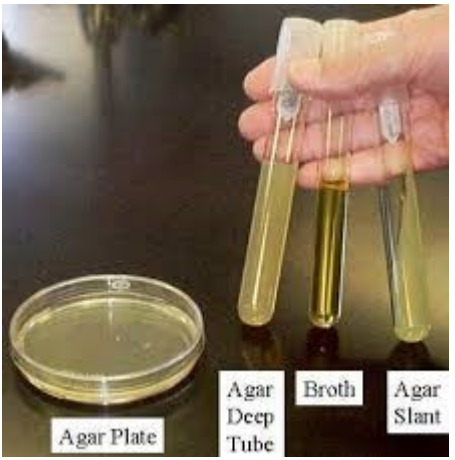

Figure 1. Types of Growth Media. Broths lack agar and are therefore liquid while slants, deeps, and plates all use agar to solidify the media. Image credit: Prokaryotes (Biology 102). Authored by: Michael J. Gregory, Ph.D. Provided by: LibreText. Located

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The specific introduction of an organism to a culture media is called inoculation. Aseptic Technique is used to inoculate media without contamination so that only the introduced organism is studied. Pure cultures are cultures that contain one organism (e.g., one genus species such as E. coli) while mixed cultures are cultures that contain multiple organisms intentional mixed. Examples of mixed cultures would include biofilms, most cultures isolated in nature, the human microbiome, and the culture used in next week's lab. It is important to observe media used in the lab before inoculating it – if any defect or growths are observed on or in media, please ask the instructor to confirm contamination. Inoculating a contaminated medium will only provide mixed results that cannot be used in the microbiology laboratory.

Method (Lab@Home)

For this lab you will need:

Prepared agar plates
Water
Cotton Swab

Candle
Disinfectant
Paper towels

Clean water
.Sharpie pen

Instructions for Aseptic Technique:

Before beginning this lab exercise, you should thoroughly wash your

hands with warm soap and water and wipe down your workspace with a disinfectant. If you have long hair or clothing, be sure to tie them back as you will be working close to a flame.

1. Prepare the lab work space by taking out the materials used in today's lab:

___ Experimental methods ___ Candle and lighter

___ Disinfectant & paper towels ___ Cotton swab

___ Sharpie ___ Clean water

___ Prepared agar plate ___ An area to work

2. Light the candle. You will want to work near a lit candle so that warm air currents are generated which will help to keep microbes away from your work space.

3. Dip a clean cotton swab into clean water and swab an area that you would like to **culture** (grow microbes). Example locations would include the skin behind your ear, a plant, or a pet's food bowl.

4. Transfer the microbes to the agar by gently streaking the swab across the surface of the agar. Be sure to hold the lid of the plate over the plate so as to minimize **contamination** (unwanted growth) from the air.

5. Label your plate with the date and what you swabbed on the bottom of the plate. You may want to put your plate in a ziplock back (partially sealed) and set the plate aside for a few days to grow. The use of a ziplock bag prevents the plate from drying out. Note: Plates are incubated "bottoms up" – that is the plate should be inverted so that the medium is upside down to prevent condensation from dropping onto the medium.

6. Clean up your work area by:

- Blowing out the candle
- Disinfecting your work space
- Washing your hands.

7. After your plate has growth on it, record the growth below in the Results section.

Notes about Labeling Media:

- Always label your plates/tubes BEFORE you start your inoculations. You can use Sharpies on the plates.
- Labels should include: your initials or name, the culture name, the inoculation date, and your lab section. (ex. LaB8.23.2021/ EC/ BI302-A)
- Make sure you label the **bottom** of the plates (the part of the plate that holds the agar).
- Place plates inverted (upside down) for incubation. This prevents condensation from falling on the surface of the agar and disrupting the streaking pattern.
- Below is an image showing how aseptic technique is done by flaming test tubes in the lab. While you won't be doing this in your home, it is good to note how the tubed media is handled a bit differently than agar plates.

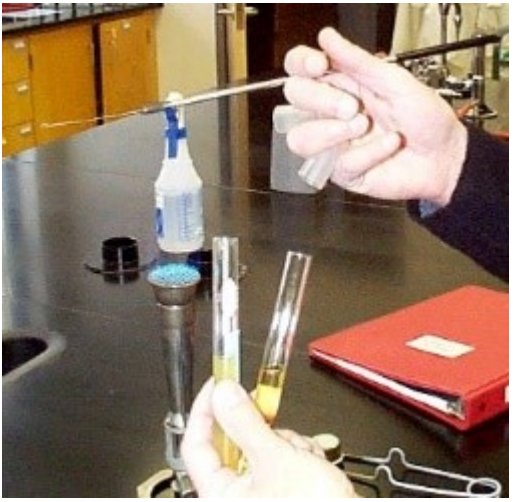

Figure 2 Transfer of a culture from a stock culture (broth) to a broth or slant. Image source: Prokaryotes (Biology 102). Authored by: Michael J. Gregory, Ph.D. Provided by: LibreText. Locatedat: http://bio.libretexts.org/Under_

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Results

Because microbes require time to grow, you will view your results in the next lab period.

Media Form	Growth (+/-)	Contamination	Growth Description
Plate			

Growth description terms:

Agars: Colony – be sure to include color, if colonies are isolated, and relative size

2. Lab 2: Isolation Streak and Types of Media

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to transfer microbes to different media types without contamination.
- Apply the isolation streak to separate a mixed culture into pure colonies.
- Describe when general purpose, chemically defined, enrichment, selective, and differential media should be used.
- Describe how the isolation streak method is an example of sample dilution.

Introduction

The isolation streak, like aseptic technique, is a key method in microbiology that is used to separate mixed cultures through a dilution process so that pure cultures can be obtained. In this lab, you will learn how to perform a four-quadrant isolation streak though it should be noted that two-, three-, and even five-quadrant streaks may be used within the clinical microbiology lab setting. The number of quadrants streaked is dependent upon the suspected number of bacteria in a sample – if a sample it though to have low numbers of bacteria (e.g. CSF sample) then a two-quadrant streak may be used verses a sample that has a high bacterial cell density (e.g. fecal sample) which may use a five-quadrant streak.

It should also be noted that bacterial growth can be further influenced by the type of culture media. Not only does culture media come in different forms (broths, deeps, plates, or slants) as

discussed in Lab: Aseptic Technique, but it also comes in different types or classifications. While media cannot exactly mimic the

- **Chemically defined (synthetic) media** – a media where the exactly chemical composition is known and ingredients are typically salts (not just NaCl). Chemically defined media are useful for determining the nutritional growth requirements of microbes.
- **General purpose (complex or rich) media** – a media where the exactly chemical composition is not known due to differences in some ingredients that can vary by preparation. General purpose media is often used to grow a wide variety of microbes and would include such media as nutrient broth/ agars or tryptic soy broths/ agars. A key give away that a medium is a general purpose medium would be the use of digests, tryptone, or extracts to make the media.

General purpose media and chemically defined media can be difficult to separate. The key is in that general purpose media will contain extracts, digests, or tryptone while chemically defined media are very detailed ingredients typically in the from of salts. Here's a comparison to help illustrate this difference.

Table 1. A comparison of a chemically defined Simmons Citrate Agar and general purpose Nutrient Agar.

Medium	Simmons Citrate Agar[1]	Nutrient Agar2
Type	Chemically defined	General purpose
Ingredients	Ammonium dihydrogen phosphate Dipotassium phosphate Sodium chloride Sodium citrate Magnesium sulfate Bromthymol blue Agar	Pancreatic digest of gelatin Beef extract Agar
What makes it the type	All ingredients with the exception are agar – that is cation + anion.	Digest and extract will vary by lot due to processing therefore the exact nutrients will not be the same each time the medium is made.

Sources: 1BD. (2015). BBL Simmons Citrate Agar Slants. 2BD. (2014). BBL Nutrient Agar product Information.

General purpose media can be further divided into more specific classifications based on the function of the media.

- **Differential media** allows for the classification or grouping of microbes based on their ability to utilize nutrients in the medium and/ or change colony appearance of the microorganism or of the medium. Common examples of Differential media that you may encounter are blood agar, eosin methylene blue (EMB) agar, MacConkey (MAC) agar, or mannitol salt agar (MSA).
- **Enrichment media** encourages the growth of some species

while omitting selective pressure on other species. In this media type, blood or serum products are frequently used to support the growth of fastidious organisms – those that grow only when specific nutrients are present. A good example of an enrichment medium would be blood agar or chocolate agar (blood agar that uses lysed blood).

- **Selective media** actively selects against some species by containing ingredients that limit or prohibit the growth of some microbes. Examples of selective media would be MSA, EMB, or MAC. For selective media, good growth will produce easy to see colonies while stunted growth will produce pinpoint sized colonies that show the organisms is having difficulty growing under selective pressure of the medium. Some organisms may not be able to grow at all under the selective pressure, thus producing no growth or no colonies.

You may have noticed that some agars such as blood agar, MSA, EMB, and MAC can serve more that one role. Let's take a look at how these media meet the criteria of being more than one type of general purpose media.

Table 2. Breakdown of differential, enrichment, and selective properties of selected medias.

Medium	Differential	Enrichment	Selective
Blood agar	Blood hemolysis pattern	Serum in blood	———
Chocolate agar	———	Serum in lysed blood	———
EMB	Lactose fermentation	———	Bile salts + eosin dye
MAC	Lactose fermentation	———	Bile salts + crystal violet
MSA	Mannitol fermentation	———	NaCl concentration

In today's lab, you will be using the four-quadrant isolation streak.

Method (Lab@Home)

For this lab you will need:

Petri plate
- Cotton swabs
- Sharpie marker

Candle
- Lighter or matches
- Paper towel

Instructions for Isolation Streak of Mixed culture onto Nutrient Agar.

Before beginning this lab exercise, you should thoroughly wash your hands with warm soap and water, wipe down your work area with a disinfectant, and light a candle.

Because you will be working around a flame, be sure to tie back any long hair or clothing.

1. **Label a NA plate with your culture information ("mixed culture"), date, and initials.** You may also find it helpful to label four quadrants on your plate I-IV while you are learning to streak for isolations.

- Using a clean cotton swab, collect a small amount of growth (colonies) from your bacterial plate from the last lab.
- Open the lid (the side without the growth medium) of the plate and carefully hold over the Petri plate while you streak the agar surface in the first quadrant. You should streak three or four times across the **first quadrant**. ****Agar can be somewhat soft – think of jello – try not to rip or tear the agar with your cotton swab.**** Replace the plate lid after inoculating.
- Set the used cotton swab on a piece of paper toweling to be disposed of at the end of the lab.
- Beginning in **first quadrant**, use a second cotton swab to pull cells into the **second quadrant**. You will streak two times across the **second quadrant** pulling cells from the **first quadrant**.
- Place the used cotton swab on the paper toweling.
- Beginning in **second quadrant**, use a third cotton swab to pull cells into the **third quadrant**. Again, you will streak two times across the **third quadrant** pulling cells from the **second quadrant**.
- Set aside the used (third) cotton swab for later disposal.
- Beginning in **third quadrant**, use a fourth clean cotton swab to pull cells into the **fourth quadrant**. Again, you will streak three or four times across the **fourth quadrant** pulling cells from the **third quadrant**. Be careful not to overlap the **first** and **fourth quadrant** as this often prevents isolated colonies from forming.
- Place the plate bottoms up (inverted) in a warm area where it can grow for 2-3 days.
- Wrap the used cotton swabs in the paper toweling. Wipe down

your work area with disinfectant. The paper toweling containing the cotton swabs can then be wrapped in the disinfectant wipe (or sprayed with disinfectant) before disposing of in the trash.

- Wash your hand and blow out the candle.

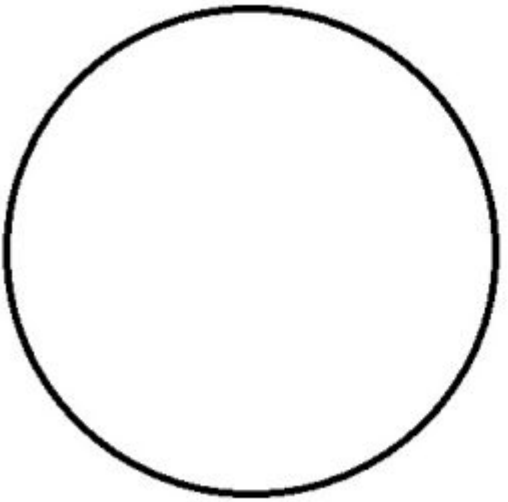
Figure 1. Four Quadrant Isolation Streak Method. Image source: James Gathany, CDC 2005. Image number 7925.

Results

Because microbes require time to grow, you will view your results in 2-3 days.

In the space below, draw a picture of your isolation streak (after it has grown). You may want to use colored pencils/ pens/ highlighters to distinguish between the different colonies.

Did you achieve isolation (of colonies)? Yes No



These data tables are included as a summary of the different media mentioned in this lab's background material. You will want to be familiar with these reactions to help answer questions on your lab report and lab quiz.

Table 1. Interpretation of the results from Selective and Differential Media Inoculations.

Plate	Selective Interpretation	Differential Interpretation
EMB	Gram negatives = growth	Metallic green, purple, black, or pink colonies = Lac fermented
	Gram positives = no growth or stunted colonies	Colorless colonies = no Lac fermented
MAC	Gram negatives = growth	Pink colonies = Lac fermented
	Gram positives = no growth or stunted colonies	Colorless or tan colonies = no Lac fermented
MSA	Halophiles = growth	Yellow ring around agar = Man fermented
	Nonhalophiles = no growth or stunted colonies	No change in media = no Man fermented

Table 2. Interpretation of the results from Enrichment and Differential Media Inoculations.

Medium	Enrichment	Differential
SBA	Growth (no selective pressure)	Hemolysis pattern • gamma hemolysis (no clearing) – agar is red around colony • beta hemolysis (complete clearing) – agar is yellow around colony • alpha hemolysis (partial clearing) – agar is greenish around colony

3. Lab 3: Enumeration and CFU/ml Calculation

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to prevent contamination during serial dilution.
- Use aseptic technique to capture a "clean catch" urine specimen.
- Explain serial dilution.
- Apply the CFU/ml calculation (CFU/ml, CFU/g, PFU/ml, or PFU/g) to cell counts obtained from a serially diluted stock culture or specimen to determine the total number of viable organisms in the specimen.
- Compare and contrast each of the following terms related to dilution and enumeration: isolation streak with serial dilution, total counts with viable counts.
- Calculate CFU/ml (CFU/g, PFU/ml, or PFU/g) based on experiment plate counts.

Introduction

Enumeration techniques allow for microbiologists to count the number of viable cells within a sample. Oftentimes samples will contain too many cells to count within the stock culture or original patient sample, thus requiring serial dilution before conducing cell counts. Common enumeration methods include:

- **Standard Plate Count Method** – which combines serial dilution with either spread plate technique or pour plate technique to serial dilute a sample and make it more

countable. This method allows for viable counts in that only living cells can divide and produce colonies.

- **Direct microscopic counts** – use a microscope and a Petroff Houser counting chamber which is a special microscope slide that contains a grid. A sample is loaded into the Petroff Houser counting chamber and a specific number of grids are counted which the total number of cells being counted in that number of grids and the average number of cells being used to calculate the cell density (number of cells) of the source specimen. Direct counts give total, rather than viable, cell counts because it is difficult to determine if all cells are alive within a sample under phase contrast microscopy, especially if the sample contains nonmotile cells.
- **Spectrophotometer measurements** – rely upon the use of a spectrophotometer to determine total cell counts within a sample. For best results, spectrophotometric assays should be combined with viable plate counts (e.g., the standard plate count method). A spectrophotometer works by detecting the amount of light that can pass through a sample – the more light that passes through the sample, the fewer cells are in that sample. Likewise, a dense sample that contains many cells would block most of the light from passing through there by giving a low percent transmission (%T).

Viable vs. total cell counts are helpful in determine effectiveness of treatment and stage of disease progression. Recall from lecture that pathogens would increase in cell number until the crisis point is reached. The crisis point will decide if the patient's immune system can overcome the infection, often with the use of chemotherapy (medication). If the patient's immune system cannot over the pathogen then pathogen numbers would continue to increase and the patient would not recover from illness. Likewise, scientists can use viable counts to determine if a chemotherapy or physical control method can reduce the number of cells within a microbial population. We'll explore this theme a bit later on in the semester

in the physical and chemical control labs. In terms of treatment studies, viable counts are of greater value than total cell counts.

The standard plate count method can use either pour plates or spread plates. The different lies in how the sample is inoculated into/ onto the agar and the amount of sample that can be inoculated. **Pour plates** use tempered agar which is still liquid but cooled to around 45-50^{0}C so as not to kill microbial cells when the sample is inoculated. Pour plates typically have 1ml of inoculum added to the molten agar which is then mixed and quickly poured into empty sterile petri plates. The plates are allowed to cool and solidify, which happens around 40^{0}C before inverting and incubating. **Spread plates** use traditional solid agar plates and have 0.1ml of inoculum added to the plate before the sample is spread or smeared across the agar surface using a **cell spreading rod** which is a bent rod made of autoclavable plastic, metal, or glass that is used to spread the inoculum across the surface of the plate. Spread plates only take a few seconds to "dry" once the inoculum has been smeared before the plates can be inverted and incubated. The amount of inoculum to be added to the plate often is the determining factor between spread plates and pour plates though other factors such as the amount of oxygen needed by cells within the sample should also be considered. Because cells will be embedded with the pour plate methods, this method would not be idea for strict or obligate aerobes and the spread plate method should be used instead.

Dilution allows for samples that contain a high number of cells to be reduced to countable numbers. Current CDC (2015) standards recommend that a plate count range of 25-250 cells per plate be used. This means that plates that have over 250 colonies are reported as either TMTC (too many to count) or TNTC (too numerous to count) due to the likelihood of missing a colony in the counting process as the plate would be overcrowded. Plates that contain less than 25 colonies would be reported as TFTC (too few

to count) due to the statistical likelihood that a dilution error would inflate counts.

It should be noted that the isolation streak, discussed in a previous lab, also allows for sample dilution. However, rather than depending on setting up a series of dilution tubes to achieve the dilution, the original source material (stock culture or specimen) is diluted as it is streaked across the surface of a plate.

While both plate counts and isolation streaks can be carried out on a variety of agar depending on the type of cell growth desired (refer to Lab: Aseptic Technique for a review of media types), specific general purpose agars such as PCA (plate count agar), TSA (tryptic soy agar), R2A (Reasoner's 2A agar), or NA (nutrient agar) tend to be used for the standard plate count method. These general purpose agars tend to have sufficient nutrients to grow most non-fastidious organisms within a sample. Of course, enrichment agars would need to be used for patient samples from throat, sputum, or blood specimens, and selective agars would need to be used for fecal samples.

Now that the basics of what a serial dilution is, what it does, and how to make one have been reviewed, let's discuss the CFU/ml calculation. It should be noted that **CFU/ml** is used to determine the number of viable bacterial cells within a liquid sample while CFU/g would be used to determine the number of viable bacterial cells within a solid sample. Likewise, bacteriophage counts can also be done from liquid cultures of bacteria (PFU/ml) and solid cultures of bacteria (PFU/g).

The equation for determining the counts/ volume (e.g., CFU/ml, CFU/g, PFU/ml, or PFU/g) is the same as are the counting limits (25-250 colonies for bacteria counts or plaques for phage counts) regardless of the original source material.

Equation 1: CFU/ml (CFU/g, PFU/ml, or PFU/g) Equation.

CFU/ml = average number of colony or plque counts(amount

plated*overall dilution)/average number of colony or plque counts(amount plated*overall dilution)

It should be noted that plate counts are most commonly done in replicates – that is the same dilution would be plated onto more than one plate so that an average count per dilution could be obtained. For the purposes of this lab, we will only be using one plate per dilution so the averaging of plate counts would not apply but you may need to average plates in CFU/ml problems on assessments.

Remember that the amount plated will vary depending on if spread plates (0.1ml plated) or pour plate (1.0ml plated) method is used.

The overall dilution would be the dilution of the sample plated compared to the stock culture.

Here is an example problem:

A urine specimen was obtained from a patient. It was noted that the specimen contained traces of blood and appeared turbid indicating infection. A culture and count were ordered. The urine specimen was streaked for isolation on the MAC (MacConkey agar) using a four quadrant isolation streak to determine if fecal contamination was present. One ml of the urine specimen was transferred to a 9ml dilution tube (**1:10 or 10-1 or 0.1ml**) was made and five subsequent dilutions were made in like manner. From each dilution, two plates were inoculated with 0.1ml and the plates were grown overnight at 37^0C before counting colonies. Colony counts are reported in Table 1. This dilution can be illustrated as follows in Table 1.

Page Break

Table 1. Serial Dilution of a Patient Urine Specimen.

Tube	Stock	I	S1	S2	S3	S4	S5
Amt. transferred to dilution blank	0ml	1ml	1ml	1ml	1ml	1ml	1ml
Dilution	0	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
Overall Dilution	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Amt. plated	0.1ml	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
Avg. colony counts	TMTC	TMTC	TMTC	218	27	TFTC	0

According to this data, plates from dilution S2 and S3 are within the countable range of 25-250 colonies so either of these counts could be used to determine the CFU/ml. It doesn't matter which dilution that is counted, they will be similar counts. Because bacterial populations can be quite large and change rapidly, counts that share the same power of 10 are considered "equal" in the sense that the counts are close enough to convey needed information without causing statistical problems. For example, a count of 1.37*105 and 7.8X*105 would both be useful in conveying that the cell density of the original urine specimen is 105.

Let's calculate the CFU/ml of this urine specimen using the S2 dilution where S2 has an average colony count of 218.

CFU/ml = 218/(10−1*10−3)

CFU/ml =218/(10−4)

CFU/ml = 218 * 10^4 [Remember that the exponent will change signs due to division.]

CFU/ml = 2.18*10^6 [It is conventional to write the answer in scientific notation.]

What would be the CFU/ml if the S3 dilution were used instead? Use the space below to calculate the CFU/ml using S3 counts.

It is important to practice aseptic technique in both obtaining a urine specimen and in processing a urine specimen. The **clean catch** technique is used to obtain a urine specimen for a non-catharized patient. If a patient is catharized then aseptic technique would have been met at the time of catheter insertion. For a urine specimen to be collected using the clean catch method, the area surrounding the urinary meatus (the area where urine leaves the body) should be cleaned with an antimicrobial or obstetrical wipe to remove any debris such as dead skin or microbial cells that could potentially contaminate and inflate bacterial counts. Once the urinary meatus is cleaned, a small amount of urine should be voided into the toilet before collecting a urine specimen. It should be noted that the entire urine specimen cup does not need to be filled – only enough to provide a sample for testing so aim for about 1/3 to 1/2 of the sample container.

Urine specimens can be used to determine quite a bit of information including, but certainly not limited to, leukocytes, urobilinogen, proteins, pH, blood, specific gravity, ketones, bilirubin, and sugars, all of which could indicate a diseased state.

In today's lab, we will be testing our urine specimens to fecal contamination using a MAC plate, counting bacterial cells in our clean catch samples to determine if infection is present and if we have carried out the clean catch method correctly, and testing urine for leukocytes, nitrates, urobilinogen, protein, pH, blood, specific gravity, ketone, bilirubin, and glucose. Students will work with their own samples to minimize health hazards. Be sure to practice good aseptic technique throughout this lab.

Method (Lab@Home)

For this lab you will need:

Bathroom cups
Water
Dark beverage

Lab Methods
Paper
Cell phone

Instructions for serially diluting a specimen

You will need to disinfect your work space and wash your hands before completing this lab. It is not necessary to work near a lit candle as you will not be handling cultures.

1. **Using paper, make a label to sit in front of five different bathroom cups**. Your labels should include:

- Stock – this will be the undiluted sample
- D1 – this will be the first diluted sample
- D2- this will be the second diluted sample
- D3- this will be the third diluted sample
- D4 – this will be the fourth diluted sample

2. **For D1-D4 cups, add enough water so that the cups are half full.** You will use these "dilution blanks" in a moment.

3. **Pour a volume of dark liquid into the bathroom cup until the cup is approximately half filled.** The choice of liquid is up to you but might include coffee, a dark colored soda, or juice. The purpose of the dark liquid is to create contrast.

4. **Pour half of the volume from the "Stock" cup into the "D1" cup.** You may want to gently swirl the D1 cup to mix the contents.

5. **Pour half of the volume from the "D1" cup into the "D2" cup.** Gently swirl the D2 cup to mix the contents.

6. **Pour half of the volume of from the "D2" cup into the "D3" cup.** Gently swirl the D3 cup to mix the contents.

7. **Pour half of the volume from the "D3" cup into the "D4" cup.** Gently swirl the D4 cup to mix the contents.

8. **Using the camera application on your phone (or other device), take a picture of your serial dilution.** You will want to submit this image along with the writing up of serial dilution to the assignment "Tech Exam: Serial Dilution".

Work area clean up:

Once you have completed the activity,

- Pour out the stock and dilution samples.
- Throw away used cups.
- Disinfect your bench.
- Wash your hands.

Results

The results for this assignment will be uploaded into the assignment "Tech Exam: Serial Dilution".

Table 3. Results of serial dilution of urine specimen.

Tube	Stock	I	S1	S2	S3	S4	S5
Amt. transferred to dilution blank	0ml	1ml	1ml	1ml	1ml	1ml	1ml
Dilution	0	10-1	10-1	10-1	10-1	10-1	10-1
Overall Dilution	0	10-1	10-2	10-3	10-4	10-5	10-6
Amt. plated	0.1ml	10-1	10-1	10-1	10-1	10-1	10-1
Colony counts							

Use the space below to calculate the CFU/ml of your urine specimen.

Figure 2. Results for UriTest urine test strips. Source: https://healthywiser.com/products/0urinalysis-reagent-strips-urs-150ct

Circle your results for the UriTest strip in Figure 2 and record the purpose (meaning) for each of the urinalysis tests. In other words, if the urine test is positive for leukocytes in the urine specimen, what does this indicate? You may need to use the internet or other sources to answer this question.

Table 4. Results of UriTest strip.

Test	Meaning
Leukocytes	
Nitrate	
Urobilinogen	
Protein	
pH	
Blood	
Specific gravity	
Ketone	
Bilirubin	
Glucose	

References

CDC (Centers for Disease Control and Prevention). (2015). Infection Control: Appendix C. Water. https://www.cdc.gov/infectioncontrol/guidelines/environmental/appendix/water.html

4. Lab 4: Microscopy

Lab Objectives

After completing this lab, the student should be able to:

- Calculate total magnification of a specimen.
- Relate changes in field of view, working distance, amount of light needed, and magnification to image size and clarity.
- Examine microscopic specimens to determine cell shape and arrangement.
- Summarize the major differences between prokaryotes and eukaryotes and between fungi, algae, and protozoa.

Introduction

Microscopy is one of the key concepts in the field of microbiology. In this lab we will review the basic parts of a microscope, how to produce a good image, and how to proper use a microscope, include the oil immersion lens.

The Microscope and its Parts: Most microscopes that are used in teaching labs today are compound light microscopes which indicates that the microscope contains at least two or three lens systems including the ocular lens, objective lenses, and the condenser lens. Below is a list of common parts found on most compound microscopes and their functions. Be sure to refer to the "Labeled Microscope" diagram provided on Canvas.

- Ocular lens (eyepiece) – the lens that the user looks through to view the specimen; this lens magnifies the sample 10x
- Microscope arm (body tube) – to properly carry a microscope, place one hand on the arm and a second underneath the microscope.
- Slide holder – the is a lever that acts to bracket the slide and

allow for smooth, easy movement of the slide across the stage by the stage control (X-Y stage control) knobs.

- Coarse focus knob (coarse adjustment knob) – controls movement of the stage up and down such that large height changes are produced quickly. The course adjustment knob should only be used with the 4x objective lens.
- Fine focus knob (fine adjustment knob) – controls movement of the stage up and down such that small height changes are produced. The fine adjustment knob can be used with any objective lens though is favored for use with the 10x, 40x, and 100x objectives.
- Power switch (on-off switch) – the switch that turns on the microscope and may be combined with the **rheostat** which increases the amount of light produced by the lamp. Power switches are located at the base or the lower center back of most microscopes.
- Illumination (lamp) source – the light source for a microscope. Light intensity is controlled by the rheostat and the Iris Diaphragm.
- Iris Diaphragm – the lever located underneath the Condenser that regulates the amount of light that reaches the condenser lens and thus the specimen.
- (Abbe) Condenser – a lens that focuses light on the specimen. The condenser is located just underneath the stage.
- Objective lenses – a series of lenses (usually 4) that provide the majority of specimen magnification.

Table 1: Lens name, color and magnification for common used objective lenses.

Objective Lens	Color	Magnification
Scanning	Red	4x
Low power	Yellow	10x
High dry	Blue	40x
Oil immersion	White	100x

- (Revolving) Nosepiece – the part of the microscope that holds the objective lenses.

Microscopic Images: When viewing a specimen under the microscope, it is important to remember four terms that will help to produce a good image – light, resolution, magnification, and working distance.

- The circle where the specimen is seen through a microscope is termed the **field of view** and it changes as magnification and working distance change.
- Light is going to be key in producing a good image. As a specimen is viewed through the field of view, the light should not be too bright (it can cause damage to the eyes) or too dark (poor resolution and contrast). Adjust the rheostat to provide sufficient light for create contrast between the specimen and the background. The amount of light necessary to create contrast will vary between slides, especially when chromophores (charged ions that make stains) are used which further help to create contrast.
- Resolution is the ability to distinguish two cells that are spatially close to each other as separate objects. Each type of microscopy will have limits to resolution based on the illumination source. Light microscopes have lower resolution that electron microscopes. Resolution can be increased by creating contrast through the manipulation of light and

staining specimens.

- Magnification refers to the visual increase in size of an image and is presented by the unit "x" (or "X"). Each lens contributes to image magnification. When a microscope uses more than one lens system, each lens's contribution to image size must be considered using the equation:

Total Magnification = Ocular Magnification * Objective Magnification

- For the microscopes that are used in our lab, the ocular provides 10x magnification and the objective magnification will range from 4x to 100x.
- Working distance describes the space between the stage and the objective lens. The higher the magnification of the objective lens, the longer the objective lens will be thus the shorter the working distance. Care must be taken when working with the longer 40x and 100x objectives.

The **oil immersion lens** represents a special case in combatting the problems associated with microscopy. First the lens itself is so long and has such as small opening that not much light can actually pass through the sample and be collected by the lens thus oil immersion lenses often require an increase in the amount of light used to view the specimen and create the necessary contrast to produce a good image. Second, the glass of the microscope will bend light from the illuminator differently than the air which causes problems with light scattering. To decrease light scatter and thereby increase the likelihood of light reaching the oil immersion lens, a drop of immersion oil is added to the specimen before moving the oil immersion objective into position. This creates a continual "lens system" from the glass of the microscope slide, the oil, through the oil immersion lens so that most of the light is bent into the oil immersion objective. Some microscope may be use a blue or green

filter over their illuminators to help reduce light refraction but the use of such filters is not always necessary.

In this lab we will be viewing images of both eukaryotes and prokaryotes. Instructions for how to use the microscope are found in the Methods section of the lab. Be sure to read through the protocol and ask your lab instructor if you have any questions.

Method (Lab@Home)

For this lab you will need:

Compound light microscope
Colored pencils
Prepared slides

Blank slides
Toothpicks
Bacterial colony

Instructions for Microscope Observation of Prepared Slides

Before beginning this lab exercise, you should thoroughly wash your hands with warm soap and water and wipe down your bench using the provided disinfectant.

1. Prepare the lab work space by taking out the materials used in today's lab

___ Prepared slides ___ Microscope

___ Colored pencils

2. Turn on the Microscope

- Depending on your microscope, this may be different than instructions described here. These instructions are for the

IQCrew Microscope.

- Add batteries (provided with microscope kit) to the base of the microscope. You may need a small star end screwdriver to remove the base plate. Once batteries have been added, return the base plate and tighten the screws.
- Add one of the prepared slides to the stage of the microscope.
- Flip the microscope mirror up to where the light shine through the condenser.

3. Observe Eukaryotic Slides

Begin by viewing the eukaryotic slides as the specimens are easier to find and can be clearly seen on either at 300x or 600x total magnification (30x or 60x objective lenses).

- Take a slide from the prepared slide case. Be sure that the stage clips rest on top of the slide.
- Begin by making sure the 30x objective is in position on the revolving nosepiece. Raise the stage with the coarse adjustment knob as far up as possible. Slowly rotate the course adjustment knob in the opposite direction while looking through the ocular lens until the image comes into view.
- Rotate the 60x objective lens into position using the coarse adjustment knob to make the image clear.
- Record your field of view in the provided circles on the Results page. Be sure to label which specimen you are drawing in each circle.
- View these slides:
    - Broad bean leaf
    - Cotton stem
    - Goldfish scale
    - Apple
    - Onion bulb epidermis
    - Note: If you are using a different microscope, then other prepared slides may have been included. It is fine to

substitute the prepared slides in your microscope kit but please update your Results section to reflect what you are drawing.

4. Observe Prokaryotic Slides

Prokaryotic slides often require the use of the 100x oil immersion objective lens and immersion oil. Because our microscope kits do not have an oil objective, we will be using the 120x provided objective and a condenser filter to provide contrast.

- Take a blank slide from the slide box.
- Add a small drop of water to the slide.
- Using a clean toothpick, mix a small amount of bacteria from one of your petri plates into the drop of water.
- Set the slide aside until it has completely air dried.
- Begin by making sure the 30x objective is in position on the revolving nosepiece. Raise the stage with the coarse adjustment knob as far up as possible. Slowly rotate the course adjustment knob in the opposite direction while looking through the ocular lens until the image comes into view.
- Rotate the 60x objective lens into position using the coarse adjustment knob to make the image clear.
- Rotate the 120x objective lens into position and carefully adjust the image using the coarse adjustment knob to make the image clear. Note that you may need to adjust the condenser filters (located under the stage) to create greater contrast with the microscope.
- Record your field of view in the provided circles on the Results page. Be sure to match the correct organism to its circle. Note that all bacterial specimens with the exception of "Algae Blue Green" or "Cyanobacteria" will be viewed with the 100x objective.
- View these slides:
- Prepared bacterial slide

Lab clean up

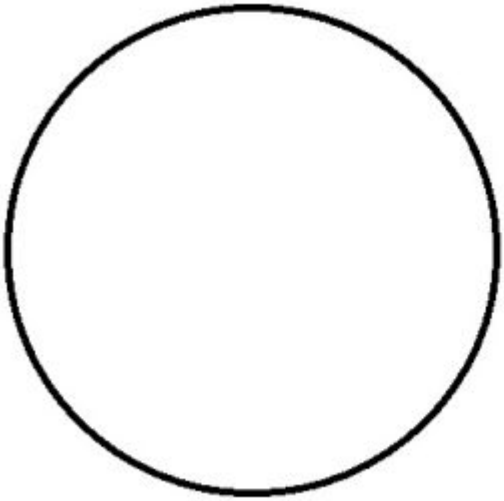
Once you have completed the lab be sure to:

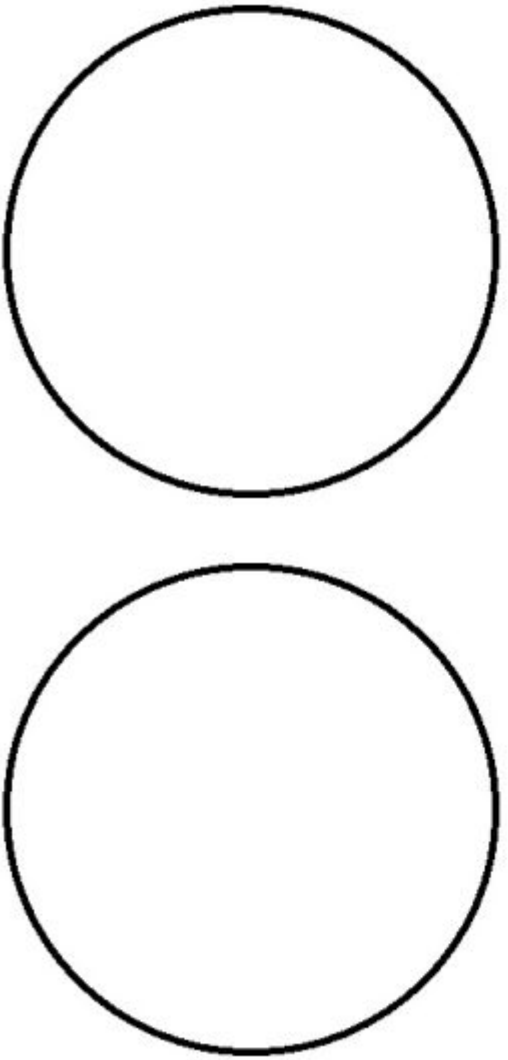
- Clean your bacterial slide with warm soapy water
- Return the slides to the slide box and put away slide box, color pencils, and microscope
- Disinfect your bench.
- Wash your hands.

Results

In the section below, draw example organisms in your field of view. You do not have to fill up the field of view, only communicate what you are observing. Be sure to include total magnification, any necessary labels, and match the correct organism to its location on the Results sheet.

Eukaryotic Cells

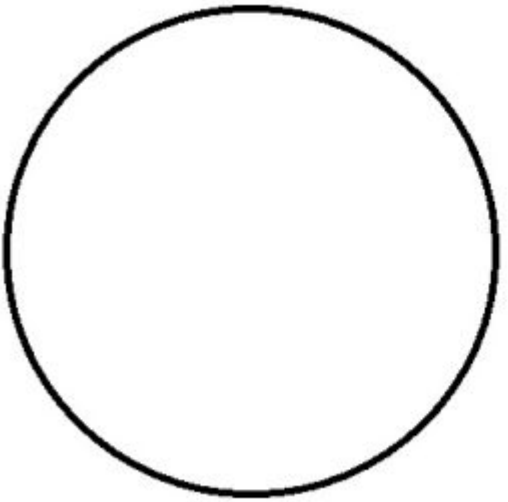


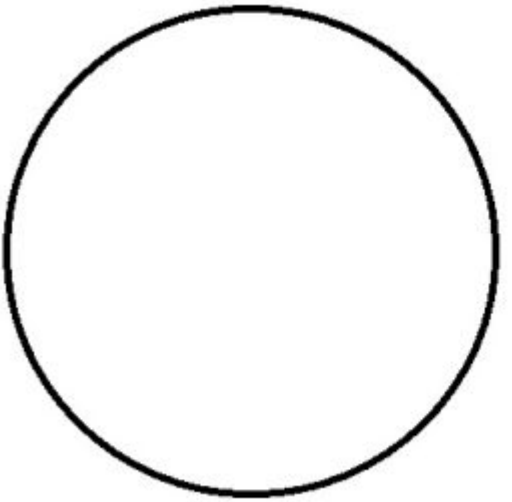


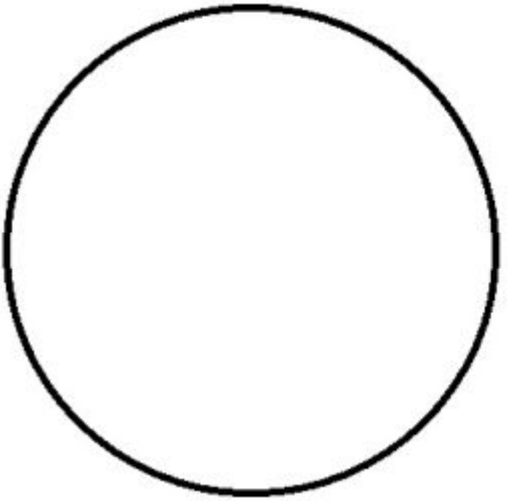
Slide: Broad bean leaf Cotton stem Goldfish scale

Total Magnification _____ _____ _____

Organism: Apple Onion bulb epidermis Bacterial colony [Prokaryotic]







Total Magnification _____ _____ _____

5. Lab 5: Staining

Lab Objectives

After completing this lab, the student should be able to:

- Use Aseptic Technique to prepare smears for staining.
- Examine smears to determine bacterial morphology, arrangement, and Gram reaction.
- Determine if a cell is positively or negatively charged and if a stain is direct, indirect, simple, or differential.
- Classify stained cells based on their cell structure as either Gram positive or Gram negative.
- Apply the Gram reaction to cell wall structure.

Introduction

Staining is a key skill in microscopy work because it helps to create contrast between cells and their background. If you have previously viewed stained slides, you are already aware of the contrast difference made by staining techniques. This lab will review how to create smears, stain smears, and explain simple and differential bacterial staining techniques.

Smears: Bacterial smears are created by applying cells to a microscope slide. If the cells come from a **liquid preparation** (e.g., a broth culture) then the cells are directly applied to the slide. However, if the cells come from a **solid culture** (e.g., a slant or plate), then a small drop of water is applied to the slide first then the cells are mixed into the drop of water. The reason why cells must be in a liquid is to allow for spreading out the cells into a thin film termed a **smear**. This reduced the number of cells in a physical space so that as the smear dries, cells are more evenly distributed and are less likely to trap stains. It is also important to ensure that cells have

sufficient space between them so that cells can be easily seen to determine morphology and arrangement which becomes important in identification methods.

Before staining, cells must be "fixed" to adhere them to the glass microscope slide. Fixing slides can be accomplished in two ways: either through the use of strong fixatives (chemicals) such as alcohol or acetone in the case of human blood cells or by heat. Our lab will use heat fixing as the method of choice. To heat fix a smear, you will simply run the slide through the top portion of the Bunsen burner flame twice.

Types of Stains: Stains are classified based on charge of the chromophore (colored ion). Because the overall net charge of a bacterial cell is negative, positively charged chromophores are attracted to the bacterial cells and will directly stain the cells – thus **direct stains** are stains that contain positively charged chromophores. **Negative stains**, on the other hand, use negatively charged chromophores that are repelled by the bacterial cell and stain the slide ("background") so that the bacterial cells appear colorless on a dark background.

Negative stains **do not** require heat fixing so negatives stains are used when one wants to observe living cells for motility. Because negative stains are not heat fixed, the cells will appear crisper thus determining morphology and arrangement can be a bit easier than with direct stains.

So when would a direct stain be used? Direct stains are the most common stain type in the microbiology lab. They can be used for both simple and differential staining techniques. **Simple stains** will use one chromophore while **differential stains** use at least two chromophores in separate staining steps to differentiate bacteria based on cell wall type, structures, or even cell membrane permeability. Listed below are some common differential stains used in the microbiology lab:

Table 1. List of Common Differential Stains in Microbiology Lab

Staining Protocol	Stains (charge)	Differential for…
Gram Stain	Crystal Violet (+), Safranin (+)	Cell wall (Gram positive/ negative)
Acid Fast	Carbol Fuchsin (+), Methylene Blue (+)	Cell wall (Acid fast/ non-Acid fast)
Endospore	Malachite Green (+), Safranin (+)	Endospores and vegetative cells

Morphology and Arrangement: Bacterial morphology and arrangement are important for helping to classify and identify bacteria. Morphology refers to the shape of the bacterial cell. Common bacterial morphologies are:

- bacillus (rod)
- coccus (spherical)
- spirillum (spiral shaped).

Arrangement refers to how cells are spaced with respect to each other. Typical arrangements for bacteria include:

- strepto (chain)
- staphylo (grape-like clusters)
- tetrad (clusters of four cells)
- sarcina (clusters of eight cells)
- single/diplo (one or two cells – These terms are frequently used together because it is difficult to determine if a cell has just divided rather than truly being a pair.)

In today's lab, we will practice simple direct, simple negative, and the Gram staining techniques on our unknow culture(s). Because we will be working with bacterial unknowns, it is important to

practice good aseptic technique. Due to the small size of the cells, oil immersion will need to be used to view the stains. To prevent stain getting onto the microscope, be sure to complete all of your stains before getting out your microscope.

Method (In Lab)

For this lab you will need:

Unused slides
Compound light microscope
Bacterial culture

Eosin dye
Disposable plastic container or paper plate
Toothpicks

Instructions for Negative Stain

Before beginning this lab exercise, you should thoroughly wash your hands with warm soap and water and wipe down your bench using the provided disinfectant.

Remember that the negative stain is not heat fixed so these cells will still be alive after staining.

1. Make a smear by placing a small drop of **eosin** stain in the center of a clean glass slide.

2. Place the slide on a surface that will not take the stain or that can be thrown away such as disposable plastic container, paper plate, or piece of foil/ wax paper/ parchment paper.

3. Working close to a lit candle, collect a small amount of bacterial growth from your plate with a toothpick and mix into the drop of **eosin** and smear the drop into a large circle.

4. Set the slide aside to air dry. Be sure to place the slide in a location where it will not be splattered.

5. Observe the slide with the 60x objective lens after completing and putting away all staining materials.

6. Record your observations below.

Because these stains are not included in your microscope kit and they require more materials, you will watch videos of the Gram stain procedure and the simple direct stain procedure on Canvas. Be sure you are confident in your understanding of these techniques as they are commonly used in microbiology and clinical lab – and they'll be on your lab report and lab quiz assessments. Protocols are listed below for both the Simple Direct and the Gram Stain for your reference. You will not actually be doing these two staining procedures (Simple Direct Stain and Gram Stain).

Instructions for Simple Direct Stain and Gram Stain

1. Make a smear by adding a small drop of water to a clean slide. Use a clean inoculating loop to catch a small drop of water from the DI water bottle located in your staining drawer.

2. Aseptically transfer a small amount of S. aureus into the drop of water and smear into a large circle.

3. Allow the slide to air dry either by leaving on your bench or taking to the slide warmer.

4. Once the slide has completely dried, heat fix the slide by passing it through the top of the Bunsen burner flame twice.

5. Stain the slide:

- Place the slide on the wire staining rack in the metal staining tray.
- Apply **methylene blue** stain until the slide is covered and incubate for 60sec.
- Using the water bottle found in the staining tray, wash off excess stain.
- Blot in the bibulous paper book as demonstrated by your instructor.
- Set the slide aside until ready to view once staining has been complete.

Instructions for Gram Staining

1. Make three smears by adding three small drop of water to a clean slide. Use a clean inoculating loop to catch a small drop of water from the DI water bottle located in your staining drawer.

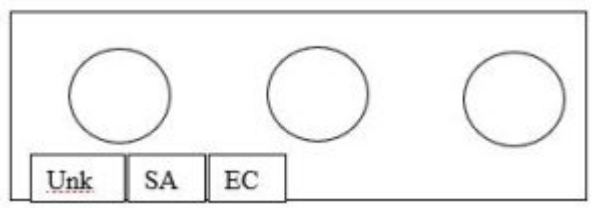


Figure 1. Slide layout for Gram Staining.

2. Aseptically transfer a small amount of cells into the respective drops of water. For this slide, you will stain all three organisms on the same slide with EC (E.coli) and SA (S. aureus) as the Gram stain controls.

3. Allow the slide to air dry either by leaving on your bench or taking to the slide warmer.

4. Once the slide has completely dried, heat fix the slide by passing it through the top of the Bunsen burner flame twice. Because there

are three smears on this slide, you may need to rotate the slide 180^0 to ensure even heating of all smears.

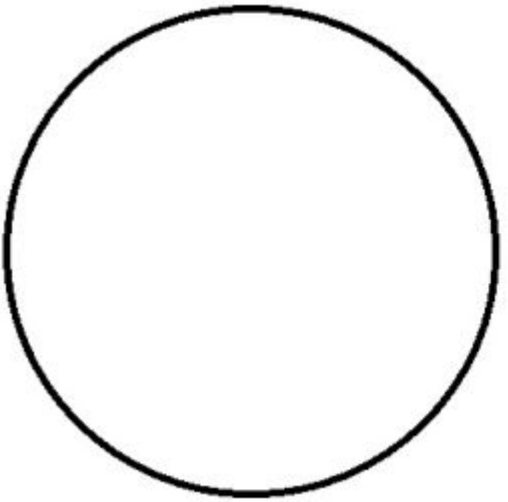
5. Stain each smear by:

- Apply **crystal violet** to cover the three smears for 60sec. Wash with water but do not blot with the bibulous paper until the end of the staining procedure. It is fine if water remains on the smears after washing.
- Apply Gram's iodine to each smear for 60sec. Wash with water.
- Apply ethanol (EtOH) to the smear for approximately 10-20 sec until large amounts of purple are no longer being rinsed out of the smears. Immediately rinse with DI water.
- Counterstain cells by applying **safranin** for 60sec. Wash with water and blot dry with bibulous paper.
- Set aside the slide until you have finished staining then view with oil immersion.

Clean up from staining

Before getting out the microscopes, make a bit more room on your work bench by putting away your staining materials. Carefully rinse out and dry the staining pans before returning them to the common second drawer. Put away stains, water and EtOH bottles, loops, Bunsen burners, striker, and bibulous paper.

Nigrosin Stain



Organism: Unknown

Morphology _____

Arrangement _____

6. Lab 6: Biochemical Tests and Dichotomous Keys

Lab: Biochemical Tests

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to inoculate biochemical media.
- Classify microbes based on their biochemical results.
- Apply the use of pH indicators to determine metabolic processes by or within a cell.
- Provided a dichotomous key, identify a bacterial unknown.

Introduction

Microbial identification is key to the clinical sciences. Based on the identification of a causative agent of disease, antimicrobial treatment can be selected that targets the specific pathogen. As you have already experienced with previous labs, microbial identification requires more than just microscopic observations. While staining and microscopy can be a great place to start when identify microbes, it doesn't provide genus or genus/ species level identification.

Most identification schemes combine Gram reaction and biochemical testing, which typically involves several different tests to narrow down the list possible organisms. There are three main systems that are used within the clinical environment that serve as biochemical testing:

- Biolog system (Biolog)
- API20-e system (bioMerieux)

- EnteroPluri Test (BD Biosciences)

Both the API system and the Biolog system require the generation of a metabolic "footprint" that is then entered into a computer database which will give a genus species level identification. It should be noted that both the Biolog and API systems are quite expensive so are cost prohibitive for many smaller clinics and hospitals thus necessitating that samples be sent to larger medical centers or clinical testing services such as LabCorp®. The Biolog system can identify approximately 3,000 different bacterial and fungal species where as the API system is limited to Gram negative Enterobacteriacaea.

A more cost-effective method for unknown identification of Enterobacteriacaea is the EnteroPluri (Entertube) system in which a Gram negative colony, typically isolated from a selective differential medium such as EMB or MAC is inoculated into one tube that contains 15 different biochemical tests. The Enteropluri tube is incubated overnight and color changes in the medium are compared to a manual that will provide genus species identification of the most common enteric pathogens and opportunists. It should be noted that while the EnteroPluri tube is the most cost effective of these three methods, it also has the most limitations in that it uses the fewest number of biochemical tests which can lead to misidentification.

Because these systems dependent upon biochemical testing rather than genetic testing, identification typically takes longer – 24 hours for the Biolog, API20e, and Enteropluri systems compared to 2-4hr for genetic identifications. Both genetic and biochemical methods can be "tricked" by microbial genetic exchange processes such as conjugation, transduction, and transformation in which non-pathogen genetic material may be taken up by the bacterial cell and result in the expression of new genetic traits. In such cases, more robust systems such as the Biolog system provide a higher confidence in genus/ species level identification.

In this lab, you will use data collected from a bacterial unknown and the associated assignment Lab: Dichotomous Key to identify a specific unknown. Because multiple types of media are being used which can be costly, data will be supplied to you via Canvas message for your unknown organism. For the Results portion of this lab, you will answer questions about the different biochemical tests.

Method (Lab@Home)

For this lab you will need:

Lab: Dichotomous Keys
Biochemicals Lab Lecture

Lab: Biochemicals
Biochemical Data sets 1 and 2

Analysis of provided data

Watch the Lab: Biochemical Tests video.

Using the two data sets (Biochemical data set 1 and Biochemical Data set 2), complete the data tables in the Results section.

Results

Table 1. Results from Carbohydrate Fermentation Tests. For Phenol Red tests record broth color and presence/ absence of bubbles in Durham tube and color change (A = acid; G= gas; AG = acid and gas; K = alkaline reaction; Neg = no reaction). For the OF glucose test, record deep color for the open and closed (with mineral oil) tubes. For MR, record color changes (if any) after the addition of reagents.

	Phenol Red Fermentation	OF Glucose	MR			
Organism	Glucose	Lactose	Sucrose	Open	Closed	Color after Reagent
E. aerogenes						
E. coli						
P. vulgaris						
S. aureus						
Negative						

Table 2. Results of Urea Hydrolysis Test.

Organism	Agar Color	Urease (+/-)
E. coli		
P. vulgaris		
Negative control		

Table 3. Results of Citrate Test.

Organism	Growth (Y/N)	Agar Color	Citrase (+/-)
C. freundii			
E. coli			
Negative control			

Table 4. Results of Catalase Test.

Organism	Bubbles (+/-)	Catalase (+/-)
E. faecalis		
S. aureus		
Negative control.		

Table 5. Results of SIM Test.

	Sulfide Test	Indole Test	Motility	
Organism	Agar Color	Agar Color	Tryptophanase (+/-)	(+/-)
C. freundii				
E. aerogenes				
E. coli				
P. vulgaris				
Negative control				

Table 6. Results of Oxidase Test.

Organism	Color	Oxidase (+/-)
E. coli		
P. vulgaris		
P. aeruginosa		
Negative Control		

Lab: Dichotomous Keys

Objectives

After completing this lab, students will be able to:

- Provided a dichotomous key, identify a bacterial unknown.

Introduction

These dichotomous keys will be used to identify microbes isolated from human samples or provided unknown data sets.

Method (Lab@Home)

For this lab you will need:

Data set (sent to your Canvas email)
Protocol (with dichotomous key)

Instructions for data collection

Complete the two tables below by adding data for each (Observations and Interpretations) for each biochemical test used. If you did not use a test then mark the "Observation" and "Interpretation" fields as NA or with a dash (—).

1. Data collected for the Unknown Bacterium. Unknown #: ——————————

Biochemical Test	Observation	Interpretation
Gram Reaction		
Morphology		
Arrangement		
Phenol Red Glucose		
Phenol Red Lactose		
Phenol Red Sucrose		
OF Glucose		
Methyl Red		
Urea		
Citrate		
Catalase		
SIM		
Oxidase		

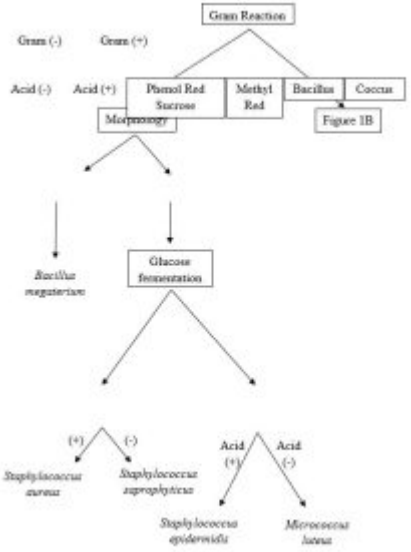


Figure 1A. Gram Positive Dichotomous Key.

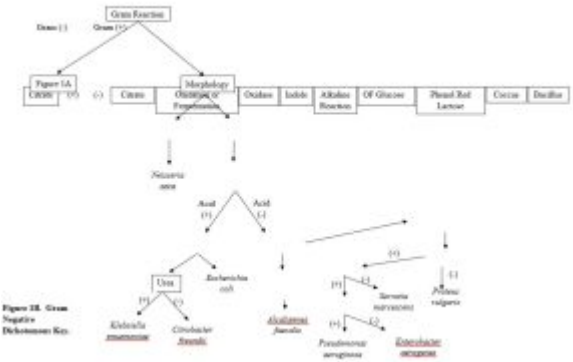


Figure 1B. Gram Negative Dichotomous Key.

7. Lab 7: Control of Microbial Growth

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to transfer specimens for the UV test, temperature test, Kirby Bauer method, and disk diffusion method.
- Interpret growth patterns to determine antimicrobial effectiveness.
- Apply microbial cell wall structure to antimicrobial control.
- Examine the effectiveness of pigments and endospores to protect against the effects of UV radiation.

Introduction

Controlling microbial growth is key to medicine and to everyday life so there are many methods used to chemically and physically limit the growth of microbes. Control methods include inhibition (**bacteriostatic**) or elimination (**bactericidal**) microbes. While elimination is ideal, not all substances or surfaces can be treated sufficient to eliminate microbes and sometimes sterilization is not a practical method. Remember that control methods can reduce microbial populations or completely eliminate microbes thus leaving a **sterile** environment, food production, or surface. While sterility is more important in the clinic, decreasing the total **microbial load** is commonly used whenever surfaces are "cleaned" or foods are "prepared".

The two main methods of controlling microbial growth are:

- **Chemical methods** which include the use of chemicals to eradicate or decrease the microbial load.
- **Physical methods** include filtration, temperature treatments, and radiation. These methods will either reduce the microbial load, completely remove microbes, or prevent microbes from reproducing thus resulting in cell death.

Chemicals are often convenient a convenient way to eliminate or reduce the number of microbes on inanimate surfaces (disinfectants) such as countertops, living surfaces (antiseptics) such as skin, or ingested (antibiotics). These chemotherapeutic agents come in a wide range of **spectrums** and thus have many uses. For examples, a compound that is a bactericide would kill bacteria but not fungi or viruses while a fungicide would kill fungi but not bacteria or viruses. Sporicides are compounds that typically kill endospores. A compound may also be **broad spectrum** where it can target multiple groups of microbes (or microbes and endospores) or **narrow spectrum** (only targeting one class or group of microbes). For examples, natural penicillins are effective against Gram positive bacteria but offer little effectivity against Gram negative bacteria and certainly no protection against fungi, viruses, endospores, or other groups of microbes.

Physical control methods are quite a bit less discriminatory in their targeted groups and are thus considered broad spectrum. Most methods of physical control target DNA, proteins, or size which are shared in common across the microbial spectrum.

- Radiation causes mutations, specifically the formation of pyrimidine or thymine dimers, which prevent DNA replication – one of the key checkpoints in the cell cycle.
- Heat breaks the hydrogen bonds that keep proteins and nucleic acids in their functional shapes. As such, many organisms cannot carry out life processes upon exposure to heat. Heat comes in two forms – **moist heat** which provides are more efficient transfer of heat from the environment to the

organism(s) and **dry heat** which does not rely upon moisture. As such, dry heat methods such as ovens typically require longer times and/or higher temperatures to kills microbes and are basically useless against endospores. Examples of moist heat include autoclaving, boiling, and pasteurization processes. [Note: Ultra high temperature (UHT) pasteurization is used to sterilize certain foods such as milk.]

- Low temperatures such as refrigeration and freezing slow or prevent microbial growth. While not true for endospores, freezing can damage the plasma membranes when ice crystals form.
- Filtration is a common method of physical control used for liquids that cannot be heat treated or air samples. During filtration, the air or liquid is passed through a filter which physically prevents particles (including microbes) from passing based on size. Not all filters are the same size so there is a limit as to the group of microbes that are targeted. For example, most bacteria would be removed from a solution if a 0.22um filter were used, however, this size would not remove viruses.

Microbes have developed some methods for counteracting physical and chemical methods of control. Some microbes are naturally resistant against chemicals and heat while others have had to develop mutations that degrade chemicals thus making the chemicals inactive. Likewise, endospore formers, bacteria that produce a pigment (color, specifically observed during the growth of colonies), or microbes that have DNA repair systems are more resistant to radiation treatment. Pigments act as a form of "sunscreen" by blocking some radiation while repair systems such as the SOS system fix any damage that radiation causes. Repair systems are limited in the amount of damage that they can fix – too many thymine dimers will result in cell death. Endospores are resistant structures due to their thick spore coats and their lack of metabolism, thus they are resistant to radiation. Heat shock

proteins are a special group of proteins that help repair/ refold damaged proteins after a stress event, including temperature extremes.

This lab explores the use of physical and chemical methods of control on microbes. Specifically, we will look at exposure to different temperatures, UV radiation, antiseptics, disinfectants, and antibiotics.

Method (Lab@Home)

For this lab you will need:

7 NA plates
Cotton swabs
Bathroom cup

Clean water
Sharpie marker
Antiseptics

Disinfectants
Antibiotic data
Antibiotic Sensitivity table

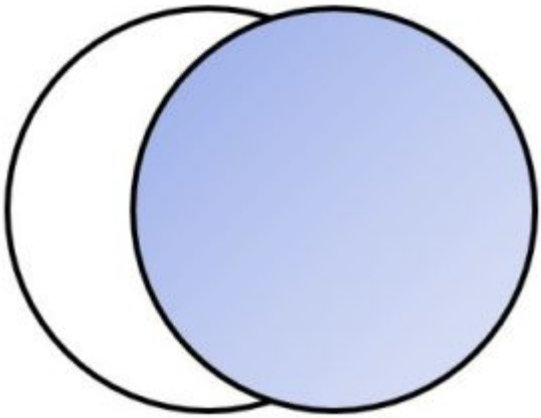
Timer/ watch
Aluminum foil
Ziplock bag

Ruler with mm

Before beginning this lab exercise, you should thoroughly wash your hands with warm soap and water and wipe down your bench using the provided disinfectant.

1. Inoculate the 4 NA plates for UV radiation test.

- Using the bathroom cup, collect a small amount of clean water. (Bottled or purified water works better than tap water.)
- Dip a clean cotton swab in the water to moisten the swab, then collect a small amount of growth from your bacterial plate from the first lab.
- Spread bacteria on the entire surface of the NA plate by streaking top to bottom, left to right, and then at a 45^0 angle across the surface of the plate. The goal is to cover every surface of the medium with the broth so that you can easily see where the treatment method damages bacterial cells and prevents growth.
- You will repeat this inoculation method for all four plates.
- Open all plates to sunshine (not at a window but in direct sunshine) or under a UV light for 2.5min or 5 min as demonstrate below. Two plates will be treated for 2.5min and two plates for 5 min.



- Cover the plates and put one plate for each time (2.5min and 5 min) in a dark cabinet or cover in foil while the cells grow. The other pair of plates will be kept in the light. [You are looking at the effect of UV exposure time and the effect of light depended repair systems.]

- Growth the plates at room temperature for 4 days.

2. Inoculate the 2 NA plates for temperature test.

- Inoculate 2 NA plates for confluent growth as described in the above procedure (Procedure 1).
- Place one plate in a zip lock bag and half seal. Incubate the plate at 4^0C refrigerator for 4 days.
- Place the second plate at room temperature and grow for 4 days.

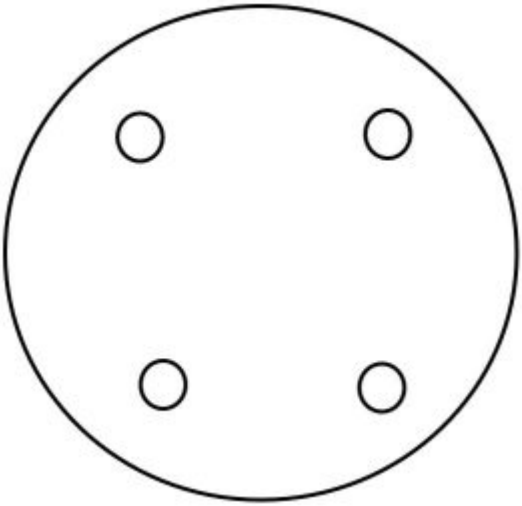
3. Inoculate a NA plate for Disinfectant and Antiseptic testing.

- Swab 1 NA plates with your organism using the streak for confluent growth method as described above (in Procedure 1).
- Select 2 antiseptics and 2 disinfectants that you have at home.
- Examples of antiseptics could be hydrogen peroxide, isopropyl alcohol, hand soap, body soap, shampoo, hand sanitizer.
- Examples of disinfectants would include surface cleaner, disinfectant wipe (or the liquid from a disinfectant wipe), Lysol, bathroom cleaner, glass cleaner.
- Record the names of the test compounds below.

Antiseptic 1 (A1): _____ Antiseptic 2 (A2): _____

Disinfectant 1 (D1): _____ Disinfectant 2 (D2): _____

- It may be helpful to pour/ spray a small amount of each antiseptic and disinfectant into a separate clean bathroom cup.
- Using a clean swab, dip the swab into the antiseptic or disinfectant compound and dot onto the inoculated plate according to the diagram below. You should have one dot for each test compound.



- Once your plate has been inoculated, incubate your plate at room temperature for 4 days then measure the zones of inhibition created by your test compounds.

4. Analyze a Kirby Bauer dataset.

- Download the data set from the Kirby Bauer test and the Antibiotic Sensitivity table from BBL. [Both documents are available on Canvas.]
- Record the zone of inhibition measurements to your table in the Results section.
- Using the Antibiotic Sensitivity table from BBL, decide if each organism is S (sensitive), I (shows intermediate sensitivity), or R (resistant) for each antibiotic tested. Record your data in the provided Table in the Results section.

5. Clean up

After you have finished your inoculations, wipe down your work

area with disinfectant and wash your hands. Blow out the candle and store for later.

Once you have read your plates (after their 4 day incubation), wrap plates in a plastic bag and dispose of in the trash. Again, wipe down your work area and wash your hands.

Results

Because microbes require time to grow, you will view your results after 4 days.

Develop a plus system to convey the amount of growth per treatment condition. How many + marks would you give the following descriptions?

No growth = _____ Little growth = _____

Moderate growth = _____ Heavy growth = _____

Table 1. Results from UV Exposure.

Plate Bacterium	Growth @ 2.5min	Growth @ 5min

Rank the growth in each plate based on your plus system.

Table 2. Results from Growth at Different Temperatures.

Organism	4^0C	21^0C
Bacterium S. marcescens		

Rank the growth in each plate based on your plus system.

Many times, chemical agents will produce zones of inhibition where an organism will not grow around a compound. For each of your plates (Disk Diffusion and Kirby Bauer tests), measure the zones of inhibition (ZOI) and report in the tables below.

Table 3. ZOI Measurements Produced by the Disk Diffusion Test. All measurements are reported in mm.

Test Compound	Bacterium
Antiseptic 1	
Antiseptic 2	
Disinfectant 1	
Disinfectant 2	

Table 4. ZOI Measurements Produced by the Kirby Bauer Test. Record the zones of inhibition measurements for each antibiotic. (R = Resistant, I = Intermediate, S = Sensitive, [Abx] = Antibiotic Concentration). This data requires the use of the provided Antibiotic dataset and the Antibiotic Sensitivity table.

Organism	Disk Code	[Abx]	Zone of Inhibition (mm)	Interpretation (R, I, S)
E. coli				
P. aeruginosa				

S. aureus

8. Lab 8: ELISA

Lab Objectives

After completing this lab, the student should be able to:

- Use aseptic technique to carry out an ELISA reaction.
- Examine how antibodies can be used to detect specific diseases such as Covid-19 or influenza in patient specimens.
- Identify if a patient has a disease based on ELISA test results.

Introduction

ELISA tests depend upon antigen (Ag) – antibody (Ab) interactions. These reactions can be used to diagnose illnesses such as Covid-19, influenza, strep throat or even changes in health states such as the secretion of pregnancy related hormones. ELISA technology with in the clinical environment serves as a rapid identification test. While not all tests are ELISA based, several common illnesses or states can be detected within a few seconds to a few minutes and new ELISA tests are being developed by medical researchers. An example of new ELISA based tests include PSA (prostate specific antigen) and TAA (tumor associated antigen) tests that are linked to the early detection of cancer, even before cancer can be detected by imaging. This is great news for patients because it means that lifestyle and chemotherapeutic changes can be made before cancer becomes problematic yet these tests do not require genetic analysis of patients thus avoiding complicated ethical and economic discussions that genetic analysis may uncover.

The ELISA reaction is fairly standard but it is helpful to identify the different reagents that are used before talking about the different types of ELISAs. There are five key components of an ELISA reaction:

- An **antigen** – This is a cell (or virus') identification card. All cells and viruses have them and for the most part, they are rather unique. The **epitope** on an antigen is a unique set of molecules, typically amino acids or simple sugar chains, that can be used to identify that organism. For example, E. coli O157:H7 has epitopes that would mark it as having O polysaccharide 157 and flagellar protein H7. Often times epitopes are just called antigens (which can make discussions in microbiology and immunology a little confusing). For the purposes of this class, we will refer to an epitope as an antigen.
- A **primary antibody** is the Ab that will bind to the Ag in the reaction. The purpose of the primary Ab is to detect the Ag of choice, depending on what the ELISA is detecting. Some ELISA methods use only one antibody in which case the indicator molecule is attached to the primary antibody. Other ELISA methods will use a **secondary Ab** which is another antibody that binds either to the primary Ab and carries the indicator molecule.
- **Indicator molecules** are used to produce some type of change or signal that an Ag has been detected. Often, indicator molecules will produce a color change that can easily be detected while other indicator molecules give off fluorescent light or radiation.
- **Blocking agents** include any large, non-reactive molecule that can be used to bind to a surface and prevent non-specific binding of the Ag or Ab depending on the type of the ELISA test. Think of blocking agents as a way to "cover up" areas on the solid substrate (card or plastic) where there is nothing to detect. For some rapid detection ELISA tests such as home pregnancy tests, the blocking agent has already been applied by the manufacturer.
- **Washing buffer** is a solution that is buffered to prevent any cross-reaction with either the Ag or Ab(s). Washes are often used after the addition of Ag or Ab to remove any unbound materials. Note that if only one sample is being added, as in

the home pregnancy test mentioned above, wash steps were completed during manufacture and thus are not usually needed though each test may vary based so always read the instructions when conducting a home test.

The three most common types of ELISA tests fall into the following groups. All of them depend on Ag-Ab interactions but there are subtill changes in how Ag and Ab are used and in what order.

- In **direct ELISA**s, Ag are fixed onto some sort of solid substrate such as card or plastic dish to prevent the sample from washing away throughout the steps of the ELISA reaction. A primary Ab conjugated (cross linked or fixed) to an indicator molecule or compound is added to bind to and thus detect the Ag being tested. A color substrate is commonly used for detection so the substrate would be added to the solution to produce a color change. Note that some ELISA test will use a fluorescent or radiographic indicator; in such cases, no color change would visually be detected without the aid of radiographic film or fluorescent detection methods (e.g., fluorescent microscopy).
- An **indirect ELISA** is very much like a direct ELISA. The Ag is bound to the solid substrate then a primary Ab is added to bind to the Ag. The difference between direct and indirect ELISAs are that indirect ELISA has the indicator molecules bound to the secondary Ab rather than the primary Ab so there is an "extra" step. Indirect ELISAs are commonly used clinical and microbiological labs and offer an added layer of sensitivity while direct ELISAs are used for rapid tests and "at home" tests.
- The **sandwich ELISA** makes an Ag "sandwich" by trapping the Ag of interest between two Ab. One Ab, typically the primary Ab, is put on the solid substrate then the Ag of interest is added, followed by adding the secondary Ab carrying the indicator molecule. Sandwich ELISAs are one way to

determine if someone has produced Ab against a specific pathogen, rather than detecting if a person has a specific pathogen. Of course, Ab titers can also be detected by serological methods in addition to ELISA testing.

In this lab, you will be performing an ELISA test to detect a specific Ag.

Method (Lab@Home)

For this lab you will need:

Disposable bathroom cup
Home ELISA test (pregnancy, ovulation, drug)

Timer
Surface cleaner (for aseptic technique)

You will want to follow the manufacturer provided instructions for your specific test. Outlined below are considerations for maintaining aseptic technique.

1. Clean the area that you will be working in with a disinfectant spray or wipe.

2. Open the home test kit (pregnancy, ovulation, drug) and lay the materials out on your work space.

3. Read the instructions carefully.

4. For tests that require the collection of a urine specimen, it is recommended that you collect a urine specimen in the disposable bathroom cup then either dip the test into the urine specimen or transfer the recommended number of drops using pipettes provided in the test kit. Each test will vary slightly between manufacturers.

5. Incubate the test for the required time (usually 1-3 min) on a flat surface.

6. Check the manufacturer instructions to determine what a positive test and negative test look like – usually there is a difference in the number of bands.

7. Record your data in the Results section.

Results (Lab@Home)

Sample	Color	Interpretation
Control		
Sample		

Lab 6: Biochemical Data Set

Lab 6 Biochemical Data set 2

Lab 7: Data Set

Lab 7 Abx Data_BI302 Control of Growth LaB7.27.22