



5g yeast extract (Fisher, #DF0127-15-1, 100g, \$32.05)  
10g Bacto-Tryptone (Fisher, #DF0123-15-5, \$22.55)  
5g NaCl  
15g agar (Fisher, #DF0140-15-4, 100g, 61.95)  
H<sub>2</sub>O to 1L

Mix the ingredients. To ensure complete dissolution, place the flask containing LB agar in a pan of water and boil the water, swirling the contents of the flask occasionally, until there are no solid particles seen in the solution. Pour the LB agar into two 500ml flasks and stopper the flasks with nonabsorbent cotton. Autoclave the agar at 121°C, 20 psi for 30 minutes. If you do not have an autoclave, you can pressure-cook it for 1 hour. Allow the media (LB agar) to cool to 55°C, then pour the plates using the sterile technique described in this lab. If you wish to make LB agar, use the following recipe for 1 liter of agar (makes 100 plates). Allow the plates to cool on the bench-top for as long as possible (at least overnight, but being out for 2-3 days will help to eliminate condensation forming on the lids). To check for contamination, you may wish to place an uninoculated dish in an incubator at 37°C overnight. No growth of bacteria means no contamination. Store the dishes in the sleeves they came in, upside down in the refrigerator. Do not store plates longer than several weeks.

- To make LB broth, use the above recipe, but omit the agar. Broth can be sterilized in half-filled and stoppered test tubes. Inoculate tubes of broth containing *Alcaligenes faecalis*, *Bacillus cereus* and *Serratia marcescens*
- several days prior to this activity for student use. Incubate the tubes overnight at 37°C. The tubes should be gently swirled prior to use to mix the bacteria with the broth. Be sure to make separate test tubes for each class.

### Correlations to State and National Standards:

- **Colorado State Standard 3:** Life Science-- Students know and understand the characteristics and structure of living things, the processes of life, and how living things interact with each other and their environment.
- **Colorado State Standard 5:** Life Science-- Students know and understand interrelationships among science, technology, and human activity and how they can affect the world.
- **Colorado State Standard 6:** Life Science--Students understand that science involves a particular way of knowing and understand common connections among scientific disciplines.
- **National Content Standard C (Life Science):** As a result of their activities in grades 9-12, all students should develop understanding of the cell; the molecular basis of heredity; biological evolution; interdependence of organisms, matter, energy, and organization in living systems; and behavior of organisms.

### **Correlation to Confronting the Microbe Menace:**

Cross reference information given on Bacteria, and Antibiotics found on the DVD 2000 and beyond confronting the microbe menace with lab 5.

General Information on Bacteria	T5C5	07:45
Size: Analogy one, ping pong ball	T5C5	07:51
Analogy two, ruler	T5C5	08:11
Gram Stain Identification	T5C6	08:55
Chart: Bacteria Are Everywhere and Numerous	T5C7	09:45
Slide: Normal Flora of the Mouth Bacteria Gram Strained	T5C9	12:11
Video: Bacteria E.Coli, show actual reproduction	T5C10	13:25
Chart: Some Bacterial cause Disease (Sometimes)	T5C14	16:52
Chart: Examples of Bacterial Diseases	T5C16	19:30
Chart: Infectious Agents Are Easily Spread	T5C18	21:50
Combat Infectious Diseases		
Chart: How to combat infectious Disease	T5C20	24:21
Chart: Antibiotics	T5C21	25:18
Chart: Antibiotic Mechanisms	T5C23	27:02
Picture E. Coli on a plate Disk Diffusion Kirby-Bauer	T5C24	27:25
Video E. Coli being lysis	T5C25	27:54
Chart: Antibiotics	T5C26	28:39
Picture: E. Coli on plate that is antibiotic resistant.	T5C27	29:30
Chart: Antibiotic Resistance	T5C38	29:38
Demonstration of Super Bug (Antibiotic Resistant)	T5C29	31:45
Chart: Antibiotic Resistance (Super Bug)	T5C29	32:09
Video: Conjugation	T5C31	34:52
Chart: Shelf Life of New Antibiotic	T5C32	35:29

### **Supplementary Materials:**

URL'S and titles of useful web sites

1. Antibiotics  
[WWW.ultranet.com/~jkimball/BiologyPages/A/Antibiotics.html](http://WWW.ultranet.com/~jkimball/BiologyPages/A/Antibiotics.html)
2. Penicillin and other Antibiotics  
[helios.bto.ed.ac.uk/bto/microbes/penicill.htm](http://helios.bto.ed.ac.uk/bto/microbes/penicill.htm)

3. Antibiotics Factory Farm Project  
[www.factoryfarm.org/antibiotics.html](http://www.factoryfarm.org/antibiotics.html)
4. Antibiotics  
[www.bact.wisc.edu/MicrotexBook/ControlG.antibiotic.html](http://www.bact.wisc.edu/MicrotexBook/ControlG.antibiotic.html)
5. Antibiotic Politics  
[helium.vancouver.wsu.edu/~kendall/politics.htm](http://helium.vancouver.wsu.edu/~kendall/politics.htm)
6. Antibiotics  
[helium.vancouver.wsu.edu/~kendall/index.htm](http://helium.vancouver.wsu.edu/~kendall/index.htm)
7. What are Antibiotics  
[helium.vancouver.wsu.edu/~kendall/whatareantibiotics.htm](http://helium.vancouver.wsu.edu/~kendall/whatareantibiotics.htm)
8. Chapter#18 Food Borne Diseases  
[www.slic2.wsu.edu:82/hurbert/micro101/pages/Chap18.html](http://www.slic2.wsu.edu:82/hurbert/micro101/pages/Chap18.html)
9. Guardian? Unlimited Special Reports/Antibiotics in food  
[www.guardian.co.uk/antibiotics/](http://www.guardian.co.uk/antibiotics/)
10. Antibiotic Attack  
[www.asklive.org.grants/lecturesbiointer...Attack/a 2.html](http://www.asklive.org.grants/lecturesbiointer...Attack/a%20.html)
11. Evolution: "Microbes: What They Do and How Antibiotics Change Them" by Maura J. Meade-Callahan, Ph. D  
[www.actionbioscience.org/evolution/meade callahan.html](http://www.actionbioscience.org/evolution/meade_callahan.html)
12. APUA: Ecology of Antibiotics  
[www.healthsci.tufts.edu/apua/Ecology/ecology.html](http://www.healthsci.tufts.edu/apua/Ecology/ecology.html)
13. Antibiotics--- Penicillins &Its Derivatives, Vancomycin derivatives  
[www.infoallglobe.com/writers/Antibiotics...term](http://www.infoallglobe.com/writers/Antibiotics...term) paper.htm
14. The Rise (and Fall) of Antibiotics  
[www.naturalrearing.com/J In Learning/Misc/Antibiotics.html](http://www.naturalrearing.com/J%20In%20Learning/Misc/Antibiotics.html)
15. Time.com: The Antibiotics Crisis  
[time.com/time/health/article/0,8599,93929,00.html](http://time.com/time/health/article/0,8599,93929,00.html)

Bibliography if print resources

## **1. Antimicrobial Use and Antimicrobial Resistance: A Population Perspective**

Emerging Infectious Diseases

April 2002

Page(s) : 347-354

Health and Human Services Department (HHS)  
 National Center for Infectious Diseases (NCID)  
 SuDoc Number : HE 20.7817/8/4

2. Natural Microbial Compounds May Control Strep and Staph Infections  
 Agricultural Research Service News, Jan. 3, 2000, 3K, SIRS Government Reporter
3. Miracle Drug Vs Superbug  
 FDA Consumer Nov./Dec. 1998, 15K SIRS Researcher
4. Antibiotics  
 World Health, Gale Group 2000
5. Antibiotics  
 U\*X\*L Science U\*X\*L 1998

**Materials Price List/ordering Information**

Carolina -1-800-334-5551, [www.carolina.com](http://www.carolina.com)

<b>Item</b>	<b>Ordering Number</b>	<b>Price</b>
Alcaligenes faecalis	Ww15-4835	\$ 9.75 per vial
Bacillus cereus	Ww 15-4780A	\$ 9.75 per vial
Serratia marcescens	15-5450A	\$ 9.75 per vial
TSA Media tubes	BA-82-7322	\$ 13.73 pack of 10
TSA Dehydrated media	BA-78-8420	\$ 17.95 /100grams

**Life Science Products**

Call for current prices 1-800-245-5774 [www.lifesciprod.com](http://www.lifesciprod.com) [lspl@lifesciprod.com](mailto:lspl@lifesciprod.com)

<i>Item</i>	<b>Ordering Number</b>	<b>Price</b>
60x15mm Petri dish (500/case)	LS-6606	\$48.70
Red 12"x24" Biohazard Bags (200/pack)	LS-4812-R3	\$32.50
Sterile Cotton-Tipped Applicator Swabs (100/pack)	AP-4304	\$6.90

**Fisher Scientific** Call for current prices 1-800-766-7000

<i>Item</i>	<b>Ordering Number</b>	<b>Price</b>
Dehydrated LB agar	DF0140-15-4	\$61.99

## **Lab #4: The Gram Stain**

### **Introduction:**

Simple stains stain biological materials indiscriminately. Differential stains stain only selected parts of the cell or certain types of cells. All differential stains require at least three components, or steps. The first stain, the primary stain, is used to stain the target cells or organelles that you want to visualize. After the application of the primary stain, a mordant is applied. The mordant reacts chemically with the primary stain and with the cell, or its component. The function of the mordant is to enhance the retention of the primary stain. Either in addition to the mordant or in place of the mordant is selective treatment. Selective treatment means applying techniques like heating the primary stain or washing it with alcohol to decolorize unstained parts. The final and third step is applying a counter stain. A counter stain is used to stain all unstained biological materials. Counter stains are usually a contrasting color to the primary stain. With the Gram stain, the primary stain colors the cells violet and the counter stained bacteria are red. Violet cells are “Gram positive” because they have reacted to the stain. The red bacteria are called “Gram negative” because they only reacted to the red counter stain.

If you want to see the shapes of various bacteria, then simple stains will work just fine. For visualizing specific structures or to help identify a specific type of bacteria, then selective differential stains are an important tool. Bacteria can be divided into two groups based on their response to the Gram stain. The Gram stain takes advantage of the composition of certain structures in the cell membrane and cell wall of many, but not all bacteria. Because of changes that take place in the cell walls of bacteria as they age, the Gram stain technique is most reliable when applied to 24-48 hour cultures. Older cultures of ordinarily Gram positive bacteria (those that are stained) may appear as Gram negative bacteria. This is because the stains do not adhere well to older structures.

The Gram stain procedure is probably the single most common staining procedure and one of the primary diagnostic tools for the bacteriologist. Bacteria are often described in terms such as “Gram negative cocci” or “Gram positive bacillus”. When working to identify an unknown bacteria, the colony morphology is noted and then the individual bacterium’s shape and reaction to Gram staining. There are other important selective staining procedures used to identify bacteria, most notably the acid-fast stain

and the metachromatic-granule stain. These are important in the rapid identification of human pathogens, so we will not perform these in this class but be aware that there are additional important stains.

**Purpose:** After reading the Introduction and the Procedure, explain the purpose in doing this lab in the space below.

**Hypothesis:**

Because this is another skills lab and not an inquiry, no hypothesis is needed.

**Materials:**

3 Microscope Slides	Oil Immersion Microscope	Soap
95% Ethanol (EtOH)	Test Tube Holder	Latex Gloves
Bunsen Burner	Matches	Clothes Pin

24-48 hr. agar and broth cultures of *Alcaligenes faecalis*, *Bacillus cereus* and *Serratia marcescens*

Stains: Gram's Crystal Violet, Gram's Iodine, Ethanol/Acetone Rinse and Gram's Safranin

**Safety:**

1. A microbiology lab is potentially a very dangerous place. For this reason it is extremely important that you follow all safety guidelines and always practice sterile technique when handling microbes, unless instructed otherwise.
2. There should be no books or papers at your workstation except this lab packet.
3. Never have any food or drink at your workstation.
4. Always thoroughly wash your hands with disinfectant soap or alcohol before leaving your workstation.
5. Never open a Petri dish after you have inoculated it and allowed it to incubate overnight.
6. Always dispose of used material in the biohazard bag, unless instructed otherwise.

**Procedure:**

1. Prepare the 3 microscope slides and apply smears as you did in the Preparation of the Smear section of the “Unstained Preparations and Simple Stains” lab.
2. Flood the smears with Gram’s crystal violet stain for 30 seconds.
3. After 30 seconds, rinse the smears gently with Gram’s iodine.
4. After the Gram’s crystal violet has been completely removed, flood the smears with Gram’s iodine for 1 minute.
5. Hold the slide over the sink and rinse with ethanol/acetone (or 95% ethanol) until the color stops washing off. As soon as the color stops flowing off the slide, rinse immediately with water. Warning: This step, decolorization, is very important. It is easy to apply too much alcohol and then rinse too much color out of the cells. The result is a faint Gram-positive stain.
6. Flood the smears with Gram’s safranin for 1 minute.
7. After 1 minute, rinse the stain from the slide as you did in the “Simple Stains” lab.
8. Allow the slide to air dry and then view under the oil immersion setting of the microscope. Complete the data table in the Results section of the lab.

**Results:**

Observations of Gram Stained Bacteria			
<u>Bacteria</u>	<u>Color</u>	<u>Shape</u>	<u>Gram + or - ?</u>

1. How is the cell wall different from the plasma membrane?
  
  
  
  
  
  
  
  
  
  
2. In what way(s) is the cell wall different between Gram positive and Gram negative bacteria?

3. In what type(s) of organisms would the Gram stain not work? Why?

With out cell walls

4. How is the Gram stain reaction by bacteria useful information to medical doctors or microbiologists?

Because of the nature of this lab, the following will not be the usual analysis/conclusion questions in this section.

*Alcaligenes feacalis*

Go to [www.sciencenet.com.au](http://www.sciencenet.com.au) on the Internet. Click on Gram negative and find Family Alcaligenaceae on the sidebar on the left side of the screen. Click on Alcaligenaceae. In the space below, summarize the morphology and biochemistry of the family.

While you are at [www.sciencenet.com.au](http://www.sciencenet.com.au), check out the uses, ecological position, pathology and virulence (if any) of *A. feacalis*. Summarize what you have discovered about this organism in the space below.

*Bacillus cereus*

You can find out about Family Bacillaceae by clicking on the Gram positive group of [www.sciencenet.com.au](http://www.sciencenet.com.au). Go to the sidebar on the left and click on Baicillaceae. In the space below, contrast (describe the differences in) the two main genera of the Family Bacillaceae, Bacillus and Clostridium.

*Serratia marcescens*

Go back to the Gram negative portion of [www.sciencenet.com.au](http://www.sciencenet.com.au) and click on Enterobacteriaceae on the sidebar. In the space below describe the characteristics of this diverse Family.

**Teachers Answers**

1. How is the cell wall different from the plasma membrane?

*The cell wall is found in both plants and bacteria. The plant cell wall is a non-living secretion made from the plasma membrane. It is composed of the following:*

- Cellulose
- Cellulose fibrils that are deposited in alternating layers for strength.
- Contains pits or openings that make its totally permeable.
- Gives cell shape to the plant cell.

*The cell wall in the bacteria is composed of different materials. Both Gram+ and Gram- bacteria cell walls are made up of peptidoglycan which is composed of overlapping lattice of 2 sugars that are cross linked by amino acid bridges. For the bacterial cell the cell wall is critical. It keeps the bacterial cell from lysis due to the different osmotic pressures. The inside of the bacterial cell has a high solute pressure, which allows for water to move into the cell, without the wall the cell would burst.*

*The cell membrane has a totally different function. Its structure is made up of phospholipids and proteins. The proteins are not in a fixed position or a rigid structure. They are of two types, peripheral proteins which lie on the surface, and two integral proteins that extend through the membrane. The function of cell membrane is to transport molecules, protect the cell, and communicate to the cell.*

2. In what way(s) is the cell wall different between Gram positive and Gram negative bacteria?

*Gram+ bacteria- the cell membrane is much thicker than the gram- bacteria containing about five times the amount of peptidoglycan and has a smooth*

*appearance on its external surface. It is also composed of polysaccharides and/or teichoic acids*

*Gram- bacteria- The gram-negative bacterial also have a second membrane which is chemically different from the plasma membrane external to the cell wall, and it also may have a gelatinous sheath external to the second membrane. The cell walls main component is lipopolysaccharide. Additionally there is a phospholipid protein, lipoprotein. It's outer appearance is convoluted.*

3. In what type(s) of organisms would the Gram stain not work? Why?

*Gram stain will not work with organisms that do not contain a cell wall but contains only a cell membrane.*

4. How is the Gram stain reaction by bacteria useful information to medical doctors or microbiologists?

The information gained from gram staining is use to diagnosis, prevent and treat bacterial infections. Gram+ Bacterial causes illness by secreting different types of toxins that affect the cells of the infected individual. Gram- bacteria cause immune reactions by lipopolysaccharides found in the cell wall.

Because of the nature of this lab, the following will not be the usual analysis/conclusion questions in this section.

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*Serratia marcescens*

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