

Oxidase Test Protocol

Created: Thursday, 11 November 2010

Author

- Patricia Shields
- Laura Cathcart

Information

History

In 1928, Gordon and McLeod (5) introduced the use of a dimethyl-*p*-phenylenediamine dihydrochloride solution to test for the presence of oxidase systems. In particular, they used the test to distinguish *Neisseria gonorrhoeae* (oxidase positive) from *Staphylococcus* spp. and *Streptococcus* spp. (oxidase negative). The sensitivity of the oxidase test was increased when Kovács found that a tetra-methyl-*p*-phenylenediamine dihydrochloride solution gave a quicker reaction (8). Gaby and Hadley developed a modified oxidase test using *p*-aminodimethylaniline oxalate with α -naphthol to detect oxidase in test tube cultures (3).

Purpose

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase (2, 10, 12). In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product (2, 4, 9).

Theory

The final stage of bacterial respiration involves a series of membrane-embedded components collectively known as the electron transport chain. The final step in the chain may involve the use of the enzyme cytochrome oxidase, which catalyzes the oxidation of cytochrome *c* while reducing oxygen to form water (10). The oxidase test often uses a reagent, tetra-methyl-*p*-phenylenediamine dihydrochloride, as an artificial electron donor for cytochrome *c* (1, 2, 15). When the reagent is oxidized by cytochrome *c*, it changes from colorless to a dark blue or purple compound, indophenol blue (5, 9). Figure 1 contains a diagram of this reaction.

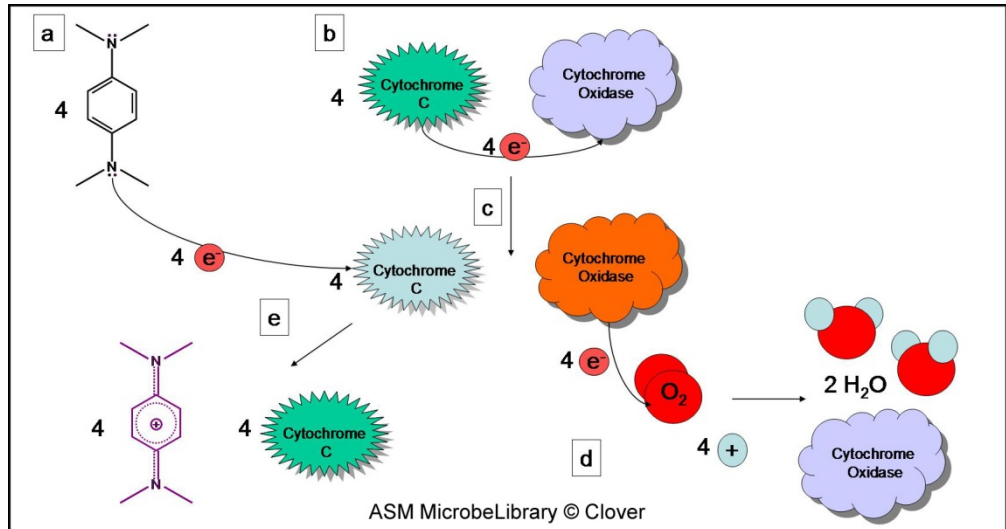


FIG. 1. (a) Tetra-methyl-*p*-phenylenediamine dihydrochloride (TMPD), the oxidase reagent, is electron rich (reduced) and has no color.

(b) In bacteria that contain the enzyme cytochrome oxidase, one electron from each of four cytochrome *c* molecules will be temporarily transferred to the enzyme.

(c) This creates four electron-poor cytochrome *c* molecules and an electron-rich cytochrome oxidase enzyme.

(d) As the final step in respiration, the cytochrome oxidase enzyme transfers four electrons to molecular oxygen and along with four protons, forms two molecules of water, returning the cytochrome oxidase enzyme to its original state.

(e) Instead of acquiring an electron from another component in the electron transport chain, an electron-rich TMPD molecule passes an electron to the electron-poor cytochrome *c*. Cytochrome *c* returns to its original state and the resulting electron-poor (oxidized) TMPD radical has a dark blue color.

In addition to a positive oxidase and negative oxidase reaction, some organisms are classified as variable oxidase or delayed oxidase positive. Variability in the oxidase reaction has been attributed to differences in cytochrome *c* composition, variability in cytochrome oxidases, and overall transport chain composition variability (6, 7, 9, 12).

Recipes

- A. Kovács oxidase reagent (8)
1% tetra-methyl-*p*-phenylenediamine dihydrochloride, in water
Store refrigerated in a dark bottle no longer than 1 week.
- B. Gordon and McLeod reagent (5)

1% dimethyl-*p*-phenylenediamine dihydrochloride, in water
Store refrigerated in a dark bottle no longer than 1 week.

- C. Gaby and Hadley oxidase test (3)
1% α -naphthol in 95% ethanol
1% *p*-aminodimethylaniline oxalate
Store refrigerated in dark bottles no longer than 1 week.

Oxidase reagents are also available commercially in droppers, impregnated disks, and test strips.

PROTOCOLS

There are many method variations to the oxidase test. These include, but are not limited to, the filter paper test, filter paper spot test, direct plate method, and test tube method. All times and concentrations are based upon the original authors' recommendations.

Filter Paper Test Method (8) (Fig. 2)

1. Soak a small piece of filter paper in 1% Kovács oxidase reagent and let dry.
2. Use a loop and pick a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate and rub onto treated filter paper (please see Comments and Tips section for notes on recommended media and loops).
3. Observe for color changes.
4. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.



FIG. 2. On the left is oxidase-positive *Pseudomonas aeruginosa* and on the right is oxidase-negative *Escherichia coli*. Both organisms were

rubbed on a filter that had been dipped in Kovács oxidase reagent and allowed to dry.

Filter Paper Spot Method (4) (Fig. 3)

1. Use a loop and pick a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate and rub onto a small piece of filter paper (please see Comments and Tips section for notes on recommended media and loops).
2. Place 1 or 2 drops of 1% Kovács oxidase reagent on the organism smear.
3. Observe for color changes.
4. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

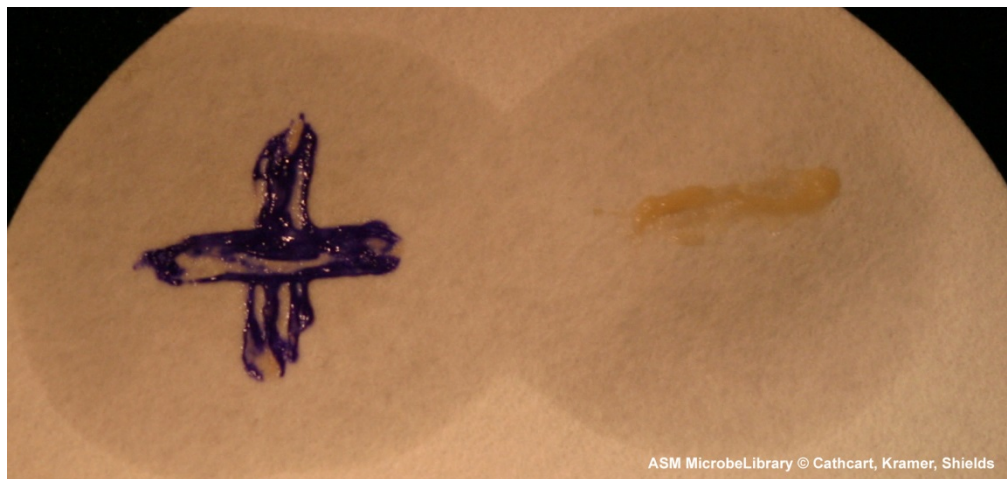


FIG. 3. On the left is oxidase-positive *Pseudomonas aeruginosa* and on the right is oxidase-negative *Escherichia coli*. Both organisms were rubbed on a dry filter that was then treated with one drop of Kovács oxidase reagent.

Direct Plate Method (7, 10) (Fig. 4)

1. Grow a fresh culture (18 to 24 hours) of bacteria on nutrient agar using the streak plate method so that well-isolated colonies are present (please see Comments and Tips section for notes on recommended media).
2. Place 1 or 2 drops of 1% Kovács oxidase reagent or 1% Gordon and McLeod reagent on the organisms. Do not invert or flood plate.
3. Observe for color changes.
4. When using Kovács oxidase reagent, microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if

the color does not change or it takes longer than 2 minutes.

5. When using Gordon and McLeod reagent, microorganisms are oxidase positive when the color changes to red within 10 to 30 minutes or to black within 60 minutes. Microorganisms are oxidase negative if the color does not change.

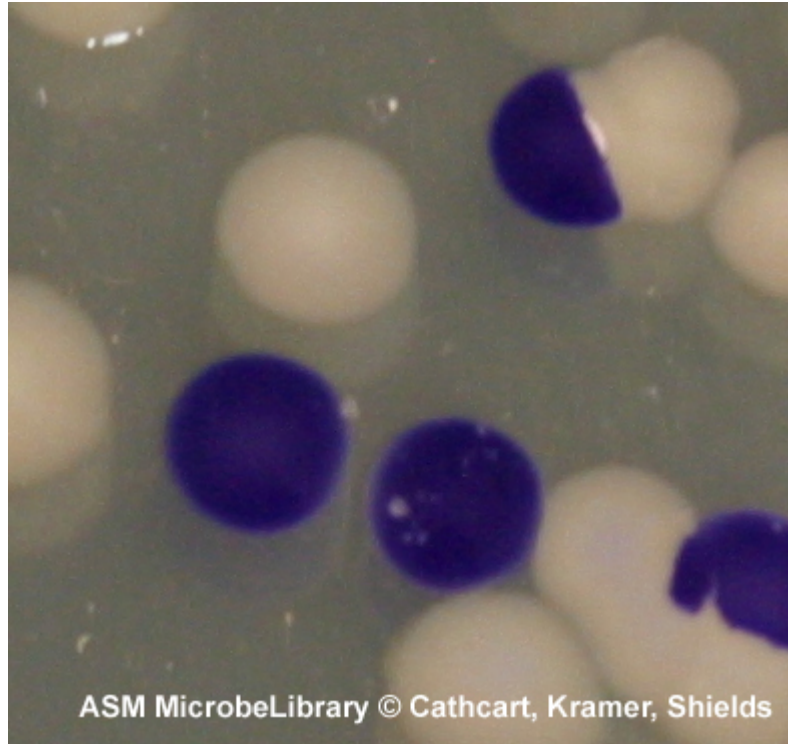


FIG. 4. This is a mixed culture of oxidase-negative *Escherichia coli* and oxidase-positive *Vibrio cholerae* showing how the direct oxidase test differentiates between the two organisms. Kovács oxidase reagent was added directly to the plate.

Test Tube Method (3) (Fig. 5)

1. Grow a fresh culture (18 to 24 hours) of bacteria in 4.5 ml of nutrient broth (or standard media that does not contain a high concentration of sugar, please see Comments and Tips section for notes on recommended media).
2. Add 0.2 ml of 1% α -naphthol, then add 0.3 ml of 1% *p*-aminodimethylaniline oxalate (Gaby and Hadley reagents).
3. Observe for color changes.
4. Microorganisms are oxidase positive when the color changes to blue within 15 to 30 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 2 to 3 minutes. Microorganisms are oxidase negative if the color does not change.

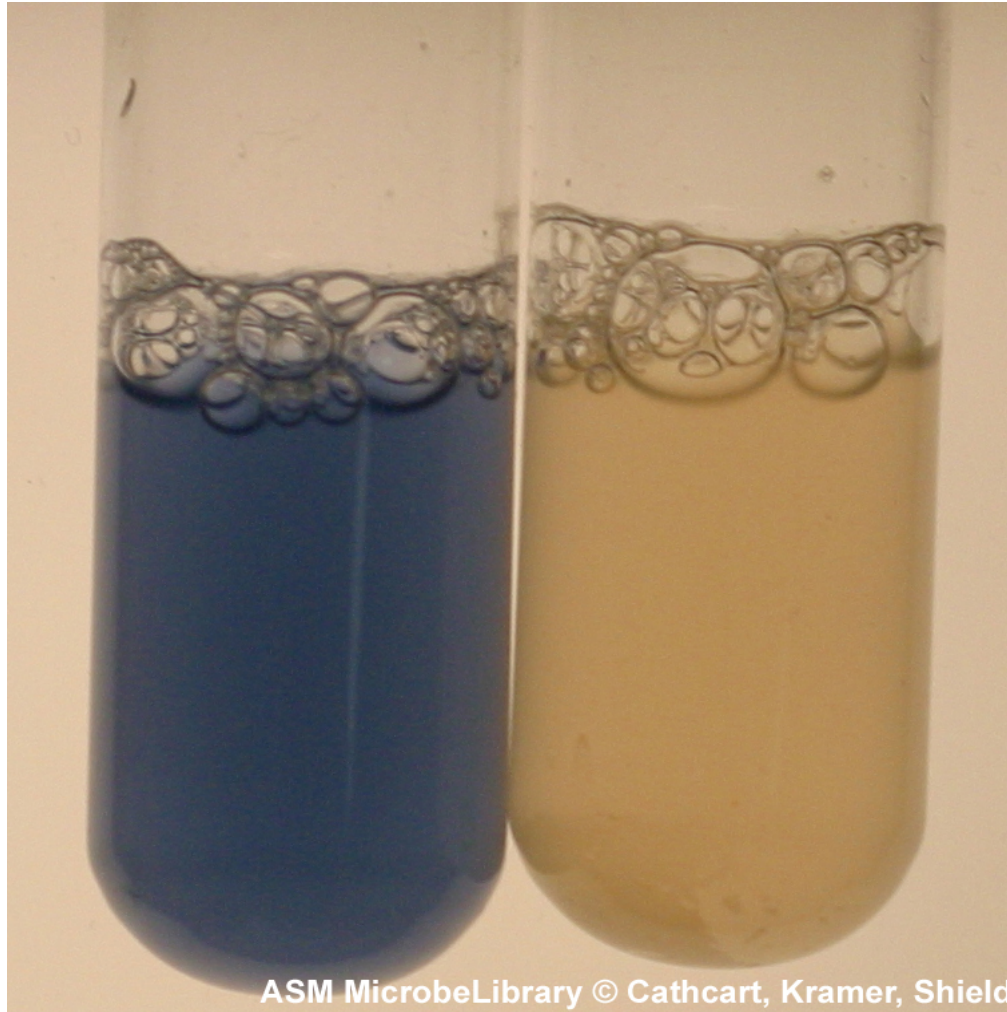


FIG. 5. The tube on the left is oxidase-positive *Neisseria sicca* and the tube on the right is oxidase-negative *Staphylococcus aureus*. After 24 hours of growth, Gaby and Hadley reagents were added to each tube (3).

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

COMMENTS AND TIPS

- The reagents used in the oxidase test have been shown to autooxidize, so it is very important to use fresh reagents, no older than 1 week (2, 3, 14, 15). Steel found that the autooxidation can be slowed by the addition of 1% ascorbic acid (14).
- Nickel, steel, and other wire loops may give false-positive results, so it is important to use only platinum or inert transfer loops, such as

sterile wood sticks commonly used in teaching laboratories (4, 13). Other acceptable examples include sterile plastic loops, sterile toothpicks, and sterile swabs.

- Both bacteria and yeast grown on media containing high concentrations of glucose show inhibited oxidase activity, so it is recommended to test colonies grown on media without excess sugar, such as nutrient agar (6, 11). Tryptic soy agar is also an excellent media.
- Bacteria grown on media containing dyes may give aberrant results.
- The test reagents will effectively kill the microorganisms (3, 5), so subculturing should be done prior to adding any reagent to an active culture.
- Older cultures are less metabolically active and are thus unreliable for this test in a clinical setting. In the classroom, if by necessity older cultures must be used, expect longer reaction times.
- All reaction times listed are based upon freshly made reagents without stabilizing agents. If you use commercially prepared reagents, these often contain stabilizing agents and thus you should follow the manufacturer's instructions.

REFERENCES

1. **Alexander, S. K., and D. Strete.** 2001. Microbiology: a photographic atlas for the laboratory. Benjamin Cummings, San Francisco, CA.
2. **Gaby, W. L., and L. Free.** 1958. Differential diagnosis of pseudomonas-like microorganisms in the clinical laboratory. *J. Bacteriol.* **76**:442–444.
3. **Gaby, W. L., and C. Hadley.** 1957. Practical laboratory test for the identification of *Pseudomonas aeruginosa*. *J. Bacteriol.* **74**:356–358.
4. **Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B Phillips.** 1981. Manual and methods for general bacteriology. ASM Press, Washington, DC.
5. **Gordon, J., and J. W. McLeod.** 1928. The practical application of the direct oxidase reaction in bacteriology. *J. Pathol. Bacteriol.* **31**:185–190.
6. **Jurtshuk, P., Jr., and D. N. McQuitty.** 1976. Use of a quantitative oxidase test for characterizing oxidative metabolism in bacteria. *Appl. Environ. Microbiol.* **31**:688–679.
7. **Jurtshuk, P., Jr., and D. McQuitty.** 1976. Survey of oxidase-positive and -negative bacteria using a quantitative Kovács oxidase test. *Int. J. Syst. Bacteriol.* **26**:127–135.
8. **Kovács, N.** 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature (London)* **178**:703.
9. **Lui, J.-K., and P. Jurtshuk.** 1986. N,N,N'-N'-tetramethyl-*p*-phenylenediamine-dependent cytochrome oxidase analyses of *Bacillus* species. *Int. J. Syst. Bacteriol.* **36**:38–46.
10. **MacFaddin, J.** 1972. Biochemical tests for the identification of medical bacteria. Williams and Wilkins Company, Baltimore, MD.
11. **Nobre, G. N., M. J. Charrua, and M. M. Silva.** 1987. The oxidase test in yeasts of medical importance. *J. Med. Microbiol.* **23**:359–361.
12. **Oser, B.** 1965. Enzymes and their action: cell respiration, p. 434-435. *In* L. Hawk (ed.), *Physiological chemistry*, 14th ed. McGraw-Hill, Boston, MA.

13. **Smith, S. K., D. C. Sutton, J. A. Fuerst, and J. L. Reichelt.** 1991. Evaluation of the genus *Listonella* and reassignment of *Listonella damsela* (Love et al.) MacDonell and Colwell to the genus *Photobacterium* as *Photobacterium damsela* comb. nov. with an emended description. *Int. J. Syst. Bacteriol.* **41**:529–534.
14. **Steel, K. J.** 1961. The oxidase reaction as a taxonomic tool. *J. Gen. Microbiol.* **25**:297–306.
15. **York, M. K., M. M. Taylor, J. Hardy, and M. Henry.** 2004. Biochemical tests for the identification of aerobic bacteria, p. 3.17.39.1. *In* H. D. Isenberg (ed.), *Clinical microbiology procedures handbook*, 2nd ed. ASM Press, Washington, DC.

REVIEWERS

This resource was peer-reviewed at the ASM Conference for Undergraduate Educators 2010.

Participating reviewers:

Shelley Aguilar
Mt. San Jacinto College, Meniffee, CA

Benita Brink
Adams State College, Alamosa, CO
Lakshmi Chilukuri
University of California San Diego, San Diego, CA

Jenny Clark
Saddleback College, Mission Viejo, CA

Bryna Clover
University of Maryland, College Park, College Park, MD

Karen Dalton
Community College of Baltimore County, Catonsville, MD

Cynthia S. Freitag
Polk State College, Lakeland, FL

Anne Hanson
University of Maine, Orono, ME

Zoe A. Hawk
Arizona Western College, Yuma, AZ

Jan Hudzicki
University of Kansas Medical Center, Kansas City, KS

D. Sue Katz
Rogers State University, Claremore, OK

Archana Lal
Independence Community College, Independence, KS

Min-Ken Liao
Furman University, Greenville, SC

Julie Oliver
Cosumnes River College, Sacramento, CA

Judy Penn
Shoreline Community College, Shoreline, WA

Michele Perchez
Grossmont College, El Cajon, CA

Jill Raymond
Mesa Community College, Mesa AZ

Karen Reiner
Andrews University, Berrien Springs, MI

Margaret Richey
Centre College, Danville, KY

Erica Suchman
Colorado State University, Ft. Collins, CO

Amy C. Vollmer
Swarthmore College, Swarthmore, PA

Ruth A. Wrightsman
Flathead Valley Community College, Kalispell, MT

Fred Zalatan
SUNY Oneonta, Oneonta, NY