

THE NITRATE REDUCTION TEST

Principle and Purpose

Many bacteria possess the ability to employ nitrate (NO_3^-) as a terminal electron acceptor during anaerobic respiration. In particular, the enzyme *nitrate* reductase reduces nitrate to nitrite (NO_2^-). In addition, some bacterial species possess *nitrite* reductase as well as other enzymes that can further reduce nitrite to either ammonia or other nitrogen containing substances (Fig. 1). The metabolic ability of these bacteria not only plays a significant role in the nitrogen cycle, but also has important agricultural, environmental, and public health implications. Moreover, the species-dependent ability to reduce nitrate can be exploited in the identification of microbes, including those of medical significance, e.g., the enteric bacteria and *Neisseria* species.

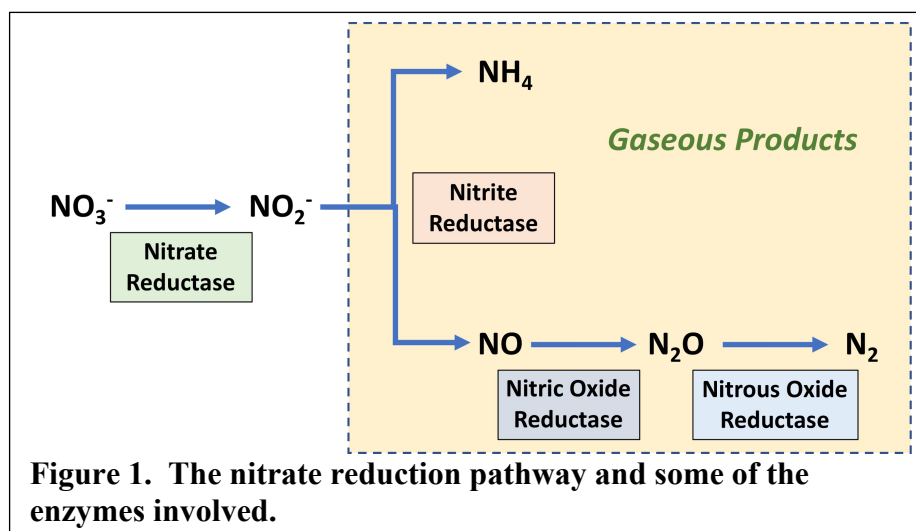


Figure 1. The nitrate reduction pathway and some of the enzymes involved.

The nitrate reduction test that students will perform in this exercise is based upon some fundamental chemical studies of dyes by Peter Griess in the mid-1850s. (For a review of Griess' contributions and the chemistry behind the current nitrate reduction test, see the following URL: <http://www.asmscience.org/content/education/protocol/protocol.3660>). In short, the foundational essence of this test is the detection of NO_2^- . To summarize, nitrites react with sulfanilic acid and N,N-dimethylnaphthylamine to form a red dye. In a test tube reaction using nitrate broth, the appearance of the red dye indicates the presence of NO_2^- . Hence, NO_2^- was a product of the reduction of NO_3^- and was not reduced further by other enzymes (see Fig. 1). If no red dye is formed, the result is presumptively negative. Yet, if zinc is added to a presumptive negative tube, any remaining NO_3^- is catalyzed to form NO_2^- , which then reacts to form the red dye complex. The zinc-induced formation of red dye indicates that the bacterium never reduced nitrate, i.e., it does not possess nitrate reductase.

The nitrate reduction test that will be conducted by students in this exercise uses a commercially-available medium and diagnostic reagents (Hardy Diagnostics, Santa Maria, CA). The following URL contains a document that describes this medium and its use:

https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/NitrateBrothDT.htm

Descriptions of the diagnostic reagents associated with this medium can be found at the following URL:

https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/NitrateReagent.htm

Students are strongly encouraged to review these latter two documents which will help them understand how to conduct the test and interpret the results.

To briefly summarize, the nitrate reduction test is performed by growing a bacterial species in a broth containing potassium nitrate (KNO_3) and a Durham tube (Fig. 2). (A Durham tube is essentially an upside-down test tube that is used to capture any gas produced by the microbe.) Following incubation, if the microbe generated gas as part of the reduction process, the liquid in the Durham tube will be displaced by a gas bubble. The gas is a mixture of carbon dioxide and nitrogen (N_2) that results from the reduction of nitrate and citric acid (the latter is from the citric acid [Krebs] cycle). The presence of nitrite is determined by a red color complex that develops upon the addition of sulfanilic acid and N,N-dimethyl-1-naphthylamine (Fig. 3).

If a red color does not appear after the addition of the above two reagents (hence, a presumptive negative test), there are two possibilities: 1) the bacterial species being tested may have further reduced nitrite to ammonia or nitrogen gas and 2) the species does not possess the reductase enzyme. In the latter instance, nitrate would remain present in the medium and can be detected by the application of zinc dust. Zinc would reduce nitrate to nitrite to form the notable red color complex. Hence, zinc-induced production of the red dye indicates that the test is negative for nitrate reduction. If, though, the addition of zinc does not produce a color change, then the test is considered positive for nitrate reduction. For a summary of these reaction results, see Fig. 4.

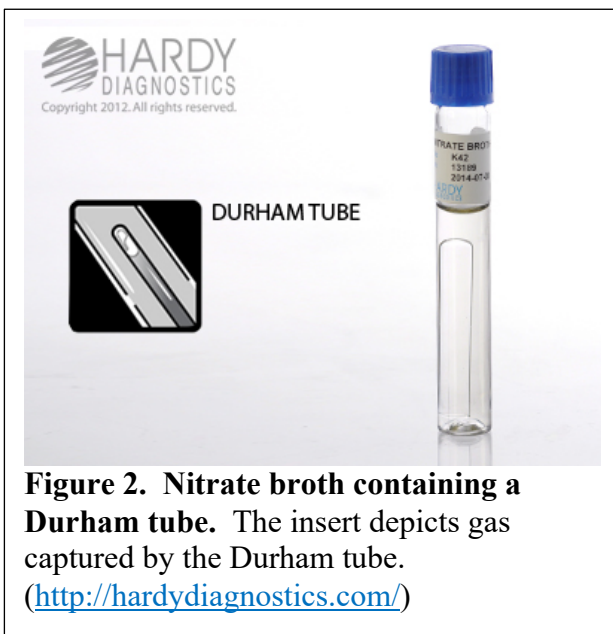


Figure 2. Nitrate broth containing a Durham tube. The insert depicts gas captured by the Durham tube. (<http://hardydiagnostics.com/>)

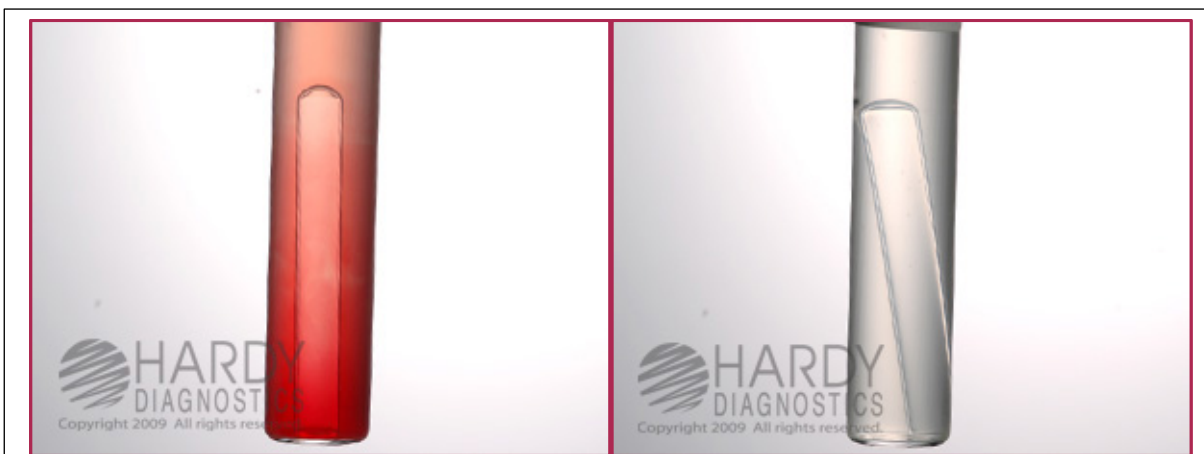


Figure 3. Positive (left image) and presumptive negative (right image) nitrate reduction test results. The positive results indicate nitrate was reduced to nitrite, whereas the absence of a red color in the tube in the right image would demonstrate a presumptive negative result that can be confirmed by adding zinc dust. Notice that no gas was captured in the Durham tube in either test result. (<http://hardydiagnostics.com/>)

In the present exercise, the nitrate reduction test that will be conducted by students uses a commercially-available medium and diagnostic reagents (Hardy Diagnostics, Santa Maria, CA). The students will conduct the nitrate reduction test using known organisms as well as non-sterile soil. The known organisms should provide various results, whereas one might expect a single type of result from the microbes in the soil sample. Can you speculate what that may be?

A video demonstrating the use of nitrate broth and the interpretation of results is available at the following URL: <https://www.micro.iastate.edu/video/microbiology-012-nitrate-reduction-test>. The methodology shown in this video is similar, but not necessarily identical to that described in the exercise presented herein.

Learning Objectives

Upon completion of this exercise, a student should be able to:

- Understand the underlying chemical mechanisms of the nitrate reduction test;
- Properly conduct the nitrate reduction test; and
- Accurately interpret the results of this test and how these results distinguish different metabolic types of bacteria.

Materials Required

The following materials are necessary to successfully conduct this exercise:

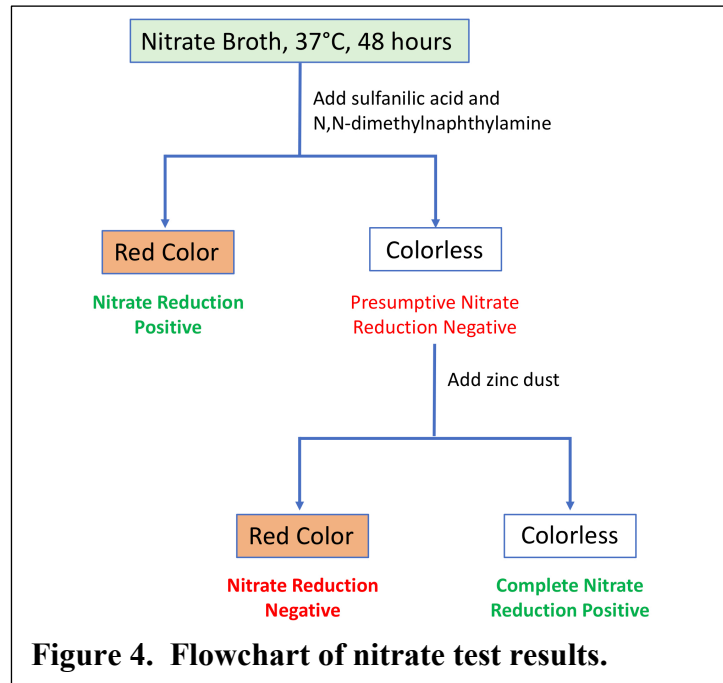
Organisms - The following organisms should be provided as 24-48 hour-old TSA slant cultures:

- *Staphylococcus epidermidis* (ATCC 12228) [abbreviated as *S. epidermidis*]
- *Pseudomonas aeruginosa* (ATCC 27853) [abbreviated as *P. aeruginosa*]
- *Escherichia coli* (ATCC 25922) [abbreviated as *E. coli*]

Media and Reagents

- Nitrate Broth (Cat. No. K42; Hardy Diagnostics; https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/NitrateBrothDT.htm)
- Nitrate Reagent A (Cat. No. Z71; Hardy Diagnostics [0.8% w/v sulfanilic acid in 5N acetic acid])*
- Nitrate Reagent B (Cat. No. Z72; Hardy Diagnostics [0.6% v/v N,N-dimethylnaphthylamine in 5N acetic acid])*
- Nitrate Reagent C (Cat. No. Z73; Hardy Diagnostics [zinc powder])*
- Freshly collected soil (several grams)

*https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/NitrateReagent.htm



Procedures

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

Notes of Precaution: Use appropriate handling procedures with Nitrate Reagents A, B, and C. If either Nitrate Reagent A or B comes in contact with skin, thoroughly wash the area immediately with soap and water. In addition, do not expose Nitrate Reagent C to an open flame. It may cause a small explosion. Also, do not breath the dust from this reagent.

- 1) Obtain five (5) tubes of Nitrate Broth, loosen (but do not remove) the caps on each, and allow the media to come to room temperature.
- 2) Label one of the tubes as 'Control', a second as '*E. coli*', another as '*S. epidermidis*', a fourth as '*P. aeruginosa*', and the remaining tube as 'Soil'. Be sure to add other identifying information as appropriate.
- 3) Inoculate the media as indicated below:
 - a) Using a loopful of inoculum from a culture of *E. coli*, inoculate the broth in the appropriately labeled tube.
 - b) Using a loopful of inoculum from a culture of *S. epidermidis*, inoculate the broth in the appropriately labeled tube.
 - c) Using a loopful of inoculum from a culture of *P. aeruginosa*, inoculate the broth in the appropriately labeled tube.
 - d) Using a spatula, place a small amount of soil (about a quarter size of a finger nail) in the appropriately labeled tube.
 - e) Do not add any microbe or soil into the tube labeled 'Control'.
- 4) Incubate all the tubes with caps remaining loose (but not so loose that they fall off) in the 35-37°C incubator for 48 hours.
- 5) Remove the tubes from the incubator and observe each for growth and the presence of gas bubbles in the Durham tube.

Interpretation of Results:

- If there is no significant growth in the nitrate broth, continue incubation for another 24-48 hours. If there still is no growth, repeat the inoculation with a new tube of nitrate broth. If growth is still absent, consult with your laboratory instructor.
- If the organism does show growth and if gas is present in the Durham tube, then the microbe has reduced nitrate to nitrogen gas – a positive test result (see Fig. 4). **FOR THIS PARTICULAR TUBE, STOP HERE!**
Record your observations on the report sheet attached to this exercise.
- In contrast, if the organism does show growth, but no gas is present in the Durham tube, then the microbe may have reduced nitrate to nitrite or another non-gas end product or no reaction occurred at all. Therefore, if no gas is present in the Durham tube, first *record your observations on the report sheet attached to this exercise*, then proceed with the following steps to determine which of these possibilities.

- 6) Add five drops of Nitrate Reagent A and five drops of Nitrate Reagent B to the culture in the nitrate broth (see Fig. 5 on the next page).



Note: Be sure to add the reagents in the order noted above. Also, do not allow the dropper bottle tip to touch the sides of the tube when adding a reagent.

Procedural Note: Nitrate Reagents A, B, and C are stored in black/dark brown, plastic snap-cap tubes (Fig. 5). The tubes are clearly labeled as to their contents. Inside the appropriately labeled tubes, Reagents A and B are stored in labeled dropper bottles. Reagent C is contained in a small screw-capped vial that is also labeled. Be sure that the correctly labeled reagent is being used in a particular step of the procedure detailed below. Importantly, after using these reagents, be sure to return them to the appropriately labeled tube.



Figure 5. Nitrate reagents in snap-cap tubes. The left image depicts how Reagents A and B come in a dropper bottle, whereas Reagent C is contained in a small screw-capped vial. (<http://hardydiagnostics.com/>)

- 7) Gently shake the reaction tube to mix the reagents.
- 8) Observe the tube for the development of a deep red color within two minutes following the addition and mixing the Reagents A and B.

Interpretation of Results: The development of a red color indicates that a positive nitrate reduction reaction, i.e., nitrate was reduced to nitrite (see Fig. 4).

Record your observations on the report sheet attached to this exercise.

- 9) If a red color does not develop within two minutes, use a spatula to sparingly add a small amount (a visible ‘pinch’ of dust) of Nitrate Reagent C to the medium.



Figure 6. Red color development after the addition of zinc to a presumptive negative test result. (<http://hardydiagnostics.com/>)

Note: The addition of zinc verifies negative nitrite reactions, thereby avoiding false-negative results (Fig. 6). However, do not add excessive amounts of zinc which may cause false-positive results. [Question: why might the control tube be used as a guide to knowing how much zinc to add?]

- 10) Observe the tube for the development of a red color within 5-10 minutes following the addition of Nitrate Reagent C.

Interpretation of Results:

- A red color indicates that nitrate was ***not*** reduced (Figs. 4 and 6), i.e., the nitrate is still present in the medium indicating that the microbe did not reduce it to another product. [continued on next page]
- The absence of a red color reaction indicates that nitrate was reduced beyond nitrite.

Record your observations on the report sheet attached to this exercise.

Practical Hints for a Successful Nitrate Test

- Interpret the color reactions should be made immediately. The color reaction of a positive test may fade quickly.
- If air bubbles are present in the Durham tube prior to inoculation, the tube should be inverted until the air is released from the Durham tube. **BE SURE THE LID IS TIGHTENED SECURELY TO PREVENT LEAKAGE PRIOR TO INVERTING THE BROTH TUBE.**
- A faint pink color produced following addition of the nitrate reagents is not a positive result. Only the production of a deep-red color is indicative of a positive reaction.

Student Name: _____

COMPLETE THE FOLLOWING TABLE BASED UPON YOUR OBSERVATIONS.

Bacteria/Soil	Significant Growth (yes or no)	Gas Produced (yes or no)	Color after Reagents A and B (red or none)	Color after Reagent C (red or none)	Nitrate Reduction (yes or no)	End Product(s)
<i>S. epidermidis</i>						
<i>P. aeruginosa</i>						
<i>E. coli</i>						
Soil						
Control						

Discussion Questions:

- Which bacteria were positive for nitrate reduction? Which bacteria were negative for the reduction of nitrate?
- One would expect soil to contain a mixture of nitrate and non-nitrate reducing bacteria. Explain the results obtained from the soil sample.
- Explain the results of the control sample. What is the purpose of this tube?