Nitrate and Nitrite Reduction Test Protocols

Created: Tuesday, 01 November 2011

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Information   **History**

Current tests for nitrate and nitrite reduction are based on the Griess diazotization reaction described in 1858 by Peter Griess.

Peter Griess, the son of a blacksmith, was raised on a farm in Prussia, but "…tilling the soil was little to his liking, and on more than one occasion his father found him in a corner of the field, deep in a book, seated on the plough" (30). In his early attempts at higher education, he was far from a model student, spending time in the institution's prison and eventually expelled for a year. Finally, in his 6th year at university he began to seriously study chemistry. He obtained employment in the coal-tar distillery where the senior chemists discovered and developed the aniline dye industry. Even though the distillery was destroyed by fire, Griess had become obsessed with the chemistry of dye making. He was recommended for a position at the Royal College of Chemistry in Great Britain on the very day that his first article on possible diazo compounds, "A Preliminary Notice on the Influence of Nitrous Acid on Aminonitro- and Aminodinitrophenol," appeared in print.

Griess' first several attempts at diazotization exploded, but his commission at the Royal College was to investigate his new nitrogen intermediates, with the result that diazobenzoic acid was isolated and an entirely new class of compounds was discovered (23, 30). Because many of these compounds were found to be stable and could be used for dying fabric without needing a mordant, Griess is heralded as the father of the modern azo dye industry (8, 13, 34).

More colorful details of Griess' life can be found in articles from the February 1930 and June 1959 *Journal of the Society of Dyers & Colourists* and April 1958 *Journal of Chemical Education* (8, 23, 30).

In 1879, Griess developed a reagent for the detection of nitrite in solutions. The reagent, an acid solution of sulfanilic acid and alpha-naphthylamine, undergoes a diazotization reaction with nitrites, forming a red azo dye (17). Many variations of the so-called Griess test can be found in chemistry, medicine, and industry, but all are based on the production of an azo dye via the diazotization of nitrite.

Crime scene investigation uses one such interesting application of the reaction. The nitrites of gun powder residue can be visualized with a

modified Griess test (33, http://www.firearmsid.com/A_distanceExams.htm, http://www.firearmsid.com/A_diststandards.htm). (Figures 1 and 2 are presented with the permission of J. Scott Doyle, Forensic Scientist Specialist, Kentucky State Police.)

FIG. 1. This shirt, from a case investigation, has a bullet entrance hole in the front chest. The shirt has been tested for nitrite and lead residues.

FIG. 2. Results of the modified Griess test for the shirt shown in Fig. 1.

For many years, adaptations of the Griess test were suggested as a means of testing the urine of asymptomatic patients, especially women during pregnancy, for the presence of nitrites as an indication of bacteriuria (1, 17, 37, 45). Similar chemistry is now employed in commonly-used "dipstick" urine chemistry tests for nitrites (18, 45).

The Griess reaction has more recently been employed to detect nitrite and nitrate as products of nitric oxide synthase in human cells and biological systems. These include a constitutive, low-output, endothelial isoform that modulates vascular tone; a constitutive, low-output, neuronal isoform that modulates synaptic plasticity; and a cytokine-inducible, high-output, immune inflammatory isoform that functions as

an effector component of the cell-mediated immune response. Nitric oxide is difficult to quantitate because it is produced in small amounts under most conditions and has a short half-life, however, measuring the accumulation of nitrite and nitrate is a useful way to quantitate nitric oxide synthase activity (22).

While all applications of the Griess reaction are interesting background for the student and the instructor (25) including those involving analysis of water (9) and plant physiology (10), the current protocol will focus on the reduction of nitrates and nitrites by bacteria in artificial media.

PURPOSE

Standard tests for reduction of nitrate, NO_3^-, and nitrite, NO_2^-, can be useful components of biochemical test batteries for identification of bacteria (15), including separating members of the family *Enterobacteriaceae* from other gram-negative bacilli, identifying species of *Neisseria* and separating them from *Moraxella* and *Kingella* species (21, 26),and facilitating species identification of *Corynebacterium* (16) and other asporogenous gram-positive bacilli (36).

Nitrate reduction by bacteria is mediated by nitrate reductase and indicates that the organism can use NO_3^- as an electron acceptor (2, 44) during anaerobic respiration (2). Nitrite may be reduced to a variety of nitrogen products (44) including NO, N_2O, N_2, and NH_3, depending on the enzyme system of the organism and the atmosphere in which it is growing. Reduction of nitrate often indicates a shift to or facilitation of anaerobic metabolism, as some organisms can use nitrate as an electron acceptor during anaerobic respiration or anaerobic chemolithotrophy (2).

THEORY

Nitrites react with an acid solution of sulfanilic acid and alpha-naphthylamine to form a red azo dye (1). In each of the test reactions the appearance of the red dye indicates the presence of NO_2^- in the test tube, whether as an unreduced primary substrate, a product of the reduction of NO_3^- by the test organism, or a product of the forced reduction of NO_3^- with a reducing agent (zinc) for control purposes. The essence of each reaction is the ability to detect NO_2^-.

In the presence of NO_2^-, the color reaction begins with the acidification of NO_2^- by the acetic acid in the combined reagents A and B to produce HNO_2. The reaction below (27, 43) demonstrates the color development that follows:
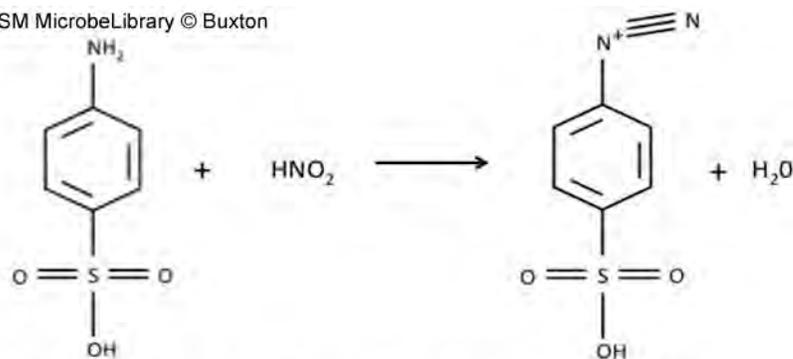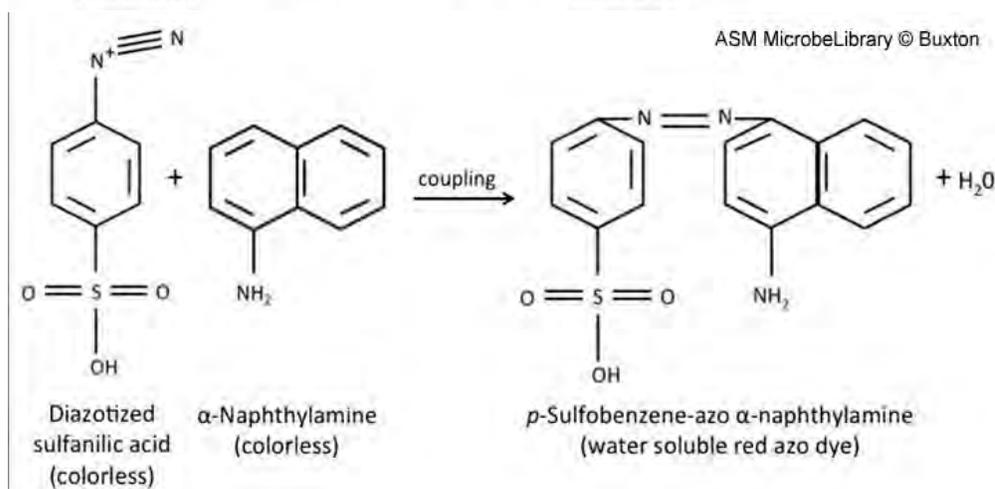
| Sulfanilic Acid (colorless) | Nitrous Acid | Diazotized sulfanilic acid (colorless) |

| Diazotized sulfanilic acid (colorless) | α-Naphthylamine (colorless) | p-Sulfobenzene-azo α-naphthylamine (water soluble red azo dye) |

The -N=N-azo group linkage yields a colored compound via a nitroso reaction. Diazonium dye compounds are formed by coupling through an azo link of an aromatic amine with a phenolic-type compound usually at the *para* position to a hydroxyl (OH) or amino group (NH_2). In this case coupling occurs *para* to an amino group (27).

An overview of nitrate reduction and the nitrogen cycle can be found in Richardson's brief introduction (42). The complexity of nitrate reduction pathways is discussed in depth in Moreno-Vivian's excellent review (32).

RECIPES

Several formulations of substrate broth can be found in the literature and are available commercially (3, 7, 19, 38, 41, 46). It is most important to choose a medium that is free from fermentable carbohydrates and in which the organism in question grows well (27). Heart infusion broth with 0.1% KNO_3 or KNO_2 added is preferred by some authors over the broths described below (11).

Nitrate reduction medium

Beef (meat) extract	3.0 g
Gelatin peptone	5.0 g

Potassium nitrate (KNO$_3$)	1.0 g
Deionized water	1,000 ml

Nitrite reduction medium

Beef (meat) extract	3.0 g
Gelatin peptone	5.0 g
Potassium nitrite (KNO$_2$)	1.0 g
Deionized water	1,000 ml

For either broth substrate, carefully weigh the ingredients and heat gently into solution. Dispense into test tubes and add inverted Durham tubes. Autoclave for 15 minutes at 121°C, 15 psi. The pressure of the autoclave will drive the broth into the Durham tube. Cool before use. Refrigerate for storage at 4°C to 10°C. Shelf life is approximately 6 months. Figure 3 shows 4 ml of broth in a 13 mm x 100 mm tube.

FIG. 3. The pressure of autoclaving forces broth into the Durham tube.

There should be no bubbles visible in the Durham tube when the broth is inoculated. Use a heavy inoculum and incubate overnight before adding reagents. Some strains need up to 5 days for full reduction of the substrates.

Reagent A

Several formulations of reagent A are described and available commercially. The one described below is not a proven carcinogen and produces a relatively stable color (12, 20, 22, 27, 39, 40).

N,N-Dimethyl-α-naphthylamine	0.6 ml
Acetic acid (5N)[a]	100 ml

Note: fresh reagent has a very slight yellowish color.

Reagent B

Sulfanilic acid	0.8 g
Acetic acid (5N)[a]	100 ml

Note: fresh reagent is colorless

[a]5N acetic acid is prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water.

Reagents A and B should be protected from light and stored in the refrigerator. Discard the reagents if they become discolored.

FIG. 4. Reagent A, N,N-dimethyl-a-naphthylamine; reagent B, sulfanilic acid.

Zinc dust
Zinc dust must be nitrate- and nitrite-free.

FIG. 5. Zinc dust will reduce nitrate to nitrite, but will not further reduce nitrite to nitrogen gas or other nitrogenous by-products when used sparingly.

PROTOCOL

For either substrate, NO_3^- or NO_2^-, inoculate the medium with a heavy inoculum from well-isolated colonies of the test organism. Incubate at 35°C for 12 to 24 hours. Rarely, incubation up to 5 days may be required. When sufficient growth is observed in the tube, test the broth for reduction of the substrate.

For NO_3^- substrate

1. Observe for gas production in the Durham tube.

2. Mix two drops each of reagents A and B in a small test tube (12 mm x 75 mm).

3. Add approximately 1 ml of the broth culture to the test tube and mix well.

If the test organism has reduced the NO_3^- to NO_2^-, a red color will usually appear within 2 minutes, indicating the presence of NO_2^- in the tube.

$2e^- + 2H^+ + \mathbf{NO_3^-} \rightarrow \mathbf{NO_2^-} + H_2O$
Nitrate reduced to nitrite
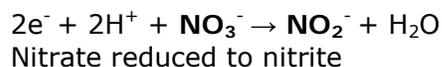
If no color change is seen within 2 minutes, there are several possible reasons. Either the organism (i) was unable to reduce NO_3^- at all, (ii) was capable of reducing NO_2^-, or (iii) reduced NO_3^- directly to molecular nitrogen.
(i) NO_3^-
Nitrate is unchanged, negative reaction.

(ii) $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$
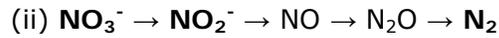
Nitrate reduced to nitrite to nitric oxide or further to nitrous oxide or further to nitrogen gas; exact pathways vary.

(iii) $2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$
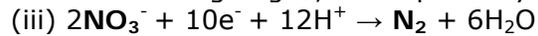
Nitrate reduced directly to molecular nitrogen.

Zinc is a powerful reducing agent. If there is any NO_3^- remaining in the tube (option (i) above), a small amount of zinc dust will rapidly reduce it to NO_2^-. Therefore the appearance of a red color after the addition of zinc dust to a colorless reaction tube indicates a negative reaction, i.e., the organism has failed to reduce NO_3^-. Zinc is added to the tube by dipping a wooden applicator stick in nitrate- and nitrite-free zinc powder, just enough to get the stick dirty, and then dropping it into the tube containing the culture broth and the reagents. If too much zinc is added, the color reaction may fade rapidly.

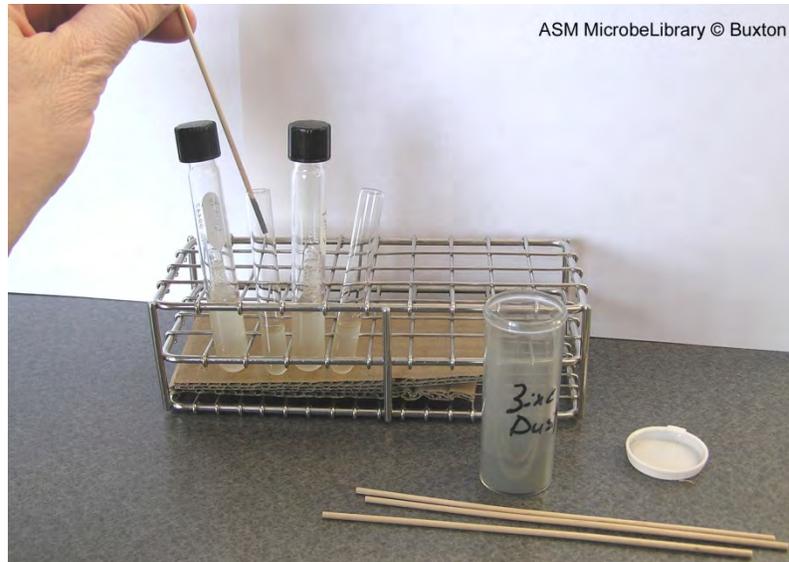FIG. 6. "Dirty" a wooden stick with zinc dust.

FIG. 7. Drop the zinc-dusted stick into tubes for nitrate reactions that show no change after the addition of reagents. There is no need to add zinc to reactions that began with a nitrite substrate.

If the broth remains colorless after the addition of zinc, the organism has also reduced the NO_2^- intermediate product to N_2 gas or some other nitrogenous product. N_2 gas is usually visible in the Durham tube. In the absence of gas, the product is assumed to be other than N_2 gas.

Occasionally a lighter pink color will appear after the addition of zinc dust (Fig. 16) because of partial reduction, i.e., some of the primary NO_3^- substrate remains in the tube. The original tube may be reincubated and retested the following day (Fig. 17).

For NO_2^- substrate

1. Observe for gas production on the surface and in the Durham tube.

2. Mix two drops each of reagents A and B in a small test tube (12mm x 75 mm).

3. Add approximately 1 ml of the broth culture to the test tube and mix well.

If the test organism has reduced the NO_2^-, there will be no color change, indicating that all of the original NO_2^- is gone, i.e., reduced. Reduction is often confirmed by the presence of N_2 gas in the Durham tube or on the surface of the broth, but other nitrogenous products may be produced. Therefore the absence of gas does not rule out reduction of NO_2^-.
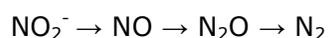
$NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$
Nitrite reduced to nitric oxide or further to nitrous oxide or further to nitrogen gas

If a red color appears, it indicates the presence of NO_2^- and therefore a

negative reaction.

Occasionally a lighter pink color will appear because of partial reduction, i.e., some of the primary NO_2^- substrate remains in the tube. The original tube may be reincubated and retested the following day. There is no need to add zinc dust to this reaction.

EXAMPLES OF RESULTS

Nitrate negative and negative controls
(uninoculated nitrate broth)

FIG. 8. With the addition of reagents to uninoculated nitrate broth (or growth of organisms failing to reduce nitrate), no color change is seen.

FIG. 9. The addition of zinc dust to the uninoculated broth in Fig. 8 forces the reduction of the NO_3^- to NO_2^-. Reagents A and B are already present, therefore the reagents react with NO_2^- resulting in a red color change.

Nitrite negative and negative controls
(uninoculated nitrite broth)

FIG. 10. The appearance of a red color with the addition of reagents A and B to an uninoculated nitrite broth indicates the presence of NO_2^-.

Reminder: in all cases, a red color change reaction indicates the presence of nitrites in the reaction tube, whether reduced by the organism from nitrate, a result of forced reduction of nitrate by zinc, or as the primary substrate.

Reduction of nitrate and nitrite with production of nitrogen gas
Pseudomonas aeruginosa

FIG. 11. Growth in both the nitrate and nitrite broth. Gas production is indicated by gas bubbles in the Durham tubes and on the surface of the broth.
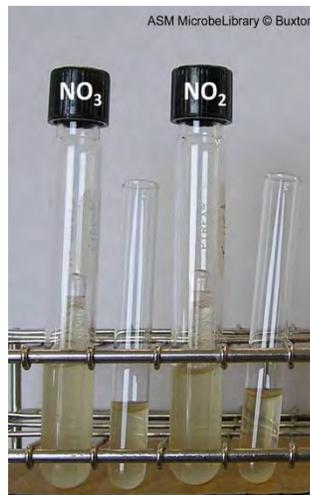
FIG. 12. Addition of reagents A and B to both the nitrate and nitrite broth results in no color change in either broth. These results indicate reduction of the NO_2^-, but whether reduction of NO_3^- occurred cannot yet be determined.

FIG. 13. Addition of zinc to the NO_3^- broth results in no color change. This result indicates reduction of NO_3^-.

Reduction of nitrate and nitrite without gas production
Moraxella catarrhalis

FIG. 14. Growth in both the nitrate and nitrite broth. No gas production.

FIG. 15. Addition of reagents A and B to both the nitrate and nitrite broth results in no color change in either broth. These results indicate the reduction of the NO_2^-, but whether

FIG. 16. Addition of zinc to the nitrate broth incubated for 24 hours results in a weak color. This result indicates partial reduction of NO_3^-.

FIG. 17. Additi zinc to the nit broth incubat 48 hours resu no color chan This result indicates the complete redu of NO_3^-.

reduction of NO_3^-
occurred cannot
yet be determined.

Reduction of nitrate, but not nitrite
Escherichia coli

FIG. 18. Growth in both the nitrate and nitrite broth. No gas production.

FIG. 19. Addition of reagents A and B to both the nitrate and nitrite broth results in a red color change in both broths. This indicates the presence of NO_2^- in both tubes. Nitrate in the nitrate broth has been reduced to NO_2^- but NO_2^- was not further reduced.

Reduction of nitrite but not nitrate
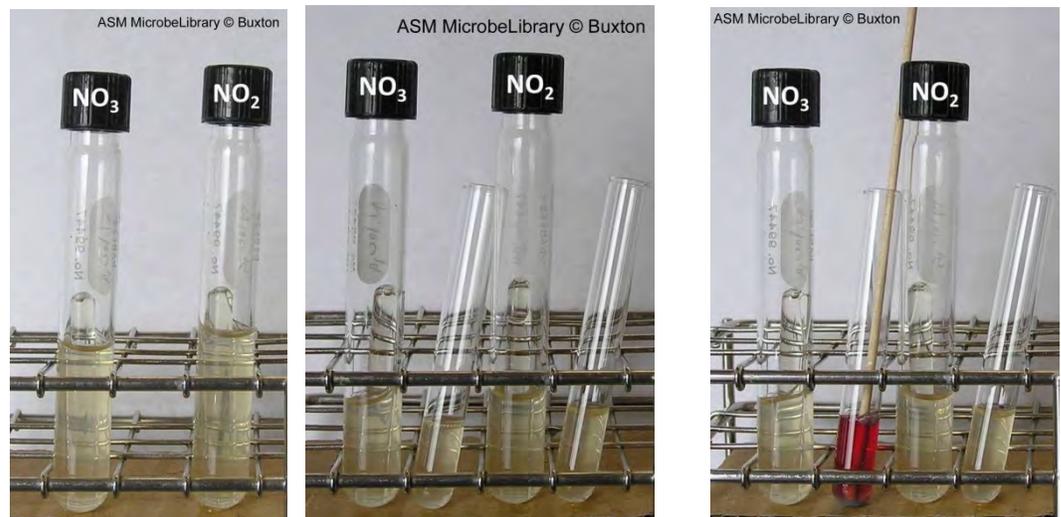*Neisseria lactamica*

FIG. 20. Growth in both the nitrate and nitrite broth. No gas production.

FIG. 21. Addition of reagents A and B to both the nitrate and nitrite broth results in no color change in either broth. These results indicate reduction of the NO_2^-, but whether reduction of NO_3^- occurred cannot yet be determined.

FIG. 22. Addition of zinc to the nitrate broth produces a red color change. This result indicates no reduction of NO_3^-.

QUALITY CONTROL

Pseudomonas aeruginosa reduces NO_3^- to N_2.
Escherichia coli reduces NO_3^- to NO_2^-.
Acinetobacter baumanii does not reduce NO_3^- or NO_2^-. *Acinetobacter baumanii* should give the same reaction as an uninoculated broth.
Alcaligenes faecalis and *Neisseria lactamica* reduce NO_2^- but do not reduce NO_3^-.

SAFETY

Reagents A and B are poisonous. They may be harmful or fatal if swallowed. They are also corrosive and may cause burns or irritation to skin, eyes, and the respiratory tract. Avoid breathing vapors and having contact with the eyes or skin. In case of contact with eyes, rinse immediately with water and seek medical advice (5, 39).

Zinc dust in contact with water liberates extremely flammable gases. Keep container tightly closed and dry. In case of fire use sand, carbon dioxide, or powdered extinguishing agent to put out flames; never use water (3).

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratorytechniques. 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

1. Some authors, including those of many commonly used text books (21, 28, 35, 45), prefer adding reagents directly to the primary culture tube, but because some organisms can be slow to reduce the substrates, the small aliquots are preferred to enable testing on a second or third day (6, 36).

2. The original formula for reagent B contained alpha-naphthylamine. Because it is a known carcinogen (14), it is now replaced with N,N-Dimethyl-α-naphthylamine. Fortunately, this formula is also less prone to fading of the color reaction (27).

3. Some authors recommend adding zinc to colorless NO_2^- reactions that do not contain gas to make sure that the NO_2 has not been oxidized to

NO_3 rather than having been reduced to a nitrogen product other than N_2 gas (21), but that reaction is rare.

4. Similar procedures can be employed in the identification of some fungi and mycobacteria, but they are not addressed here (24, 29).

5. Because reduction of NO_3^- is assumed to be anaerobic, many published procedures warn that the medium needs to be anaerobic or deep enough to support an anaerobic process. However, later experiments have shown that the metabolism on the surface of the broth for most organisms that grow well in the broth will reduce enough dissolving oxygen for the reaction to take place (25, 26). Four to five milliliters of broth in a 13 mm x 100 mm tube provide a sufficiently small surface to volume ratio and sufficient volume to repeat the test if extended incubation is necessary.

6. Filter paper disk tests are commercially available for detecting nitrate reduction by anaerobic species grown on solid-plated media in an anaerobic atmosphere (6).

7. In order to reinforce personal and laboratory safety, the instructor may wish to dispense the zinc dust. This may present an opportune time for the instructor to assess student understanding of the exercise.

8. Be sure to run a negative control, uninoculated broth, to illustrate that the remaining NO_2 will be reduced by zinc dust, producing a red color.

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