HISTORY

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

Griess was raised on a farm in Prussia and was the son of a blacksmith, 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.” (25) 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 soon 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 appeared in print: “A Preliminary Notice on the Influence of Nitrous Acid on Aminonitro- and Aminodinitrophenol.”

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 (18, 25). Because many of these compounds were found to be stable and could be used for dying fabric without needing a mordant, he is heralded as the father of the modern azo dye industry. (3, 8, 28)

(More colorful details of Griess’ life can be found in articles from the February 1930 and June 1959 Society of Dyers &Colourists and April 1958 Journal of Chemical Education. (3, 18, 25)

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. (12) 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 applies one such interesting application of the reaction. The nitrites of gun powder residue can be visualized with a “Modified Griess Test.” (13, 14, 27) (Figures 1 and 2 are presented with the permission of J. Scott Doyle, Forensic Scientist Specialist, Kentucky State Police.)
Nitrate and Nitrite Reduction Test Protocols

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 nitrates as an indication of bacteriuria. Similar chemistry is now employed in commonly-used “dipstick” urine chemistry tests for nitrates.

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

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

PURPOSE

Standard tests for reduction of nitrate (NO₃⁻) and nitrite (NO₂⁻) can be useful components of biochemical test batteries for identification of bacteria, including separating members of the family Enterobacteriaceae from other gram-negative bacilli, identifying species of Neisseria and separating them from Moraxella and Kingella species, and lending species identification to Corynebacterium and other asporogenous gram-positive bacilli.

Nitrate reduction by bacteria is mediated by nitrate reductase and indicates that the organism can use NO₃⁻ as an electron acceptor during anaerobic respiration.

Nitrite may be reduced to a variety of nitrogen products including NO, N₂O, N₂, and NH₃ depending on the enzyme system of the organism and the atmosphere in which it is growing.

THEORY

Nitrites react with an acid solution of sulfanilic acid and alpha-naphthylamine to form a red azo dye.
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 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 $^{(22)}$ demonstrates the color development that follows:

$$\text{NH}_2\text{H}_2\text{SO}_3 + \text{HNO}_2 \rightarrow \text{H}_2\text{SO}_3 + \text{H}_2\text{O}$$
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 (NH₂). In this case coupling occurs para to an amino group. (22)

An overview of nitrate reduction and the nitrogen cycle can be found in Richardson’s brief introduction. (32) The complexity of nitrate reduction pathways is discussed in depth in Moreno-Vivian’s excellent review. (26)

**RECIPE**

Several formulations of substrate broth can be found in the literature and are available commercially. (36, 39, 40, 41, 44, 47) It is most important to choose a medium that is free from fermentable carbohydrates and in which the organism in question grows well. (22) Heart Infusion Broth with 0.1% KNO₃ or KNO₂ added is preferred by some authors to the broths described below. (6)

**Nitrate Reduction Medium:**

Beef (meat) extract 3.0 g  
Gelatin Peptone 5.0 g  
Potassium Nitrate (KNO₃) 1.0 g  
Deionized water 1000.0 ml

**Nitrite Reduction Medium:**

Beef (meat) extract 3.0 g  
Gelatin Peptone 5.0 g  
Potassium Nitrite (KNO₂) 1.0 g  
Deionized water 1000.0 ml
For either broth substrate, carefully weigh the ingredients and heat gently into solution. Dispense into tubes and add inverted Durham tubes. (Figure 3 shows 4ml of broth in a 13mm x 100mm tube.) 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.
Store refrigerated (4-10°C)
Shelf life is approximately 6 months.

Figure 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.
**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.\(^{(7, 17, 22, 42, 45, 46)}\)

N,N-Dimethyl-α-naphthylamine 0.6 ml
Acetic Acid (5N)* 100 ml
(Fresh reagent has a very slight yellowish color)

**Reagent B**
Sulfanilic Acid 0.8g
Acetic Acid (5N)* 100 ml
(Fresh reagent is colorless)

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

Reagents A and B must be protected from light and stored in the refrigerator. They must be discarded if they become discolored.

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

---

Figure 4.
Reagent A: N,N-dimethyl-a-naphthylamine
Reagent B: Sulfanilic Acid
Zinc dust will reduce nitrate to nitrite, but will not further reduce nitrite to nitrogen gas or other nitrogenous by-products when used sparingly.

**Figure 5.**
Nitrate and Nitrite Reduction Test
Protocols

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 12-24 hours. Rarely, incubation up to 5 days may be required.

When growth is observable in the tube, the broth is tested for reduction of the substrate.

Nitrate Reduction Medium:

1. Observe for gas production in the Durham tube.
2. Mix two drops each of reagents A and B in a small (12mm x 75 mm) tube.
3. Add approximately one ml of the broth culture 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.

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \\
\text{Nitrate reduced to Nitrite}
\]

If no color change is seen within 2 minutes, there are several possibilities: Either (1) the organism was unable to reduce NO$_3^-$ at all, or (2) it was also capable of reducing NO$_2^-$, or (3) it reduced NO$_3^-$ directly to molecular nitrogen.

1. NO$_3^-$
   Nitrate (unchanged/negative reaction)

2. NO$_3^-$ $\rightarrow$ NO$_2^-$ $\rightarrow$ NO $\rightarrow$ N$_2$O $\rightarrow$ N$_2$
   Nitrate reduced to nitrite to nitric oxide or further to nitrous oxide or further to nitrogen gas

3. $2\text{NO}_3^- + 10e^- + 12H^+ \rightarrow \text{N}_2 + 6\text{H}_2\text{O}$
   Nitrate reduced directly to molecular nitrogen
Zinc is a powerful reducing agent. If there is any NO$_3^-$ remaining in the tube [option (1) 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 NO$_3^-$ / NO$_2^-$/free zinc powder, just enough to get the stick dirty, and then dropping it in the tube containing the culture broth and reagents A and B. If too much zinc is added the color reaction may rapidly fade.)

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 a nitrogenous molecule other than N$_2$ gas.

Occasionally a lighter pink color will appear after the addition of zinc dust (Figure 16) because of partial reduction. (i.e., some of the primary NO$_3^-$ substrate remains in the tube.) The original tube may be re-incubated and retested the following day (Figure 17).
Nitrate and Nitrite Reduction Test

Protocols

Nitrite Reduction Medium:

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 (12mm x 75 mm) tube.
3. Add approximately one ml of the broth culture and mix well.

If the test organism has reduced the NO\textsuperscript{2-}, there will be no color change, indicating that all of the original NO\textsuperscript{2-} is gone, i.e., reduced. Reduction is often confirmed by the presence of N\textsubscript{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\textsuperscript{2-}.

\[
\begin{align*}
\text{NO}_2^- & \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2 \\
\text{Nitrite reduced to Nitric Oxide or further to Nitrous Oxide or further to Nitrogen gas}
\end{align*}
\]

If a red color appears, it indicates the presence of NO\textsuperscript{2-} and therefore a negative reaction.

Occasionally a lighter pink color will appear because of partial reduction. (i.e., some of the primary NO\textsuperscript{2-} substrate remains in the tube.) The original tube may be re-incubated and retested the following day.

There is no need to add zinc dust to this reaction.
EXAMPLES OF RESULTS

**Nitrate Negative/Negative Controls**
(uninoculated nitrate broth)

**Figure 8.** No color change is observed with the addition of reagents A and B to uninoculated nitrate broth.

**Nitrite Negative/Negative Controls**
(uninoculated nitrite broth)

**Figure 9.** The addition of zinc dust to the uninoculated nitrate broth in figure 8 forces the reduction of NO₃⁻ to NO₂⁻. Reagents A and B are already present therefore the reagents react with the NO₂⁻ resulting a red color change.
Figure 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 reaction indicates the presence of nitrites in the reaction tube, whether reduced by the organism from nitrate, forced reduction of nitrate by zinc, or as the primary substrate.
Nitrate and Nitrite Reduction Test
Protocols

Reduction of nitrate and nitrite with production of nitrogen gas

*Pseudomonas aeruginosa*

![Figure 11](image1.png)

**Figure 11.** Growth in both nitrate and nitrite broth. Gas production is indicated by gas in the Durham tubes and on the surface of the broth.

![Figure 12](image2.png)

**Figure 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 $\text{NO}_2^-$, but whether reduction of $\text{NO}_3^-$ occurred cannot yet be determined.

![Figure 13](image3.png)

**Figure 13.** Addition of zinc to the $\text{NO}_3^-$ broth results in no color change. This result indicates reduction of $\text{NO}_3^-$. 

Reduction of nitrate and nitrite without gas production

*Moraxella catarrhalis*

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

Figure 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 reduction of NO$_3^-$ occurred cannot yet be determined.

Figure 16. Addition of zinc to nitrate broth incubated for 24 hours results in a weak color. This result indicates partial reduction of NO$_3^-$. 

Figure 17. Addition of zinc to the nitrate broth incubated for 48 hours results in no color change. This result indicates the complete reduction of NO$_3^-$. 
Nitrate and Nitrite Reduction Test Protocols

Reduction of nitrate, but not nitrite
*Escherichia coli*

![Image of nitrate and nitrite test tubes showing growth and no gas production.]

**Figure 18.** Growth in both nitrate and nitrite broth. No gas production.

Reduction of nitrite but not nitrate
*Neisseria lactamica*

![Image of nitrate and nitrite test tubes showing red color change.]

**Figure 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 NO₂⁻ in both tubes. Nitrate in the nitrate broth has been reduced to NO₂⁻ but NO₂⁻ was not further reduced.
**Figure 20.** Growth in both nitrate and nitrite broth. No gas production.

**Figure 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 the reduction of the NO₂⁻, but whether reduction of NO₃⁻ occurred cannot yet be determined.

**Figure 22.** Addition of zinc to the nitrate broth produces a red color change. This result indicates no reduction of NO₃⁻.
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 does 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 respiratory tract. Avoid breathing vapor and eye/skin contact. In case of contact with eyes, rinse immediately with water and seek medical advice. ($^{37,45}$)

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. Never use water. ($^{36}$)

The American Society for Microbiology advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, visit the ASM Curriculum Recommendations: Introductory Course in Microbiology and read the section on laboratory safety.

Three additional articles provide important information:

*Biosafety Levels—What We Need to Know About Them in Teaching Labs* by Christina Thompson (2004)

*Update of Biosafety Level Designations* by Erica Suchman (2004)

*Safety Recommendations from the Concurrent Sessions on Safety in the Microbiology Teaching Laboratory at the Undergraduate Microbiology Education Conference 2003* by Jackie Laxon (2003)

COMMENTS AND TIPS

1. Some authors, including many commonly-used text books, ($^{16,23,29,34}$) 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 subsequent days. ($^{30,39}$)

2. The original formula for Reagent B contained alpha-naphthylamine. Because it is a known carcinogen, ($^{36}$) it is now replaced with N,N-Dimethyl-alpha-naphthylamine. Fortunately, this formula is also less prone to fading of the color reaction. ($^{22}$)
3. Some authors recommend adding zinc to colorless NO₂⁻ reactions that do not contain gas to make sure that the NO₂⁻ has not been oxidized to NO₃⁻ rather than having been reduced to a nitrogen product other than N₂ gas, 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. (19, 24)

5. Because reduction of NO₃⁻ 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. (20, 21) Four to five milliliters of broth in a 13x100mm 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. (38)

REFERENCES


Nitrate and Nitrite Reduction Test
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Nitrate and Nitrite Reduction Test Protocols


Bayer Multistix

36. BBL Nitrate Broth with Durham Tube
http://bd.com/ds/technicalCenter/inserts/L007480%2807%29%280506%29.pdf

37. BBL Nitrate Reagent A & B
http://bd.com/ds/technicalCenter/inserts/L001190%281206%29.pdf

38. BBL TaxoDifferention Discs Nitrate
http://bd.com/ds/technicalCenter/inserts/8820281%281106%29.pdf

39. Biomerieux Nitrate, Nitrite Media
http://www.pmlmicro.com/assets/TDS/555.pdf

40. Difco & BBL Manual, Nitrate Broth
http://bd.com/ds/technicalCenter/inserts/Nitrate_Broth.pdf

41. Fluka Analytical Nitrate Broth

42. Fluka Analytical Nitrate Reduction Test

43. Molecular Probes Griess Reagent for Nitrite Determination
http://probes.invitrogen.com/media/ps/mpt/7921.pdf

44. Remel Nitrate Broth
document R061536

45. Remel Nitrate Reagent A
document R21239
Nitrate and Nitrite Reduction Test
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46. RemelNitrateReagent B
document R21242

47. RemelNitriteBroth
document R061552