



## Nitrogen Oxides in Exhaled Breath Condensate

Multiple nitrogen oxides (NOx) have been reported in EBC.

NO <sub>2</sub> -	Nitrite
NO <sub>3</sub> -	Nitrate
SNO	S-nitrosothiols
NO <sub>2</sub> Tyr	Nitrotyrosine

Nitrogen oxides in EBC are likely derived from two compartments of the exhaled air:

1. aerosolized particles of airway lining fluid (which contains many nitrogen oxides)
2. Gas phase nitric oxide, which then is oxidized in the EBC to higher oxides of Nitrogen.

The relative contributions of these sources of NOx in EBC have not been clarified, and conceivably vary in certain diseases.

### Nitrite (NO<sub>2</sub>-)

#### Advantages

- Relatively easy to measure.
- Recognizable value given its chemical relationships with nitric oxide
- Multiple studies report its presence in EBC
- Complementary to exhaled NO and other NOx assays.

#### Disadvantages

- Anatomic source not elucidated, but risk of upper airway contamination is present
- Not amenable to long storage. Nitrite is a reactive molecule. It is not stable at low pH and can readily be converted to nitric oxide, which then can evolve out of EBC as a gas. Alternatively, in EBC can be oxidized to NO<sub>3</sub>- or can be consumed by reaction with reduced thiols (to form SNOs) or tyrosine residues (to form nitrotyrosine).
- Laboratory contamination is ubiquitous. Nitrite can be found on almost all surfaces, including the insides of microcentrifuge tubes (which are commonly used to store EBC), pipette tips, etc. Many materials may leech out NO (and therefore the higher oxides). This occurs, for example, in Mylar bags used for off-line exhaled NO assay. Large nitrite contamination is present on the surface of latex gloves (irregardless of sterility, of course). Low extracable ion gloves are available that have minimal surface contamination. Please contact us for information on how to obtain these gloves.

#### Expected levels

- In health EBC NO<sub>2</sub>- levels are approximately 0.5 - 1 micromolar(1, 2)
- In disease, levels 2-3 fold higher are commonly reported. Much higher levels have also been reported occasionally.

#### Assays

- Greiss Reaction
  - o available as kits through many lab supply companies-however the reagents can be readily made in a research lab for approximately 1/100th of the cost of the kits).
  - o limit of detection is approximately 0.5 micromolar(1)
- Chemiluminescence
  - o after reduction in potassium iodide/acetic acid, nitrite will evolve as NO which can be analyzed in a nitric oxide analyzer. The Sievers NOA is perhaps the most used for aqueous phase NOx assays. Level of detection 0.1 micromolar (with 20 ?L sample). Can be improved substantially if more EBC sample is used.
- Several other colorimetric assays are available, but generally with higher detection limits.

### Recommendations

1. Rinse with deionized water everything that will contact your EBC sample, and do so as soon as possible before use. Be meticulous about this. Trust nothing to be nitrite-free.
2. Be circumspect about the Griess assay, as you will be working near its detection limit.
3. Minimize storage time. Assay as soon as possible after collection, especially if dealing with illnesses in which EBC pH is often low (CF, asthma, COPD, and others)
4. It is unclear what the plural term "nitrites" indicates when dealing with aqueous solutions. Nitrites are salts, and this is a reasonable term for solid phase. But in aqueous phase, the preferred is the singular, "nitrite."

## Nitrate (NO<sub>3</sub><sup>-</sup>)

### Advantages

- Relatively easy to measure.
- Recognizable value given its chemical relationships with nitric oxide
- Multiple studies report its presence in EBC
- Stable. Can be stored indefinitely. (conceivably rises slightly in storage as lower oxides of Nitrogen slowly oxidize.)
- Complementary to exhaled NO and other NO<sub>x</sub> assays.

### Disadvantages

- Anatomic source not elucidated, but our unpublished data reveal that the overwhelming amount of NO<sub>3</sub><sup>-</sup> is derived from the lower airway. There are a small number of patients in whom the upper airway appears to be relevant to NO<sub>3</sub><sup>-</sup>
- Laboratory contamination is ubiquitous. Nitrate can be found on almost all surfaces, including the insides of microcentrifuge tubes (which are commonly used to store EBC), pipette tips, etc. Many materials may leech out NO (and therefore the higher oxides). This occurs, for example, in Mylar bags used for off-line exhaled NO assay. Large nitrate contamination is present on the surface of latex gloves (irregardless of sterility, of course). Low extracable ion gloves are available that have minimal surface contamination. Please contact us for more information regarding such gloves.

### Assays

- Griess Reaction
- Modified by the addition of an initial step of enzymatic nitrate reductase. This assay will identify NO<sub>3</sub><sup>-</sup> as well as NO<sub>2</sub><sup>-</sup>. Necessary to perform both the basic Griess and the modified Griess in order to quantitate NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> individually. Level of detection approximately 1 μM
- Chemiluminescence
- after reduction in vanadium chloride in hydrochloric acid at 95°, nitrate (and nitrite) will evolve as NO which can be analyzed in a nitric oxide analyzer. The Sievers NOA is perhaps the most used for aqueous phase NO<sub>x</sub> assays. Level of detection 0.1 micromolar (with 20 μL sample). Can be improved substantially if more EBC sample is used. Note that this assay also will read all SNOs, and possibly nitrotyrosine (the latter only to a small extent).

### Recommendations

1. Rinse with deionized water everything that will contact your EBC sample, and do so as soon as possible before use. Be meticulous about this. Trust nothing to be nitrate-free.
2. The term NO<sub>x</sub> refers to all nitrogen oxides. Many nitrate assays are actually identifying several oxides of nitrogen, and this should be recognized.
3. For best understanding of NO<sub>x</sub> in the lung, consider assaying all the forms you can. Exhaled NO is but a very small part of the NO<sub>x</sub> picture.

## S-nitrosothiols

These compounds are endogenously formed small polypeptides or full proteins with an NO<sup>+</sup> group replacing a sulfhydryl H<sup>+</sup> on a cysteine residue. The process of replacing the H<sup>+</sup> with NO<sup>+</sup> is called nitrosation, or nitrosylation for proteins.

S-nitrosoglutathione (GSNO) is a nitrosylated tripeptide that is an endogenous bronchodilator. GSNO is reported to be elevated in the EBC of asthmatic patients, although it is low in the tracheobronchial secretions of severe acutely ill asthmatics.

Assays are best performed by chemiluminescence. Detailed information about S-nitrosothiol assays are available from us. Simply send an email or give us a call, and we will provide whatever information that you may need.

## Nitrotyrosine

Nitrotyrosine is formed by reaction of tyrosine residues with peroxynitrite, acidic nitrite, or nitrogen dioxide. Carbon dioxide presence accelerates the formation of nitrotyrosine. The presence of this molecule is indicative of an oxidative environment with nitrogen oxides available, and the term nitrative stress has been coined on the reasonable assumption (and some data) that nitration adversely affects protein function. Nitrotyrosine has been reported to be identified in EBC.

Nitrotyrosine is assayed primarily by ELISA, kits for which are available commercially. CAUTION: these are standard sandwich ELISAs, and therefore cannot be measuring Nitrotyrosine as a single amino acid, which is simply too small to be measured in sandwich ELISA, but rather are assuredly identifying nitrated proteins. This is not made clear in the labeling, but should be kept in mind, especially as manuscripts are being prepared. An additional caveat is that proteins that are nitrated only at one site should not be identifiable with the currently available commercial sandwich ELISA kits. Of course, with all immunoassays, the specificity of the antibodies employed must always be considered, and non-specific background controlled for.

Mass spectroscopic assays after protein digestion have been developed, although there is minimal information published to date.

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