GE Water & Process Technologies
Analytical Instruments

Sievers Nitric Oxide Analyzer NOA 280i

Operation and Maintenance Manual

Firmware Version 3.00 and later

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DLM 14291 Rev C

IDENTIFICATION RECORDS

Record the following numbers as they are listed on the identification labels located on the back panel of the NOA and the front of the vacuum pump.

Analyzer serial no. _____

Pump serial no. _____

Warranty Start Date _____

Date of receipt/Installation _____

REVISION HISTORY

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Standard Limited Warranty

GE Analytical Instruments warrants its products (Sievers™, GE Analytical™ and Leakwise™) against defects in materials and workmanship. GE Analytical Instruments will, at its option, repair or replace instrument components that prove to be defective with new or remanufactured components (i.e., equivalent to new). The warranty set forth is exclusive and no other warranty, whether written or oral, is expressed or implied.

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The GE Analytical Instruments warranty term is thirteen (13) months ex-works, or twelve (12) months from installation or start up by GE Analytical Instruments certified service personnel. In no event shall the standard limited warranty coverage extend beyond thirteen (13) months from original shipment date.

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Warranty Service is provided to customers through telephone support (1-800-255-6964), Monday - Friday, from 8:00 a.m. to 5:00 p.m. (Mountain Time), excluding all company and legal holidays. Telephone support is provided for troubleshooting and determination of parts to be shipped from GE Analytical Instruments to the customer in order to return the product to operation. If telephone support is not effective, the product may be returned to GE Analytical Instruments for repair or replacement. In some instances, suitable instruments may be available for short duration loan or lease. GE Analytical Instruments warrants that any labor services provided shall conform to the reasonable standards of technical competency and performance effective at the time of delivery. All service interventions are to be reviewed and authorized as correct

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Repair components (parts and materials), but not consumables, provided in the course of a repair, or purchased individually, are warranted for 90 days ex-works for materials and workmanship. In no event will the incorporation of a warranted repair component into an instrument extend the whole instrument's warranty beyond its original term.

Consumables (e.g., dilution standards, verification solutions, and UV lamps, etc.) are warranted to the extent of their stated shelf life, provided these items are maintained within the stated environmental limitations. Warranty claims for consumables and verification standards are limited to the replacement of the defective items, prorated from the time of claim to the expiration of shelf life.

Shipping

A Repair Authorization Number (RA) must be obtained from the Technical Support Group before any product can be returned to the factory. GE Analytical Instruments will pay freight charges, exclusive of any taxes and duties, for replacement or repaired products shipped to the customer site. Customers shall pay freight charges, including all taxes and duties, for all products returning to GE Analytical Instruments. Any product returned to the factory without an RA number will be returned to the customer.

Declaration of Conformity

A copy of the Declaration of Conformity for this product is available under the Products link on our web site (http:///www.geinstruments.com).

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1 INTRODUCTION

The Model 280i Nitric Oxide Analyzer (NOA™) from GE Analytical Instruments is a high-sensitivity detector for measuring nitric oxide based on a gas-phase chemiluminescent reaction between nitric oxide and ozone:

$$NO + O_3 \rightarrow NO_2^* + O_2$$
$$NO_2^* \rightarrow NO_2 + h\nu$$

Emission from electronically excited nitrogen dioxide is in the red and near-infrared region of the spectrum, and is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube. The detection limit of the NOA for measurement of gas-phase NO is ~0.5 part per billion by volume. The detection limit for measurement of NO and its reaction products in liquid samples is ~ 1 picomole.

In biological systems, nitric oxide is produced from the enzymatic oxidation of arginine. Three isoforms of the enzyme nitric oxide synthase (NOS) have been identified in many cell types: endothelial NOS, neuronal NOS, and inducible NOS. The biological functions of NO include action as a vasodilator, neurotransmitter, cytotoxic agent, inhibit of platelet aggregation, and activator of smooth muscle proliferation. Nitric oxide is also present in exhaled breath and may be a useful marker of airway inflammation.

In solution, nitric oxide reacts with molecular oxygen to form nitrite (NO^-_2), and with oxyhemoglobin and superoxide anion (O_2^-) to form nitrate ($NO^{-)}$. NO also reacts with thiols to form S-nitroso compounds, amines to form nitrosamines, and metals to form metal-nitrosyl complexes. In the gas phase, NO reacts with high concentrations of oxygen to form nitrogen dioxide.

UNITED STATES REGULATORY REQUIREMENTS

CAUTION – INVESTIGATIONAL DEVICE

Limited by United States law to investigational use.

EXHALED BREATH AND LIQUID APPLICATIONS

For research use ONLY. Not for Use in Diagnostic Procedures.

SAFETY WARNINGS

WARNING

High voltage is present in the instrument when power cord is connected. To avoid potentially dangerous shock, disconnect the power cord before removing the cover.

WARNING:

This is a safety Class I product provided with a protective earthing ground incorporated into the power cord. The mains plug shall only be inserted in a socket outlet provided with a protective earth contact. Any interruption of the protective conductor, inside or outside the instrument is likely to make the instrument dangerous. Intentional interruption is prohibited.

WARNING:

This symbol indicates that to comply with European Union Directive 2002/96/EC for waste electrical and electronic equipment (WEEE). The Analyzer should be disposed of separately from standard waste.

ENGLISH

WARNING
Any operation requiring access to the inside of the equipment could result in injury. To avoid potentially dangerous shock, disconnect from power supply before opening the equipment.

WARNING:
For continued protection against fire hazard replace fuse with same type and rating.

WARNING
This symbol on the instrument indicates that the user should refer to the manual for operating instructions.

WARNING:
This is a safety Class I product. It must be wired to a mains supply with a protective earthing ground incorporated into the power cord. Any interruption of the protective conductor, inside or outside the equipment, is likely to make the instrument dangerous. Intentional interruption is prohibited.

WARNING
If this instrument is used in a manner not specified by Sievers Instruments Inc. USA, the protection provided by the instrument may be impaired.

WARNING:
Disposal of RAM Card Lithium batteries must follow the local environmental regulations.

ESPAÑOL

ATENCION
Cualquier operación que requiera acceso al interior del equipo puede causar una lesión. Para evitar peligros potenciales, desconectarlo de la alimentación a red antes de abrir el equipo.

ATENCION:
Para protección contínua contra el peligro de fuego, sustituir el fusible por uno del mismo tipo y características.

ATENCION
Este símbolo, en el instrumento indica que el usuario debería referirse al manual para instrucciones de funcionamiento.

ATENCION:
Esto es un producto con clase I de seguridad. Debe conectarse a una red que disponga de tierra protectora en el cable de red. Cualquier interrupción del conductor protector, dentro o fuera del equipo, puede ser peligroso. Se prohibe la interrupción intencionada.

ATENCION
Si este instrumento se usa de una forma no especificada por Sievers Instruments, Inc., USA, puede desactivarse la protección suministrada por el instrumento.

ATENCION:
Las pilas de litio de la RAM Card deshechadas deben seguir las regulaciones medioambientales locales.!

FRANÇAIS

ATTENTION
Chaque opération à l'intérieur de l'appareil, peut causer du préjudice. Afin d'éviter un shock qui pourrait être dangereux, disconnectez l'appareil du réseau avant de l'ouvrir.

ATTENTION:
Afin de protéger l'appareil continuellement contre l'incendie, échangez le fusible par un fusible du même type et valeur.

ATTENTION
Le symbol, indique que l'utilisateur doit consulter le manuel d'instructions.

ATTENTION:
Ceci est un produit de Classe de sécurité I. L'instrument doit être branché sur l'alimentation secteur par un fil de secteur prévu d'une prise de masse. Chaque interruption du conducteur protégeant, à l'intérieur ou á l'extérieur de l'appareil peut rendre l'instrument dangereux. Interruption intentionnelle est interdite.

ATTENTION
Si l'instrument n'est pas utilisé suivant les instructions de Sievers Instruments, Inc., USA, les dispositions de sécurité de l'appareil ne sont plus valables.

ATTENTION:
Les batteries RAM Card Lithium doivent être déposés suivant les régulations d'environnement locales.

DEUTSCH

WARNHINWEIS
Vor dem Öffnen des Gerätes Netzstecker ziehen!

WARNHINWEIS:
Für kontinuierlichen Schutz gegen Brandgefahr dürfen bei Sicherungswechsel nur Sicherungen der gleichen Stärke verwendet werden!

WARNHINWEIS
Dieses, auf dem Gerät weist darauf hin, dab der Anwender zuerst das entsprechende Kapitel in der Bedienungsanleitung lesen sollte.

WARNHINWEIS:
Dies ist ein Gerät der Sicherheitsklasse I und darf nur mit einem Netzkabel mit Schutzleiter betrieben werden. Jede Unterbrechung des Schutzleiters auβerhalb oder innerhalb des Gerätes kann das Gerät elektrisch gefährlich machen. Absichtliches Unterbrechen des Schutzleiters ist ausdrücklich verboten.

WARNHINWEIS
Wenn das Gerät nicht wie durch die Firma Sievers Instruments, Inc., USA, vorgeschrieben und im Handbuch beschrieben betrieben wird, können die im Gerät eingebauten Schutzvorrichtungen beeinträchtigt werden.

WARNHINWEIS:
Die Entsorgung der Lithium-Batterie in der RAM-Karte darf nur nach den geltenden Umweltschutzregeln erfolgen.

ITALIANO

ATTENZIONE
Qualsiasi intervento debba essere effettuato sullo strumento può essere potenzialmente pericoloso a causa della corrente elettrica.
Il cavo di alimentazione deve essere staccato dallo strumento prima della sua apertura.

ATTENZIONE:
Per la protezione da rischi da incendio in seguito a corto circuito, sostituire I fusibili di protezione con quelli dello stesso tipo e caratteristiche.

ATTENZIONE
Il simbolo, ⚠ sullo strumento avverte l'utilizzatore di consultare il Manuale di Istruzioni alla sezione specifica.

ATTENZIONE
Questo strumento è conforme alle specifiche per I prodotti in Classe I - Il cavo di alimentazione dalla rete deve essere munito di "terra". Qualsiasi interruzione del cavo di terra all'interno ed all'esterno dello strumento potrebbe risultare pericolòsa. Sono proibite interruzioni intenzionali.

ATTENZIONE
Se questo strumento viene utilizzato in maniera non conforme alle specifiche di Sievers Instruments, Inc. USA, le protezioni di cui esso è dotato potrebbero essere alterate.

ATTENZIONE
Le batterie al Litio sulla RAM CARD, quando sono esaurite, devono essere gettate secondo le regolamentazioni vigenti localmente.

DUTCH

⚠ ***OPGELET***
Iedere handeling binnenin het toestel kan beschadiging veroorzaken. Om iedere mogelijk gevaarlijke shock te vermijden moet de aansluiting met het net verbroken worden, vóór het openen van het toestel.

OPGELET:
Voor een continue bescherming tegen brandgevaar, vervang de zekering door een zekering van hetzelfde type en waarde.

OPGELET
Het symbool, ⚠ geeft aan dat de gebruiker de instructies in de handleiding moet raadplegen.

OPGELET:
Dit is een produkt van veiligheidsklasse I. Het toestel moet aangesloten worden op het net via een geaard netsnoer. Bij onderbreking van de beschermende geleider, aan de binnenzijde of aan de buitenzijde van het toestel, kan gebruik het toestel gevaarlijk maken. Opzettelijke onderbreking is verboden.

OPGELET
Indien het toestel niet gebruikt wordt volgens de richtlijnen van Sievers Instruments, Inc., USA gelden de veiligheidsvoorzieningen niet meer.

OPGELET:
RAM kaart Lithium batterijen dienen volgens de lokale afvalwetgeving verwijderd te worden.

The Model 280i NOA is used for measurement of NO in exhaled breath and measurement of nitrite, nitrate/nitrite and nitrosothiols in biological fluids, cell culture media, and other liquid samples. A schematic of the 280i NOA is shown in Figure 1-1 and consists of the following major components:

- Sampling Inlet Systems
- Sample Flow Control Device
- Ozone Flow Control Module
- Ozone Generator
- Chemiluminescence Reaction Chamber and Optical Filter
- Photomultiplier Tube and Cooled Housing
- Vacuum Pump and Chemical Trap
- Front Panel Display
- Four Button Keypad
- PMT Amplifier
- Power Supply
- Analog to Digital Converter
- Microprocessor and Output Electronics
- Analog, Printer, and RS-232 Outputs
- Exhalation Pressure Transducer
- Optional Thermal Mass Flowmeter

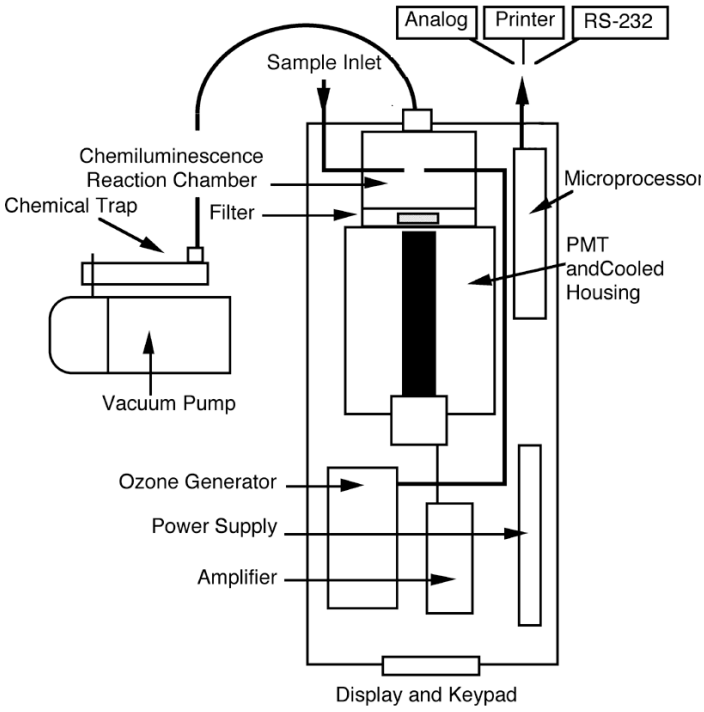


Figure 1-1: ***Schematic of Model 280i Nitric Oxide Analyzer***

Sample Inlet Systems

The NOA 280i has a complete range of sample inlet systems for measurement of NO and its reaction products including:

- Gas Sampling Kit for measurement of gas-phase NO. The kit includes a Nafion® drier, 0.45 µm particle filter, PVC sampling lines with Luer® adapters and a calibration tee.
- Purge vessel for the measurement of NO, nitrite, nitrate and other reaction products in liquid samples. The purge vessel can also be used for headspace analysis with a gas-tight syringe.
- Accurate NO™ Exhaled Breath Kit for on-line measurement of exhaled NO using elevated pressure to close the soft pallet and constant low exhalation flow (30 – 250 mL/s BTPS)
- Bag Collection and Sampling Kit for off-line measurement of exhaled NO using collection in Mylar® bags.

Sample Flow Control Device

The vacuum pump continuously draws gas into the analyzer at a constant flow rate. A porous metal frit restrictor sealed in a 1/8" adapter is connected to a Swagelok® bulkhead union at the rear of the NOA. The standard restrictor provides a flow rate of ~200 mL/min and restrictors for other flow rates are available from GE Analytical Instruments.

Ozone Flow Control Module

The connection for the gas supply for the ozone generator (oxygen or 95% O_2/ 5% CO_2) is made using Teflon tubing and a Swagelok bulkhead connector. The gas must be a regulated supply from an external cylinder, lecture bottle or house oxygen. The flow rate of gas into the ozone generator (~30 mL/min) is controlled using a regulator and small diameter tubing restrictors. The regulator and bulkhead connectors are located

on the back of the NOA. The regulator is adjusted to 6 psi pressure, which is measured by a pressure transducer, and monitored on the front panel display.

Ozone Generator

An electrostatic ozone generator and high voltage transformer are used to generate ozone at a concentration of ~2% by volume from oxygen. This large excess of ozone is sufficient for measurement of NO up to 500 ppm.

Chemiluminescent Reaction Chamber and Optical Filter

Nitric oxide and ozone are mixed in a small volume (~20 mL) reaction cell. This small volume permits measurement of low concentrations of NO at low flow rates and produces sharp peaks for analysis of liquid samples. For maximum sensitivity, the reaction cell is operated at low pressure (typically 4-7 torr). A few other chemicals, such as sulfur-containing compounds, undergo a chemiluminescent reaction with ozone but emit light at shorter wavelengths. To minimize interference from these species, an optical filter that transmits only red wavelengths (>600 nm) is installed between the reaction cell and the photomultiplier tube.

Photomultiplier Tube and Cooled Housing

The light from the chemiluminescent reaction of NO with O_3 is measured using a red-sensitive photomultiplier tube. For maximum sensitivity, the PMT is cooled to -12 °C using a thermoelectric cooled housing. The cooler is operated continuously whenever the main power switch is on. The temperature of the cooled housing is measured using a K-type thermocouple and monitored on the front panel display.

Vacuum Pump and Ozone Destruction Trap.

A vacuum pump is used to draw the sample into the NOA and maintain the reaction cell at low pressure. The exhaust from the reaction cell exits the analyzer at the rear of the instrument using a metal tube connected to Tygon tubing. Ozone in the exhaust is removed using a chemical trap containing Hopcalite™. This material reacts with ozone, removing it from the exhaust before the gas reaches the vacuum pump. Since the Hopcalite is consumed, the chemical trap must be periodically replaced. The oil used in the vacuum pump is a synthetic motor oil (Mobil 1™ weight 10W-30), which

provides better protection than conventional pump oil. For long pump lifetime, the oil must be changed at regular intervals. The exhaust from the pump contains some oil mist, which can be removed using a charcoal trap. The microprocessor keeps track of the trap and oil lifetimes, and notifies the user when it is time to replace the traps or change the oil.

Electronics

There are 7 circuit boards in the NOA:

- PMT amplifier
- Analog to digital converter (ADC) board
- Microprocessor and Outputs board
- Power supply board
- 24 V power supply
- Front panel display
- Keypad.

The PMT amplifier processes the signal from the PMT. To provide both high sensitivity and wide dynamic range, the amplifier has two gain ranges. High Gain is used for measurements requiring high sensitivity (liquid samples and exhaled breath). The amplifier in high gain has a linear response up to ~1 ppm of NO gas or ~400 picomoles of NO_2^- or NO_3^- for liquid samples. The low gain decreases the sensitivity of the amplifier, permitting measurement of up to ~500 ppm of NO gas or ~200 nanomoles of NO_2^- or NO_3^- for liquid samples. The analog output signal (mV only) is obtained from the amplifier. A switch on the amplifier sets the full-scale voltage. When the switch is in the down position the output range is 0–1V. When the switch is in the up position, the output range is 0–10V.

The amplifier is also connected to the ADC board that also monitors three pressure transducers (cell, supply and exhalation), the cooler thermocouple and the thermal mass flowmeter. The microprocessor and firmware calculate gas concentration, monitor the performance of the analyzer, keep track of maintenance items, and control the output of data. The power supply board and 24V power supply provides the high voltage for the PMT, power to the PMT cooler and the DC power for the electronics. The front panel display and keypad are used for display of the data and the operation of the analyzer.

Analog, Printer and RS-232 Outputs

In addition to displaying data on the front panel, data can also be: sent to a recorder or integrator using the analog output, sent to a computer using the RS-232 output, and printed using the parallel printer port. The analog output can be set to 0-1 V or 0-10 V full-scale. The printer output shows the minimum, maximum and average value for a selected print interval ranging from 5 seconds to 10 minutes. The RS-232 output provides data at sampling rates from 32 samples per second to 6 samples per minute.

Exhalation Pressure Transducer

An exhalation pressure transducer is present in the analyzer for use with the Accurate NO breath kit and for detection of exhalations during breath-by-breath measurements.

Thermal Mass Flow Meter

On-line measurement of exhalation flow rates can be performed using an optional flow meter. The signal from the flow meter is included in the RS-232 output.

This completes the introduction to the NOA and its components:

- Chapter 2 lists the specifications of the analyzer.
- Chapter 3 has an overview of the firmware and controls.
- Chapter 4 contains the basic installation procedures.
- Chapter 5 contains the installation and setup for gas-phase measurements.
- Chapter 6 describes how to calibrate the NOA for gas-phase measurements.
- Chapter 7 describes on-line measurement of NO in exhaled breath.
- Chapter 8 describes off-line measurement of exhaled NO.
- Chapter 9 describes breath-by-breath and chamber sampling for exhaled NO measurements.
- Chapter 10 describes measurement of nasal nitric oxide.
- Chapter 11 contains the installation and setup liquid measurement.
- Chapter 12 describes the setup and measurement of nitrite in liquid samples.

- Chapter 13 describes the setup and measurement of nitrate in liquid samples
- Chapter 14 describes the measurement of nitrosothiols and other reaction products in liquid samples.
- Chapter 15 describes maintenance of the NOA.
- Chapter 16 lists troubleshooting procedures and error and warning messages.

2 SPECIFICATIONS

Sensitivity

	Gas	< 1 ppb
	Liquid	~1 picomole

Range

	Gas	< 1 – 500,000 ppb
	Liquids	nanomolar to millimolar

Response Time

	Electronics	67 msecs to 90% full scale
	Lagtime	1 second

Repeatability

	Gas	± 5%
	Liquid	± 5 10%

Sample Size

	Gas	10 – 300 mL/min
	Liquid	0.001 – 5 mL

Display	Back-lit LCD screen
	ppb/ppm or mV

Outputs

	Analog	0 – 1V, 0 – 10 V
	Digital	RS-232 (9600-38.4K baud)
	Printer	parallel port

Data Sampling Rate	0.002 - 32 samples/second

Power Requirements	120 V, 60 Hz (6A)
	100 V, 50 or 60 Hz (7A)
	230 V, 50 Hz (3A)

NOA

.....	Height	16 in.	(41 cm)
.....	Width	6.2 in.	(16 cm)
.....	Length		20 in.(51 cm)
.....	Weight		35 lbs. (16 kg)

Vacuum Pump with installed trap

.....	Height	14.5 in.	(37 cm)
.....	Width	7.5 in.	(19 cm)
.....	Length		19 in.(48 cm)
.....	Weight		47 lbs. (21.5 kg)

Operating Environment

Ambient Temperature .....32°F to 86°F (0°C to 30°C)

Relative Humidity ..... 0% to 90%

Fuse Requirements

Main Fuse .....100 VAC model: T, 10A, 250V	
	120 VAC model: T, 5A, 250V
	230 VAC model: T, 5A, 250V
Ozone Fuse.....100VAC model: T, 200mA, 250V	
	120 VAC model: T, 200mA, 250V
	230 VAC model: T, 100mA, 250V

3 MENUS AND CONTROL OVERVIEW

Operation of the NOA 280i is performed using the four front panel buttons (UP Arrow, DOWN Arrow, ENTER, and CLEAR) to run the menu-based firmware. Use the UP or DOWN Arrow buttons to scroll through the menu options and select values. The ENTER Button is used to select menu options and to save set points. The CLEAR Button is used to exit menus and clear entries. The CLEAR Button is also used to display the Status Menu from the Main Menu, select the Main Menu from the Measurement Menu or to return to the Measurement Menu from the Main Menu. A cursor is used for selection of the menu options and the location of the cursor is indicated by a highlighted menu option.

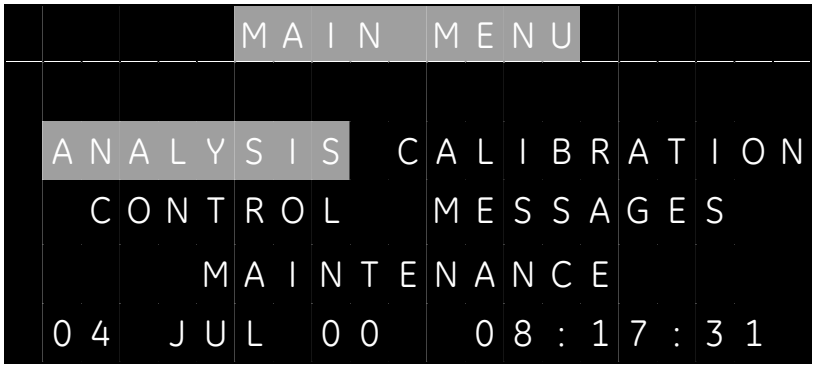


Figure 3-1: *Main Menu*

Main Menu

There are five options in the Main Menu: Analysis, Control, Calibration, Messages, and Maintenance. A title field is located at the top of the menu, and the date and time are displayed in the message line at the bottom of the display. Select an option by using the Arrow buttons to highlight the desired option, and press the ENTER button.

Status Screen

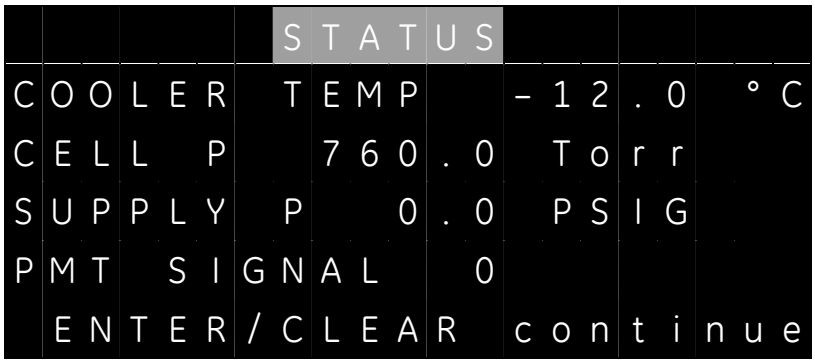


Figure 3-2: *Status Screen (PMT Off).*

Press the CLEAR button from the Main Menu to display the Status Screen. This screen shows the current values for the PMT cooler temperature, reaction cell pressure, oxygen supply to the ozone generator, and the PMT signal, (counts at analog to digital converter). Before starting the NOA, the status screen should be checked to confirm that the cooler temperature, cell and supply pressures are within the specifications required for the start-up tests.

Pressing either the ENTER or CLEAR button will return to the Main Menu.

Analysis

The Analysis option is used to start and stop the NOA. The NOA has three modes of operation: Start, Stand-by and Stop. From the Main Menu, pressing the ENTER button with the Analysis option highlighted will display the Analysis Menu.

Figure 3-3: *Analysis Menu with NOA in Stop Mode.*

Selecting the Start option will:

- Switch to the Startup Screen.
- Check the cell pressure to see if it is above 300 torr, and then turn on the pump.
- Check the PMT cooler temperature to see if it is –12 ± 2 °C.
- Wait until the reaction cell pressure is < 100 torr.
- Check the ozone supply pressure is >4 psig (6 psig recommended).
- Turn on the PMT and record an ozone-off baseline signal.
- Turn on the ozone generator, and wait for an increase in the PMT signal due to the background chemiluminescence from ozone.
- Display the PMT signal in the Measurement Display.

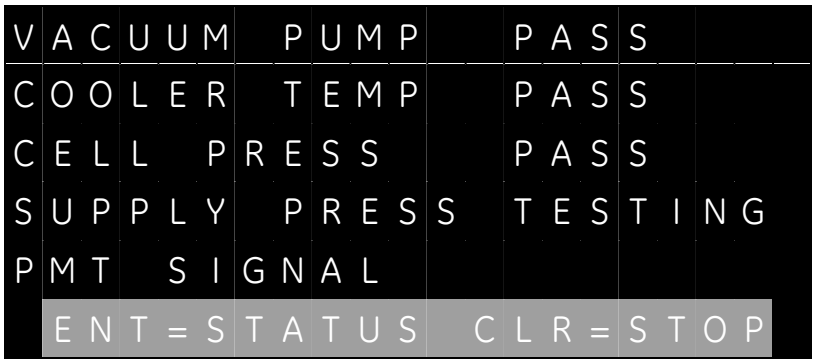


Figure 3-4: *Start-up Screen.*

If any of the above conditions are not met, the start-up screen will display FAILED for that test. Pressing ENTER from the Start-up Screen will display the Status Screen to aid in troubleshooting failed tests. Pressing CLEAR will return to the Analysis Menu.

Measurement Menu

Once the start-up testing is completed, the Measurement Menu is displayed. This menu shows that NOA's mode (Nitric Oxide or Exhalation), the PMT amplifier's setting (HI or LO sensitivity) and the signal from the PMT (mV or gas concentration). Two shortcuts are available from the Measurement Menu: DATA and WARN.

Figure 3-5:*Measurement Menu (Nitric Oxide Mode)*

When DATA is highlighted, pressing the ENTER button moves to the Data Output Menu. The data output (Com port and printer) are enabled at start-up, but can be disabled to pause data collection by pressing the ENTER button.

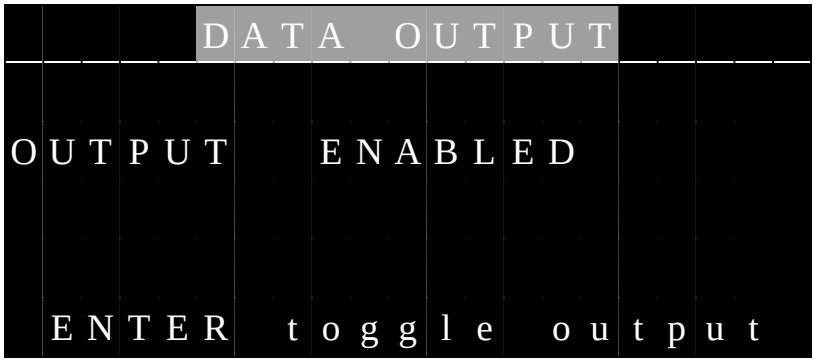


Figure 3-6: *Data Output Menu.*

The outputs are re-enabled by pressing the ENTER button. Pressing CLEAR returns to the Measurement Menu.

The firmware keeps track of usage and when maintenance is required, a WARN shortcut is displayed in the Measurement Menu. The UP or DOWN Arrow buttons can be used to scroll between DATA and WARN. With the WARN shortcut highlighted, pressing the ENTER button will display the Warning Menu with a list of the current warnings (see Chapter 14 for information on maintenance and warnings).

In the Nitric Oxide mode the current values for the cooler temperature, cell pressure and ozone supply pressure are displayed at the bottom of the Measurement Menu. In the Exhalation mode, a bar graph of the exhalation pressure is displayed.

From the Measurement Menu, pressing the CLEAR button will return to the Main Menu and from the Main Menu, pressing CLEAR will return to the Measurement Menu.

When the NOA is in the Start mode, selecting Analysis from the Main Menu will display two options: Stand-by and Stop.

The Stand-by option is always available from the Analysis Menu. If the analysis has been started, selecting the Stand-by option will turn off the PMT and ozone

generator, but leave the vacuum pump on. If the analysis was stopped, selecting the Stand-by option will turn on the vacuum pump.

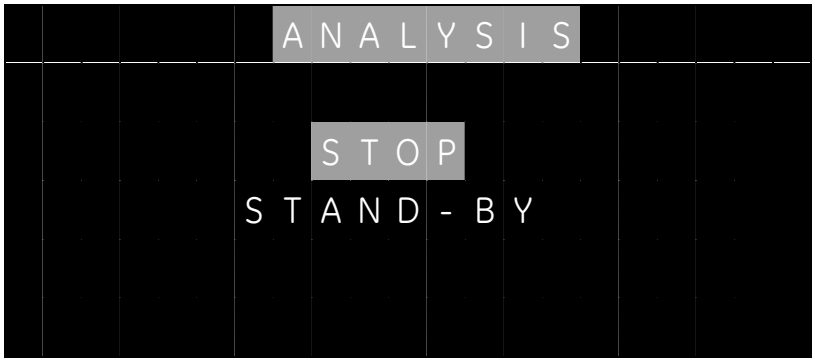


Figure 3-7: *Analysis Menu (NOA in Start Mode).*

When the NOA is in the start mode, selecting Stop will:

- Display a confirmation screen "Are you sure? continue / stop"
- If the Stop option is selected, turn off the PMT, and ozone generator.
- Run the vacuum pump for 2 minutes to clear residual ozone from the system.
- Turn off the vacuum pump.

Main Menu Options

The other options from the Main Menu are used to configure the NOA for the different applications, perform the calibration for gas-phase measurements, view warnings and errors, and view and install consumables.

Control

The Control Menu is used to setup the NOA for liquid or gas-phase measurements, set the output intervals and the sensitivity of the NOA. The setup can be stored as a method to permit easy switching between applications. The Control Menu also permits viewing of the cooler temperature and pressure via the Status Screen.

Calibration

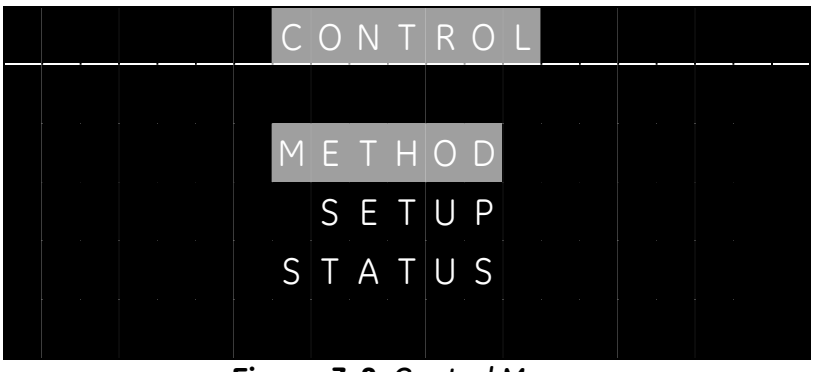


Figure 3-8: *Control Menu.*

The Calibration Menu is used to perform the calibrations for gas-phase measurements and can be used to view and print the gas and pressure transducer calibration values.

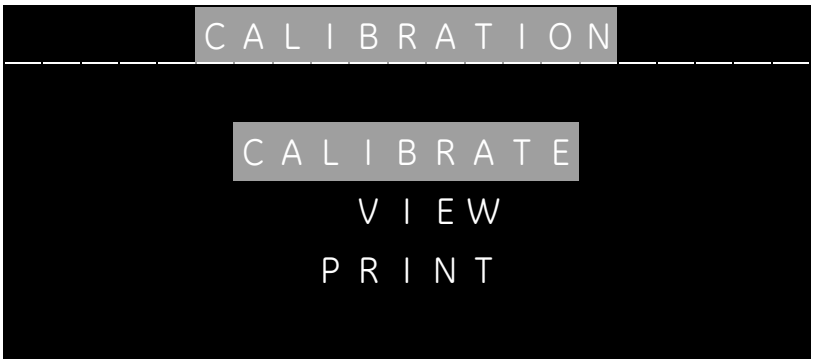


Figure 3-9: *Calibration Menu.*

Messages

The Messages Menu is used to view warnings and errors. Warnings indicate that maintenance is required and a WARN shortcut is displayed in the Measurement Menu. Errors indicate that the cooler temperature or the supply or cell pressures are out of range. When an error is detected, the NOA is placed in the Stop Mode and the firmware switches to the Errors menu.

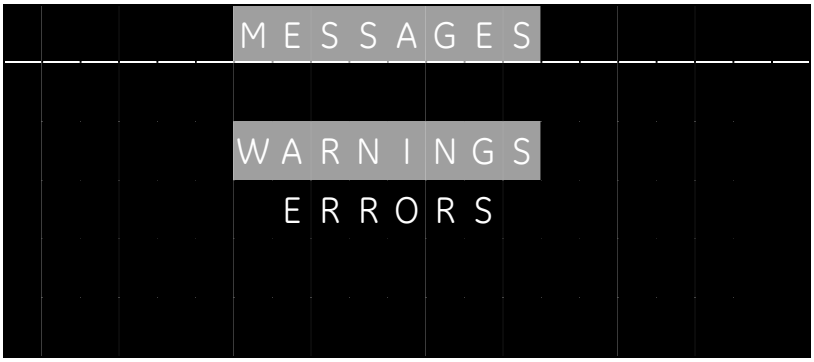


Figure 3-10: *Messages Menu.*

Maintenance

The Maintenance Menu is used to setup the security, view and install consumable and delete old warnings and errors.

Figure 3-11: *Maintenance Menu.*

Time-out Function

The NOA's firmware monitors keyboard activity and if no button pushes are detected for 10 minutes, the firmware will return to the Main Menu, if the NOA is in the Stop or Standby Mode or the Measurement Menu if the NOA is in the Start Mode.

4 INSTALLATION

Location

Place the analyzer on a clean, unobstructed surface approximately 25" (60 cm) deep by 6.2" (16 cm) wide that can support at least 35 pounds (16 kg) in addition to existing equipment. For proper heat dissipation, ensure that an additional 6" (16 cm) is available at the rear and on both sides of the detector. Leave ~ 24" (60 cm) of additional space on one side of the detector for the purge vessel. Additional space will be required for computers, printers and integrators.

Place the vacuum pump on a space of nearby floor or bench 7.5" (19 cm) by 19" (48 cm) with a minimum height clearance of 14.5" (37 cm). Pump weight is 47 lbs. (21.5 kg).

The analyzer and pump can also be placed on a cart for mobile operation. The cart should have a bottom shelf with enough clearance for the vacuum pump and should be sturdy enough to support the total weight of the analyzer and pump (82 lbs. 38 kg), plus any additional equipment or gas cylinders that will be placed on the cart.

Power Requirements

The detector and vacuum pump are powered from a standard, 15 amp, 120 VAC 60 Hz grounded AC outlet. The NOA and pump will draw ~6 amps in normal operation, and slightly higher instantaneous current with the pump running.

For 230 VAC versions of the detector, a standard (230 VAC, 50 Hz) grounded AC outlet (~3 amps) is required.

For 100 VAC versions, a standard (100 VAC, 50 or 60 Hz) grounded AC outlet (~7 amps) is required.

Environmental Considerations

Operate the NOA 280 in an environment comfortable for human habitation with reasonably constant temperature and humidity. Avoid elevated temperatures; operating at temperatures greater than 85° F (30° C) may cause problems with the LCD display and the PMT cooler.

Tools and Additional Supplies

The following items will be needed to install and operate the NOA 280:

Tools

The following tools will be required for all applications:

- Open End Wrenches - 1/4", 5/16", 7/16", 1/2", 9/16", 13/16" and 7/8" (Adjustable wrenches can also be used)
- Adjustable wrench or 11/8" open-end wrench is required for connecting regulators to gas cylinders.
- Phillips-head Screw Driver
- Hexdriver or regular screwdriver

Gases

A cylinder of oxygen or 95% oxygen, 5% CO2 equipped with a two stage regulator is required for the ozone generator. House oxygen and a flow controller may also be used. If oxygen is not available, air can be used, however, the NOA will need to be recalibrated for gas measurements if air is used for the ozone generator.

Data Collection

A computer (PC or Macintosh) is required for collection of data using the RS-232 output. For PCs, a Pentium is required. For Mac's a PowerPC is required. The computers should have at least 32 megabytes of RAM. For computers without an internal serial port, a USB-Serial adapter is required.

For real-time display of the analog signal, a strip chart recorder or integrator may be used. The analog signal can also be sent to a computer using an analog-to-digital

converter. For the printer output, any Centronics® style printer can be used. Contact GE Analytical Instruments with any questions regarding data collection equipment.

Vacuum Pump Setup

Open the vacuum pump box and remove the pump, pump oil (Edwards Ultra Grade), and the power cord. The accessories kit (pump fittings, Tygon tubing, hose clamps and mounting bracket) is shipped in a separate box.

Step 1 – Add Oil to the pump

Remove one of the two oil fill plugs from the top of the pump (see Figure 4-1) and add oil until the oil level just reaches the MAX mark on the pump at the top of the sight-glass (Do NOT overfill).

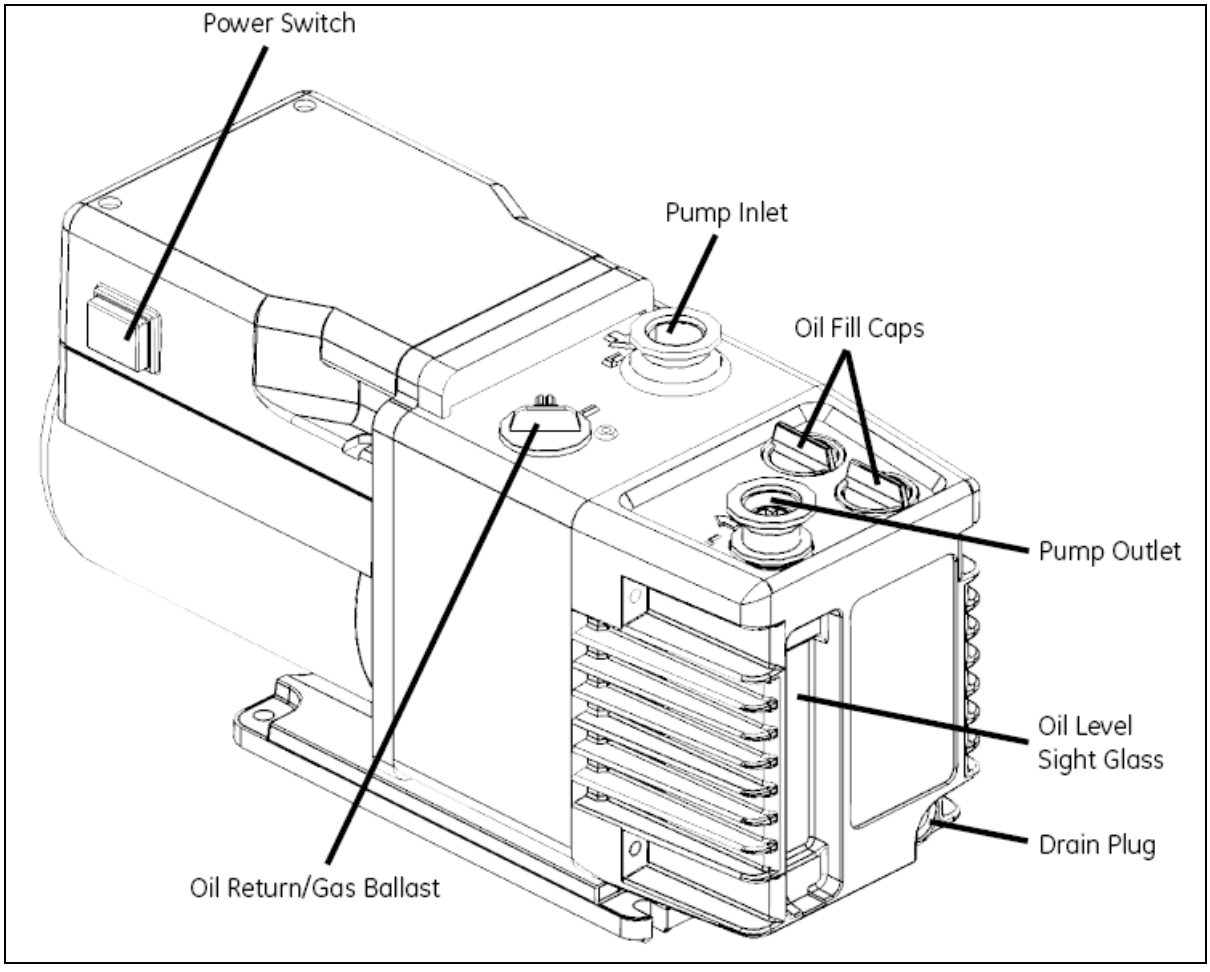


Figure 4-1: *Schematic of RV 3 Pump*

Step 2 - Install Pump Inlet Fitting

Remove the plastic caps from inlet and outlet of the pump. Locate the barbed inlet fitting and clamp in the pump accessories kit. Place the barbed fitting on the o-ring on the pump inlet. Place the clamp over the inlet fitting, o-ring and barbed fitting and secure with the screw and wing nut finger-tight.

Step 3- Install the Chemical Trap Mounting Bracket

Locate the Allen wrench, Allen screws and trap mounting bracket in the pump accessories kit. Use the Allen wrench to remove the two screws on the rear of the top plate of the pump. Insert the Allen screws through the holes in the bracket and connect to the pump as shown in Figure 4-2: *RV3 Pump with Chemical and Charcoal Traps*.

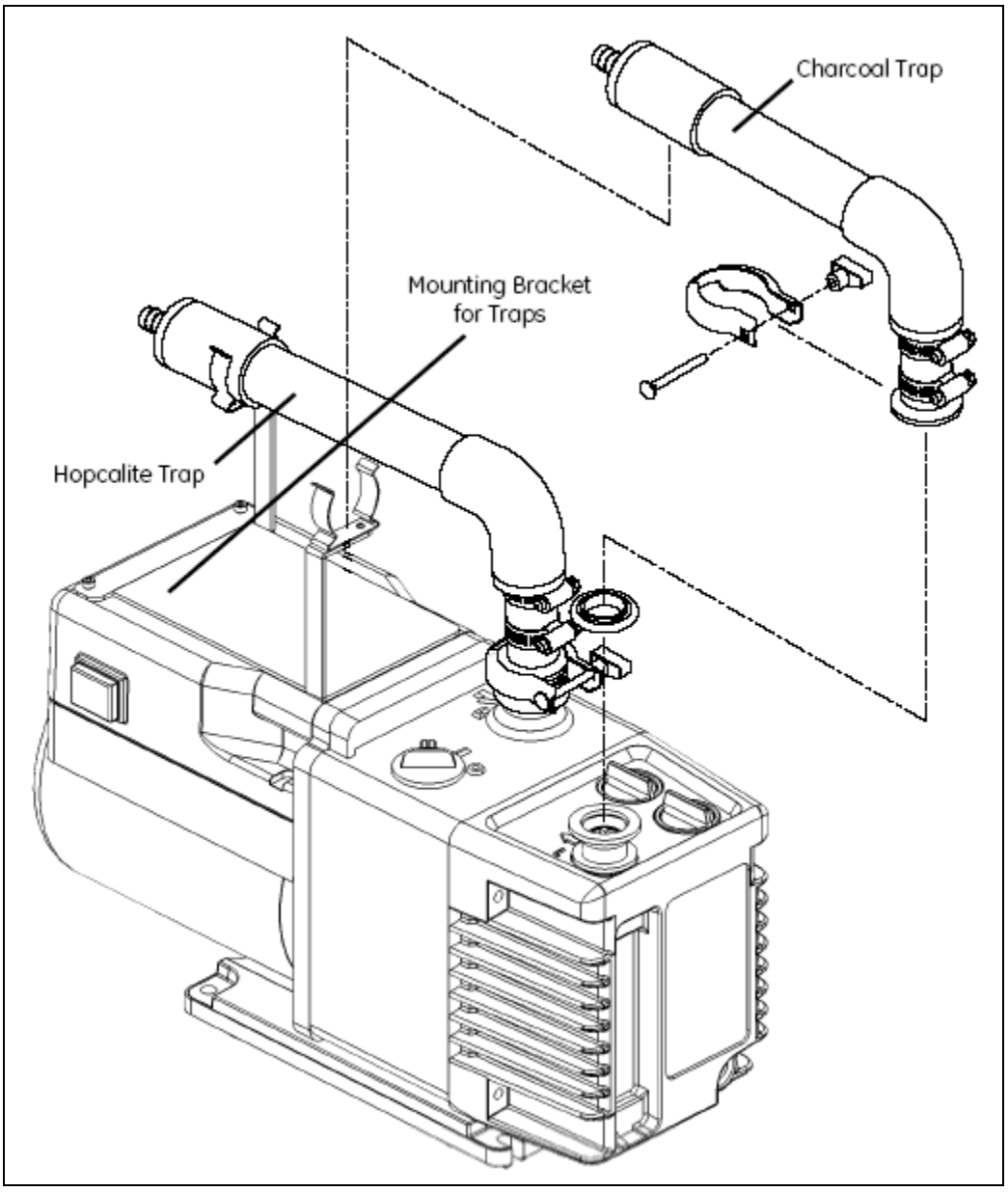


Figure 4-2: *RV3 Pump with Chemical and Charcoal Traps.*

Locate the Hopcalite trap in the NOA accessories box and the short length of clear Tygon tubing and two hose clamps in the pump accessories kit. Place the hose clamps over the tubing, and connect one end of the tubing to the pump inlet barbed fitting and the other end to the barbed fitting on the elbow of the chemical trap. Use a 5/16" hexdriver or a regular screwdriver to tighten the hose clamps.

Step 4 - Install the Pump Outlet Fitting

The RV3 pump can be equipped with either a charcoal filter to remove oil mist from the pump exhaust or a barbed outlet fitting for connecting tubing to the pump exhaust for venting to a fume hood or house vacuum.

Place the centering O-ring on the pump outlet fitting, and then place the barbed fitting on the O-ring. Place the clamp over the outlet fitting, O-ring and barbed fitting and secure with the screw and wing nut. If the charcoal trap is not used, connect a length of Tygon tubing over the barbed outlet fitting and place the other end of the tubing in a fume hood.

Locate the charcoal trap, the short length of clear Tygon tubing and two hose clamps. The charcoal trap is shorter than the Hopcalite trap used on the pump inlet. Place the hose clamps over the tubing, and connect one end of the tubing to the pump outlet barbed fitting and the other end to the barbed fitting on the elbow of the charcoal trap. Use a 5/16" hexdriver or a regular screwdriver to tighten the hose clamps.

Step 5 - Install the Chemical Trap and Vacuum Hoses

Place a hose clamp over the end of the vacuum hose (Tygon tubing with black heat shrink and a metal fitting on one end), and connect the hose to the barbed fitting on the straight end of the Hopcalite trap. Use a 5/16" hexdriver or a regular screwdriver to tighten the hose clamp.

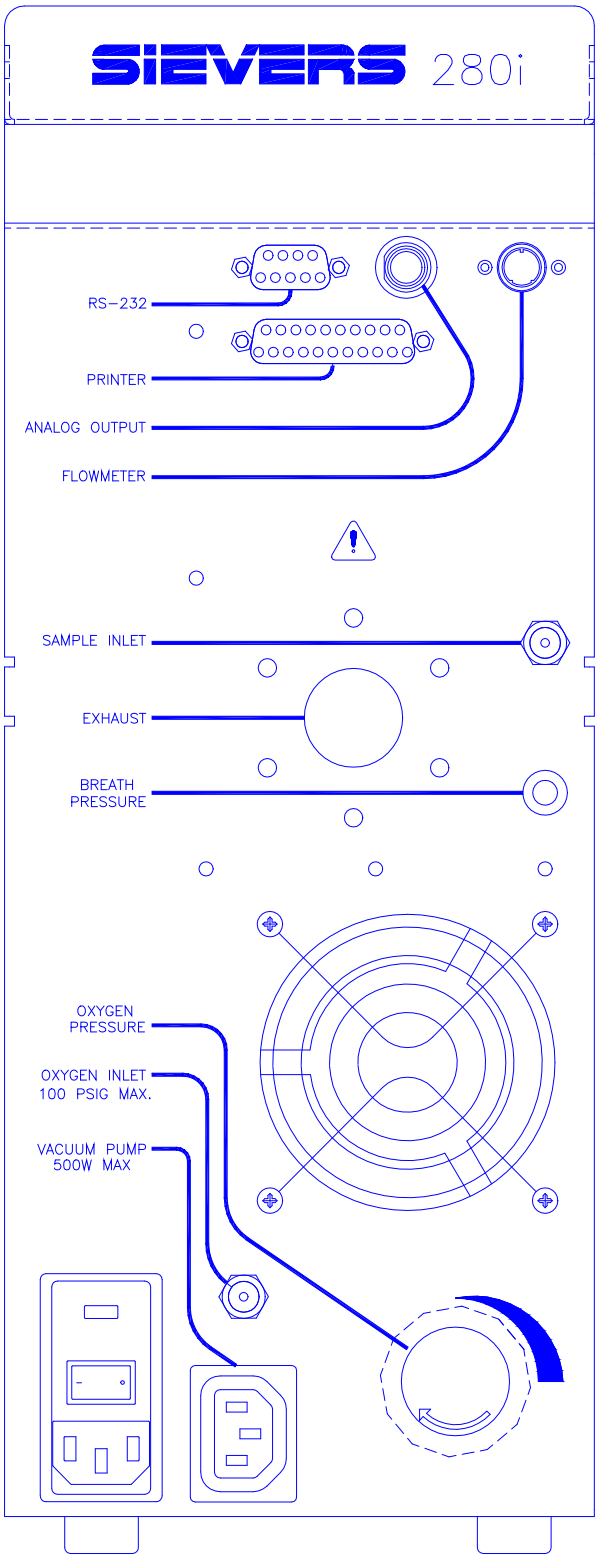


Figure 4-3: *NOA Back Panel.*

Step 6 - Connect Power Cord to Vacuum Pump and Turn On Pump Power Switch

Locate the pump power cord and connect one end to the power cord inlet on the side of the pump. Turn the pump power switch on the other side of the pump to the ON position.

Connections to NOA

All of the connections for gases, the vacuum pump and outputs are at the back of the NOA (Figure 4-3). Place the NOA on a bench in a position so there is access to the back of the analyzer. Connect the power cord to the analyzer and plug the cord into an AC Outlet.

Vacuum Pump Power Cord and Vacuum Hose

Plug the female plug on the power cord from the vacuum pump into the socket labeled "Vacuum Pump" on the back of the NOA. In the center of the back panel is a protective cap on the exhaust of the NOA. The cap protects the photomultiplier tube from exposure to light during shipping and anytime the vacuum hose is removed from the analyzer.

Remove the protective cap from the exhaust port of the NOA. Anytime the vacuum hose is removed from the NOA this cap should be replaced. Carefully thread the 1/2" Swagelok nut on the vacuum hose to the exhaust fitting. Place a 13/16" wrench on the fitting on the NOA and use a 7/8" wrench to fully tighten the nut. This will require some force to get a good seal.

Vacuum Test

Once the vacuum pump has been connected, test that all connections are tight by performing a vacuum test. Make sure the power switch on the vacuum pump is in the

ON position and the protective caps are on the sample and oxygen inlets. To perform the test:

- Use a 7/16" wrench to tighten the protective caps on the sample inlet and ozone supplies.
- Turn on the Main Power switch located at the rear of the NOA.
- With the Analysis option highlighted, press the ENTER Button to display the Analysis Menu.
- Use the Down Arrow Button to scroll to Stand-by and press ENTER. The vacuum pump will start.
- Allow the pump to operate for ~10 minutes and record the reaction cell pressure by selecting the Control option from the Main Menu and pressing the ENTER Button.
- From the Control Menu, use the Down Arrow Button to scroll to Status and press ENTER. The cell pressure will be displayed.

Reaction Cell Pressure Vacuum Test _____ torr

If the vacuum pump connections are tight, the reaction cell pressure should decrease to 1-2 torr within 10 minutes. If the pressure decreases to <2 torr, the connections are tight.

If the pressure is higher check the connections to the vacuum pump, retighten the hose clamps and the connection of the vacuum pump to the back of the analyzer. If the pressure remains above 1-2 torr after retightening the connection, contact GE Analytical Instruments at (303) 444-2009 or (800) 255-6964 for assistance.

After completing the vacuum test, turn the vacuum pump off by pressing CLEAR to return to the Main Menu, scroll to Analysis and press ENTER and select Stop and press ENTER. The display will change with Continue and Stop options. Select Stop to turn off the vacuum pump.

Leave the main power switch ON while completing the installation to cool the PMT.

Gas for Ozone Generator

The NOA requires oxygen for the ozone generator. The connection to the NOA is made via a 1/8" Swagelok bulkhead in the back panel, just above the vacuum pump plug. If oxygen is supplied from a gas cylinder, the cylinder must be equipped with a two-stage regulator with a shutoff valve on the outlet and an adapter for connection of 1/8" tubing with Swagelok-brand connections. A 1/4" female NPT to 1/8" Swagelok union and a 1/4" male NPT to 1/8" Swagelok union are provided with the analyzer. Wrap Teflon tape on the outlet of the shutoff valve or the male NPT union to prevent leaks on the NPT connection.

A 6 ft length of 1/8" OD Teflon tubing with brass nuts and ferrules is supplied with the NOA for connecting the oxygen supply to the bulkhead fitting on the NOA back panel. Use a 7/16" wrench to remove the protective cap from the bulkhead fitting "Oxygen Inlet" on the back panel. Connect the Teflon tubing to the bulkhead fitting. Connect the other end of the tubing to the adapter on the regulator. Use a 7/16" wrench to tighten the nuts 1/4 turn past finger tight. Turn on the main valve on the oxygen tank and adjust the outlet pressure on the regulator to 10 psig (~700 mBar, 520 torr or 0.7 kg/cm^2).

The flow of gas through the ozone generator is ~30 mL/min and is controlled by a pressure regulator located on the back panel of the NOA. The black knob on the regulator has two positions; locked and adjust. When pulled out away from the back panel, the knob can be turned to adjust the ozone gas pressure, when pushed in toward the back panel, the knob will be locked and the knob will not turn. To set the pressure, from the NOA Main Menu, press CLEAR to display the Status Screen. Adjust the regulator until the Supply Pressure is 6.0 ± 0.2 psig (419 mBar, 314 torr or 0.4 kg/cm^2) .

Oxygen can also be supplied from an E-size cylinder or from house oxygen. Normally these are equipped with flow controllers instead of a pressure regulator. Oxygen tubing with 22 mm connectors, universal oxygen tubing or bubble tubing can be used to connect the barbed connector of the flow controller to the NOA. In the accessories kit is a brass 1/8" Swagelok to barbed adapter. Remove the cap from the oxygen inlet

at the back of the NOA and attach the barbed adapter. Use a 7/16" wrench to tighten the nut 1/4 turn past finger tight. Connect one end of the oxygen tubing to the barbed adapter on the NOA and the other end to the barbed adapter on the oxygen tank or house supply. Since the flow through the ozone generator is only 30 mL/min, a low-flow flow controller should be used and the flow rate adjusted to the lowest flow possible. **If the flow is set too high, the tubing will come off the barbed adapters!** Hose clamps can be used to help secure the tubing. Turn on the oxygen, press CLEAR to view the Supply Pressure and adjust the NOA's regulator to 6 psig. If the controller cannot be set to a low enough flow, a pinhole in the oxygen tubing will prevent the tubing from coming off of the barbed adapters.

Frit Restrictor

The flow into the NOA is controlled by a metal-frit restrictor in an adapter attached to the Sample Inlet bulkhead fitting on the back panel of the NOA (Figure 4-3). The standard restrictor has a flow rate of ~200 mL/min and restrictors with other flow rates are available. Remove the protective cap and locate the frit restrictor (stainless steel 1/8" male/female adapter in the accessories kit). Connect the restrictor to the bulkhead fitting labeled Sample Inlet and use a 7/16" wrench to tighten the nut 1/4 turn past finger tight.

Computer, Printer and Analog Signal Connections

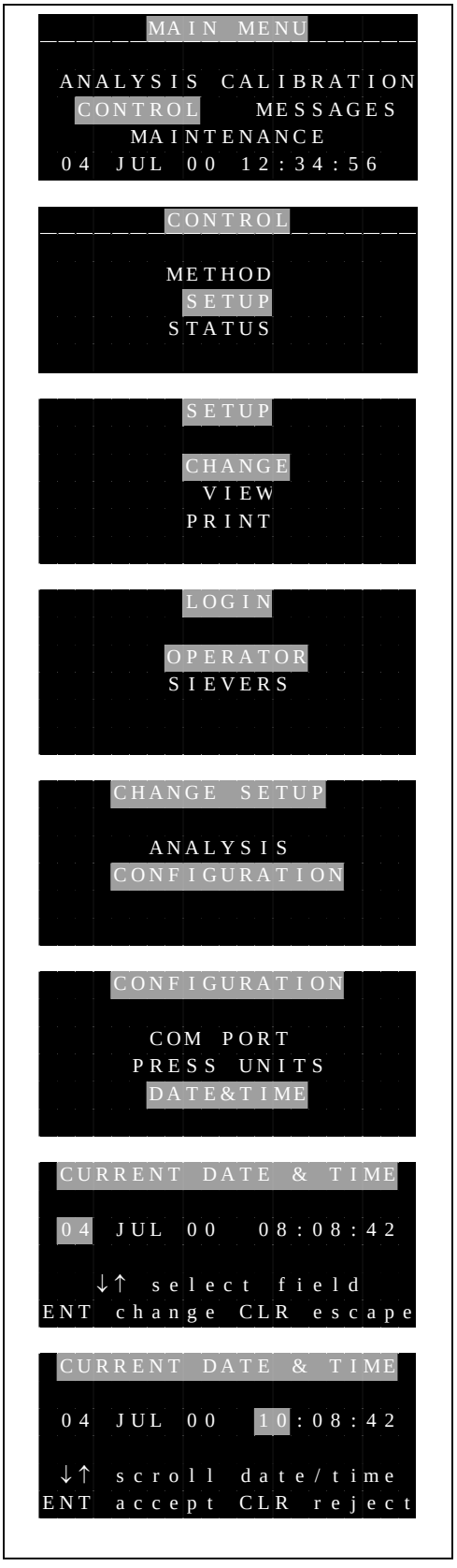
The connections for the computer, printer and analog signal are at the back of the NOA.

Computer – the RS-232 connector is the 9-pin connector near the top of the back panel. Two cables are available for connecting the NOA to PCs and Macs. The cable for PCs is a 9-pin male/9-pin female cable (ACH 09010). Connect the male end to the NOA and secure with the thumbscrews. Connect the 9-pin female end to the computer's COM Port. Some desktop PCs have 25-pin Com Ports and require a 9-pin to 25-pin Serial adapter for use with the NOA. These adapters are available from most computer stores. For Apple computers, the cable is a 9-pin male to 8-pin DIM (ACH 14000). Connect the 9-pin male end to the NOA port and secure with a screwdriver. Connect

the DIM end to either the printer or modem port on Macintosh computers. Newer Macs do not have serial ports and either a USB to serial adapter or a serial port card is required. Contact GE Analytical Instruments for more information on these devices.

Printer – A Centronics 25-pin printer connector is located below the RS-232. The NOA can be connected to any 80-column printer using the cable that came with the printer.

Analog Output – The analog signal from the NOA is the BNC connector to the right of the RS-232 output. This is a 0-1V output directly from the PMT amplifier and can be set to a 0-10V output using a switch on the amplifier. A cable (ASM 00165) with a BNC connector and spade lugs is available from GE Analytical Instruments.



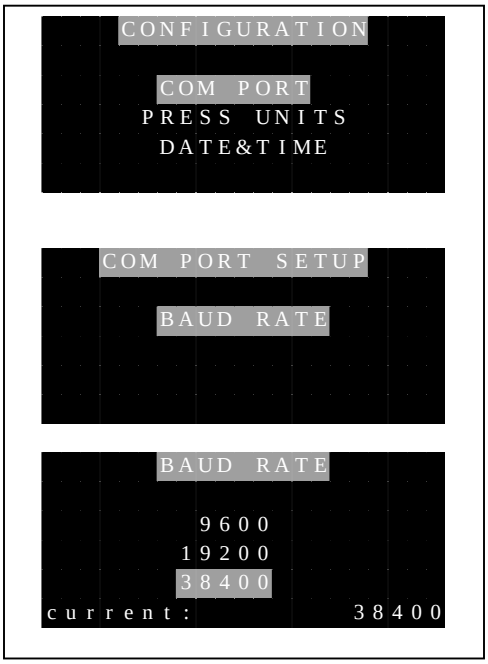
Setting the Clock

To set the clock on the NOA:

- From the Main Menu use the Arrow buttons to scroll to Control and press ENTER to display the Control Menu.
- Use the Down Arrow to scroll to Setup and press ENTER to display the Setup Menu.
- Select the Change option and press ENTER to display the Login Menu.
- Select Operator and press ENTER to display

the Change Setup Menu.

- Scroll to Configuration and press ENTER.
- Scroll to DATE and TIME and press ENTER.
- To change the time, use the Down Arrow button to scroll to the hour field and press ENTER.
- Use the Arrow buttons to scroll to the current hour and press ENTER to save.
- To change the minutes, use the Down Arrow button to scroll to this field, and then press ENTER. Use the Arrow buttons to change the minutes, and then press ENTER to save.
- After setting the time, press CLEAR to return to the Configuration Menu.



Configuration Menu Options

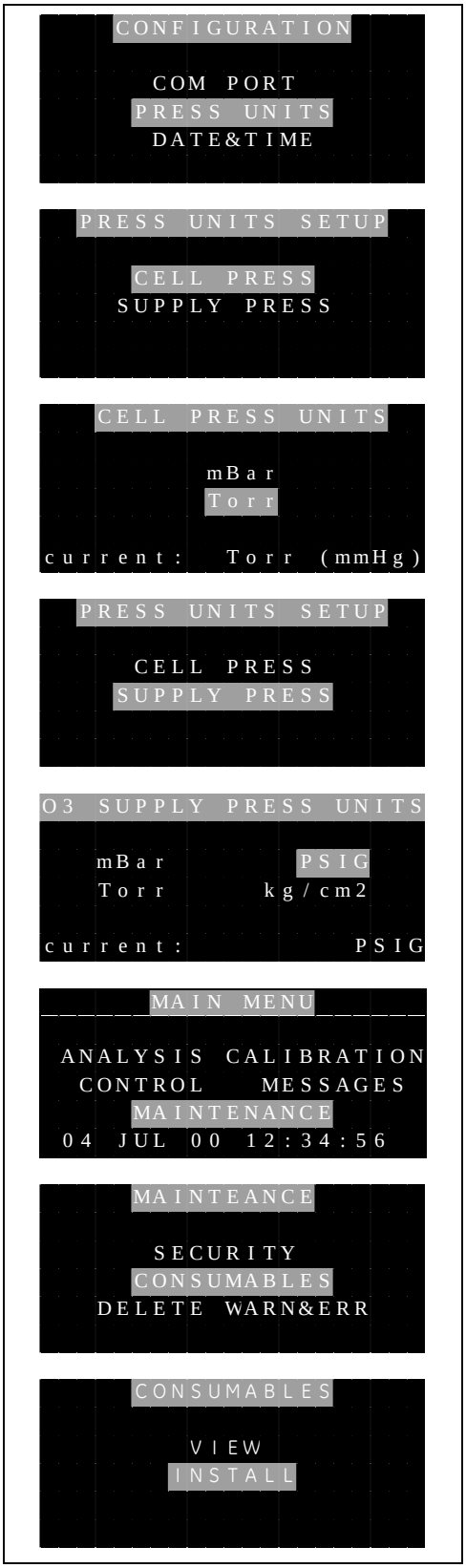
The Configuration Menu contains two other menu options: Com Port and Pressure Units. These options, along with the clock, and only be changed when the NOA is in the Stop or Standby mode.

Com Port

The Com Port option is used to set the baud rate for communications with the computer, with a default setting of 38400. This rate should be used for all application—unless the computer used for data acquisition cannot be used at this baud rate. Lower baud rates will limit the Com port intervals that can be used. At 19200 or 9600, the 1/32 interval cannot be used and some data loss may be observed at an interval of 1/16.

To change the Baud Rate:

- From the Configuration Menu select Com Port.
- Select Baud Rate and use the Arrow buttons to scroll to the desired rate.
- Press ENTER to display the Confirmation screen (not shown) and press ENTER to save the new rate. Press CLEAR to return to the Configuration Menu.



Pressure Units

The units for the cell and supply pressures shown in the Status menu and in the Measurement Menu for the Nitric Oxide Mode can be changed using the Press Units option. The default units are Torr (mm Hg) for the cell pressure and PSIG (pounds per square inch gauge) for the supply pressure.

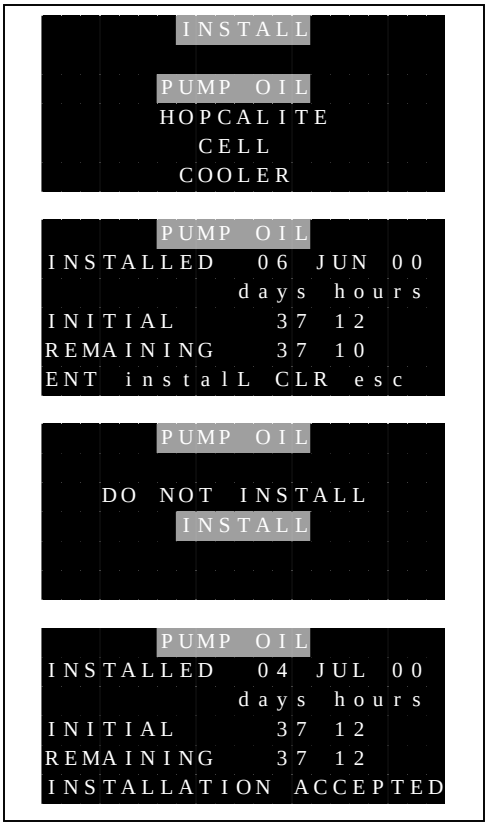
To change the Cell Pressure Units:

- From the Configuration Menu, select Press Units and press Enter to display the Pressure Units Setup Menu.
- Select Cell Pressure and press ENTER.
- From the Cell Press Units Menu, use the Arrow buttons to scroll to the desired units, press Enter and when the confirmation screen is shown, press ENTER to save the new units.

To change the Supply Pressure Units

- From the Pressure Units Setup Menu, select Supply Press.

- From the O_3 Supply Press Units Menu, use the Arrow buttons to scroll to the desired units and press ENTER.
- From the Confirmation Screen, press ENTER to save the new units.
- Press CLEAR five times to return to the Main Menu.



Setting the Consumables Installation Data

To set the installation dates:

- From the Main Menu, select Maintenance.
- From the Maintenance Menu, select Consumables.
- From the Consumables Menu select Install.
- From the Install Menu, select Pump Oil.
- From the Pump Oil Menu, press ENTER, scroll to Install and press ENTER. The Pump Oil Menu will indicate that the installation was accepted and then return to the Install Menu.
- Repeat the installation process for the Hopcalite, Cell and Cooler by selecting each item from the Install menu, pressing ENTER, then selecting Install.

Start-up

This completes the initial setup of the NOA. Instructions for installing the purge vessel for liquid samples are in Chapters 1012. Instructions for installation of the gas-sampling package are in Chapter 5 and instructions for assembly of the exhaled

breath accessories are in Chapters 79. Before continuing with the installation, start the NOA to verify proper operation.

To Start the NOA:

- From the Main Menu, press CLEAR to display the Status Screen.
- Verify that the Cooler Temperature is –12 ± 0.2 °C, the Supply Pressure is 6 ± 0.2 psig and the Cell Pressure is > 300 torr.
- Press ENTER or CLEAR to return to the Main Menu.
- With the Analysis option highlighted, press ENTER.
- From the Analysis Menu, select Start.

The Startup screen is displayed and the NOA should pass all tests and display the Measurement Menu. If the NOA fails any tests, consult the Troubleshooting section (Chapter 14) for assistance.

5 INSTALLATION AND SETUP: GAS-PHASE MEASUREMENTS

Installation of Gas Sampling Package

The gas sampling package is used for all gas-phase NO measurements. It includes a Nafion drier, a white 0.45 µm Teflon filter, a 6 ft PVC sampling line with male Luer adapters, a bacterial filter, a 6 ft PVC pressure line with white male Luer adapters, a calibration tee, tubing clamp and a male Luer cap.

To install the gas sampling package:

- Connect the 1/8" Swagelok nut on the Nafion drier (tubing with outer mesh) to the frit restrictor on the sample inlet on the NOA's back panel. It is not necessary to use a wrench to tighten the nut. Finger-tight is acceptable.
- Connect the white Teflon filter to the female Luer fitting on the Nafion drier.
- Connect the NO sample line (clear PVC tubing without the white Luer fittings) to the Teflon filter.
- Connect the bacterial filter to the Luer bulkhead fitting labeled “Breath Pressure” on the NOA back panel.
- Connect the Pressure line (clear PVC tubing with white Luer adapters on ends) to the bacterial filter.

- The Luer tee is used for calibration only. If the Calibration Kit was ordered, the tee is installed on the outlet of the NO calibration regulator.

- The clamp is used to securing the sampling and pressure tubing when not in use. Remove the adhesive backing and secure the clamp to the upper left-hand side at the rear of the NOA's top cover, near the sample inlet.

Installation of Thermal Mass Flowmeter

For on-line measurement of exhaled NO, the subject's exhalation flow rate can be measured using the optional Thermal Mass Flowmeter. To install the flowmeter:

- Locate the flow meter cable with 8 pin mini-DIN connectors on both ends. On the connector is a small positioning arrow.
- Hold the connector with the positioning arrow on top and plug the cable into the Flowmeter connector on the NOA's back panel (see Figure 4-3).
- Hold the flowmeter with the serial number tag facing up and adjust the end of the cable so that the positioning arrow is on top and plug the cable into the flowmeter.
- Remove the Phillips-head screw from the front, middle position on the NOA's left side cover.
- Locate the metal flow meter clamp and Phillips-head screw from the flow meter kit and install the clamp on the NOA's side panel.
- Install the flow meter in the clamp.

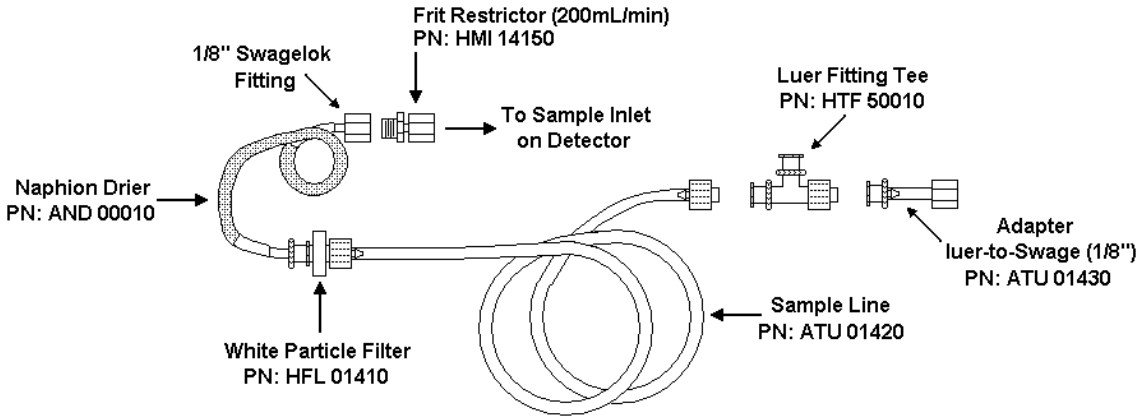
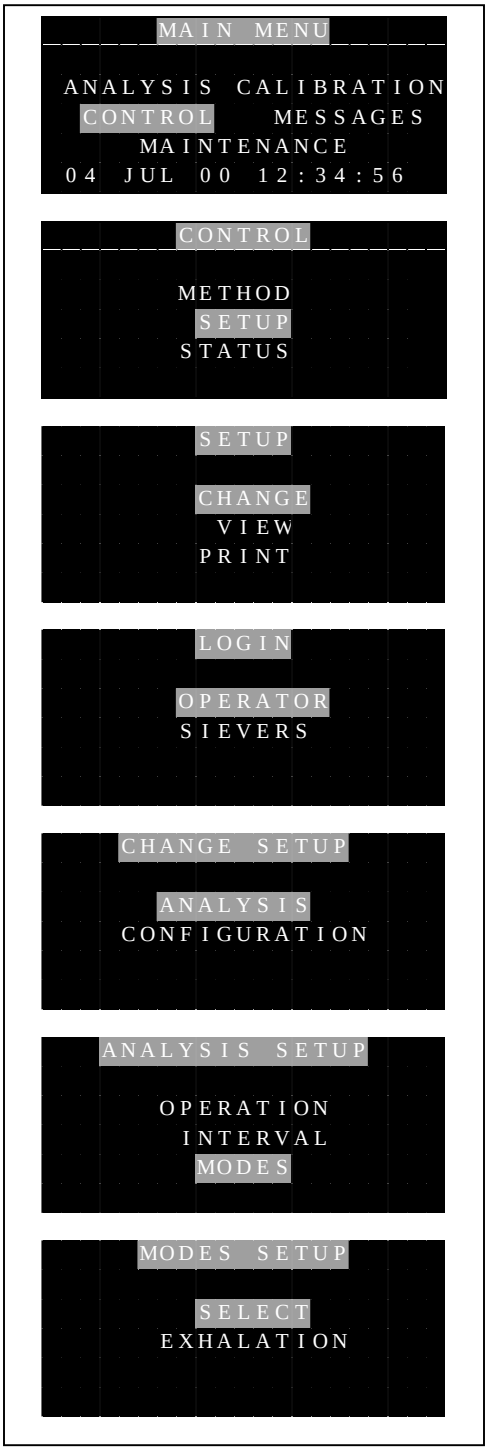


Figure 5-1: *Gas Sampling Package*

- Locate the plastic cable clamp for the flowmeter cable.
- Remove the adhesive backing and secure the cable clamp to the NOA's top cover, near the back (below the gas sampling clamp).
- Secure the flow meter cable using the clamp.

NOA Setup for Gas-Phase Measurements

Gas-phase NO can be measured using either the Nitric Oxide or Exhalation Modes. In the exhalation mode, the NOA outputs the signal from the breath pressure transducer and the thermal mass flowmeter. The Exhalation Mode must be used for on-line and breath-by-breath NO measurements. The Nitric Oxide Mode is used for measurements that do not require the flowmeter or breath pressure such as off-line exhaled NO measurements.

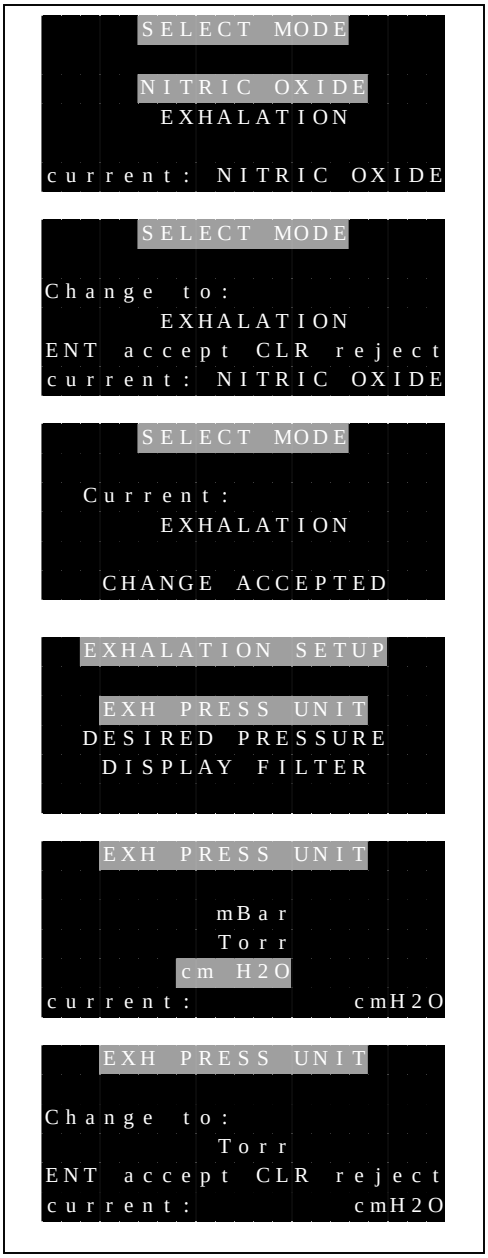


Exhalation Mode

To setup the NOA for the exhalation mode (press ENTER to select Menu Option):

- From the Main Menu, select Control.
- From the Control Menu, select Setup.
- From the Setup Menu, select Change.
- From the Login menu, select Operator.
- From the Change Setup Menu, select Analysis.
- From the Analysis Setup Menu, select Modes.
- From the Modes Menu, select Select.
- From the Select Modes Menu, select Exhalation

- From the Confirmation screen, press ENTER to change the Mode.
- The menu will change briefly to indicate that the Mode has been changed.



The Exhalation Mode has three additional parameters that are set in the Modes Setup Menu: Pressure Units, Desired Pressure and Display Filter. Pressure Units determines the units for the Breath Pressure transducer in the Com Port output. The Desired Pressure and Display Filter selections only affect the bar graph in the Measurement Menu. Desired Pressure sets the value of pressure at the arrow and display filter is used to filter the displayed pressure bar graph.

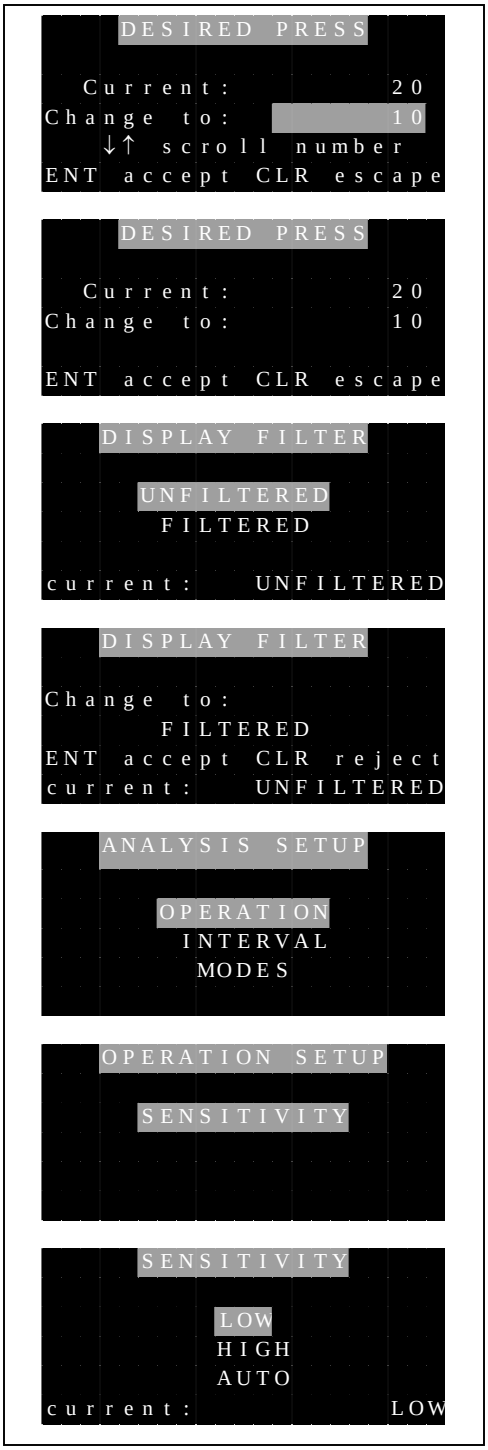
The default unit for the exhalation pressure is cm H_2O that is acceptable for most applications. To change the unit:

- From the Modes Setup Menu, select Exhalation.
- From the Exhalation Setup Menu select EXH PRESS UNIT.
- From the Exh Press Unit Menu, scroll to the desired pressure unit and then press ENTER.
- A confirmation screen displays. Press ENTER to change the unit.

To change the desired pressure (pressure value for arrow on Measurement Menu):

- From the Exhalation Setup Menu, select Desired Pressure.

- Use the Arrow buttons to select the desired value and press ENTER.
- A confirmation screen is shown, press ENTER to change the value.



The exhalation pressure is shown in the Measurement Menu in bar graph form and updated four times a second. The pressure can be displayed as unfiltered pressure or the value filtered to produce a smoother signal. The default setting is unfiltered. To change the Display Filter:

- From the Exhalation Setup Menu, select Display Filter.
- Select the desired option and press ENTER.
- A confirmation screen is shown, press ENTER to change the value.

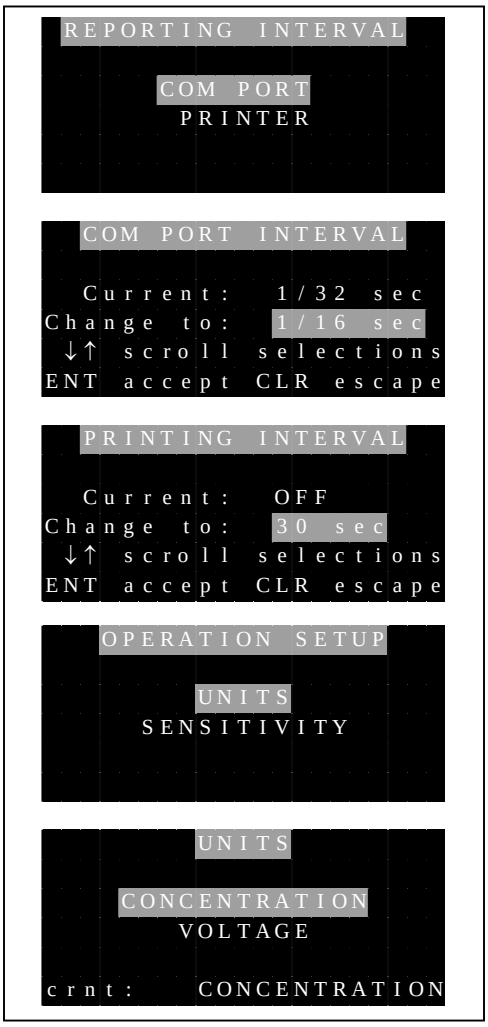
Two final items need to be set for the Exhalation Mode: Sensitivity and Interval.

The sensitivity sets the range of the NOA's PMT amplifier. To set the sensitivity:

- From the Modes Setup Menu, press CLEAR to return to the Analysis Setup Menu.
- From the Analysis Setup Menu, select Operation
- From the Operation Setup Menu, select Sensitivity.
- Scroll to the desired sensitivity and press ENTER.

High sensitivity corresponds to parts per billion (ppb), Low sensitivity corresponds to part per million (ppm) and Auto will automatically switch between ppb and ppm

depending on the concentration of gas being measured. The recommended setting is Auto. In some instances, such as measurement of a sample near 1 ppm or for integration of a signal, it may be desirable to force the NOA into high or low sensitivity.



The interval determines how often data is outputted to the computer and/or printer. The recommended Com Port intervals depend on the application

On-line Exhaled NO	1/16 or 1/8 sec.
Off-line Exhaled NO	1/2 or 1/4 sec.
Breath-by-breath	1/32 or 1/16 sec.

The printer is not used for most gas-phase measurements and should be set to OFF.

To change the Com Port Interval

- From the Analysis Setup Menu, select Interval
- From the Reporting Interval Menu select Com Port.
- Scroll to the desired interval and press ENTER.

Nitric Oxide Mode

The NOA can also be used to measure gas-phase NO in the Nitric Oxide mode. The Nitric Oxide mode has one additional parameter (units) in the Analysis Setup. For gas measurements, the Units must be set to Concentration. To set the Units

- With the NOA in the Nitric Oxide Mode (see above for setting the mode), from the Analysis Setup Menu, select Operation.
- From the Operation Setup Menu, select Units
- From the Units Menu select Concentration.

The Sensitivity can be set to High, Low, or Auto as described previously.

6 CALIBRATION

To use the NOA for measurement of gas-phase NO, a calibration is required. The stability of the calibration depends on many factors including ambient temperature humidity, flow into the NOA, line voltage fluctuations, and contamination of the reaction chamber. Most laboratories calibrate the analyzer at least once a day or each time they make measurements. The calibration consists of two parts; measurement of an offset for the ppb and ppm ranges using a gas containing <1 ppb NO ("zero air") and measurement of response factors for the ppb and ppm ranges using a gas that contains a known concentration of NO. The calibration should be done using the gas-sampling package with the Nafion drier. Use of the drier will ensure that the relative humidity of the calibration gases is the same as the humidity in the sample gas.

Note:
The NOA must be in the Start Mode to perform the Calibration.

Zero Gas Calibration

There is always a background signal from the PMT and the ozone in the NOA. This background or offset is measured by running a gas that contains < 1 ppb NO, and is subtracted from the PMT signal for standards and samples. Air containing < 1 ppb NO can be produced using the Zero Air Filter from GE Analytical Instruments (Sievers Part No. ACT 01400). The NOA draws room air through a bed containing $KMnO_4$ and activated carbon. NO is oxidized to NO_2 by the $KMnO_4$ and NO and NO_2 absorbed by the carbon. The $KMnO_4$ is converted to MnO_2 (brown color) and the filter should be replaced when all of the purple $KMnO_4$ has turned brown. The lifetime of the filter will depend on the frequency of use and the levels of NO in ambient air, but typically the filter is good for 5 years.

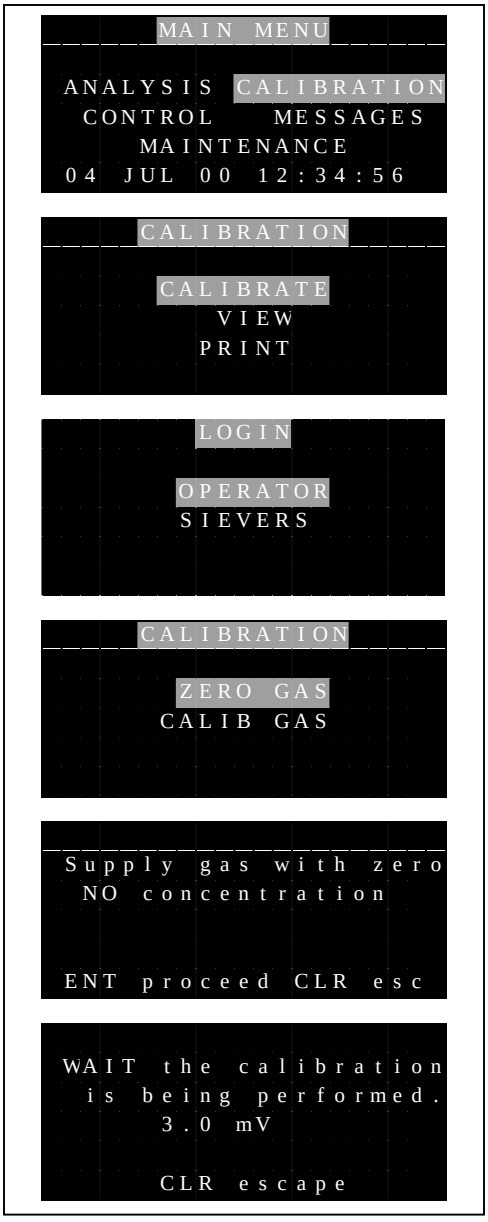
Most gas companies supply so-called "zero air" that can also be used for the zero gas calibration. Cylinders of breathing air, nitrogen or oxygen may or may not contain NO at ppb levels and should not be used for the Zero Gas Calibration unless they contain < 1 ppb of NO.

Calibration with Zero Air Filter

To calibrate the zero offset using the filter, remove the Luer plug and brass Swagelok cap from the zero air filter. Connect the Luer fitting on the gas sampling line directly to the Luer adapter on the outlet of the filter and allow the NOA to draw ambient air through the filter for ~5 minutes.

Calibrating with Zero Air Cylinder

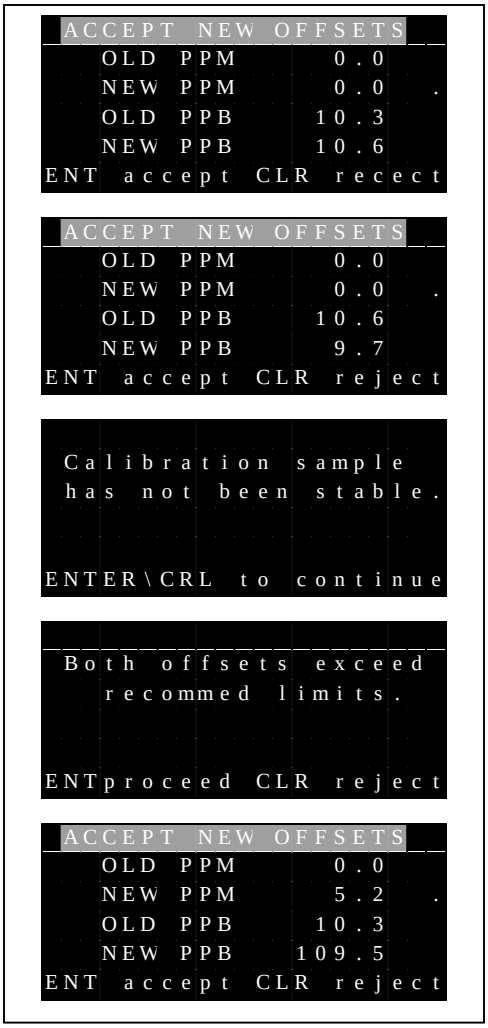
The setup for sampling gas from a pressurized gas source is shown in Figure 7-1. The Luer adapter tee included in the gas sampling package is connected to the outlet of the regulator on the gas cylinder, the PVC gas sampling line connected to one leg of the tee and the remaining leg is open to the atmosphere. The flow rate of gas from the pressurized source is adjusted to provide >200 mL/min with the excess gas flowing out the open leg of the tee. Using this setup will ensure that the calibration is performed at the same flow rate as the measurements. Allow the NOA to sample the zero air for ~ 5 minutes.



To calibrate of offset:

- Start the NOA and from the Measurement Menu, press CLEAR to switch to the Main Menu.
- Use the arrow buttons to scroll to Calibration and press ENTER.
- The Calibration Menu is displayed with three options: Calibrate, View and Print.
- Select Calibrate to display the Login menu.

If the security is not enabled, then the menu will display Operator and Sievers options. If the security features are enabled, a list of the administrators is displayed and a password is required to calibrate the NOA (see Chapter 14).



- Select Operator to display the Calibrate Menu.
- Select the Zero Gas option.
- The zero gas calibration instruction menu is displayed, after the zero gas has been flowing into the NOA for ~5 minutes, and then press ENTER to start the zero gas calibration.

The analyzer switches displays and shows a moving average of the PMT signal in millivolts with the amplifier in the low sensitivity range. The moving average starts at 0 mV and slowly increases. To ensure an accurate calibration, the firmware compares the most recent measurement of the ozone background signal to the moving average until a stable measurement is obtained. The analyzer then switches to the high sensitivity setting, resets the moving average to 0 mV and measures background signal until a stable signal is obtained. The ppb and ppm offsets are then calculated and displayed along with the previous calibration. The units for the offsets are counts at the analog-to-digital converter, with each count being ~ 0.2 ppb or 0.1 ppm.

- Press ENTER to accept the new calibration values and return to the Calibrate Menu. Press Clear to reject the new calibration.
- Two or three successive Zero Gas calibrations should be performed to make sure that the calibration is not drifting up or down. Replicate zero gas calibrations should agree within 1 or 2 counts. If the calibrations are drifting, continue sampling the zero air until stable replicate calibrations are obtained.

Zero Gas Calibration Warnings

If a stable zero gas calibration in either low or high sensitivity cannot be obtained, an unstable calibration warning is displayed. This situation can occur if the zero air sample was not sampled for 5 minutes before starting the calibration, if the caps are not removed from the zero air filter (no flow into NOA) or if the flow rate of the zero air cylinder is < 200 mL/min and room air is being drawn into the detector.

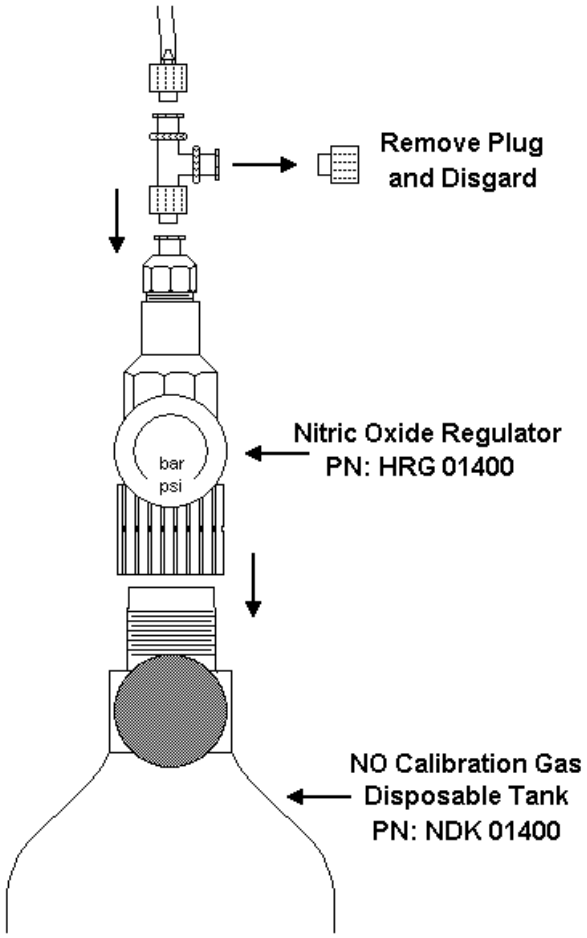


FIGURE 7-1: *Setup for Gas Calibration*

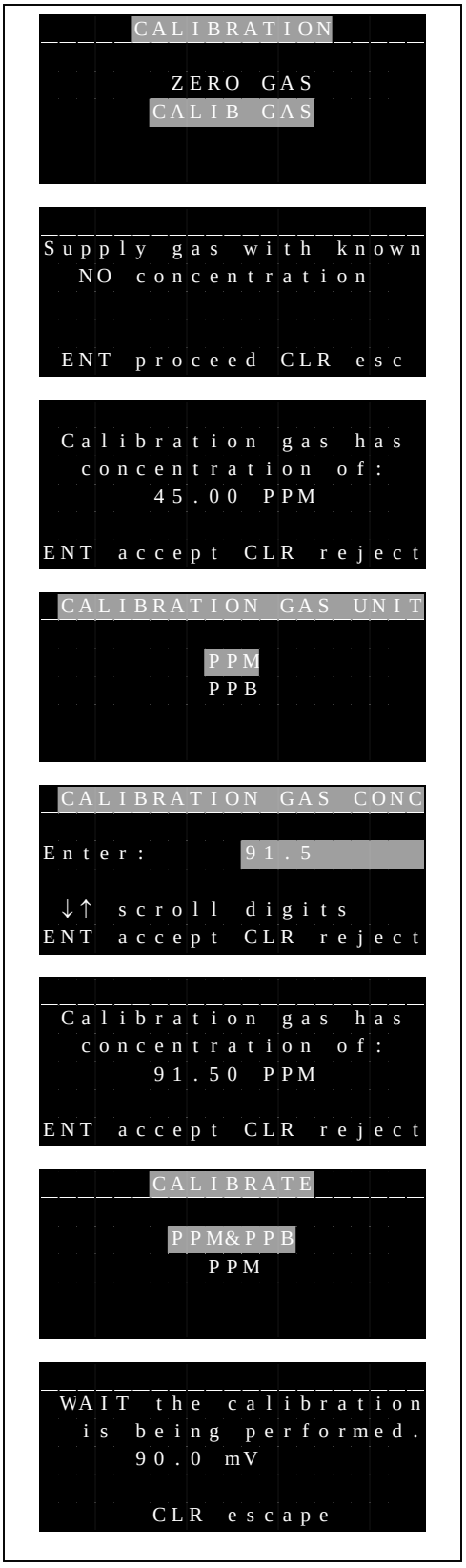
If the ppb offset is more than 100 counts or the ppm offset is more than 5 counts, the display will change to indicate that the zero calibration exceeded the recommended limits. This usually indicates that the gas being used for the zero calibration contains > 1 ppb NO or there is a light leak or other cause of the high background. When the offsets are out of range, a warning message will be displayed. Press ENTER to view the out of range offsets values (values may be rejected from the Accept Menu) or press CLEAR to return to the Calibration Menu. In general, do not accept out of range calibrations. Chapter 14 lists procedures to be followed to determine the cause of the high background.

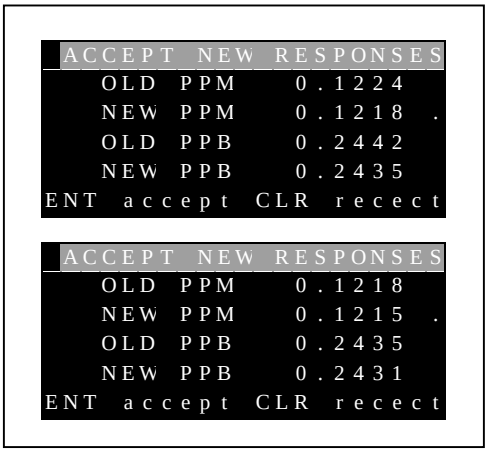
If the offsets are OK, pressing the ENTER button from the Accept Menu will store the new calibration values in the battery-backed RAM.

NO Calibration Gas

The second step in the calibration is to determine the response of the NOA for a known concentration of nitric oxide. The preferred method is to use a gas containing >1 ppm (typically 10-100 ppm) to calibrate the ppm response and have the analyzer automatically calculates the response for the ppb range. A disposable cylinder

containing 45 ppm NO in N_2 and regulator is available from Sievers. Connect the gas sampling line to one leg of the tee (see Figure 7-1) and adjust the gas flow rate to provide >200 mL/min, so that excess gas flows out the open leg of the tee. For the Sievers calibration gas, install the tee on the Luer adapter on the outlet of the regulator and open the valve on the cylinder. The flow rate is set at 300 mL/min by the regulator.





To calibrate the response:

- From the Calibration Menu select Calib Gas to display the instruction screen.
- After the calibration gas has been flowing for ~5 minutes, press ENTER to display the gas concentration screen.

The concentration and units for the calibration gas are stored in the NOA battery-backed RAM. The default concentration is 45 ppm. If a different concentration is used, it is necessary to enter the units and concentration and these values will be stored for future use.

- If the 45 ppm gas is being used, press ENTER and skip to the next section. If another concentration is being used, press CLEAR.
    - Select the gas units (PPM) and press ENTER to display the Calib Gas Conc Menu.
    - Use the arrow buttons to scroll to the value of the first digit of the concentration and press ENTER to save this digit and move the cursor. Press CLEAR to delete an incorrect value.
    - Use the arrow buttons to scroll to the value of the second digit and press ENTER to save this value.
    - Continue entering the digits or a decimal place until the concentration of the calibration gas is entered. Press ENTER to accept the concentration.
    - The menu will change to indicate that the concentration value was accepted and then will display the concentration and units.
    - Press ENTER to accept the concentration. Press CLEAR to reject the concentration and return to the Calibrations Menu.

- Select PPM&PPB to calibrate both ranges and press ENTER to start the calibration.

The display changes and shows the moving average for the PMT signal in millivolts, starting at zero. The firmware waits until the signal stabilizes and then calculates the PPM response based on the final PMT signal and the concentration of the calibration gas. The NOA is set at the factory to have a response of 2 mV/ppm. For a 45 ppm calibration gas, the final PMT signal should be ~ 90 mV. The NOA then calculates the response for the PPB range based on the new PPM calibration.

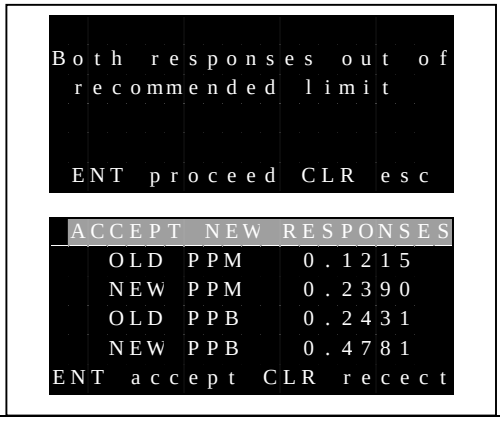
- The new PPM and PPB response factors (ppm/ADC counts and ppb/ADC counts) are then displayed. Press ENTER to save the new response factors.
- Two or three successive gas calibrations should be performed to make sure that the calibration is not drifting up or down. Replicate gas calibrations should agree at the third decimal place. If the calibrations are drifting, continue sampling the calibration gas until stable replicate calibrations are obtained.

Calibration Gas Warnings

The ppm response factor should be in the range 0.09768 – 0.1465 and the ppb response factor should be in the range 0.1953 – 0.293. If the calibration results are outside of these limits, the display will indicate that the response factors are out of range. In general, do not accept out of range response factors. To view the out of range response factors, press ENTER (the response factors can be rejected by pressing CLEAR in the Accept Responses Menu).

If the response factors exceed the recommended limits (ppm response >0.1465, ppb response > 0.293), it usually means that the reaction cell is dirty or the frit restrictor is partially clogged.

Chapter 15 contains a procedure for checking the flow through the frit restrictor and cleaning the reaction cell and frit. An out of range message can also mean the flow of the NO calibration gas is <200 mL/min, so that ambient air is being drawn into the tee and the concentration of NO reaching the detector is less than the entered concentration. If the NO standard is older than 12 months, then the actual concentration of NO

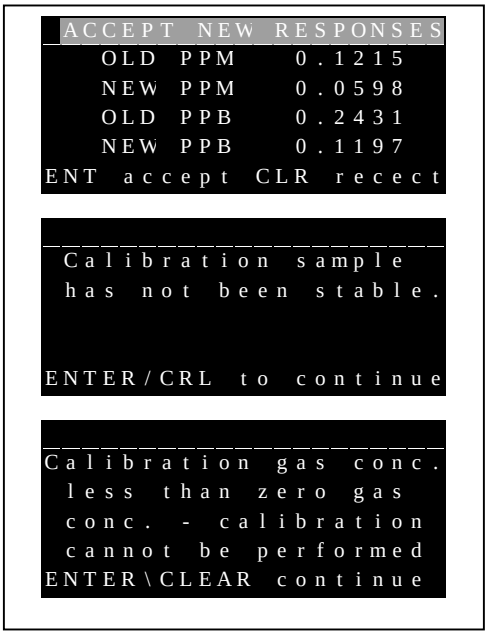


may be less than the reported concentration resulting in response factors that are too high. This can also occur with brand-new regulators and NO cylinders. Check to make sure that there is excess gas flowing out the open leg of the tee and make sure the age of the gas standard has not exceeded the recommended lifetime. For new regulators, allow the gas to flow for several minutes and repeat the calibration.

A less common occurrence is getting response factors that are too low (ppb response <0.195, ppm response <0.0977). This can occur if there is a light leak or if the concentration of NO reaching the detector is greater than the entered concentration. Make sure that the correct gas concentration was entered. Chapter 14 has a procedure for testing and correcting light leaks.

If the PMT signal is not stable during the calibration, an unstable calibration warning will be displayed. This can be caused by too low of cal gas flow or loose connections for the frit restrictor, gas sampling package or vacuum pump or a problem with the ozone generator or electronics.

If the NO cal gas is not turned on when a calibration is performed, a warning screen indicating that the NO is lower than the zero gas will be displayed. Check that the NO cal gas is turned on and excess gas is flow out the open leg of the tee.



Calculation of Gas Concentration

To calculate the concentration of NO in a gas sample, the NOA reads the PMT signal from the Analog to Digital converter, subtracts the offset and then multiples by the response factor. If the calculated value is less than zero, the NOA will display a value of <0.0 ppm or <0.0 ppb. If the display shows a <0.0 reading, this indicates that the offset value is too large and the instrument must be recalibrated.

Because the offset is subtracted from the PMT signal, the range of the NOA will always be less than 1000 ppb for high sensitivity, and less than 500 ppm for low sensitivity. The maximum signal for the ppb range can be calculated using the formula (4095 - ppb offset) X ppb scaling. When the signal from the PMT exceeds the maximum signal, the NOA will display the greater than sign (>) and the current maximum value. For example, if the ppb offset is 20 and the ppb scaling is .24, the maximum value that can be measured is 978 ppb. If the sample contains a higher concentration of NO, the display and outputs, will show greater than 978 ppb. .

Independent Calibration of ppb and ppm Ranges

For NO standards that contain <1 ppm NO, or if accurate standards in the ppb range can be prepared, the ppb range can be calibrated independent of the ppm range. In most cases, there is no advantage in independently calibrating the ppb range. The best method for preparing ppb level standards is to use mass flow controllers to dilute ppm level NO standards with zero air. For low ppb standards, a two-stage dilution is usually required. For example, a setup can be done with a 1:100 dilution of a 10 ppm NO standard with zero air to prepare a 100 ppb standard, and then dilute this gas 1:10 with zero air to prepare a 10 ppb standard.

To calibrate the ppb range, connect a gas containing <1 ppm NO to the sampling tee, let it flow for 5 minutes, then select PPB for the Calibration Gas Units Menu. To calibrate the ppm range, connect a gas containing >1 ppm NO to the sampling tee, let it flow for 5 minutes, then select the PPM option in the Calibration Range Select Menu (Figure 7-15). When the ranges are calibrated independently, only one response factor will be calculated and displayed.

Accuracy of ppb level Measurements using ppm level Calibration

Chemiluminescence has been conclusively shown to provide a linear response over 6 orders of magnitude (0.5 ppb to 500 ppm for the NOA). This means that the error associated with measurement of ppb levels of NO when calibrating with a ppm level gas will be low (for the NOA typically <5% at 10 ppb). While gas standards are commercially available in the 100-500 ppb range, they are expensive and not as stable as gas standards containing >1 ppm NO. Gas standards containing <100 ppb NO can be prepared by dilution, but special equipment is required to accurately dilute gases.

Most laboratories will get the most accurate (and less expensive) measurements by using a 10-100 ppm level calibration gas and using the calibrate PPM&PPB option.

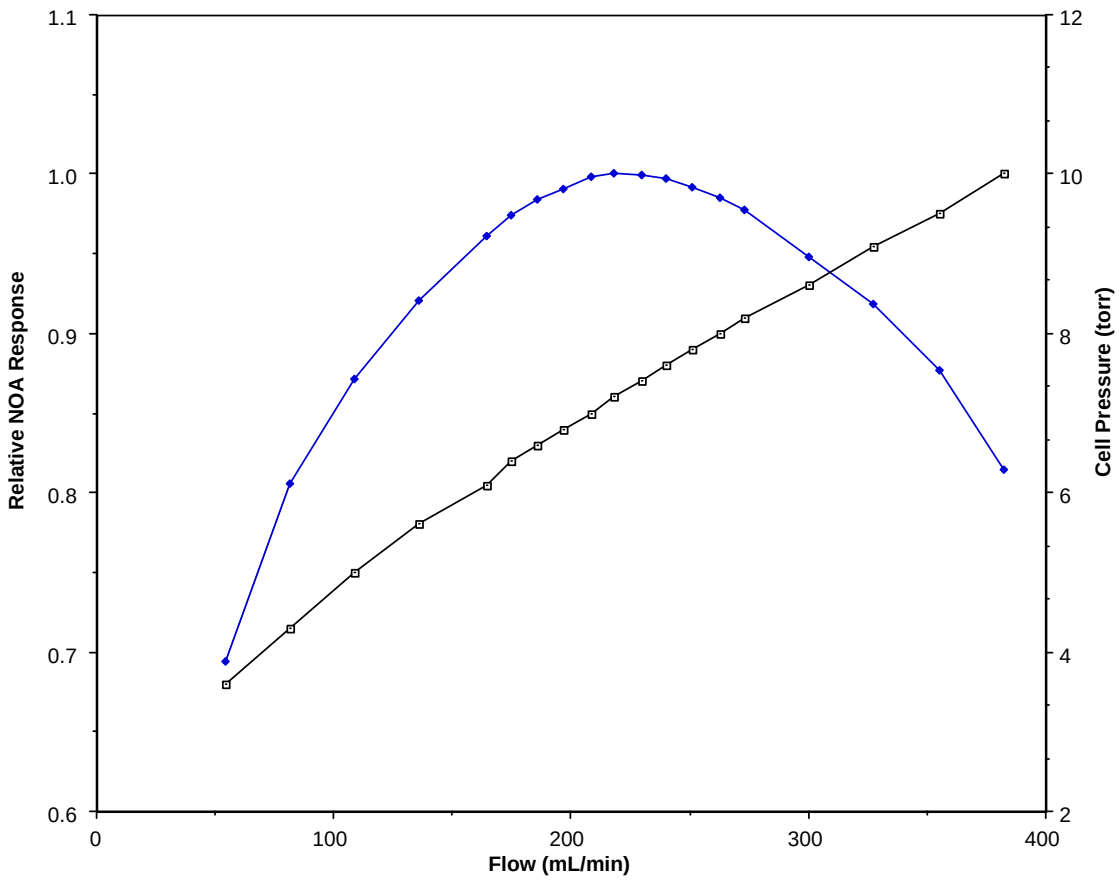


FIGURE 7-2: *Flow/Response/Cell Pressure Characteristics of NOA*

Flow/Response Characteristics of NOA 280i

Figure 7-2 shows the relative response and reaction cell pressure of the NOA as a function of gas flow (in mL/min at 760 torr and 25 °C) into the analyzer. The optimum flow rate into the analyzer is ~175 to ~250 mL/min. Over this flow range, the response of the NOA is independent of flow into the analyzer (<2% deviation from maximum) and small changes in flow rate will not cause changes in the response of the NOA. At higher flow rates, sensitivity of the NOA decreases due to quenching at higher reaction cell pressures. At lower flow rates, sensitivity also decreases as fewer NO molecules enter the reaction cell and react with ozone. The standard flow restrictor provides a flow of ~200 mL/min into the analyzer.

For measurement of exhaled NO in small animals and some other applications, operation at lower flow rates may be required. For these applications, flow restrictors that provide a lower flow into the analyzer are available. Table I lists the restrictors available from GE Analytical Instruments.

Table I. Available Flow Restrictors

Part No.	Flow Rate (mL/min)*	Cell Pressure (torr)	Color Code
HMI 14140	100	5.5	Black
HMI 14120	55	4.0	Blue
HMI 14110	30	3.2	Red
HMI 14100	10	2.5	Green

* - flow rates into NOA at atmospheric pressure of 630 torr

To use the NOA at a lower flow rate, remove the standard flow restrictor. Install the new flow restrictor and recalibrate the NOA at the lower flow rate.

Calibration at Lower Flow Rates

Since the response of the NOA at flow rates below 200 mL/min will be lower than with the standard flow rate, the analyzer must be recalibrated for the lower flow rates. In most cases, high voltage for the PMT will need to be adjusted to achieve good results. Contact GE Analytical Instruments for the procedure to set the high voltage with the lower flow restrictors. If the high voltage is not adjusted, the NOA will report a response out of recommended limits" message during calibration.

7 ON-LINE EXHALED NITRIC OXIDE

On-line measurement of exhaled NO is performed by having the subject exhale orally at a constant flow rate through a restriction to elevate the soft pallet and eliminate nasal contamination. With the NOA 280i, this is performed using the patented Accurate NO breath kit††, the exhalation pressure transducer in the NOA and the NOAnalysis REB program. Exhalation flow rate can also be directly measured using a thermal mass flowmeter.

The American Thoracic Society has published "Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide in Adults and Children-1999" (*Am J Respir Crit Care Med* Vol 160. pp 2104–2117, 1999). The recommended flow rate for on-line measurements is 0.05 L/s (±10%) at Body Temperature and Pressure, Saturated with water vapor (BTPS). For adults, the duration of exhalation should be at least 6 seconds in order to obtain a stable NO value for 3 seconds. Repeated, reproducible exhalations are performed until three NO values are obtained that agree within 10% of the mean value. Exhaled NO ($FE_{NO\ 0.05}$) is the mean of these three values. Instantaneous flow should not be < 0.045 L/s or > 0.055 L/s at any time during the exhalation. If it is not possible for the subject to keep within these values, the results should still be recorded and the failure to achieve this flow criterion noted in the record.

For children < 12 years old, the duration of exhalation should be at least 4 seconds in order to obtain a stable NO value for 2 seconds. Repeated exhalations are performed until three measurements are obtained that agree within 10% of the mean value or two measurements agree within 5% of the mean.

Assembly of the Accurate NO Breath Kit

† U.S. Patent Nos 5,795,787 and 6,010,459.Under license from Aperon Biosystems, Inc.

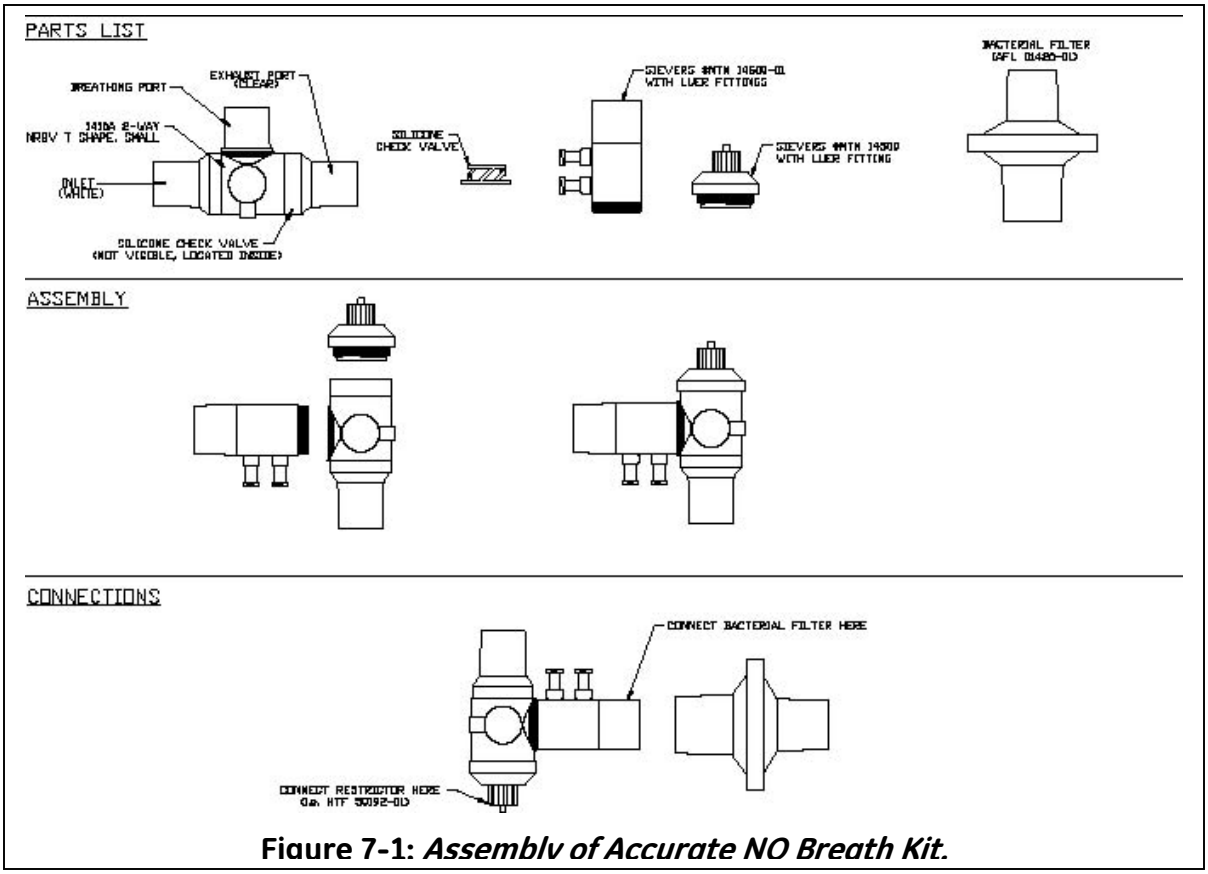


Figure 7-1: ***Assembly of Accurate NO Breath Kit.***

The breath kit is used for on-line exhaled NO measurements and must be assembled before use. To assemble the breath kit:

- Remove the Hans Rudolph® non-rebreathing valve from its package.
- Unscrew the white mouthpiece from the non-rebreathing valve.
- Install the white Sievers mouthpiece with two Luer ports by screwing the mouthpiece into the valve clockwise until secure.
- Unscrew the clear exhaust port fitting from the valve, making sure the spiral diaphragm valve remains inside the non-rebreathing valve.
- Install the Sievers exhaust port with a Luer adapter by screwing the port into the valve until secure.
- The kit includes restrictors that permit exhalation at flow rates ranging from 30 – 250 mL/s. Select the desired restrictor (50 mL/s recommended by ATS) and connect to the Luer port on the exhaust port.

For measurements, a single subject, disposable viral and bacterial filter is installed on the mouthpiece. Also included in the kit is a sterilizable blue mouthpiece (not shown), which can be installed on the inlet of the viral and bacterial filter. **The blue mouthpiece should be sterilized before use.**

Connection of Thermal Mass Flowmeter

If the thermal mass flowmeter will be used for measurement of the exhalation flow rate, connect the large end of the flowmeter to the Sievers mouthpiece on the non-rebreathing valve and connect the bacterial and viral filter and blue mouthpiece to the other end of the flowmeter. The flow direction arrow on the flowmeter will point towards the non-rebreathing valve.

Rotate the flowmeter or valve so that the signal cable from the flowmeter to the NOA is aligned with the Luer fittings on the mouthpiece of the valve.

Connection of Gas Sampling and Pressure Tubing

Connect the male Luer fitting on the gas sample line to one of the female Luer ports on the mouthpiece of the valve. To avoid kinks in the tubing, rotate the male Luer fitting 1/2 turn counterclockwise, then secure the tubing to the mouthpiece by rotating the Luer fitting 1/2 turn clockwise. After the gas sampling line is attached, connect the pressure line (PVC tubing with white adapter on end) to the other female Luer port on the mouthpiece, again rotating the tubing 1/2 turn counterclockwise before securing the pressure line to the mouthpiece with a 1/2 clockwise turn.

For ease of use, cable ties or twist ties can be used along the length of the gas sampling and pressure lines to connect the two pieces of tubing. If the flowmeter is being used, the flow meter's signal cable and the gas sampling and pressure lines can be connected together using cable or twist ties. The pressure line must be connected even if the flowmeter is used.

Inspiratory Gas Connections

The Accurate NO breath kit normally uses ambient air as the inspiratory gas. For on-line measurement of exhaled NO in adults, ambient NO levels do not affect expired NO values, however, high ambient NO levels will require longer exhalations to achieve a stable NO plateau. For children < 12 years old, inspired NO can affect exhaled NO levels and the ATS recommends that inspired gas contain < 5 ppb NO. If ambient air is used for the inspiratory gas, ATS recommends measuring and recording ambient NO levels.

Two options are available for use with the Accurate NO Breath Kit to provide low NO air: an inspiratory gas filter and a 15 L Tedlar bag and adapter.

Inspiratory Gas Filter

This filter is part of the Bag collection and sampling kit, but can also be used with the Accurate NO Breath Kit. It consists of two parts, a charcoal filter (AFL 01410) and an adapter (MTM 01461). To install the filter:

- Screw the male threaded end of the adapter into the charcoal filter.
- Connect the female end of the adapter either by sliding it over the white inlet adapter on the non-rebreathing valve or by unscrewing the white inlet adapter on the non-rebreathing valve (make sure the spiral diaphragm valve remains in the valve). Then carefully screw the black adapter onto the valve body.

NOA Setup

For the on-line NO measurements, the NOA must be in the Exhalation Mode, Sensitivity set to Auto and the Com Port Interval set to 1/16 or 1/8. See Chapter 5 (INSTALLATION AND SETUP: GAS-PHASE MEASUREMENTS) for configuring the NOA. The NOA should be calibrated (Chapter 6–CALIBRATION) prior to measurement.

Performing the Maneuver

On-line measurements of exhaled NO are best performed using the NOAnalysis REB program. Consult the NOAnalysis manual for program instructions. The maneuver

consists of having the subject inhale to total lung capacity (TLC) and immediately exhale while maintaining the targeted exhalation pressure or exhalation flow rate for the required duration (6 seconds of adults, 4 seconds for children. For 50 mL/s at 760torr, the target pressure is 16 cm H_2O.

It is usually necessary to have the subject practice a few times before good exhalations can be achieved. Subjects that are used to spirometry usually will exhale at too high of flow rate as the on-line measurement uses a very slow exhalation. When a subject exhale too fast, there is a tendency to stop exhaling rather than continuing to exhale, but at a lower flow rate.

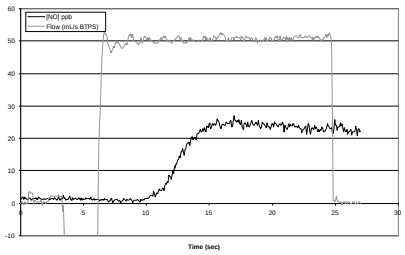


Figure 7-2: *Plateau with Low Inspired NO.*

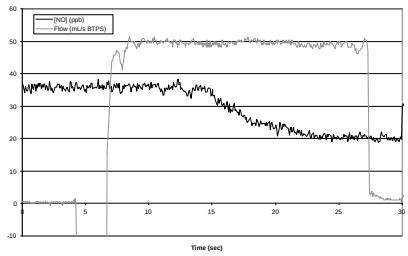


Figure 7-3: *Plateau with High Inspired NO.*

While the subject is exhaling, monitor the NO signal and have the subject continue exhaling until the NO signal is stable for the required duration (3 seconds for adults, 2 seconds for children). Figure 7-2 shows a typical exhalation when inspiratory NO is low. The NO signal slowly increases, then reaches a plateau after a ~10 seconds. Figure 7- shows a typical exhalation when ambient NO is high. NO levels start at ambient levels, and then slowly decrease until a stable NO plateau is obtained (~ 20 seconds after start of exhalation). Figure 7-4 shows an exhalation with high ambient NO, but using the inspiratory filter. NO levels start high, drop during inhalation, then increase to reach a plateau after ~ 10 seconds.

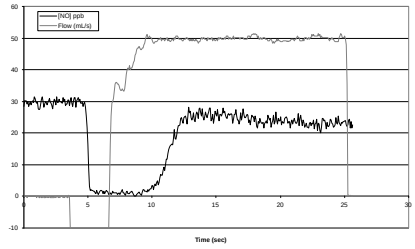


Figure 7-4: *Plateau with High Inspired NO and Filter.*

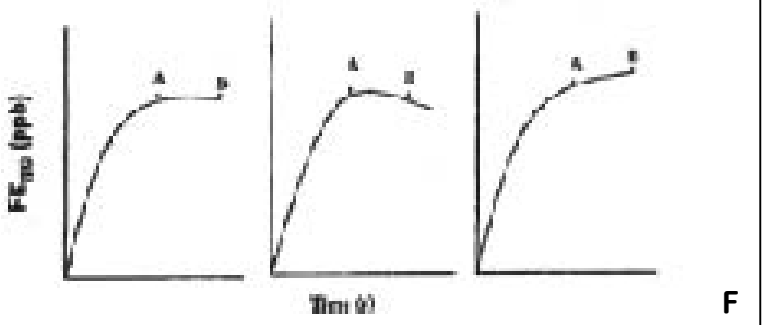


Figure 7-5: *Possible NO Plateaus.*

To measure F exhaled NO:

- Have the subject comfortably seated with a clear view of the computer monitor or the NOA's front panel display. For children < 12, ATS recommendations suggest the subject breath room air for 5 minutes prior to the test.
- Install a new bacterial and viral filter and a sterile blue mouthpiece on the breath kit.
- Instruct the subject on how to perform the maneuver, in particular, it is important to have the subject inhaled through the breath kit and they must inhale through the breath kit if the inspiratory gas filter or Tedlar bag is being used.
- Have the subject inhale to TLC and immediately exhale while maintaining the pressure at 16 cm H_2O (should be between 13 – 19 cm H_2O), adjusting the force of exhalation as required to maintain the desired pressure/flow.
- After the NO levels have reached a stable plateau for the required duration, have the subject stop the exhalation and rest for at least 30 seconds between measurements.
- After 3 replicate measurements that agree within 10% are obtained, remove and discard the bacterial and viral filter and sterilize the blue mouthpiece before the next subject.

Selection of NO Plateau

The ATS recommendations define the plateau as the first 3 second interval (2 seconds for children < 12) in which the difference between the [NO] at the beginning and the [NO] at the end of the interval varies by < 10% (based on the lowest [NO]). At no time during the interval should the [NO] exceed the maximum NO at the beginning or end. For exhaled NO levels < 5 ppb, the beginning and end NO values should not differ by more than 1 ppb.

The REB program uses these criteria to select the value for the NO plateau. Three exhalation profiles can be observed; constant NO versus time, increasing NO versus time and decreasing NO versus time.

For increasing NO plateaus, the end [NO] minus the beginning [NO] divided by the beginning [NO] must be < 10%, the [NO] during the interval must be less than the end [NO] and the end [NO] is selected as the exhaled NO value.

For decreasing NO plateaus, the beginning [NO] minus the end [NO] divided by the end

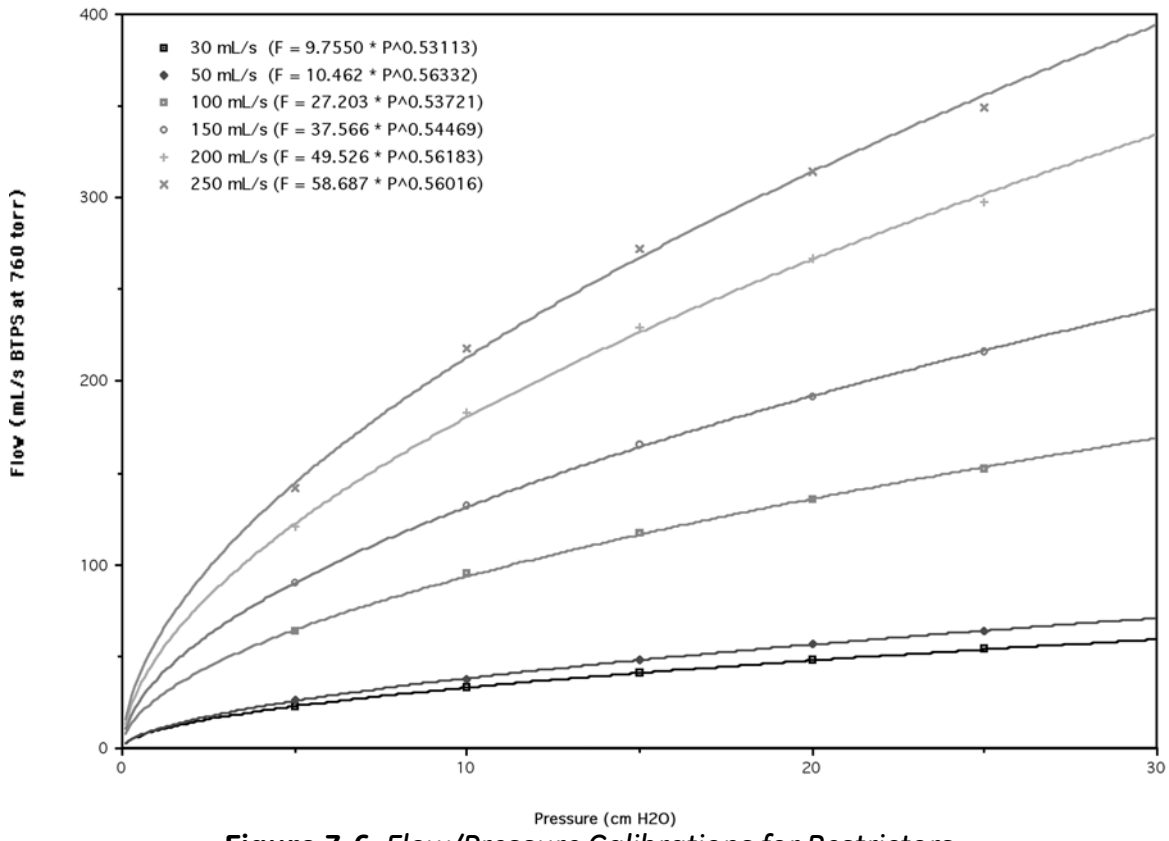


Figure 7-6: *Flow/Pressure Calibrations for Restrictors*

[NO] must be < 10%, the [NO] during the interval must be less than the beginning [NO] and the beginning [NO] is selected as the exhaled NO value.

Flow/Pressure Characteristics of Accurate NO Restrictors

The flow rates (mL/s BTPS at 760 torr) obtained during exhalation as a function of mouth pressure from 5 to 25 cm H_2O for the restrictors in the Accurate NO Breath kit are shown in Figure 7-6. The data can be fit to a logarithmic curve and the equations for each restrictor shown in the figure. The REB program uses these curves to calculate the flow for a given mouth pressure. Table II lists the mouth pressure required to obtain the target flow for each restrictor.

Table II. Pressures Required to achieve Target Flows for Restrictors

Restrictor #/Target Flow	Required Pressure (760 torr Atmospheric Pressure)		
	cm H_2O	torr	mbar
#1 (30 mL/s)	8-9	6	8-9
#2 (50 mL/s)	16	12	16-17
#3 (100 mL/s)	11-12	8-9	11-12
#4 (150 mL/s)	12-13	9-10	13
#5 (200 mL/s)	12	8-9	12-13
#6 (250 mL/s)	13-14	9-10	13-14

Models of Nitric Oxide Production in the Airways

Several groups have published mathematical models of NO production in the airways. By measuring the concentration of exhaled NO at different expiratory flow rates, these models permit calculation of additional parameters such as diffusing capacity, wall concentration and alveoli NO concentration. The restrictors included with the Accurate NO breath kit permit measurement at different flow rates from 30 to 250 mL/s BTPS for use with the following models. The published models include:

> Tsoukias, N.M., George, S.C. "A two-compartment model of pulmonary nitric oxide exchange dynamics". *J Appl Physiol* 1998; 85(2): 653-666.

Pietropauoli, A.P., Perillo, I.B., Torres, A., Perkins, P.T., Frasier, L.M., Utell, M.J., Frampton, M.W., and Hyde, R.W. "Simultaneous Measurement of Nitric Oxide Production by Conducting and Alveolar Airways of Humans." *J Appl Physiol* 1999; 87(4): 1532- 1542.

Silkoff P.E., Sylvester J.T., Zamel N., and Permutt S. "Airway Nitric Oxide Diffusion in Asthma: Role in Pulmonary Function and Bronchial Responsiveness." *Am J Respir Crit Care Med* 2000; 161(4 Pt 1): 1218-28.

Jörres, R. A. "Modeling the production of nitric oxide within the human airways." *Eur. Respir. J.* 2000, 16: 555-560.

Cleaning the Accurate NO Breath Kit and Flowmeter

The procedures for cleaning the non-rebreathing valve, restrictor and tubing are outlined below. Consult your institution's infection control procedure for recommended frequency For the valve and flowmeter, avoid using denatured alcohol or alcohol based solutions as they may cause cracking. Also, avoid temperatures in excess of 45 °C (113 °F). Autoclaving, pasteurization, and ethylene oxide gas sterilization are not recommended. Do not try to clean or sterilize the inspiratory gas filter or Tedlar bag.

Disassemble the Valve

Remove the flowmeter and inspiratory gas filter or Tedlar bag from the breath kit. To clean the breath kit, start by disassembling all of the components:

- Remove the mouthpiece from the modified mouth port and unscrew the mouth port from the valve body.
- Remove the restrictor from the Luer adapter and unscrew the modified exhalation port.
- Remove the exhalation diaphragm from the valve and remove the diaphragm from its ring.
- Unscrew the inhalation tube, remove the diaphragm from the valve body and remove the inhalation diaphragm from its ring.

- Do not remove the Luer adapters from the modified mouth tube or exhalation port.

Prewash the Components

Use a mild detergent and water to clean the components. Metrizyme™, a proteolytic enzymatic detergent from Metrex Research Corporation, can also be used. Rinse thoroughly with warm water.

Sterilization

The following glutaraldehyde solutions are recommended by the valve's manufacturer.

Cidex™, Cidex 7™ (Surgikos, Inc.)
Metricide™, Metricide 28™ and ColdSpor™ (Metrex Research Corp.)
Glutarex™ (3M Company)

Follow the manufacturer's instructions for sterilization. Glutaraldehyde is also available in spray form for spot decontaminations.

Rinsing

After sterilization, rinse all parts thoroughly with warm water.

Drying

A thorough drying is a necessity using a heated chamber to prevent bacterial growth. Do not exceed 45 °C (113 °F) when drying the components.

After drying, inspect all components to ensure that they are free from residue, not deformed or cracked. Reassemble the components by:

- Placing the diaphragms back on their rings, making sure the flange is seated in the ring groove.
- Place the inhalation diaphragm in the valve body and screw the inhalation tube onto the valve body.
- Place the exhalation diaphragm in the modified exhalation port and connect it to the valve body.

- Reconnect the restrictor to the Luer adapter on the exhalation tube.
- Screw the modified mouth tube into the valve body and reconnect the mouthpiece.

The PVC tubing used for the sample and pressure lines can be cleaned as described above. The green bacterial filter on the pressure inlet of the NOA cannot be cleaned and should be replaced when visibly wet or when the valve and tubing are cleaned.

Checking the Inspiratory Gas Filter

The lifetime of the inspiratory filter will depend on the levels of NO in ambient air and the frequency of use. The performance of the filter to remove NO should be tested periodically to determine when it is no longer removing NO. The NOA must be in the Start Mode and should be calibrated before performing this test. To test the filter:

- With the filter installed on the Accurate NO breath kit, connect the NO sampling and pressure lines from the NOA to the breath kit.
- Cover the mouthpiece of the breath kit with the palm of the hand to prevent room air for entering the breath kit (a tight seal is required).
- Note the [NO] on the NOA's front panel. If the filter is operating properly, the [NO] should be < 5 ppb.

If the [NO] is > 5 ppb, replace the inspiratory filter.

8 *OFF-LINE EXHALED NITRIC OXIDE (BAG SAMPLING)*

The concentration of nitric oxide in exhaled breath can also be measured by collecting expired air in a Mylar balloon or Tedlar gas sampling bag and analyzing the contents of bag. Sievers offers two systems for the off-line collection and analysis exhaled NO.

The Vital Capacity Bag Collection and Sampling Kit (BSK 01410) meets the ATS "Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Nitric Oxide in Adults and Children-1999" (*Am J Respir Crit Care Med* Vol 160. pp 2104–2117, 1999). Subjects exhale their vital capacity at a flow rate of 0.35 L/s (±10%) at Body Temperature and Pressure, Saturated with water vapor (BTPS) into a 12L Mylar bag.

The Deadspace Discard Bag Collection and Sampling Kit (BSK 01400) permits collection of exhaled breath at the same flow rate (0.05 L/s) employed for on-line measurements. A divert value in the filler permits discarding of the first portion of the exhalation (physiological deadspace) and then a small sample (~300 mL) of exhaled breath is collected in a 1.5 L Mylar bag equipped with a self-sealing valve.

Assembly of Vital Capacity Bag Collection Kit

The bag collection kit must be assembled before use (refer to Figure 8-1). To assemble the kit:

- Remove all packages from the shipping container. Remove the Body of the Sampler (the check valves and adapters are already installed).
- Screw the pressure gauge into the bag filler body (Teflon tape is not required).
- Inspect the pressure gauge. If the needle does not read 0 cm H_2O, unscrew the clear plastic cover of the gauge and adjust the screw at the base of the gauge with a screwdriver until the needle points to 0 cm H_2O. Replace the clear plastic cover of the gauge.
- Connect the Inspiratory Filter to the filler body. The lifetime of the filter will depend on the concentration of nitric oxide in the inspired air and the frequency of use. A

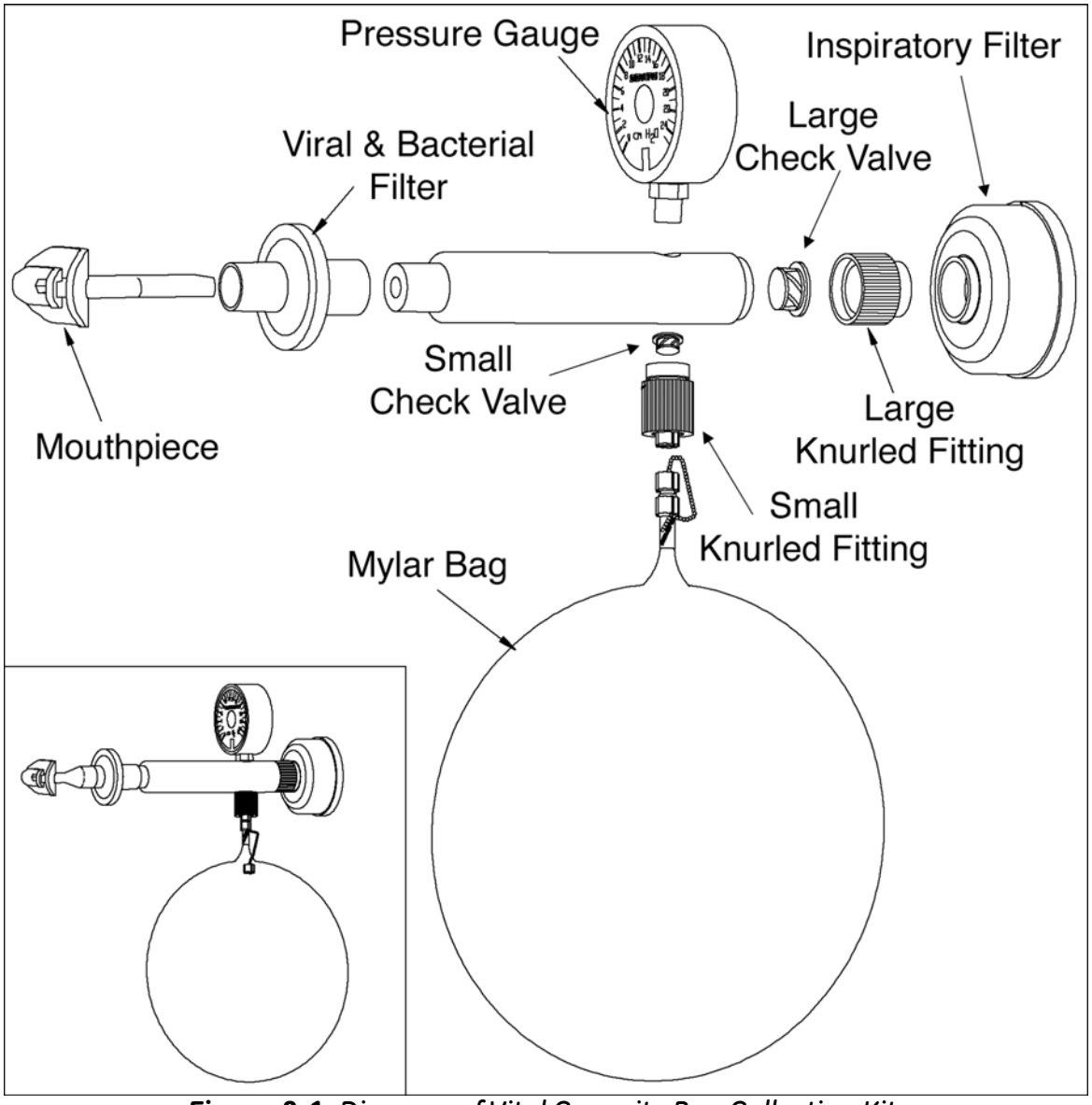


Figure 8-1: *Diagram of Vital Capacity Bag Collection Kit.*

reasonable estimate is three months from the date it is installed. Calculate three months from the day the filter is installed and write that expiration date in the space provided on the filter. Screw the filter onto the threaded end of the body. The Bag Kit is now ready for use.

Assembly of Deadspace Discard Bag Collection Kit

The bag collection kit must be assembled before use (refer to Figure 8-2). To assemble the kit:

- Remove all packages from the shipping container. Remove the Body of the Sampler (the valves, pin, screw and adapters are already installed).
- Screw the pressure gauge into the bag filler body (Teflon tape is not required).
- Inspect the pressure gauge. If the needle does not read 0 cm H_2O, unscrew the clear plastic cover of the gauge and adjust the screw at the base of the gauge with

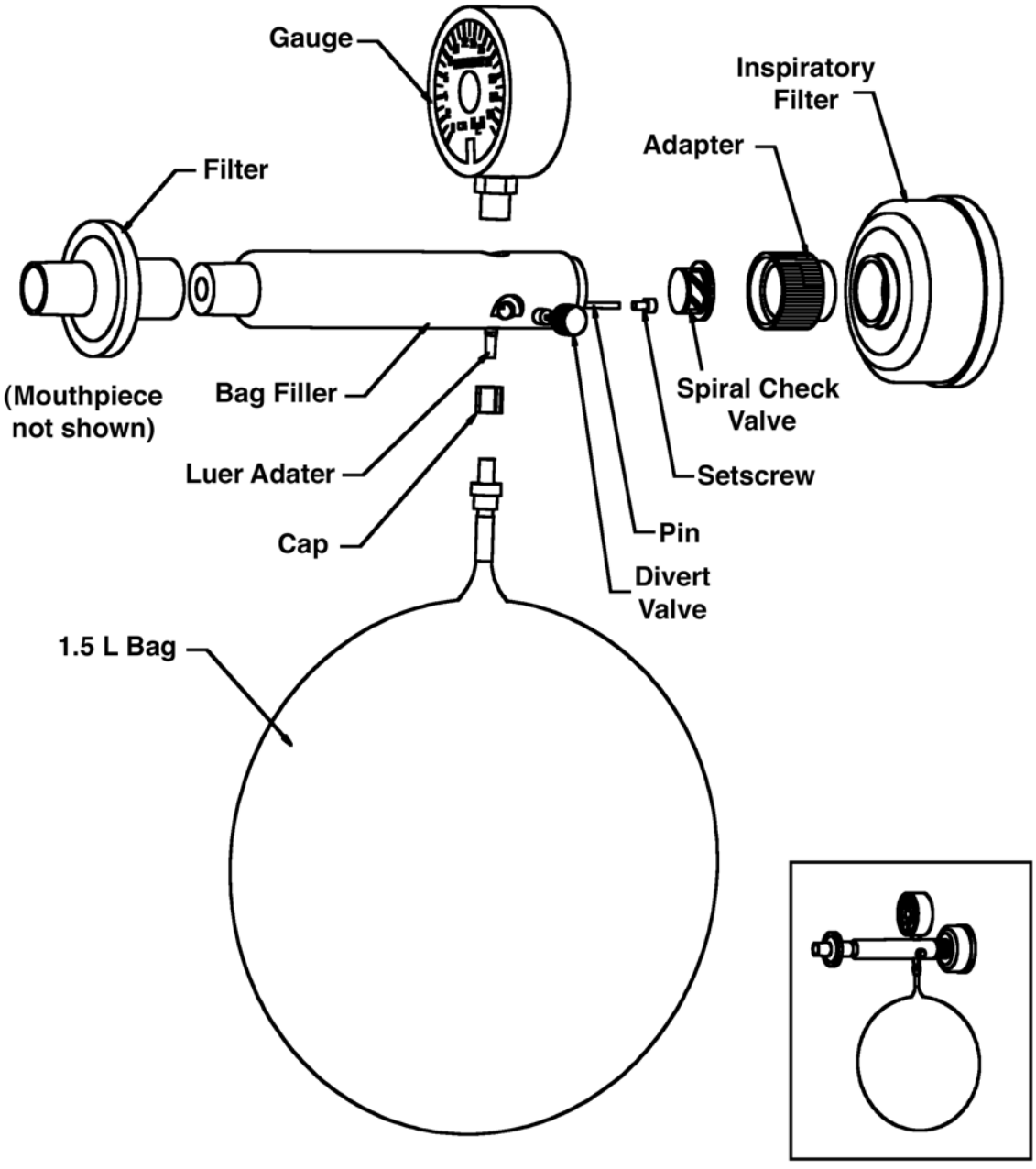


Figure 8- 2: *Diagram of Deadspace Discard Bag Collection Kit.*

a screwdriver until the needle points to 0 cm H_2O. Replace the clear plastic cover of the gauge.

- Connect the Inspiratory Filter to the filler body. The lifetime of the filter will depend on the concentration of nitric oxide in the inspired air and the frequency of use. A reasonable estimate is three months from the date it is installed. Calculate three months from the day the filter is installed and write that expiration date in the space provided on the filter. Screw the filter onto the threaded end of the body. The Bag Kit is now ready for use.

Cleaning the Bags

Mylar bags should be cleaned prior to use and this same procedure can be used to reuse the bags. The vacuum pump on the NOA or another vacuum source is used to withdraw all of the gas from the bag and the bag is then filled with a dry gas containing low levels of NO. Suitable gases include cylinder nitrogen, air or oxygen. House air or oxygen can also be used if the NO level in these gases is < 5 ppb. The gas is again removed using the vacuum pump on the NOA. Usually a single flush with low NO gas is sufficient to remove any NO absorbed on the bag's walls, although multiple flushes with low NO gas can also be performed. The flow rate on the NOA 280i is limited to 200 mL/min by the frit restrictor. This means a full bag (~12 L) will take about 60 minutes to empty. If the frit restrictor is removed and the gas sampling package connected directly to the NOA inlet, the bags can be evacuated in ~ 10 minutes. Alternately, another vacuum source such as house vacuum or a vacuum pump can be used to evacuate the bags.

WARNING:

ALWAYS USE THE VACUUM PROCEDURE TO REMOVE GAS FROM THE BAG. DO NOT ATTEMPT TO REMOVE GAS FROM THE BAGS BY HAND, AS SQUEEZING THE BAGS MAY INTRODUCE PINHOLE LEAKS.

After evacuation, the bags will be under a slight vacuum and when disconnected from the vacuum source, room air will be drawn into the bag. If ambient NO levels are high, this can contaminate the bag. The best procedure is to use a 3-way valve, with one leg

connected to the zero air filter, one leg to the vacuum source and the final leg connected to the bag (Figure 8-3). As outlined below, this setup allows zero air to be drawn into the bag.

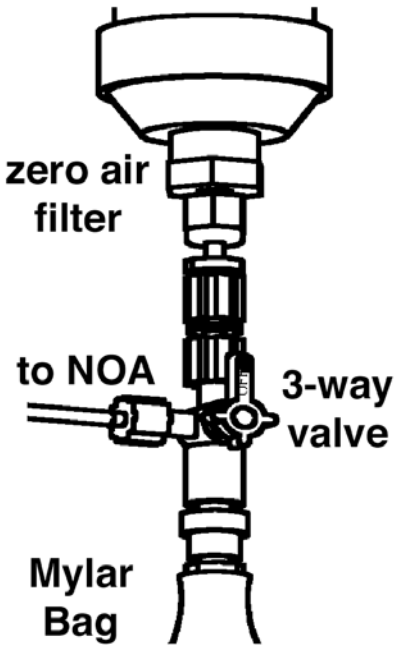


Figure 8-3: *Setup for Cleaning Bags*.

- Remove the frit restrictor from the NOA and connect the Nafion drier directly to the NOA sample inlet. With 3-way valve and zero air filter connected to the NOA, turn the valve so that OFF is positioned toward the Zero Air Filter and allow the NOA to completely evacuate the gas from the bag. Disconnect the bag and repeat for the remaining bags.

- Connect the bag to a source of dry, low NO gas and slowly fill the bag. It is not necessary to completely fill the bag.

- Reconnect the bag to the 3-way valve. Turn the valve so that OFF points toward the zero air filter. Allow the NOA to completely evacuate the contents of the bag.

- After the bag is evacuated, turn the 3-way valve so that OFF points toward the sampling line connected to the NOA. This will allow gas from the zero air filter to be drawn into the Mylar bag and bring the pressure inside the bag back to atmospheric. Disconnect the bag, and seal the bag with the cap. Reposition the 3-way valve so that OFF points toward the Zero Air Filter. Repeat for remaining bags. Store the bags in a clean, dry and secure location. **Reinstall the restrictor before using the NOA for gas analysis.**

Bags cleaned by this procedure will remain low in NO for approximately 24 hours.

Collecting the Samples – Vital Capacity Bag Kit

Sample collection using the Vital Capacity Bag kit consists of three steps:

- Connecting a pre-cleaned bag to the filler.
- Instructing the subject on how to perform the maneuver and collecting the samples
- Disconnecting the bag from the filler and sealing the bag.

Connecting the bag to the filler

To prepare the filler for sample collection:

- Stand the filler on the inspiratory filter.
- Remove the cap from the bag and grasp the cap, the top of the bag and internal tube with one hand while holding the bag filler with the other hand.
- Twist the bag slightly counterclockwise, then insert the Luer fitting on the bag into the Luer fitting on the filler and rotate fully clockwise until the o-ring seals against the base of the bag filler.
- Rotate the bag so that there are no folds or restrictions at the end of the tube.
- Open a new viral and bacterial filter and connect the filter to the tapered end on the top of the filler.
- Connect a sterile mouthpiece to the filter.

Instructing the Subject and Collecting the Samples

To perform the maneuver:

- Have the subject comfortably seated and holding the bag filler level.
- Have the subject place the mouthpiece in their mouth.
- Instruct the subject to inhale orally through the inspiratory filter to total lung capacity.
- Have the subject immediately exhale while watching the pressure gauge and adjusting the force of exhalation to maintain a pressure of 13 cm H_2O to give an

expiratory flow rate of 0.35 L/s.* During the exhalation, the pressure should always be greater than 10.5 cm H_2O (0.315 L/s) but less than 15 cm H_2O (0.385 L/s).

- The subject must continue exhaling to residual capacity while maintaining this pressure.

If ambient NO levels are greater than 20-40 ppb, subjects should breath from a source of low NO air or through the inspiratory filter for 15 s (or a minimum of two tidal breaths) before the collection of the sample.

Make sure the o-ring on the bag is tightly sealed against the filler and that there are no folds or restrictions in the bag.

WARNING:

IF THE FILLER IS NOT HELD LEVEL, THE BAG MAY FOLD AGAINST THE END OF THE INTERNAL TUBE, RESTRICTING THE FLOW OF AIR INTO THE BAG.

Disconnecting the bag from the filler and sealing the bag.

After the subject has completed the maneuver:

- Stand the bag filler on the inspiratory filter.
- Grasp the Luer fitting and tube on the bag, rotate counterclockwise to disconnect the bag from the filler.
- Seal the Mylar Bag with the cap.

Repeat the above procedures to connect another bag to the filler for replicate measurements. Normally, subjects will fill three bags, and the average NO concentrations in these three samples is used for the subject's $FE_{NO,\ 0.35.}$ Subjects should rest a few minutes between exhalations.

* at 760 torr, for other pressures, calculate the required pressure from the equation: Flow = $(760/P_{Bar}\ (torr)) * 79.3 * P_{exh}^{0.5851}$

After the subject has filled the replicate bags, remove the mouthpiece and filter. The filter should be discarded, while the mouthpiece can be reused after sterilization.

Collecting the Samples – Deadspace Discard Bag Kit

Sample collection using the Deadspace Discard Bag kit consists of five steps:

- Connecting a pre-cleaned bag to the filler.
- Instructing the subject on how to perform the maneuver.
- Exhaling to discard the gas in the physiological deadspace.
- Continued exhalation to collect the sample.
- Disconnecting the bag from the filler and sealing the bag.

Connecting the bag to the filler

To prepare the filler for sample collection:

- Stand the filler on the inspiratory filter.
- Push the female Luer adapter on the bag onto the male Luer fitting on the filler and secure the bag by rotating the nut on the filler. The male Luer fitting on the filler must contact and slightly depress the blue piece in the self-sealing valve to allow gas to flow into the bag.
- Pull the valve stem on the filler to the out position (see Figure 8-4).
- Open a new viral and bacterial filter and connect the filter to the tapered end on the top of the filler.
- Connect a sterile mouthpiece to the filter.

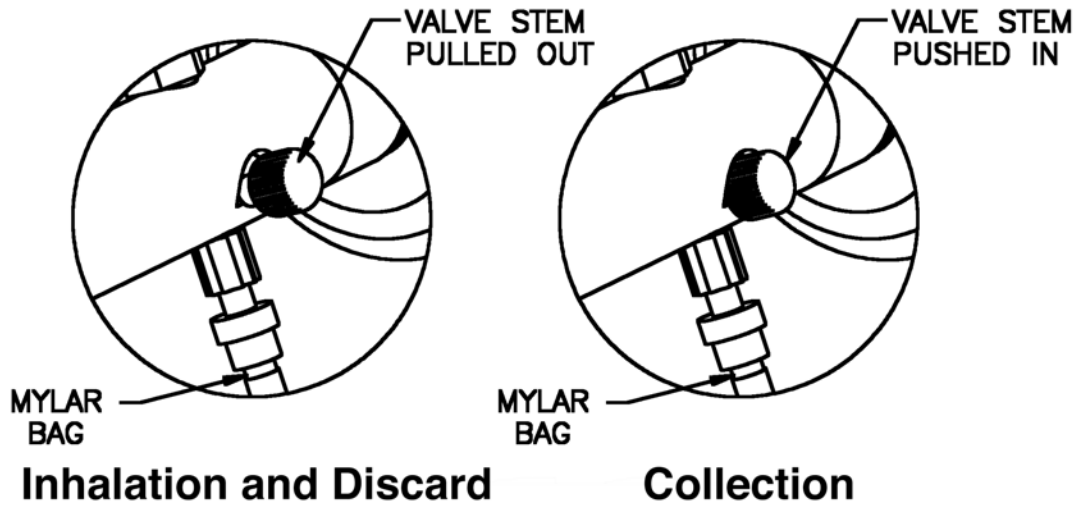


Figure 8-4: *Divert Valve Operation*

Instructing the Subject and Collecting the Samples

- Have the subject comfortably seated and holding the bag filler level.
- Have the subject place the mouthpiece in their mouth and position their hand on the divert valve.
- With the valve pulled out, have the subject inhale orally through the inspiratory filter to total lung capacity.
- Have the subject to immediately exhale while watching the pressure gauge and adjusting the force of exhalation to maintain a pressure of 5 cm H_2O for a flow of 50 mL/s BTSP*
- After exhaling for 4 – 6 seconds, have the subject push the divert valve to the in position to start collecting gas in the bag.
- The subject must continue exhaling, while maintaining a pressure to 5 cm H_2O for 5 to 6 seconds to collect 250 to 300 mL of gas in the bag.

* at 760 torr, for other pressures, calculate the required pressure from the equation: Flow = $(760/P_{Bar}\ (torr)) * 21.1 * P_{exh}^{0.533}$

Disconnecting the bag from the filler and sealing the bag.

After the subject has completed the maneuver:

- Stand the bag filler on the inspiratory filter.
- Loosen the nut on the filler and remove the bag. The self-sealing valve will close and seal the bag. Do not use caps to seal the bags.

Repeat the above procedures to connect another bag to the filler for replicate measurements. Normally, subjects will fill three bags, and the average NO concentrations in these three samples is used for the subject's $FE_{NO,\ 0.05}$. Subjects should rest a few minutes between exhalations.

After the subject has filled the replicate bags, remove the mouthpiece and filter. The filter should be discarded, while the mouthpiece can be reused after sterilization.

Analyzing the Samples

The same procedure is used to analyze samples collected using either the vital capacity or deadspace discard bag kits. The NOAnalysis Bag program is designed for collecting data during analysis of these samples (consult the NOAnalysis manual for instructions). Alternatively, the NOA's front panel display can be used for viewing the concentration of NO in the samples.

Analysis Setup

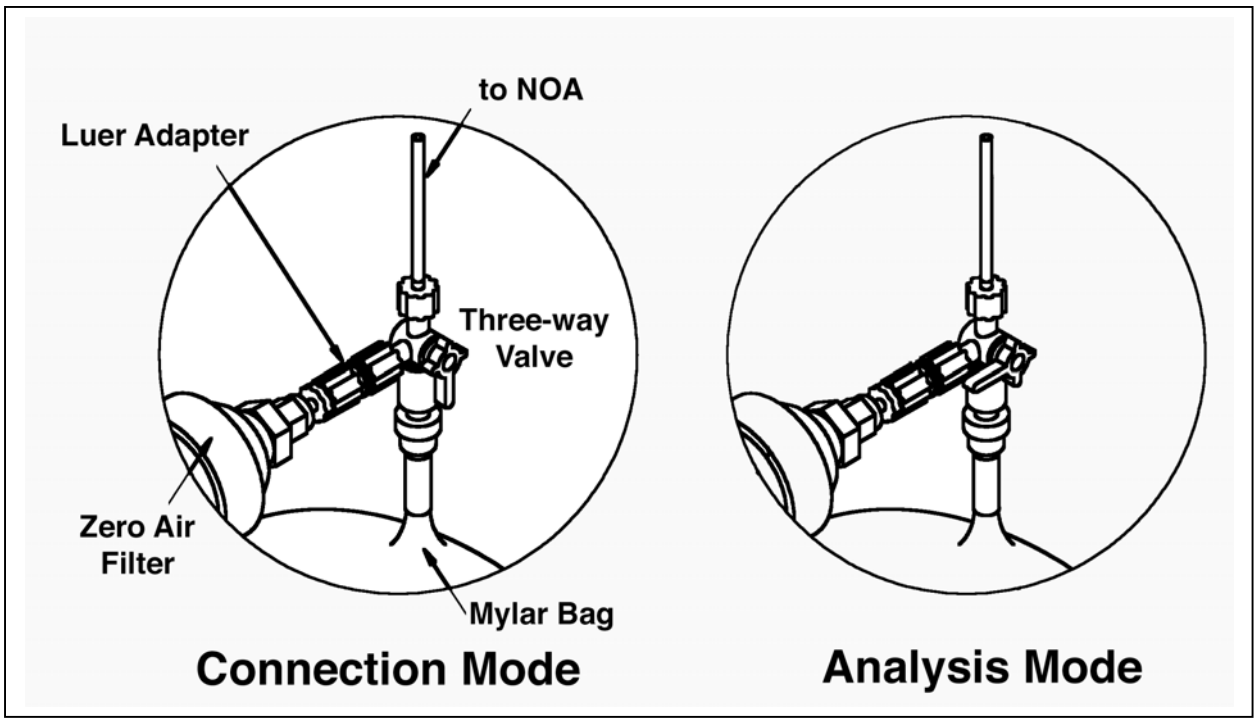
The setup for the analysis of Bag samples is shown in Figure 8-5 and consists of a three-way valve, a male-male Luer adapter, the zero air filter and the gas-sampling package. Connect the Luer adapter to the outlet of the zero air filter and to one leg of the three-way valve. Connect the NO sample line to the other female Luer leg of the three-way valve. The bags will be connected to the male Luer leg of the valve.

NOA Setup

For the off-line NO measurements, the NOA can be in either the Exhalation Mode or the Nitric Oxide mode with the Units set to Concentration. Sensitivity should be set to Auto and the Com Port Interval set to 1/2 or 1/4. See Chapter 5 (INSTALLATION AND SETUP:

GAS-PHASE MEASUREMENTS) for configuring the NOA. The NOA should be calibrated (Chapter 6 – CALIBRATION) prior to measurement.

- Start the measurements with the three-way valve positioned so that OFF points toward the bag or open leg of the valve so that zero air is drawn into the NOA.
- Connect the first bag to the three-way valve making sure that the Luer connection between the valve and bag is secure.
- Turn the three-way valve so that OFF points toward the zero air filter. Gas from the bag is now being drawn into the NOA.
- Allow the NOA to sample gas from the bag for at least 15 seconds.
- Record the NO value from the display or select the Add NO value button in the Bag program.
- Turn the valve back so that OFF points toward the bag.
- Remove the bag from the three-way valve and connect the next sample bag.
- Repeat these steps to analyze the remaining samples.



After the samples have been analyzed, follow the Bag cleaning procedures (page 72) to reused the bags.

Cleaning the Bag Kits

The body of the Bag Sampling Kit, the mouthpiece and spiral check valve(s) should be cold sterilized. Consult your institution's infection control procedure for recommended frequency. **Do not sterilize the bags, pressure gauge or inspiratory gas filter.** The brass fitting on the pressure gauge that screws into the body can be cleaned using an alcohol wipe.

Vital Capacity Bag Kit

Consult Figure 8-1 for disassembly of the bag filler. To disassemble the filler:

- Unscrew the small knurled fitting opposite the pressure gauge, and remove the small spiral check valve.
- Remove the Inspiratory Filter and pressure gauge from the bag filler.
- Unscrew the large knurled fitting and remove the large spiral check valve from the body.

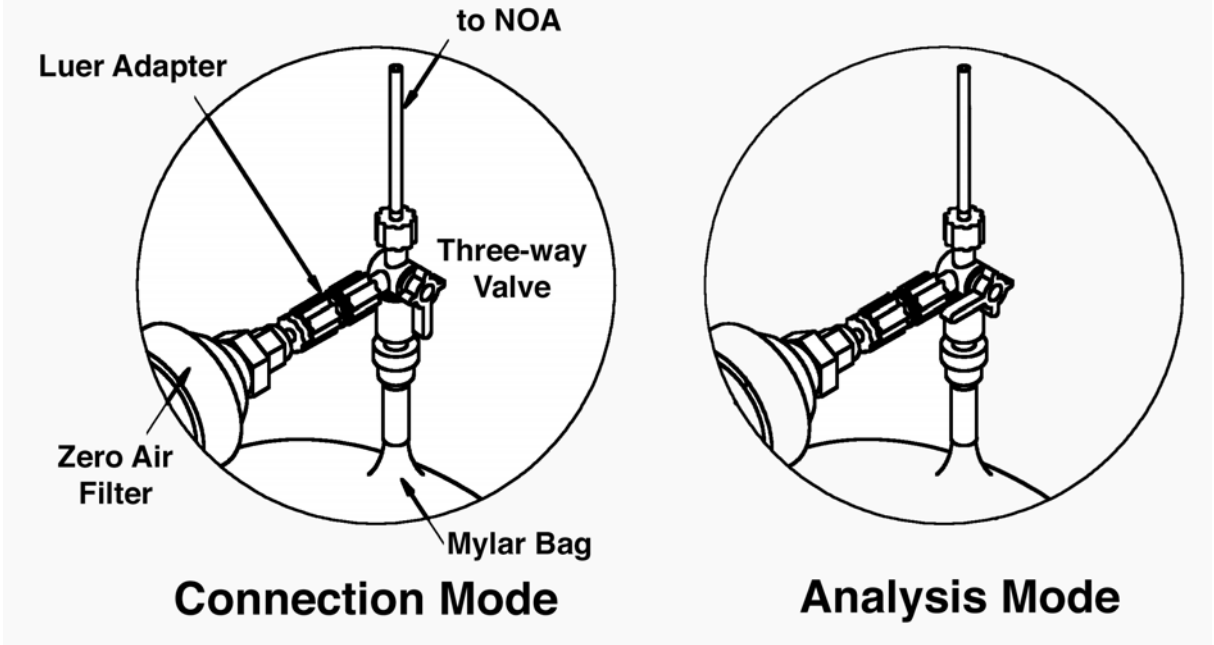


Figure 8-6: *Setup for Analysis of Bag Samples.*

Clean the two knurled fittings, the check valves, and the body using your institution's approved cold sterilization procedure for respiratory equipment.

After sterilization and drying, reassemble the body:

- Insert the large spiral check valve in the threaded end of the body with the spirals facing into the body and the white plastic support flush with end of body.
- Screw the large knurled fitting onto the threaded end of the body.
- Screw the inspiratory filter onto the knurled fitting.
- Screw the pressure gauge into the bag filler body.
- Insert the small spiral check valve into the small knurled fitting with the spirals facing into the fitting.
- Screw the small knurled fitting into the body, opposite the pressure gauge.

Deadspace Discard Bag Kit

Consult Figure 8-2 for disassembly of the bag filler. To disassemble the filler:

- Remove the Inspiratory Filter and pressure gauge from the bag filler.
- Unscrew the large knurled fitting and remove the large spiral check valve from the body. After the valve is removed, the setscrew that secures the divert valve will be visible.
- Use the Allen wrench provided with the kit to remove the setscrew. The pin and divert valve can now be removed from the filler body.

Clean the knurled fitting, check valve, divert valve, pin, setscrew and the body using your institution's approved cold sterilization procedure for respiratory equipment.

After sterilization and drying, reassemble the body:

- Insert the divert valve fully in the body (Discard position in Figure 8-4).
- Use forceps to insert the pin in the hole of the body.
- Place the setscrew on the Allen wrench and screw into the body to secure the divert valve and pin.

- Inserting the large spiral check valve in the threaded end of the body with the spirals facing into the body and the white plastic support flush with end of body.
- Screw the large knurled fitting onto the threaded end of the body.
- Screw the inspiratory filter onto the knurled fitting.
- Screw the pressure gauge into the bag filler body.

Flow/Pressure Characteristics of Bag Kits

The exhalation pressures reported above permit collection of exhaled NO at a flow rate of 350 mL/s for the Vital Capacity and 50 mL/s for the Deadspace Discard bag kits. Figure 8-6 shows the exhalation flow as a function of pressure for the two kits from 5 to 25 cm H_2O along with logarithmic curve fit of the data. For the Vital Capacity bag kit, flows ranging from 200 to 475 mL/s are obtained over this pressure ranges. For the Deadspace Discard kit, flows range from 50 to 125 mL/s for these pressures.

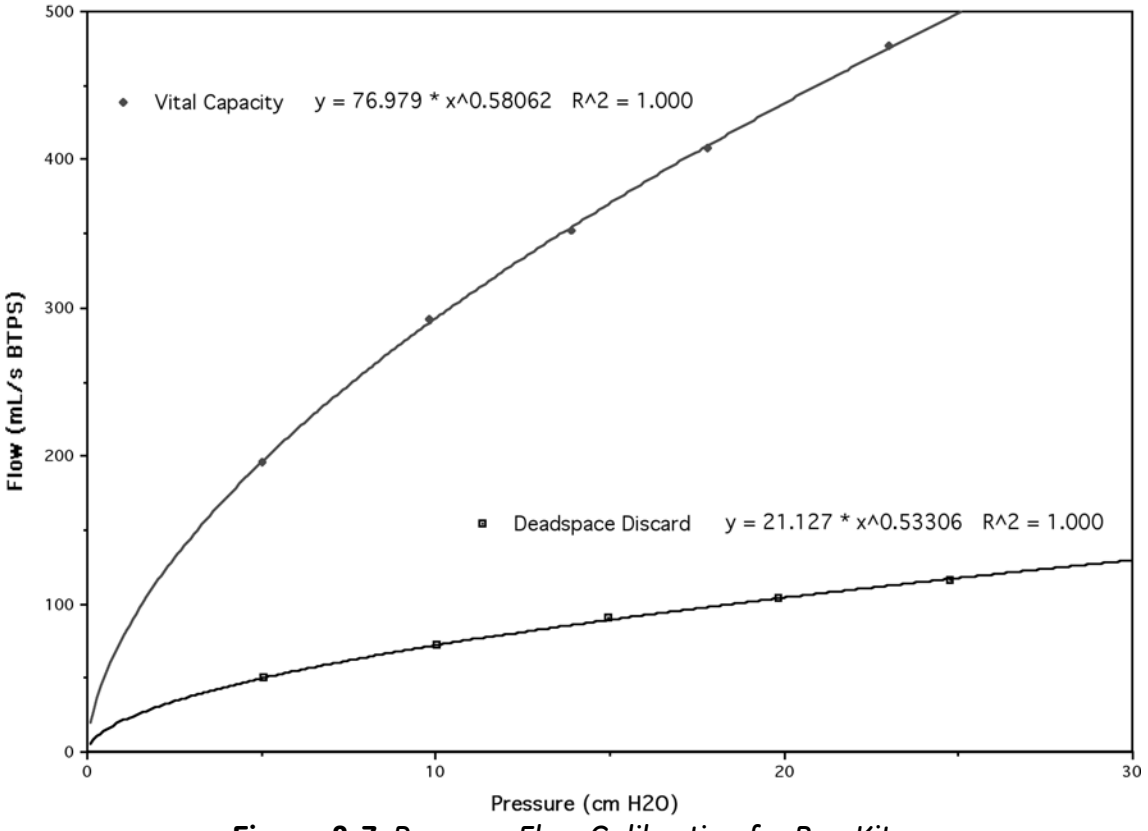


Figure 8-7: *Pressure Flow Calibration for Bag Kits.*

Stability of NO in Mylar Bags

It is best to analyze the samples as soon after collection as possible. In the ATS guidelines it is reported that the concentration of NO in new Mylar bags is stable for up to 48 hours (Massaro *et. al., Am. J. Respir. Crit Care Med* 1995, **152**, 800-803). GE Analytical Instruments testing has shown that NO concentrations slowly increases over time with a small but significant increase (1-2 ppb) observed after 8-12 hours. This increase continues as long as the samples are stored in the bag, reaching ~ 5 ppb after 48 hours.

Examples of the observed increase are shown in Figure 8-7. For these tests, a single subject filled multiple bags (13 Vital Capacity Bags, 6 Deadspace Discard Bags), which were then analyzed over time. In these box plots, the median value (the middle value in an ordered listing of the data) is the center line of the box, the box is the interquartile range (2nd and 3rd quartiles) and includes half of the measurements. The "hinges" are 1.5 times the interquartile range and outliers are identified using asterisks (outside of ± 1.5 times the interquartile range) or open circles (outside of ± 3 times the interquartile range).

There are a couple of reasons for this observed increase. Bags filled with NO-free air show a small increase (~1 ppb in 24 hours), which suggests that NO or some other chemical that undergoes a chemiluminescent reaction with ozone is coming from the bags. Bags containing exhaled breath show an additional increase due conversion of nitrite or some other component of exhaled breath or breath condensate to NO.

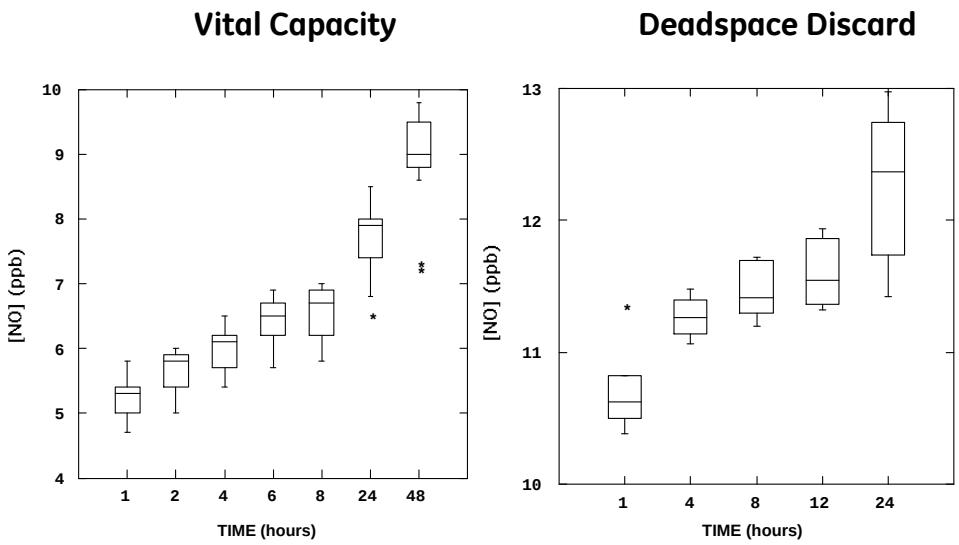


Figure 8- 8: *Box Plots showing Stability on NO in Bags.*

One way to monitor possible increases in the concentration of NO in the bags, particularly when there is a long time between collection and analysis of samples is to use a control. Collect exhaled breath from a subject and analyze the bag immediately after collection. Save this bag and then re-analyze the control along with the samples. The control will thus provide a measure of how much of a change in NO might be expected.

Off-Line versus On-Line Exhaled NO Measurements

When the Deadspace Discard Bag Kit is used to collect exhaled NO at an expiratory flow rate of 50 mL/s, there is no significant difference between off-line and on-line exhaled NO measurements. When the Vital Capacity Bag Kit is used, an expiratory flow rate of 350 mL/s gives a significantly lower exhaled NO value than on-line measurements at 50 mL/s. A linear relationship between off-line and on-line measurements has been reported (see Silkoff, *et al.*, "A Method for the Standardized Offline Collection of Exhaled Nitric Oxide." *Chest* 1999; 116:754-759 and Canady, *et al.*, "Vital Capacity Reservoir and Online Measurement of Childhood Nitrosopnea Are Linearly Related." *Am J Respir Crit Care Med*. 1998; 159:311-314), but the values are not interchangeable.

Testing Bags for Pinhole Leaks

Mylar bags have been reused over 50 times without problems. The most common problem in reusing the bags is the development of pinhole leaks usually as a result of emptying the bags by hand or rough handling. This can be avoided by always using the vacuum technique to empty the bags and gently handling the bags. The recommended procedure to test the integrity of the bags is to fill the bags with varying concentrations of NO, such as exhaled breath from different individuals, and measuring the NO concentration in the bags over a period of several hours while also measuring the ambient NO concentration.

WARNING:

DO NOT FILL THE BAGS WITH PPM LEVELS OF NO FOR THESE TESTS. HIGH CONCENTRATIONS OF NO WILL ABSORB ON THE WALLS OF THE BAGS AND IS DIFFICULT TO REMOVE.

If there are leaks in the bag, ambient air will diffuse into the bag, resulting in a change in the concentration of NO over time. If ambient NO is higher than the NO concentration in the bags, an increase in NO (above the small increase normally observed with the bags) will be detected. If ambient NO is lower than the NO concentration in the bag a decrease in NO can be observed. Discard any bags that show large changes in NO concentration.

9 BREATH-BY-BREATH AND CHAMBER SAMPLING FOR EXHALED NITRIC OXIDE

Breath-by-breath measurement of exhaled nitric oxide is only used in those situations where the subject cannot perform the on-line or off-line maneuvers (*e.g.*, children) and for ventilated subjects. For spontaneously breathing subjects, this technique provides no control of expiratory flow rate and for primates, contamination from nasal nitric oxide may occur.

The chamber sampling technique is a simple and rapid method for measuring exhaled NO from small animals (*e.g.*, rats and mice) using a head-out, double chamber plethysmograph. The head chamber is flushed with NO-free air, then sealed for a few seconds to allow exhaled breath to accumulate, the contents of the chamber are then flushed into the NOA for measurement.

Breath-by-Breath Measurements

Spontaneously Breathing Subjects

A face mask and non-rebreathing valve equipped with female Luer sampling ports is used for the measurements. The NOA's NO sampling and pressure lines are connected to the ports or a Luer tee is used to connect the lines if the mask has a single Luer port. Ambient air or a source of low NO air can be used for the inspiratory gas and the sampling port must be positioned to allow alternate sampling of inspired

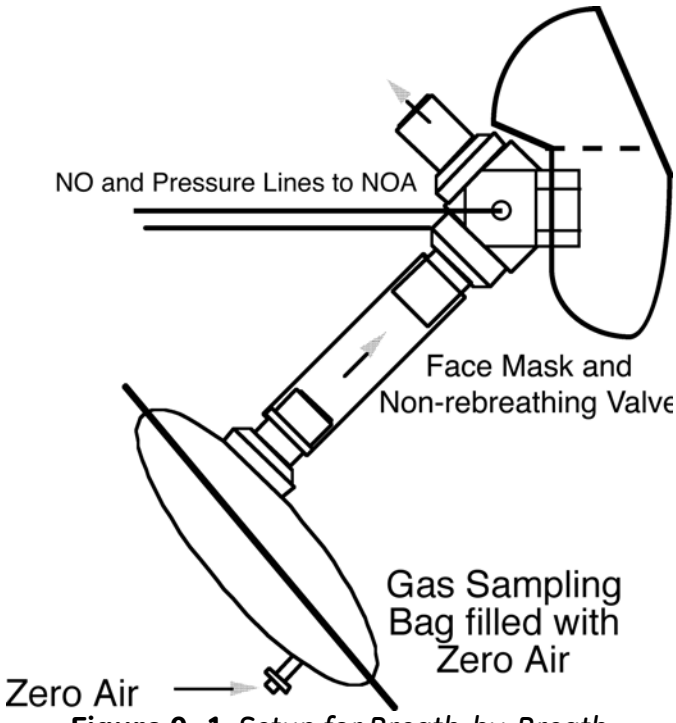


Figure 9- 1: *Setup for Breath-by-Breath Sampling (spontaneously breathing subject).*

and expired gas. The NOA exhalation pressure transducer is used to determine the beginning and end of exhalation using the NOAnalysis Breath program.

Ventilated Subjects

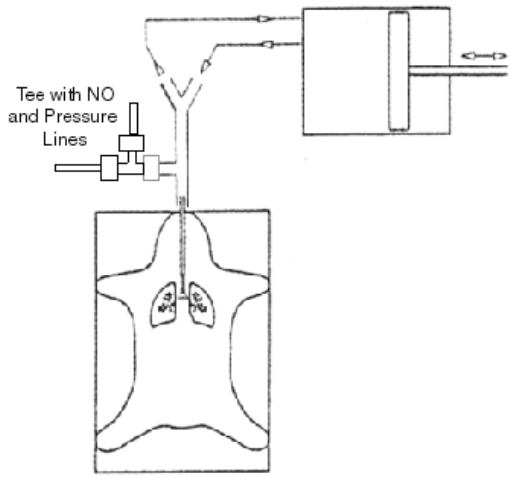


Figure 9- 2: *Setup for Breath-by-Breath Measurement on Ventilated*

An ET tube adapter equipped with a female Luer sampling port is installed between the ventilator Y-piece and the ET tube. For small animals, an adapter with a female Luer port is positioned between the ventilator Y-piece and the tracheal cannula. A three-way Luer stopcock is suitable and provides a means to isolate the sampling port while continuing to ventilate the animal. A Luer tee is used to connect the NO sampling and pressure lines to the circuit. Since the NOA using the standard frit restrictor is drawing 200 mL/min of gas from the circuit, the ventilator must be set so that the product of minute ventilation times tidal volume is greater than 200 mL/min. Lower flow frit restrictors can be used if the ventilator cannot be adjusted to provide > 200 mL/min of gas.

The inspired gas must be low in nitric oxide, preferably <1 ppb. In most cases, ambient air cannot be used and hospital air may contain high levels of NO. The best sources are compressed gas cylinders of air or oxygen that are known to be low in NO. In some cases, the gas can be connected directly to the ventilator or for piston-type ventilators, a reservoir can be filled with low NO gas and connected to the inlet of the ventilator.

The NOAnalysis Breath program detects the beginning and end of exhalations based on changes in the exhalation pressure and reports maximum, minimum and end-tidal NO values for each breath.

NOA Setup

For the breath-by-breath NO measurements, the NOA must be in the Exhalation Mode, sensitivity should be set to Auto and the Com Port Interval set to 1/16 or 1/32. For subjects with high respiratory rates, such as rats and guinea pigs, an interval of 1/32 should be used. See Chapter 5 (INSTALLATION AND SETUP: GAS-PHASE MEASUREMENTS)for configuring the NOA. The NOA should be calibrated (Chapter 6 – CALIBRATION) prior to measurement.

NO/Pressure Offset

The pressure signal travels down the NOA's pressure line at the speed of sound, while the NO signal travels at a slower speed. It is necessary to measure this offset in the signals to align the NO and pressure signals. This is done using the NOAnalysis Breath program and an NO//Pressure Offset Calibration syringe (AAK 01410). The NO and Pressure lines are connected to the syringe, the syringe is filled with NO calibration gas, pressurized and then injected. The program then determines the difference (~1 second) between the NO and pressure signals.

Humidified Circuits

When the NOA is used to measure exhaled NO in humidified circuits, water will condense in the NO sample line and can block the Teflon filter, restricting flow into the analyzer. This can be avoided by disconnecting the NO sampling line from the circuit and letting the NOA sample room air, drying the sampling lines and filters or by installing an additional Nafion drier (AND 00010A) at the connection to the circuit.

The NOA's cell pressure can be used to monitor possible restriction in the sampling line due to condensations. Normally, the cell pressure is 4-7 torr and will drop to < 4 torr if the filter is blocked by liquid water. The Nafion drier after the filter eliminates any problems in the sensitivity of the NOA due to water vapor in the circuit.

Chamber Sampling

Chamber sampling can be used to measure exhaled NO from small animals such as mice, rats and guinea pigs. The setup for chamber sampling is shown in Figure 9-3 and consists of:

- A head-out, double chamber plethysmograph
- A supply of low NO air
- Two three-way Luer stopcocks
- 1/8" Teflon tubing with male Luer fittings

A tee is installed on the zero air inlet to the chamber. The flow rate of zero air is adjusted so that a small excess of gas exits the open leg of the tee and the vacuum pump on the NOA draws the gas through the chamber.

The a head-out, double chamber plethysmograph is preferred to a single chamber system as the head-out configuration helps to minimize NO from urine and feces. The measurement consists of four steps:

- The animal is placed in the chamber.
- The stopcocks are positioned to flush the head-out chamber with low NO air.

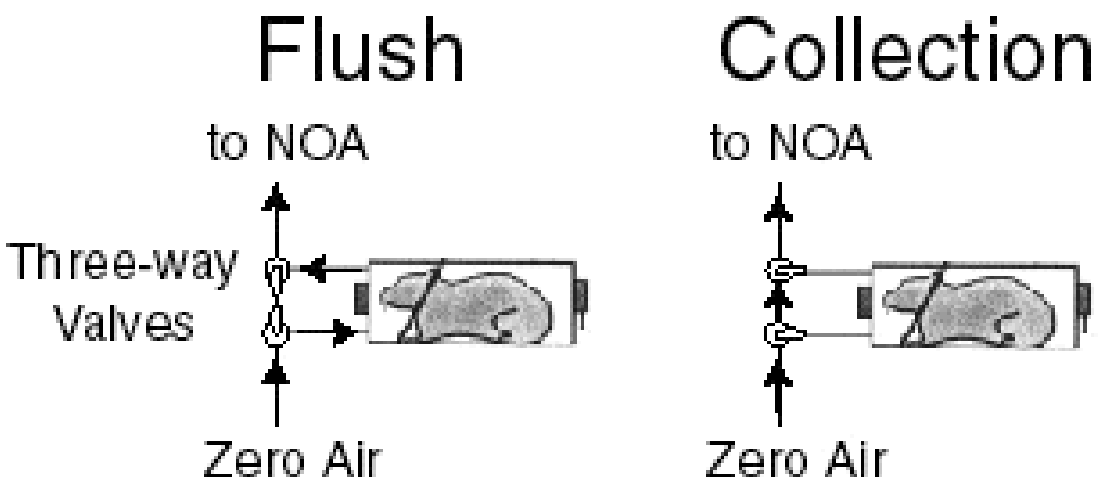


Figure 9- 3: *Chamber Sampling Setup.*

- The stopcocks are positioned to seal the chamber for short period of time (typically 5-15 seconds depending on the size of the head-out chamber). During this time, zero air flows directly to the NOA.
- The stopcocks are repositioned to flush the accumulated NO to the NOA.

Replicate measurements can be performed by repeating these steps while the animal is in the chamber.

Since the chamber will fill with ambient air when the animal is inserted or removed from the chamber, sufficient time to thoroughly flush the chamber before collection is required. The NOA can be used to determine when all of the NO has been removed from the chamber.

The duration of the collection period will depend on: the size of the chamber, the size of the animal and the tidal volume/respiratory rate of the animal. Since the chamber is sealed, the animal can become hypoxic if the collection period is too long. The best procedure is to start with a short collection period to see if enough NO accumulates to give a significant increase above the background NO and increase the period if not enough NO accumulates.

The NOAnalysis Bag program can be used for collecting data during analysis of the chamber sampling (consult the NOAnalysis manual for instructions). Alternatively, the

NOA's front panel display can be used for viewing the concentration of NO in the samples.

NOA Setup

For the off-line NO measurements, the NOA can be in either the Exhalation Mode or the Nitric Oxide mode with the Units set to Concentration. Sensitivity should be set to Auto and the Com Port Interval set to 1/2 or 1/4 second. See Chapter 5 (INSTALLATION AND SETUP: GAS-PHASE MEASUREMENTS) for configuring the NOA. The NOA should be calibrated (Chapter 6 – CALIBRATION) prior to measurement.

10 *NASAL NITRIC OXIDE*

The concentration of nitric oxide in the nasal cavities, paranasal sinuses, and other cavities is measured using a trans-nasal flow of low NO air while the subject performs an oral exhalation against resistance to elevate the soft palette, eliminating air from the lower airways. With the NOA 280i, this is performed using an external vacuum pump with a flow control device, a nasal olive, and a sampling line equipped with a tee for sidestream sampling by the NOA

The American Thoracic Society has published "Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide in Adults and Children-1999" (*Am J Respir Crit Care Med* Vol 160. pp 2104–2117, 1999).

The recommended procedure uses two nasal olives with a central lumen that are securely placed in the nares. The subject inserts a mouthpiece with expiratory resistance, inhales to total lung capacity, and then orally exhales while maintaining a mouth pressure of 10 cm H_2O. While the oral exhalation is occurring, air is aspirated at a flow rate of 3 L/min via one of the nasal olives while a supply of low NO air is provided via the other olive.

An NO plateau should be achieved under these conditions within ~20 seconds If an NO plateaus is not obtained at 3 L/min, higher flows (3–6 L/min) can be used. The transnasal flow used for the collection should be recorded with each nasal NO measurement.

Recommended Setup

The setup for nasal nitric oxide measurements consists of:

- A vacuum source capable of drawing 3-6 L/min.
- A rotometer or other flow control device for regulating the flow into the vacuum source.
- Tubing to connect the vacuum source.

- A Luer tee for connection of the NOA's gas sampling line.
- A nasal olive equipped with tubing to connect to the Luer tee.
- A second nasal olive connected to a supply of low NO air.
- The Accurate NO Breath Kit or another device such as a balloon for elevating the soft palette.

Suitable vacuum sources include house vacuum or vacuum pump capable of drawing gas at the required flow rates. Flow into the pump must be controlled using some type of flow control device. Rotometers are available from a number of sources (*e.g.* Aalborg Instruments & Controls, Inc., Alltech Associates, Inc., Supelco Inc.) and provide for both flow control and measurement. Needle valves and other flow controllers can be used, but require some flow measurement system for adjusting the flow.

The nasal olives should be composed of a soft, non-traumatizing material. Soft rubber one-hole stoppers have also been used in place of nasal olives.

Performing the Maneuver

- Connect the vacuum line to one leg of the Luer tee, connect the NOA's gas sampling line to another leg and the line to the nasal olive the final leg of the tee.
- Adjust the flow rate into the vacuum source to give 3 L/min.
- Connect a low NO air source to the second nasal olive and adjust the gas flow to ~3 L/min.
- With the subject comfortably seated, insert the nasal olives, making sure flow to the vacuum source is maintained (olive not sealed against the skin).
- If the Accurate NO breath kit is used for expiratory resistance, connect the Pressure line to one of the Luer port on the mouthpiece and use a Luer cap to seal the other port. The oral exhalation flow rate is not important. The 50 mL/s restrictor is suitable for most subjects. ATS recommends a target pressure of 10 cm H_2O to ensure vellum closure.

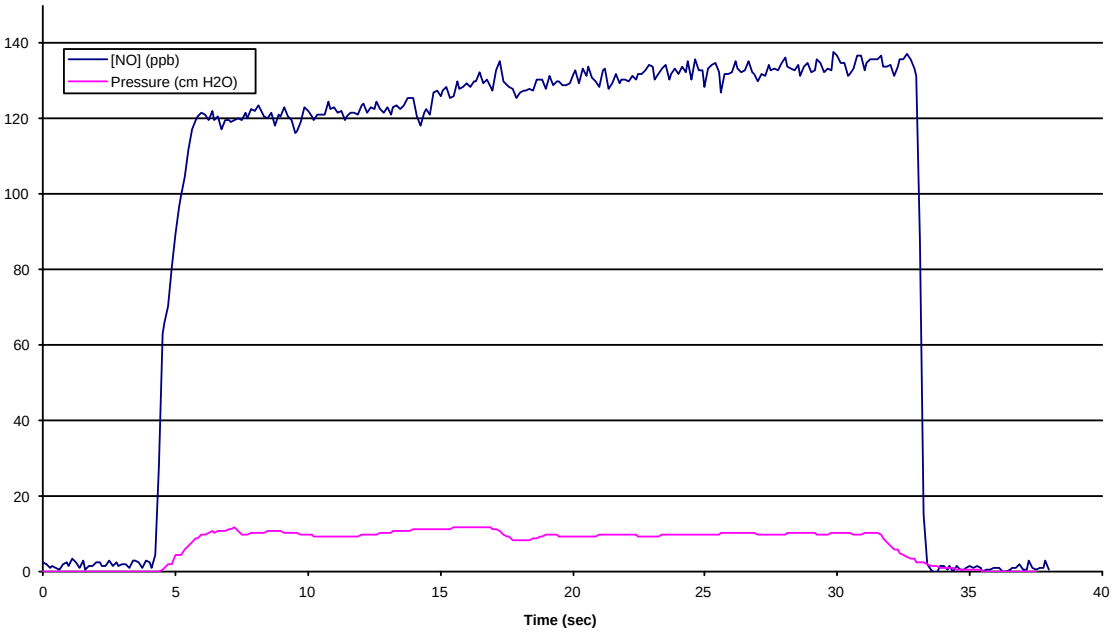


Figure 10-1: *Nasal NO Plateau.*

- Have the subject inhale orally to total lung capacity and exhale orally while maintaining a mouth pressure of 10 cm H_2O. The NOA's signal will slowly increase as air is drawn through the nose, reaching a plateau within a few seconds (Figure 10-2).
- Allow the subject to rest between replicate measurements. The ATS recommendations do not specify how many replicates should be performed or what repeatability should be achieved.

NOA Setup

For the nasal NO measurements, the NOA should be in the Exhalation Mode with the Sensitivity should be set to Auto and the Com Port Interval set to 1/16 or 1/8 second. See Chapter 5 (INSTALLATION AND SETUP: GAS-PHASE MEASUREMENTS) for configuring the NOA. The NOA should be calibrated (Chapter 6 – CALIBRATION) prior to measurement.

The NOAnalysis REB or Bag program can be used for data acquisition. If the Bag program is used, the subject must watch the NOA's front panel to maintain the target

exhalation pressure of 10 cm H_2O. See the NOAnalysis manual for operation of these programs.

11 *INSTALLATION AND SETUP: LIQUID MEASUREMENTS*

Measurement of nitric oxide and its reaction products in liquid samples is performed using a purge vessel. An inert gas bubbles through chemical reducing agents, to convert oxidation products back to nitric oxide. Samples are injected through a septum into the purge vessel and the inert gas carries the NO over to the NOA for detection. Calibration is performed by injection of standard solutions of the analyte.

Supplies

The following items are required to perform liquid measurements:

Gases

A cylinder of Helium, Nitrogen or Argon equipped with a two-stage regulator is required for the purge vessel. The outlet pressure of the regulator is < 5 psi, typically 1-2 psi.

Reagents

Deionized water containing < 1 µM nitrite/nitrate.

Nitrite – Glacial acetic acid, sodium or potassium iodide, and sodium or potassium nitrite.

Nitrate – Concentrated hydrochloric acid or 1 M HCl, vanadium (III) chloride, sodium or potassium nitrate, 1 M sodium hydroxide, sodium hydroxide pellets or 50% $NaOH_{(aq)}$.

Nitrosothiols – Thiols (glutathione, N-acetyl cysteine, albunin, hemoglobin)
PBS, Cu(I)Cl, cysteine, or I_2, CuSO4, $HgCl_2$, reagents for nitrite

Iron-bound NO – KCN, K_3FeCN_6

Lab Equipment

- A small ring stand and clamps will be required to support the glass purge vessel.
- Two, 3-finger clamps work well for holding the purge vessel and gas bubbler.

- Volumetric flasks (10 mL) and pipettes (100 and 1000 µL) are useful for preparing the standards and reagents.
- Syringes are required for injecting the samples into the purge vessel. Microliter syringes (10 µL, 25 µL, 100 µL) with beveled needles are particularly useful.
- Gas-tight syringes are required for headspace analysis. Recommended sizes for gas-tight syringes are 125 µL, 1 mL and 5 mL.

Circulating Water Bath for Nitrate Reduction –A circulating water bath capable of delivering >90°C water is required. Cooling water (<15 °C) is also required. At some locations, tap water can be used as the cooling water. At other locations, a second circulating water bath or ice bath and peristaltic pump is needed if the tap water is not cold.

Setup of Purge Vessel

The Purge Vessel consists of five components:

- Gas inlet line (ASM 00430)
- Purge vessel with heating jacket and condenser (ASM 03292)
- NaOH gas bubbler trap with Teflon sleeve (ASM 04000)
- Connection tubing from the purge vessel to the gas bubbler (ASM 00440)
- Outlet tubing with IFD filter (ASM 00450)

There are two different configurations of the purge vessel depending on whether measuring nitrite only, using iodide and acetic acid, or measuring nitrate and nitrite, using vanadium (III) and hydrochloric acid. Acetic acid is less corrosive than hydrochloric acid and, therefore, it is not necessary to use the gas bubbler/NaOH trap when measuring nitrite. The gas bubbler/NaOH trap must be used for nitrate measurement. Nitrite reduction is performed at room temperature and a circulating water bath is not required. Nitrate reduction is performed at> 90° C and requires a circulating water bath and cold water for the condenser.

- Support the purge vessel on a ring stand using a three-finger jaw clamp to secure the middle of the purge vessel. The side of the clamp with a single finger fits in the gap between the body of the purge vessel and the needle valve. The purge vessel should be high enough that a small beaker can be placed under the drain stopcock.

- The gas bubbler-NaOH trap consists of three pieces with a ground-glass joint. Install the Teflon sleeve in the base of trap, insert the top piece and secure with the plastic clamp. Support the gas bubbler-NaOH trap on the same ring stand using a clamp.

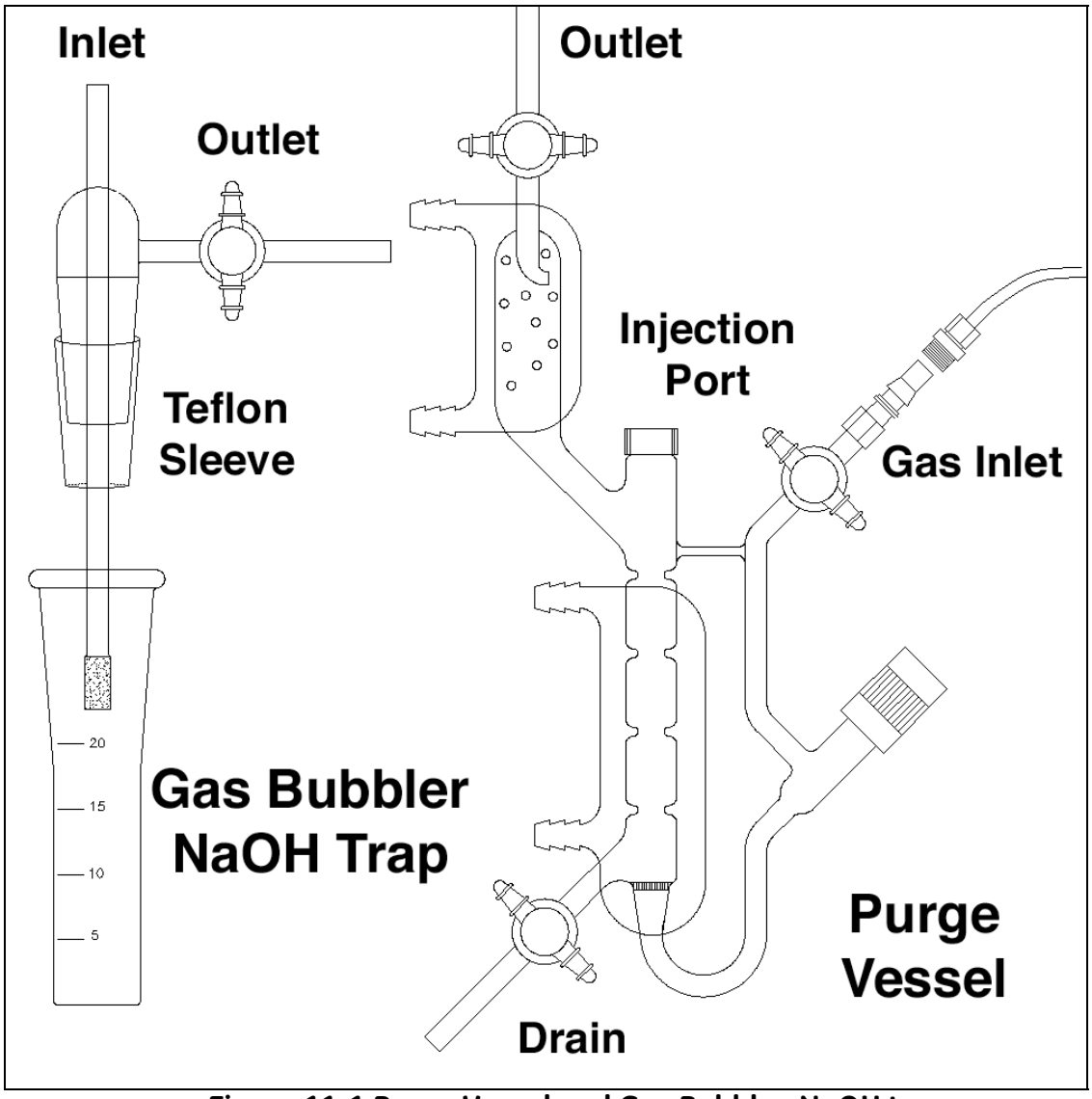


Figure 11-1:Purge Vessel and Gas Bubbler-NaOH trap.

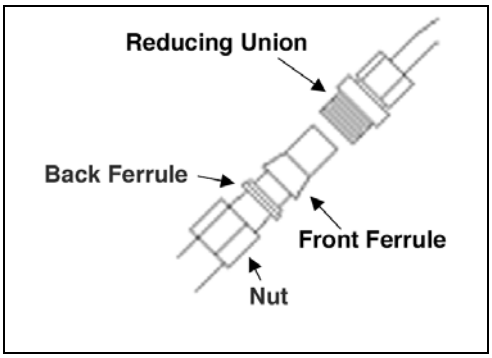


Figure 11-2: Connections to Glassware

Connections of tubing to glassware

The connections to the purge vessel are made using Swagelok® brand connectors. The connection consist of four parts (see Figure 11-2):

- A 1/4" stainless steel nut
- A 1/4" Teflon back ferrule (HTF 24034)
- 1/4" Teflon front ferrule (HTF 24033)
- 1/4" to 1/8" reducing union (HTF 44200)

Three different 1/8" Teflon lines are used with the purge vessel:

- Purge Line (ASM 00430) – a 6 ft length of 1/8" Teflon tubing with a 1/8" to 1/4" SS reducing union on one end and an 1/8" nut and ferrules on the other end.
- Bubbler Line (ASM 00440) – a 1 ft length Teflon tubing with 1/8" to 1/4" SS reducing unions on both ends.
- Filter Line (ASM 00450) – a 3 ft length of Teflon tubing with an in-line filter, a 1/4" nut and Teflon ferrules on one end and an 1/8" nut and ferrules on the other end.

Install the Purge Line by loosening the 1/4" nut and carefully sliding the 1/4" nut and reducing union containing the Teflon ferrules over the 1/4" OD glass on the gas inlet of the purge vessel (see Figure 11-2). In some cases, the OD of the glass is slightly larger than 1/4" and it may necessary to disassemble the 1/4" nut and ferrules and slip the nut and ferrules over the glass on the purge vessel.

Procedure for Tightening Swagelok Fittings

- Grasp the reducing union with one hand.
- Carefully screw the 1/4" nut onto the reducing union with the other hand until finger tight
- When the fitting is loose, the reducing union can be easily rotated using two fingers. When the fitting is tight, the union cannot be easily rotated with two fingers. Since the Teflon ferrules will slide on the glass, the union can be rotated when the fitting is tight, but some force will be required to turn the union.
- When tightening the fittings, hold the union in-line with the glass. If torque is applied to the union or the union is misaligned, the end of the glass may break.

WARNING:

DO NOT USE WRENCHES TO TIGHTEN THE FITTINGS.

OVERTIGHTENING THE NUT AND FERRULES WILL BREAK THE GLASS!

For nitrate measurements, repeat this procedure to attach the Bubbler line to the outlet of the purge vessel to the top inlet of the NaOH trap.

To prevent liquid from entering the chemiluminescence reaction cell, a polypropylene filter disk (IFD) is installed on the outlet of the purge system. One side of the IFD has two ports, a center port for the gas inlet and a side port to permit removal of liquid from the filter. The side with two ports faces the purge vessel. Install the 1/8" Teflon line with the IFD filter to the side port outlet of the NaOH trap for nitrate, or the outlet of the purge vessel for nitrite and tighten the nut fingertight.

Connect the 1/8" Teflon tubing from the outlet of the IFD to the inlet of the frit restrictor on the Sample Inlet of the NOA and tighten fingertight.

The purge vessel requires an inert gas supply (helium, nitrogen, or argon) from a gas cylinder. The cylinder must be equipped with a two-stage regulator, a shutoff valve, and an adapter for connection of a 1/8" Swagelok nut. A 1/4" female NPT to 1/8" Swagelok union is provided with the analyzer and permits connection to most shutoff valves. Wrap Teflon tape on the outlet of the shutoff valve to prevent leaks on the NPT connection.

Open the main valve on the gas cylinder and adjust the regulator to give 1-2 psi on the outlet of the regulator. Do not open the shutoff valve. The procedures for adjusting the gas flow into the purge vessel are in Chapters 12 – 14.

For nitrate measurements, a cold water supply is connected to the barbed fittings on the condenser of the purge vessel and a hot water supply is connected to the barbed fittings on the heating jacket of the purge vessel (Figure 11-3).

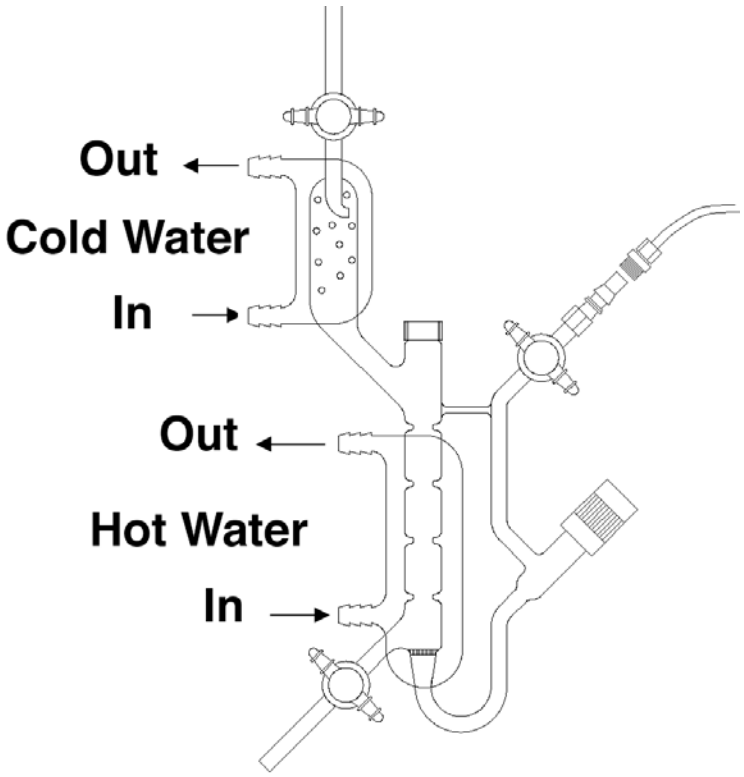


Figure 11-3: *Connections for Hot and Cold Water for Nitrate Reduction.*

To secure the tubing to the purge vessel, hose clamps are provided with the purge vessel. In most locations, cold tap water can be used for the condenser. Connect a length of Tygon or rubber tubing to a barbed fitting on a faucet, place a hose clamp over the end of the tubing and connect the tubing to the bottom barbed fitting on the condenser. Wetting the barbed fitting on the condenser with water will aid in sliding the tubing over the fitting. Slide the tubing over as much of the barbed fitting as possible. Once the tubing is installed, carefully tighten the hose clamp to secure the tubing. Do not overtighten the hose clamp; the glass will break.

Connect a second piece of tubing to the barbed fitting on the top of the condenser, secure the tubing with a hose clamp, and place the other end of the tubing in a drain.

The hot water supply for nitrate measurement must be supplied from a circulating water bath capable of heating to ~95 °C. Use the silicon tubing provided with the purge vessel to make the connections between the water bath and the barbed fittings on the purge vessel. Tygon tubing expands when heated and is not recommended. Connect a piece of silicon tubing from the outlet of the water bath, place a hose clamp over the tubing and connect the tubing to the bottom barbed fitting on the purge vessel heating jacket. Wetting the barbed fitting on the condenser with water will aid in sliding the tubing over the fitting. Push the tubing on the barbed fitting as far as possible and secure the tubing with a hose clamp. Connect a second piece of silicon tubing to the top barbed fitting on the heating jacket, pushing the tubing as far as possible onto the barbed fitting and securing with a hose clamp. Connect the other end of the tubing to the inlet fitting on the water bath.

This completes the installation of the Purge Vessel. Procedures for preparing the chemical reducing agents, adjusting the purge gas flow rate and analysis of samples are in Chapters 12 – 14.

Dilution of Anti-foaming Agent

Included with the purge vessel is a small vial containing a silicon emulsion, anti-foaming agent. This material must be diluted 1:30 with deionized water and 100 µL of the diluted anti-foaming agent is always added to the purge vessel to minimize foaming from protein the samples. To dilute the anti-foaming agent:

- Vortex or thoroughly mix the concentrated anti-foaming agent.

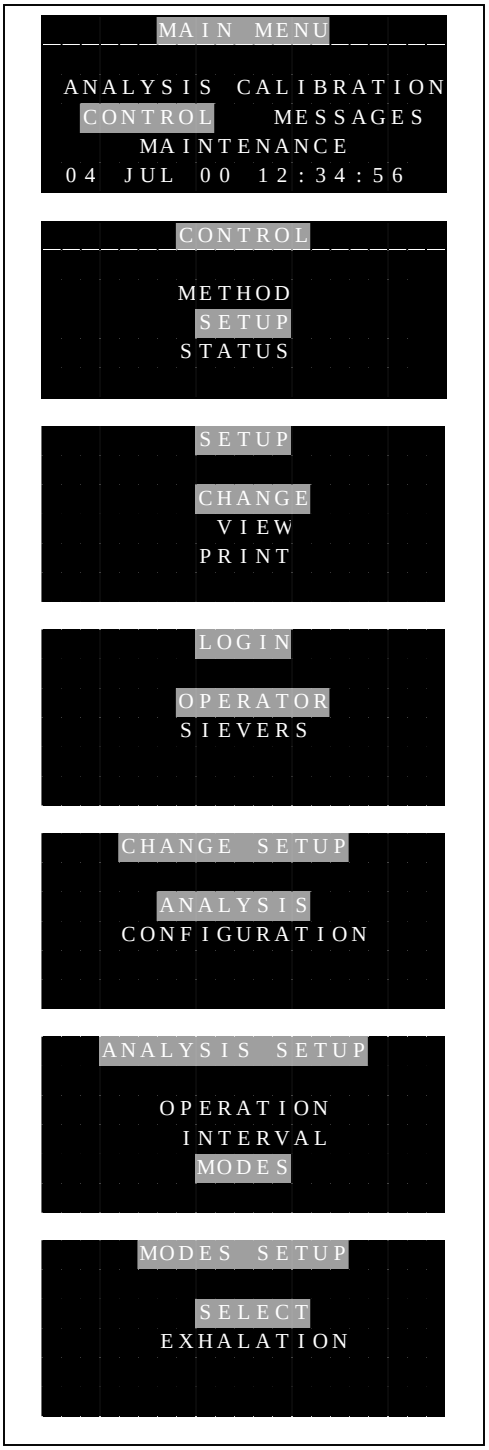
- Add 3 mL of deionized water to a vial, falcon tube or other container.
- Use a pipette to withdraw 100 µL of the antifoaming agent. Since the material is very viscous, allow sufficient time for the agent to be drawn into the pipette.
- Add the agent to the water, rinsing the pipette tip with water to completely transfer the agent.

The diluted anti-foaming agent is stable for several months at room temperature. The emulsion will separate from the water and the diluted anti-foaming agent should be thoroughly mixed before use.

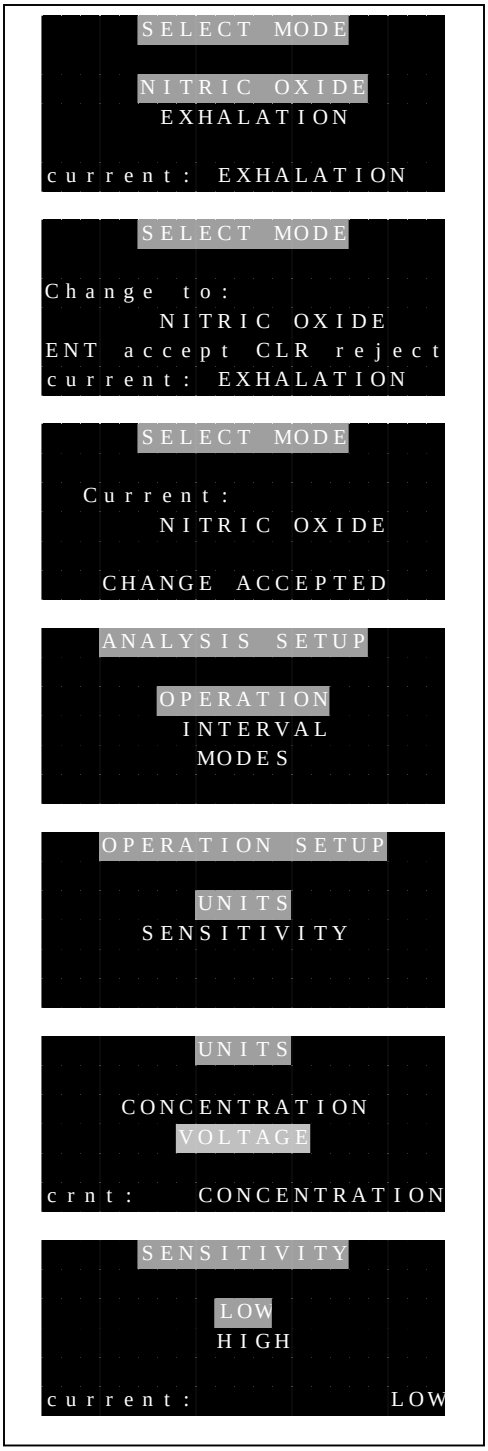
NOA Setup for Liquid Measurements

For liquid measurements the NOA is operated in the Nitric Oxide Mode with Units set to Voltage. Sensitivity is normally set to High, but for samples containing >100 µM nitrite or > 250 µM nitrate, the sensitivity can be set to Low or the sample diluted. To set to the NOA to the Nitric Oxide mode (press ENTER to select Menu Option):

- From the Main Menu, select Control.
- From the Control Menu, select Setup.
- From the Setup Menu, select Change.
- From the Login menu, select Operator.

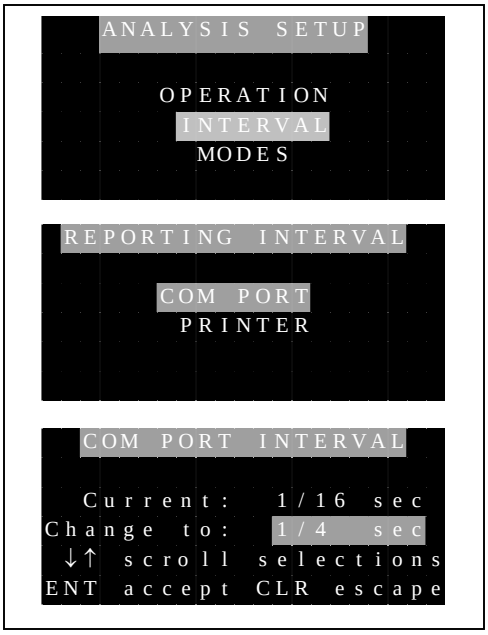


- From the Change Setup Menu, select Analysis.
- From the Analysis Setup Menu, select Modes.
- From the Modes Menu, select Select.
- From the Select Modes Menu, if not already select, select Nitric Oxide.
- From the Confirmation screen, press ENTER to change the Mode.
- The menu will change briefly to indicate that the Mode has been changed.
- Press CLEAR to return to the Analysis Setup Menu.
- To set the Units, scroll to Operation.
- From the Operation Setup Menu, select Units.
- From the Units Menu, Select Voltage.
- Press CLEAR to return to the Operation Setup Menu.
- Select Sensitivity.
- Select High or Low Sensitivity depending on the application (in most cases sensitivity should be set to High).



The NOAnalysis Liquid program should be used for the collection and processing of data from the NOA. The COM Port Interval is normally set at 1/4 second for data collection. Using a faster interval (1/8 or 1/16 second) does not provide any significant

advantage and results in larger data files. Slower intervals (1/2 or 1 second) will not provide sufficient resolution to accurately fit the peak and should only be used if the computer cannot collect data at 1/4 second. To set the COM port interval:



- Press CLEAR to return to the Analysis Setup Menu.
- Select Interval.
- From the Reporting Interval Menu, select COM PORT.
- Use the Arrow buttons to scroll to 1/4 second and press ENTER to save.

The printer is not used for liquid measurements and should be set to OFF.

Deproteinization Procedures

For both the Nitrite Measurement (Chapter 12) and Nitrate Measurement (Chapter 13) samples containing high concentrations of protein can either be directly analyzed and the reagent replaced when foaming occurs or the samples can be deproteinized prior to analysis. Any deproteinization procedure can be used and two techniques that have been successfully employed are listed. Contamination from nitrite or nitrate can occur during these procedures, especially if the reagents are contaminated. A blank should always be included to check for contamination.

Cold ethanol precipitation

- Rinse all glassware and tubes with deionized water to remove any contamination.
- Chill the required volume of ethanol to 0° C.
- Place 0.5 mL of the sample in a 1.5 mL microcentrifuge tube, add 1 mL of cold ethanol and vortex.

- Let stand at 0° C for 30 minutes.
- Centrifuge at ~14,000 RPM for 5 minutes.
- Remove the supernatant for analysis. (Remember to account for 3-fold dilution in the calculations).

Zinc Sulfate/Sodium Hydroxide precipitation

- Rinse all glassware and tubes with deionized water to remove any residual nitrate.
- Add 200 µL of sample to microcentrifuge tube.
- Add 400 µL of 0.5 N NaOH and 400 µL of 10% by weight aqueous $ZnSO_4$.
- Vortex for 30 seconds then let stand at room temperature for 15 minutes.
- Centrifuge at ~14,000 RPM for 5 minutes.
- Remove supernatant for analysis (remember to account for 5-fold dilution in the calculations).

12 MEASUREMENT OF NITRIC OXIDE AND NITRITE IN LIQUID SAMPLES

Nitric oxide reacts with dissolved oxygen to form nitrite (NO_2^-). In the absence of oxyhemoglobin or superoxide anion, nitrite will be the major oxidation product of NO. This includes most cell culture systems, perfusates and other liquid samples. To measure nitrite, the purge vessel contains a reducing agent (1% wt/vol of NaI or KI in acetic acid) to convert nitrite to nitric oxide.

$$I^- + NO_2^- + 2H^+ \rightarrow NO + 1/2I_2 + H_2O$$

For most applications, ~5 mL of the reducing agent is prepared in the purge vessel and this volume is sufficient for measurement of 20-50 samples, depending on the volume of sample injected. As the reagent is depleted, the solution will turn yellow due to formation of I_3^-

Apparatus for Nitrite Reduction

Figure 12-1 shows the setup of the purge vessel for nitrite reduction. Since acetic acid is volatile and less corrosive than mineral acids, a gas bubbler containing NaOH is not required. The outlet of the purge vessel is connected to the IFD filter to prevent liquid from entering the chemiluminescence reaction cell. The outlet of the IFD is connected to the inlet of the standard flow restrictor (do not use the Nafion drier). The reduction is performed at room temperature, and it is not necessary to connect a circulation

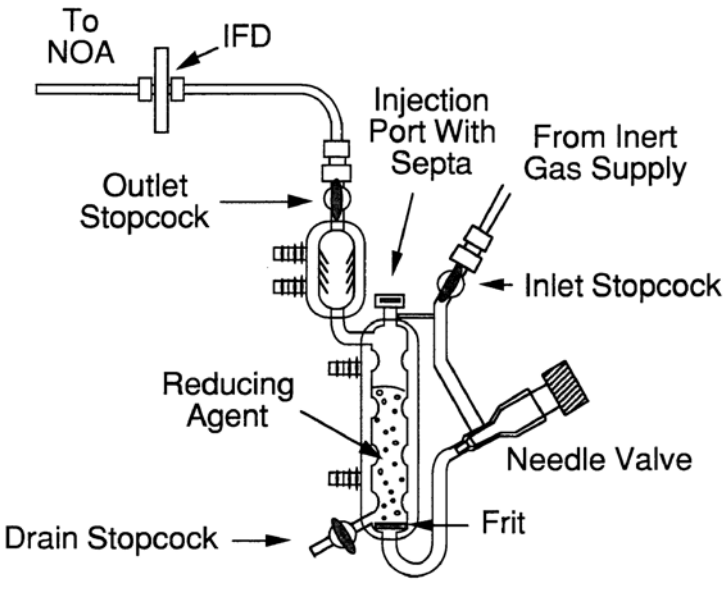


Figure 12-1: *Setup for Nitrite Measurement*

water bath or cold water supply to the purge vessel.

Preparation of the Nitrite Reducing Agent

To minimize reaction of the reducing agent with atmospheric oxygen, it is best to prepare the reagent directly in the purge vessel. To prepare the reagent:

- Close the drain stopcock, gas inlet stopcock and outlet stopcock on the purge vessel and remove the septum and cap from the top of the purge vessel. Screw the needle valve on the purge vessel all the way into the glassware to stop the gas flow.
- Add 4 – 6 mL of glacial or concentrated acetic acid to the purge vessel. This volume should be sufficient to fill the first bulb of the purge vessel. Do not replace the septum and cap at this time. The volume of the purge vessels will vary and it may be necessary to experiment to determine the best volume of acid to use in the purge vessel.
- Adjust the outlet pressure on the inert gas cylinder's regulator to 1-2 psi.
- With the needle valve on the purge vessel fully closed, open any shutoff valves on the inert gas regulator, and open the gas inlet stopcock on the purge vessel.
- Slowly open the needle valve on the purge vessel to allow gas to flow into the acetic acid. A slow, gentle bubbling of gas through the acid to remove any dissolved oxygen is desired. Let the acid purge for a few minutes while preparing the iodide solution.
- Use a balance to weigh approximately 50 milligrams of NaI or KI. Accurate weighing is not necessary since the requirement is an excess of the reagent and the conversion efficiency is not affected by the iodide concentration.
- Dissolve the iodide in 1-2 mL of deionized water.
- Transfer the iodide solution to the purge vessel containing the acid and continue to purge for a few minutes to aid in mixing the reagent.
- Add 100 µL of the dilute antifoaming agent to the purge vessel.
- Install the screw cap and septum on the purge vessel. The septum has a Teflon lining on one side of the septum, and this Teflon side should face down so that the

reagent does not come in contact with the silicone in the septum. The Teflon coating is always a different color from the silicone in the septum. Depending on the supplier of the septum it may be red, brown, or another color, but can be identified by the shiny, hard surface in contrast to the soft, dull silicone.

Adjustment of Purge Gas Flow Rate

The flow rate of inert gas into the purge vessel must be carefully adjusted. If the flow rate is too low, the purge vessel will be under a relatively high vacuum. This will cause rapid loss of the reducing agent and NO from room air can be drawn into the purge vessel resulting in a high background signal. If the flow rate is too high, NO from the samples can leak out into the atmosphere, causing reduced sensitivity and poor repeatability. To adjust the flow rate:

- Disconnect the 1/8" stainless steel nut on the IFD filter line from the frit restrictor on the NOA's sample inlet.
- With the frit restrictor open to the atmosphere and the NOA in the Start mode, record the cell pressure. The cell pressure can be viewed at the bottom of the measurement menu or from the Control/Status screen.
- Reconnect the IFD filter line to the frit restrictor and tighten the nut fingertight.
- Open the outlet stopcock and adjust the gas flow into the purge vessel using the needle valve so that the cell pressure with the purge vessel connected is the same as recorded when the frit restrictor was open to the atmosphere (typically 4-7 torr).

Adjustment of Liquid Level

When the gas flow rate is properly adjusted, the purge vessel will be under a slight vacuum and the level of the reducing agent should be near the top of the purge vessel. When samples are injected into the purge vessel, the needle will be below liquid level. If the liquid level is not near the top of the purge vessel, injections may hit the wall of the purge vessel resulting in a broad peak. To raise the level, use a syringe to add water through the septum until the level is near the top. Water/acetic acid mixtures will foam (even with the antifoaming agent) and this foaming should be near the top of the purge vessel but not into the condenser.

If the liquid level is too high, close the outlet stopcock while continuing to allow gas to flow into the purge vessel. Once the bubbling slows, close the gas inlet stopcock. Release any pressure in the purge vessel by slowly opening the screw cap and then drain some of the reagent using the drain stopcock.

Preparation of Nitrite Standard Solutions

An analytical balance capable of weighing to ± 0.1 mg, a supply of reagent grade sodium or potassium nitrite, nitrite-free, deionized water, volumetric flasks and pipettes are required to calibrate the NOA for nitrite measurements.

Preparation of Stock Solution

A standard solution of ~100 mM NO_2^- is prepared and dilutions of the standard used for constructing the calibration curve.

To prepare 10 mL of 100 mM NO_2^-:

- Weigh out ~69 mg of $NaNO_2$ or ~85 mg of KNO_2 directly into a 10 mL volumetric flask.
- Record the weight of $NaNO_2$ or KNO_2.
- Dilute to the mark with nitrite-free, deionized water.
- Invert the flask several times until the solid dissolves.
- Transfer the stock solution to air-tight storage container.
- Calculate the concentration of the stock solution. For $NaNO_2$, the concentration is

$$\text{Conc. of Stock} = (\text{mg}NaNO_2/69\ \text{mg/mmol})/10\ \text{mL} \times 1000\ \text{mL/L}$$

Use this stock 100 mM standard to prepare dilutions to construct a standard curve for the NOA. The concentrations of the dilutions will depend on the nitrite levels in the samples. Prepare dilutions that contain nitrite at concentrations greater than the samples, concentrations less than the samples and concentrations approximately the same as the samples. For most cell culture work, nitrite concentrations will be in the nanomolar to micromolar range. Analysis of standard solutions containing 10 nM, 50 nM, 100 nM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM will allow construction of a calibration curve suitable for most cell culture work. For stimulated macrophages and other cell types that produce high levels of NO, the standard curve may be extended to include standards with nitrite concentrations greater than 100 µM.

Preparation of Dilute Standards

The best way to prepare dilute standards is by serial dilution. The containers for the dilute standards must be free of nitrite contamination (rinse with nitrite-free water before use). Microfuge tubes (1.5 mL) or similar disposable containers are suitable containers. To prepare the 1 mL of the dilute standards:

- Arrange 7 or 8 tubes in a rack and add 900 µL of nitrite-free water to each tube.
- Set one tube aside and label the tube Water Blank.
- Add 100 µL of the stock solution to the first tube, mix thoroughly and label the tube 10 mM (Note: the actual concentration will depend on the concentration of the stock solution and the tube should be labeled accordingly).
- Using a new pipette tip, transfer 100 µL of the 10 mM standard to the next tube, mix thoroughly and label the tube 1 mM.
- Using a new pipette tip, transfer 100 µL of the 1 mM standard to the next tube, mix thoroughly and label the tube 100 µM.
- Using a new pipette tip, transfer 100 µL of the 100 µM standard to the next tube, mix thoroughly and label the tube 10 µM.
- Repeat these steps to prepare 1 µM and 100 nM standards or lower concentration standards if necessary.
- Intermediate dilutions can be prepared from the dilute standards. For example, 50 µM, 5 µM and 0.5 µM standards can be prepared by 1:1 dilutions of the 100, 10 and 1 µM standards (100 µL of the standard plus 100 µL of nitrite-free water.
- Set aside or discard the two most concentrated standards (10 mM and 1 mM) as these will not be used.

WARNING:

ALWAYS USE NEW PIPETTE TIPS FOR EACH DILUTION. SOME NITRITE REMAINS IN THE TIP AND WILL CONTAMINATE SUBSEQUENT DILUTIONS.

The 100 mM nitrite standard is stable for several weeks, if stored in an air tight container, refrigerated (4 °C or lower), and not exposed to light. The lifetime can be

extended by storing the solution headspace free. The dilute standards are not stable and should be prepared fresh each day.

After preparing the dilute standard solutions, the calibration curve is constructed by injection of the standards into the Purge Vessel, and measurement of the peak area for each standard. To prepare a concentration-based calibration curve, inject the same volume for all of the standards and samples. For amount-based calibrations, different volumes can be injected, but the volume must be included in the sample name. For a 10 µL syringe, injection of 5 µL is a good injection volume and will allow construction of calibration curves down to ~0.2 µM. For best results using the 10 µL syringes, injection volumes should be between 2 to 8 µL. For measurement of lower concentrations, injection of 10 to 100 µL will be required using larger syringes. For a 100 µL injection, calibration curves down to 10 nM can be constructed.

Water Blanks

Before analyzing the standards, check the nitrite levels in the water used to prepare the standards by injecting water from the sample tube created during standard preparation. Inject the water sample into the purge vessel, being sure that the liquid is injected into the reducing agent. If the solution does not contact the reducing agent or contacts the glass on the side of the purge vessel, nitrite will not be rapidly converted, resulting in a broad peak.

Figure 12-2 shows a typical result from several injections of water. With freshly prepared iodide/acid reducing agent, the first injection of water results in a relatively large peak, presumably due to nitrite in the reagents. The peak size decreases with additional water injections and depending on the nitrite contamination in the water may not be detectable. If the water is contaminated, large peaks will continue to be observed; another source of water should be used.

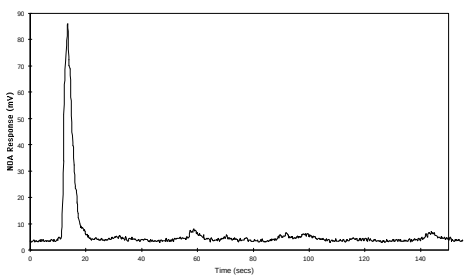


Figure 12-2: *Typical Peaks from Injections of Water*

Injection Technique

For best results, develop a standard procedure for the injections. One approach that works well is:

- Rinse the syringe once or twice with the sample and then pump the syringe several times in the sample to remove any air bubbles.
- Draw the plunger up to the desired volume, remove the needle from the sample and then draw the plunger back to pull the sample into the barrel of the syringe.
- Use a Kimwipe® or paper towel to wipe the outside of the needle.
- To insert the syringe into the purge vessel, it helps use to one hand to help guide the needle through the septum on the purge vessel.
- Push the syringe all the way down until the barrel rests on the top of the screw cap. The end of the needle should be in the liquid reducing agent.
- Rapidly depress the plunger to transfer the liquid into the purge vessel.
- Withdraw the syringe, wipe the needle to remove any reducing agent and then rinse the syringe two or more times with deionized water.
- While the peak is returning to baseline, load the next sample into the syringe.

Preparation of Calibration Curve

After obtaining a clean water blank, inject the standards to prepare a calibration curve. It is best to start with the most dilute standard and make duplicate injections for each standard.

Linear Range and Off-scale Peaks

If the NOA output signal reaches 1000 mV, the linear range for the High Sensitivity setting of the amplifier has been exceeded and the top of the peak has been "cut-off". If off-scale peaks are obtained for standards, the standard should not be included in the calibration curve. There are three options for off-scaled peaks: inject a smaller volume, dilute the sample or change the NOA's sensitivity to Low. The Low Sensitivity is 500 times less sensitive than High. The linear range for the NOA for nitrite measurements will depend on how sharp the peaks are, but typically the NOA has a

linear response from the detection limit of ~1 picomole to ~400 picomoles in the High Sensitivity and ~0.1 nanomoles to 200-250 nanomoles in the Low Sensitivity. In terms of concentration, 1 nM is 1 femtomole/µL, 1 µM is 1 picomole/µL, and 1 mM is 1 nanomole/µL. For a 100 µL injection, the linear range is 10 nM to 4 µM for the High Sensitivity, For a 5 µL injection the linear range is 0.2 µM to 80 µM.

The NOAnalysis Liquid program will automatically multiply the signals collected in the Low sensitivity by 500 to account for the difference in the PMT amplifier gain. However, Sensitivity cannot be changed while collecting data with the Liquid program and then have the program correct for the gain difference. Stop the acquire program and collect data in either the High Sensitivity setting or the Low Sensitivity setting.

Repeatability

The repeatability for nitrite measurements should be 5-10% depending on the concentration of the samples and standards (lower repeatability will be obtained for low concentration samples). If the repeatability is >5% or a linear response is not achieved, identify the problem before analyzing the samples (see the Troublshooting Chapter 16 for solutions to repeatability problems).

Nitrite Contamination

The most common problem, particularly for low level (<1 µM) nitrite measurement is contamination. If the glassware, pipettes, and other equipment are contaminated with nitrite, then the actual concentration of NO_2^- in the standards will be higher than expected. Rinse all equipment with nitrite-free deionized water before use to minimize nitrite contamination. Another common problem is nitrite contamination in the water used to prepare the standards. Check for contaminated water simply by injecting a sample of the water. Most laboratory water systems can produce water with <1 µM nitrite if properly maintained. Ion exchange resins must be replaced at regular intervals, and the manufacturer's recommendations should be followed to ensure high purity water is produced. Water exposed to the atmosphere can absorb nitric oxide,

which will be converted to nitrite. It is best to use water fresh from the purification system and minimize its exposure to air to avoid contamination.

Sample Analysis

The procedure for sample analysis is the same as described above. If the samples contain protein, the reducing agent will begin to foam as more protein is added to the vessel. The anti-foaming agent will reduce the amount of foaming; however, after injecting a large number of samples, foaming from the protein will begin to reach the condenser of the purge vessel. Once the foaming becomes severe, or if there is liquid in the outlet of the purge vessel (the line connected to the IFD filter), drain the reducing agent, clean the purge vessel and prepare fresh reducing agent. While running samples containing protein, observe the IFD filter to make sure that liquid is not collecting in the filter. The filter will initially block the liquid from getting into the NOA, but since the filter is under vacuum eventually the liquid will pass through the filter into the reaction chamber. If there is liquid in the filter, stop the purge vessel, clean and replace the reducing agent and clean the filter as described below. (see page 122).

Foaming problems can also be minimized by keeping the injection volumes small. The amount of protein injected into the purge vessel will be the same for one injection of 100 µL or 100 injections of 1µL, thus more samples can be analyzed before replacing the reducing agent by keeping the injection volumes small. The best injection volume will depend on the concentration of nitrite being measured and the amount of protein in the samples.

Some culture media contain high levels of protein and should be deproteinized before injection into the purge vessel. For example, media with 10% BSA or fetal calf serum will start to foam after only a few injections. To determine if the media has too much protein for direct analysis, inject several different sample sizes (for example, 1 µL, 10 µL and 100 µL) and observe how much foaming occurs in the purge vessel. Deproteinization procedures are described in Chapter 11 (page 106).

Background Nitrite

All cell culture media, the buffers used for perfusates or organ baths, will contain some nitrite. This background contamination will determine the minimum concentration of

nitrite that can be measured in the samples. If a commercial media is used, talk to the supplier about purchasing media low in nitrite. If preparing the media or buffers, check the chemicals used in the preparation for nitrite contamination. In some cases, the nitrite contamination is introduced during filtration or storage of the media. Rinsing all equipment, filter paper, etc. with nitrite-free, deionized water prior to use will help minimize contamination.

Replacing the Reducing Agent and Opening the Purge Vessel

Under normal operating conditions, the purge vessel is under a slight vacuum. In order to open the purge vessel or drain the reducing agent from the vessel, it is necessary to bring the pressure in the purge vessel back to near atmospheric pressure using the procedure below. To open the purge vessel:

- Close the outlet stopcock on the purge vessel.
- Continue purging the solution with the inert gas until the flow of gas, as indicated by the gas bubbles, has stopped or has slowed considerably.
- Close the gas inlet stopcock to stop the flow of gas into the purge vessel.
- Slowly unscrew the cap on the top of the purge vessel to release any pressure that might have built-up during the purging.
- To drain the reducing agent, open the drain stopcock.
- To refill the purge vessel with fresh reducing agent.
- Close the drain stopcock, add 4 to 6 mL of acetic acid, open the gas inlet stopcock and purge for a few minutes.
- Dissolve 50 mg of NaI or KI in 1to 2 mL of nitrite-free deionized water and add the solution to the purge vessel along with 100 µL of the dilute antifoaming agent.
- Replace the screw cap and septum, the open the outlet stopcock and purge for a few minutes before analyzing water blanks, then samples or standards.

Septum Replacement

After 50-100 injections, the septum on the purge vessel will need to be replaced. The best procedure is to replace the septum on a regular basis after a fixed number of

injections. The number of injections will depend on the gauge of the needle used, and whether the needle is bent or distorted. Large gauge needles or needles with bent tips will core the septum and require more frequent replacement than small gauge needles with sharp tips. To some extent the ease of insertion can be used to tell when it is time to replace the septum. With a new septum, some force is required to insert the needle, but after many punctures, the septum loses its integrity and the needle can be inserted without force. To replace the septum, follow the procedures to open the purge vessel, then remove the old septum from the screw cap and discard. Install the new septum with the Teflon side facing the reducing agent. The lifetime of the septum can be extended by varying the location on the septum when injecting.

Cleaning the Purge Vessel

After running samples, follow the procedure below to clean the purge vessel. By following this procedure each time the purge vessel is used, clogging of the glass frit in the purge vessel will be prevented. To clean the purge vessel:

- Follow the procedures above to drain the reducing agent from the purge vessel.
- Remove the screw cap on the purge vessel.
- Use a squeeze bottle filled with deionized water to rinse the purge vessel several times with water.
- To clean the condenser, loosen the 1/4" stainless steel nut on the IFD filter line on the outlet of the purge vessel, and remove the union from the glass. Do not completely remove the nut from the union, simply loosen the nut and remove the nut, ferrules and union as a unit.
- Open the outlet stopcock and use a squeeze bottle to rinse the sides of the condenser with water.
- If the condenser cannot be totally cleaned by rinsing, loosen the clamp so that the purge vessel can be tilted. Fill the purge vessel with water then replace the screw cap a few turns so that it is not tightly sealed, and will allow air to escape. Tilt the purge vessel to fill of the condenser with water then return to the upright position. Repeat until all of the contamination has been removed.

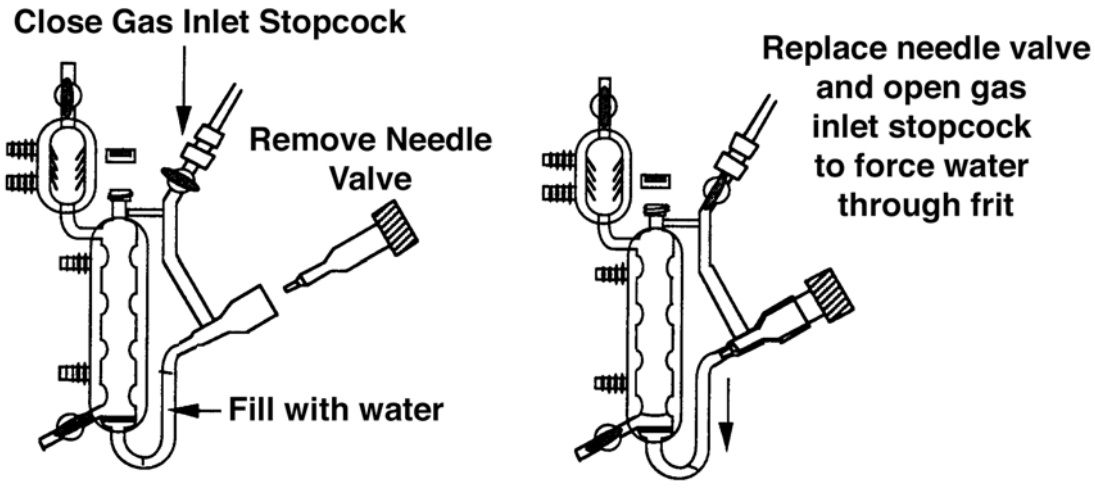


Figure 12-3: *Cleaning the Purge Vessel Frit.*

- To clean the frit, turn off the gas inlet stopcock and unscrew and remove the needle valve on the purge vessel. Use a wash bottle or pipette to fill the glass tube between the needle valve connection and the frit with water.
- Replace the needle valve, and open the gas inlet stopcock to allow the gas to force the water through the frit. Once the water has been passed through the frit, turn off the gas inlet stopcock, remove the needle valve, and add more water to the tube. Replace the needle valve, and turn on the gas to force the water through the frit. Repeat this procedure 3 or 4 times to completely clean the frit and tubing.
- After cleaning the frit, replace the reducing union on the outlet of the purge vessel. Tighten the 1/4" nut fingertight. Do not use a wrench to tighten the nut because the glass will break if the nut is overtightened (see Procedure for Tightening Swagelok Fittings page 101).

Cleaning of the IFD Filter

If the liquid passes out of the purge vessel, it will be blocked by the IFD filter. Most often this is the result of injection of samples containing protein, causing the reagent to foam into the filter. While running samples, observe the IFD filter to check if liquid is present in the filter. While the filter will initially block liquid from the reaction cell, the vacuum will eventually draw the liquid through the filter. If there is liquid in the tubing

on the outlet of the IFD filter (and therefore liquid in the reaction cell) it will be necessary to clean the reaction cell as described in Chapter 15 to remove the contamination. Whenever there is liquid in the IFD filter, remove the liquid and clean the filter as described below.

- Remove the plastic nut and tubing on the inlet of the filter (the side with two ports) to bring the filter to atmospheric pressure and then remove the nut and tubing from the outlet of the filter. Remove the cap on the Luer port and use a syringe (10 – 50 cc) with a Luer adapter to draw the liquid out of the filter.
- Use a wash bottle filled with water to rinse the filter by squirting water in the port where the tubing is connected, letting the water flow out the Luer port.
- After rinsing, use the syringe to remove the remaining liquid.
- Repeat the rinsing and withdrawal of liquid until the filter is clean
- Allow the filter to dry before reinstalling the filter in the NOA.
- After drying, reconnect the tubing to the filter and replace the Luer cap.
- Make sure the inlet side of the filter (the side with two ports) is connected to the tubing coming from the purge vessel.

It is strongly recommended to have a supply of replacement filters. Replacement filters are available from GE Analytical Instruments (AFL 01400) and also available from most laboratory supply houses. Two styles of IFD filters are sold by the supply houses, the polypropylene filter used in the NOA and a nylon filter. Always use the polypropylene filter with the NOA.

Long-term maintenance of the purge vessel and bubbler

Cleaning the purge vessel after use will help to prevent clogging of the frits or contamination of the glassware. Periodically, it is a good idea to clean the glassware thoroughly in a laboratory detergent to remove any material that cannot be rinsed from the purge vessel. Turn off the inert gas supply and remove the 1/4" to 1/8" reducing unions on the gas inlet and the outlet of the purge vessel. Remove the needle valve from the purge vessel and the three stopcocks. Soak the purge vessel, needle

valves and stopcocks in warm detergent for several hours. Use an ultrasonic cleaner to assist in cleaning. Rinse the purge vessel thoroughly in deionized water and allow to air dry before re-assembling the purge vessel.

13 MEASUREMENT OF NITRATE, NITRITE AND NITRIC OXIDE IN LIQUID SAMPLES

Nitric oxide reacts with oxyhemoglobin and superoxide anion to form nitrate. Nitrate is the major oxidation product of NO in some cell culture systems and in animals and human samples. To measure nitrate, vanadium (III) chloride in hydrochloric acid is used to convert nitrate to nitric oxide.

$$2NO_3^- + 3V^{+3} + 2H_2O \rightarrow 2NO + 3VO_2^+ + 4H^+$$

To achieve high conversion efficiency, the reduction is performed at ~90° C. To prevent damage to the NOA from the hydrochloric acid vapor, a gas bubbler filled with aqueous sodium hydroxide is installed between the purge vessel and the NOA. Vanadium (III)/HCl will also convert nitrite and S-nitrosocompounds to NO. Nitro-compounds such as nitroarginine, sodium nitropruside, nitroglycerin are slowly converted to NO by the reagent. This conversion is not quantitative, but will result in an elevated baseline as the nitro group is slowly converted to NO. Use of nitroarginine or the methyl ester L-NAME is not recommended when using VCl_3/HCl. Methyl arginine and other NOS inhibitors that do not contain nitro groups should be used.

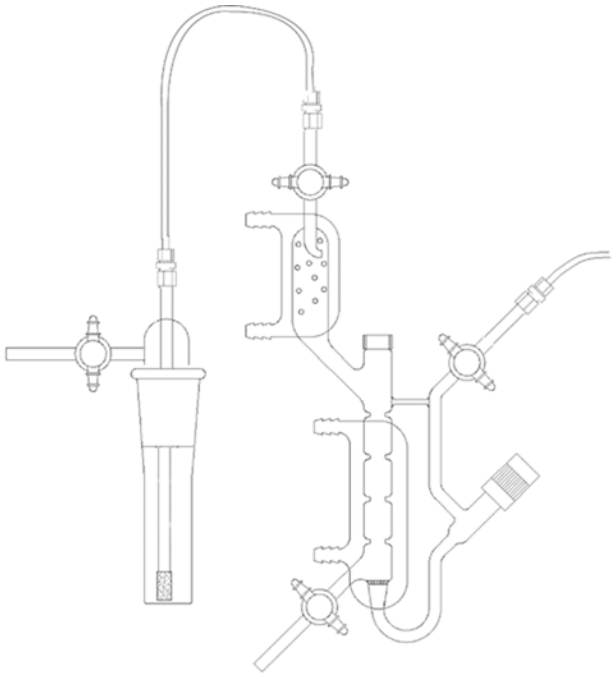


Figure 13- 1: *Setup for Nitrate Reduction*

Apparatus for Nitrate Reduction

Figure 13-1 shows the setup for nitrate reduction. The outlet of the purge vessel is connected to the gas bubbler/NaOH trap and the outlet of the bubbler connected to the inlet of the NOA with the IFD filter in-line

(not shown) to prevent liquid from entering the reaction cell.

Silicon tubing is connected to the circulating water bath and the heating jacket of the purge vessel. Hose clamps are used to secure the tubing to the purge vessel and the fittings on the circulating water bath. Cold water from the tap, a second circulating water bath or ice bath and peristaltic pump is connected to the condenser on the purge vessel using rubber tubing and the tubing secured with hose clamps.

Preparation of the Nitrate Reducing Agent

A saturated solution of VCl_3 in 1 M HCl is prepared and then filtered before use. Vanadium (III) is oxidized slowly in air, and it is best to prepare small volumes of the reagent to ensure that the reagent is active. Some researchers report that the reagent may be stored with refrigeration for 2-4 weeks. To prepare 100 mL of the reagent:

- Use a balance to weigh approximately 0.8 grams of VCl_3 into a clean dry 100 mL flask. When water is added to solid VCl_3, an exothermic reaction occurs and the glassware will get hot. Use caution when rinsing the glassware with water or adding the acid to the vanadium.
- Carefully add 1 M HCl to bring the level of the solution to the 100 mL mark. Because this is a saturated solution, the dilution does not have to be exact.
- Cap the flask and invert several times. Not all of the solid will dissolve, but the solution should turn blue.
- The solution MUST be filtered before use. Any filter paper (such as Whatman #1) can be used to filter the solution. Store the solution in a clean screw-cap bottle with a Teflon or aluminum foil seal.

Preparation of 1M NaOH

Aqueous sodium hydroxide is used in the gas bubbler to prevent HCl vapors from entering the NOA. The reagent (1M NaOH) may be purchased from most chemical

supply houses. The solution can be prepared from solid NaOH (4 grams/100 mL) or from 50% NaOH (5 mL/100 mL).

Startup Procedures for Nitrate Reduction

After preparing the VCl_3/HCl reagents and assembling the purge vessel, circulating water bath, cold water supply and inert gas supply the following procedures are used to setup the nitrate reduction system. The NOA should be in the Start Mode.

- Set the temperature of the water bath to 95°C, turn on the circulating pump and check for any leaks. If water is leaking, turn off the bath, reposition the tubing and tighten the hose clamps to stop the leak.
- Turn on the cold water supply to the condenser and check for any leaks. If water is leaking, reposition the tubing and tighten the hose clamps to stop the leak.
- Open the Gas Bubbler and add 15 mL of 1 M NaOH to the bubbler base. The gas bubbler has a Teflon sleeve that prevents the ground-glass joint from being etched by the NaOH. Make sure the Teflon sleeve is in place and there are no holes in the sleeve.
- Replace the bubbler top, and seal the bubbler by pressing the bottom onto the top and twisting to achieve a tight seal. When correctly tightened, it is not possible to turn the top of the bubbler without some force. Secure the bubbler pieces with the green plastic clamp and mount the bubbler on the ring stand using a clamp.
- Connect the IFD filter line to the outlet of the bubbler and tighten the nut (see Procedure for Tightening Swagelok Fittings page101) and open the outlet stopcock on the bubbler. **Do not connect the IFD line to the NOA.**
- Connect the bubbler line tubing from the outlet of the purge vessel to the inlet of the bubbler.
- Close the gas inlet stopcock and outlet stopcock on the purge vessel and screw the needle valve on the purge vessel all the way in (fully closed).
- Close the drain stopcock on the purge vessel. Open the screw cap on the top of the purge vessel, and add 5 mL of the filtered VCl_3/HCl reagent.

- Add 100 µL of the dilute antifoaming agent to the purge vessel. Leave the screw cap off while adjusting the gas flow into the purge vessel.
- Open the main valve on the inert gas cylinder, adjust the regulator to give 1-5 psi, and open any shutoff valve on the regulator.
- Open the gas inlet stopcock on the purge vessel and slowly open the needle valve to start the flow of gas into the purge vessel. Obtain a slow, gentle bubble of gas through the reagent.
- Replace the screw cap, open the outlet stopcock on the purge vessel and slowly open the outlet stopcock on the gas bubbler while keeping an eye on the level of NaOH in the bubbler. When opened slowly, the NaOH will not bump as the gas is added. If the gas is added too fast, the NaOH will bump into the IFD filter. Gas should now be bubbling through the purge vessel and gas bubbler.

Adjustment of Purge Gas Flow Rate

The flow rate of inert gas into the purge vessel must be carefully adjusted. If the flow rate is too low, the purge vessel will be under a relatively high vacuum. This will cause the VCl_3/HCl to vacuum distill (see 129) If the flow rate is too high, NO from the samples can leak out into the atmosphere, causing reduced sensitivity and poor repeatability. To adjust the flow rate:

- The IFD filter line should not be connected to the NOA.
- With the frit restrictor open to the atmosphere and the NOA in the Start mode, record the cell pressure. The cell pressure can be viewed at the bottom of the measurement menu or from the Control/Status screen.
- Connect the IFD filter line to the NOA's frit restrictor and tighten the nut fingertight.
- Open the outlet stopcock on the purge vessel and on the gas bubbler.
- Adjust the gas flow into the purge vessel using the needle valve so that the cell pressure with the purge vessel connected is the same as recorded when the frit restrictor was open to the atmosphere (typically 4-7 torr).

The vanadium reagent should turn from blue to green when the water bath reaches 80-90 °C. If the reagent does not change color, the vanadium has been oxidized and fresh reagent should be prepared.

The background signal on the NOA will usually go offscale on the high sensitivity setting when fresh VCl_3/HCl reagent is purged. The background will drop with continued purging, with a final baseline of 10-50 mV.

Leak Check for Purge Vessel

The best way to ensure that all of the connections to the purge vessel and gas bubbler are tight is to see if the VCl_3/HCl reagent will vacuum distill when the gas flow is turned off. The water bath should be at 95 °C for this test. To perform the leak check:

- Close the needle valve on the purge vessel to stop the flow of gas.
- After a few seconds, the VCl_3/HCl reagent should begin to vacuum distill as indicated by the violent boiling with large bubbles.
- If the reagent does vacuum distill, all of the connection are tight. Open the needle valve and adjust the gas flow rate to match the cell pressure determined above.
- If the reagent does not vacuum distill, there is a leak that must be fixed. The most common location is the Teflon sleeve in the gas bubbler. Make sure the top and bottom connections are tight. Other sources include the Swagelok connections (make sure the ferrules are in place and the connections are tight) and the connections at the IFD filter.

Adjustment of Liquid Level

When the gas flow rate is properly adjusted, the purge vessel will be under a slight vacuum and the level of the reducing agent should be near the top of the purge vessel. When samples are injected into the purge vessel, the needle will be below liquid level. If the liquid level is not near the top of the purge vessel, injections may hit the wall of the purge vessel resulting in a broad peak. To raise the level, use a

syringe to add 1 M HCl through the septum until the level is near the top. The reagent will foam (even with the antifoaming agent) and this foaming should be near the top of the purge vessel but not into the condenser.

If the liquid level is too high, close the outlet stopcock while continuing to allow gas to flow into the purge vessel. Once the bubbling slows, close the gas inlet stopcock. Release any pressure in the purge vessel by slowly opening the screw cap and then drain some of the reagent using the drain stopcock.

WARNING:
THE VCl_3/HCl REAGENT IS HOT!

Preparation of Nitrate Standard Solutions

An analytical balance capable of weighing to ± 0.1 mg, a supply of reagent grade sodium or potassium nitrate, nitrate-free, deionized water, volumetric flasks and pipettes are required to calibrate the NOA for nitrate measurements.

Preparation of Stock Solution

A standard solution of ~100 mM NO_3^- is prepared and dilutions of the standard used for constructing the calibration curve.

To prepare 10 mL of 100 mM NO_3^-:

- Weigh out ~85 mg of $NaNO_3$ or ~101 mg of KNO_2 directly into a 10 mL volumetric flask.
- Record the weight of $NaNO_3$ or KNO_3.
- Dilute to the mark with nitrate-free, deionized water.
- Invert the flask several times until the solid dissolves.
- Transfer the stock solution to air-tight storage container.
- Calculate the concentration of the stock solution. For $NaNO_3$, the concentration is:

Conc. of Stock = (mg$NaNO_2$/85 mg/mmol)/10 mL x 1000 mL/L

Use this stock 100 mM standard to prepare dilutions to construct a standard curve for the NOA. The concentrations of the dilutions will depend on the nitrate levels in the samples. Prepare dilutions that contain nitrate at concentrations greater than the samples, concentrations less than the samples and concentrations approximately the same as the samples. For most cell culture work, nitrate concentrations will be in the nanomolar to micromolar range. Analysis of standard solutions containing 10 nM, 50 nM, 100 nM, 1 µM, 5 µM, 10 µM, 50 µM and 100 µM will allow construction a calibration curve suitable for most samples. For urine and other samples that contain high levels of NO, the standard curve may be extended to include standards with nitrate concentrations greater than 100 µM.

Preparation of Dilute Standards

The best way to prepare dilute standards is by serial dilution. The containers for the dilute standards must be free of nitrate contamination (rinse with nitrate-free water before use). Microfuge tubes (1.5 mL) or similar disposable containers are suitable containers. To prepare the 1 mL of the dilute standards:

- Arrange 7 or 8 tubes in a rack and add 900 µL of nitrate-free water to each tube.
- Set one tube aside and label the tube Water Blank.
- Add 100 µL of the stock solution to the first tube, mix thoroughly and label the tube 10 mM (Note: the actual concentration will depend on the concentration of the stock solution and the tube should be label accordingly).
- Using a new pipette tip, transfer 100 µL of the 10 mM standard to the next tube, mix thoroughly and label the tube 1 mM.
- Using a new pipette tip, transfer 100 µL of the 1 mM standard to the next tube, mix thoroughly and label the tube 100 µM.
- Using a new pipette tip, transfer 100 µL of the 100 µM standard to the next tube, mix thoroughly and label the tube 10 µM.

- Repeat these steps to prepare 1 µM and 100 nM standards or lower concentration standards if necessary.
- Intermediate dilutions can be prepared from the dilute standards. For example 50 µM, 5 µM and 0.5 µM standards can be prepared by 1:1 dilutions of the 100, 10 and 1 µM standards (100 µL of the standard plus 100 µL of nitrate-free water.
- Set aside or discard the two most concentrated standards (10 mM and 1 mM) as these will not be used.

WARNING:

ALWAYS USE NEW PIPETTE TIPS FOR EACH DILUTION. SOME NITRATE REMAINS IN THE TIP AND WILL CONTAMINATE SUBSEQUENT DILUTIONS.

The 100 mM nitrate standard is stable for several weeks, if stored in an air tight container, refrigerated (4 °C or lower), and not exposed to light. The lifetime can be extended by storing the solution headspace free. The dilute standards are not stable and should be prepared fresh each day.

After preparing the dilute standard solutions, the calibration curve is constructed by injection of the standards into the Purge Vessel, and measurement of the peak area for each standard. To prepare a concentration-based calibration curve, inject the same volume for all of the standards and samples. For amount-based calibrations, different volumes can be injected, but the volume must be included in the sample name. For a 10 µL syringe, injection of 5 µL is a good injection volume and will allow construction of calibration curves down to ~0.2 µM. For best results using the 10 µL syringes, injection volumes should be between 2 to 8 µL. For measurement of lower concentrations, injection of 10 to 100 µL will be required using larger syringes. For a 100 µL injection, calibration curves down to 10 nM can be constructed.

Water Blanks

As with the nitrite reducing agent, injection of water into fresh VCl_3/HCl will initially result in a large peak with additional injections yielding smaller peaks. Nitrate is a

common contaminant in water and the level of nitrate in the water will determine the lowest concentration of nitrate that can be measured in samples. Before analyzing standards or samples, several water injections should be made until small, repeatable peaks are obtained.

Injection Technique

For best results, develop a standard procedure for the injections. One approach that works well is:

- Rinse the syringe once or twice with the sample and then pump the syringe several times in the sample to remove any air bubbles.
- Draw the plunger up to the desired volume, remove the needle from the sample and then draw the plunger back to pull the sample into the barrel of the syringe.
- Use a Kimwipe® or paper towel to wipe the outside of the needle.
- To insert the syringe into the purge vessel, it helps use to one hand to help guide the needle through the septum on the purge vessel.
- Push the syringe all the way down until the barrel rests on the top of the screw cap. The end of the needle should be in the liquid reducing agent.
- Rapidly depress the plunger to transfer the liquid into the purge vessel.
- Withdraw the syringe, wipe the needle to remove any reducing agent and then rinse the syringe two or more times with deionized water.
- While the peak is returning to baseline, load the next sample into the syringe.

Preparation of Calibration Curve

After obtaining a clean water blank, inject the standards to prepare a calibration curve. It is best to start with the most dilute standard and make duplicate injections for each standard.

Analysis of Samples and Standards

Under normal operating conditions, the level of the VCl_3 reagent will be at the top of the purge vessel and refluxing into the condenser. Remember the purge vessel is

HOT! Because of the extra volume of the gas bubbler and the slower conversion of nitrate to nitric oxide, the peaks will appear about 30 seconds after injection. For sera, plasma, or other sample containing high levels of protein, the reagent will begin to foam as samples are injected. When the foaming becomes severe, as indicated by foam exiting the condenser, replace the reagent and clean the purge vessel. The reagent should be replaced before the foaming reaches the NaOH. If the reagent foams into the gas bubbler, the gas bubbler should be cleaned and fresh NaOH added to the bubbler. The samples can also be deproteinized as described on page 106.

Serum and Plasma Samples

Serum and plasma samples are also quite viscous and can be difficult to draw into microliter syringes and can clog the needles. Often these samples will also contain suspended solids and a solid film over the top of the liquid. Injecting these solids will result in poor repeatability for the measurement of nitrate. Diluting serum or plasma samples 1:1 with nitrate-free water will make it easier to analyze these samples and yield better reproducibility.

Samples containing protein will also change the viscosity of the VCl_3/HCl causing the level of the reagent to drop. Usually the level will return to near the top of the purge vessel after purging for a minute. If it does not, add more VCl_3/HCl to bring the level of the liquid near the top of the purge vessel. A tuberculin syringe with a small needle can be used to inject the reagent through the septum without having to open the purge vessel. Before analyzing samples, allow the reagent to purge for a few minutes while the NOA signal drops.

Nitrate Contamination

The most common problem, particularly for low level (<1 µM) nitrate measurement is contamination. If the glassware, pipettes, and other equipment are contaminated with nitrate, then the actual concentration of NO_3^- in the standards will be higher than expected. Rinse all equipment with nitrite-free deionized water before use to minimize nitrite contamination. Another common problem is nitrate

contamination in the water used to prepare the standards. Check for contaminated water simply by injecting a sample of the water. Most laboratory water systems can produce water with <1 µM nitrate if properly maintained. Ion exchange resins must be replaced at regular intervals, and the manufacturer's recommendations should be followed to ensure high purity water is produced. It is best to use water fresh from the purification system and minimize its exposure to air to avoid contamination.

Replacing the Reducing Agent and Opening the Purge Vessel

In order to open the purge vessel or drain the reducing agent from the vessel, it is necessary to bring the pressure in the purge vessel back to near atmospheric pressure using the procedure below. To open the purge vessel:

- Close the outlet stopcock on the purge vessel.
- Continue purging the solution with the inert gas until the flow of gas, as indicated by the gas bubbles, has stopped or slowed considerably.
- Close the gas inlet stopcock to stop the flow of gas into the purge vessel.
- Slowly unscrew the cap on the top of the purge vessel to release any pressure that might have built up during the purging.
- To drain the reducing agent, open the drain stopcock (CAUTION: HOT HYDROCHLORIC ACID).

To refill the purge vessel with fresh reducing agent:

- Close the drain stopcock, add 4-5 mL of VCl_3/HCl and 100 µL of dilute antifoaming agent.
- With the Outlet stopcock closed, replace the screw cap and open the gas inlet stopcock. Gas will begin to flow into the purge vessel
- Since the gas bubbler is still connected to the NOA, it is under reduced pressure. If the outlet stopcock on the purge vessel is opened rapidly, the NaOH will bump into the IFD filter. If the stopcock is opened slowly, the NaOH will not bump.
- Slowly open the outlet stopcok while watching the level of the NaOH.

The NOA signal will initally increase with fresh VCl_3/HCl, but the signal will return to baseline after a few minutes.

Opening the Gas Bubbler

The gas bubbler containing NaOH is also under a vacuum and if not opened properly, the NaOH can backup into the tubing to the purge vessel. To open the gas bubbler:

- Close the outlet stopcock on the purge vessel, leaving the stopcock on the bubbler open.
- Loosen the 1/4" nut on the Swagelok union connected to the outlet of the purge vessel.
- Once loose, slowly pull the union off the purge vessel while keeping an eye on the level of NaOH in the bubbler. As the union is removed, air will be drawn into the bubbler, bringing the pressure in the bubbler back to atmospheric pressure.
- When the union is removed and the bubbler is at atmospheric pressure, disconnect the IFD line from the NOA frit restrictor.

Septum Replacement

After 50-100 injections, the septum on the purge vessel will need to be replaced. The best procedure is to replace the septum on a regular basis after a fixed number of injections. The number of injections will depend on the gauge of the needle used, and whether the needle is bent or distorted. Large gauge needles or needles with bent tips will core the septum and require more frequent replacement than small gauge needles with sharp tips. To some extent the ease of insertion can be used to tell when it is time to replace the septum. With a new septum, some force is required to insert the needle, but after many punctures, the septum loses its integrity and the needle can be inserted without force. To replace the septum, follow the procedures to open the purge vessel, then remove the old septum from the screw cap and discard. Install the new septum with the Teflon side facing the

reducing agent. The lifetime of the septum can be extended by varying the location on the septum when injecting.

Cleaning the Purge Vessel

After running samples, follow the procedure below to clean the purge vessel. By following this procedure each time the purge vessel is used, clogging of the glass frit in the purge vessel will be prevented. For nitrates, turn off the circulating water bath and allow the glassware to cool before cleaning the purge vessel. To clean the purge vessel:

- Follow the procedures above to drain the reducing agent from the purge vessel.
- With the outlet stopcock on the purge vessel closed, loosen the 1/4" nut on the Swagelok union connected to the outlet of the purge vessel.
- Once loose, slowly pull the union off the purge vessel while observing the level of NaOH in the bubbler. As the union is removed, air will be drawn into the bubbler, bringing the pressure in the bubbler back to atmospheric pressure.
- When the union is removed and the bubbler is at atmospheric pressure, disconnect the IFD line from the NOA frit restrictor.
- Remove the screw cap on the purge vessel.
- Use a squeeze bottle filled with deionized water to rinse the purge vessel several times with water.
- Open the outlet stopcock and use a squeeze bottle to rinse the sides of the condenser with water.
- If the condenser cannot be totally cleaned by rinsing, loosen the clamp so that the purge vessel can be tilted. Fill the purge vessel with water then replace the screw cap a few turns so that it is not tightly sealed, and will allow air to escape. Tilt the purge vessel to fill of the condenser with water then return to the upright position. Repeat until all of the contamination has been removed.
- To clean the frit, turn off the gas inlet stopcock and unscrew and remove the needle valve on the purge vessel. Use a wash bottle or pipette to fill the glass tube between the needle valve connection and the frit with water.

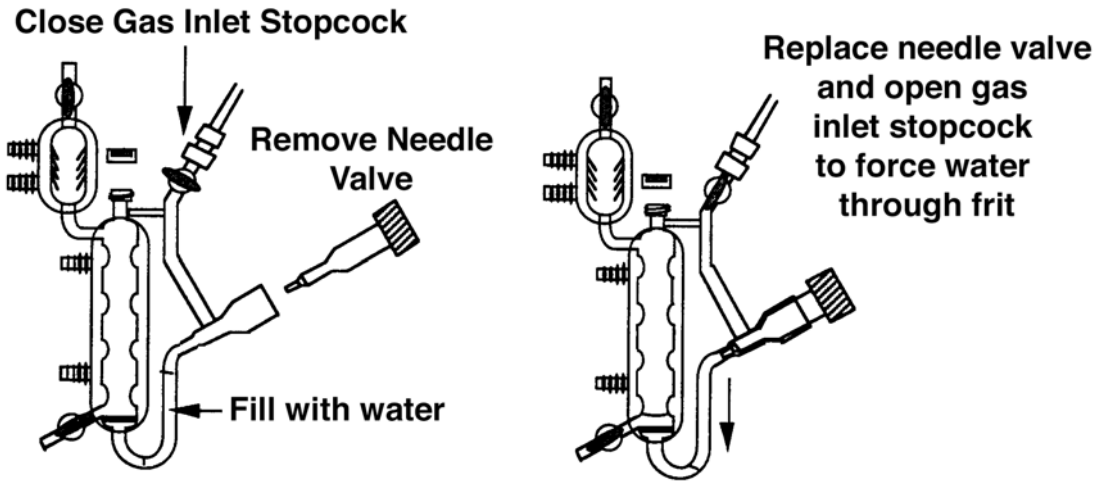


Figure 13-2: *Cleaning the Purge Vessel Frit.*

- Replace the needle valve, and open the gas inlet stopcock to allow the gas to force the water through the frit. Once the water has been passed through the frit, turn off the gas inlet stopcock, remove the needle valve, and add more water to the tube. Replace the needle valve, and turn on the gas to force the water through the frit. Repeat this procedure 3 or 4 times to completely clean the frit and tubing.
- After cleaning the frit, replace the reducing union on the outlet of the purge vessel. Tighten the 1/4" nut fingertight. Do not use a wrench to tighten the nut; the glass will break if the nut is overtightened (see Procedure for Tightening Swagelok Fittings page 101).

Cleaning the Gas Bubbler

The gas bubbler should also be cleaned after running samples or whenever the VCl_3/HCl reagent gets into the bubbler. To clean the bubbler:

- Follow the procedures listed above to open the gas bubbler and remove the Swagelok unions on the inlet and outlet of the bubbler.
- Remove the green clamp and twist the bubbler's top to open the bubbler.
- Discard the NaOH and rinse the bubbler bottom thoroughly with water.

- To clean the frit, close the bubbler stopcock, fill the bubbler bottom with water, point the bubbler away from you and quickly immerse the top into the water. The water will be forced into the frit and squirt out the bubbler inlet tubing.
- Repeat this process several times to remove any material inside the frit and tubing.

After cleaning the inside of the bubbler top, open the outlet stopcock and rinse the bubbler top with water.

Cleaning the Bubbler Tubing

The tubing and fittings from the purge vessel to the bubbler should be rinsed with water to remove HCl and prevent corrosion of the fitting. To clean the tubing and fittings:

- Hold the tubing with the fittings still attached (including the ferrules) over a sink.
- Use a squeeze bottle filled with deionized water to thoroughly rinse the tubing and fittings (do not take the fittings apart).
- After cleaning the bubbler and tubing, reassemble the bubbler and reconnect the tubing. (see Procedure for Tightening Swagelok Fittings page 101).

Cleaning of the IFD Filter

If the level of the NaOH in the gas bubbler gets too high it will be drawn into the tubing and blocked by the IFD filter. Most often this is as a result of injection of a sample containing protein, causing the VCl_3 reagent to foam into the bubbler and subsequent foaming of the NaOH. While running samples, observe the IFD filter to check if liquid is present in the filter. While the filter will initially block liquid from getting into the reaction cell, the vacuum will eventually draw the liquid through the filter. If there is liquid in the tubing on the outlet of the IFD filter (and therefore liquid in the reaction cell) it will be necessary to clean the reaction cell as described in Chapter 11 to remove the contamination. Whenever there is liquid in the IFD filter, remove the liquid and clean the filter as described below.

- Remove the plastic nut and tubing on the inlet of the filter (the side with two ports) to bring the filter to atmospheric pressure and then remove the nut and tubing from the outlet of the filter. Remove the cap on the Luer port and use a syringe (10 – 50 cc) with a Luer adapter to draw the liquid out of the filter.
- Use a wash bottle filled with water to rinse the filter by squirting water in the port where the tubing is connected, letting the water flow out the Luer port.
- After rinsing, use the syringe to remove the remaining liquid.
- Repeat the rinsing and withdrawal of liquid until the filter is clean
- Allow the filter to dry before reinstalling the filter in the NOA.
- After drying, reconnect the tubing to the filter and replace the Luer cap.
- Make sure the inlet side of the filter (the side with two ports) is connected to the tubing coming from the purge vessel.

It is strongly recommended to have a supply of replacement filters. Replacement filters are available from GE Analytical Instruments (AFL 01400) and also available from most laboratory supply houses. Two styles of IFD filters are sold by the supply houses, the polypropylene filter used in the NOA and a nylon filter. Always use the polypropylene filter with the NOA.

Long-term maintenance of the purge vessel and bubbler

Cleaning the purge vessel and bubbler after use will help to prevent clogging of the frits or contamination of the glassware. Periodically, it is a good idea to clean the glassware thoroughly in a laboratory detergent to remove any material that cannot be rinsed from the purge vessel. Turn off the inert gas supply and remove the 1/4" to 1/8" reducing unions on the gas inlet and the outlet of the purge vessel. Remove the needle valve from the purge vessel and the three stopcocks. Remove the stopcock and Teflon sleeve from the bubbler. Soak the purge vessel, bubbler, needle valves and stopcocks in warm detergent for several hours. Use an ultrasonic cleaner to assist in cleaning. Rinse the items thoroughly in deionized water and allow to air dry before re-assembling the purge vessel and bubbler.

14 *OTHER LIQUID MEASUREMENT TECHNIQUES*

Measurement of Nitrosothiols

Several techniques have been developed to allow conversion of RSNO compounds to NO for measurement by the NOA. Nitrosothiols are generally present at low nanomolar concentrations and will require injection of larger volume (50-1000 µL) of sample for detection. A brief description of some reported techniques is given below, consult the original reference for more information.

Cu(I)/Cysteine Reagent

Fang and coworkers (Fang, K., Ragsdale, N.V., Carey, R.M., MacDonald, T., and Gaston, B. Reductive Assays for S-Nitrosothiols: Implications for Measurements in Biological Systems. *Biochem Biophys Res Comm* 1999;**252**:535-540) have reported the use of copper(I) ions in combination with cysteine for the conversion of low molecular weight nitrosothiols to NO using the purge vessel. While the efficiency of conversion is high for lower molecular weight compounds such as S-nitroso-glutathione, lower efficiencies were reported for high molecular weigh species such as S-nitroso-albumin. This regent was specific for nitrosothiols, with no response reported for nitrite, nitrate or nitro-containing compounds at pH > 6.

Preparation of Reducing Agent

The reducing agent is PBS, another buffer, or water that contains 1 mM cysteine and is staturated with CuCl. Since CuCl has low water solubility, only a few milligrams of the solid is sufficient to prepare a saturated solution. The reagent can be used at room temperature, but more rapid conversions are obtained at elevated temperatures (50 °C). The highest response for GSNO was reported at pH 4.2, but some care must be exercised to ensure that nitrite does not produce a response.

Preparation of Nitrosothiols Standards

Low molecular weight nitrosothiols are prepared by reaction of equimolar amounts of nitrite (in water) and the thiols (in 1 M HCl). Under acidic conditions, the nitrosothbiols are relatively stable, but should be kept cold and fresh standards should be prepared daily. This method for preparation is not quantitative and the concentration of RSNO formed in the reaction should be confirmed by another method such as the Saville technique (Saville, B. *Analyst* 1958, **83**, 670-672).

Copper(I)/Iodide/Iodine Reagent

Marley and co-workers (Marley, R., Feelisch, M., Holt, S., and Moore, K. A Chemiluminescence-based Assay for S-nitroso-albumin and Other Plasma S-nitrosothiols. Free Rad Res 2000:32(1):1-9) have reported a technique for measurement of S-nitroso-albumin and other plasma nitrosothiols based on alkylation of free thiols with N-ethylmaleimide, removal of nitrite by reaction with acidified sulfanilamide and reduction of RSNOs using $Cu(I)/I^-/I_2$ at 70 °C.

Preparation of the Reducing Agent

The preparation of the reagent is similar to that described in Chapter 12 for nitrite reduction. Glacial acetic acid is added to the purge vessel then an aqueous solution of KI is added to the acid. Just prior to injection of the sample, a small volume of 200 mM $CuSO_4$ is added to the purge vessel. Due in part to the large sample volumes (100 – 2000 µL)), the reagent is replaced after each injection.

Preparation of S-Nitroso-Albumin

The procedure for preparing S-nitroso-albumin is described in this reference and involves treatment of albumin with dithiothreitol and DTPA to fully reduce the thiol groups, dialysis, then trans-nitrosylation using S-nitroso-cysteine (prepared by reaction of equimolar amount of thiol and nitrite). Unreacted thiols were alkylated

with N-ethylmaleimide (1 mM) and the stock solution stored at – 20 °C. S-nitroso-albumin concentration was determined by the Saville reaction.

Treatment of Plasma Samples

Samples were collected in pre-chilled tubes containing EDTA (final concentration 2 mM) and N-ethylmaleimide (final concentration 5 mM). After centrifugation, the samples are treated with 0.5% sulfanilamide in 0.1 M HCl to remove the nitrite.

A similar method has been developed by Gladwin and co-workers (Gladwin, M.T., Shelhamer, J.H., Schechter, A.N., Pease-Fye, M.E., Waclasis, M.A., Panza, J.A., Ognibene, F.P.and Cannon III, R.O. Role of circulating nitrite and S-nitrosohemoglobiun in the regulation of regional blood flow in humans *Proc Natl Acad Sci* 2000; **97**:11482-11487) for the measurement of S-nitrosohemoglobin in red blood cells. The reagent is similar to the reducing agent for nitrite (acetic acid/sodium iodide) but a crystal of I_2 is added to the purge vessel to convert RSNOs.

To eliminate response from iron-bound NO, the samples are pretreated with 100-fold excess of 0.2 M KCN and 0.2 M $K_3Fe(CN)_6$ in 0.5 mM EDTA. Nitrite was removed through use of a Sephadex G25 column.

Measurement of Iron-bound NO

Gladwin and co-workers have also reported a technique to measure Hb(FeII)NO (Gladwin, M.T., Ognibene, F.P, Pannell, L.K., Nichols, J.S., Pease-Fye, M.E., Shelhamer, J.H., and Schechter, A.N., Relative role of heme nitrosylation and □-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation *Proc Natl Acad Sci* 2000; **97**:9943-9948).

The method employs two measurements using KI/I_3^-/acetic acid, without and without pretreatment with $KCN/K_3Fe(CN)_6$. Treatment with the CN^- releases the Fe-

bound NO, to permit detection of the S-bound NO only (after removal of the nitrite). The I_3^- reagent will also liberate Fe-bound NO, so without pretreatment, both the Fe-bound and S-bound NO are detected. The difference between the measurements is the amount of Fe-bound NO.

Headspace Measurement of Nitric Oxide

At physiological conditions, nitric oxide is a gas with limited solubility in water. In cell cultures, some of the NO produced will diffuse from the liquid into the gas above the media. Analysis of the NO in the gas above cultured cells (so-called headspace) can be used as an alternative to measuring nitrite/nitrate in the media. In headspace analysis, a gas-tight syringe is used to collect the gas above the media and this gas is injected into the NOA. The main advantage of headspace analysis is the sample is a gas rather than a liquid. This eliminates the need to deproteinize samples and reduces the problems associated with nitrite/nitrate contamination. The main disadvantage of headspace analysis is that gas-tight containers must be used to collect samples (most cell culture flasks are not gas-tight) and the sensitivity of the assay will be lower than nitrite/nitrate measurement since some of the NO produced will be oxidized and remain in the fluid.

Headspace analysis can be used for a wide range of samples including cell cultures, tissue homogenates, and when combined with reducing agents, measurement of nitrite or nitrate in serum, plasma and other fluids. Contact GE Analytical Instruments for specific references for these applications.

Apparatus for Headspace Analysis

The purge vessel is used as a injection port for headspace measurements using the same configuration as is used for nitrite analysis (page 109) except no reducing agent is used. A few milliliters of water can be added to the purge vessel to help visualize the gas flow. Adjust the flow rate of gas into the purge vessel to produce a cell pressure of 4-7 torr.

Sample Collection

In headspace analysis, the sample is allowed to equilibrate in a sealed container for a fixed period of time, typically 5-30 minutes. During the equilibration, NO will partition into the gas above the liquid and reach an equilibrium concentration. After the equilibration period, a gas-tight syringe is used to withdraw the gas sample for injection into the purge vessel. The sample sizes can be adjusted to achieve the desired sensitivity but typically 0.1 to 5 cc of gas can be removed from the sample container.

The best procedure is to first fill the gas-tight syringe with a volume of NO-free gas. The syringe is then inserted into the sealed container and the plunger depressed to inject the NO-free gas into the sealed container. The plunger is then withdrawn to remove an equal volume of headspace gas. If the container is not first pressurize, it will not be possible to withdraw gas from the sealed container. Since removal of the gas will change the equilibrium, normally only one sample of headspace can be collected from a sealed container.

The gas sample is then injected into the purge vessel and the Liquid program used to collect the data and integrate the peaks. In order to obtain a sharp NO peak, a sample should be injected as fast as possible.

Gas-tight syringes equipped with shut-off valves are ideal for headspace measurements. With these syringes, the shut-off valve can be closed and the plunger depressed to pressurize the gas in the syringe before injection. After inserting the needle through the septum of the purge vessel, the shut-off valve is then opened and the plunger fully depressed to inject the sample. Gas-tight syringes usually have large needles that can core the septum in the purge vessel, resulting in clogging of the needle or frequent replacement of the septum. Side-port needles are available for most gas-tight syringes that will reducing septum coring and clogging of the needle.

Preparation of Standards for Headspace

There are several different techniques that can be used to prepare calibrations curves for quantitating headspace samples. The simplest approach is to use NO calibration gas. By injecting different volumes of the calibration gas, a calibration of peak area versus moles NO injected can be prepared. The amount of NO injected is calculated from the volume injected, the concentration of the standard and the ideal gas law. This technique allows quantitation of the amount of NO in the headspace above the samples, but cannot be used to determine the concentration of NO in the liquid phase.

Another approach for standards is to prepare sealed containers with the volume of water or media as is present in the samples. NO from a calibration gas is then injected into the liquid phase and the container equilibrated. A sample of the gas headspace is then withdrawn and analyzed in the same manner as the samples. From the volume of the liquid in the container and the amount of NO added, the concentration of NO in the liquid phase can be calculated. This same approach can be used with NO-donor compounds rather than NO gas. A known concentration of an NO donor (*e.g.*, SNAP, SNP, nonoates) is added to the sealed container, the sample is equilibrated and a headspace sample collected as described above.

Dynamic Headspace Analysis

The technique described above is often referred to as static headspace, since the sample container is sealed, an equilibrium established between NO in the gas and liquid phases and an aliquot of the gas sampled. In dynamic headspace, the entire gas above the liquid is measured. A special container equipped with a gas inlet and outlet is required for dynamic headspace. In this technique, NO can either be continuously monitored or the sample allowed to equilibrate and then the entire gas sample is injected into the NOA. For this technique, the purge vessel is not used, instead the outlet of the apparatus is connected directly to the gas sampling package of the NOA. A source of NO free gas is connected to the container and is used to sweep the gas headspace from the container into the NOA. Contact

GE Analytical Instruments for more details and specific references on dynamic headspace analysis.

15 MAINTENANCE

There are four major maintenance items for the NOA:

- Vacuum pump oil
- Hopcalite trap
- Reaction cell
- PMT cooler.

Periodically cleaning or replacing the frit restrictor, IFD filter for the purge vessel, the in-line filter of the gas sampling package and the bacterial filter will also be required.

The microprocessor keeps track of the usage of the NOA and will report warnings when it is time to service the analyzer. There are two warnings for each maintenance item. The first warning indicates that it is near the time to service the item and is reported 10 days before the service is due. The second warning is reported when service is due and will continue to be reported until the analyzer is serviced and the timer is reset.

The maintenance schedule is 900 hours for pump oil, chemical trap and reaction cell cleaning. Service on the cooler is required after one year of operation. The pump oil timer is on whenever the vacuum pump is on. The timers for the reaction cell cleaning and Hopcalite trap are on whenever the NOA is in the Start mode.

Changing the Vacuum Pump Oil

A flat-head screwdriver, a funnel, a container for the waste oil and ~1/2 quart of Edwards Ultra vacuum pump oil will be required to change the oil. It is best to change the oil when the pump is still warm to help remove all impurities in the oil. If the NOA has been in the Stop mode, set the analyzer to the Stand-by mode and let the vacuum pump run for 1/2 hour before changing the oil. If the NOA has been in the Start or Stand-by mode pump then simply set the NOA to the Stop mode. It simplifies changing the oil if the pump is elevated. Place the pump on a lab bench or other elevated surface. If necessary, loosen the hose clamp on the vacuum tube connection from the

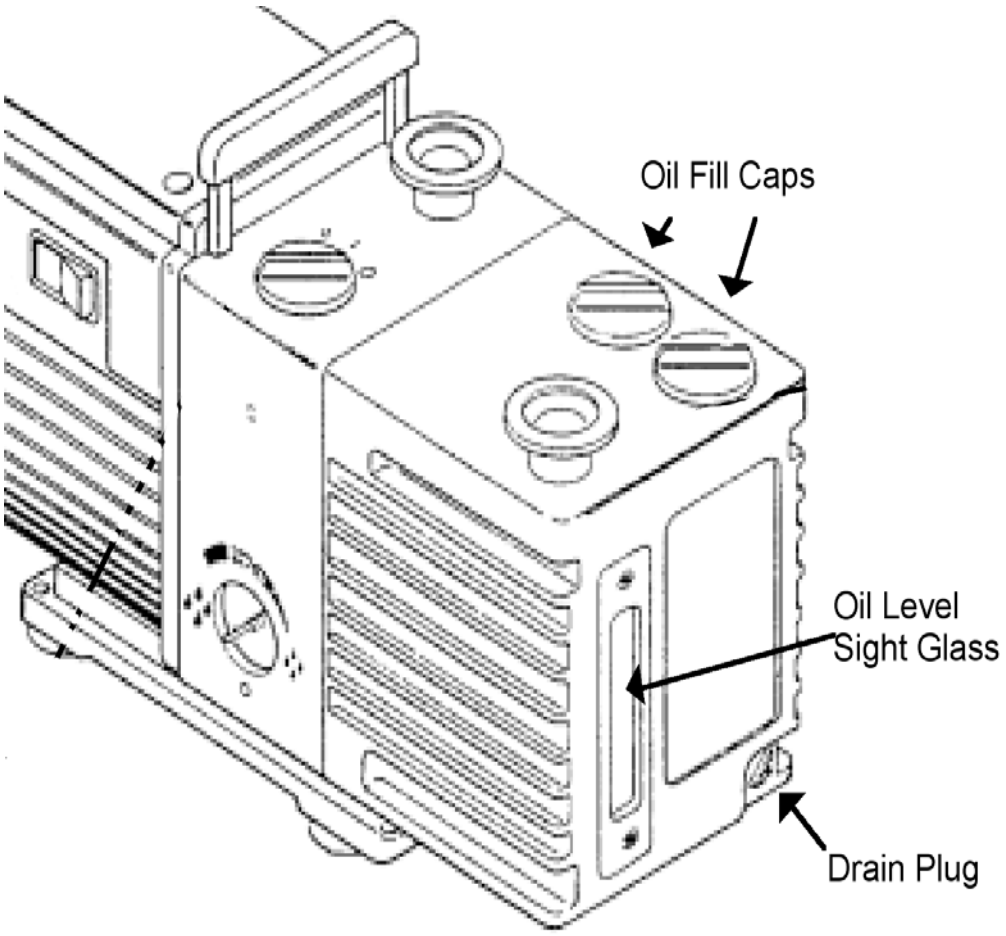


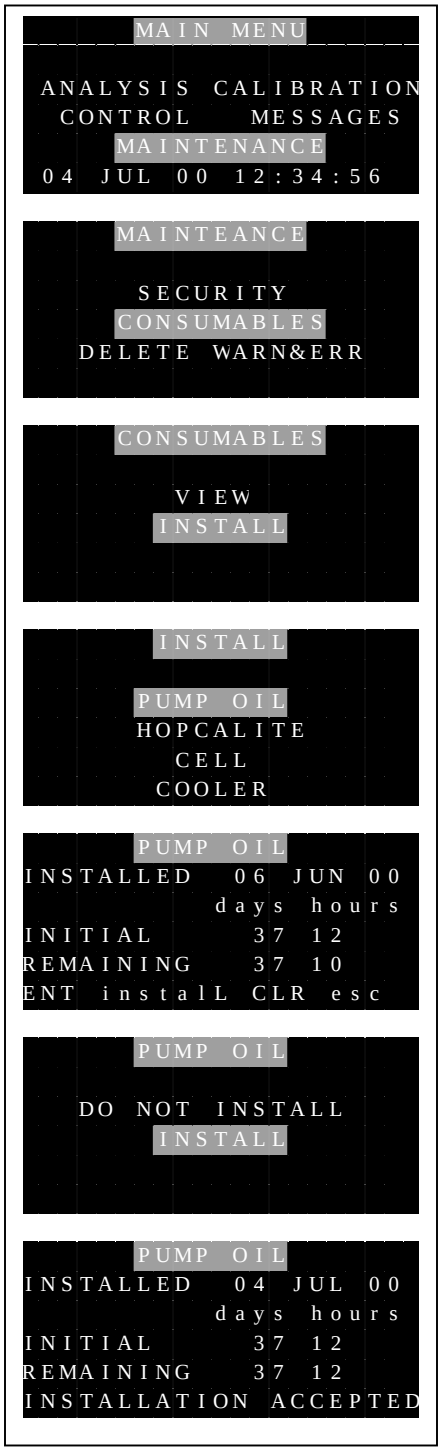
Figure 15-1: *Location of Oil Fill and Drain Plugs*

chemical trap to the NOA and remove the tubing from the trap. Figure 15-1 shows the oil fill and oil drain plugs on the vacuum pump.

To change the oil:

- Open the oil fill cap and set aside.
- Position the waste oil container and funnel under the oil drain plug.
- Use a screwdriver to slowly open the oil drain plug by turning the screwdriver counterclockwise.
- When the plug is removed, the oil will immediately flow out the drain.
- Tilt the pump to help drain all of the oil from the pump. The oil can usually be disposed at commercial motor oil recycling centers.

- Make sure the o-ring is present on the oil drain plug and reinstall the plug using a screwdriver to secure the plug.
- Fill the pump with ~1/2 quart of fresh Edwards oil. View the oil level in the window on the pump. Add enough oil so that the level is above the minimum level mark, but below the maximum level mark.
- Replace the oil fill cap.
- If the vacuum hose was disconnected, reconnect the hose and secure with the hose clamp.
- Follow the procedures below to reset the timer for the pump.



To reset the timers for the consumables, the NOA must be in the Stop or Stand-by mode. From the Main Menu, use the Arrow Buttons to scroll to Maintenance and press ENTER. The Maintenance Menu is displayed. Use the Arrow Buttons to scroll to Consumables and press ENTER. The display will change giving the option to view or install the consumables. Select Install and press ENTER to display the Consumables Menu. Select the Pump Oil option and press ENTER to display the Pump Oil Menu. Press ENTER to display a confirmation screen. Select Install and press ENTER to reset the Pump Oil Timer. The Pump Oil Menu will be displayed with the remaining time reset to 900 hours.

Changing the Hopcalite Trap

The chemical trap contains an ozone destruction material Hopcalite and is consumed when the ozone is removed. In order to protect the pump, it is vital

that this trap be replaced when indicated. A replacement trap and a 5/16" hexdriver or flathead screwdriver is required. To change the trap:

- Set the NOA to the Stop mode and wait until the pump is off.
- Use the hexdriver to loosen the hose clamps on the vacuum tube leading to the NOA, and the clamp on the clear Tygon tubing leading to the pump. Remove the trap from the securing clamp on the pump.
- Remove the old trap from the tubing. A Material Safety Data Sheet is included with the traps and provides guidelines for proper disposal of the traps. Contact your safety or environmental health department with any questions on disposal of the trap.
- Remove the plastic caps from the ends of the new chemical trap. Hold the trap vertically and gently tap on the trap to remove any dust. Turn the trap upside down and repeat the process to remove any dust from the other end of the trap.
- Place the hose clamp over the short length of clear Tygon tubing and connect the elbow end of the trap. Secure the hose clamp with the hexdriver.
- Place a hose clamp over the black vacuum hose leading to the NOA and connect the remaining end of the trap to the tubing. Secure the hose clamp with the hexdriver.
- Press the trap into the c-clamp on the vacuum pump.
- Follow the procedures below to reset the timer for the trap.

From the Main Menu, use the Arrow Buttons to scroll to Maintenance and press ENTER. The Maintenance Menu is displayed. Use the Arrow Buttons to scroll to Consumables and press ENTER. The display will change giving the option to view or install the consumables. Select Install and press ENTER to display the Consumables Menu. Select the Hopcalite option and press ENTER to display the Hopcalite Menu. Press ENTER to display a confirmation screen. Select Install and press ENTER to reset the Hopcalite Timer. The Hopcalite Menu will be displayed with the remaining time reset to 90 days.

Cleaning the Chemiluminescence Reaction Cell

Ozone will react with organic compounds in air to form an opaque film on the optical filter and the walls of the reaction cell. Over time, this film will block the transmission of light and cause a loss in sensitivity. In addition, any liquid that gets into the reaction cell will also stain the filter and cell, and reduce sensitivity. In order to restore sensitivity, it is necessary to clean the optical filter and reaction cell. The recommended schedule is 900 hours of operation with the ozone generator on. However, it may be necessary to clean the cell more frequently, particularly if any liquid is drawn into the analyzer. If measuring gas-phase NO, a calibration outside recommended limits may also indicate a dirty reaction cell. Also, any loss in sensitivity for measurement of nitrite or nitrate may indicate that it is time to clean the cell. To clean the cell, it is necessary to allow the PMT temperature to rise to ambient, and then remove the PMT from the analyzer. A Phillips-head screwdriver, the Allen wrench and PMT cap from the accessories kit for the NOA, an alcohol wipe or a clean cloth and some reagent grade methanol or ethanol will be needed. It is not necessary to disconnect the vacuum hose from the NOA to clean the cell. To clean the cell:

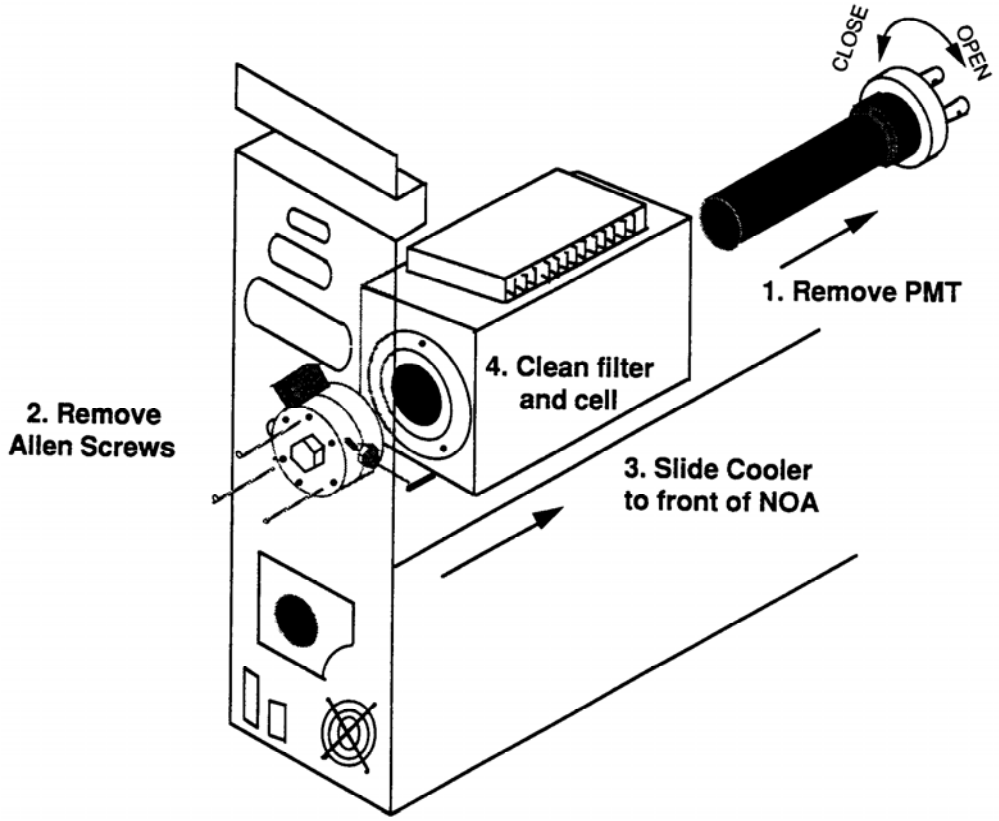


Figure 15-2: *Schematic of NOA-Procedure for Cleaning Reaction Cell.*

- Turn off the main power switch and remove the AC power cord from the power entry module.
- Allow the analyzer to stand for a couple of hours (overnight is best) so the PMT can warm to room temperature.
- Make sure the power cord is disconnected, and then remove the NOA cover by removing the Phillips-head screws on the side of the analyzer.
- Carefully lift the cover off the NOA.
- Remove the two coaxial cables connected to the PMT socket.
- In order to minimize exposure of the PMT to light, turn off the room lights before removing the PMT.
- Unscrew the PMT socket counterclockwise until the socket is clear of the cooler.
- Remove the PMT from the cooler, but leave the PMT in the socket.

- Place the black, PMT cap over the end of the PMT, being careful not to touch the face of the PMT. Once the cap is in place, the room lights can be turned back on.
- Use a paper towel to remove any moisture from the outside of the PMT and from the inside of the cooler.
- Place the PMT in a drawer or other dark location while cleaning the reaction cell.

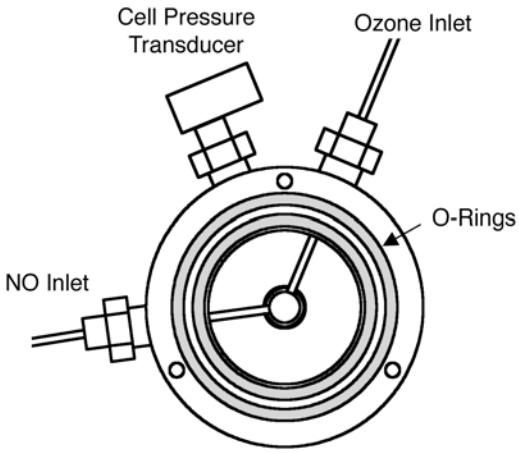


Figure 15-3: *Reaction Cell as viewed from PMT side.*

- Use the Allen wrench to loosen the three Allen screws holding the reaction chamber to the cooler. Remove the screws from the reaction cell. The cell is still attached to the back panel of the NOA by the three Phillips-head screws.
- The cooler is mounted in a slotted bracket with four screws and washers to hold the cooler in place. Carefully slide the cooler toward the front of the NOA. The optical filter is not attached at this point and may fall out of the cooler if the cooler is moved too rapidly. The cooler should slide far enough forward to give access to the filter and the reaction cell.
- With the filter still in the cooler, use an alcohol wipe or wet a clean cloth with methanol or ethanol and wipe the deposits from the surface of the red filter. If the filter is removed from the cooler, be sure to remove any fingerprints from the filter when reinstalling the filter in its holder.
- Use an alcohol wipe or wet a clean cloth with methanol and remove any deposits in the reaction cell, being careful to not bend the tubing in the cell or break the plastic pressure transducer. Typically, deposits will form on the wall opposite of where the NO and O_2 tubes enter the reaction cell.
- If NaOH has gotten into the reaction cell, it may be necessary the rinse the NO inlet tubing and bulkhead fitting with deionized water. Use a 7/16" wrench to loosen the nut from the inside of the bulkhead fitting, then use a 1/4" wrench to loosen the

1/16" nut at the reaction cell. Carefully pull the 1/16" tubing out of the reaction cell and rinse thoroughly with deionized water. Remove the frit restrictor and use a squeeze bottle to rinse the bulkhead fitting on the back of the NOA. Replace the tubing in the reaction chamber, tighten the 1/16" nut hand tight, connect the 1/8" nut to the bulkhead, then secure both the 1/8 and 1/16" nuts with wrenches. Replace the frit restrictor and tighten with a wrench.

- There are two o-rings in the reaction cell. The inner o-ring provides the vacuum seal and the outer o-ring provides the light-tight seal. These o-rings must be seated in the o-ring grooves of the reaction cell. If the o-rings fall out, carefully reseat them in the grooves. If the o-rings do not stay in the grooves, a very small amount of silicone vacuum grease can be applied to the o-rings to help secure them. Be sure to wipe any excess grease from the o-rings before replacing the cooler.
- After cleaning the filter and reaction cell, carefully slide the cooler being sure that the o-rings and red filter remain in place. Slide the cooler back until it contacts the o-rings on the reaction cell.
- Insert one of the Allen screws into the back panel and through the reaction cell. The screw should be aligned with the threaded hole in the cooler. If aligned, then carefully thread the screw by hand into the cooler. If the hole is not aligned, adjust the position of the cooler to align the hole with the screw.
- Follow the same procedure to install the remaining two Allen screws, carefully tightening the screw by hand.
- Once all three screws are started, use the Allen wrench to tighten the screws. **Do not overtighten the screws; the optical filter will break!** The best procedure is to hold the Allen wrench by the sort arm, then tighten one screw 2-3 turns, tighten the next screw 2-3 turns, then tighten the last screw 2-3 turn. Continue alternatively tightening the three screws until tight.
- If the vacuum hose was removed from the exhaust of the NOA, replace the hose and tighten with a wrench.

- Turn the room lights off, remove the PMT from the drawer and remove the black cap being careful not to touch the face of the PMT. If the face of the PMT is touched, use a clean, dry cloth to remove any fingerprints.
- Insert the PMT into the cooler and reinstall the socket by turning the socket clockwise. Tighten the socket until it can no longer be turned by hand.
- Reconnect the high voltage and signal cables to the PMT. The connectors are different sizes and the cables can only be connected one way.
- Position the cover for the NOA so that the slots on the side of the cover are pointing towards to back of the NOA. Place the cover over the NOA, being careful to clear all of the wires on both sides of the analyzer. Position the back of the cover on top of the back panel of the NOA. Position the front of the cover so that it rests on the front panel of the NOA. Move the front and rear panels a little to help align the cover.

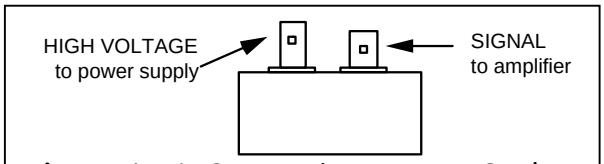


Figure 15-4: *Connections to PMT Socket.*

- With the cover resting on top of both the front and rear panels, position the front panel to align the front, right-hand screw holes in the front of the analyzer and install a screw a few turns. Repeat the process to align the top right-hand screw at the back of the analyzer and partially install a screw.
- Repeat this procedure to align the holes on the top, left-hand side of the analyzer (front and back) and partially install the screws.
- Align the bottom screw holes and partially install the screws.
- Repeat the process until all screws are partially installed. If the screw holes do not all line-up, the cover has been installed backwards. Remove all of the screws, turn the cover around and repeat the process.
- After all of the screws have been installed, use a Phillips-head screwdriver to tighten the cover.
- Reconnect the power cord and turn the main power switch on.

Vacuum Test

After the reaction cell has been cleaned, repeat the vacuum test described in Chapter 4 (page 30), to make sure the o-rings are properly positioned in the reaction cell. With the sample inlet and ozone gas supply inlet capped off, the cell pressure should drop to <1 torr. If the pressure is above 1 torr, there may be a leak due to the o-ring being out of its groove. Follow the procedures described above to remove the PMT, disconnect the cooler from the reaction cell and slide the cooler away from the cell. Check the position of the o-rings, reconnect the cooler and replace the PMT. Repeat the vacuum test after reconnecting the reaction cell.

Reset the Cell Cleaning Timer

To reset the timers for the Reaction Cell, the NOA must be in the Stop or Stand-by mode. From the Main Menu, use the Arrow Buttons to scroll to Maintenance and press ENTER. The Maintenance Menu is displayed. Use the Arrow Buttons to scroll to Consumables and press ENTER. The display will change giving the option to view or install the consumables. Select Install and press ENTER to display the Consumables Menu. Select the Cell option and press ENTER to display the Reaction Cell Menu. Press ENTER to display a confirmation screen. Select Install and press ENTER to reset the Reaction Cell Timer. The Reaction Cell Menu will be displayed with the remaining time reset to 900 hours.

Light Leak Test

After the temperature of the cooler has stabilized at –12 °C, check that the o-rings are properly positioned by checking if ambient light is getting into the reaction cell. To perform the light leak test:

- Turn on the oxygen supply to the NOA.
- From the Main Menu, press CLEAR to display the Status screen.
- Confirm that the Cooler Temperature is at –12 °C and the supply pressure is 6 psi.
- Press ENTER or CLEAR to return to the Main Menu, select Analysis and Start.
- When the Startup Screen is displayed, press Enter to display the Status Screen.

- The NOA will turn on the high voltage to the PMT and display the PMT signal (counts at analog-to-digital converter) in the Status Screen. Typically, the PMT signal will be 10 – 50 counts and should always be < 100 counts. If there is a light leak, the PMT signal will be higher than normal and the NOA may fail the PMT test.
- If the signal is higher than normal, turn off the room lights and see if the PMT signal decreases. If tuning the lights off does not change the PMT signal, then there is not a light leak.
- If the PMT signal does change when the lights are turned off, try tightening the Allen screws 1/2 to 1 turn and repeat the lights on/off test.
- If there is still a light leak after tightening the Allen screws, turn off the NOA, remove the top cover, remove the PMT and check the o-rings in the reaction cell. Reconnect the cooler to the reaction cell, install the PMT and top cover and repeat the light-leak test.

Cooler Maintenance

Over time, the cooler will pick up water from the atmosphere that reduces its efficiency. After one year of operation, it is necessary to remove the cooler from the NOA and use an oven to remove water that has accumulated in the insulation. To bake-out the cooler:

- Turn off the main power switch and remove the NOA top cover.
- Follow the procedures described above to remove the PMT and socket from the cooler and to disconnect the cooler from the reaction cell. Remove the optical filter from the cooler face.
- Slide the cooler all the way towards the front and the NOA.
- There are three electrical connections to the cooler: the fan power, power to the cooler and the thermocouple. The fan connector is located on the left side of the NOA as viewed from the front. Trace the wires from the fan to a small black connector, hold the tab on the connector down and pull the connector apart. The thermocouple is connected to the analog board on the right side of the NOA using a large green connector (See Figure 15-5). Hold the board and remove the green

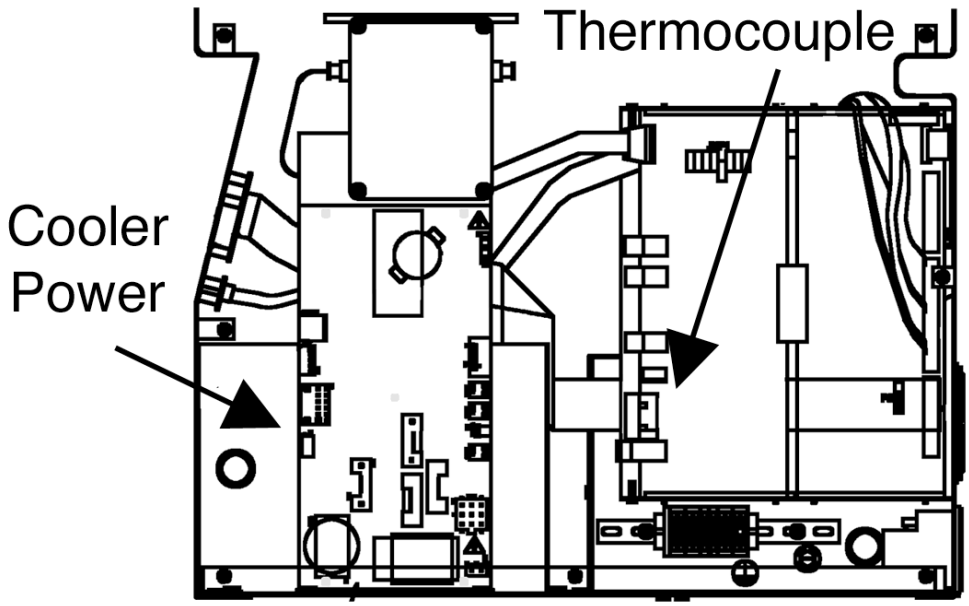


Figure 15- 5: *Electrical Connections for Cooler (fan connection not shown)*

connector from the board. The power for the cooler is connected to the Power Supply board (P5 on power supply board). Disconnect the cooler power supply by pressing the two tabs on the top and bottom of the connector and pulling the connector off the board. Feed the thermocouple connector and the cooler power connector over to the left side of the NOA.

- Lift the cooler from the chassis. It may be necessary to move the cooler slightly towards the back to the NOA to align the cooler with the holes in the chassis.
- Place the cooler in a drying oven set to 90 °C for at least 2 hours, preferably overnight. Allow the oven to cool before removing the cooler.
- To install the cooler, hold it above the chassis with the electrical connectors toward the left side of the analyzer. Set the cooler onto the chassis, ensuring that the four bolts go through the keyholes in the chassis. Make sure the cooler can slide back and forth.
- Reconnect the thermocouple to the analog board on the right-hand side of the NOA and reconnect the cooler power connector to the power supply board. Reconnect the fan to the black connector on the left side of the analyzer.

- Clean the optical filter as described in the cell cleaning section and reinstall the glass in the cooler. Slide the cooler back towards the reactions cell and install the Allen screws.
- Reinstall the PMT and connect the high voltage and signal cables.
- This completes the cooler maintenance. Replace the NOA top cover and turn on the Main Power Switch. When the Main Menu is displayed, scroll to Maintenance and press ENTER. Scroll to Consumables and press ENTER. Select Install and press ENTER to display the Install Consumables Menu. Scroll to Cooler and press ENTER Press ENTER to display a confirmation screen. Select Install and press ENTER to reset the cooler timer.
- After the PMT has cooled to -12 °C, perform a vacuum test and light-leak test as described in the cell cleaning sections.

Testing and Cleaning the Flow Restrictor Frit

Over time, the metal frit in the flow restrictor at the back of the NOA can become clogged, reducing the flow into the NOA. The flow can be checked using a flow meter or by simply monitoring the reaction cell pressure. To check the frit, disconnect the purge vessel or gas sampling package from the inlet to the restrictor so that the restrictor is open to the atmosphere. If not already in the Start or Stand-by mode, select one of these modes in the Analysis Menu and monitor the cell pressure either in the Measurement Menu (Nitric Oxide Mode only) or in the Control/Status Menu.

Normally, the cell pressure when the restrictor is open to the atmosphere will be in the range of 4-7 torr. If restrictor is clogged, the pressure will be <4 torr, typically less than 3.5 torr. If the cell pressure is low with the restrictor open to the atmosphere, attempt to clean the frit as described below.

Set the NOA to the Stop mode and wait until the purging cycle is finished and the pump is off. Use a 7/16" wrench to remove the restrictor from the sample inlet of the NOA back panel. Use a squeeze bottle to fill the metal tubing on the NOA's side of the restrictor with water, then connect the 1/8" nut to a high-pressure gas source. Turn on

the gas the force the water back through the frit. Repeat this several times to backflush the frit with water and retest the flow.

If backflushing with water does not remove the clog and an ultrasonic cleaner is available, place the restrictor in the ultrasonic bath using water or a detergent and sonicate for 10-15 minutes to dislodge any particles that may be blocking the frit. Rinse the restrictor thoroughly with deionized water. Connect the restrictor to a high pressure gas supply and backflush the restrictor with high pressure gas to remove any water and particles that remain in the restrictor. Reconnect the restrictor to the NOA and repeat the flow check. In some cases, sonication will not remove the blockage and the restrictor will have to be replaced.

Gas Sampling Particle Filter

The filter in the gas-sampling package will get clogged over time and needs to be replaced. The filter should be replaced when it becomes visibly dirty or wet. The cell pressure can be used to test if the filter is partially clogged. Place the NOA in the Start or Stand-by Mode and monitor the reaction cell pressure in the Measurement Menu or Status Menu. Note the cell pressure with the filter in-line and then remove the filter. Normally the cell pressure will increase by ~0.1 torr when the filter is removed. If a larger increase in cell pressure is observed, replace the filter. These filters are disposable and are not to be cleaned. Replacement filters are available from GE Analytical Instruments (HFL 01410) and from most laboratory supply houses. The filter is Teflon to help block liquid from getting into the restrictor and reaction cell.

Security

The security features for the NOA must be enabled by GE Analytical Instruments personnel during installation. Once enabled, passwords are required to change the setup of the NOA, delete methods, clear error and warning stacks, etc. Use of these features is not recommended.

16 TROUBLESHOOTING

The NOA's microprocessor monitors the operation and usage of the instrument and if there is a problem, reports an error or warning. Errors are serious problems and if the NOA is in the Start mode, detection of an error will cause the analyzer to switch to the Stop mode and immediately display the error stack. The cause of the problem must be determined and the situation remedied before restarting the NOA. Warnings are less severe problems and when a warning condition is detected, the NOA will indicate the problem by activating the Warning shortcut in the Measurement Menu. Use this shortcut to view the warning and when appropriate, take corrective action.

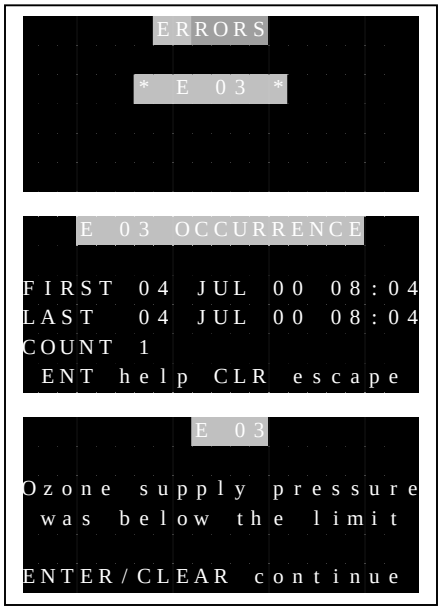
The NOA will also monitor certain parameters during the initial startup and if there is a problem, will report that the instrument has failed a startup test, without reporting an error. The cause of the problem must be determined and the situation remedied before the NOA can be started.

Errors

A list of possible error conditions and the required actions to remedy the situation have been described. When an error is detected, the NOA will switch to the Errors Menu. This menu uses asterisks to indicate active errors. Check the nature of the error condition, by scrolling to the error and pressing ENTER to display the Error Occurrence Menu.

This menu shows the first and last occurrence of this particular error and the number of times this error was detected. By pressing ENTER, a brief help screen is shown indicating the nature of the error.

After viewing the error occurrence and help screen, press ENTER or CLEAR to return to the Error Menu. After viewing an error, the asterisks in the Error Menu are removed.



A list of errors that have been detected will remain in the error stack until the error stack is cleared as described below.

Possible Errors and Remedies

E 01 – Setup Data Corrupted, Check Before Running

When the power to the NOA is first turned on, the program checks the data stored in the battery-backed RAM. If there is a problem with this data, E 01 is reported. This error may be generated when installing a new version of the firmware. In most cases, continue running after getting this error, but if the error continues, contact GE Analytical Instruments.

E 02 – Cell Pressure was Above the Limit

When the NOA is running, this error is reported if the cell pressure rises above 100 torr. Make sure that the main power switch on the vacuum pump is in the on position, the power cord from the pump is securely plugged into the rear of the NOA and the vacuum hose connections to the NOA and Hopcalite trap are secure. Select the Analysis/Stand-by Mode to start the vacuum pump without getting this error and monitor the cell pressure in the Control/Status menu to help locate any leaks.

E 03 – Ozone Supply Pressure was Below the Limit

The NOA monitors the supply pressure of oxygen to the ozone generator and if the pressure drops below 4 psi, E 03 is reported. This error will be reported when the oxygen cylinder is empty or if there is no oxygen flow into the NOA. Make sure that the oxygen tank contains gas, the main valve is turned on and the outlet pressure on the regulator is set to > 6 psig. If an E size cylinder and flow controller are being used, make sure the tank is on and the flow controller is set to a non-zero flow. Also check the tubing from the oxygen tank to the oxygen inlet at the rear of the NOA and check that the oxygen supply regulator at the rear of the NOA is adjusted.

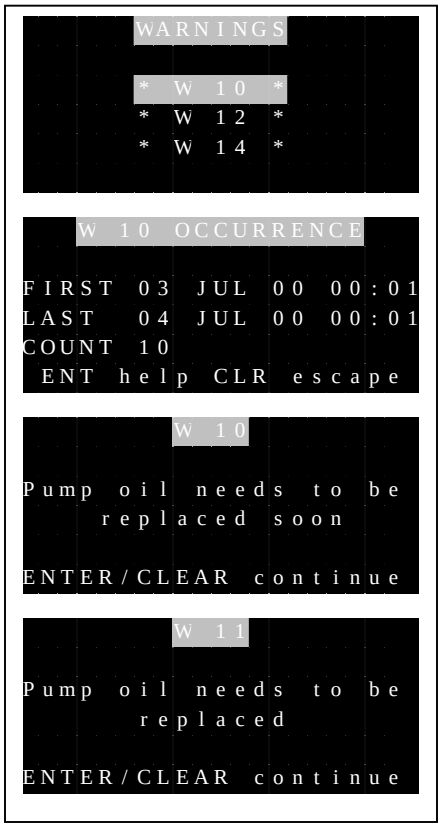
E 05 – Cooler Temperature Above the Limit

This error is reported if the temperature of the cooler is outside of the range –12 °C ± 2 °C. The most likely cause is the cooler need maintenance (see Cooler Maintenance page 159). This error can also be reported if the cooler power cable is not connected to the Power Supply Board, the thermocouple is not connected to the Analog Board or if the fan is not powered. Check all of the electrical connections and if the connections are OK, contact GE Analytical Instruments.

Warnings

Less serious problems with the NOA are indicated by Warnings. Unlike Errors that stop the analysis and require immediate attention, Warnings indicate when it is close to the time to replace the consumables and perform maintenance.

When a warning condition is detected, the NOA will display the Warning Short Cut in the Measurement Menu. To view the warning, use the DOWN arrow button to scroll to WARN and press ENTER. The History/Warning Menu will be displayed. Active Warnings are indicated by asterisks before and after the warning. After viewing the warning, the asterisks will be removed.



To view a warning, use the arrow buttons to scroll to the warning and press ENTER. An information screen will be displayed indicating the nature of the warning. Press CLEAR or ENTER to exit the information screen and return to the Warning Menu. A list of possible warnings and corrective actions are listed below.

W 09–Pump Oil Needs to be Replaced

The pump oil has exceeded its recommended lifetime. Change the oil and reset the counter. This warning will be reported each day until the oil is changed.

W 10–Pump Oil Needs to be Replaced Soon

This warning will be reported with 90% of the oil lifetime has expired. It will be reported each day until the oil is changed and the counter reset or the lifetime of the oil is reached.

W 11–Hopcalite Needs to be Replaced

The ozone destruction trap has exceeded its recommended lifetime. Replace the trap and reset the timer. This warning will be repeated each day until the trap is replaced.

W 12–Hopcalite Needs to be Replaced Soon

The ozone destruction trap has reached 90% of its lifetime. This warning will be reported each day until the trap is replaced or the lifetime is exceeded.

W 13–Reaction Cell Needs to be Cleaned

The reaction cell has not been cleaned within the recommended interval. Clean the reaction cell and reset the timer. This warning will be repeated each day.

W 14–Reaction Cell Needs to be Cleaned Soon

The reaction cell counter has reach 90% of the recommended interval. This warning will be repeated each day until the cell is cleaned and counter reset if the interval is exceeded.

W 15–Cooler Needs to be Serviced

The recommended interval for baking out the cooler has been exceeded. The warning will be repeated each day until the service is performed and the counter reset.

W 16–Cooler Needs to be Serviced Soon

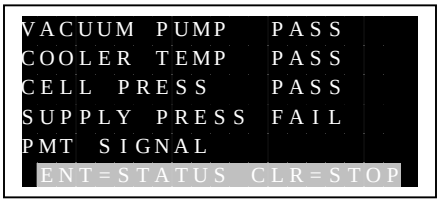
The counter for PMT cooler service has reach 90% of the recommended interval. This warning will be repeated each day until the service is performed and the counter reset or the interval is exceeded.

Clearing the Error and Warning Stacks

One of the options under the Maintenance Menus is Delete Warn&Err. This option can be used to delete any or all errors and warnings that had been detected. While it is not necessary to clear the stacks, it is usually best to delete the error to avoid any confusion from past errors. After viewing an active error or warning, the error/warning can remain in the stack. The next time the error or warning is detected, it will be activated (as indicated by the asterisks), the last occurrence date updated and the count incremented. Active errors or warnings are always listed on the top row, so it is not necessary to scroll down to see active errors or warnings. To clear the error or warning stack, select the Maintenance Menu, scroll to Delete Errors & Warnings and press ENTER. Select Delete Errors or Delete Warning.

A list of the Errors or Warnings in the stack is displayed. Select the item to be deleted, press ENTER. A confirmation screen is displayed, select Delete to remove this item from the stack.

Start-up Tests



Errors are only reported after the NOA has passed the Start-up Tests. During start-up, problems with the cooler, vacuum pump, the oxygen supply to the ozone generator will cause the NOA to display Fail in the Start-up Screen for these tests. Unlike errors, the NOA will not go into the Stop mode, but delay start-up until the problem is solved.

Vacuum Pump

Make sure that the main power switch on the vacuum pump is in the on position, the power cord from the pump is securely plugged into the rear of the NOA and the vacuum hose connections to the NOA and Hopcalite trap are secure.

Cooler Temp

Make sure that sufficient time has been given to allow the cooler to reach temperature if the NOA's power has been off. It may take 15-20 minutes for the cooler to reach –12 °C. If the cooler is not cooling, check the electrical connections to the cooler. The NOA may also fail this test when cooler maintenance is required.

Supply Pressure

Make sure that the oxygen tank contains gas, the main valve is turned on and the outlet pressure on the regulator is set to > 6 psig. If an E size cylinder and flow controller are being used, make sure the tank is on and the flow controller is set to a non-zero flow. Also check the tubing from the oxygen tank to the oxygen inlet at the rear of the NOA and check that the oxygen supply regulator at the rear of the NOA is adjusted.

PMT Signal

There are several reasons why the NOA may fail the PMT test including a light leak, a blown fuse for the ozone generator, a dirty reaction cell or problems with the PMT or electronics. A light leak will be indicated by a higher than usual PMT signal in the Status screen (see Light Leak Test, page 158). In some cases, it may be possible to get the NOA to pass this test by connecting a NO calibration gas to the sample inlet (or by blowing into the sample line). If supplying a high NO sample allows the NOA to pass the test, the reaction cell may need cleaning.

If there is no change in the PMT signal with and without ozone, the ozone fuse may be blown. To check the ozone fuse:

- Turn the Main Power Switch to OFF, remove the power cord from the power entry module and remove the NOA top cover.
- Remove the ozone generator fuse holder from the right-hand side of the NOA, near the back.
- Remove the fuse from the holder and use an ohm meter to test the resistance of the fuse. If the fuse is blown then the resistance will be infinite.
- If the fuse is blown, replace it with one of identical type and current rating. Refer to the System Specification section of this manual for a listing of fuse types and ratings appropriate for the NOA.
- If the fuse is OK, replace the fuse in the holder, reinstall the fuse holder in the back panel, replace the top cover, plug-in the power cord, and turn the Main Power Switch ON.

If the ozone fuse is OK, then the problem may be a clogged restrictor. There are two restrictors in the ozone flow path, a pre-restrictor before the ozone generator and a post-restrictor that delivers the ozone to the reaction cell. To test for a clogged restrictor:

- From the Main Menu, press ENTER to display the Status Screen.
- Note the supply pressure.
- Decrease the pressure to the ozone generator by turning the regulator on the NOA back panel clockwise (as viewed from the front).
- Check the pressure in the Status Menu, the pressure should drop as the pressure on the regulator is released.

If the pressure drops, the restrictors are not clogged and the problem is most likely the ozone generator, the PMT or the electronics. Contact GE Analytical Instruments for additional instructions on troubleshooting the problem.

Troubleshooting the NOA

In addition to start-up tests, errors and warnings, there are several other things that can cause problems with the NOA or applications (gas measurements. liquid

measurements using the purge vessel, etc.). The first step in troubleshooting is to isolate the cause of the problem to either the sample inlet system or the chemiluminescence detector. A list of possible problems with the chemiluminescence detector appears below. If the problem is not listed below or assistance is needed, contact GE Analytical Instruments.

No Power to NOA

When the main power switch is turned on, if there is no power to the analyzer, as indicated by the absence of backlighting or characters on the front panel display and the cooler and back panel fans are not operating, then the problem is most likely a blown fuse. The main fuse for the NOA is located in the power entry module at the rear of the NOA. Remove the power cord from the analyzer to open the power entry module. To check the main fuse on the NOA:

- Remove the power cord from the analyzer.
- Open the power entry module by inserting a small screwdriver behind the slot at the top of the power entry module, and lifting the cover on the power entry module back away from the NOA back panel.
- Pull the red fuse holder out of the power entry module. The holder contains two fuses.
- Remove the fuses from the holder. Use an ohm meter to test the resistance of each fuse. If a fuse is blown, then the resistance will be infinite.
- If a fuse is blown, replace it with one of identical type and current rating. Refer to the System Specification section of this manual for a listing of fuse types and ratings appropriate the NOA 280i.
- If the fuse is OK, replace the fuse, reinstall the fuse holder in the power entry module and replace the lid on the power entry module. Contact GE Analytical Instruments for additional instructions.

No Display

If there is power to the NOA as indicated by operating fans, but the display is blank then either the fuse on the Power Supply Board is blown, the cables are not connected or the contrast needs adjustment.

To check the fuse on the Power Supply Board:

- Remove the power cord from the analyzer.
- Remove the NOA top cover.
- Locate the Power Supply Board on the front right-hand side of the NOA (see Figure 15-5). The round fuse is located on the left side of the board, near the top (just below TP3 HV Adj.).
- Remove the fuses from the board. Use an ohm meter to test the resistance of the fuse. If the fuse is blown, then the resistance will be infinite.
- If a fuse is blown, replace it with one of identical type and current rating. Refer to the System Specification section of this manual for a listing of fuse types and ratings appropriate the NOA 280i.

To check the Cables:

- Remove the power cord from the analyzer and remove the NOA top cover.
- A ribbon cable connects to the LCD display and the Digital Board (last board on right-hand side of NOA). Ensure that the cables are secure in the connectors.

If the fuse is OK and the cables are secure, the contrast may need adjustment. This requires that the NOA be turned on with the top cover removed (Warning: Hazardous Volatages are present!) To Adjust the Contrast:

- Turn off the NOA power and remove the top cover.
- Locate the Contrast Adjustment on the Digital Board (small white screw near bottom, right corner of board).

- Turn the NOA Power on and use a small screwdriver to turn the contrast adjustment screw while viewing the LCD display. Adjust the screw to give a clear display.

If the contrast cannot be adjusted, then the problem is with the display, the power supply board or another electrical problem. Contact GE Analytical Instruments for assistance.

Cell Pressure too High or too Low

The NOA Cell Pressure should range from 4 to 7 torr. If the pressure is > 7 torr (typically 12 torr) then the frit restrictor is not connected to the sample inlet. See Frit Restrictor, page 33 for installation of the restrictor. If the Cell Pressure is < 4 torr, the restrictor may be clogged. See Testing and Cleaning the Flow Restrictor Frit, page 161 for assistance.

Gas Sampling Problems

The most common problem encountered in gas-phase NO measurements is out of range calibration. The ppm response factor should be in the range 0.09768 – 0.1465 and the ppb response factor should be in the range 0.1953 – 0.293. If the response factors exceed the recommended limts, possible problems include:

- A dirty reaction chamber (see Cleaning the Chemiluminescence Reaction Cell, page152).
- A clogged Frit Restrictor (see Testing and Cleaning the Flow Restrictor Frit, page 161).
- Calibration Gas flow rate is too low (make sure gas is venting out the open leg of the tee).
- Incorrect concentration entered during calibration.
- Lower than expected NO concentration reaching the analyzer.

Lower than expected NO concentration can occur if the NO calibration gas is past its expiration date and for new NO cylinders and regulators. Try letting the NO calibration gas flow for 10-15 minutes and repeat the calibration.

In some cases it may be necessary to adjust the high voltage for the PMT to achieve in-range calibrations. Contact GE Analytical Instruments for assistance.

High Background NO after Calibration

After calibration, the NOA's signal should return to the background levels within 5 to 10 minutes. If something in the gas sampling package has absorbed NO, a high background will be observed and a loner time required to return to the background levels. The most common problem is a dirty Teflon filter. Try replacing the filter and see if this solves the problem.

Liquid Measurements Problems

The most common problems observed in liquid measurements are low sensitivity and poor repeatability. Both of these problems can be caused by leaks in the purge system, a dirty reaction chamber or dirty purge vessel and problems with the syringes used for injection of the samples.

Low Sensitivity

Reduced sensitivity can be cause by:

- A dirty reaction chamber (see Cleaning the Chemiluminescence Reaction Cell, page152).
- Clogged frit restrictor (see Testing and Cleaning the Flow Restrictor Frit, page 161).
- Leaks in the purge system.
- Water bath not at >90 °C (nitrate only)
- Depleted reagents.

Leaks in Purge System

Leaks can occur at the septum, the Swagelok connections to the purge vessel and gas bubbler, at the Teflon sleeve of the gas bubbler and at the IFD filter. For nitrate measurement, the best way to test for leaks is to see if the reagent will vacuum distill (see Leak Check for Purge Vessel, page129). If the reagent does not vacuum distill, there is a leak in the system.

Leaks at the Swagelok connections can occur if the connection is not tight, if the ferrules are missing or not properly position (see Figure 11-2). Another problem is glass in the reducing union of the fitting. If the end of the glass is broken, a tight connection can still be achieved provided that the Teflon ferrules are installed over the broken end and any glass is removed from inside the reducing union.

Another common location for leaks when using the gas bubbler is at the Teflon sleeve. Make sure the connection for the bubbler is tight.

Leaks can also occur at the septum. Try replacing the septum to see if this solves the problem.

Low Conversion for Nitrate

If the VCl_3/HCl reagent is oxidized or the temperature of the water bath and purge vessel is too low, the conversion of nitrate to NO will be slow causing reduced sensitivity. Make sure the temperature of the water bath is set to 95 °C so that the purge vessel will be at least 90 °C. One test for low temperature or depleted reagents is to analyze a nitrite standard using the VCl_3/HCl reagent. Since nitrite is converted by reaction with acid at room temperature, higher response for nitrite versus nitrate indicates a problem with the reagent or the water bath.

Poor Repeatability

All of the items listed (leaks, depleted reagents, etc.) above can also result in poor repeatability for replicate injections. Other causes include syringe problems, foaming of the reagent, injection technique and contamination of the samples and standards.

Syringe Problems

Over time, the needle on the syringe will be etched, particularly by the VCl_3/HCl reagent. This results in nitrate contamination of the needle leading to large peaks even when no sample is injected (needle just inserted in the purge vessel). In some cases, the needle can be cleaned by placing it in the reagent for a few minutes, then rinsing with deionized water.

Another problem that is observed is leaks in the plunger or contamination from the barrel of the syringe. Try using a new syringe to see if this solves repeatability problems.

Foaming of theVCl_3 Reagent

When samples containing protein are injected, the reagent may foam into the condenser, cooling the reagent. When the reagent stops foaming and returns to the heated portion of the purge vessel, residual nitrate will be converted to NO. The result of this foaming is multiple peaks from a single injection. If multiple peaks are observed, clean the purge vessel and replace the reagent.

Injection Technique

If the sample is not injected below the liquid level of the reagent, poor repeatability can be observed as the sample hits the walls of the purge vessel. Make sure the liquid level is high enough so that the end of the needle is below the liquid level.

Contamination

As standards and samples sit over time, NO from the air and nitrite and nitrate from the sample containers may leach into the solutions, resulting in increased peaks over time. This is particularly true for low level (< 1 µM) samples and standards. Thoroughly wash all containers with deionzed water and it may be necessary to prepare fresh standards, particularly at the lowest concentrations.

High background Signal and Rising Baselines

If there is a leak in the purge system, room air can be drawn into the NOA resulting in a high baseline signal. High baseline signals can also be observed if the purge vessel or reaction chamber is dirty, the purge gas is contaminated with NO or if the reagents are contaminated. These possible sources can be identified by recording the background without any reagents in the purge vessel or by trying another purge gas cylinder.

The most common cause of increasing baseline signals over time is injection of materials that are slowly converted to nitric oxide such as L-NAME and other nitro-containing compounds. If possible use other NOS inhibitors such as methyl arginine or other non-nitro-containing compounds (contact GE Analytical Instruments for additional information).

Ghost Peaks

When fresh reagent is added to the purge vessel, particularly the VCl_3/HCl reagent, sometimes peaks are observed even when no injection is made. These are due to slow conversion of materials in the reagents to a species that react with ozone and will usually disappear as the reagent is purged. If ghost peaks are observed, continue purging the reagent until the baseline is stable. Extra peaks are also sometimes observed as the samples are being analyzed. These are due to compounds that are slowly converted to NO from previous injections. Purging the reagent (without injections) with usually eliminate these extra peaks.

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