



# RESPIRATORY RESEARCH, INC.

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## Welcome!

Congratulations on your purchase of the RTube™ Exhaled Breath Condensate Collection System from Respiratory Research, Inc. Our mission is simple; provide world-class EBC products and solutions to the respiratory community and serve as an information resource to further aid in the development of this rapidly expanding field. This Product Information Sheet is divided into three sections: Warnings, Cautions, and Information and contains important information regarding the safe and effective use of the RTube, as well as useful general technical information on assays and usage. For more information, please visit us online at [www.RespiratoryResearch.com](http://www.RespiratoryResearch.com).



A Warning, if unheeded, could potentially affect the patient. Warnings are identified with the following symbol:



A Caution, if unheeded, could potentially affect the quality of the sample or present a hazard to the medical and laboratory staff. Cautions are identified with the following symbol:



An Information item highlights useful scientific and product knowledge. Information items are identified with the following symbol:

Please feel free to contact your local Authorized Reseller or us directly with any questions. We can be reached at:

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The RTube is CE Marked under the Medical Device Directive (93/42/EEC) and the In Vitro Diagnostic Directive (98/79 EC).

Declaration of Conformity available upon request. Please contact us to obtain this document.



**WARNING: NOT RECOMMENDED FOR USE ON UNCONSCIOUS OR INTUBATED PATIENTS DUE TO RISK OF OBSTRUCTION INSIDE THE DEVICE.**

Respiratory Research has identified several circumstances in which the RTube may block exhalation. There are no safety risks to the conscious patient as she will naturally cease attempting to exhale through the device; however, an unconscious or intubated patient will be completely dependent upon external intervention to detect and correct obstruction within the device. This complete dependence upon external intervention introduces risks and requires strict attention and extra precautions.

Respiratory Research has identified five conditions which could obstruct airflow through the RTube. These are unlikely but worthy

of mention and include:

- Improper assembly of the device by the customer attempting to rebuild and re-use
- User error in applying the end caps prior to use instead of after use
- Entry of foreign material into the device after removing from factory-sealed pouch
- Light sticking in the duckbill valve
- Improper mating of condensation chamber with the mouthpiece.

Several of these items are mitigated by following the Cautions listed in the next section.



**WARNING: DO NOT REUSE.**

The RTube is a disposable device designed for single use. It is assembled to stringent specifications and cleaned under strictly controlled clean-room conditions to ensure proper operation, safety, and cleanliness. Any subsequent rebuilding or tampering with the device will reduce the effectiveness of the device, risk the quality of your data, and potentially pose a safety risk to the patient.

Respiratory Research strongly discourages re-use of tubes. We are very sensitive to the quality of data obtained with our device and take extraordinary steps to ensure product consistency, reliability, and quality. We are meticulous in our manufacturing and quality control process to ensure a safe, clean device upon which you can absolutely depend on for solid data. The components are designed for single use, and the abilities of our customers to restore a previously used RTube to proper functioning condition vary greatly.



**CAUTION: Verify the placement of the blue end cap when storing or shipping EBC samples inside the RTube.**

The RTube cartridge is supplied with one end-cap which should be placed on the end opposite the blue one-way valve (near the red arrow). Once the cap is properly attached, the EBC sample will be trapped inside of the collection chamber and can be stored and transported as needed. Failure to cap the correct end of the cartridge may result in sample loss during transport.

The blue one-way valve located inside of the RTube's collection chamber is designed to relieve pressure buildup during freeze/thaw cycles via a small hole at the valve's peak. The position and the size of the hole will prevent any loss of sample; however, during this process some gas exchange may occur. If gas exchange is a concern to your particular project we offer additional caps upon request. However, capping both ends of the tube instead of only one will disable the pressure relief mechanism.

With the pressure relief disabled, either end cap may pop off the RTube cartridge during thaw from deep freeze. This occurs due to the pressure increase from expansion of the air and vapors inside the cartridge as the temperature is normalized to ambient. This pressure buildup can pop off caps with enough force to become airborne and sample loss may occur.

Please contact RRI for more information regarding the safe and effective use of dual caps.



**CAUTION: Condensing surface requires rinsing soon before use if nitrogen oxides are of interest.**

Nitric oxide can diffuse through various materials, and its oxides, including nitrite and nitrate, are common and abundant contaminants on laboratory surfaces.

If nitrogen oxides are to be assayed we strongly recommend rinsing with deionized water all apparatus that will come in contact with the exhaled breath condensate sample as close as possible to the time of use. This includes the inside of the RTube condensing chamber, microcentrifuge tubes, and pipette tips. Note that many latex gloves are highly contaminated with nitrogen oxides.

We recommend plunging and removing sample from the RTube within 24 hours if nitrogen oxides are of interest. Additionally, we recommend analyzing the sample as soon as possible if nitrite is of interest.



**CAUTION: Device for investigational use only**

The RTube is an investigational device only and is designed for research purposes. Due to the non-invasive nature of the device, the RTube is exempt from the Investigational Device Exemption of the United States Food and Drug Administration. Any invasive use of the device or use outside of protocols approved by local Institutional Review Boards is not supported by Respiratory Research, Inc.



**CAUTION: Rapid or forceful manipulation of tube when inserted onto plunger may result in sample leakage**

The Rtube should be handled with a slow, steady, controlled motion when inserting onto plunger. This steady motion should be maintained even if unpredictable resistance levels are met. If excessive force is used and the resistance unexpectedly drops, the rapid motion of the Rtube as it is forced over the plunger can distort the internal parts and can result in partial loss of sample



**CAUTION: If separated, the mouthpiece and collection chamber must be properly mated to allow exhalation through the device.**

Prior to use, please check to ensure the red arrow at the end of the collection chamber is pointing AWAY from the blue mouthpiece assembly.

The RTube collection chamber houses one of the two internal one-way check valves and must be properly oriented when attached to the mouthpiece. This collection chamber is easily identified as the long semi-clear polypropylene tube sitting atop the blue mouthpiece assembly. By design, these parts can easily be separated to allow isolation and transport of the sample within the chamber. The initial mating is done at the factory and quality checked to ensure correctness. However, during handling by patients or medical staff they can become separated. This is not a problem as long as they are mated correctly prior to use.

Note: The word "UP" is printed in red letters beneath the red arrow and pertains to the overall device orientation during use, not the assembly of the collection chamber to the mouthpiece.



**CAUTION: If stored in a warm environment for an extended period of time the RTube may exhibit increased resistance upon first attempt to use.**

This resistance is due to minor adhesion internal to the duckbill valve. The duckbill valve is made of silicone rubber. Silicone rubber, especially at warm temperatures, is a naturally tacky material. We minimize this tackiness through a curing process where we bake the valve at 400 degrees Fahrenheit. A 4-hour cure is the industry standard for ensuring all volatiles are removed and that all residual reactions of the two-part reagent chemical mix used to create the compound are complete. Respiratory Research currently demands a 6-hour cure from our suppliers, or 50% more than standard, to ensure the highest quality.

Even with this 6-hour cure, the area around the slit of the valve may still experience light sticking when the RTubes are stored for longer than 6 months or stored in warm ambient temperatures. This sticking is expected and normally has no effect, but in rare cases it can be enough to impair usage of the RTube. Apply the procedure shown on the next page prior to using RTube to verify proper operation and eliminate any potential impairment. **Please Note:** It is important that all staff and patients handling the RTube be made aware of this procedure.



## Procedure to Verify Proper Operation Before Use

**i** If stored in a warm environment for an extended period of time, the RTube may offer increased resistance upon the first attempt to use. This is due to minor adhesion internal to the duckbill valve. This is expected and normally has no effect, but in rare cases it can be enough to impair usage of the RTube. Apply this procedure prior to using RTube to verify proper operation and eliminate any potential impairment.

<p><b>1</b></p> <p>Remove from package and set cap aside.</p>	<p><b>2</b></p> <p>Look down into top of RTube and note the orientation of the apex of the duckbill valve. This is the visible peak of the valve running all the way across.</p>	<p><b>3</b></p> <p>Grasp the RTube between your thumb and forefinger such that the apex of the valve is in line with finger placement. Note that valve orientation varies normally. Adjust finger position accordingly and gently squeeze the RTube.</p>	<p><b>4</b></p> <p>Look for the opening of the valve as shown. Proceed to use RTube.</p>
<p><b>5</b></p> <p>If valve does not open, ensure proper position of fingers and try again. If on successive attempts the valve does not open, do not use.</p>			<p><b>5</b></p>

## Rtube Technical Specifications

The Rtube Exhaled Breath Condensate Collector is specifically designed to meet the needs of a wide range of subjects, investigators, and clinicians. Its size, weight, materials, and performance characteristics have been carefully selected to provide the maximum safety, effectiveness, and flexibility.

Physical Specifications	RTube w/Cooling Sleeve (Configured for Use)	Rtube Transportable Cartridge
Envelope Dimensions	29.0 cm Tall X 3.5 cm Wide X 10.0 cm Long	22.2 cm Tall X 2.6 cm Wide X 2.6 cm Long
Weight	442 grams	17 grams

Material Specifications	RTube	Cooling Sleeve
Mouthpiece	Polyethylene	N/A
Tee	Polyethylene	N/A
Check Valve	Polyethylene housing	N/A
Tube	Copolymer Polypropylene	N/A
Duckbill Valve	Silicone Rubber (FDA-approved Ingredients)	N/A
O-ring	PTFE (Teflon)	N/A
Endcaps	Medical-grade Vinyl	N/A
Label *	Mylar	N/A
Sleeve *	N/A	Aluminum 6061-T6
Insulator *	N/A	Polyester/Cotton Fabric with Polyester Insulation
<b>NOTE: Parts annotated with (*) do NOT contact condensate</b>		

Performance Specifications	Adult	Child
Duration of Collection	7 Minutes	10 Minutes
Volume of Condensate Collected	1000 microliters	700 microliters
Collection Temperature	-20C	-20C
Flow Resistance	0.20 cm H <sub>2</sub> O/liter	



### INFORMATION: Assay Guidelines for pH

**pH Assay Collection:** Exhaled breath condensate can be collected in the usual manner. No special preparation of the collection system is necessary, although special equipment can be helpful (see below). Minute ventilation (including profound hyper- and hypoventilation) does not affect the pH of deaerated EBC, although it may be relevant if pH is measured without deaeration. We recommend storage of the cooling sleeve at a temperature of collection between -18 and +10°C until immediately prior to use. Please note: use of the optional filter does not affect pH measurements.

The RTube is designed to allow pH sample degassing and analysis inside the RTube. Use of this feature requires a custom-designed deaeration base, available from us. Alternatively, the RTube can be used for collection, and the sample plunged and stored in the usual manner in a microcentrifuge tube prior to assay. We strongly recommend deaeration prior to pH measurement.

Argon (approximately 300 ml/min) bubbled through approximately 200-600 uL of EBC for approximately 8 minutes is generally satisfactory to completely stabilize the pH. We recommend measuring the pH immediately after, or even during deaeration, preferably while the EBC is still under Argon. Argon is heavier than air, and so tends to serve as a shield against rapid CO<sub>2</sub> absorption from the atmosphere (which acidifies the EBC). Measurement during deaeration helps to confirm stability of the reading—which is the endpoint for the deaeration. Note that when EBC pH before deaeration is less than 5.5 or so, it is unlikely to change much during deaeration. When initial pH is above 6.5, deaeration will likely bring the pH substantially up, into the mid- to high- 7 range. As for all assays in EBC, we are very cautious about what comes into contact with the sample, although pH is very robust and does not seem to suffer seriously from lab errors.

**Note:** If you would like to study multiple biomarkers in the EBC using a non-deaerated sample, simply aliquot a portion of the EBC into a separate microcentrifuge sample before deaerating the sample and testing the pH.

**Storage for pH:** no special conditions are needed. We store all samples either in the RTube or pHTube itself (both of which are constructed from polypropylene, Teflon and silicon rubber), or in a polypropylene microcentrifuge tube until assays are performed. We tend to freeze samples at -20° to -80° C, but have found excellent intrasample pH assay reproducibility after storage at room temperatures for years.



### **INFORMATION: Assay Guidelines for Ammonia**

**Ammonia Assay:** Much of the ammonia trapped in EBC is derived from the upper airway. We believe that the reason EBC ammonia is so low in acute asthma is because the native airway lining fluid is acidified, thus trapping ammonia (and perhaps facilitating diffusion into the blood stream). Ammonia serves as a very sensitive and readily measurable indicator of low EBC pH, but is subject to significant variability in healthy subjects (yet minimal variability in acutely ill asthmatics, all of whom have very low or undetectable ammonia levels). Rigorous studies have not been performed on ammonia stability in EBC, but extensive anecdotal experience suggests that EBC ammonia concentration is stable at room temperature for weeks at least, and at -20 for years. Ammonia in EBC rises by a few percent during deaeration, likely because of evaporation of water from the EBC. Ammonia has a potential advantage in that it can be measured in unprocessed samples with a simple commercially available colorimetric dipstick. This assay is fully usable at home by an individual subject. However, in the laboratory, we use a commercial spectrophotometric ammonia assay kit from Sigma. We have modified this commercial assay to be much faster and use less sample and less reagent. We can provide instructions for this modification on request.



### **INFORMATION: Assay Guidelines for Nitrate and Nitrite**

**Nitrate and Nitrite Collection:** Cautious techniques need to be used for EBC collection, storage and assay when nitrite and/or nitrate are the ions of interest. Nitrite and nitrate are ubiquitous laboratory contaminants that are carried in humidity and land on every available surface. These ions are abundant on fingertips. Protection by covering lab surfaces is not sufficient because nitrite and nitrate are also formed from nitric oxide (NO) oxidation, and NO gas is in almost all labs and clinics—especially respiratory clinics—at variable, but relevant concentrations. NO can travel through plastic coverings as well. Latex gloves may have extremely high levels of nitrogen oxides on their surface. Microcentrifuge tubes and other test tubes are almost always substantially contaminated by nitrogen oxides.

It seems to us very difficult to eliminate some contamination of EBC with nitrogen oxides from the collection system and the storage containers. This is an important issue particularly for nitrite, because nitrite is found in low- to sub-micromolar ranges in EBC, and thus contamination, though unavoidable, can overwhelm the signal from the subject. Additionally, nitrite can oxidize to nitrate in aqueous solution (for example, using hydrogen peroxide as the oxidant), or be converted to NO (through protonation and release of NO from decomposed nitrous acid). These processes can decrease nitrite levels during storage over time.

As a result of these concerns, we make the following recommendations for EBC collection when nitrogen oxides are of interest:

1. Be very diligent about not allowing fingers to come into contact with any surface that will contact EBC. This includes the insides of the covers of microcentrifuge tubes (a commonly overlooked site of contamination), and the insides of the

caps on the RTube system. When handling any surface that might touch the condensate, we recommend that you put on gloves, then wash your gloved hands with deionized water. Preferably, use gloves that have been pre-rinsed by the manufacturer and have low extractable ion content. These gloves are readily available from research supply catalogues.

2. We strongly urge rinsing the RTube with distilled/deionized (DDI) water as soon as possible before use if you are planning on doing assays for nitrogen oxides. A simple rinse eliminates almost all nitrite and most nitrate that may be present from environmental contamination. We have an effective method available for cleaning and drying the RTube components that contact the condensate. Please contact us for the specifics of this method.
3. When nitrogen oxides are of interest, we recommend plunging the RTube and removing EBC sample soon after collection (preferably within hours), and placement of EBC sample in a polypropylene microcentrifuge tube that has been pre-rinsed with DDI water and dried with forced air.

**Nitrite Assay:** We recommend assay for nitrite as soon as possible after collection. Loss of nitrite to protonation (and NO evolution) will occur most actively in acidified EBC (such as is found in acute asthma, cystic fibrosis, COPD, bronchiectasis, and immediately after infection with the common cold). Thus nitrite can be rapidly lost from EBC (over days) when the patient is sickest. Furthermore, the higher the initial nitrite levels are, the more rapidly the nitrite is lost. Given that nitrite in EBC is highest in sick patients—and EBC pH is lowest—if assays are not performed rapidly, the substantial differences that might otherwise be seen in these patients tend to not be identifiable.

Overall, we recommend performing the nitrite assay within 48 hours, if at all possible, while keeping samples frozen at  $-80^{\circ}\text{C}$  until then. One possible, but only partly explored solution to the acid-induced loss of nitrite, is to aliquot 100  $\mu\text{L}$  of EBC into a microcentrifuge and add 1-2  $\mu\text{L}$  of 1M sodium hydroxide (NaOH) immediately after collection. Again, be cautious that the NaOH used is not contaminated by nitrite that leached out of the container in which it was stored. Nitrite can be assayed by the Griess reaction, which with great caution and careful optimization, may be accurate down to approximately 500 nM. Unfortunately, this puts the bottom range of this assay right at the levels of nitrite seen in healthy subjects. Also, such optimization requires larger volumes of sample (often 300  $\mu\text{L}$  or more).

Alternative assays are available. Using chemiluminescent NO detection, EBC nitrite can be reduced by potassium iodide and the NO produced measured. Nitrite assays are reproducible to the low nanomolar range, and only 10  $\mu\text{L}$  of sample are needed. Also, ion chromatography has been reported by one group to be very effective for nitrite quantification in EBC.

**Nitrate Assay:** Nitrate is often measured as total nitrogen oxides (NO<sub>x</sub>). We first request that NO<sub>x</sub> results be more clearly reported as Total nitrogen oxides, or nitrate plus nitrite, or nitrate by itself. Nitrate does not appear to suffer from instability during storage, although levels can rise slightly as nitrite is oxidized during storage. Nonetheless, because storage containers can leach out NO, we recommend processing the samples as soon as possible.

A modified Griess reaction is commonly employed in which the sample is treated first with E coli nitrate reductase, and then the Griess reaction quantifies the nitrite subsequently formed. Thus this assay quantifies nitrite + nitrate. Again, for this reaction, larger volumes of EBC are required. Nitrite must be subsequently assayed and subtracted from the result of the initial nitrite+nitrate assay.

Sample can be assayed by chemiluminescence after reduction of nitrate and nitrite (and low molecular weight s-nitrosothiols) to NO using vanadium chloride. This assay requires only 10-30  $\mu\text{L}$  of sample, but quantitates Total NO<sub>x</sub>. Assays for nitrite and s-nitrosothiols should be subsequently performed in order to specifically quantify the nitrate concentration. We recommend great caution with the vanadium chloride (which is toxic), primarily because of nitrogen oxide laboratory contamination concerns. The needle used to inject the EBC sample into the assay chamber will be reduced by the powerful Vanadium reductant, and can generate an impressive NO signal if caution and speed are not employed.

We would like to strongly reiterate that some or all of these issues will almost certainly be relevant in any effort to do EBC nitrogen oxide measurements.



#### **INFORMATION: Assay Guidelines for Cysteinyl-leukotrienes**

**Cysteinyl-leukotriene collections:** We have not confidently identified any special collection concerns when the substances of

interest are Cys-LT's. Nonetheless, there is the possibility that repeated freeze-thaws may decrease the stability of the Cys-LT's, but that is uncertain. If this IS a problem, then collection of EBC at a temperature of 0? or higher may optimize stability as below 0?, EBC will constantly freeze to the condenser while no air is flowing (i.e. during inhalation) and thaw somewhat during exhalation of warm air through the condenser. We have not rigorously studied these issues.

**Cys-LT assays:** Difficulties that we have had with this assay in the past have stemmed, we believe, from problems with accuracy and reproducibility of the commercial ELISA assays that we, and most all others, have employed. Recently, the Cys-LT standards available seemingly have been improved. Mass spectroscopy offers promise as a more accurate, but less available, method to confirm findings of the more simple ELISA tests. We are following the recent trend to report these ELISA results as "cysteinyl-leukotriene-like immunoreactivity"; accepting the possibility that the ELISA may not be perfectly specific in the EBC setting.



**INFORMATION: For more information please visit our website at [www.RespiratoryResearch.com](http://www.RespiratoryResearch.com)**