

Video Article

Breath Collection from Children for Disease Biomarker Discovery

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Abstract

Breath collection and analysis can be used to discover volatile biomarkers in a number of infectious and non-infectious diseases, such as malaria, tuberculosis, lung cancer, and liver disease. This protocol describes a reproducible method for sampling breath in children and then stabilizing breath samples for further analysis with gas chromatography-mass spectrometry (GC-MS). The goal of this method is to establish a standardized protocol for the acquisition of breath samples for further chemical analysis, from children aged 4-15 years. First, breath is sampled using a cardboard mouthpiece attached to a 2-way valve, which is connected to a 3 L bag. Breath analytes are then transferred to a thermal desorption tube and stored at 4-5 °C until analysis. This technique has been previously used to capture breath of children with malaria for successful breath biomarker identification. Subsequently, we have successfully applied this technique to additional pediatric cohorts. The advantage of this method is that it requires minimal cooperation on part of the patient (of particular value in pediatric populations), has a short collection period, does not require trained staff, and can be performed with portable equipment in resource-limited field settings.

Introduction

Biomarkers can yield valuable information about normal and pathological biological processes that may contribute to clinically identifiable disease. Recently, there has been increasing interest in the evaluation of breath volatiles as biomarkers for a variety of disease states, including infection, metabolic disorders, and cancer¹. Exhaled breath contains quantifiable levels of volatile organic compounds (VOCs), semi-volatile organic compounds, and microbially derived material (e.g., nucleic acids from bacteria and viruses). The central goal of exhaled breath analysis is to gain insight into the status of a medical condition and/or environmental exposures non-invasively. There are various methods for collecting and analyzing exhaled breath, depending on the constituents of interest. Currently there is no standardized exhaled breath collection method, which complicates comparative analysis of results across studies. Standardizing breath collection procedures is essential, as the sampling procedure itself has a considerable effect on the downstream results of breath analyses.

In many studies, late respiratory breath sampling is employed^{2,3}. This sampling involves discarding the initial portion of exhaled breath ("dead space"), in order to preferentially capture the air at the end of the breath cycle. The advantage of this strategy is that it minimizes the levels of exogenous VOC (e.g., environmental VOCs), while enriching for endogenous, patient-specific VOCs. This method excludes the first few seconds of exhalation from an individual before collecting the breath sample. Other investigators have employed a pressure sensor to activate sampling during a predefined phase of expiration^{4,5}. Because pressure sensors require complex engineering, this alternative method requires a dedicated and relatively costly sampling device.

Pediatric breath sampling can be particularly challenging. A key concern is that young children may be unable to cooperate with protocols for voluntary exhalation of "dead space" air. For this reason, it is easier to obtain mixed respiratory breath from children. However, a major caveat with mixed respiratory breath samples is the risk of environmental and material contamination. Therefore, the feasibility of pediatric collection is a driving concern in the field.

In addition, to collection methods, storage of breath samples can also influence sample quality. The high humidity in breath exhalate and the ultra-low concentrations (parts-per-trillion) of volatile organic breath compounds make breath samples particularly susceptible to problems related to storage^{6,7}. Despite the great potential of real-time techniques like proton transfer reaction-mass spectrometry (PTR-MS), GC-MS remains the gold standard for the analysis of breath samples. Since GC-MS analysis of breath samples is an offline technique, it is coupled with pre-concentration methods such as thermal desorption (TD) tubes, solid phase micro-extraction, and needle trap devices. Prior to pre-concentration, breath samples need to be temporarily stored in polymer bags⁸. Polymer bags are popular because of their moderate price, relatively good durability, and reusability. While bags may be reused, time and effort are required to ensure efficient cleaning^{7,8}. Each specific bag type also requires empirically determined and standardized procedures for quality control, reusability, and recovery.

TD tubes are widely used for breath pre-concentration because they capture a large number of volatiles and can be customized. The absorbent materials used for packing TD tubes may be adapted to particular applications and particular target volatiles of interest. TD tubes substantially

improve the convenience of breath biomarker studies, especially at remote field sites, because TD tubes safely store breath volatiles for at least two weeks and are easy to transport³.

In an effort to standardize pediatric breath collection for biomarker discovery, here we describe a simple method to collect breath from young children. To illustrate the representative results of the implemented protocols, de-identified data are presented from an on-going cohort of children (age 8-17) undergoing evaluation for nonalcoholic fatty acid liver disease (NAFLD). Full results and analysis of this study will be reported in a later publication. In this work, we report a sub-set of data to demonstrate application of our protocol. In brief, children are instructed to exhale normally via mouthpiece into a polymer bag, as if "blowing a balloon." The process is repeated 2-4 times until 1 L of breath is collected. The sample is then transferred into a TD tube and stored at 5 °C prior to GC-MS analysis.

Protocol

The study has been approved by the Institutional Review Board of Washington University School of Medicine (#201709030). Informed consent was obtained from a parent or legal guardian prior to inclusion in the study. Photographs in **Figure 2** reproduced with written informed parental consent.

1. Breath sampler assembly

1. Using disposable gloves, attach a cardboard mouthpiece to the breath sampler, as shown in Supplemental **Figure 1**. Attach a short length of large diameter tubing to the other extreme of the breath sampler, as shown in **Supplemental Figure 1**. Use new tubing for each patient.
2. Attach the breath connector to the bag valve via the tubing. See **Figure 1** for photo of the breath sampler and bag connected.
3. Push the stem of the bag valve down, to open the inlet fitting for sampling.
4. Lock the valve open, by turning the knurled thumbscrew on the side of the inlet fitting clockwise.
5. Confirm that the blue valve on the breath sampler is open (parallel with the connector).
6. Write patient ID, date, and time on the label of the polymer bag.
7. Condition TD sorbent tube prior to breath collection using recommended procedures (available from individual manufacturers). Cap and store thermal desorption tubes at 4–5 °C prior to breath collection to minimize artifacts.

2. Breath collection

1. Perform a demonstration of breath exhalation to the child by using a breath sampler (without a bag). Explain to the child that they should breathe out like they would do when "blowing up a balloon" and continue breathing out as far as they can comfortably. Put the cardboard mouthpiece between your lips and exhale as far as you can.
2. Provide the child with a new breath sampler attached to a bag and ask them to exhale as in the demonstration, as illustrated in **Figure 2**.
3. Close the blue valve on the breath sampler device as soon as the child has finished breathing out. Reopen the valve as needed prior to additional exhalations.
4. Repeat step 2.2 and 2.3 until at least 1 L of breath has been collected. For a healthy child this may take 2 exhalations, and for a sick or younger child 2–4 exhalations. 1 L of breath is the minimum analytical requirement. Note on the bag label how many breaths were collected from the patient. See **Supplemental Figure 2** for a photo of bag containing different volumes of breath.
5. Before detaching the bag from the breath sampler, make sure to loosen the knurled thumbscrew on the side of the inlet fitting by turning it anticlockwise and push the stem of the valve up to close the inlet fitting. See **Supplemental Figure 3** for photo of the bag valve in the open and closed position.
6. Lock the bag valve closed by turning the knurled thumbscrew on the side of the inlet fitting clockwise.
7. Detach the bag from the breath sampler.
8. Dispose of the mouthpiece and put aside the breath sampler for cleaning prior to use with a different patient.

3. Breath transfer to thermal desorption tubes

1. Remove the TD tube from the refrigerator. Remove the long-term storage caps of the sorbent tube, using the manufacturer-provided tube capping/uncapping tool.
2. Attach the grooved end of the TD sorbent tube to the sampling bag using tubing. Note that tube orientation is important, as TD tubes are designed to have air flowing in one direction only, starting from the grooved end. Note that the transfer of breath from bag to TD should be done within 1 hour of breath collection.
3. Insert the other end of the TD tube into the tubing, which is connected to a pump.
4. Turn the pump on and set to run at 100 mL/min for 10 min.
5. Open the valve on the bag by turning anticlockwise the knurled thumbscrew on the side of the inlet fitting and push the stem of the valve down to open the inlet fitting. This is illustrated in **Supplemental Figure 4**, which demonstrates breath transfer into a TD sorbent tube using a pump.
6. Start the pump, which will stop after 10 min of collection.
7. Remove the TD sorbent tube and tighten the caps onto both ends using the tube capping/uncapping tool. Long-term storage caps must be firmly tightened in order to ensure a leak-tight seal.
8. Place a sticker on the end of one cap to indicate the tube has been used. On the sticker, indicate patient study identification (ID) number and date.
9. Place tube in a small re-sealable plastic bag. Store sorbent tube at 4–5 °C. Press the rest of the breath out of the bag and discard bag. Record patient study ID, TD tube serial number, collection day, time of breath collection, time of breath transfer, and food intake (time of food intake prior to breath collection and meals consumed).

4. Ambient air collection

1. Collect the ambient air samples in patient's environment immediately after breath collection.
 1. Attach the bag to the pump outlet port by using tubing, as illustrated in **Supplemental Figure 5**.
 2. Push the stem of the bag valve down to open the inlet fitting for sampling.
 3. Lock the valve open by turning clockwise the knurled thumbscrew on the side of the inlet fitting.
2. Turn the pump on and set to run at 100 mL/min for 12 min. The pump will collect 1,200 mL of ambient air.
3. After the requested volume has been collected, loosen the knurled thumbscrew on the side of the inlet fitting by turning it anticlockwise and push the stem of the valve up to close the inlet fitting.
4. Lock the bag valve closed by turning clockwise the knurled thumbscrew on the side of the inlet fitting.
5. Detach the bag from the pump.
6. Follow the same steps as in section 3. The only difference is that ambient air VOCs will be transferred, not those from breath.

5. Sample and data analysis

NOTE: Conditions for analysis of breath and air samples have been described previously⁹.

1. Analyze collected data and detect compounds in the chromatograms. Use typical software programs to find and identify all compounds detected by the instrument (**Figure 3A**). For example, use a deconvolution feature to identify compounds. Filter data using retention window size factor of 80, mass heights filters ≥ 100 counts, and compound absolute area filter ≥ 500 counts.
2. Use chemical standards to identify compounds in breath and air samples. Extract the base ion peak area of compounds of interest, such as isoprene and β -pinene (**Figure 4**), and compare the levels of volatiles in breath and air.

Representative Results

In our study, breath samples were collected from 10 children (8-17 years old) undergoing evaluation at St. Louis Children's Hospital. Breath samples and ambient air samples ($n = 10$) were collected as described above. Samples were analyzed using gas chromatography quadrupole time-of-flight mass spectrometry (GC-QToF-MS) and thermal desorption, as previously described⁹. After removal of background contaminants, the implemented protocols yielded an average of 311 volatile organic compounds (VOCs) in each of the mixed exhaled breath samples. On average, significantly more VOCs were found in breath samples compared to ambient/environmental controls (311 ± 11.5 versus 190 ± 12.6 , $p < 0.0001$) (**Figure 3A**). The increased number of VOCs in breath, compared to ambient air, are visibly distinguished by comparing representative total ion chromatograms (TICs) (**Figure 3B**).

As a quality control measure of successful breath collection, the levels of two common breath VOCs (isoprene and β -pinene) were compared to room air controls (**Figure 4**). Isoprene, one of the most abundant VOCs in breath, is normally found in parts-per-billion (ppb) levels (131 ppb) while β -pinene is found in sub-ppb levels (0.59 ppb)⁶. Both compounds are well established to be enriched in breath of healthy adults, as compared to the low levels present in room air, which indicates normal physiologic processes as a primary source of these analytes in breath⁶. Isoprene (m/z 67) was found at retention time 2.12 min and β -pinene (m/z 93) was found at retention time 14.4 min. We find that the abundance of isoprene was 10-fold higher in pediatric breath samples than in room air controls (**Figure 4**; mean abundance \pm SEM are $4.2 \times 10^5 \pm 1.0 \times 10^5$ and $3.9 \times 10^4 \pm 0.9 \times 10^4$ for breath and air respectively, $p = 0.0003$) and β -Pinene exhibited 3-fold higher abundance in breath than air (mean abundance \pm SEM are $3.0 \times 10^4 \pm 1.3 \times 10^4$ and $9.1 \times 10^3 \pm 1.6 \times 10^3$ for breath and air respectively, $p = 0.007$), confirming successful breath collection. Full descriptive analysis of biomarker discovery findings from this study will be reported in a future publication.



Figure 1: Assembled breath sampler and bag for exhaled breath collection. Breath sampler (with blue valve open, i.e., parallel with the connector as indicated by the double-sided red arrow) and bag connected with tubing, ready for breath collection. [Please click here to view a larger version of this figure.](#)

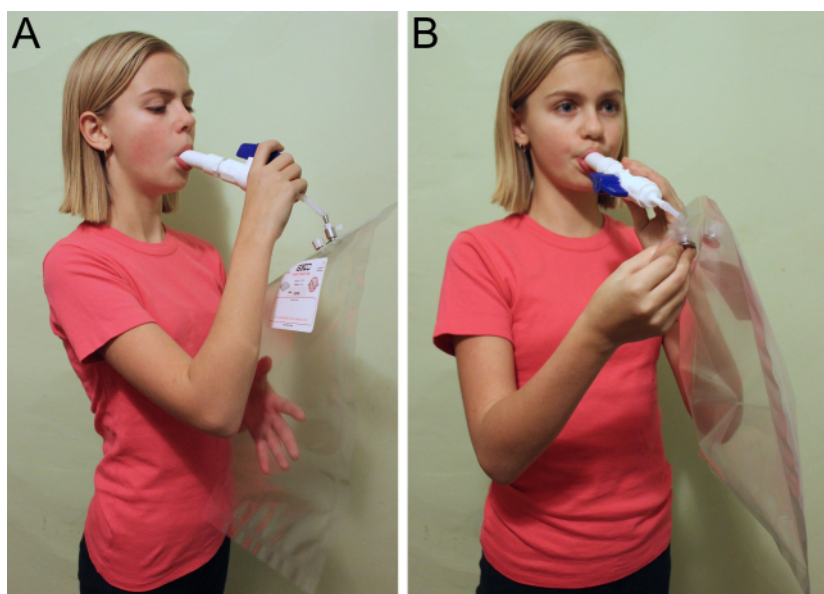


Figure 2: Child exhaling breath into a breath sampling bag. (A) Child holds breath sampler, exhales, and (B) provides breath sample into the bag. Photograph with permission. [Please click here to view a larger version of this figure.](#)

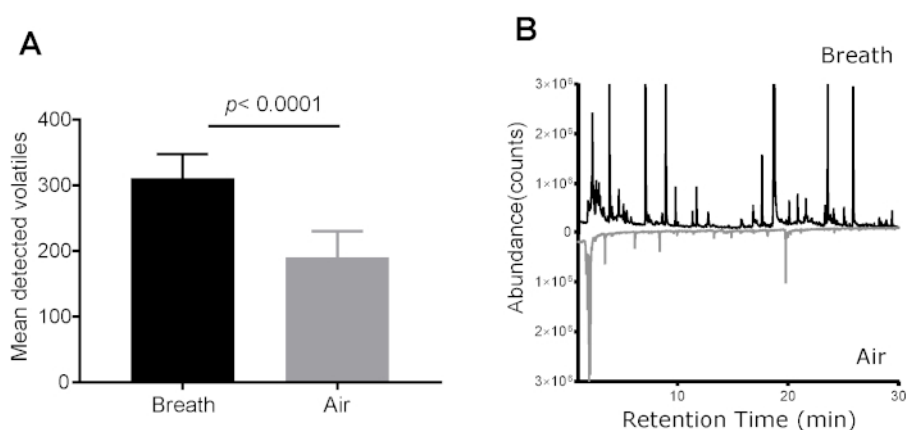


Figure 3: Exhaled Breath Volatiles. (A) Number of distinct volatile compounds in each breath sample from pediatric subjects ($n = 10$) and ambient air controls ($n = 10$). Displayed are the means and standard error of the mean (SEM). (B) Total ion chromatograph (TIC) of representative pediatric breath samples versus air control, for visualization. [Please click here to view a larger version of this figure.](#)

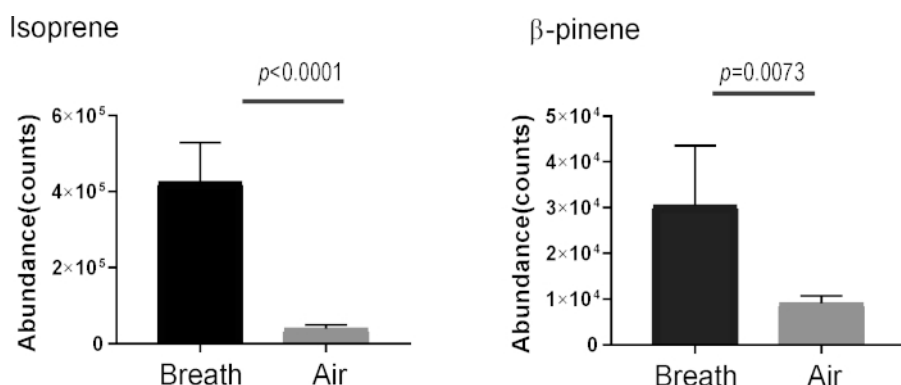


Figure 4: Abundance of two exhaled breath volatiles. Abundance of isoprene and β -pinene in breath samples from pediatric subjects ($n = 10$) and room air controls ($n = 10$). Abundance quantified by the base ion peak area. Mean and SEM are shown. [Please click here to view a larger version of this figure.](#)

Supplemental Figure 1: Breath sampler. Left: A) assembled breath sampler: 1) male adapter + 2) two-way ball valve connector + 3) Teflon male adapter. B) Cardboard mouthpiece. C) Small and large diameter tubing. Right: breath sampler with mouthpiece and tubing attached. [Please click here to download this file.](#)

Supplemental Figure 2: Different breath volumes. Above are pictures of a sampling bag filled with different volumes of air (1 L, 2 L and 2.5 L), as a visual representation of approximate breath volumes to be collected. [Please click here to download this file.](#)

Supplemental Figure 3: Valve on the bag. *Left:* stem of the valve is down (bag valve is open). Lock the bag valve closed by turning clockwise the knurled thumbscrew on the side of the inlet fitting. Bag is ready for breath collection. *Right:* stem of the valve is up (bag valve is closed). [Please click here to download this file.](#)

Supplemental Figure 4: Breath transfer. *Left:* sorbent tube (1) attached to one end of the bag using small and large diameter tubing and, on the other end, to the pump. *Right:* note grooved end on the sorbent tube; grooved end should point towards the sampling bag. [Please click here to download this file.](#)

Supplemental Figure 5: Ambient air collection. *Left:* pump with two ports: inlet and outlet. The outlet port is attached to sampling bag. The inlet port will draw ambient air and transfer it to the bag. *Right:* ambient air collection system assembled. [Please click here to download this file.](#)

Discussion

Despite considerable progress in breath research over the last decade, standardized practices for the sampling and analysis of breath gas volatiles remain undefined¹⁰. A primary reason for this lack of standardization has been the diversity of breath collection methods, which have direct impact on the resulting chemical diversity present in any given exhaled breath sample. Breath exhalate contains an extensive range of volatile organic compounds at highly varied concentrations⁶. Therefore, changing collection methods alters not only the abundance but also the diversity of compounds that may be present in a given sample.

Breath gas sampling is surprisingly complex. While subjects need only to exhale into the mouthpiece of a breath collector or into a gas-tight container prior to analysis, breath sampling must account and control for a number of potential variables. In this work, we detail a specific, validated protocol for breath gas sampling in children. We have previously successfully implemented this protocol with febrile children as young as 4 years of age, in a field scenario in a resource-limited setting (Malawi), demonstrating the feasibility of our breath collection and analysis pipelines for biomarker discovery⁹. Subsequently, we have also implemented and evaluated our protocols for the collection of breath samples from children under evaluation in a modern pediatric subspecialty clinic in the United States. Our results suggest that for discovery of pediatric breath biomarkers, the collection of mixed air is critical, as it provides a true "breathprint" of a given individual. In addition, mixed expiratory breath is also the simplest type of breath that can be obtained, because all phases of exhaled air (mouth and nasal) are acquired³.

In the field, and especially when subjects are acutely ill, it may be difficult to control for common confounders such as diet, body temperature, and/or the use of fragrance or creams by a given subject. These factors may have a profound impact on the breath levels and quality. For this reason, we recommend investigators not only record the time of breath collection and transfer to sorbent tubes, but also note additional patient-specific factors such as diet (e.g., 24-hour dietary recall), use of mouthwash, and medication use, in order to evaluate specifically for the effects of these potential confounders during biomarker discovery and downstream analyses.

Compounds inhaled from the ambient air can also influence the composition of exhaled breath, which may pose a challenge to breath biomarker discovery efforts. Therefore, analysis and collection of ambient air is a critical control, yielding important insights regarding the origin of the exhaled breath volatiles. For example, ambient air volatile profiles have been used to establish whether a given breath volatile is at higher or lower abundance in breath compared to surrounding air¹¹. A particular breath compound is thus considered to be derived from inside the body (e.g., endogenous origin) if the concentration is higher in breath than in ambient air, while reduced concentration in breath indicates that compound was derived from the environment (e.g., exogenous origin). Comparing volatile abundance in breath versus ambient air also serves as an important positive control for whether breath collection is adequate. As demonstrated in our representative data (**Figure 4**), the volatile compound isoprene is of endogenous origin and should be present in breath samples at concentrations >10 times that of ambient air⁶.

For biomarker discovery, volatile profiles from individuals with conditions of interest must be compared to a matched healthy control individuals, such that patterns can be identified using statistical techniques like machine learning and multivariate analyses¹². The breath collection method described here can be applied to a wide range of pathological states; the only requirement is that the child is able to cooperate with breath sampling voluntarily. Because breath testing is non-invasive, easily repeated, and closely reflects the arterial concentrations of biological substances, it holds great promise for implementation in point-of-care testing for clinical use.

Future work will focus on the development of new methods for breath collection in young infants and children (< 4 years of age), who are developmentally unable to exhale on command.

Disclosures

The authors have nothing to disclose.

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