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Breath collection from children for disease biomarker discovery

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Cover Letter

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29 November 2018

Benjamin Werth
Senior Science Editor of the JoVe journal

Please find attached our revised manuscript, **"Breath collection from children for disease biomarker discovery,"** for consideration for publication in *JoVE*. All authors (Amalia Z. Berna, Brian DeBosch, Janis Stoll, Audrey R. Odom John) support this publication, have reviewed the manuscript, and concur with each of its parts. This work is not under consideration elsewhere.

We were grateful for the thorough and thoughtful reviews of our initial submission (JoVE59217). The queries and comments by both reviewers were well received. In response, we have edited the main text and figure legends to improve clarity, as requested by the reviewers, and now provide an updated illustration of our equipment in use (updated Figure 2). Please find submitted our point-by-point response to reviewers, as well as unmarked and marked copies of our revised manuscript. We believe our work has been strengthened and hope that it will now be suitable for publication in *JoVE*. Thank you again for your time and consideration.

Sincerely,

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TITLE:

Breath Collection from Children for Disease Biomarker Discovery

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KEYWORDS:
Breath, pediatric, collection, children, gas chromatography, mass spectrometry, thermal desorber tubes, biomarker, diseases

SUMMARY:
This protocol describes a simple method for the acquisition of breath samples from children. Briefly, samples of mixed air are pre-concentrated in sorbent tubes prior to gas chromatography-mass spectrometry analysis. Breath biomarkers of infectious and non-infectious diseases can be identified using this breath collection method.

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 cancer [1]. 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 bags[8]. 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 transport[3].

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

1.1. 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.

1.2. Attach the breath connector to the bag valve via the tubing. See **Figure 1** for photo of the breath sampler and bag connected.

1.3. Push the stem of the bag valve down, to open the inlet fitting for sampling.

1.4. Lock the valve open, by turning the knurled thumbscrew on the side of the inlet fitting clockwise.

1.5. Confirm that the blue valve on the breath sampler is open (parallel with the connector).

1.6. Write patient ID, date, and time on the label of the polymer bag.

1.7. 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

2.1. 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.

2.2. 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**.

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.

2.4. 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.

2.5. 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.

2.6. Lock the bag valve closed by turning the knurled thumbscrew on the side of the inlet fitting clockwise.

2.7. Detach the bag from the breath sampler.

2.8. 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

3.1. 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.

3.2. 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.

3.3. Insert the other end of the TD tube into the tubing, which is connected to a pump.

3.4. Turn the pump on and set to run at 100 mL/min for 10 min.

3.5. 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.

3.6. Start the pump, which will stop after 10 min of collection.

3.7. 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.

3.8. 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.

3.9. 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**

4.1. Collect the ambient air samples in patient's environment immediately after breath collection.

4.1.1. Attach the bag to the pump outlet port by using tubing, as illustrated in Supplemental **Figure 5**.

4.1.2. Push the stem of the bag valve down to open the inlet fitting for sampling.

4.1.3. Lock the valve open by turning clockwise the knurled thumbscrew on the side of the inlet fitting.

4.2. 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.

4.3. 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.

4.4. Lock the bag valve closed by turning clockwise the knurled thumbscrew on the side of the inlet fitting.

4.5. Detach the bag from the pump.

4.6. 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 previously[9].

5.1. 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.

5.2. Use chemical standards to identity 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 described[9]. 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 breath[6]. 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 ± SEM are $4.2x10^5 \pm 1.0x10^5$ and $3.9x10^4 \pm 0.9x10^4$ for breath and air respectively, p = 0.0003) and β-Pinene exhibited 3-fold higher abundance in breath than air (mean abundance ± SEM are $3.0x10^4 \pm 1.3\ x10^4$ and $9.1x10^3 \pm 1.6x10^3$ 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 AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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.

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).

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.

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.

DISCUSSION:

Despite considerable progress in breath research over the last decade, standardized practices for the sampling and analysis of breath gas volatiles remain undefined[10]. 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

concentrations[6]. 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 discovery[9]. 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 acquired[3].

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 air[11]. 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 air[6].

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 analyses[12]. 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.

ACKNOWLEDGMENTS:

We express our gratitude to the children and families of St Louis Children's Hospital who participated in this study. We acknowledge the unique efforts of Ms. Stacy Postma and Ms. Janet Sokolich during the breath collection. This work is supported by the St. Louis Children's Hospital Foundation.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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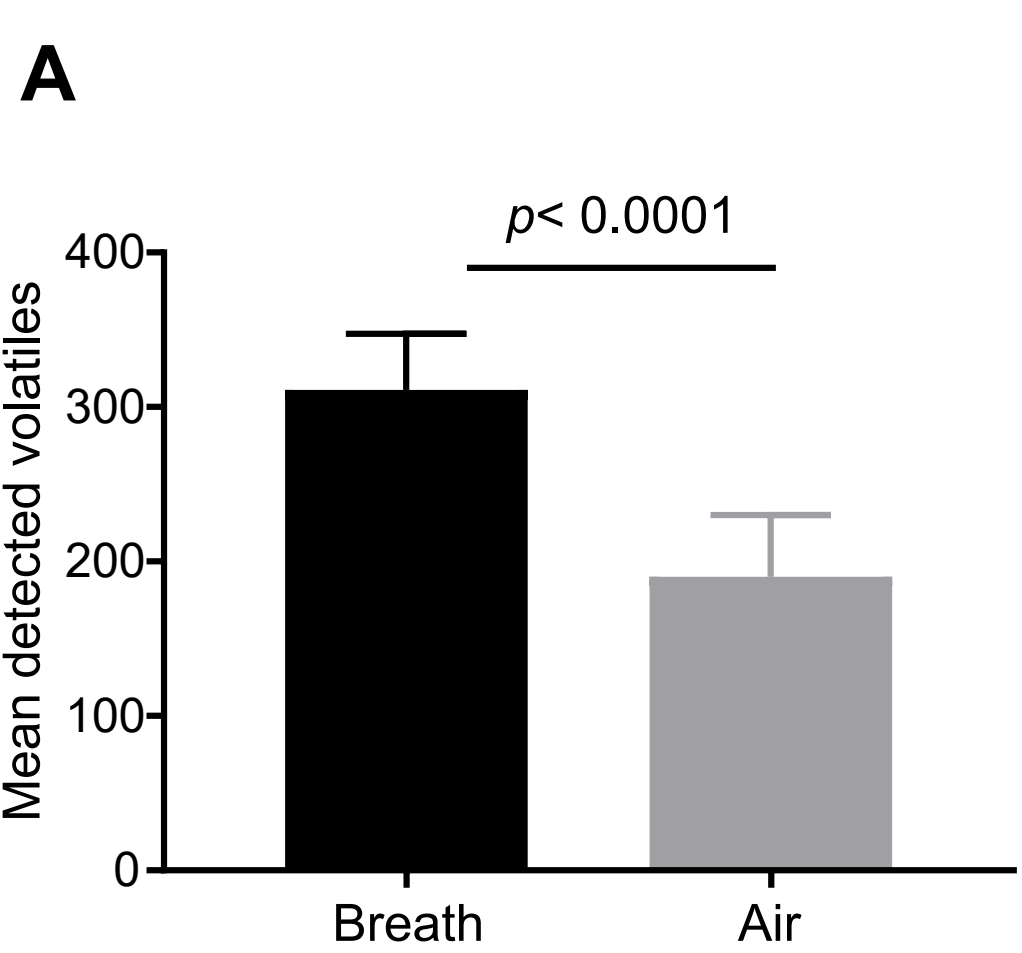
A


B



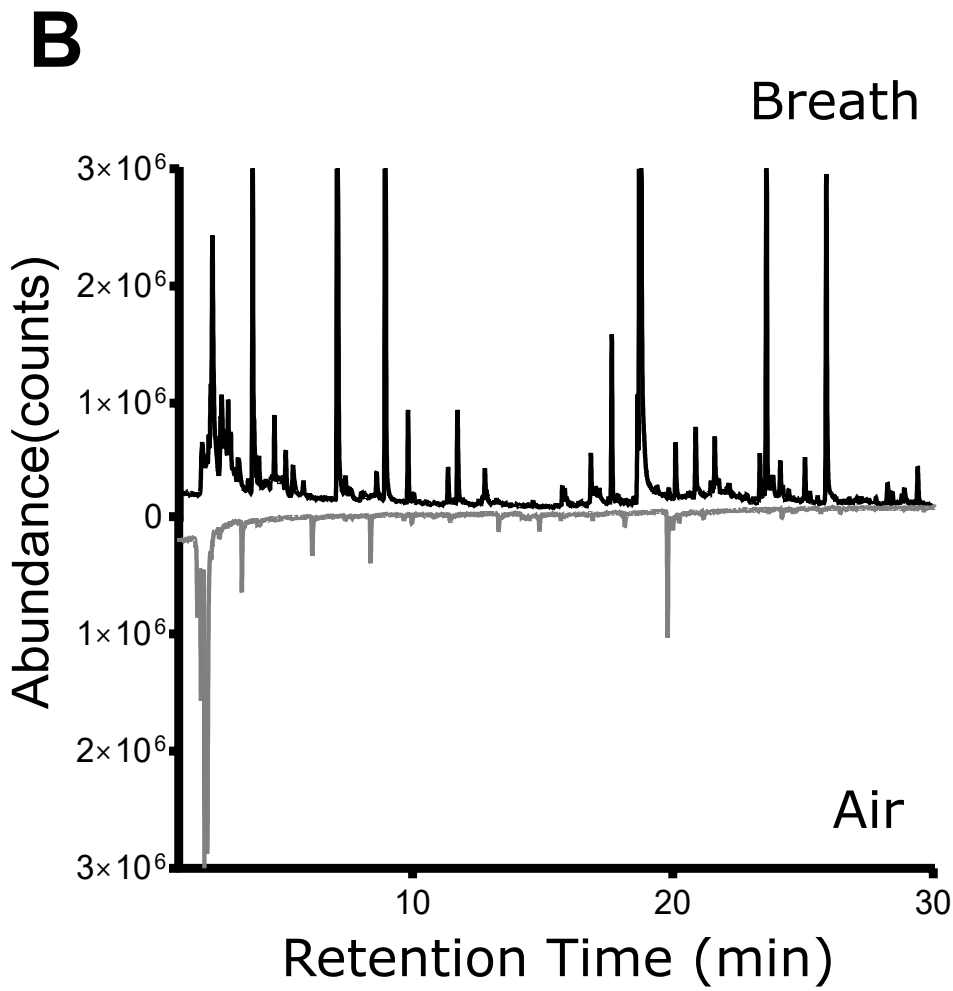
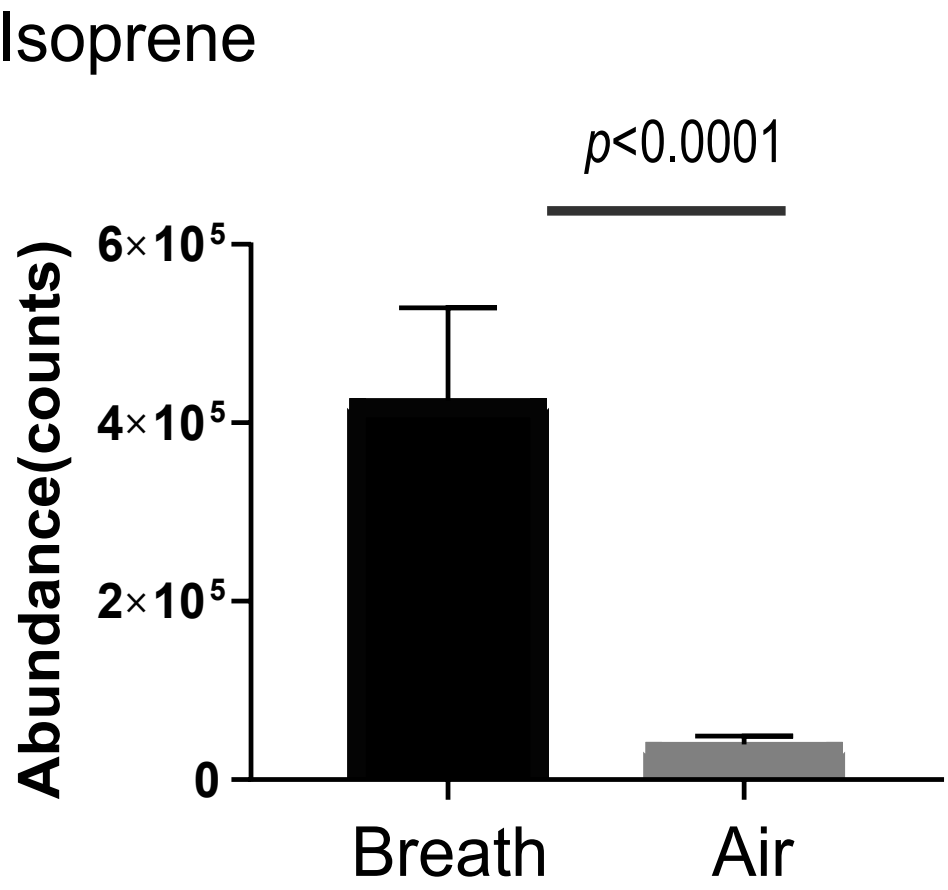


Figure 4



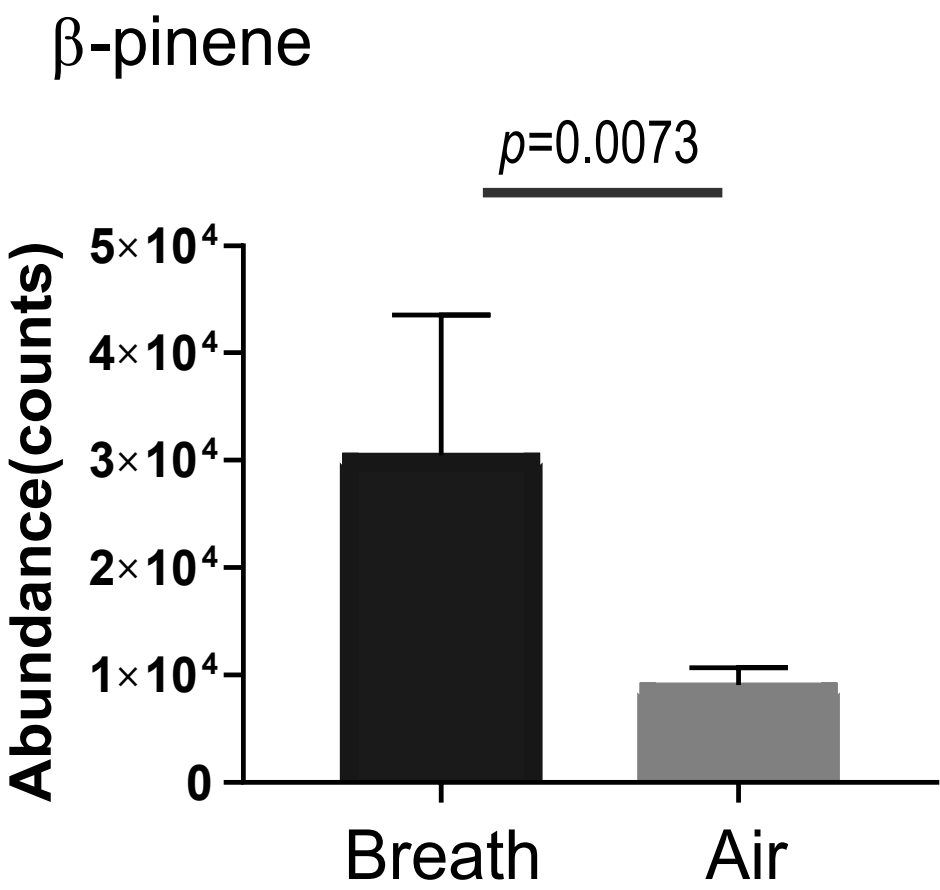


Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Breath bag	SKC	237-03
Cardboard mouthpiece	A-M systems	161902
Large diameter tubing	Cole Parmer	95802-11
Long-term storage caps	Markes International	C-CF010
Male adapter	Charlotte Pipe	2109
Male adapter (made from Teflon)	In-house built	
Pump	SKC	220-1000TC-C
Small diameter tubing	Supelco	20533
Thermal desorption tubes	Markes International	C2-CAXX-5314
Tube capping/uncapping tool	Markes International	C-CPLOK
Two-way ball valve connector	Homewerks Worldwide	VBV-P40-E3B

Comments/Description
These are 3 L bags
0.86" OD, 2.00" L
Silicone Tubing, 1/4"ID x 5/16"OD,
Brass storage cap ¼" & PTFE ferrule, pk 10
Part 1/3 of breath connector (1/2" Universal part No. 436-005)
Part 3/3 of breath connector (1/4" ID x 1/2" MIP). This part was specially machined from rods made from virgin Teflon
Pocket PumpTouch with Charger
Teflon tubing L × O.D. × I.D. 25 ft × 1/4 in. (6.35 mm) × 0.228 in. (5.8 mm)
Tube, inert, TnxTA/Sulficarb, cond/cap, pk 10
Part 2/3 of breath connector (1/2")

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Manuscript ID: JoVE59217

TITLE: Breath collection from children for disease biomarker discovery

We sincerely thank the reviewers for their thoughtful consideration and overall comments. Resulting manuscript changes have been indicated on the marked-up version of the revised manuscript. Responses (in blue) below to specific reviewer concerns:

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript again in hopes of minimizing spelling and grammar errors.

2. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

We have adjusted the numbering of the Protocol.

3. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined some of the shorter Protocol steps (for example, see 2.1 and 3.8 on pages 3 and 4).

4. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have checked that single-line spaces are used between all paragraphs, headings, etc.

5. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the pages of the Protocol in yellow that identify the essential steps of the protocol for the video.

6. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We have highlighted complete sentences.

7. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have included all relevant details in the highlighting.

8. Please also consider describing briefly in the Protocol how to obtain data presented in Figures 3 and 4.

We have described briefly in the Protocols (page 4, lines: 213-222) how the data in Figures 3 and 4 may be obtained.

9. Please remove the titles and Figure Legends from the uploaded supplemental figures. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Titles and Figure Legends have been removed from Supplemental Figures, and we have included all Figure Legends at the end of the Representative Results in the manuscript.

10. A minimum of 10 references should be cited in the manuscript. For instance, please include applicable references to previous studies when describing advantages over alternative techniques.

We have added four references (references: 7, 8, 10 and 12).

11. Table of Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We have sorted the items in alphabetical order according to the Name of Material/Equipment.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a protocol to collect exhaled breath from children to be used in diseases biomarker discovery. The protocol seems very simple, what would be very relevant as the subjects are children.

Major Concerns:
The protocol described is just too simple and its efficiency is poorly supported:
- the protocol refers to the collection of "at least 1L of breath". This is a very large volume of exhaled breath to collect in children and so there are obvious issues in this point, mainly if children are somehow debilitated.

We have found that 1L of breath is the minimum volume we require to capture sufficient numbers of volatiles for GC-MS analysis and data interpretation (data unpublished). For most children, this requires 2-4 exhalations and our experience in multiple studies has established that this is not burdensome. For example, we have previously demonstrated that this volume is readily and safely collected, even from febrile children as young as 4, in a malaria-endemic field setting (Schaber et al, *JID* 2018). To improve clarity on this point, we have added further clarifying information in the text of the manuscript (see page 3; lines 151-153).

- the authors used a simple Breath sampler and 3L bag but there are many reports claiming many weakness about these collection bags. Have the authors assayed for potential contamination from the breath sampler and collection bag?

The reviewer raises an important point. It is well known that sampling bags, tubing and connectors contain some contaminants. For this reason, we removed all artifacts (especially silicon-containing compounds) from the data and only the remaining breath volatiles were considered. We have added in the introduction the advantages and disadvantages of the use of bags (page 2, lines 95-98) and reported the removal of contaminants in the discussions (page 5, lines 229-230).

- the authors refers that over 300 VOCs where identified in the subjects. Where they are reported? Were all those 300 VOCs identified in all samples? If they are not present in, at least, over 90% of the patients, then their use as biomarkers is not viable.

For this study, we did not focus on the identity of the volatiles (except for the reported isoprene and β-pinene) and whether or not they were present consistently in all samples. The main objective of this paper was to report and carefully describe the developed protocol to collect

breath in children for methodological standardization. The biomarkers identified in the population under study will be reported in a future manuscript.

- the number of samples, n=10, is very low when compared with the >300 VOCs identified in breath. Moreover, there are no subject controls? Obtain a statistical correlation from this ratio is totally biased and most certainly only voodoo correlations will be found under these conditions. The authors will obtain a different set of potential biomarkers if they apply the current protocol to a different set of 10 subjects.

The current manuscript is intended as a guide to establish reproducible protocols for pediatric breath collection. Instead of re-reporting published data from a prior study, we report a sub-set of data from an on–going study, in order to demonstrate that a breath volatiles are adequately collected from children with these protocols. In addition, we have provided guidelines as to what quality controls are necessary to evaluate for successful breath collection.

We agree with the reviewers that this current report would be considered insufficient as a biomarker discovery project. Description of the potential biomarkers of non-alcoholic liver disease (the disease under study), biomarker validation, and the statistical testing used to characterize these biomarkers will be reported in full in a later publication. As expected, our larger studies do include a study population and a control group for comparison.

- the authors sampled NAFLD children as a cohort to discover characteristic NAFLD biomarkers in the exhaled breath. Where are the results of this assay? What was the statistical analysis performed? Which VOCs were found statistically relevant and potential NAFLD biomarkers? Where those potential biomakers assayed with a new group of subjects? Overall, In my opinion the authors failed to support they "have successfully applied this technique to a cohort of children diagnosed with non-alcoholic fatty liver disease." The quality control presented is not suitable for the claims the authors refer. The number of samples analysed (n=10) is too low to support any meaningful chemical analysis.

Please see our answer to the previous question. We apologize for any confusion that the statement of our aims may have created. We have provided additional clarification that the main goal of the current report is to demonstrate successful application of this breath collection protocol (page 2, lines 112-115 and page 7, lines 337-339).

Minor Concerns:
Review of the literature has to be more consistent. The experimental conditions used have to be more detailed.

We hope the reviewer will find our review of literature now improved. We have also provided additional details of the experimental conditions, which include sample and data analysis.

Reviewer #2:

Manuscript Summary:
There are several and different methods for collecting alveolar or/and breath air: usually the analytical methods impose the collection way. I suggest to include this concept in the summary. This article is quite interesting because it reports a standardized method for breath collection when the analyses are going to be performed with GC/MS after thermal desorption.

We agree with the reviewer, and in response we have edited the manuscript to indicate that the method we are presenting requires a pre-concentration step followed by analytical GC/MS, as well as additional clarification in the Introduction [see pages 1&2 (lines 84-95)].

Major Concerns:
Line 109: Why do you suggest of storing thermal desorption tubes at 4-5 °C for at least one hour prior to breath collection? I suggest to include how to be sure that the thermal desorption tubes are clean before the breath transfer to the tubes.

We apologize for the lack of clarity on this point. We have edited these sentences and now include additional clarification that the sorbent tubes need to be conditioned, capped and stored at low temperature prior to breath collection, in order to minimize artifacts [see page 3 (lines 138-140)] [1,2].

Minor Concerns:
The valve of the breath sampler has got different colours in figure 1 (bleu) and 2 (red): I suggest to use the same colour.

We have provided a new Figure 2 with blue two-way valve.

Bag connector in figure 1 seems made in steel; on the contrary in figure 2A and 2B the same connector seems made in plastic. Why?

To improve consistency, we have provided a new Figure 2 with metal fittings.

Also in supplemental figure 2 the valve seems in plastic, while in supplemental Figure 3 the Valve on the bag seems in steel: I suggest to use figures in order not to favour easy misunderstanding.

For clarification, we have updated Supplemental Figure 2 (with metal fittings).

Reviewer #3:

Manuscript Summary:
The manuscript by Berna Z.A et al. entitled Breath collection from children for disease biomarker discovery describes a protocol to capture and assess breath volatile organic compounds. Overall it is well written, the method is easy to understand and the figures are adequately informative.

Major Concerns:
Since the title of the manuscript refers to the use of this method for biomarker discovery , authors should expand the discussion. It is recommended to describe how this method will be applied in biomarkers discovery and in clinical practice. How this ''breathprint'' will facilitate diagnosis and personalized therapy.

The control group they are using is ambient air and the authors describe in detail the advantages of using this control. However in pathological conditions, how do they discriminate changes in VOC from healthy group? Is there a method to normalize the results to the ambient group? It would add strength to the method if they could provide evidence that VOC levels are altered in disease state compared to healthy.

We have added a paragraph in the Discussion section on page 7 (lines 343-345) regarding the advantages of breath and how breath biomarkers may be used in the future to facilitate point-of-care diagnosis in clinical settings.

To the reviewer's point on control group, to demonstrate one strategy that may be used for quality control, we have provided a comparison of the number and levels of compounds in breath against control (ambient air). However, for breath biomarker discovery, we agree that samples from the "diseased" group should be compared to a representative "healthy" group. We have clarified this point in the manuscript on page 7 (lines 337-339).

Minor Concerns:
There are some English and punctuation mistakes that should be corrected and below are

some examples.
We have proofread the manuscript again in hopes of minimizing spelling and grammar errors.

Line 52. ''to non-invasively gain insight into the status of a medical condition'' Non-invasively must be placed at the end of the sentence.
We have fixed this.

Line 71 ''contamination, feasibility of collection is a driving concern'. Must add "the" before feasibility
We have fixed this.

Line 78 ''larger'. Should be large
We have fixed this.

Line 93 ''The studies is approved''. Replace ''is'' with ''are''.
We have fixed this.

Line 94 ''Informed consent were obtained'' Replace ''were' with ''was''
We have fixed this.

Line 242 ''Mixed expiratory breath is also simplest type of''. Add ''the'' before simplest
We have fixed this.

Line 270 ''to voluntarily cooperate with breath sampling'' Voluntarily must be placed at the end of the sentence.
We have fixed this.

Line 272 '' who are developmentally unable to consistently exhale on command.'' Consistently must be placed at the end of the sentence.
We have fixed this.

Figure legend 2. '' A child exhaling breath into the bag.'' Add ''illustration of a child…''
We have fixed this.

Reviewer #4:

Manuscript Summary:
The manuscript 'Breath collection from children for disease biomarker discovery' by Berna and colleagues is concisely written and explains a method to collect exhaled breath volatile organic compounds in children. The sampling procedure is explained in great detail and with adequate figures to support the understanding of the practical aspects of the technique. The validity of the method is supported by the presented results. In the discussion a special focus is given to the practicability of the presented procedure.

Major Concerns:
No major concerns

Minor Concerns:
- Please make sure that informed consent has been given to publish an identifiable picture of the child in Fig. 1

We have obtained informed consent from parent of the child to take and use this picture. We have added a statement in the legend to reflect this.

- Language needs improvement

Our apologies, and in response we have edited the spelling, grammar, and flow of the document.

References

1 Woolfenden, E. Monitoring VOCs in Air Using Sorbent Tubes Followed by Thermal Desorption-Capillary GC Analysis: Summary of Data and Practical Guidelines. *Journal of the Air & Waste Management Association.* **47** (1), 20-36, (1997).

2 Brown, V. M., Crump, D. R., Plant, N. T. & Pengelly, I. Evaluation of the stability of a mixture of volatile organic compounds on sorbents for the determination of emissions from indoor materials and products using thermal desorption/gas chromatography/mass spectrometry. *Journal of Chromatography A.* **1350** 1-9, (2014).

Supplemental Figure 1

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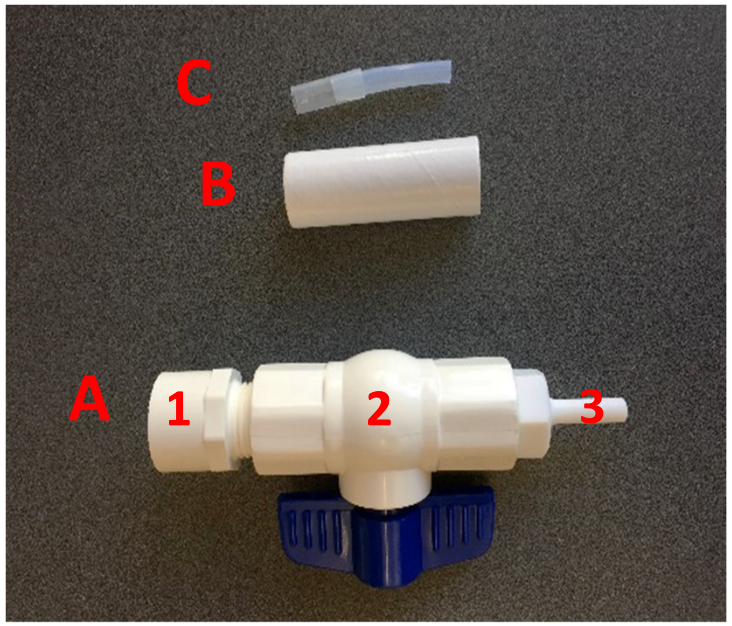

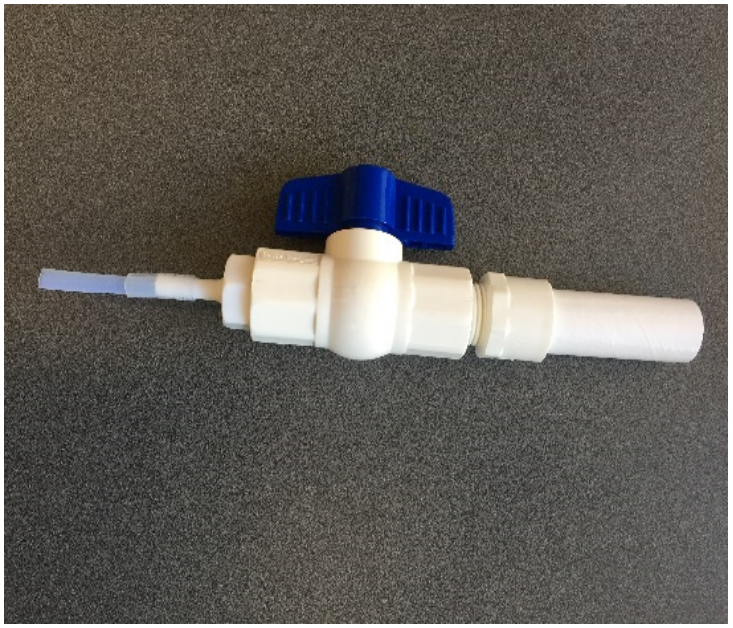

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1 liter

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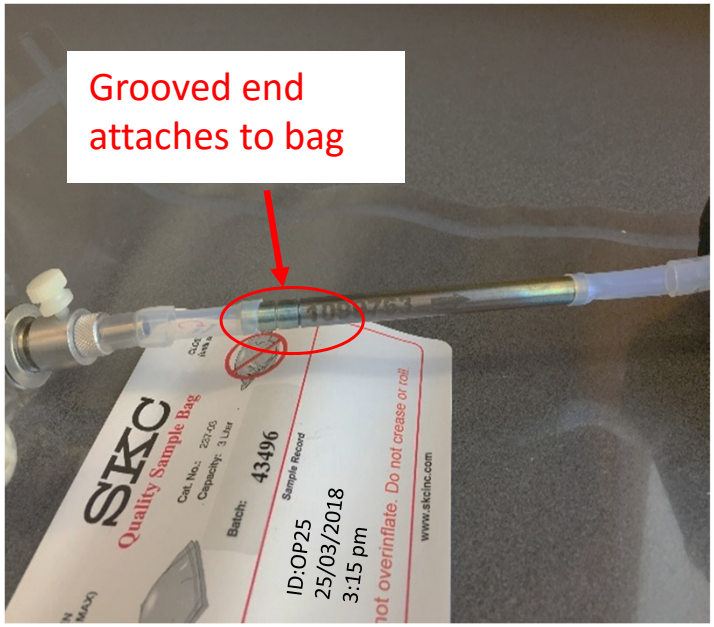

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