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).