Dear Prof. Lee,

Your manuscript JoVE54144R2 "Sugar content assessment using a portable FRET analyzer: a prototype of a widely applicable point-of-care testing tool" has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, e.g., if formatting or other changes were made, commercial language was removed, etc.

Please track the changes in your word processor (e.g., Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

Your revision is due by Dec 16, 2015. Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays.

To submit a revision, go to the JoVE Submission Site and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

Sincerely,

Jaydev Upponi, Ph.D.

Science Editor

JoVE

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Editorial comments:

NOTE: Please download this version of the Microsoft word document (File name: 54144\_R2\_101315) for any subsequent changes.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

•Formatting:

-Please define all abbreviations at their first use in the manuscript (IPTG).

[Step 1.12] Done

-Please include DOI information for references, if available.

•Grammar:

-1.3- “incubate at 37 °C shaker”

[Step 1.3] Done

-1.6-Please clarify the language. What constitutes the source here? The resuspension? Only the glycerol?

[Step 1.6] Revised the sentence

-1.17- “perform chromatography assay using a fast protein liquid chromatography”

[Step 1.17 – 1.22] Revised and updated the steps 1.17 ~ 1.22

•Additional detail is required:

-The protocol relies on the use of a custom made FRET analyzer; however, no step-wise details are provided regarding how to construct the analyzer. In reference 7, is there sufficient detail for viewers to build the device? If so, include a note at the beginning of Section 2 stating: Note: The FRET analyzer was constructed using the method described in…”. If not, we suggest including an additional section detailing the construction of the device.

At the end of Introduction section, a note was added for clarifying the aim of this protocol which is to promote the use of the portable FRET analyzer with FRET based sensors in lab-scale experiments.

-2.4-In what is the cuvette placed? The analyzer? Please clarify.

[Step 2.4] Changed the sentence

-2.5-What constitutes the “detection solution”? Please clarify.

[Step 2.5] The constitutes of the detection solution was described in Step 2.1. We added an indicator “(See 2.1)” in the sentence.

-2.7-Is any criteria used to choose the commercial beverage samples?

[Step 2.7] There was no specific criterion for the beverage selection. The types of beverages were indicated in the Materials/Equipment list.

-In the Materials/Equipment list, for beverages E-I, please indicate what general type of beverages these are (e.g., soda/soft drink, juice, water).

Done

•Unnecessary branding and commercial language should be removed:

-1.17-Resource Q

[Step 1.17] Removed

-Discussion-Cary Eclipse (?)

[Line 286] Changed the word (Cary Eclipse 🡪 Fluorescence Spectrophotometer)

-Please remove the commercial language from the last sentence of the Discussion: “…this device…will promote commercialization in the POCT market.”

Removed

•Results: In the Figure 2 legend, please define the error bars (e.g., SD or SEM).

[Figure 2 Legend] Done

•Discussion: Please expand on the future directions of this method.

[Line 350] There is a description about the further extension of our research

If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

We updated the results and figures which were not used in any of previous publications

\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

too little attention to accuracy, precision, error bars. Explain error bars shown in figure 2.

Updated the whole results and figures including error bars

Major Concerns: N/A

Minor Concerns:

"Silicon photodiode" is misspelled twice (silicone is not silicon)

[Line 89, 282] Corrected

Additional Comments to Authors: N/A

**Reviewer #2:**

Manuscript Summary:

The authors describe a method to measure the concentration of sugar using periplasmic-binding proteins (PBP) that provide a FRET signal when a conformational change due to the binding of sugars alters the distance of an enhanced cyan fluorescent protein (ECFP) from an enhanced yellow fluorescent protein (EYFP) attached to the PBP. This structural change results in a low intensity signal and in order to address this problem, the authors use the ratio of the emissions of both fluorescent proteins (ECFP and EYFP). The protocol includes the steps to the preparation of the biosensor and the steps to measure the sugar content using the FRET analyzer.

Major Concerns:

None.

Minor Concerns:

\*Given that the measurement of the fluorescence ratio is an important part of the method and it is key to improve the obtained signal, I think it would be convenient to include a mention to the fluorescent ratios in the (long) abstract.

[Line 50] Updated the sentence

\*Line 114. Seeing the following steps in the protocol, it seems that it is more important to allow optical density to arrive to 0,5 at 600 nm than to let the culture incubate for 3 hr. Therefore, I think that it might be clearer if it was worded saying " [...] incubate at 37ºC until an optical density at a wavelength of 600 nm (OD600) reaches 0,5 (it will take about 3 hr)."

[Step 1.3] Done

\*Step 1.6.) The important point in this step seems to be to have an OD600 of 100. However, I can imagine a situation when some experimenter resuspended the pellet and it didn't yield this optical density initially. So, I think it would help if the authors stated in the text whether it would be valid to "[r]esuspend the pellet in ice-cold 10% (v/v) glycerol, which is a source of electrocompetent cells, and take the solution to an OD600 of 100)". I.e. to adjust the final OD600 to the desired concentration regardless of what the initial concentration is.

[Step 1.6] Revised the sentence

\*Step 2.2.) I would appreciate if the authors stated explicitly the rationale behind using 55ºC for the FRET measurement and if they discussed how this may affect the binding of the analyte to the sensor.

[Step 2.2] There had been a major update throughout the manuscript. We mainly added descriptions about the temperature control which is exactly the same issue pointed out in this comment. Note that the optimal temperature was changed to 53 °C. Please see the updates highlighted in red.

\*Line 201 refers to values A1 and A2, but I suspect they mean rmax and rmin. In any case, A1 and A2 have not been defined earlier. So, this point should be clarified. In addition, we can imagine that x refers to the sugar concentration in the sample, but this should aslo be defined.

[Line 237, 243] Corrected the sentence. rmax and rmin were defined in line 243 and Figure 1B showed the measured values (0 and 1mM maltose concentrations) with the CMY-BII sensor at 53°C.

\*Line 217 refers to a "simplified model", but this is the first mention to two models one that would be more "elaborate" or "complex model" and a "simplified" one. I would appreciate if the authors stated what they mean when they refer to the "simplified model" or to indicate which other (more complex) model they are comparing it with. If they mean that the one model that they use is a simplified version of what could more accurately account for the physicochemical processes happening in the system, the authors should state it.

[Page 7, Line 278, Figure 2 Legend] Updated the figures including the figure legends

\*The authors state that ratiometric FRET measurements have advantages when the sensor concentration is not easy to control. However, beyond concentration, I think it would be good to comment on whether their method depends at any rate on the possible degradation of the sensor. In addition, I would appreciate if the authors mentioned how long the detection solution can be stored to be used in their method and whether measurements are affected by using a non-fresh detection solution.

[Line 307] We have not yet performed the analysis handling the FRET sensor degradation and storage issues. But we agreed that these issues are essential for the further reliable and stable applications of the FRET sensors and the device. A brief description was added in the Discussion section along with the quality control issue. Thank you for this comment.

\*Figure 2 is missing units in the Y axis.

Figures were updated.

Additional Comments to Authors:

(Bio)chemical or other materials are often made available for other researchers to use in their research. This can be done as collaboration or under other terms agreed by the involved institutions or groups. In this case, given that the FRET measurement instrument that the authors used is a prototype (although they explain the method for other instruments), I think it would help the researcher community if the authors offered to share their prototype for research purposes. However, this is just a suggestion. If the authors have reasons not to want to share their prototype at this stage of development, I don't think they need to do it in order for this article to be published. I just think it may help others become aware of the utility of this instrument.

Thank you for your helpful comments and suggestions. In fact, we’re making this protocol to promote the custom made FRET device along with optimal temperature control can be widely available in detecting various small molecules without expensive fluorescence detection devices. So any collaboration for research purposes would be more than welcome for us. As you said, this is a prototype but an upgraded FRET analyzer which is much lighter and less cost than the prototype is expected soon or later.

**Reviewer #3:**

Manuscript Summary:

The manuscript, titled "Sugar content assessment using a portable FRET analyzer: a prototype of a widely applicable point-of-care testing tool" describes an experimental procedure to determine sugar (or other solutes) concentrations in a complex solution using a prototype FRET analyzer developed by the authors. Closely following their previous publication, they demonstrate the procedure, using sugar content analysis in commercial beverages as an example.

Major Concerns:

The manuscript seems to have been revised twice prior to my assignment as a reviewer, but I still have a number of concerns with this manuscript. The main points that need to be addressed are followings,

1) I have doubts as to whether the procedure really benefits from publishing in a visual format. The protocol is fairly straight forward (bacterial expression followed by FPLC, then cuvette-based measurement of FRET ratio), and their previous publication describe the procedure in a sufficient detail. The most technically involving part might be the FPLC purification, but that procedure is mostly left out from this protocol (I don't even find the solutions used for this procedure).

There has been a major update to the script. This protocol mainly claims to support the wide applicability of the custom made FRET analyzer with optimal temperature control. FRET signal (ratio) intensity dramatically increases in the optimal temperature (reference 8 in the main script) and the custom made FRET analyzer with the FRET sensors can be used to quantifying target molecules in a simple manner. Please see the updates highlighted in red. The FPLC purification procedure was included in steps 1.17-1.22.

2) It is not clear to me how matrix effects are controlled. Fluorescent proteins (FPs) based sensors are innately sensitive to solutes, especially the ones that can function as quenchers such as phosphate ions (which happens to be abundant in commercial beverages). The protocol presented seems to provide a single point measurement. This would work only if other solutes contained in the beverage do not interfere with rmin and rmax. The effect on rmax can easily be assessed by adding a saturating concentration of maltose in addition to the diluted beverage. While assessing of rmin is impossible, using rmax value with the matching matrix would be better than not correcting for the matrix effect at all.

[Line 313] We agreed that the FRET signal will be interfered by the ingredients in the commercial beverage samples. Including the phosphate ions you mentioned, other types of sugars can also affect to the signal since FRET sensor engineering for substrate specificity resulted in broaden the specificity (reference 2 in the main text). So it is required further to investigate FRET sensors responding to various cases of sugar mixtures for the sugar quantification. Also collaboration with companies that produce the beverages will be helpful to confirm and to adjust the calibration of the FRET analyzer. We added it in the Discussion section.

3) The estimated concentration would be accurate only if the substrate concentration happens to fall within the dynamic range (usually two orders of magnitude) of the particular sensor. This can be evaluated by selecting a threshold (such as 10-90% saturation). If the r value does not fall within this range a different the concentration should be measured at a different dilution.

[Line 213] We agreed that our device also only detect the FRET signal within the sugar concentration is in between 0 to 1mM of maltose. So it is critical to dilute the beverage sample properly. In our case, 1000 fold diluted for the measurement. We suggest that one can estimate the target sugar concentration approximately by referring the sugar contents labeled on the beverage products.

4) For the particular application discussed here (measuring sugar content in beverages), it seems that the end user would want to know the content of all sugars, including fructose and glucose that are often used in very high concentrations. It would be possible to measure those sugars using this system provided there is a suitable FRET sensor, but as it stands those more common sugars in beverages are not discussed. Also the authors should discuss the substrate specificities of sensors.

[Line 313, 341] The substrate specificity issues were discussed with the FRET interference on Page 8 Line 318. It is true that people nowadays have an interest of the glucose and fructose concentration in beverages for the reason of healthcare. We discussed this issue with glucose FRET sensors on Page 8 Line 341.

5) Since the main advantage of this procedure (compared to using a high-end spectrofluorometer) seems to be the affordability of the instrument, it would be nice to know the predicted price range of the instrument. Also, the authors do not discuss other technology available for determining sugar content (including the ones that do not uase FRET measurement).

Since the journal policy strictly limits the use of commercial languages, the related contents were not discussed in this protocol. But the price range was discussed in our previous paper (reference 7 in the main text). We focused on the availability of custom made FRET device with optimal temperature control of FRET sensors and sugar detection is an example. So please understand that it seems not appropriate to compare sugar detection technologies in this protocol.

Other points;

1) Figure 1 is exactly the same as a figure included in a manuscript the authors previously published (ref.7). The authors should acknowledge this and sort out the copyright issues.

2) Likewise, the data in Figure 2 seems to be identical to what has been published in ref 7.

We changed the figures.

3) Line 201- A1 and A2 are not used in the equation. Is it rmin and rmax instead? Also provide the unit for x0.

[Line 240] Revised the equations

4) The last page of the manuscript seems to have a formatting issue.

Done

I would like to see the above points revised before I recommend the manuscript for publication.

We have carried out a major update of the manuscript including the results and figures. Thank you for your helpful discussions and comments.

Minor Concerns:

- FRET is an acronym Foerster Resonance Energy Transfer to be precise.

Changed

- There are many other types of FRET sensors than what they discuss - PBP is certainly not the only type of scaffold, and there are many different types of FPs too although CFP and YFP derivatives are most common. The authors should acknowledge that.

Thank you

- The readers won't know which one of the instruments listed is the "FRET analyzer" mentioned in the protocol.

We haven’t determined the proper noun of our device yet. But we revised the text to minimize readers’ confusion.

- line 204 rate or ratio?

Ratio is correct.

Additional Comments to Authors:

N/A