**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 to ensure correct spelling and grammar.**

2. Please revise lines 37-39 to avoid previously published text.

**We have revised the lines to avoid text that was previously published**

3. 1.1.1 and 1.1.2: Please revise so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

**We have included sub-steps to meet the requirements of the journal.**

4. 1.3.3: Please specify the antibiotics used here.

**We have specified the required antibiotics.**

5. 3.1.2, 4.1.3, 4.2.2: Please specify the culturing/growth conditions.

**We have specified the conditions mentioned in the cited paper.**

6. Lines 187: Please specify the previous step that makes the calibration curve.

**We have specified the step involved.**

7. 4.1.4: Please specify the earlier steps (step 2.1?).

**We have specified the step.**

8. 4.1.5, 4.2.1, 4.3.1, 4.3.2: Please specify the incubation temperature.

**We have specified all the incubation temperatures.**

9. 4.3.5: What is the pore size of the filter paper?

**We have specified the filter paper grade and pore size.**

10. Table of Materials: Please merge sheet 1 and sheet 2, and then sort the items in alphabetical order according to the Name of Material/Equipment.

**We have merged the tables and sorted them alphabetically.**

**Reviewers' comments:**

Reviewer #1:

Manuscript Summary:

The manuscript "Analysis of Fucosylated Human Milk Oligosaccharides in Biotechnological Context Using Genetically Encoded Biosensors" from Enam and Mansell, investigates a high-throughput method to detect HMO using whole-cell biosensor. The aim of this research is very interesting and valuable. The authors faced a major hurdle of false positive while adapting HMO production strains. Three alternative methods were introduced in the manuscript.

Major Concerns:

Authors have sufficiently addressed my concerns.

Minor Concerns:

Authors have sufficiently addressed my concerns.

**We thank the reviewer for the previous comments which has helped improve our manuscript.**

Reviewer #3:

Manuscript Summary:

This work describe a high-throughput screening method for detection and quantification of specific human milk oligosaccharides, in this case 2-fucosyllactose, using a whole cell biosensor.

Lactose produced by the biosensor trigger the fluorescence response that allow the HTS but there is a concern about noise signal due lactose naturally present in the samples. This work also addressed that problem with four different strategies.

The experiments are appropriately designed and performed, and the results support conclusions in general. There is, however, a minor concern about the method and result of the experiment related to Fig. 3C that need further explanation.

Minor Concerns regarding experiment related to Figure 3C.:

Regarding the methodology. It is not clear the meaning of "a mixture 1:1 (0,2% lactose equivalent)". Is this 0,1% lactose + 0,15% 2'-FL; 0,2% lactose + 0,3% 2'-FL, or some different concentrations?

**A mixture 1:1 (0.2 % lactose equivalent) implied 0.1 % lactose + 0.15 % 2'-FL. We have clarified that in the text.**

The clarification of this will be helpful to better understand the result of that figure regarding the fluorescence level reached after 3h incubation with the E. coli BL21 (DE3) in LB + the mixture 1:1, comparing either with Fig 1A or 3E. Otherwise with that scale it would seam that E. coli is able to also metabolize 2'FL after growth with lactose, because the fluorescence is much closer to the level reached in the LB + 0,2% lactose than to the level of LB + 0,3% 2'-FL.

**We have previously shown that *E. coli* lacking fucosidase expression cannot utilize 2’-FL as was seen by lack of growth on 2’-FL in minimal media (Enam and Mansell, *Cell Chem. Biol.,* 2018). With the fluorescence being expressed in arbitrary units, we cannot compare the fluorescence values obtained with the other graphs.**

It would be nice to have in the same figure, another line with LB + the same concentration of 2'-FL used in the 1:1 mixture, to use as a control, showing that E.coli consume all the lactose, but nothing of the 2'-FL, reaching the same fluorescence level after 3h.

**The question of whether *E. coli* BL21 is acting on 2’-FL *in the presence* of lactose is interesting. In the whole cell biosensor, the relevant fucosidases are expressed in the periplasm because we were unsure if HMO trisaccharides would cross the inner membrane. However, growth in the presence of lactose in a Lac+ strain would increase LacY expression which *might* (maybe?) transport 2’-FL as well, sequestering it inside the cell and leaving (at least the lactose moiety) subject to degradation by LacZ. Alternatively, at longer times (3 h) some lysis or leakage from BL21 might release LacZ into the media (compare selectivity at 2h and 3h timepoints). Thus, we have a similar issue as in the case of exogenously added LacZ (Figure 3B). However, we feel that exploring 2’-FL transport/degradation is interesting, but beyond the scope of this methods paper. Like any fluorescent biosensor, a standard curve under the relevant conditions is advisable.**

Query/suggestion for author: Considering that production of GDP-L-fucose can be achieved by overexpression of manB, manC, gmd and wcaG genes, and that would not be neccesary to add fucose to the 2'-FL producing strains, would be useful to change the biosensor to use Fucose as a trigger instead of lactose ? In this way you will not have noise issues due the presence of lactose from your producing strains.

**We thank the reviewer for the insightful comment. The producer strain we used in fact has the de novo synthesis pathway integrated and does not require addition of L-fucose. We are currently exploring this angle, but for this work, our goal was to develop a platform to detect not only fucosylated HMOs but all others (the original paper features fucosylated, sialylated, and N-acetylglucosaminated trisaccharides), and hence, our strategy was based on detecting the common lactose core.**

Reviewer #4:

Manuscript Summary:

The manuscript does not contain the fact that E.coli strains are in use as biosensors. Moreover the fact that fluorescence is measured from lactose-dependent gene expression of a fluorescent protein (GFP) needs to be mentioned.

**We have clarified the nature of the biosensor in the text by including “whole cell biosensor” in the description. While we mentioned the T7 promoter driving expression of GFP, we did not explicitly mention its inducibility by lactose. We have added details in line 252.**

Major Concerns:

The method has its merits for the detection of the simplest HMO, 2´-fucosyllactose, being produced by a recombinant E.coli strain which needs to be LacY+ and LacZ+. The title of the manuscript, however, gives the promise that "fucosylated human milk oligosaccharides" can be detected. Only one example is given thus. Detection of difucosylated HMOs in the presence of monofucosylated HMO would be difficult as the readout is based on lactose formation.

**Here, we demonstrate biosensors for 2’-FL and 3-FL, and thus have changed the title to “Analysis of Fucosylated Human Milk Trisaccharides in Biotechnological Context Using Genetically Encoded Biosensors”**

Minor concerns:

line 49: please give a quotation for this chapter.

**We have cited the relevant paper.**

line 84: pET28:GFP and pT7-GFP are used in the text. Are these the same or different ones?

**They are the same plasmids. We have replaced it with pET28:GFP throughout the text.**

line 83-85. Please give the origin of plasmids pAfcA and pAFCb.

**We have cited the reference for the plasmids.**

line 158: are you sure you have used 100g/l of sodium citrate? Is this then the C source of E.coli? How about glycerol?

**We apologize for the miscalculation and thank the reviewer for pointing it out. The sodium citrate concentration is in fact 75 mg/L. 100 g/L was the concentration of the stock solution. Glycerol (1 % w/v) was used as the carbon source in these experiments. Aerobic citrate metabolism is not natively present in *E. coli*.**

line 161: as the reader of JOVE might not have access to Baumgartner et al. : please describe briefly, how cells have to be cultivated

**We have detailed the steps needed to be carried out.**

lines 252/253: J23110 promoter is not explained or referenced; pG9m-2 vector is neither explained nor referenced.

**To remove ambiguity, we have replaced the names with description of the promoter and vector. The details can be found in the cited reference.**