We tried to address all of the comments to the best of our possibilities.

**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We proofread the manuscript.

2. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

3. Please expand the Summary to briefly describe the applications of this protocol.

Summary expanded.

4. Please define all abbreviations before use.

Abbreviations defined.

5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.:

Done.

6. For culture media and buffer such as RPMI, DMEM, PBS, etc., please spell out at first use and provide composition. If they are purchased, please cite the materials table.

We defined the composition of PBS, other media are purchased, Materials table cited.

7. 1.3-1.9: How to adjust pH? What type of filter is used? Roughly what volume is needed? Please specify throughout.

We specified the filter and volumes in text.

8. 1.10: Is a water bath used?

Yes, water bath is used.

9. 2.1/3.1: Please include this as a “Note” instead of a step.

Done.

10. 3.2/5.4: Please use subscripts in chemical formulae to indicate the number of atoms: CO2.

Done.

11. 6.1: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action.

This step only explains that you need to seed the cells in two XFp plates.

12. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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.

Done.

13. 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.

Done.

14. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Corrected.

**Reviewers' comments:**  
  
  
  
**Reviewer #1:**   
Manuscript Summary:  
The manuscript titled 'Assessment of metabolic profile of primary leukemia cells employing Seahorse XFp Analyzer' is a very clear protocol to perform the procedure. It is well written and most of the steps are very clearly explained. I have a couple of minor comments listed below to possibly further improve the clarity of presentation.  
  
Major Concerns:  
It should be commented on how quickly after the bone marrow collection the procedure should be initiated, to let interesting parties know if there is a possible wait/transportation window or not.  
Note added (line 127): Ideally, measurement of metabolic state of primary leukemia cells should start immediately after bone marrow collection and cell isolation. Nevertheless, we were able to get relevant data also from cells isolated after transportations from other hematology centers from Czech Republic.

Minor Concerns:  
Line 47: Glucose uptake does not need to be measured radioactively, NBDG is not radioactive.

We added NBDG method as another procedure to be used for glycolysis function measurement.

Line 55: 'These methods allow to measure only one metabolic parameter in one sample' Given the reference 11 listed in the section above, where respiration and mitochondrial membrane potential are measured simultaneously, this statement is perhaps not completely correct.

We corrected the statement.

Line 84: Would the new Agilent-recommended procedure to use Hepes in the medium for pH stability, i.e. not having to set the pH before every experiment, be applicable to the procedure? Maybe this should be commented on.

We use HEPES only in the Cell mito stress test media, not in the Glycolysis stress test medium. And because setting the pH to 7.4 is quite important, we recommend to adjust the pH before every experiment and we do not recommend to use Hepes supplemented media in Glycolysis stress test.

Line 100 Point 1.12, it is not entirely clear when BSA should be added.

The sentence is corrected since in the point 1.12 we are describing the preparation of the Mito cell stress medium without BSA.

Line 110: Maybe provide info on how to identify blasts to make sure they form 80% of cell population?

Leukemic blast cells are identified by flow cytometry using specific immunophenotypic markers. First, we adjust the total cell number by syto-16 dye which marks only nuclear cells. After that we use CD markers typical for each type of leukemia, B-ALL (CD19+, CD45 dim/-), T-ALL (CD3, CD4, CD5, CD8, CD7, CD99) and in case of AML (CD33 and other myeloid markers). We tried to addressed it also in the text.

Line 153: Better perhaps: 'Let the plates with Cell-TAK sit in the hood for about 20 minutes.'

Corrected.

Line 156: 'with THE rim wrapped…'

Corrected.

Line 189: maybe state the optimal and lowest acceptable number of replicates, e.g. number of wells used.

Sentence added: The optimal number of replicates is six, as is described here. We do not recommend using less replicates, since primary cells could sometimes behave erroneously.

Line 237: 'After subtracting the lowest ECAR value'. While this is expected to be the value after 2DG addition which represent non-glycolytic acidification, this should be clearly stated.

Sentence added to the point 9.1: This lowest value represents the non-glycolytic acidification.

Lina 238: the first three measurements (omit the first ECAR value if it significantly differs from the other two): Use the expression 'measurement points' instead of 'measurements', to make it clearer.

Measurement points are used instead measurements.

Line 252: the same as above

Measurement points are used instead measurements.

Line 291: Consider adding something like: 'Please note that the first measurement point can significantly differ from the rest and should be excluded from the analysis in that case'.  
Note added.  
  
**Reviewer #2:**  
Manuscript Summary:  
In this manuscript Hlozkova K and Starkova J provide and describe a protocol to, first, isolate primary blast leukemic cells from the bone marrow´s patients and second, to study the metabolic status (oxigen consumption rate and extracellular acidification) of these cells using Seahorse analyzer XFp  
  
Major Concerns:  
No major concerns  
  
Minor Concerns:  
1.- We encourage the authors to revise the abstract writing, specifically regarding "the need of metabolically characterize the leukemic cell population"- for example, the idea of the first three sentences could be rephrase for a better comprehension

We revised the abstract.

2.- Provide the formulation of the Glycolysis stress media (as provided for the cell mito stress)

Formula was added. It is XF Base medium with the pH adjusted to 7.4.

3.- Why authors use BSA only in the Cell Mito Stress Test, but not in the Glycolysis. An explanation would be useful for the scientific community.

BSA is used because the cells response better to FCCP with BSA in the medium. And FCCP is used only in the Cell mito stress test.

Sentences added at the end of the point 1: NOTE: BSA is added to the Cell mito stress test medium because the cells response better to FCCP when the medium is supplemented with BSA. Cell Mito stress test medium without BSA is used for loading the ports as the manufacturer does not recommend using BSA there.

4.- Why authors add pyruvate to the Seahorse media? We ask because we think that the cells need to be monitored by the Seahorse in the same conditions as they grow (RPMI-no pyruvate)

We use pyruvate only in the Cell mito stress test, it is recommended by the manufacturer. Anyway, the cells are not monitored in the same conditions as they grow because, among other things, during the measurement, only atmospheric CO2 is present and no FBS is added to the media.

5.- We encourage the authors to include a protein/cell number control in parallel (for example, seed 500.000 cells in a separate plate), as the number of cells/protein content is critical for the normalization of the measurements.

Since we count cells prior to measurement and we measure them half hour after seeding we are sure about the cell number. In case of adherent cells which are seeded day before it could be a relevant point but in case of suspension cells we measure the number of cells added into the well.

6.- Step 6.8, we advice to increase the incubation time to 1 hour.

We cannot agree with this advice since primary cells die rapidly in a non-CO2 environment.

7. Which is the final concentration of the drugs in the wells?

Final concentrations added to the Table 1.

8.- Step 9.1: it would be better to subtract the average of the measurement after 2DG/rotenone&antimycinA addition, instead of only consider the lowest value?

It is also possible but we think that using the lowest values is better and more correct way to calculate the non-mitochondrial respiration and the non-glycolytic acidification.

9.- Why in the cycles authors do not include a "wait" step? Generally the normal setting used in Seahorse are 3 cycles of: 2min mix, 2 min wait and 4 min measure.

We did not include wat step in the measurement since it is not recommended my manufacturer of this specific Analyzer XFp. Details of the run we used are mentioned in the text. It is 3 cycles of 3 min mix and 3 min measure for each parameter.

10.- Although we understand that the cell material is limited, we were wondering if a only-DMSO port should be added as a control for the FCCP treatment.

Since the DMSO in the wells is approximately 200 times diluted, we decided to omit the wells with an only-DMSO port.