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TITLE:

Assessment of the Metabolic Profile of Primary Leukemia Cells

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KEYWORDS:

leukemia, bone marrow, metabolism, glycolysis, mitochondrial respiration, extracellular flux, metabolic profile, analyzer

SUMMARY:

Here we present a protocol for the isolation of leukemic cells from leukemia patients bone marrow and analysis of their metabolic state. Assessment of the metabolic profile of primary leukemia cells could help to better characterize the demand of primary cells and could lead up to more personalized medicine.

ABSTRACT:

The metabolic requirement of cancer cells can negatively influence survival and treatment efficacy. Nowadays, pharmaceutical targeting of metabolic pathways is tested in many types of tumors. Thus, characterization of cancer cell metabolic setup is inevitable in order to target the correct pathway to improve the overall outcome of patients. Unfortunately, in a majority of cancers, the malignant cells are quite difficult to obtain in higher numbers and the tissue biopsy is required. Leukemia is an exception, where a sufficient number of leukemic cells can be isolated from the bone marrow. Here, we provide a detailed protocol for the isolation of leukemic cells from leukemia patients bone marrow and subsequent analysis of their metabolic state using extracellular flux analyzer. Leukemic cells are isolated by the density gradient, which does not affect their viability. The next cultivation step helps them to regenerate, thus the metabolic state measured is the state of cells in optimal conditions. This protocol allows achieving consistent, well-standardized results, which could be used for the personalized therapy.

INTRODUCTION:

The metabolic profile is one of the main characteristics of cells and altered bioenergetics are now considered one of the hallmarks of cancer[1–3]. Moreover, changes in the metabolic setup could be used in the treatment of cancer by targeting signal transduction pathways or enzymatic

machinery of cancer cells[4–6]. Knowing the metabolic predisposition of cancer cells is thus an advantage and can help improve the current therapy.

There are a plenty of already established methods which can assess the metabolic activity of cells in culture. Regarding glycolysis, glucose uptake can be measured by the radioactive labeling, using 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) or extracellular lactate levels measured enzymatically[7,8]. Fatty acid oxidation rate is another metabolic parameter measured by isotopically labeled palmitate[9,10]. Oxygen consumption rate is a method widely-used for determining mitochondrial activity in cells[11,12], together with the mitochondrial membrane potential evaluation[13,14], ATP/ADP (adenosine 5'-triphosphate/Adenosine 5'-diphosphate) ratio measurement[15] or total intracellular ATP measurement[16]. Signaling pathways known to regulate metabolic processes could be determined by protein quantifications and can improve the understanding of metabolic measurements[17–19].

However, all these methods measure only one or, in the best scenario, a few metabolic parameters in one sample simultaneously. Importantly, simultaneous measurement of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) can be achieved by the extracellular flux analysis by, for example, Seahorse XFp Analyzer. OCR is an indicator of mitochondrial respiration and ECAR is mainly the result of glycolysis (we cannot ignore CO_2 production possibly elevating ECAR of cells with high oxidative phosphorylation activity)[20]. So far, various cell types have been studied using these analyzers[21–23].

Here we describe the protocol for the extracellular flux analysis of primary blasts (leukemia cells derived from the immature hematopoietic stage) from leukemia patients. To the best of our knowledge, a specific protocol for primary blasts is not available yet.

PROTOCOL:

All samples were obtained with the informed consent of the children's parents or guardians and approval of Ethical committee of Charles University in Prague, Czech Republic, the study no. NV15-28848A.

1. Preparation of Reagents

1.1 Prepare 500 mL of PBS by dissolving 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, in ddH_2O. Adjust the pH to 7.4 with HCl. Sterilize by autoclaving.

1.2 Prepare 100 mL of RPMI medium: RPMI-1640 medium with L-Alanyl-Glutamine supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL).

1.3 Prepare 50 mL of 0.1 M $NaHCO_3$ in distilled water. Adjust the pH to 8.1, filter sterilize (0.22

μm) and store at 4 °C.

Note: For two 8-well extracellular flux analyzer plates, prepare 250 μL of 0.1 M $NaHCO_3$ pH 8.1.

1.4 Prepare 1 mL of 2 M D-glucose in distilled water. Filter sterilize (0.22 μm) and store at -20 °C.

1.5 Prepare 100 μL of 1 mM Oligomycin A in ethanol. Filter sterilize (0.22 μm) and store at -20 °C.

1.6 Prepare 250 μL of 1 M 2-deoxy-D-glucose (2-DG) in Minimal DMEM (Dulbecco's Modified Eagle's medium). Warm to 37 °C and adjust the pH to 7.4 (carry out pH measurement at 37 °C). Filter sterilize (0.22 μm) and store at -20 °C.

1.7 Prepare 100 μL of 1 mM FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) in DMSO. Filter sterilize (0.22 μm) and store at -20 °C.

1.8 Prepare 100 μL of 1 mM Rotenone in ethanol. Filter sterilize (0.22 μm) and store at -20 °C.

1.9 Prepare 100 μL of 1 mg/mL Antimycin A in ethanol. Filter sterilize (0.22 μm) and store at -20 °C.

1.10 Just before the use, prepare 10 mL of Glycolysis stress test medium. Warm Minimal DMEM to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).

1.11 Prior to the use, prepare 10 mL of Cell Mito stress test medium with BSA. Supplement Minimal DMEM with 2 mM L-glutamine, 10 mM D-glucose, 1 mM HEPES (pH 7.4), 1 mM pyruvate and 0.1 % BSA (bovine serum albumin). Warm to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).

1.12 Prior to the use, prepare 10 mL of Cell Mito stress test medium without BSA. Supplement Minimal DMEM with 2 mM L-glutamine, 10 mM D-glucose,1 mM HEPES (pH 7.4) and 1 mM pyruvate. Warm Cell Mito stress test medium without BSA to 37 °C in a water bath and adjust the pH to 7.4 (carry out pH measurement at 37 °C).

Note: BSA is added to the Cell Mito stress test medium because the cells respond better to FCCP when the medium is supplemented with BSA (different conditions were tested). Cell Mito stress test medium without BSA is used for loading the ports as the manufacturer does not recommend using BSA in the medium.

2. Isolation of Mononuclear Cells from Bone Marrow

Note: Ideally, measurement of metabolic state of primary leukemia cells should start immediately after bone marrow collection and cell isolation. Nevertheless, relevant data could be also obtained from cells isolated after transportation from other hematology centers in the Czech Republic. Perform all sub-steps in a sterile tissue culture hood.

2.1 Warm up PBS and the density gradient medium to room temperature.

2.2 Dilute the bone marrow sample of leukemia patient with PBS, in a ratio of 1:1. Make sure that the sample contains at least 80% of leukemic blasts.

2.3 Determine the percentage of blasts by flow cytometry using specific CD markers for immunophenotype characterization of cell types. After density gradient separation, detect nucleic acid positive events with a nuclear dye in order to establish the overall cell count.

2.4 Then, determine leukemia cells using specific CD markers for each type of leukemia: B-ALL (CD19, CD45), T-ALL (CD3, CD4, CD8, CD5, CD7, CD99) and AML (CD45, CD33, and specific myeloid markers)[24]. Divide the number of leukemia cells positive for specific CD markers by all nuclear cells to determine the percentage of leukemia blast cells.

Note: The bone marrow has to be collected into the tubes with anticoagulants.

2.5 Carefully layer 6 mL of diluted bone marrow sample over 6 mL of the density gradient medium in a 15 mL conical tube. Centrifuge at 400 × g for 35 min at 4 °C in a swinging-bucket rotor without the brake.

Note: Larger volume of the sample could be divided into more aliquots or 50 mL conical tube can be used with the larger amount of density gradient medium.

2.6 Using a Pasteur pipette, carefully transfer the interphase layer which consists of mononuclear cells (**Figure 1**) to a new 50 mL conical tube with 5 mL of PBS. Centrifuge at 400 × g for 10 min at 4 °C in a swinging-bucket rotor.

Note: Transfer all mononuclear cell layers into a single 50 mL conical tube with 5 mL of PBS.

2.7 Aspirate the supernatant and resuspend the cell pellet in 2 mL of sterile PBS. Count the cells using a hemocytometer.

Note: The number of leukemia cells in one mL of aspirated bone marrow differs significantly among patients[25].

3. Overnight Cultivation of Mononuclear Cells

Note: Perform all sub-steps in a sterile tissue culture hood.

3.1 Prepare two T75 flasks with 20 mL of RPMI. To each flask, add 30 x 10^6 isolated mononuclear cells. Incubate the cells with the flask standing up for 16- 24 h at 5% CO_2 and 37 °C.

4. Preparation of Cell Adhesive-coated Plates

4.1 Coat two 8-well extracellular flux analyzer plates.

Note: Perform the coating in a sterile tissue culture hood.

4.2 Add 2.2 µL of cell adhesive (density: 2.54 mg/ml) to 250 µL of 0.1 M $NaHCO_3$, pH 8.1, and pipette immediately 12.5 µL of the solution into each well.

Note: Cell adhesive stock solutions can differ in their density, adjust the volume added to $NaHCO_3$ accordingly.

4.3 Let the plates sit in the hood for about 20 min, then aspirate the cell adhesive and wash each well twice using 200 µL of sterile water. Let it sit in the hood with the lid open until wells are dry.

4.4 Use the plates right away or save up to 1 week at 4 °C with the rim wrapped in paraffin film to avoid condensation. Ensure that the plates are warmed up to room temperature (for about 20 min) in the hood before seeding the cells.

5. Hydration of Sensor Cartridge

Note: Hydrate two 8-well extracellular flux analyzer cartridges.

5.1 Separate the utility plate and the sensor cartridge. Place the sensor cartridge upside down on the lab bench.

5.2 Fill each well of the utility plate with 200 µL calibrant. Fill each moat around the outside of the wells with 400 µL of calibrant.

5.3 Return the sensor cartridge to the utility plate that now contains the calibrant.

5.4 Place the cartridge assembly in a humidified, non-CO_2, 37 °C incubator overnight.

5.5 Turn on the extracellular flux analyzer and let it warm to 37 °C overnight.

6. Seeding Cells in Cell Adhesive-coated Plates

Note: For Glycolysis stress test, use Glycolysis stress test medium. For Cell Mito stress test, use Cell Mito stress test medium with BSA.

6.1 Seed cells for the Glycolysis stress test and the Cell Mito stress test in separate plates. For each test, use cells from one flask with overnight culture.

6.2 Centrifuge cells at 200 × g for 5 min at room temperature. Resuspend cells in 1 mL of the

appropriate medium and count them.

6.3 Add 4 x 10^6 of live cells to the final volume of 400 µL (use appropriate medium).

6.4 Plate 50 µL of the cell suspension into wells B-G. Ensure 500,000 cells are seeded in one well.

Note: It is crucial to seed exactly 500,000 cells per well as no other normalization is performed. That way, the results from different patients can be compared. The optimal number of replicates is six, as is described here. Using less replicates is not recommended since primary cells could sometimes behave erroneously.

6.5 Add 180 µL of the appropriate medium into wells A and H (these wells will serve as a background correction).

6.6 Centrifuge the plate at 400 × g for 5 min at room temperature with brake set to 1.

6.7 Add 130 µL of appropriate medium to wells B-G in two 8-well extracellular flux analyzer plates slowly and carefully. Visually confirm that the cells are stably adhered to the bottom of the wells by viewing under the microscope.

6.8 Place the plate into a humidified, non-CO_2, 37 °C incubator for 30 min.

7. Loading the Sensor Cartridge

7.1 For the Glycolysis stress test, prepare 250 µL each of 100 mM glucose, 20 µM Oligomycin A and 1 M 2-DG, all in Glycolysis stress test medium.

7.2 For the Cell Mito stress test, prepare 250 µL each of 20 µM Oligomycin A, 15 µM FCCP, 30 µM FCCP and a mixture of 10 µM Rotenone and 10 µg/ml Antimycin A, all in Cell Mito stress test medium without BSA.

Note: Injecting two concentrations of FCCP in one assay is recommended since there is not enough patients' material for the FCCP titration. Nevertheless, the concentrations should be determined by the researcher.

7.3 Load the compounds into the appropriate injector ports of the cartridge as follows (**Table 1):**

8. Setting Up the Program

8.1 For the Glycolysis stress test, set up the program as described in **Table 2**.

8.2 For the Cell Mito stress test, set up the program as described in **Table 3**.

8.3 Start the program. Replace the calibrant plate with the assay plate (when prompted).

9. Evaluation and Interpretation of the Results

9.1 In the Glycolysis stress test results, subtract the lowest ECAR value after 2-DG injection from all other ECAR values.

Note: This lowest value represents the non-glycolytic acidification. Usually, the lowest value is from the 4^{th} measurement.

9.2 Calculate Basal acidification, Glycolysis, Maximal glycolysis and Glycolytic reserve parameters from glycolytic function **(Figure 2A)**. After subtracting the lowest ECAR value, calculate Basal acidification as a mean of ECAR from the first three measurement points (omit the first ECAR value if it significantly differs from the other two), calculate Glycolysis as a mean of ECAR from three measurement points after glucose injection and calculate Maximal glycolysis as a mean of ECAR from three measurement points after Oligomycin A injection. Calculate Glycolytic reserve as Maximal glycolysis minus Glycolysis.

Note: Alternatively, for Maximal glycolysis calculation, use the highest ECAR value from the three points measured.

9.3 In the Cell Mito stress test results, subtract the lowest OCR value after Rotenone/Antimycin A injection from all other OCR values.

9.4 Calculate Basal respiration, ATP production, Maximal respiration and Spare capacity parameters from mitochondrial function (**Figure 2B**). After subtracting the lowest OCR value, calculate Basal respiration as a mean of OCR from the first three measurement points.

Note: Maximal respiration is the highest OCR value after FCCP injection.

9.5 For ATP production calculation, subtract the mean of the three OCR measurement points after Oligomycin A injection from Basal respiration. Calculate Spare capacity as the Maximal respiration minus the Basal respiration.

Note: Always calculate the Maximal respiration from the highest OCR value, regardless of the FCCP concentration used.

REPRESENTATIVE RESULTS:

Figure 3 shows the curves after Glycolysis stress test and Cell Mito stress test measurements of leukemic blasts from the BCP-ALL (B-cell precursor acute lymphoblastic leukemia) and AML (acute myeloid leukemia) patients. The calculation of metabolic parameters from these measurements is also indicated. 500,000 cells per well were seeded and all measurements were done in hexaplicates.

In the Glycolysis stress test, the only basal medium is used, so that the cells are deprived of nutrients. The first parameter obtained is the Basal acidification, which should reflect the amount of glucose stored in cells. After the first injection, ECAR is increased since cells utilize glucose and can ferment it to lactate. Oligomycin A in the second injection inhibits ATP-synthase and thus directs the cells to produce ATP mainly via glycolysis. This should cause further elevation of ECAR. Injection of 2-DG completely inhibits glycolysis and ECAR drops.

In the Cell Mito stress test, a medium supplemented with glutamine and glucose are used, so that the cells are not deprived of all nutrients and the Basal respiration parameter reflects their basal metabolic state. After first injection with Oligomycin A, cells inhibit mitochondrial respiration and switch to glycolysis which is represented as a decrease of OCR. FCCP (the second and third injection), on the other hand, uncouples ATP production from respiration, so that the cells now consume oxygen at a maximal rate and OCR rise to its highest value. The last injection of Rotenone and Antimycin A mixture completely inhibits mitochondrial respiration and OCR is decreased close to zero.

FIGURE AND TABLE LEGENDS:

Figure 1: Density gradient centrifugation of the bone marrow sample. Mononuclear cells enriched for leukemic cells are separated by density gradient medium.

Figure 2: Outline of Glycolysis stress test and Cell Mito stress test. A. Exemplary result of Glycolysis stress test. **B.** Exemplary result of Cell Mito stress test. Parameters are indicated within the curves.

Figure 3: Extracellular flux analysis of leukemic blasts from BCP-ALL (A, B) and AML (B, C) patient. A and C. Glycolysis stress test results. Please note that the first measurement point can significantly differ from the rest and should be excluded from the analysis in that case. **B and D.** Cell Mito stress test results. Parameters are indicated within the curves.

Table 1: Compound volumes.

Table 2: Program for Glycolysis stress test.

Table 3: Program for Cell Mito stress test.

DISCUSSION:

The above-described protocol allows for the measurement of the metabolic activity assessed by OCR and ECAR values in primary leukemic blasts derived from patients with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML). The advantage of measurement using an extracellular flux analyzer is that it enables the detection of metabolic profile in the real time in the live cells. Essentially, every step in the provided protocol could be adjusted depending on the cell type one plans to study. Here, we will discuss the most important parameters which could affect the results and could provide less than optimal values.

The first step towards optimization was a comparison of data obtained from fresh material vs frozen material. The ability to measure the metabolic activity from the frozen material would allow for retrospective studies of patients' samples stored in liquid nitrogen bank. In case of ALL samples, we were able to detect consistent metabolic activity only from the fresh material whereas AML cells were measured also after de-freezing with optimal results.

The second step towards optimization is the cultivation of primary leukemic blasts. We have tested the metabolic activity of the cells straight after the density gradient separation (without culturing) or after culturing overnight. Even if the cells after the density gradient separation looked viable and vital under a microscope, their metabolic activity was impaired. Overall ECAR and OCR values were lower and also, after injection, OCR or ECAR values did not respond optimally as they did in cultivated cells.

Cultivation under different conditions can also influence the results. Using insulin transferrin sodium selenite supplement (ITS) is considered a good practice when cultivating primary blasts[8], but this supplement interferes with the metabolic activity of the cells. During Cell Mito stress test, leukemic blasts cultivated with ITS did not respond to Oligomycin A (OCR should decrease in order to calculate ATP-linked respiration). We also tried to co-cultivate the cells with mesenchymal stem cells (MSC), but in this case, OCR and ECAR values have been lower compared to the cells cultivated without MSCs. In summary, cultivating primary blasts from leukemia patients in RPMI medium with 10 % FBS is the best option.

Patients suitable for the characterization of their metabolic profile must meet certain criteria which on one hand limit the number of tested samples but on the other will yield relevant results. We measured the metabolic function of patients with high cellularity (for one measurement we seeded 500,000 cells/per wells in hexaplicate) and only samples with 80 and a higher percentage of leukemic blasts could be measured to avoid detection of unspecific metabolic activity of other cell types present in the suspension.

One of the crucial steps in data analysis is the normalization, so that metabolic parameters between different leukemic samples could be compared. According to our previous experiments performed with leukemic cell lines, we found that normalization to the number of the cells gives the best results. The specific number of cells per well needs to be determined by the researcher and depends on the size and metabolic activity of tested cells.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1. DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G. & Thompson, C. B. The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metabolism.* **7,** 11–20 (2008).
2. Ward, P. S. & Thompson, C. B. Metabolic Reprogramming: A Cancer Hallmark Even Warburg Did Not Anticipate. *Cancer Cell.* **21,** 297–308 (2012).
3. Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell Metabolism.* **23,** 27–47 (2016).
4. Wise, D. R. & Thompson, C. B. Glutamine addiction: a new therapeutic target in cancer. *Trends in Biochemical Sciences.* **35,** 427–433 (2010).
5. Kroemer, G. & Pouyssegur, J. Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell.* **13,** 472–482 (2008).
6. Liberti, M. V. *et al.* A Predictive Model for Selective Targeting of the Warburg Effect through GAPDH Inhibition with a Natural Product. *Cell Metabolism.* **26,** 648–659.e8 (2017).
7. Lundgaard, I. *et al.* Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. *Nature Communications.* **6,** 6807 (2015).
8. Hermanova, I. *et al.* Pharmacological inhibition of fatty-acid oxidation synergistically enhances the effect of l-asparaginase in childhood ALL cells. *Leukemia.* **30,** 209–18 (2016).
9. Malandrino, M. I. *et al.* Enhanced fatty acid oxidation in adipocytes and macrophages reduces lipid-induced triglyceride accumulation and inflammation. *American Journal of Physiology-Endocrinology and Metabolism.* **308,** E756–E769 (2015).
10. Patella, F. *et al.* Proteomics-based metabolic modeling reveals that fatty acid oxidation (FAO) controls endothelial cell (EC) permeability. *Molecular & Cellular Proteomics : MCP.* **14,** 621–34 (2015).
11. Chowdhury, S. R., Djordjevic, J., Albensi, B. C. & Fernyhough, P. Simultaneous evaluation of substrate-dependent oxygen consumption rates and mitochondrial membrane potential by TMRM and safranin in cortical mitochondria. *Bioscience Reports.* **36,** e00286 (2015).
12. Simonnet, H., Vigneron, A. & Pouysségur, J. Conventional Techniques to Monitor Mitochondrial Oxygen Consumption. *Methods in Enzymology.* **542,** 151–161 (2014).
13. Martínez-Reyes, I. *et al.* TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. *Molecular Cell.* **61,** 199–209 (2016).
14. Sukumar, M. *et al.* Mitochondrial Membrane Potential Identifies Cells with Enhanced Stemness for Cellular Therapy. *Cell Metabolism.* **23,** 63–76 (2016).
15. Wundenberg, T., Grabinski, N., Lin, H. & Mayr, G. W. Discovery of InsP6-kinases as InsP6-dephosphorylating enzymes provides a new mechanism of cytosolic InsP6 degradation driven by the cellular ATP/ADP ratio. *The Biochemical Journal.* **462,** 173–84 (2014).
16. Morciano, G. *et al.* Use of luciferase probes to measure ATP in living cells and animals. *Nature Protocols.* **12,** 1542–1562 (2017).
17. Chau, M. D. L., Gao, J., Yang, Q., Wu, Z. & Gromada, J. Fibroblast growth factor 21 regulates energy metabolism by activating the AMPK-SIRT1-PGC-1alpha pathway. *Proceedings of the National Academy of Sciences of the United States of America.* **107,** 12553–8 (2010).
18. Lee, K.-H. *et al.* Targeting energy metabolic and oncogenic signaling pathways in triple-negative breast cancer by a novel adenosine monophosphate-activated protein kinase (AMPK) activator. *The Journal of Biological Chemistry*. **286,** 39247–58 (2011).
19. Godlewski, J. *et al.* MicroRNA-451 Regulates LKB1/AMPK Signaling and Allows Adaptation

to Metabolic Stress in Glioma Cells. *Molecular Cell.* **37,** 620–632 (2010).
20. Zhang, J. *et al.* Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nature Protocols.* **7,** 1068–85 (2012).
21. Kaplon, J. *et al.* A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. *Nature.* **498,** 109–112 (2013).
22. Pardee, T. S. *et al.* A phase I study of the first-in-class antimitochondrial metabolism agent, CPI-613, in patients with advanced hematologic malignancies. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research.* **20,** 5255–64 (2014).
23. Stein, M. *et al.* A defined metabolic state in pre B cells governs B-cell development and is counterbalanced by Swiprosin-2/EFhd1. *Cell Death and Differentiation.* **24,** 1239–1252 (2017).
24. Hrušák, O. & Porwit-MacDonald, A. Antigen expression patterns reflecting genotype of acute leukemias. *Leukemia.* **16,** 1233–1258 (2002).
25. Amin, H. M. *et al.* Having a higher blast percentage in circulation than bone marrow: clinical implications in myelodysplastic syndrome and acute lymphoid and myeloid leukemias. *Leukemia.* **19,** 1567–1572 (2005).

Figure 1

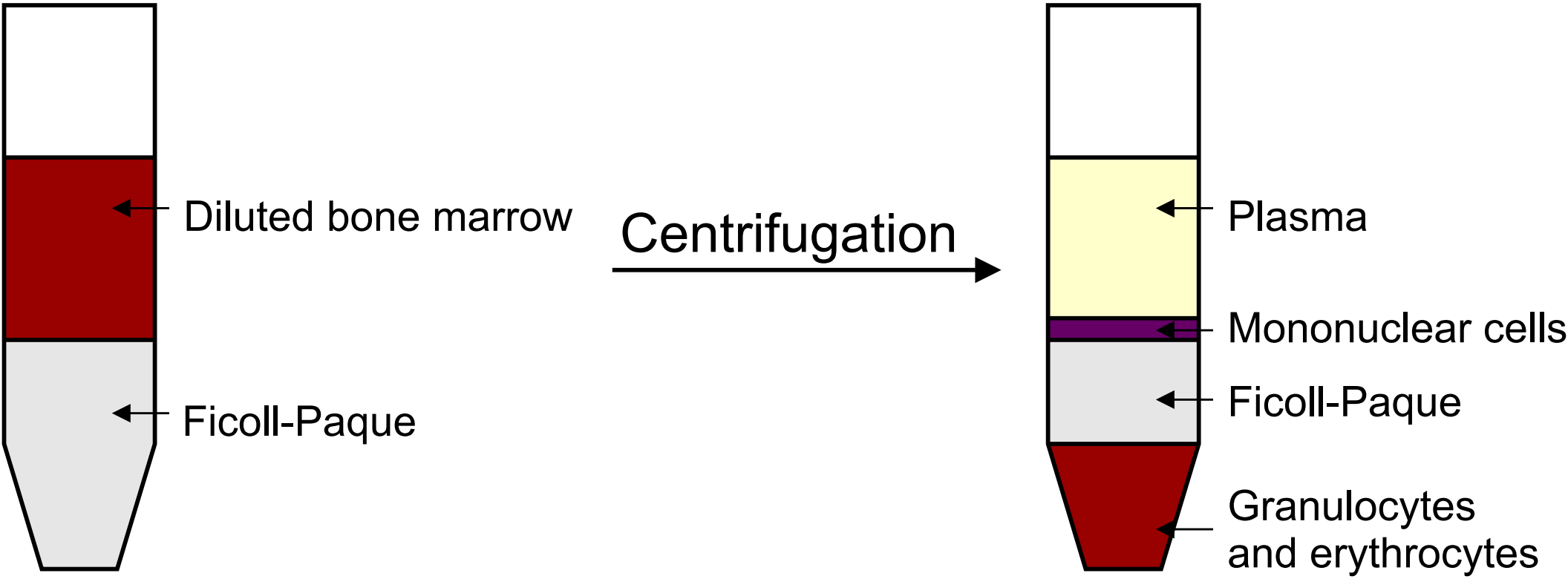

Figure 2

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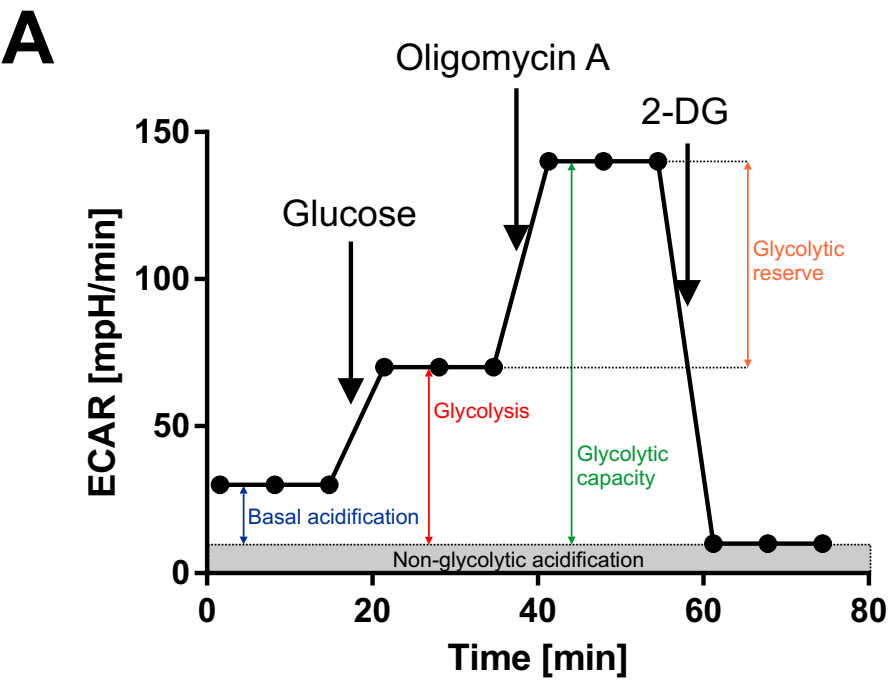

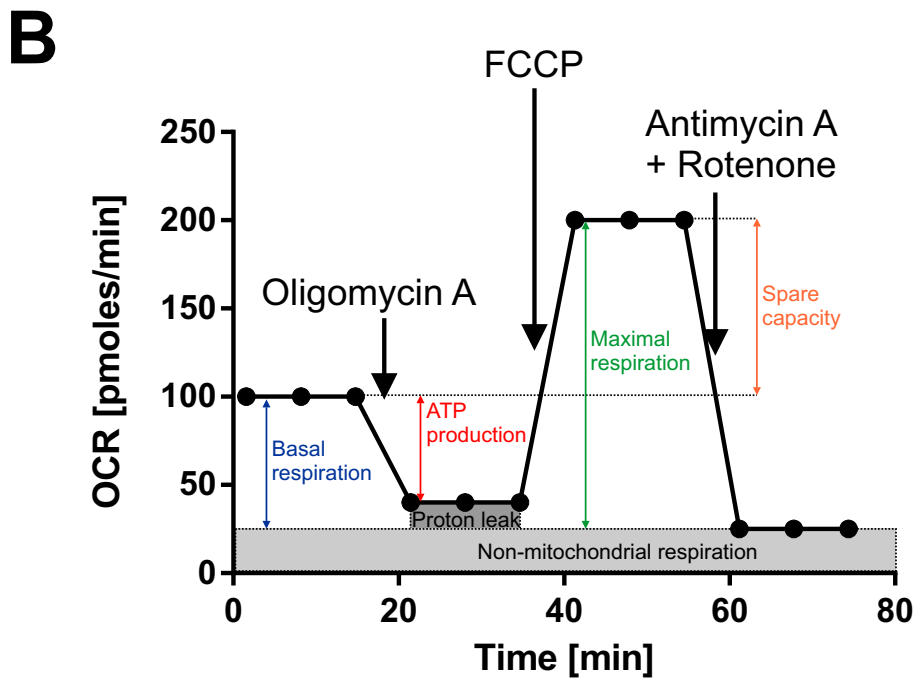

Figure 3

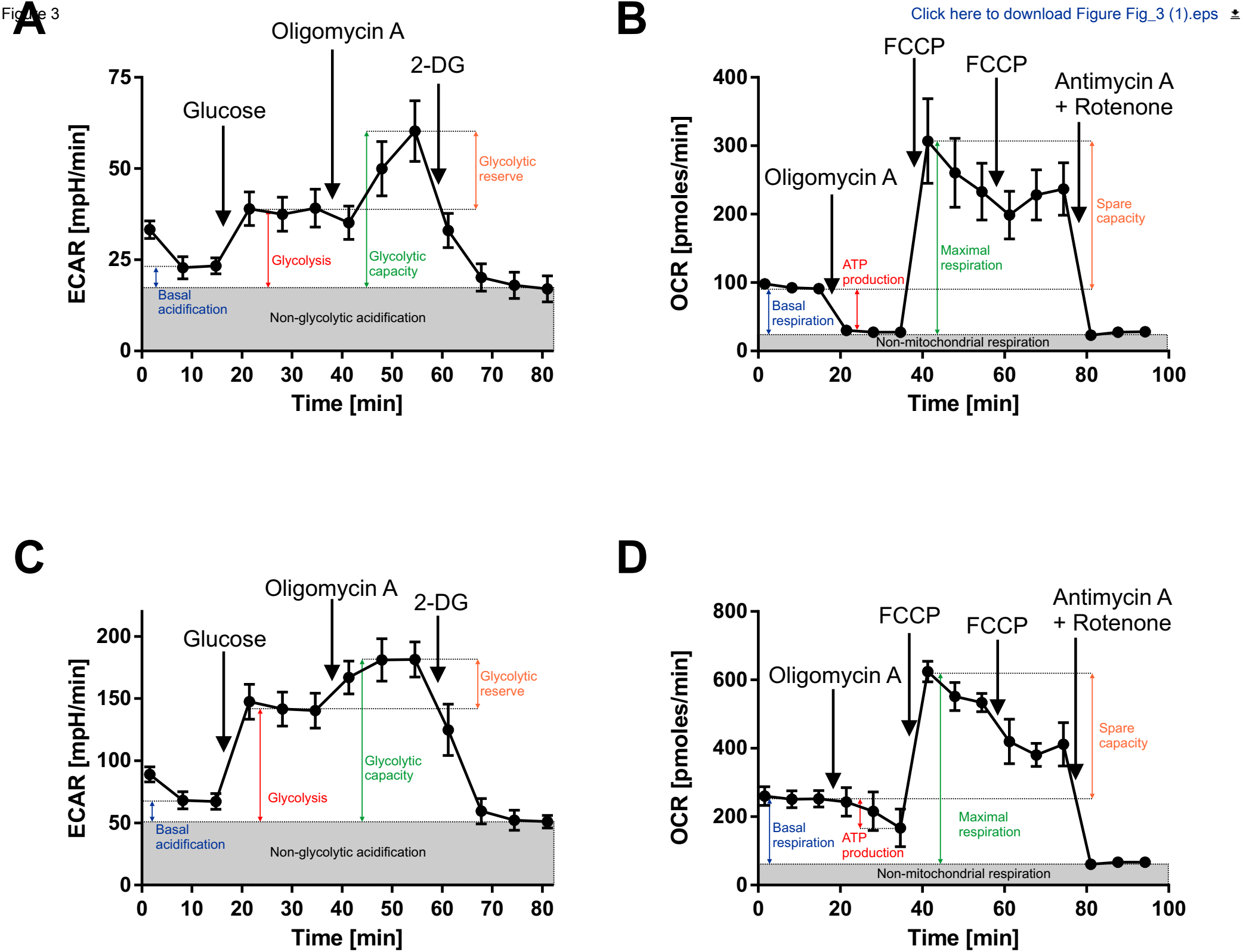

Table 1

Port	Glycolysis stress test	
	Load to the port	**Final concentration in the wells**
A	20 µL of 100 mM glucose	10 mM glucose
B	22 µL of 20 µM Oligomycin A	2 µM Oligomycin A
C	25 µL of 1 M 2-DG	100 mM 2-DG
D	X	

Cell mito stress test	
Load to the port	**Final concentration in the wells**
20 µL of 20 µM Oligomycin A	2 µM Oligomycin A
22 µL of 15 µM FCCP	1.5 µM FCCP
25 µL of 30 µM FCCP	4.5 µM FCCP
25 µL of 10 µM Rotenone and 10 µg/ml Antimycin A	1 µM Rotenone and 1 µg/ml Antimycin A

Table 2

Step	Settings
Calibration	Automatic
Equilibration	Automatic
Baseline measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port A	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port B	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port C	Injection
Measurement	Four times: Mix – 3 min, Wait – 0 min, Measure – 3 min

Table 3

Step	**Settings**
Calibration	Automatic
Equilibration	Automatic
Baseline measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port A	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port B	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port C	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min
Injection of the port D	Injection
Measurement	Three times: Mix – 3 min, Wait – 0 min, Measure – 3 min

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
RPMI 1640 Medium, GlutaMAX Supplement	Gibco, ThermoFisher Scientific	61870-010	
Fetal Bovine Serum	Biosera	FB-1001/100	
Antibiotic-Antimycotic (100X)	Gibco, ThermoFisher Scientific	15240-062	
Sodium bicarbonate	Sigma-Aldrich	S5761-500G	
D-(+) Glucose	Sigma-Aldrich	G7021-100G	
Oligomycin A	Sigma-Aldrich	75351-5MG	
2-Deoxy-D-glucose	Sigma-Aldrich	D8375-1G	
FCCP	Sigma-Aldrich	C2920-10MG	
DMSO	Sigma-Aldrich	D8418-100ML	
Rotenone	Sigma-Aldrich	R8875-1G	
Antimycin A from *Streptomyces* sp.	Sigma-Aldrich	A8674-25MG	
Seahorse XF Base Medium, 100 mL	Agilent Technologies	103193-100	
L-glutamine solution, 200 mM	Sigma-Aldrich	G7513-100ML	
HEPES solution, 1 M, pH 7.0-7.6	Sigma-Aldrich	H0887-100ML	
Sodium pyruvate	Sigma-Aldrich	P5280-25G	
Bovine Serum Albumin	Sigma-Aldrich	A2153-10G	
Ficoll-Paque Plus	Sigma-Aldrich	GE17-1440-02	Density gradient medium
Seahorse XFp FluxPak	Agilent Technologies	103022-100	
Corning™ Cell-Tak Cell and Tissue Adhesive	ThermoFisher Scientific	CB40240	
Seahorse Analyzer XFp	Agilent Technologies	S7802A	
Seahorse XFp Cell Culture Miniplate	Agilent Technologies	103025-100	

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Title of Article: ASSESSMENT OF METABOLIC PROFILE OF PRIMARY LEUKEMIA CELLS EMPLOYING SEAHORSE XFp ANALYZER

Author(s): KATERINA HLOZKOVA, JULIA STARKOVA

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Signature: [signature] Date: 4.5. 2018

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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 CO_2 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-CO_2 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.