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## Analysis of Hematopoietic Stem Progenitor Cell Metabolism

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**Nandita Singh, Ph.D.**  
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RE: **Revision of JoVE 60234**

Dear Dr. Singh,

Thank you very much for facilitating the review of our manuscript entitled "Analysis of Hematopoietic Stem Progenitor Cell Metabolism" for your review. We are thankful to reviewers' constructive feedback, and we are glad that our revised the manuscript is suitable for publication at JoVE. Thank you in advance for considering our manuscript.

Sincerely,

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**TITLE:**

Analysis of Hematopoietic Stem Progenitor Cell Metabolism

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**KEYWORDS:**

hematopoietic stem progenitor cells, metabolism, mitochondrial respiration, glycolysis, extracellular flux analysis, non-adherent cells

**SUMMARY:**

Hematopoietic stem progenitor cells (HSPCs) transition from a quiescent state to a differentiation state due to their metabolic plasticity during blood formation. Here, we present an optimized method for measuring mitochondrial respiration and glycolysis of HSPCs.

**ABSTRACT:**

Hematopoietic stem progenitor cells (HSPCs) have distinct metabolic plasticity, which allows them to transition from their quiescent state to a differentiation state to sustain demands of the blood formation. However, it has been difficult to analyze the metabolic status (mitochondrial respiration and glycolysis) of HSPCs due to their limited numbers and lack of optimized protocols for non-adherent, fragile HSPCs. Here, we provide a set of clear, step-by-step instructions to measure metabolic respiration (oxygen consumption rate; OCR) and glycolysis (extracellular acidification rate; ECAR) of murine bone marrow-Lineage<sup>neg</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> (LSK) HSPCs. This protocol provides a higher amount of LSK HSPCs from murine bone marrow, improves the viability of HSPCs during incubation, facilitates extracellular flux analyses of non-adherent HSPCs, and provides optimized injection protocols (concentration and time) for drugs targeting oxidative phosphorylation and glycolytic pathways. This method enables the prediction of the metabolic status and the health of HSPCs during blood development and diseases.

**INTRODUCTION:**

Since the lifespan of most mature blood cells is short, the homeostasis of blood relies on the self-renewal and differentiation of a long-lived but rare population of hematopoietic stem cells (HSPCs)<sup>1</sup>. HSPCs are quiescent, but they are quick to proliferate and undergo differentiation upon stimulation to sustain demands of the blood system. As each HSPC cellular state requires a unique bioenergetic

demand, the metabolic changes are key drivers of HSPC fate decisions. Therefore, the loss of metabolic plasticity, by altering the equilibrium between quiescence, self-renewal, and differentiation of HSPCs, often leads to myelo- or lympho-proliferative disorders. Together, the understanding of metabolic regulation of HSPC development is critical to uncover mechanisms underlying hematologic malignancies<sup>2-5</sup>.

Mitochondrial respiration and glycolysis generate ATP to drive intracellular reactions and produce the building blocks necessary for macromolecule synthesis. Since HSPCs have low mitochondrial mass compared to differentiated cells<sup>6</sup> and they sustain quiescence in hypoxic bone marrow niches, HSPCs primarily rely on glycolysis. Activation of HSPCs enhances their mitochondrial metabolism that leads to the loss of quiescence and their subsequent entry into the cell cycle. Such metabolic plasticity of HSPCs allows the maintenance of the HSPC pool throughout adult life<sup>6-12</sup>. Therefore, it is critical to investigate their metabolic activities, such as the oxygen consumption rate (OCR; index of oxidative phosphorylation) and the extracellular acidification rate (ECAR; index of glycolysis) to analyze the HSPC activation and the health status. Both the OCR and the ECAR can be measured simultaneously, in real time, using an extracellular flux analyzer. However, the current method requires large numbers of cells and is optimized for adherent cells<sup>13</sup>. Since HSPCs cannot be isolated in large quantities from mice<sup>14</sup>, require sorting to obtain a pure population, are non-adherent cells<sup>15</sup>, and cannot be cultured overnight without avoiding differentiation<sup>16</sup>, it has been difficult to measure the OCR and the ECAR of HSPCs. Here, we provide a set of clear, step-by-step instructions to accompany video-based tutorials on how to measure metabolic respiration and glycolysis of few thousands murine bone marrow-Lineage<sup>neg</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> (LSK) HSPCs.

## PROTOCOL:

This protocol was approved by Nationwide Children's Hospital Animal Care and Use Committee (IACUC).

NOTE: The protocol is described in chronological order that spans over the period of two days. Use fresh reagents as described in the protocol below.

### 1. Preparation of reagents on the day prior to the assay

#### 1.1. Hydrate the sensor cartridge.

1.1.1. Incubate 5 mL of the calibrant (**Table of Materials**) in a non-CO<sub>2</sub> 37 °C incubator overnight.

1.1.2. Open the flux assay kit (**Table of Materials**) and separate the sensor cartridge (green; top) from the utility plate (transparent; bottom). Next, place the sensor cartridge upside down and adjacent to the utility plate.

1.1.3. Using sterile water, fill the wells of the utility plate (200 µL) and the chambers around the wells (400 µL).

1.1.4. Submerge the sensor cartridge into the utility plate making sure that the sensors are completely covered by water. Tap 3x to avoid the formation of bubbles.

1.1.5. Place the cartridge submerged in the utility plate in a non-CO<sub>2</sub> 37 °C incubator overnight. To prevent evaporation of the water, make sure that the incubator is properly humidified. Place an open beaker containing H<sub>2</sub>O as an extra precaution next to the cartridge-utility plate, particularly if using a regular oven.

## 1.2. Prepare the assay plate.

1.2.1. Sterilize the surface of biosafety cabinet class 2 using 70% ethanol. Open the assay plate under the hood.

1.2.2. Add 40 µL of commercially available 0.01% (w/v) poly-L-lysine (PLL) solution to each well of the assay plate under the hood.

1.2.3. Cover the assay plate with the lid provided in the kit. Incubate the closed assay plate at room temperature (RT) under the hood for 1 h.

NOTE: The goal of the incubation is to let PLL coat the surface of the assay plate to facilitate adhesion of suspension cells to the surface of the assay plate.

1.2.4. After 1 h incubation of the assay plate, remove the excess solution with a sterile vacuum-based aspirator and air dry the well under the hood.

NOTE: It takes ~30–60 min to air-dry the wells of the assay plate following the excessive PLL removal using the aspirator.

## 2. Day of the assay

### 2.1. Prepare the cartridge.

2.1.1. Lift the sensor cartridge. Place it upside down in the tissue culture hood and discard the water from the utility plate.

2.1.2. Fill the utility plate wells with 200 µL of the pre-warmed calibrant.

2.1.3. Fill the chambers around the wells with 400 µL of the calibrant.

2.1.4. Submerge the sensor cartridge into the utility plate, making sure that the sensors are completely covered by the calibrant. Tap 3x to avoid the formation of bubbles.

2.1.5. Equilibrate the sensor cartridge submerged in the utility plate in a non-CO<sub>2</sub> 37 °C incubator for 45–60 min.

### 2.2. Harvest murine bone marrow-derived LSK HSPCs.

2.2.1. To accommodate sufficient biological replicates, plan to use HSPCs derived from one mouse per well of the assay plate.

143 NOTE: This bone marrow-harvesting method provides ~50,000–80,000 LSK HSPCs from each mouse.  
144 This protocol to measure extracellular flux is optimized for ~70,000 LSK HSPCs per well of a 96 well-  
145 plate.

146  
147 2.2.2. Euthanize mice using CO<sub>2</sub> overdose and cervical dislocation, following local IACUC approved  
148 methods.

149  
150 2.2.3. Sterilize the surface of dissecting tools and the bench using 70% ethanol.

151  
152 2.2.4. For each mouse, pre-fill one Petri dish with 1x phosphate-buffered saline (PBS) containing 2%  
153 heat inactivated fetal bovine serum (FBS) at RT.

154  
155 NOTE: Do not use pre-chilled PBS as it will create clumps in the following steps.

156  
157 2.2.5. Spray 70% ethanol (v/v) on the entire euthanized mouse. Isolate all bones, including upper  
158 and lower limbs, hip bones, sternum, rib cage, and spine, from the mouse<sup>17,18</sup>. Pull out white matter  
159 from the spinal cord of the mouse as it can contaminate LSK cells. Place all bones in 1x PBS (+ 2%  
160 FBS), as they are being collected, in a Petri dish until further use.

161  
162 2.2.6. Invert a 50 mL conical tube (new each time) and use it to triturate bones submerged in 1x PBS  
163 (+ 2% FBS) in the Petri dish.

164  
165 2.2.7. Using a 10 mL serological pipette, pipette up and down (~10x) to uniformly flush out cells  
166 from bones, after crushing bones.

167  
168 2.2.8. Place a 40 µm cell strainer on a 50 mL conical tube. Pre-wet the surface of the strainer by  
169 passing 1 mL of 1x PBS (+ 2% FBS).

170  
171 2.2.9. Harvest bone marrow-derived cell suspension from step 2.2.7 and pass it through the pre-  
172 wet surface of the cell strainer to remove bone debris.

173  
174 2.2.10. Repeat steps 2.2.6 through 2.2.9 until all bone materials turn white as a marker that most of  
175 bone marrow cells are collected in the 50 mL conical tube.

176  
177 NOTE: It often takes two 50 mL conical tubes per mouse to collect all bone marrow-derived cells.

178  
179 2.2.11. Centrifuge 50 mL conical tubes containing bone marrow-derived cells for 5 min at 500 x g  
180 and RT.

181  
182 2.2.12. Remove the supernatant. Resuspend bone marrow-derived cells (combine contents of both  
183 50 mL tubes) in 5 mL of 1x PBS (+ 2% FBS) as a final volume and keep cells at RT.

## 184 185 2.3. Harvest mononucleated murine bone marrow cells.

186  
187 2.3.1. Add 5 mL of density gradient medium (i.e., Ficoll) to a 15 mL conical tube. Then slowly add 5  
188 mL of the bone marrow cell suspension. Make sure that cells remain as a layer above the density  
189 gradient medium.

190

2.3.2. Centrifuge for 30 min at 500 x *g* and RT. Do not use a brake in the centrifuge. Make sure the centrifuge is at the lowest possible acceleration (e.g., 1 acceleration and 0 deceleration).

2.3.3. Harvest the middle interface of mononucleated cells (white color) following centrifugation into a fresh 15 mL conical tube.

2.3.4. Wash cells, harvested from density gradient medium, with 5 mL of 1x PBS (+ 2% FBS). Centrifuge for 5 min at 500 x *g* and 4 °C. Remove the supernatant.

2.3.5. Repeat step 2.3.4.

2.3.6. Resuspend cell contents of the tube in 300 µL of 1x PBS (+ 2% FBS). Aliquot 10 µL of cell suspension for unstained or single-color control in a FACS tube.

2.4. Harvest LSK HSPCs from mononucleated murine bone marrow cells.

2.4.1. Make a cocktail of biotin-antibodies by mixing 3 µL per sample of the following antibodies: Gr1, Cd8a, Cd5, B220, Ter119. Add 15 µL of the biotin-antibody cocktail to 300 µL of mononucleated bone marrow cells.

NOTE: Each antibody is used at 1:100 dilution.

2.4.2. Incubate cells with the biotin-antibody cocktail for 30 min at 4 °C with agitation to avoid cells clumping in the bottom of the tube.

2.4.3. Add 10 mL of pre-chilled 1x PBS (+ 2% FBS) to cells mixed with the biotin-antibody cocktail.

2.4.4. Centrifuge the tube for 5 min at 500 x *g* and 4 °C. Discard the supernatant and resuspend the cell pellet in 400 µL of 1x PBS (+ 2% FBS). Aliquot 10 µL for streptavidin-single color control.

2.4.5. Briefly vortex anti-biotin microbeads (**Table of Materials**) before use. Add 80 µL of microbeads to each cell sample (of 400 µL). Mix well and incubate for additional 20 min at 4 °C, with agitation.

2.4.6. Add 10 mL of pre-chilled 1x PBS (+ 2% FBS) to cells. Centrifuge the tube for 5 min at 500 x *g* and 4 °C.

2.4.7. Discard the supernatant and resuspend the cell pellet in 1 mL of 1x PBS (+ 2% FBS). Store at 4 °C while setting up magnetic separation unit.

2.4.8. Place a column (**Table of Materials**) in the magnetic field of the magnetic assisted cell sorting (MACS) separator at 4 °C. Prepare the column for magnetic separation by rinsing it with 3 mL of 1x PBS (+ 2% FBS) under the gravity flow at 4 °C.

2.4.9. Add the cell suspension from step 2.4.7 to the pre-wet column at 4 °C. Allow the cells to pass through the column at 4 °C and collect effluent in a 15 mL conical tube.

238 NOTE: The fraction with unlabeled cells in such effluent represents the enriched lineage negative  
239 cells.

240  
241 2.4.10. Wash column with 3 mL of 1x PBS (+ 2% FBS) at 4 °C. Repeat 3x. Collect the flow-through  
242 and keep it at 4 °C. Count the eluted viable cells by trypan blue exclusion using a hemocytometer.

243  
244 2.4.11. Centrifuge the 15 mL conical tube containing the flow-through for 5 min at 500 x *g* and 4 °C.  
245 Discard the supernatant. Resuspend cells in 0.5 mL of 1x PBS (+ 2% FBS) and transfer the contents  
246 to a FACS tube.

247  
248 2.4.12. Add 24 µL of the LSK antibody cocktail to each 10<sup>7</sup> cells. The antibody cocktail contains equal  
249 concentration of 450-streptavidin antibody, PE-CY7-Sca1 antibody, and APC-c-Kit antibody.

250  
251 2.4.13. Incubate for 1 h at 4 °C with agitation under dark (covered with tin foil).

252  
253 2.4.14. Add 3 mL of 1x PBS (+ 2% FBS) to the FACS tube. Centrifuge for 5 min at 500 x *g* and 4 °C.  
254 Discard the supernatant.

255  
256 2.4.15. Resuspend antibody-labelled cells in 1 mL of 1x PBS (+ 2% FBS). Add 1 µL of 1 mg/mL  
257 propidium iodide to cell suspension just before sorting.

258  
259 2.4.16. Filter contents of the FACS tube using a 40 µm strainer right before sorting LSK cells.

260  
261 2.4.17. Collect LSK cells, via FACS sorting, into 1.5 mL tube containing 0.5 mL of complete media  
262 supplemented with 2 mM glutamine, 3 mg/mL glucose, 1 mM pyruvate, 1x thrombopoietin (TPO),  
263 1x stem cell factor (SCF), 0.5x penicillin/streptomycin (P/S), pH 7.4 (**Table 1**).

### 264 265 **3. Mitochondrial respiration and glycolysis assays of LSK HSPCs**

#### 266 267 **3.1. LSK seeding in the assay plate**

268  
269 3.1.1. Centrifuge LSK cells from step 2.4.17 for 5 min at 500 x *g* and RT. Discard the supernatant.

270  
271 3.1.2. Resuspend cells in complete media to a final concentration of at least 70,000 cells/40 µL.

272  
273 3.1.3. Seed contents of 40 µL media (containing 70,000 cells) in the PLL-coated 8-well plate from  
274 step 1.2.4. Leave all corner wells empty.

275  
276 3.1.4. Centrifuge for 1 min at 450 x *g* and RT. Do not apply the brake. Make sure that cells are  
277 attached to the well bottom using the inverted microscope.

278  
279 3.1.5. Add 135 µL of complete media to the cells in each well for a final volume of 175 µL. Add 175  
280 µL of complete media to the 2 corners of the plate as blanks.

281  
282 3.1.6. Incubate the cells in the non-CO<sub>2</sub> incubator for 2 h at 37 °C.

283  
284 3.1.7. Set up a program to add drugs to each well of the well plate in the analyzer using a metric  
285 described in **Table 2** (mitochondrial stress test) and **Table 3** (glycolysis stress test).



NOTE: While cells are incubating, turn on the instrument and make sure it is at 37 °C.

### 3.2. Mitochondrial stress test

3.2.1. Prepare 45  $\mu$ M stock solutions of oligomycin, 50  $\mu$ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and 25  $\mu$ M stock solutions of rotenone/antimycin A. To prepare 45  $\mu$ M oligomycin stock solution, dissolve the contents of the commercially available pouch in 280  $\mu$ L of the complete media. To prepare 50  $\mu$ M FCCP stock solution, dissolve the contents of the commercially available pouch in 288  $\mu$ L of the complete media. To prepare 25  $\mu$ M rotenone/antimycin A stock solution, dissolve the contents of the commercially available pouch in 216  $\mu$ L of the complete media.

3.2.2. Take the sensor cartridge in the utility plate out from the incubator and load its ports (A, B, and C) such that each well would have final concentration of 2  $\mu$ M oligomycin, 1.5  $\mu$ M FCCP, and 0.5  $\mu$ M of rotenone/antimycin A as needed, following dilution metrics described in **Table 4** (for oxygen consumption rate).

3.2.3. Remove the lid from the sensor cartridge assembled in the utility plate and place it on the instrument tray. Start the calibration that will take 20 min.

3.2.4. After the calibration, remove the utility plate and substitute it with assay plate containing LSK cells, which are now adhered to the bottom of the well.

3.2.5. Press **Continue** to start the program described in **Table 2**. After the completion of the program, retrieve the data and analyze them using the Wave Desktop software.

NOTE: The data generated from the extracellular flux assays can be plotted using the dot plot from the Wave Desktop software or exported to web-based statistics program.

### 3.3. Glycolysis stress test

3.3.1. Reconstitute glucose in 300  $\mu$ L (100 mM), oligomycin in 288  $\mu$ L (50  $\mu$ M), 2-D glucose (2-DG) in 300  $\mu$ L (500 mM) of complete media from the commercially available pouch.

3.3.2. Gently pipette up and down (~10x) to solubilize the compounds. Vortex the 2-DG for approximately 1 min to ensure proper dissolution into media.

3.3.3. Remove the sensor cartridge out from the incubator. Load ports A through C following dilution metrics described in the **Table 5** to obtain a final concentration of 10 mM glucose, 2  $\mu$ M oligomycin, and 50 mM 2-DG.

3.3.4. Repeat steps 3.2.3 and 3.2.4.

3.3.5. Press **Continue** to start the program described in **Table 3**. After the completion of the program, retrieve the data and analyze them using the Wave Desktop software.

### REPRESENTATIVE RESULTS:

Our extraction method allowed us to harvest up to ~80,000 LSK HSPCs per mouse. The viability and numbers of LSK cells were improved with our method, because we: (1) combined bone marrow from upper and lower limbs, hip bones, sternum, rib cage, and spine, (2) avoided using red cell lysis buffer that would have increased cell-death and clumping, (3) used the density gradient medium separation of mono-nucleated cells, and (4) avoided using pre-chilled buffer that would have caused the loss of cells-of-interest in clumps.

Although extracellular flux analysis has been traditionally used for adherent cells, our use of the PLL coating of wells, followed by centrifugation of cells on it, facilitated adherence of LSK HSPCs to the surface of the well. This allowed us to measure the extracellular flux, and thus metabolic health of LSK HSPCs. Considering the limited number of cells that can be harvested from a mouse and the long duration of the protocol for their isolation, our use of the analyzer with its 8 well format has emerged as the most cost-effective and feasible solution (**Figure 1**).

Cells use glycolysis and mitochondrial respiration to replenish their energy requirements and to produce intermediates needed for their proliferation and growth<sup>19</sup>. The hexokinase enzyme converts glucose in glucose-6-phosphate and that is subsequently transformed into pyruvate<sup>20</sup>. Pyruvate can then be processed into lactate and is exported from the cell with protons<sup>21</sup>. ECAR measures the acidification of the media and is thus an indicator of glycolysis. Pyruvate can also be transported into the mitochondria and transformed in acetyl coenzyme A (CoA). Acetyl CoA enters the TCA cycle, which provides energy intermediates to drive the electron movements of the Electron Transport Chain (ETC) and generates a proton gradient in the mitochondrial inter-membrane space<sup>22</sup>. Oxygen acts as the final electron acceptor, and protons move back to the mitochondrial matrix through the ATP synthase complex while generating ATP<sup>23</sup>. OCR measures the oxygen consumption and it is therefore used to quantify the mitochondrial respiration.

In order to analyze the OCR and the ECAR in basal and stressed conditions, we used sequential injection of drugs that interfere with glycolysis and mitochondrial respiration. We used glucose and 2-deoxyglucose (2-DG), a glucose analogue, to initiate and block glycolysis respectively<sup>24</sup>. We used Rotenone (a complex I-specific inhibitor of the ETC), antimycin A (a complex III-specific inhibitor of the ETC), oligomycin (inhibitor of ATP synthase), and the uncoupling agent carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) to block specific events of the ETC<sup>25</sup>. We titrated such reagents to find the optimal concentration for LKS HSPCs (**Figure 2A,B**).

To perform the glycolysis stress test, we cultured the LSK HSPCs in a glucose/pyruvate deprived media (as recommended by the manufacturer), or in glucose/pyruvate containing media. As expected, we found the basal level of the ECAR was higher for LSK HSPCs cultured in glucose/pyruvate+ media compared to LSK HSPCs cultured in glucose/pyruvate- media. The first injection with glucose did not change the basal level of ECAR for LSK HSPCs cultured in glucose/pyruvate+ media while it boosted glycolysis in LSK HSPCs cultured in glucose/pyruvate- media. However, the basal level of the ECAR, after the injection with glucose, remained lower compared to the glucose/pyruvate+ group. The second injection with oligomycin, which could block the production of ATP through oxidative phosphorylation, activated the glycolysis at its maximum level of LSK HSPCs in glucose/pyruvate+ media, but it did not affect the glucose/pyruvate- group. The last injection with the glucose analogue 2-DG returned the ECAR to its non-glycolytic level (**Figure 2C**).

381 For the mitochondrial stress test, we measured the basal level of OCR of LSK HSPCs in  
382 glucose/pyruvate+ media. We first injected oligomycin that initially hyperpolarized the  
383 mitochondrial membrane, prevented more proton pumping through the ETC complexes, and thus  
384 reduced the rate of mitochondrial respiration. The second injection of FCCP ionophores pushed ETC  
385 and OCR levels to their maximum as cells tried to recover the mitochondrial membrane potential.  
386 The final injection with other two of the ETC inhibitors (antimycin A and rotenone) caused the  
387 complete stop of mitochondrial respiration and thus the OCR reverted to its minimum level (**Figure**  
388 **2D**).

390 **FIGURE AND TABLE LEGENDS:**

392 **Figure 1: Schema demonstrating isolation of Lineage<sup>neg</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> (LSK) hematopoietic stem**  
393 **progenitor cells from mouse bone marrow.** Bone marrow is extracted from bones and mononuclear  
394 cells (MNCs) are isolated through density gradient medium gradient separation. Next, cells are  
395 incubated with biotinylated Lineage<sup>+</sup> antibodies and streptavidin-conjugated magnetic beads to  
396 elute Lineage negative (Lin<sup>-</sup>) cells following their magnetic separation. Lin<sup>-</sup> cells are subsequently  
397 incubated with LSK antibodies and LSK cells isolated by cell sorting.

399 **Figure 2: Extracellular flux analyses of murine Lineage<sup>neg</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> (LSK) hematopoietic stem**  
400 **progenitor cells. (A,B)** Mechanistic description of drugs utilized for extracellular flux analyses during  
401 glycolysis and mitochondrial respiration. **(C)** Representative results of glycolysis stress test on  
402 murine LSK HSPCs in presence or absence of glucose/pyruvate in the media. **(D)** Representative  
403 results of mitochondrial stress test on murine LSK HSPCs. Error bars represent the standard  
404 deviation of the mean (S.D.)

406 **Table 1: Contents and preparation of the complete XF media.**

408 **Table 2: Injection protocol for the mitochondrial stress test.**

410 **Table 3: Injection protocol for the glycolysis stress test.**

412 **Table 4: Dilution metrics to obtain optimum drug concentration for mitochondrial stress test in**  
413 **each well of the analyzer.**

415 **Table 5: Dilution metrics to obtain optimum drug concentration for glycolysis stress test in each**  
416 **well of the analyzer.**

418 **DISCUSSION:**

419 Here, we demonstrate the isolation of a maximum amount of pure and viable murine LSK HSPCs  
420 population as well as the measurement of their glycolysis and mitochondrial respiration with an  
421 extracellular flux analyzer. Specifically, the protocol overcomes the following technical issues for the  
422 use of LSK HSPCs : i) the low frequency of LSK HSPCs in murine bone marrow<sup>14</sup>, ii) low basal metabolic  
423 activity of LSK HSPCs<sup>26</sup>, iii) the fragility of LSK HSPCs<sup>27</sup>, and iv) the non-adherence of LSK HSPCs to  
424 culture vessels<sup>15</sup>. In addition, we have optimized the drug concentrations and media composition  
425 for the optimum performance of the extracellular flux assays.

427 Bone marrow-derived HSPCs reside in the hypoxic niche and they display a glycolytic phenotype,  
428 which is crucial for the maintenance of their stemness<sup>28</sup>. Conversely, respiration is essential for HSPC

differentiation<sup>6</sup>. As metabolic dysfunction of HSPCs leads to blood diseases; here, we have described the protocol and video-based tutorials to measure the hallmarks of metabolic functions, such as the OCR and the ECAR, for murine bone marrow-derived LSK HSPCs.

Contrary to manufacturer recommendations for adherent cells, we found that the glycolysis stress test results on LSK HSPCs are optimum when cells are cultured in glucose/pyruvate+ media compared to glucose/pyruvate- media. We realized that the lack of glucose in media for LSK HSPCs results in cell death during the ~2 h equilibration period in a non-CO<sub>2</sub> incubator. As the glucose injection did not change the basal level of ECAR for LSK HSPCs cultured in glucose/pyruvate+ media, our modification of the protocol not only preserves the essence of the glycolytic stress test, but it also allows us to distinguish between the basal glycolysis (before any injections), glycolytic capacity (after oligomycin injection) and non-glycolytic acidification (after injection with 2-DG) of LSK HSPCs.

Our mitochondrial stress test of LSK HSPCs showed that the ATP produced by oxidative phosphorylation in LSK HSPCs is minimal as seen with the small reduction in the OCR after the oligomycin injection. Conversely, the elevation in the OCR upon the FCCP injection affirms that LSK HSPCs are able to respond to higher energy demand.

Together, the goal of this protocol was to provide a set of clear, concise instructions to accompany video-based tutorials on how to measure the metabolic functions, such as the OCR and the ECAR, of HSPCs. With key modifications and additional recommendations for harvesting higher numbers of healthy LSK HSPCs, making them adherent to the surface of the well, as well as the optimization of the incubation time and drug concentration, this protocol will empower investigators to analyze glycolysis and mitochondrial respiration status of HSPCs as well as non-adherent hematopoietic cells during blood development and diseases.

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#### DISCLOSURES:

The authors have nothing to disclose.

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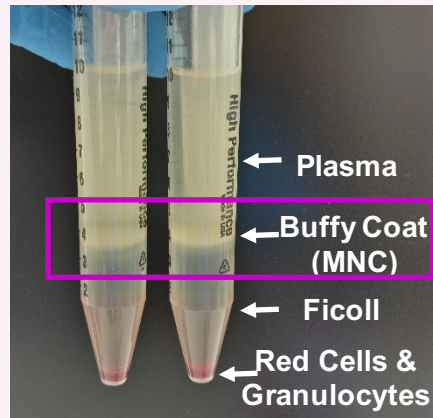
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Figure 1

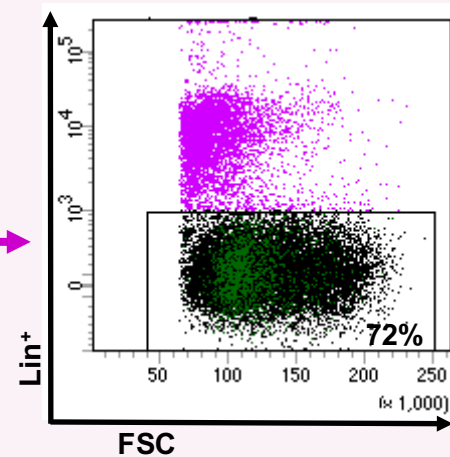
### Bone Marrow Isolation



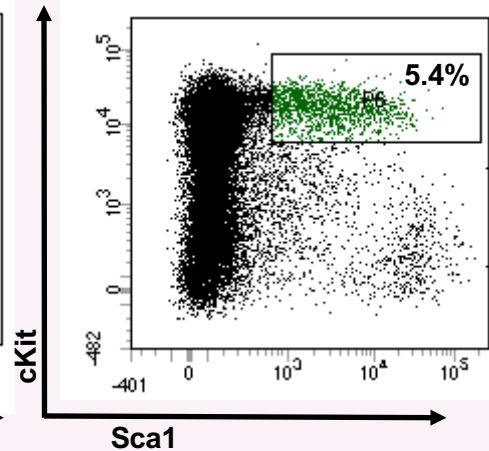
### Ficoll Gradient Separation



### Elution of Lin<sup>-</sup> Cells

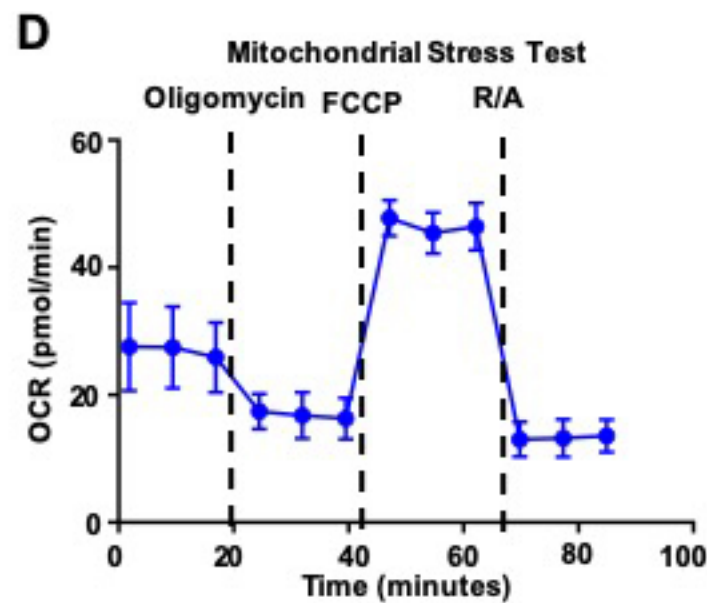
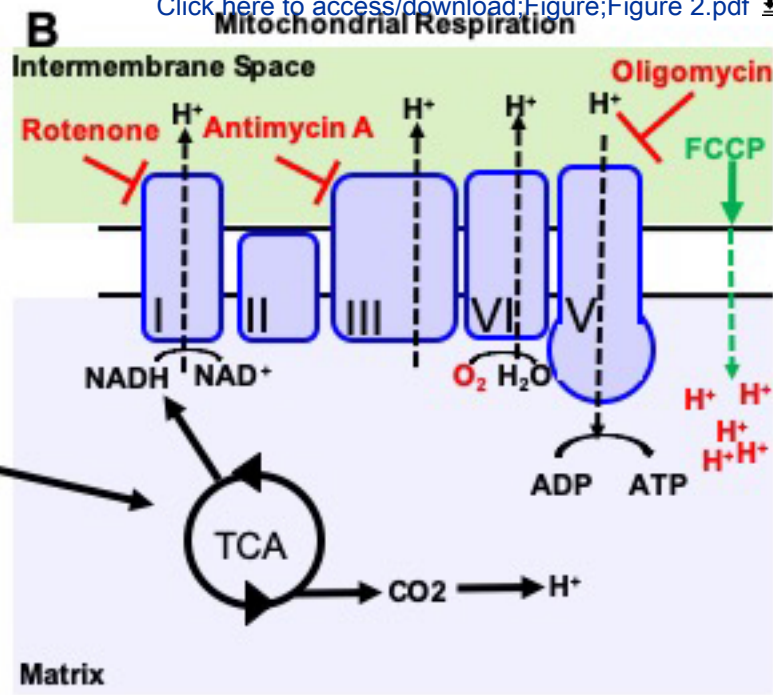


### Sorting of Lin<sup>-</sup> Sca1<sup>+</sup> cKit<sup>+</sup> (LSK) Cells



[Click here to access/download;Figure;Figure 1.pdf](#)

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	Stock	Final concentration	Volume for 30 mL
Complete media			28.691 mL
P/S	100x	0.5x	150 µL
L-Glutamine	200 mM	2 mM	300 µL
Pyruvate	100 mM	1 mM	300 µL
Glucose	1 M/180.2 mg/mL	3 mg/mL	499.4 µL
TPO	100 µg/mL	100 ng/mL	30 µL
SCF	100 µg/mL	100 ng/mL	30 µL



	Basal	Oligomycin (2 µM)	FCCP (1.5 µM)	R/A (0.5 µM)
Repetition	3 times	3 times	3 times	3 times
Mix	3 min	3 min	3 min	3 min
Wait	0 min	0 min	0 min	0 min
Measure	4 min	4 min	4 min	4 min

	Basal	Glucose (10 mM)	Oligomycin (2 µM)	2-DG (50 mM)
Repetition	3 times	3 times	3 times	3 times
Mix	3 min	3 min	3 min	3 min
Wait	0 min	0 min	0 min	0 min
Measure	7 min	7 min	7 min	7 min

Port	Drug	Final well concentration (μM)	Stock solution volume (μL)
Port A	Oligomycin	2	100
Port B	FCCP	1.5	100
Port C	Rotenone/antimycin A	0.5	60

Media volume (μL)	Port solution (μM)	Volume added to port (μL)
181.25	16	25
270.4	13.5	25
240	5	25

Port	Drug	Final well concentration	Stock solution volume (μL)	Media volume (μL)
Port A	Glucose	10 mM	300	75
Port B	Oligomycin	2 μM	108	192
Port C	2-DG	50 mM	300	0

Port solution	Volume added to port (μL)
80 mM	25
18 μM	25
500 mM	25

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.01% (w/v) poly-L-lysine solution	Sigma	P8920	Used for LSK attachment
40 µm cell strainer	Fisher Scientific	22-363-547	Used for cell filtration after bone
Anti-Biotin MicroBeads	Miltenyi	130-090-485	Used for Lin- separation
Biotin Rat Anti-Mouse CD45R/B220 Clone RA3-6B2	BD Biosciences	553086	Used for Lin- separation
Biotin Rat Anti-Mouse CD5 Clone 53-7.3	BD Biosciences	553019	Used for Lin- separation
Biotin Rat Anti-Mouse CD8a Clone 53-6.7	BD Biosciences	553029	Used for Lin- separation
Biotin Rat Anti-Mouse Ly-6G and Ly-6C Clone RB6-8C5	BD Biosciences	553125	Used for Lin- separation
Biotin Rat Anti-Mouse TER-119/Erythroid Cells Clone TER-119	BD Biosciences	553672	Used for Lin- separation
CD117 (c-Kit) Monoclonal Antibody (2B8), APC	eBioscience	17-1171-83	Used for LSK sorting
Falcon 15 ml Conical Centrifuge Tubes	Falcon-Fischer Scientific	14-959-53A	Used in cell isolation
Falcon 50 ml Conical Centrifuge Tubes	Falcon-Fischer Scientific	14-432-22	Used in cell isolation
Falcon Round-Bottom Polypropylene Tubes	Falcon-Fischer Scientific	14-959-11A	Used for LSK sorting
Fetal Bovine Serum	Neuromics	FBS001-HI	Used in FACS buffer
Histopaque-1083	Sigma	10831	Used for ficoll gradient separatio
L-glutamine 100x	Fisher Scientific	25-030-081	Used for the assay media
LS Column	Miltenyi	130-042-401	Used for Lin- separation
Ly-6A/E (Sca-1) Monoclonal Antibody (D7), PE-Cyanine7	eBioscience	25-5981-82	Used for LSK sorting
Murine Stem Cell Factor (SCF)	PeproTech	250-03-100UG	Used for the assay media
Murine Thrombopoietin (TPO)	PeproTech	315-14-100UG	Used for the assay media
PBS 1%	Fisher Scientific	SH3002802	Used for FACS buffer
Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	15140122	Used for the assay media
Propidium Iodide	Fisher Scientific	P1304MP	Used for LSK sorting
Seahorse XFp Cell Culture Miniplate	Agilent Technologies	103025-100	Used for LSK seeding
Sodium Pyruvate (100 mM)	ThermoFisher	11360070	Used for the assay media
Streptavidin eFluor 450 Conjugate	eBioscience	48-4317-82	Used for LSK sorting
XF Calibrant	Agilent Technologies	100840-000	Used for cartridge equilibration
XF media	Agilent Technologies	103575-100	Used for the assay media
XFp Glycolysis Stress Test Kit	Agilent Technologies	103017100	Drugs for glycolysis stress test
XFp Mitochondrial Stress Test Kit	Agilent Technologies	103010100	Drugs for mitochondrial stress te
XFp Sensor Cartridge	Agilent Technologies	103022-100	Used for glycolysis and mitochon

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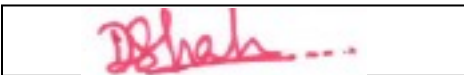
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## Responses to Reviewers' Comments

We are grateful to reviewers' extremely positive comments for our manuscript. Reviewer # 1 & # 2 have generously accepted the manuscript as it is. We have addressed reviewer # 3's comments, and we believe that our manuscript has significantly improved for the publication at the JoVE.

**Reviewer # 1:** No edits or comments required.

**Reviewer # 2:** No edits or comments required.

### Reviewer # 3

#### Major Concerns:

1. The standard glycolysis stress test involves incubating cells for 45 min-1 h in glucose-free medium without glucose or pyruvate. The authors indicate that a 2 hr incubation period resulted in LSK cell death. Did they try shorter incubation times. Also, it is expected that ECAR is higher in cells in glucose+ media than cells in glucose- media; in other words, not contrary to manufacturer's notes. Note the non-glycolytic extracellular acidification is higher in cells starting in glucose+ media.

Responses: It takes ~8 hr from harvesting bones to FACS sorting Lin<sup>neg</sup>Sca1<sup>c</sup>-Kit<sup>+</sup> (LSK) cells prior to our use. Unlike other cells growing in the incubator, the LSK cells go through intense physical stress during harvesting from bones and FACS sorting and they need time to recover before being utilized for the experiment. Our previous attempts to use LSK cells within 45 min-1 hr failed, and we determined that 2 hr is optimum incubation time for cells to recover from the physical stress and homogenously respond to drug treatments.

Agreed. We have made changes in the text to accurately reflect that ECAR is higher in cells in glucose+ media. See Lines # 329-331

#### Minor Concerns:

1. In protocol section 1.1, lines 4-6 refer to XF Calibrant solution, while water is actually used for hydration.

Response: We have edited the text as suggested. See Lines # 87-96; 112-113.

2. In protocol section 2.1.3, "lead" should be "lid"?

Response: We have edited the text as suggested. See Line # 102.

3. mitochondrial "inter-membrane" space rather than "intra-membrane" space

Response: We have edited the text as suggested. See Line # 315.

4. FCCP doesn't create pores, it is an ionophore

Response: We have edited the text as suggested. See Lines # 343.

5. Fig 2A, cytoplasm label is in the extracellular space

Response: We have edited Fig. 2A.

**Editor's Comments:** We have made sure that our manuscript is compliant with JoVE's policies.

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.

Response: Done

2. Please revise lines 92-94 and 126-134 to avoid textual overlap with previously published work.

3. Keywords: Please provide at least 6 keywords or phrases.

Response: See Line # 21-22.

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Response: Done.

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7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Please refrain from using bullets, dashes, or indentations.

Response: Done.

8. Please include single line spacing between each numbered step or note in the protocol.

Response: Done.

9. Lines 106, 148: Please describe how to sterilize the surface.

Response: Done.

10. Line 145: Please specify the euthanasia method.

Response: Done.

11. Lines 156-157: Please describe how to perform this step or provide a relevant reference here.  
Response: This is an obvious method. Detailed description would take up huge section. So we have decided to keep it succinct and cite the reference of earlier papers (refs # 17-18).

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Response: Done.

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Response: Done.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: Done.

15. 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.

Response: Done.

16. Figure 2: Please define error bars in the figure legend.

Response: Done.

17. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response: Done.

18. Tables: Please use the micro symbol  $\mu$  instead of u and include a space between all numbers and the corresponding unit (15 mL, 5 mM, etc.).

Response: Done.

19. References: Please do not abbreviate journal titles; use full journal name.

Response: Done.

20. Table of Materials: Please remove any <sup>TM</sup>/<sub>®</sub>/<sub>©</sub> symbols. Please sort the materials alphabetically by material name.

Response: Done

## Responses to Editor's Comment: JoVE 63234\_R1

### Editorial comments:

1. Please note that the editor has made some minor changes (e.g., combined some shorter steps and updated the numbering of protocol steps according to JoVE guidelines). Please review for accuracy.

Response: Done.

2. Table 4 and Table 5: Please specify the concentration unit for stock solution and unit for volume (i.e., columns "Media Volume" and "Media Volume").

Response: Done.

3. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove any <sup>TM</sup>/<sup>®</sup>/<sup>©</sup> symbols.

Response: Done.

4. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript.

5. After you have made all the recommended changes to your protocol section, please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text. Please note that some of the shorter protocol steps can be combined; however, please ensure that there are no more than 2-4 actions per step.

Response: Done.

6. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Response: Done.

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.

Response: Done.