Analysis of Hematopoietic Stem Progenitor Cell Metabolism

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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^{neg}Sca1⁺c-Kit⁺ (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.

Keywords

Developmental Biology; Issue 153; hematopoietic stem progenitor cells; metabolism; mitochondrial respiration; glycolysis; extracellular flux analysis; non-adherent cells

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)¹. 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

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Video Link

The video component of this article can be found at <https://www.jove.com/video/60234/>

Disclosures

The authors have nothing to disclose.

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^{2,3,4,5}.

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 cells⁶ 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^{6,7,8,9,10,11,12}. 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 cells¹³. Since HSPCs cannot be isolated in large quantities from mice¹⁴, require sorting to obtain a pure population, are non-adherent cells¹⁵, and cannot be cultured overnight without avoiding differentiation¹⁶, 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^{neg}Sca1⁺c-Kit⁺ (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. Hydrate the sensor cartridge.

1. Incubate 5 mL of the calibrant (Table of Materials) in a non-CO₂ 37 °C incubator overnight.
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.
3. Using sterile water, fill the wells of the utility plate (200 µL) and the chambers around the wells (400 µL).
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.

5. Place the cartridge submerged in the utility plate in a non-CO₂ 37 °C incubator overnight. To prevent evaporation of the water, make sure that the incubator is properly humidified. Place an open beaker containing H₂O as an extra precaution next to the cartridge-utility plate, particularly if using a regular oven.

2. Prepare the assay plate.

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

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

1. Prepare the cartridge.

1. Lift the sensor cartridge. Place it upside down in the tissue culture hood and discard the water from the utility plate.
2. Fill the utility plate wells with 200 µL of the pre-warmed calibrant.
3. Fill the chambers around the wells with 400 µL of the calibrant.
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.
5. Equilibrate the sensor cartridge submerged in the utility plate in a non-CO₂ 37 °C incubator for 45–60 min.

2. Harvest murine bone marrow-derived LSK HSPCs.

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

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

2. Euthanize mice using CO₂ overdose and cervical dislocation, following local IACUC approved methods.

3. Sterilize the surface of dissecting tools and the bench using 70% ethanol.
4. For each mouse, pre-fill one Petri dish with 1x phosphate-buffered saline (PBS) containing 2% heat inactivated fetal bovine serum (FBS) at RT.
NOTE: Do not use pre-chilled PBS as it will create clumps in the following steps.
5. Spray 70% ethanol (v/v) on the entire euthanized mouse. Isolate all bones, including upper and lower limbs, hip bones, sternum, rib cage, and spine, from the mouse^{17,18}. Pull out white matter from the spinal cord of the mouse as it can contaminate LSK cells. Place all bones in 1x PBS (+ 2% FBS), as they are being collected, in a Petri dish until further use.
6. Invert a 50 mL conical tube (new each time) and use it to triturate bones submerged in 1x PBS (+ 2% FBS) in the Petri dish.
7. Using a 10 mL serological pipette, pipette up and down (~10x) to uniformly flush out cells from bones, after crushing bones.
8. Place a 40 µm cell strainer on a 50 mL conical tube. Pre-wet the surface of the strainer by passing 1 mL of 1x PBS (+ 2% FBS).
9. Harvest bone marrow-derived cell suspension from step 2.2.7 and pass it through the pre-wet surface of the cell strainer to remove bone debris.
10. Repeat steps 2.2.6 through 2.2.9 until all bone materials turn white as a marker that most of bone marrow cells are collected in the 50 mL conical tube.
NOTE: It often takes two 50 mL conical tubes per mouse to collect all bone marrow-derived cells.
11. Centrifuge 50 mL conical tubes containing bone marrow-derived cells for 5 min at 500 x *g* and RT.
12. Remove the supernatant. Resuspend bone marrow-derived cells (combine contents of both 50 mL tubes) in 5 mL of 1x PBS (+ 2% FBS) as a final volume and keep cells at RT.

3. Harvest mononucleated murine bone marrow cells.

1. Add 5 mL of density gradient medium (i.e., Ficoll) to a 15 mL conical tube. Then slowly add 5 mL of the bone marrow cell suspension. Make sure that cells remain as a layer above the density gradient medium.
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).
3. Harvest the middle interface of mononucleated cells (white color) following centrifugation into a fresh 15 mL conical tube.
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.

5. Repeat step 2.3.4.
6. Resuspend cell contents of the tube in 300 μ L of 1x PBS (+ 2% FBS). Aliquot 10 μ L of cell suspension for unstained or single-color control in a FACS tube.

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

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

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

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.
3. Add 10 mL of pre-chilled 1x PBS (+ 2% FBS) to cells mixed with the biotin-antibody cocktail.
4. Centrifuge the tube for 5 min at 500 x *g* and 4 °C. Discard the supernatant and resuspend the cell pellet in 400 μ L of 1x PBS (+ 2% FBS). Aliquot 10 μ L for streptavidin-single color control.
5. Briefly vortex anti-biotin microbeads (Table of Materials) before use. Add 80 μ L of microbeads to each cell sample (of 400 μ L). Mix well and incubate for additional 20 min at 4 °C, with agitation.
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.
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.
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.
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.

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

10. Wash column with 3 mL of 1x PBS (+ 2% FBS) at 4 °C. Repeat 3x. Collect the flow-through and keep it at 4 °C. Count the eluted viable cells by trypan blue exclusion using a hemocytometer.
11. Centrifuge the 15 mL conical tube containing the flow-through for 5 min at 500 x *g* and 4 °C. Discard the supernatant. Resuspend cells in 0.5 mL of 1x PBS (+ 2% FBS) and transfer the contents to a FACS tube.

12. Add 24 μL of the LSK antibody cocktail to each 10^7 cells. The antibody cocktail contains equal concentration of 450-streptavidin antibody, PE-CY7-Sca1 antibody, and APC-c-Kit antibody.
13. Incubate for 1 h at 4 °C with agitation under dark (covered with tin foil).
14. Add 3 mL of 1x PBS (+ 2% FBS) to the FACS tube. Centrifuge for 5 min at 500 x *g* and 4 °C. Discard the supernatant.
15. Resuspend antibody-labelled cells in 1 mL of 1x PBS (+ 2% FBS). Add 1 μL of 1 mg/mL propidium iodide to cell suspension just before sorting.
16. Filter contents of the FACS tube using a 40 μm strainer right before sorting LSK cells.
17. Collect LSK cells, via FACS sorting, into 1.5 mL tube containing 0.5 mL of complete media supplemented with 2 mM glutamine, 3 mg/mL glucose, 1 mM pyruvate, 1x thrombopoietin (TPO), 1x stem cell factor (SCF), 0.5x penicillin/streptomycin (P/S), pH 7.4 (Table 1).

3. Mitochondrial Respiration and Glycolysis Assays of LSK HSPCs

1. LSK seeding in the assay plate

1. Centrifuge LSK cells from step 2.4.17 for 5 min at 500 x *g* and RT. Discard the supernatant.
2. Resuspend cells in complete media to a final concentration of at least 70,000 cells/40 μL .
3. Seed contents of 40 μL media (containing 70,000 cells) in the PLL-coated 8-well plate from step 1.2.4. Leave all corner wells empty.
4. Centrifuge for 1 min at 450 x *g* and RT. Do not apply the brake. Make sure that cells are attached to the well bottom using the inverted microscope.
5. Add 135 μL of complete media to the cells in each well for a final volume of 175 μL . Add 175 μL of complete media to the 2 corners of the plate as blanks.
6. Incubate the cells in the non-CO₂ incubator for 2 h at 37 °C.
7. Set up a program to add drugs to each well of the well plate in the analyzer using a metric 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.

2. Mitochondrial stress test

1. Prepare 45 μM stock solutions of oligomycin, 50 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and 25 μM stock solutions of rotenone/antimycin A. To prepare 45 μM oligomycin stock solution, dissolve the contents of the commercially available pouch in 280 μL of the complete media.

To prepare 50 μM FCCP stock solution, dissolve the contents of the commercially available pouch in 288 μL of the complete media. To prepare 25 μM rotenone/antimycin A stock solution, dissolve the contents of the commercially available pouch in 216 μL of the complete media.

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 μM oligomycin, 1.5 μM FCCP, and 0.5 μM of rotenone/antimycin A as needed, following dilution metrics described in Table 4 (for oxygen consumption rate).
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.
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.
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. Glycolysis stress test

1. Reconstitute glucose in 300 μL (100 mM), oligomycin in 288 μL (50 μM), 2-D glucose (2-DG) in 300 μL (500 mM) of complete media from the commercially available pouch.
2. Gently pipette up and down ($\sim 10\times$) to solubilize the compounds. Vortex the 2-DG for approximately 1 min to ensure proper dissolution into media.
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 μM oligomycin, and 50 mM 2-DG.
4. Repeat steps 3.2.3 and 3.2.4.
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 $\sim 80,000$ 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 growth¹⁹. The hexokinase enzyme converts glucose in glucose-6-phosphate and that is subsequently transformed into pyruvate²⁰. Pyruvate can then be processed into lactate and is exported from the cell with protons²¹. 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 space²². Oxygen acts as the final electron acceptor, and protons move back to the mitochondrial matrix through the ATP synthase complex while generating ATP²³. 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 respectively²⁴. 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 ETC²⁵. 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).

For the mitochondrial stress test, we measured the basal level of OCR of LSK HSPCs in glucose/pyruvate+ media. We first injected oligomycin that initially hyperpolarized the mitochondrial membrane, prevented more proton pumping through the ETC complexes, and

thus reduced the rate of mitochondrial respiration. The second injection of FCCP ionophores pushed ETC and OCR levels to their maximum as cells tried to recover the mitochondrial membrane potential. The final injection with other two of the ETC inhibitors (antimycin A and rotenone) caused the complete stop of mitochondrial respiration and thus the OCR reverted to its minimum level (Figure 2D).

Discussion

Here, we demonstrate the isolation of a maximum amount of pure and viable murine LSK HSPCs population as well as the measurement of their glycolysis and mitochondrial respiration with an extracellular flux analyzer. Specifically, the protocol overcomes the following technical issues for the use of LSK HSPCs : i) the low frequency of LSK HSPCs in murine bone marrow¹⁴, ii) low basal metabolic activity of LSK HSPCs²⁶, iii) the fragility of LSK HSPCs²⁷, and iv) the non-adherence of LSK HSPCs to culture vessels¹⁵. In addition, we have optimized the drug concentrations and media composition for the optimum performance of the extracellular flux assays.

Bone marrow-derived HSPCs reside in the hypoxic niche and they display a glycolytic phenotype, which is crucial for the maintenance of their stemness²⁸. Conversely, respiration is essential for HSPC differentiation⁶. 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-CO₂ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Schema demonstrating isolation of Lineage^{neg}Sca1⁺c-Kit⁺ (LSK) hematopoietic stem progenitor cells from mouse bone marrow.

Bone marrow is extracted from bones and mononuclear cells (MNCs) are isolated through density gradient medium gradient separation. Next, cells are incubated with biotinylated Lineage⁺ antibodies and streptavidin-conjugated magnetic beads to elute Lineage negative (Lin⁻) cells following their magnetic separation. Lin⁻ cells are subsequently incubated with LSK antibodies and LSK cells isolated by cell sorting.

Figure 2: Extracellular flux analyses of murine Lineage^{neg}Sca1⁺c-Kit⁺ (LSK) hematopoietic stem progenitor cells.

(A,B) Mechanistic description of drugs utilized for extracellular flux analyses during glycolysis and mitochondrial respiration. (C) Representative results of glycolysis stress test on murine LSK HSPCs in presence or absence of glucose/pyruvate in the media. (D) Representative results of mitochondrial stress test on murine LSK HSPCs. Error bars represent the standard deviation of the mean (S.D.)

Table 1:

Contents and preparation of the complete XF media.

	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

Table 2:

Injection protocol for the mitochondrial stress test.

	Basal	Oligomycin (2 μ M)	FCCP (1.5 μ M)	R/A (0.5 μ M)
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

Table 3:

Injection protocol for the glycolysis stress test.

	Basal	Glucose (10 mM)	Oligomycin (2 μ M)	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

Table 4:

Dilution metrics to obtain optimum drug concentration for mitochondrial stress test in each well of the analyzer.

Port	Drug	Final well concentration (μM)	Stock solution volume (μL)	Media volume (μL)	Port solution (μM)	Volume added to port (μL)
Port A	Oligomycin	2	100	181.25	16	25
Port B	FCCP	1.5	100	270.4	13.5	25
Port C	Rotenone/antimycin A	0.5	60	240	5	25

Table 5:

Dilution metrics to obtain optimum drug concentration for glycolysis stress test in each well of the analyzer.

Port	Drug	Final well concentration	Stock solution volume (μL)	Media volume (μL)	Port solution	Volume added to port (μL)
Port A	Glucose	10 mM	300	75	80 mM	25
Port B	Oligomycin	2 μM	108	192	18 μM	25
Port C	2-DG	50 mM	300	0	500 mM	25

Materials

Name	Company	Catalog Number	Comments
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 crushing
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 separation
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 test
XFp Sensor Cartridge	Agilent Technologies	103022-100	Used for glycolysis and mitochondrial stress test