Standard Operating Procedure for the DK U54 Cooperative Center of Excellence in Hematology (CCEH) Hypoxia Core at the Indiana University School of Medicine

NOTE: The below methodology may require some changes in the future as we learn more from our work and the work of others.

Bone Marrow Flushing in Hypoxic Chamber

Materials:

NOTE: Our chambers are set to 3% O_2 unless otherwise directed. It is important to always include extra supplies other than just what you calculated needing. Additional material cannot be added into chamber at time of experiment as it will not have been acclimated to the lower O_2 levels. All hardware and reagents (e.g. culture media, antibodies, etc.) must be pre-equilibrated to hypoxia chamber at least 18 hours before the chamber is to be used for experiments. Be advised that very small volumes left to equilibrate overnight might result in loss of volume due to the evaporation caused by the moving gasses.

- 5 mL or 10 mL syringes
- Assorted needles: 18/25/26 gauge
- Dissecting scissors and forceps
- Tubes (assorted)
    - We suggest using 12 mL polystyrene snap-cap tubes for collection of bone marrow. We collect marrow in one tube but split to second tube for air equilibration: one tube to be exposed to ambient air [~21% O_2] and one to remain in hypoxia [3% O_2]. We also recommend dispensing PBS into a 50 mL conical for aspiration into the syringes, rather than dipping into the supply bottle.
- Paper towels
- 70% ethanol spray (old hood only)
- 5 mL combitips
    - This is used to break up the bone marrow to ensure single cell suspension.
- Sterile 1X D-PBS or preferred flushing media

NOTE: Mice are euthanized prior to placing them in the hypoxia chamber where the cells/tissues are removed. Cells collected in hypoxia can then be split into two parts: one part remains in hypoxia for processing and further experimentation and one part is placed into ambient air for 1-4 hours before processing.

Procedure

NOTE: When placing materials in the hypoxia chamber, unlock the airlock, place all materials in the airlock, and relock the airlock. Allow time for oxygen to be flushed from airlock chamber. If using the smaller Coy Lab Products hypoxia chamber (Fig. 1), press the "Purge Airlock" button. After the timer has completed, use the hypoxia chamber gloves to open the airlock from inside the chamber. Move the materials to the chamber and close the airlock. If using the larger BioSpherix hypoxia chamber (Fig. 2), wait ~5 minutes prior to removing the material from the airlock to give time for the system to automatically recalibrate to the set O_2 concentration. Then move the materials to the chamber as described above. Make sure that all glove sleeves are airtight when using so that there is no large leakage of air into/out of the chambers. This is crucial. Also, for safety make sure hands fit well into the gloves.

Figure 1: Modified Coy Lab Products chamber.

1. <u>To repeat, at least 18 hours in advance, ensure all necessary materials are available within the hypoxia chamber so they can acclimate to the hypoxic environment.</u>
    a. Items that are individually wrapped for sterility should be left slightly open to allow them to fully acclimate to the lowered O_2 tension in the hypoxia chamber.
    b. Materials may include pipettes, pipette tips, microcentrifuge tubes, 15 mL conical centrifuge tubes, 50 mL conical centrifuge tubes, tube racks, needles, syringes, 1X D-PBS, etc.
    c. Caps on fluids should be left loose. Vortex fluids in chamber to ensure fluids have been exposed to air with lower O_2.
2. Clean the chamber. For the smaller chamber (Fig. 1), clean with 70% ethanol. For the larger chamber (Fig. 2), clean with the wipes provided.
3. Prepare syringes with needles and 1X D-PBS.
4. Euthanize mice according to IACUC standard procedure. IMPORTANT: If any live mice are brought into either 304 (small chamber) or 312A (large chamber) these rooms MUST be added and approved on your IACUC protocol.
5. Completely submerge euthanized animal(s) in a beaker of 70% ethanol. Transfer to airlock on a paper towel and follow describe

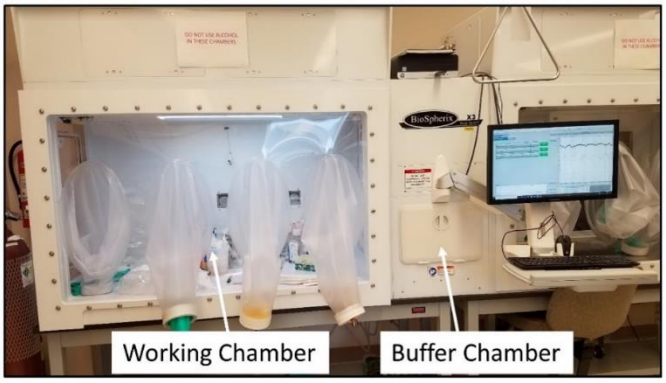

Figure 2: Modified BioSperix chamber.

protocol above to move animal(s) into working chamber.

6. Remove the femur(s) from the mouse. For each femur:
    a. Make a small snip in skin on outside of ankle. Grab foot with fingers and peel the skin up past the hip using forceps. Repeat for other leg.
    b. One the leg is “skinned”, use the dissecting scissors to trim the muscle off and away from the bone (both hamstring and quadricep, or the mouse equivalent).
    c. Hyperextend the knee over a blade of the scissor and transect the knee through the soft tissue connecting the tibia to the femur.
    d. Place the scissors in the hip joint and seat into the joint by pulling the femur superiorly while pulling the scissor inferiorly into the joint and transect.
    e. Open the femur by removing as little bone as possible from each end.
7. Flush the bone marrow.
    a. Using forceps, hold the femur over collection tube and gently insert the needle (25/26) of the syringe (filled with 1X D-PBS) in the newly made hole at the top of the femur.
    b. Run the needle completely through the femur ensure an opening at the other end.
    c. Inject the D-PBS into the bone, rotating the needle and raising and lowering the bone to ensure the entire bone is flushed. The bone should whiten.
8. Clean tools and prepare for the next cage of mice.
9. When all mice have been sampled, use the combitip repeater pipette syringe to break up clumps of bone marrow.
    a. Ensure the sides of the tubes are checked for clumps of cells.
    b. Pull up and depress the plunger about five times unless there is a large clump that is difficult to break up.
10. If performing air vs hypoxia experiment, transfer approximately half of each sample to a new tube labeled “air”. Remove the “air” tubes to a biosafety cabinet and let them acclimate to the ambient oxygen levels by leaving caps lightly closed and vortexing the samples approximately every half-hour. **NOTE:** Air acclimation should be performed for 1-4 hours.
11. Cell counts can be performed on the sample moved to air using a hemacytometer or other counting method (if available).

References

Aljoufi A, Cooper S, Broxmeyer HE. Collection and Processing of Mobilized Mouse Peripheral Blood at Lowered Oxygen Tension Yields Enhanced Numbers of Hematopoietic Stem Cells. Stem Cell Rev Rep. 2020 Oct;16(5):946-953. PMCID: PMC7484397 (available on 2021-10-01)

Broxmeyer HE, Capitano ML, Cooper S, Potchanant ES, Clapp DW. Numbers of long-term hematopoietic stem cells from bone marrow of fanca and fancc knockout mice can be greatly

enhanced by their collection and processing in physioxia conditions. Blood Cells Mol Dis. 2021 Feb;86:102492. PMID: 32896825; PMCID: PMC7686233.

Capitano ML, Mohamad SF, Cooper S, Guo B, Huang X, Gunawan AM, Sampson C, Ropa J, Srour EF, Orschell CM, Broxmeyer HE. Mitigating oxygen stress enhances aged mouse hematopoietic stem cell numbers and function. J Clin Invest. 2021 Jan 4;131(1):e140177. PMID: 33393491; PMCID: PMC7773345.

Mantel CR, O'Leary HA, Chitteti BR, Huang X, Cooper S, Hangoc G, Brustovetsky N, Srour EF, Lee MR, Messina-Graham S, Haas DM, Falah N, Kapur R, Pelus LM, Bardeesy N, Fitamant J, Ivan M, Kim KS, Broxmeyer HE. Enhancing Hematopoietic Stem Cell Transplantation Efficacy by Mitigating Oxygen Shock. Cell. 2015 Jun 18;161(7):1553-65. PMID: 26073944; PMCID: PMC4480616.