Equine Tissue Collection - Whole Animal SOP

Materials:

General:

- □ 3, 6, 12, 20, & 60 cc regular tip syringes
- □ Labeling tape
- □ Permanent markers and pencils
- □ -80°C freezer
- □ Ice
- □ PBS (Phosphate Buffered Saline)
- □ PPE (Personal Protective Equipment)
- □ Weigh boats
- □ Razor blades
- □ Microcentrifuge tube racks

Blood:

- □ Vacutainers with EDTA, heparin, and serum separator
- □ 20 gauge needles
- □ 20 cc syringes
- □ Heparin flush

CSF Tap, Synovial Fluid & Skin Biopsies:

- □ Cup for catching urine
- □ 15 mL conical tubes (approx. 15)
- □ Vacutainers with no additive
- □ Dermal biopsy punch
- □ Hair clippers
- □ PBS solution
- □ 50 mL and 15 mL conical tubes

Necropsy:

- □ Scalpel blades, forceps, scissors
- □ Large dissecting instruments
  - Saws of varying sizes
  - Bandsaw
  - Bolt cutter
- □ Pre-labeled 2.0 mL cryotubes
- □ 30+lbs dry ice
- □ Large necropsy table for dissection
- □ 5 medium necropsy tables for aliquoting and preserving
- □ 5 dewars for liquid nitrogen
- □ Liquid nitrogen (copious amounts)
- □ Cutting boards and trays
- □ Record sheets for times and information
- □ Tissue labels
- □ 10% buffered formalin
- □ 5 Styrofoam coolers of ice
- □ Cardboard freezer boxes
- □ 50 mL conical tubes for nuclei prep
- □ RNase Away

ChIP and Nuclei Prep:

- □ 16% formaldehyde
- □ Dounce homogenizers
- □ 1.5 mL microcentrifuge tubes
- □ Microcentrifuge (4°C, 2000 xg)
- □ 2.5 M glycine
- □ Vacuum line
- □ P1000, 200 and 20 pipettes and tips
- □ Wide-bore pipette tips
- □ Cryogenic vial cooling rack
- □ 50 mL & 15 mL conical tubes
- □ Hybri-Max grade DMSO
- □ D-sucrose
- □ Molecular biology grade sterile H_2O
- □ cOmplete™, EDTA-free Protease Inhibitor Cocktail (04693132001 ROCHE)
- □ 100 µM Steriflip 50 mL vacuum filter system
- □ 37°C & 55°C water baths
- □ Gentle-MACS dissociator and tubes
- □ Graduated pipettes
- □ Phase contrast microscope
- □ 500 mL and 1 L Corning 0.2 µM filter system
- □ 20 µM Steriflip 50 mL vacuum filter system
- □ Rocker platform
- □ Sucrose buffer
  - 250 mM D-sucrose (0.5 M stock)
  - 10 mM Tris-HCl (stock 1 M pH 7.5)
  - 1 mM $MgCl_2$ (1 M stock)

Personel:

- □ 3-5 Veterinarians to identify tissues and perform antemortem phenotyping
  - Internist, Orthopedic Surgeon, and Ophthalmologist,
- □ 6 people with knowledge about equine anatomy to collect tissues at the stations
- □ Team of 2-3 people for tissue cross-linking
- □ Team of 2-3 people for nuclei isolation
- □ 1-2 people for gathering of supplies, labeling of tubes that were forgotten, and other miscellaneous tasks
- □ 1 Veterinary Pathologist
- □ 1-2 necropsy technicians for removal of tissues from the body.

Ante-mortem Procedures

Phenotyping

1. Physical and neurological examination by ACVIM Internist
2. Physical and orthopedic examination by DACVS Surgeon
3. Physical and ophthalmic examination by DACVO Ophthalmologist
4. Bloodwork by the Veterinary personnel

Blood

1. Have a veterinarian place a jugular catheter
2. Pull 6 mL of blood from the jugular catheter and discard this blood.
3. Pair two 20 mL syringes with 20 gauge needles. Draw 20 mL of blood from the catheter at a time, swapping syringes with an assistant (one person draws blood with one syringe while the assistant is transferring blood from the previously filled syringe to vacutainers) until the desired number and type of blood collection tubes have been filled in the order listed:
   - 4 serum separator tubes – (12 mL draw)
   - 5 plasma tubes containing EDTA – (3 mL draw)
   - 5 plasma tubes containing EDTA – (5 mL draw)
   - 5 plasma tubes containing heparin – (5 mL draw)
4. Flush the catheter with 5-10 mL heparin flush.
5. Wrap all tubes in foil and place all NON-SERUM tubes on ice until centrifugation.

Peripheral Blood Mononuclear Cell Collection and Preservation

1. Use the 3 mL draw tubes of EDTA blood to isolate PBMCs. Transfer the 3 mL of blood into a clean 15 mL conical tube and then add 2 mL of PBS. Mix gently.
2. Warm histopaque 1077/1119 to room temperature. Add 3 mL of 1119 to a 15 mL conical tube and then overlay 3 mL of 1077 followed by the 5 mL blood/PBS mixture. Minimize mixing during this layering set.
3. Centrifuge at 700 xg and 18-20^{o}C for 30 minutes. Mononuclear cells will be at the top interface with the histopaque 1077 while neutrophils will be at the 1077/1119 border.
4. Transfer the cell layers at any interface and any higher bands to a clean 15 ml conical tube.
5. Add 6 mL of PBS to these transferred cell layers.
6. Centrifuge at 600 xg and 18-20^{o}C for 10 minutes. Discard the supernatant being careful to not disturb the cell pellet.
7. Add another 6 mL of PBS to the cleaned cells and repeat centrifugation in step 6.

8. Discard supernatant and re-suspend in 1.5 mL of RNAlater. Transfer suspension to a screw top tube and store at 4°C for 3 days.
9. Remove RNAlater and transfer cells to -80°C for long term storage.

Euthanasia

1. This should be performed by a veterinarian.
2. Use a cup or other container to collect urine via free catch after the animal goes down. Using a large syringe (60CCs), transfer any urine collected to 15 mL conical tubes. Alternatively, urinary catheterization can be performed following euthanasia.

Post-Mortem Procedures

Cerebrospinal Fluid Collection

1. A veterinarian should perform an atlanto-occipital puncture and collect cerebrospinal fluid (CSF) while an assistant flexes the animal's neck at the joint between atlas and the occiput.
2. After the CSF needle is placed by the veterinarian, use 15 mL conical tubes to collect the fluid from an extension set attached to the needle. Ensure that the fluid does not come into contact with the surrounding environment. Fill the tubes to the 12 mL mark. You should be able to fill approximately 5 tubes. Cover the tubes with foil and place on ice as they are filled.

Synovial Fluid Collection

1. A veterinarian should collect fluid from the synovial strucutes using sterile technique.
2. Transfer the fluid to plain (no additive) vacutainers. Ensure the tubes are individually labeled with the joint from which the synovia was collected from including its location (i.e. forelimb vs hindlimb, and left vs right). Cover tubes with foil and place on ice as they are filled.

Skin Biopsy Collection

1. After the animal is deceased, shave an area above the gluteal muscle and on the inside of the proximal hind limb for skin biopsy collection. Using Betadine® and alcohol, sterilize the area.
2. Using a dermal biopsy punch, collect full thickness skin biopsies. Rinse the biopsy in PBS and then place in media appropriate for the type of cells to be isolated (epidermal keratinocytes or dermal fibroblasts).

Fluid Sample Preservation

1. Separate 4 mL of CSF for cytological analysis. Save labeled Samples in fridge (4°C) until delivered to histology lab.
2. Spin blood (serum and plasma) and CSF in a tabletop centrifuge for 10 minutes at 2,000 xg and 4°C to pellet any cells in the CSF and separate the serum and plasma from the cellular constituents of the whole blood.
3. Make 1 mL aliquots of serum, plasma, urine, CSF, and synovial fluid and flash freeze with liquid nitrogen. For fluids of larger volumes (CSF and urine), 15 mL conical tubes can be flash frozen but should not be filled with more than 12 mL of fluid.

Tissue Collection

1. Prior to euthanasia of the animal, the necropsy area should be prepared with all labeled tubes, dry ice, storage containers, liquid nitrogen, sampling tools, RNase away, PBS, permanent markers, pencils, spare tubes of all sizes, gloves, formaldehyde, sample tags, and ice.
2. Tissues are divided into stations based on organ systems and the order in which they are removed from the animal during the necropsy. Each station needs its own set of dissection tools and storage containers of ice and liquid nitrogen as well as personnel (1-2) assigned to tissue collection and storage at that station only (to minimize sample contamination).
3. As veterinarians and necropsy technicians remove organs and tissues from the body, samples are brought to the appropriate stations.
4. Before collection, a pathologist must examine the tissue and select a section for preservation in formaldehyde and subsequent histologic examination.
5. As the pathologist selects and sections off samples, a recorder takes pictures of the sample location in relation to the entire organ. These pictures are also used as time stamps for when the samples were collected.
6. Immediately following sample selection by the pathologist, additional samples are collected by the personnel assigned to that particular station, as close to the pathology section as possible. Samples directly touching the pathology section should be marked as proximal samples and the more distal samples as such.
7. Two of the proximal samples are placed on ice for cross-linking of nuclear proteins to DNA before cryopreservation.
8. For any tissue to be used in nuclei isolation, two dime-sized pieces are collected in a 50 mL conical tube and placed on ice for further processing and nuclei extraction.