

File No. 16632 Appendix E: Hawaiian Monk Seal Epidemiology Sampling and Sample Processing Protocols

Hawaiian Monk Seal Epidemiology Sampling Protocol

All persons coming into direct contact with the seal or collecting specimens must be in good health and **wear gloves**. Eye protection (sunglasses or other) is recommended (see “Tagging and Handling” section of HMS manual). Before handling a seal, a vet assistant should be identified and given instructions.

TOOLBOX SUPPLY LIST:

Bleeding supplies:

6ml and 12 ml syringes
18g x 1.5” needles
18g x 3.5” spinal needles
Pre-assembled vacutainer hub/adapters in clean ziplocs
Extra vacutainer adapters
7.5 ml LTTs
3ml LTTs
10ml GNTTs
10ml RTTs
PAX gene RNA tubes

Biopsy supplies:

Sterile surgical gloves (sizes 6.5, 8.5)
Gauze
6mm Acuderm biopsy punches
5ml cryovials
2.5 ml precleaned teflon vials (NIST)
Precleaned instruments (NIST) wrapped in foil (scissors, forceps)

Fecal supplies:

C&S media & swabs

Viral swab supplies:

Dacron fiber tipped swabs w/ plastic applicator
1.8ml cryovials pre-labeled with 2 each: eye, nasal, oral, rectal, genital

Additional supplies:

Crash kit with fluids and emergency drugs (See Appendix A)
Pole syringe
Sedative(s) – at veterinary discretion (midazolam, concentrated midazolam, diazepam)
Cooler and blue ice
Towel
Ceftiofur (Excede)

25 or 30 mL syringes
16g x 3 1/2" needles
Powder free gloves
Data sheets: Epidemiology Sampling Form, Tagging Data Sheet
Clipboard, pens for labeling, pencils for data recording
Betadine-soaked gauze
Alcohol-soaked gauze
Teflon squirt bottle of high purity alcohol (isopropanol)**
Tape measure
Sharps container
Trash bag/container
Rectal thermometer w/ extra batteries
Sterile lubricant (KY gel or other)

****Always replace teflon bag over squirt bottle! Please be mindful that this is an expensive bottle to replace and should be used specifically for this purpose.**

ORDER OF FIELD SAMPLING PROCEDURES:

1. Sedation and restraint
2. Blood collection order:
 1. Fill big (7.5mL) LTT ***ALWAYS FIRST**
 2. Fill one of each RTT and GNTT
 3. Fill PAX gene tube
 4. Fill remaining RTTs and GNTTs in any order
 5. Fill small (3ml) LTT
3. Swab collection (eye, nose, mouth, genital, rectal, fecal culture)
4. Blubber biopsy
5. Tag(s)

SAMPLE COLLECTION:

I. PATIENT MONITORING

A veterinarian must be present for all procedures requiring chemical immobilization. During restraint, vital signs, particularly respirations, should be monitored continuously. In the event of an emergency, the attending veterinarian will abort all sampling efforts and direct emergency procedures. One individual should be designated as the veterinary assistant and tasked with vital sign monitoring/communication with the veterinarian and assistance in the event of an emergency (drawing up emergency drugs, etc., see Appendix A).

II. SEDATION

Positioning: ventral recumbency with foreflippers tucked to the sides.

1. Diazepam (Valium) (5mg/ml): 0.1-0.25 mg/kg IV

- *Route:*
 - Diazepam is most effective when administered IV.
 - Use the extradural or hind flipper veins (see Fig. 1).

- Draw up appropriate amount of drug in a sterile syringe (a 6mL syringe is usually best).
- *Preparation:*
 - Clean the area with betadine solution and alcohol-soaked gauze.
 - Last, do a final spray rinse with the high purity alcohol in the teflon vial. This step is important for ensuring that NIST blood samples are not contaminated and are collected consistently.
- *Venipuncture:*
 - Palpate the vertebral column and pelvis and move your fingers cranially 2 or more vertebral spaces, feeling for a “divot” lateral to the spinous processes of the vertebrae.
 - Needle choice:
 - Pups and thin/average weaners: use an 18 or 20g x 1 ½ to 2” needle.
 - Adults and fat weaners: use a 3.5” spinal needle.
 - Before insertion, remove the stylet, holding needle from hub only.
 - Inform restrainers that you are ready to insert the needle.
 - The angle of the needle may vary from a 45 - 90 degree angle to the dorsal surface of the animal.
 - As the needle is inserted, feel it moving through skin, blubber, and muscle until you feel it pop through the membrane of the extradural sinus. You should now see blood rising to the hub of the needle. Attach the syringe containing the diazepam and inject.
 - Draw back using the same syringe to collect 2-3ml of “waste blood” and dispose of syringe/blood in the sharps container. It is collected to avoid contaminating blood samples with residual diazepam from the needle.
 - Remove syringe and attach the vacutainer hub/adaptor setup to the needle.
 - Fill tubes in order specified above.

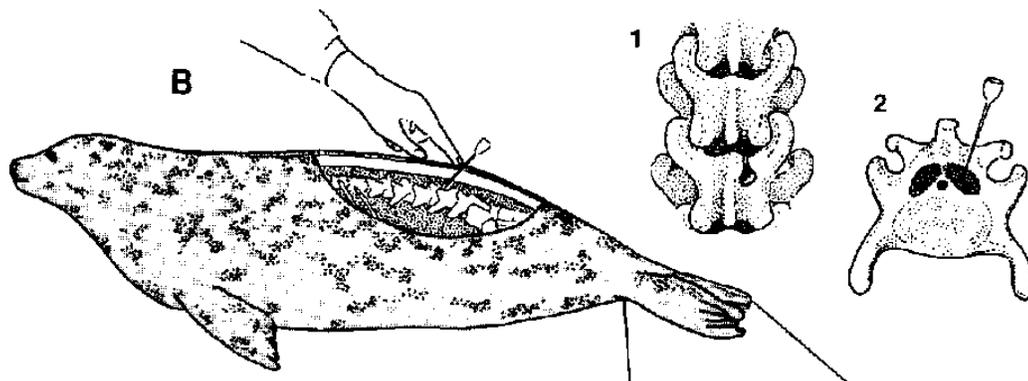


Figure 1.

2. Midazolam (5 mg/ml or 50mg/ml): 0.1 – 0.15mg/kg IM

- Some situations may require capture and manual restraint before drug administration. Other times, it may be sufficient to sneak up to an animal, administer the injection be prepared with boards/nets in case the animal approaches the water. The decision to restrain or corral an animal is to be made between with the handling team and veterinarian on a case-by-case basis.
- *Route:*
 - Midazolam should be administered IM. Draw up the appropriate amount of drug in a small syringe for accuracy.
 - Pole syringes are ideal for IM drug administration, as you can sneak up on a sleeping animal and inject in the hindquarters rapidly, causing minimal stress.
 - Instructions for loading the Dan-Inject Jab Stick pole syringe are included in the syringe carrying case.
 - Special needle sizes (16g x 3 ½” needles) are available for use with large adults.
 - For hand injections, transfer appropriate dose to a large (20mL or larger) syringe to speed drug administration. Use an 16 to 18g x 2“ needle.
- *Administration without immediate restraint:*
 - As the vet approaches the seal to administer sedation, 2-3 people with boards should be standing by (quietly, low to the ground and as out of sight as possible). Ideally, the animal’s reaction to the injection will be brief and if everyone else remains beyond the flight distance of the seal, the best scenario is that it will not move far and go back to sleep.
 - If the seal moves toward the water (or other danger such as boulders, ledges), boarders should approach the head of the animal to prevent the animal from it from reaching the danger or water.
 - A person with a net should be ready in case the boarders cannot contain the animal adequately.
- It will take 10-15 min. for the sedation to take effect.
- Record respiration rate and activity (movement, head position, etc.) at least every 5 min. on the Epi Sampling Form. Use these trends to gauge the alertness of the seal. Do not approach the animal too soon after administering the sedative.
- Once you approach the animal, do so quietly. Consider wetting down a towel and place it gently over the seal’s eyes (only), as this will help keep it calm. Proceed to blood sampling.

3. Reversal of sedative: Flumazenil (0.1mg/ml): 0.1 - 0.2mg/kg IM or IV

- Midazolam and diazepam can be reversed if an accidental overdose is given, if the animal is not responding well to the sedation and emergency procedures are instituted, or if the animal is too slow to wake up following a procedure. IV administration will work rapidly, but IM administration is also fast (minutes) and can be used if IV access is not immediately available.

III. BLOOD SAMPLING

- *Assistant duties:*
 - Before capture, set the vacuums in the RTT and LTT vacutainer/syringe tubes by pulling back on the plunger until it locks into place and snapping it off. This step should **not** be done at the lab ahead of time, as syringes can lose their vacuum.
 - The assistant should ensure that the veterinarian receives the blood tubes in the correct order (see below).
- Attach vacutainer tubes (they will automatically fill if you are in the vessel).
- Fill tubes at least halfway to allow for proper ratio of blood to anti-coagulant.
- Gently rock the tubes 10-15 times to thoroughly mix additive and blood.
- Fill tubes as follows:
 1. Big (7.5mL) LTT
 2. One of each RTT and GNTT
 3. PAX gene tube
 4. Remaining RTTs and GNTTs in any order
 5. Small (3ml) LTT
- Immediately place all tubes **upright** (in particular, the RTTs) in the styrofoam tube holder in the cooler. Tubes should not come into direct contact with the ice, which will cause blood cell lysis.
- The PAX gene tubes must be cooled gradually. They should be stored upright at room temperature for 2 hours before freezing (in the cooler with blue ice is fine, provided that they do not come in direct contact with ice). When freezing, the PAX gene tube should be frozen for 24 hours at -20C before being transferred to the -80C freezer. Long-term storage in a -20C freezer is acceptable as well.
- If the large LTT is not filled first, please note this on the datasheet.

III. SWAB COLLECTION

Use sterile dacron polyester fiber tipped swabs with plastic applicators. Swabs should be collected from the following locations: rectum, genital orifice, nares, medial canthus (corner) of eye, lateral commissure (corner) of the mouth. Collect 2 swabs from each location, place in pre-labeled cryovials, break off tips of swabs in sterile manner, and preserve in liquid nitrogen.

Collect 2 fecal swabs and place both in one vial of C&S media.

IV. BLUBBER BIOPSY

Preparation of biopsy site

Wear sterile surgical gloves. The blubber biopsies should be collected from the lateral aspect of the seal's pelvic girdle, 5-15cm cranial to the wing of the ileum. Before inserting the punch, clean the area with betadine and 70% isopropyl alcohol (take precautions in windy situations to avoid getting in eyes of personnel). **After scrubbing, do a final rinse of the area with the high purity isopropanol in the teflon squirt bottle** (This is important for NIST sampling).

Blubber biopsy collection and disposition

Collect two full-thickness biopsies (2-5 cm). Use the pre-cleaned, foil wrapped (provided by NIST) thumb forceps or scissors as necessary. **Do not allow gloves to contact tissue.** After samples have been collected, clean the biopsy sites with betadine solution. The wound can be left to heal through second intention.

The first blubber sample should be stored in a 7mL teflon vial for toxicology analysis. The second sample should have the skin removed (as aseptically as possible). The skin should be placed in a 2mL cryovial containing DMSO. The blubber should be placed in a cryovial for fatty acid radioactive isotope analysis. Freeze both samples in liquid nitrogen as soon as possible.

SAMPLE COLLECTION GUIDE

See Sample Processing Protocol for details on what to do back at the lab.

Item (#/seal)	Tube Vol.	Anti-coagulant	Blood Fraction	Short-term	Processing instructions	Long-term	Purpose (Investigator)
LTT (1)	7.5 mL	EDTA	Whole	Blue ice	Divide into 1mL aliquots and place in cryovials	LN	Archive (NIST/Trace Elements)
LTT (1)	3 mL	EDTA	Whole	Blue ice	IDEXX	Do not store	Blood chemistry (HMSRP)
PAX gene tube (1)	2.5mL	RNA preservative	Whole	Store upright at room temp for 2 hours	Divide into 1 or 2mL aliquots in cryovials after 2 hours at room temp. Then transfer to -20C freezer for 24 hours. Then transfer to LN/UF if desired.	-20/ LN/ UF	Biotoxins (NOS)
GNTT (3)	10 mL	NaHep	Plasma	Blue ice	a) Spin down, transfer 2.5 mL from tube into 7 mL Teflon jar b) Divide remainder into 1 mL aliquots and place in cryovials	LN	a) Contaminants (NIST) b) Archive (min. 4 aliquots each to NIST & HMSRP)
GNTT (1)	4 mL	NaHep	Plasma	Blue ice	Spin down and aliquot into cryovial(s)	LN/ UF	a) Biotoxins (NOS)

Item (#/seal)	Tube Vol.	Anti-coagulant	Blood Fraction	Short-term	Processing instructions	Long-term	Purpose (Investigator)
RTT (4)	10 mL	None	Serum	Upright Blue ice	Divide into 1mL aliquots and place in cryovials	LN/ UF	a) Tier 1 (HMSRP) b) HMSRP/ NIST Tissue Bk (minimum 4 aliquots each to NIST & HMSRP)
Viral swabs				Blue ice		LN/ UF	Tier 1 (HMSRP)
Rectal swabs				Blue ice		Fridge	Culture (<i>Vibrio</i>) (UC Davis)
Blubber biopsy #1				7 mL vial (Teflon) Blue ice		LN/ UF	Contaminants (NWFSC)
Blubber biopsy #2				2 mL cryovial	a) Remove skin from biopsy at the lab and place in DMSO cryovial b) Freeze biopsy	LN/ UF	a) Skin: archive/stable isotopes (HMSRP) b) Fatty acids (S. Iverson/Dalhousie; CITES permit required)

Hawaiian Monk Seal Sample Processing Protocol

LABELING

Use nalgene cryoware markers for labeling vials. Label all samples as follows:

Seal ID Collection date (YYYYMMDD) Specimen # and subnumber (ex: 1000A) Specimen Type – Subtype (ex: PS-GT) HMS Island

Specimen Types: PS - plasma; SE - serum; WB - whole blood; BB - blubber biopsy

Specimen Subtypes: GT - green top; LT - lavender top; RT - red top

Please refer to the cheat sheet in the lab for additional sample codes.

IDEXX Samples only need to be labeled with Seal ID, Date, Specimen # and Account #1689 (for routine clinical samples; see Angie for IDEXX research account number if applicable).

BLOOD TUBE PROCESSING

Note: Whenever you start a new lot number of blood tubes, you must make a field blank for NIST. See Epidemiology Sampling Protocol for instructions.

1. Every effort should be made to process blood **within 4 hours of collection**.
2. If sending samples to IDEXX, call 1-800-444-4210 and tell them you are calling from acct #1689 and have samples to be picked up. Confirm pick-up time. See IDEXX Shipment Instructions for more details.
3. Put on a clean pair of powder free gloves, disinfect your work surface and/or put down one of the tex wipes provided by NIST. Refer to biosafety documents.
4. Brush sand off of tubes and make sure tubes are properly balanced in centrifuge (use water-filled tubes to balance as necessary). Spin the RTTs and GNTTs for 10 minutes at 2000 rpm.
5. While RTTs and GNTTs are spinning, assign specimen numbers and ensure that samples and containers are labeled:
 - Cryovials w/ swabs
 - Blubber biopsies
 - Cryovials for serum, buffy coat and plasma aliquots
 - C&S media for fecal culture
6. Place each animal's samples in a separate cryobox. Then put the plastic box in a plastic bag along with the two Teflon vials. Label bag with seal ID and date, and then place in UF. Record location of cryobox on specimen data sheet.

Lavender-top EDTA tubes (whole blood):

7.5mL tube

- Wearing clean gloves, unscrew the blood tube top
- Pour 1mL aliquots of whole blood into 2mL cryovials. To prevent contamination, do not remove aliquots from these vials.
- Label each aliquot as directed above
- Place in cryobox and transfer to UF

3mL tube

- Label tube as directed above.
- Refrigerate.
- Send to IDEXX (see IDEXX Shipment Instructions)

Red-top tubes (serum):

- Using a clean, plastic pipette, place 1 mL aliquots of serum into 2 mL cryovials.
- Be sure to record states of hemolysis and/or lipemia in the notes column of the specimen log and lab data sheet.

- Assign specimen numbers and label serum vials as shown above.
- In most cases, 1.0 ml of **refrigerated** serum will be sent with the 3mL LTT to IDEXX for chemistry. **Use serum sub # E.** See IDEXX Shipment Instructions.
- Place remaining serum aliquots in cryobox and store in UF.
- Dispose of remainder of RTT after all serum is collected.

PAX gene tube (whole blood):

- After blood is collected, rock tube gently to mix additive with blood.
- Store at room temperature for at least 2 hours (up to 72 hours).
- Transfer to 1 or 2mL cryovial aliquots and place in -20C freezer for at least 24 hours.
- Long-term storage in -20C or -80C freezer is appropriate. If storing in -80C freezer, samples should be frozen at -20C for 24 hours before transfer to -80C freezer.

Green-top tubes (buffy coat, plasma):

- Transfer plasma from the 4 mL GNTT into a cryovial for biotoxin analysis. Assign specimen number and label cryovial. Freeze in UF or LN.
- Using a NIST-provided pipette, transfer 2.5 mL of plasma into a 7 mL Teflon jar (Contaminants). Label the side of the jar and the paper Nomex tab and place tab in recessed lid of the Teflon jar.
- Use a clean glass Pasteur pipette to aliquot 1mL of plasma into 2mL cryovials.
- Then, re-spin the tube in the centrifuge for 10 min.
- Use a clean plastic pipette to transfer the buffy coat to a separate cryovial.
- Place all samples in a cryobox and store in UF.

OTHER SAMPLES:

The C&S medium sample should go in the refrigerator to be shipped to UC Davis.

Emergency contact #s:

Liz work c 721-5344, personal c 285-4578

Angie work c 343-6249, personal c 512-773-7341

Dr. Bob Braun 783-6565

Dr. Gregg Levine 358-5311

Dr. Michelle Barbieri c: 443-834-8612, w: 808-983-3706

SAMPLE SUMMARY

Bold = Tier 1 Sampling

LAB	Screening
IDEXX	CBC/chemistry
Jerry Saliki, Athens Diagnostic Lab, UGA	Morbillivirus serology and PCR
Terry Spraker, CSU	Histopathology
J. Dubey, USDA	Toxoplasma serology
WADDL	Canine Adenovirus, Feline Calicivirus and in MHI, Parvovirus
Athens Diagnostic Laboratory, UGA	Phocid herpesvirus 1 serology
NVSL	Chlamydophila psittaci
Renee Galloway, CDC	Leptospirosis
UC Davis	Fecal culture
Mike Grigg, Nat'l. Institutes of Allergy/Inf. Dz	Toxoplasma PCR/genotyping
Marine Biotoxins Program, NOS	Biotoxins
Jenny Schultz, UH Manoa	Genetics
Mote Marine Lab	Fertility potential
Mystic Aquarium	Brucella (cELISA serology)
NVSL	Brucella (culture and isolation if indicated)
Jeff Stott, Tracy Goldstein (UC Davis)	Herpesvirus
NIST, archive	Toxicology