

**PROTOCOLS FOR DRAWING BLOOD FROM
THE BRACHIAL PLEXUS OF DESERT TORTOISES:
Instructions for Ordering Equipment and Culture Media;
Summary of How to Draw Blood and
Information on How to Contract for Laboratory Analysis
and Ship Laboratory Samples**

**Appendix on New Technique for Conducting Nasal Lavages
by Dr. Lori Wendland**

Prepared by

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for Desert Tortoise Fieldwork in 2005**

For research conducted under Federal Fish and Wildlife Service Permit No. TE-006556-12
California Department of Fish and Game Scientific Collecting Permit SC-003623 to Kristin H.
Berry, including Additional Conditions and Authorizations

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OVERVIEW OF THE PROTOCOL

Blood can be drawn from desert tortoises from several sources, such as the jugular vein, heart (cardiocentesis), sinus associated with the eye, and a subcarapacial venipuncture site (Hernandez-Divers et al., 2002). For research on hematological and plasma biochemical values of tortoises, the jugular vein is the appropriate source to obtain pure blood, without lymph (see Christopher et al., 1999, Christopher et al., 2003). For enzyme-linked immuno assay (ELISA) tests, blood can be drawn from the brachial plexus, providing that lymph is avoided (Berry et al., 2005). The ELISA tests are for assessing exposure to herpes virus (Origgi et al., 2001), *Mycoplasma agassizii* (see Jacobson et al., 1991; Schumacher et al., 1993; Brown et al., 1994, 1995, 2002), and *M. testudineum* (Brown et al., 2004; Brown et al., personal communication). *Mycoplasma agassizii* is the bacterial pathogen that causes upper respiratory tract disease (URTD). *Mycoplasma testudineum* has been identified in desert tortoises with URTD and is also a pathogen in the gopher tortoise (Brown et al., 2004). However transmission studies have not been completed for the desert tortoise on *M. testudineum* to confirm whether it is a pathogen similar to *M. agassizii*.

The ELISA tests do not require pure blood samples from the jugular vein. However, samples with lymph should be avoided because antibody levels are lower than for plasma (Berry et al., 2005). Our protocol is designed to obtain blood from the brachial plexus in the forelegs of large immature, subadult, and adult desert tortoises, i.e., tortoises >140 mm in carapace length at the midline. The protocol also includes a nasal lavage to assess the presence of *Mycoplasma* species in the upper respiratory tract. The nasal protocol was prepared by Lori Wendland, DVM, and is a separate document (Appendix 1). The subcarapacial venipuncture site is also available (Hernandez-Divers et al., 2002), is easy to learn, and may be preferred in projects when permitted by government agencies. Samples obtained from the subcarapacial venipuncture site also may contain lymph.

The Protocol shown below starts with acquisition of equipment and ends with delivery of plasma and nasal lavage samples to the laboratory. It is designed to be used in conjunction with modified and expanded health profile data forms (modified from Berry and Christopher, 2001). The Protocol is also associated with Kristin Berry's research permit with the U. S. Fish and Wildlife Service, TE-006556-12.

LIST OF EQUIPMENT TO ACQUIRE, CARRY IN FIELD PACK AND VEHICLE

Equipment for Day Pack (Lunch Carrying Case with Slots for Ice):

Frozen ice packs (several)
Tubes of frozen tryptose broth (SP4)
Microtainer Brand Tube with Lithium Heparin
Heparin
1 cc syringes with needles (25 gauge); for the subcarapacial venipuncture site, a 1 1/2 inch

22 gauge needle is necessary
10 cc syringes for nasal flush (20 gauge x 1.5 inches)
22 gauge x 1 inch IV catheters
Sterile urine cups
0.9% saline solution
Betadine mix (10:1, specifically 9 parts water to one part Betadine)
Rubbing alcohol
Foamed alcohol hand scrub (Alcare)
Bleach solution (diluted 1:20, specifically one part bleach [see below] to 19 parts water))
Q-tips
Disposable latex gloves
Gauze and/or cotton balls
Rubber bands
Small zip-lock bags
Nalgene Lab Markers or similar for writing on glass/plastic tubes
Disposable aprons for handling tortoise
Clip board with pen with permanent black ink
Data sheets
Standard equipment for processing tortoises in the field (scales, calipers, ruler, etc.),
including 35 mm camera with close-up lens or digital camera (depending on
requirements of permit holder and principal investigator)

Equipment for Field Vehicle Must be Sufficient for Entire Field Trip:

Centrifuge and inverter for running centrifuge off of vehicle cigarette lighter
Ice chest with dry ice; also includes space for several small ice packs for day packs
Frozen vials of tryptose broth (keep frozen in ice chest) for nasal lavage, culturing
Mycoplasma
Microtainer brand tube with lithium heparin
Heparin
Cryotube vials (external threads on cap)
Syringes/needles of three sizes (four sizes are necessary if the subcarapacial venipuncture
site is to be used)
Syringes and catheters for nasal flush
Saline solution
Sterile urine cups
Bottles of Betadine
Bottles of rubbing alcohol
Alcare (foamed alcohol hand scrub)
Box of Q-tips
Box of gauze pads and bags of cotton balls
Disposable latex gloves
Rubber bands
Small zip-lock bags

Nalgene lab markers or similar for writing on glass/plastic tubes
 Disposable aprons for handling tortoise
 Supply of data sheets on archival paper
 Disinfectant (dilute solution of bleach, specifically sodium hypochlorite solution at 0.175%, made fresh every three days) for disinfecting equipment

SOURCES of above equipment:

- (1) Fisher Scientific Company: (1-800-766-7000)
 Be certain to check all catalog numbers; they may change. You will need to set up an account.
- | | |
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| Latex gloves, small: powder-free. | Catalog No.: 11-394-5A |
| Latex gloves, medium: powder-free. | Catalog No.: 11-394-5B |
| Latex gloves, large: powder-free. | Catalog No.: 11-394-5C |
| Microtainer with lithium heparin, 50/pk
without separator OR | Catalog No: 0266875 |
| Microtainer with lithium heparin ,
50/pk with separator | Catalog No. 0266939 |
| Cryotube 1.8 ml, 500/pk (externally threaded) | Catalog No: 12565166N |
| Syringe/needle TB, 1cc, 25GX5/8", 100/pk | Catalog No: 1482688 |
| Syringe TB 21GX1", 1cc, 100/pk | Catalog No: 1482910D |
| Syringe/needle, 10 cc, 20G x 1.5", 100/pk | Catalog No: 1482918G |
| Syringe/needle, 3 cc, 22G x 1.5", 100/pk
(for subcarapacial venipuncture) | Catalog No. 02-669-39 |
| Sterile 100 cc urine collection container | Catalog No: 14375410 |
| Isopropyl alcohol 70%, 500 ml | Catalog No: LC157601 |
| Betadine solution, 16 oz | Catalog No: 19027133 |
| Gauze pads, sterile 3 x 3, 100/pk | Catalog No: 19027227 |
| Nalgene Lab Markers (fine point) | Catalog No. 13-382-50, 6310-0010 |
| Polyethylene Apron, 50/pk | Catalog No. 01-351-15 |
- (2) From a veterinarian:
 Heparin
 Terumo Surflew 22G x 1" IV Catheter
 Sterile 0.9% NaCl (saline) IV Fluid (250 or 500 ml bags)
- (3) Frozen sterile tryptose /SP4 broth for culturing *Mycoplasma*: Order directly from Dr. Mary Brown, Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611-0633, (352) 392-4700, ext. 3970, (Barbara Crenshaw is at 352-392-4700, ext. 3968). Email address for Dr. Brown: mbbrown@nersp.nerdc.ufl.edu. Dr. Brown will arrange for a FEDEX of the frozen tryptose broth; you will need to be present to place it in the freezer when it arrives.

Dr. Brown has provided the frozen broth free of charge.

INSTRUCTIONS FOR COLLECTING BLOOD FROM THE BRACHIAL PLEXUS AND CONDUCTING NASAL LAVAGES

1. For the Fish and Wildlife Service research permit No. TE-006556-12 held by Kristin H. Berry, two field workers are required to work together on drawing blood and conducting nasal lavages. One field worker must be named on the portion of the permit described for drawing blood. In addition, Dr. Berry must issue a special letter authorizing this activity. Only the individuals listed on the cover letter are authorized to participate in the field work. The exception to this rule is if Dr. Berry is present and authorizes the action.
2. Prepare Centrifuge for Use. The centrifuge, if one of the more basic and sturdy models, can be run by using an inverter attached to the cigarette lighter of a vehicle. Be sure to test the centrifuge system with the vehicle prior to leaving for the field, because the centrifuge/inverter system may not work on some of the older, smaller vehicles. The centrifuge should be kept cool in the field. A good place to put the centrifuge is on the floor of the car in front of the front seat (in the shade).

Balance the centrifuge on the opposite side, e.g., put water in microtainers.

3. Prepare Day Pack For Use. Drawing on the field equipment and ice packs in the car, prepare all necessary equipment for drawing blood and collecting nasal lavages away from the vehicle. Keep needles, gauze, and other equipment sterile. Have disposable apron available to keep tortoise from clothes and limbs. Use a fresh pair of gloves for each tortoise. Prepare clipboard with pen (with permanent black ink) and data sheets for Live Desert Tortoises and Health Profiles (expanded format).

Dilute Betadine 10:1, specifically 9 parts water and 1 part Betadine, in a container for use in the day pack. Take care to ensure that the Betadine does not get on clothes and pack, because it stains.

If ice packs thaw, return to the vehicle to get fresh ice (and centrifuge the blood).

4. Temperature limitations. The drawing of blood and nasal lavages requires careful attention to air and surface temperatures and other weather conditions, because the desert tortoise can easily become stressed and overheated on warm spring days. Desert tortoises generally are not active above ground when air temperatures at 1.0 – 1.5 meters exceed 33° C. Once air temperatures reach this point, field workers must take great care not to stress the tortoise with overheating. The U. S. Fish and Wildlife Service has a new protocol for handling tortoises on warm to hot days. It is described below.

"During all handling procedures, desert tortoises must be treated in a manner to ensure that they do not overheat, exhibit signs of overheating (e.g., gaping, foaming at the mouth) or are placed in a situation where they cannot maintain surface and core temperatures necessary to their well-being. Desert tortoises must be kept shaded at all times until it is safe to release them. For the purposes of this permit, ambient air temperature must be measured in the shade, protected from the wind, and at a height of 2 inches above the ground surface.

"No desert tortoise shall be captured, moved, transported, released, or purposefully caused to leave its burrow for whatever reason when the ambient air temperature is above 95 degrees Fahrenheit (35 degrees Celsius). No desert tortoise shall be captured if the ambient air temperature is anticipated to exceed 95 degrees Fahrenheit before handling or processing can be completed. If the ambient air temperature (2 inches above the ground surface) exceeds 35 degrees Celsius during handling or processing, the desert tortoises shall be kept shaded in an environment which does not exceed 35 degrees Celsius."

5. Reducing Stress to the Desert Tortoise. Go to the desert tortoise in the field, carrying field equipment in the day pack. Do not plan to bring the tortoise to the vehicle, carry it in a pack, drive it in the car to a laboratory, or otherwise remove it from the vicinity of its capture point. The system described in this protocol will function best when the field workers travel to the tortoise at its cave, burrow, or shrub, or other location in the field. In our experience, the tortoise is less likely to void if not moved more than 10-30 m from the site of capture.

Once the tortoise is observed, prepare a shady place for the tortoise and the field workers away from the cover site or capture site, so that the cover site is not disturbed. This can be done by placing an umbrella, small light-weight blanket, canvas, shade cloth, or other similar light-weight material over a large shrub or group of shrubs, thereby creating a block of shade. Soil temperatures, if warm or hot, can be reduced by clearing away the top few inches. Do not disturb the area around the cover site, the burrow mound, or initial resting place of the tortoise. Instead, prepare the shady site a few to 30 m away. Ensure that the area where tortoise will be handled is clean. Lay out equipment in the order that it will be used. Determine who will hold the tortoise, take notes, and draw blood. The process of conducting a health evaluation and drawing blood works best with 3 individuals: two to work on the tortoise and one to record the data and assist with labeling tubes, etc.

6. Fill out the Health Profile Data Sheets. Once the tortoise is located in the field, the data sheets should be filled out. The data sheets are an integral and essential part of the health assessment. The eyes and nares should be evaluated prior to drawing blood and taking the nasal lavage, preferably when the tortoise has its head and beak naturally exposed.

7. Photograph the tortoise. Close-up views of the carapace, plastron, beak, and eyes (both sides of the face) should be taken with a 35-mm camera equipped with close-up lenses. Each slide must be labeled with the tortoise identification number, date, study site name, observer, and location details (UTMs or latitude/longitude in decimal degrees, county, state).

8. Drawing blood. Prepare the 25 gauge needle by drawing heparin into the needle and syringe and injecting it directly back into the heparin vial. Expel any additional heparin remaining in the syringe. Gently hold and warm the forelimb, squeezing it to improve circulation; consider using a rubber band as a tourniquet posterior to the tendon. Swab the posterior side of one forelimb, either right or left, with diluted Betadine. Rinse with rubbing alcohol. Palpate the area, feeling for the brachial vein. After locating the vein, insert the 25 gauge needle at a 45 degree angle into the brachial vein, anterior to the tendon and below the muscle. Slowly draw out at least 0.5 cc blood. Be careful not to push the plunger into the syringe: air bubbles must be avoided. If the needle stick is dry, you may attempt to draw blood with a maximum of 4 needle sticks in each foreleg. If eight attempts are unsuccessful, do not continue. Another method is also possible. If the location of the needle appears to be correct or almost so, then the needle may be withdrawn to the skin surface and redirected to another site. Such a withdrawal and redirection of the needle counts as one of the sticks. However, once the needle is inserted into the foreleg, do not move it about to search for the vessel.

Lymph or a mixture of blood and lymph may be withdrawn into the syringe. Observe very closely the material flowing into the syringe. Lymph is not desirable, because it does not have the same antibody titer as plasma (Berry et al., 2005). Therefore, every effort should be made to avoid collecting lymph. However, if lymph is collected or the sample contains a mixture of blood and lymph, note this observation on the data sheet. Continue to work with the tortoise to the maximum of eight sticks to ensure a good blood sample.

When blood (or lymph, or a combination thereof) has been successfully drawn, apply a gauze pad or cotton balls to the puncture site and apply pressure to prevent bleeding. Tightly press the gauze to the leg for a few minutes. Remove the needle from the syringe, inject the blood into a Microtainer brand tube, and gently mix about 10-15 times. Label as RBC, or if the sample contains lymph or a combination of lymph and blood, place this information on the Microtainer (see item 9 below). Place the sample in a labeled plastic bag and into the ice chest. Chill, but do not freeze because the blood must be centrifuged prior to freezing.

Nasal lavages. See procedure prepared by Dr. Lori Wendland dated May 2004.

9. Required procedure for labeling vials: use a Nalgene Lab Marker Pen. Other markers will not retain the ink on the plastic and the specimen will be useless. Place

the following information on the vials:

G. agassizii

Tortoise number and sex

Date

Study site name

RBC, plasma, lymph, combinations of plasma and lymph, or nasal sample

Name of Principal Investigator, e.g., Berry

After the blood is centrifuged, there should be two and possibly three identically labeled tubes: one for red blood cells (in the Microtainer Brand Tube), one for plasma (cryotube) and, if lymph was collected, one for the lymph sample (cryotube). (Samples with a combination of blood and lymph should be labeled in a similar manner as described above). Place the tubes in labeled ziplock bags (see item 10 below) and immediately place on dry ice in the ice chest.

Take exceptional care that all lids of tubes are tightly in place. Numerous samples have been compromised or lost due to loose lids.

10. Sorting the tubes and labeling the ziplock bags. Tubes should be sorted by study site and by sample type and placed in separate, labeled small ziplock bags. For example, samples should be separated into 2 to 3 bags labeled Sand Hill Red Blood Cells (Sept. 2005), Sand Hill Plasma (Sept. 2005), and potentially, Sand Hill Lymph (Sept. 2005). The county and state should follow. Ziplock bags should be labeled directly on the surface using the Nalgene marker. Small bags can be placed in large ziplock bags.
11. Return the tortoise to the cover site or shady site adjacent to where collected.
12. Disinfect all equipment/materials/your hands or arms that have come in contact with the tortoise, the tortoise cover site (probes), or similar using a dilute solution of bleach. Do not use alcohol or other substances. The bleach solution must be made fresh every three days and kept out of the sun.

Hands may be disinfected using Alcare foamed alcohol hand scrub.

13. Centrifuging the blood. The blood and nasal lavages must be kept on ice at all times. If the ice melts, the fieldworker must return to the vehicle and get fresh ice. The blood must be centrifuged as soon as possible, preferably within 2 to 3 hours and sooner if the weather is hot. The longer the blood sits, the more likely it is to clot and have the sample spoiled. We have experienced clotting within 15 minutes. Before starting the centrifuge, ensure that the centrifuge is properly balanced. Spin the blood for 3 minutes. The centrifuged blood in the Microtainer tube may have a layer of plasma on the top, a layer of clay in the middle, and a layer of blood at the

bottom, depending on the type of Microtainer tube. With a 22 gauge needle and syringe, draw out the plasma only, without getting clay or blood/red blood cells. Inject the plasma into a cryotube, labeling the tube as plasma or lymph or a mixture of plasma and lymph (see item 9 for labeling). Label the Microtainer, cryotubes, and ziplock bags as described in items 9 and 10. Freeze the samples immediately. Do not leave the tubes exposed where they can heat up or warm at any time. Do not hold samples for more than a few seconds in warm fingers.

LABORATORY EVALUATION OF THE BLOOD AND NASAL LAVAGES

1. Evaluation of the blood samples with the ELISA test and the nasal lavage using cultures and the polymerase chain reaction (PCR) tests. For those individuals not involved with Dr. Berry and her FWS Scientific Research permit, a contract will need to be established with Dr. Mary Brown at the University of Florida . The research scientists and the University of Florida hold the ELISA tests under a patent or similar. In the past, they have provided outstanding service. We recommend requesting a price for all tests for each tortoise. (Department of the Interior agencies have paid about \$50 for the set of *Mycoplasma* tests for each tortoise in the past, as well as additional fees for the herpes virus tests.) The University of Florida does not want to handle charges on a single tortoise basis, so it is most effective to set up a one- to two-year contract for ≥ 50 sets of tests at a time.

The USGS has a contract with the University of Florida for ELISA tests, PCR tests, and cultures for *Mycoplasma* species and for herpes virus ELISA tests. Dr. Berry manages this contract and will manage the blood and nasal samples sent to the University of Florida under her research permit.

2. Preparing the samples for shipping to Dr. Berry. Samples should be stored in layers of zip-lock bags and kept frozen until shipping. For those individuals working on Dr. Berry's permit and projects, samples will be delivered directly to Dr. Berry. Vials should be carefully packed to avoid breakage (we have lost vials and samples to breakage), layered in paper towels when necessary, and must be very carefully labeled with the Nalgene pen. We have lost valuable data because the ink washed off sample vials during shipment! Samples should be personally delivered at the office or shipped by FEDEX on dry ice by overnight mail only after Dr. Berry or her staff confirm that they are ready to receive the samples and will track the status and arrival. You do not want to have the samples accidentally lost or thawed. For this reason, only plan to ship Monday through Wednesday for arrival dates of Tuesday through Thursday. Check the shipping address in advance with Dr. Berry and staff.

Each shipment to Dr. Berry should be accompanied by a letter and list of the samples with details (see Table 1). The letter and packing list should be placed in a zip-lock bag with the samples.

3. For individuals working under other Scientific Research permits, shipping may be directly to the University of Florida and Dr. Brown. Be sure to follow procedures described above, and notify Dr. Brown and her staff (Dr. Wendland, Dina Demcovitz, Barbara Crenshaw) prior to shipping because the shipping address differs from the mailing address. The advance announcement of arrival should also be sent via electronic mail and/or facsimile message to Dr. Brown or Ms. Demcovitz on the date of the FEDEX shipment.
4. For individuals who are making arrangements for the ELISA herpesvirus tests with Dr. Elliott Jacobson, procedures will be similar to the above.

Table 1. Blood and nasal samples collected from tortoises at the Tiefert Mountain Potential Release Plot in San Bernardino County, California, during the spring of 1999.

Tortoise number	Date mmddyyyy	Sex	MCL (mm)	Weight (grams)	Samples collected		
					Nasal lavage	blood	lymph
23	04251999	F	208	2050	Yes	No	Yes
26	05241999	M	259	3850	Yes	Yes	No
42	04251999	M	265	4000	Yes	Yes	No
52	04251999	F	241	2800	Yes	No	No
72	04251999	F	244	2600	Yes	No	Yes
96	03301999	M	268	3600	Yes	Yes	Yes
103	03311999	F	238	2500	Yes	Yes	No
128	05241999	F	228	2950	Yes	Yes	No

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