

**Protocol for the Prevention of the Transmission of Disease
among Desert Tortoises (*Gopherus agassizii*) at the
Desert Tortoise Conservation Center and Transfer and Holding Facility**

Introduction

This protocol was developed by Southern Nevada Environmental Incorporated (SNEI) and the Bureau of Land Management (Bureau) for handling, housing and caring, processing, and disposing of tortoises. This protocol describes the minimum requirements necessary to minimize the spread of disease among captive tortoises at the Desert Tortoise Conservation Center and the Clark County Desert Tortoise Transfer and Holding Facility (Facility). Implementation of these protocols will assure upper respiratory tract disease (URTD) is not transmitted among tortoises during testing, or once tested, ensure that tortoises will not be exposed to URTD or other potential diseases. SNEI is under contract with Clark County to pick-up, process, and care for tortoises collected under incidental take permit PRT-801045, as described in the Clark County Desert Conservation Plan and section 7 of the Endangered Species Act of 1973, as amended (Act). The Bureau processes and cares for tortoises collected under section 10(a)(1)(A) permit PRT-747182 issued to The Nature Conservancy, Nevada Division of Wildlife, and the Bureau. On September 6, 1996, the U.S. Fish and Wildlife Service (Service) issued a section 10(a)(1)(A) permit to the Bureau to determine if tortoises collected under PRT-747182 have been exposed to *Mycoplasma agassizii*, the causative organism for URTD, using the enzyme-linked immunosorbent assay (ELISA) method.

The Bureau will identify tortoises as either (1) available for release/transfer, or (2) being held for research. Tortoises held for research will be isolated from those available for release/transfer to assure URTD is not spread from research tortoises to those awaiting release/transfer.

Appendix A includes detailed protocols for moving and holding tortoises, drawing blood, and sanitizing equipment and pens.

Disposition of Tortoises

Unless part of a research group, any tortoise exhibiting visual signs of URTD or exposed to *M. agassizii* as determined by ELISA (seropositive) shall be euthanized and disposed of as directed by the Service. Euthanasia of injured or ill tortoises is authorized under sections 7 and 10 of the Act. Visual signs of URTD include nasal discharge, puffy eyelids, conjunctivitis, and eyes recessed into the orbits. Seropositive tortoises or tortoises with visual signs of URTD and involved in research will be euthanized upon termination of their usefulness as research animals unless directed differently by the Service.

I. Tortoise Handling and Housing (General)

Tortoise handling protocol shall apply to all procedures such as weighing, measuring, and transporting tortoises which involves any physical contact between tortoises and people, utensils, or equipment. Throughout this protocol, the term “sanitary” is used to mean: “Incapable of transmitting disease”. Technicians will sanitize their footwear prior to entering unknown, suspect, or seronegative pens or whenever a technician questions the sanitary status of his/her footwear. Footwear will be sanitized following the protocol outlined in section IV. of Appendix A. Whenever possible, seronegative pens shall be entered first, then unknown, suspect, and seropositive pens.

Pens shall be cleaned at least twice per year, preferably in mid-summer and fall prior to hibernation. Pen cleaning shall consist of collecting and sanitizing food and water dishes/bowls (section IV., Appendix A). In addition, alfalfa and scat will be removed and discarded. All food and water dishes/bowls will be stored over winter. Clean food and water bowls shall be placed in each active pen in the spring following tortoise emergence from hibernation.

Tortoises which are not part of a research project may be temporarily housed in cardboard boxes or plastic totes up to ten (10) days except tortoises smaller than 80 millimeters, which may be housed in these containers for up to six (6) months.

A. Tortoises of Unknown Status

This group consists of incoming tortoises and other tortoises of unknown exposure to *M. agassizii* as determined by ELISA. Each tortoise shall be handled with sanitary gloves and will not come into contact with other tortoises. These tortoises shall be maintained in their pens until tested for exposure to *M. agassizii* or kept in the cardboard box or tote in which the tortoise was received or placed in a sanitary container (box, tote, or pen). Only sanitary equipment and utensils will come in contact with these tortoises.

B. Research tortoises

This group consists of tortoises involved in ongoing research or held in pens at the Facility for future research (e.g. those tortoises held by Dr. Dave Rostal). Research tortoises shall be placed in pens or holding containers which are isolated from all other tortoises. These tortoises shall not be assessed by ELISA using section 7 funds until they are no longer needed for research and are available for transfer or release. Tortoises in this group which show signs of URTD or were previously determined to be seropositive but must be maintained at the Facility for research purposes, shall be considered URTD-exposed including all penmates. Some of these tortoises are known to have URTD based on visual signs and shall be either handled with gloves or all other tortoises shall be handled previous to URTD tortoises. Otherwise, handling procedures for tortoises in this group which have not shown signs of URTD, may be handled without gloves. However, personnel shall wear one pair of sterile gloves and an apron (disposable or laundered after use) while handling tortoises in this group if they anticipate

handling any tortoise outside this group. In this case, the gloves shall be disposed of in a designated receptacle and apron disposed of likewise or laundered upon completion of tortoise handling activities.

Upon completion of research involving these tortoises, the tortoises shall be evaluated by ELISA. Only tortoises that are seronegative will be considered for release. Subsequently, these tortoises will be handled in accordance with I.C. if seropositive and I.D. if seronegative.

C. Seropositive for URTD, Suspect, and Other Unhealthy Tortoises

This group consists of seropositive tortoises and tortoises with uncertain or borderline antibody titers as determined by ELISA (suspect) or shown signs of other diseases. Sanitary gloves and aprons shall be worn while handling these tortoises to prevent the spread of disease to healthy tortoises. The apron should be disposable but if not, it shall be laundered after handling these tortoises. One set of protective clothing (sanitary gloves, apron) may be used to handle all seropositive animals without changing between individual tortoises. Seropositive tortoises being transferred for euthanasia will be placed into a cardboard box which does not need to be sanitary. All cardboard boxes shall be disposed of following use.

D. Seronegative for URTD (Presumed Healthy) Tortoises

This group consists of tortoises which have not been exposed to *M. agassizii* as determined by ELISA (seronegative). All seronegative tortoises shall be handled with a new pair of sanitary gloves per animal and placed in a sanitary container (box or tote) or pen. Only sanitary equipment and utensils will come in contact with these tortoises. These tortoises shall be placed individually in pens separated from seropositive, suspect, or unknown tortoises (non-seronegative tortoises) by a minimum of ten feet. If available pen space is limited at the Facility, multiple seronegative tortoises may be housed in a single pen, upon authorization by the Service by telephone or in writing.

After the processing of ELISA results and before tortoises are placed into pens, each tortoise with a carapace length greater than 80 millimeters will receive AVID[®] integrated transponders. The implanting process requires the use of sanitary gloves and sanitary/sterile transponder injection needles, transponder injection syringes, forceps, aprons, AVID[®] transponders, and an AVID[®] transponder reader. Prior to use upon each tortoise utensils will be sanitized as described under section IV. of this document. Under no circumstances should seronegative, suspect, or unknown tortoises be housed in pens adjacent (i.e, physically connected) to pens containing seropositive tortoises.

II. Attaching External Accession Number Tags

Each incoming tortoise to the Facility will receive an accession number. Only those tortoises without visual signs of URTD will have the accession number affixed to the carapace.

The process of external tagging requires attachment of a numbered tag to the carapace using a clear-drying water-proof epoxy applied with sanitary wooden or plastic toothpicks. Each tortoise will either be left in its plastic tote or set on a sanitary surface (as described in section IV. of Appendix A) for tagging. Technicians will wear sanitary gloves and use a new toothpick and individualized number tag for each tortoise.

III. Feeding & Monitoring

Tortoises are fed in accordance with research study protocol or once to twice per week during the active tortoise season (between March 1 and October 31). Non-research tortoises will not be handled during feeding without the use of sanitary gloves. Unless tortoises are fed and watered in accordance with research protocol, tortoises will be fed a 20-percent protein pellet diet developed by Smithsonian Institute and Bermuda grass. Dried alfalfa may be used as a supplement if deemed necessary. Pens will be checked during the hibernation period and at other times when precipitation conditions exist which could cause severe flooding of burrows. Water dishes will be filled in conjunction with the activation of the sprinkler system for the Bermuda sod in each pen. During feedings, tortoises are monitored for visual changes in behavior and health status. Tortoises which are not feeding during the tortoise active season and appear to be lethargic or losing weight will be examined by a veterinarian.

Tortoises shall be weighed and measured at least twice per year- once after the tortoise emerges from hibernation (March-May) and again prior to hibernation in the fall (September- October). Each tortoise will be visually observed during the weighing and

measuring period to determine overall condition. Information such as feeding habits, general tortoise health, visual signs of URTD, and necessary pen maintenance is also recorded.

Unless part of a research group, tortoises exhibiting visual signs of URTD will be removed from the pen within 24 hours. If seropositive tortoises must be maintained for research, technicians will designate the pen as “URTD Exposed” and note this designation on the feeding and monitoring sheets. Before traveling to any other pen, technicians will sanitize their footwear and any items which may have come in contact with non-seronegative tortoises or items within their pen (see section IV. of Appendix A).

IV. Collecting/returning tortoises from/to pens

When tortoises are pulled from burrows with the tortoise extraction tool (TET), the TET will be sanitized between uses on individual tortoises. Prior to placing the tortoise in the container, the technician will examine the tortoise for visual signs of URTD and will verify the tortoise accession number. The technician will write, in either black marker (for cardboard boxes) or dry erase marker (for totes), the tortoise's accession number on the container, then place the tortoise in its container. Cardboard boxes containing tortoises will have a layer of paper placed on the bottom and will be sealed with packaging tape to prevent tortoise fecal matter and urine from leaking from the boxes and to prevent tortoises from escaping from their boxes. While collecting tortoises, technicians will properly sanitize footwear if traveling between pens with tortoises of different status (e.g., seropositive to seronegative). After collection, tortoises will be kept in a temperature controlled climate until successfully transferred or returned to pens.

Utensils will be sanitized between placement of each tortoise. Unsanitary totes will be stacked together in a "to be sanitized" pile until such time as they can be sanitized.

Appendix A. Movement and Holding of Tortoises, Invasive Techniques, and Sanitation

I. Transfers to/from Facility

A. Clark County Pick-Up Service

The Clark County Desert Tortoise Hotline and pick up service provides the means of disposition for tortoises displaced by development or found in harm's way. Upon pick up, tortoises will be placed in sanitary cardboard boxes or plastic totes. The container will then be labeled with the name, address, and phone number of the person who found the tortoise and called the pick up service. The label will also include the dates and times of the call and pick up and whether or not the tortoise is showing visual signs of URTD. Tortoises collected under section 7 or 10 will be delivered in sanitary cardboard boxes to the entity contracted to operate the Facility. Each box will be sealed with packaging tape and labeled with the site name from which the tortoise was removed and biological opinion number, if applicable. The label will also include the date and time of removal as well as the finder's name and whether or not the tortoise has visual signs of URTD.

Tortoise showing visual signs of URTD will be given an accession number, measured, euthanized, and disposed of as directed by the Service. Until it is euthanized, the tortoise, will be kept in its container and physically separated from other tortoises.

Tortoises not showing signs of URTD will be processed. Processing a desert tortoise consists of the following: Tortoises will be given an accession number, measured, and tested for exposure to URTD. After being measured,

tortoises in cardboard boxes will be placed in sanitary plastic totes and labeled for testing for exposure to URTD (ELISA). They will be held in the boxes or totes until ELISA results have returned. Seropositive tortoises will be euthanized. If borderline or suspect after being active for a minimum of 8 weeks they shall be euthanized; if during the inactive season, the tortoise shall be held for a second ELISA. At no time during the pick up process will any individual tortoise come into contact with any other individual tortoise. If seronegative, the tortoise shall be given an AVID[®] integrated transponder.

B. Transfers from Facility

All boxes containing seropositive tortoises will be marked with the tortoise accession number and the label "URTD+" to prevent confusion of healthy tortoises with URTD positive tortoises. Tortoises will be kept in a climate controlled location until the transfer is complete. Tortoises being euthanized for URTD will be physically separated from other animals being transferred. This measure will prevent the accidental transfer of fecal matter or urine between boxes containing seropositive tortoises and boxes containing healthy tortoises.

II. Holding of Tortoises

A. Exposure to Other Tortoises

Tortoises can have a negative ELISA test and be exposed to URTD if they are not an incoming tortoise and have been in a pen with a tortoise which has either had a positive ELISA test or shown visual signs of URTD. When multiple tortoises are picked up at one location, via the pick up service, those tortoises are considered as exposed to one another. If any of those tortoises shows visual signs of URTD or has a positive ELISA result, the other tortoises will be labeled "URTD

exposed." Any individual tortoise which has been ELISA tested and has been exposed to URTD will not come into contact with any other individual tortoise.

Tortoises with one negative ELISA result, and not URTD exposed will not be exposed, under any circumstances, to non-tested tortoises, seropositive, or twice tested tortoises.

B. Holding and Placement of Tortoises in Pens

- 1) **Incoming.** Prior to being ELISA tested any individual tortoise will not come into contact with any other tortoise through either direct contact or latent contact of unsanitary handling (e.g., placed on contaminated table). Tortoises coming into the Facility will be completely quarantined until the time when they can be ELISA tested.

- 2) **Tortoises tested for exposure to URTD during the active season and have a negative result** will be placed in pens having appropriate size burrows. Each tortoise will be placed in a pen containing no other tortoises unless there are no sanitary empty pens available with burrows of the necessary size. If available pen space is limited at the Facility, multiple seronegative tortoises may be housed in a single pen, upon authorization by the Service. Tortoises being placed with other tortoises (same designation only) will only be placed in the following sex ratios and order of preference: Female to female, female to unknown, juvenile to juvenile, unknown to unknown, or male to female. This measure will prevent fighting and injury among male tortoises and minimize reproduction.

3) **Tortoises tested for exposure to URTD during the inactive season and have a negative or suspect result** will be completely quarantined, preferably in totes, until a second negative ELISA result is obtained, a minimum of 8 weeks following the first ELISA. These tortoises must be kept warm (above 75 degrees Fahrenheit) to be considered active and eligible for the second ELISA. In the case of juvenile and hatchling tortoises which are kept several per pen, those URTD exposed tortoises which were together in one pen prior to ELISA may remain in the same pen together until the time of their second ELISA.

III. Bleeding for ELISA

The bleeding process requires the use of sterile butterfly needles, syringes, heparin tubes, and Sarstedt tubes; and sanitary gloves, aprons, utensils, and equipment. Tortoises collected from their pens shall be stored in individual sanitary containers in a climate controlled room for the duration of the bleeding and result processing. When traveling from one pen to the next, each team of biologists will carry a sheet containing information regarding the status of each pen (e.g., sanitized, URTD exposed, etc.).

Each tortoise will be kept separately and will not come into contact with any other tortoise during this time. After ELISA results are received and processed, tortoises with negative results will be returned to the pens from which they came or to other sanitary pens or containers. Seropositive tortoises will be collected, euthanized, and disposed of as directed by the Service. Pens which have held seropositive tortoises will be sanitized prior to re-use.

IV. Sanitation

Throughout this protocol, the term “sanitary” is used to mean: “Incapable of transmitting disease”. The following lettered sections outline the manner in which items need to be either cleaned or covered to make them sanitary. Novalsan[®] (chlorohexiderm) is a recommended bactericidal solution and should be the solution of choice for sanitizing equipment and utensils.

A. Equipment

1. **Calipers** will be sanitized by wiping the edges and tips of the calipers with solution. Anywhere the calipers may touch a tortoise will be disinfected thoroughly with a bactericidal wipe.
2. **Scales, lab table or any other surface** upon which a tortoise has been placed or has touched will be sanitized by wiping the surfaces and edges with bactericidal solution. Any surfaces which may come into contact with a tortoise will be disinfected thoroughly with a bactericidal wipe or covered with a sanitary, disposable plastic covering.
3. **Sanitary aprons** will be worn over the technician's clothes to prevent the contact of a tortoise with a technician's clothes during the handling of a tortoise. Aprons are specifically necessary during the bleeding and AVID[®] integrated transponder implanting processes. Aprons will consist of a sanitary disposable apron or sanitary and previously unused plastic sheet to be placed over the lap and/or chest of the technician. This sheet will be discarded after contact with each tortoise.
4. **Totes.** Unsanitary or "used" plastic totes will be kept in a stack noted "to be sanitized." Plastic totes will be sanitized in a three step process. First,

each tote will be rinsed with water and, when necessary, scrubbed with a scouring pad. This step removes any urine or fecal matter from each tote. Second, each tote's interior and exterior will be thoroughly sprayed with a 1:10 or greater concentration of bleach and water or bactericidal solution to ensure that the entire surface of the totes is in contact with the solution for a minimum of 20 minutes or in accordance with product instructions. Third, each tote will be rinsed with water and set in the sun to dry. After appropriate drying time, the totes will be stacked and placed in areas designated for clean totes. Totes not found in designated "clean tote" areas will be considered unsanitary and be placed in the "to be sanitized" stack.

5. **Tortoise Extraction Tool** is a long pole of wood, aluminum or plastic with a padded, blunt-ended curved metal rod attached to one end. They are used in cases where it is necessary to remove a tortoise from a burrow and arm's length does not allow the technician to reach the tortoise. Between uses each tortoise extraction tool must be sanitized in the following manner. The rod and two feet of the pole beginning with the hook and moving downward will be sprayed with 1:10 or greater concentration of bleach and water or bactericidal solution and allowed to air dry without rinsing.
6. **Water bowls** will be sanitized by first rinsing and scrubbing out any dirt, algae or fecal matter. After rinsing each bowl will be sprayed with a bactericidal solution or 1:10 or greater concentration of bleach and water. After spraying each water bowl, they will be rinsed again.
7. **Cardboard boxes.** A sanitary cardboard box is any cardboard box not previously used for biological storage. Any box which has previously held animals, including but not exclusive to desert tortoises, is not sanitary

and will not be used to contain desert tortoises when a sanitary container is required.

8. **AVID[®] integrated transponders** are considered sanitary when they are stored within a container filled with bactericidal solution and touched by only sanitary items.
9. **AVID[®] integrated transponder reader** can be sanitized by wiping the flat active reader and the handle with bactericidal solution.

B. Medical Supplies

1. **Butterfly needles**, used during bleeding, are sanitary when they are stored in their original packaging and have not been previously used or exposed to any other blood, plasma or tortoises. Each butterfly needle is disposed of after a single use.
2. **Heparin and Sarstedt tubes**, used to hold blood or plasma, are sanitary when they are stored with their original seal intact, and have not been previously used or exposed to other blood or plasma. Tubes shall be stored with original seal intact. Tubes are used to hold the blood or plasma of a single tortoise and are discarded when the blood is either transferred to another container or no longer needed.

3. **Syringes**, used to extract blood from tortoises, are sanitary when they have not previously held or been exposed to other blood or plasma. Syringes are disposed of after being exposed to any blood or plasma.
4. **AVID[®] transponder injection syringes and needles** may be used multiple times on multiple tortoises. Each syringe and needle must be stored in a container submerged in 70% ethanol or bactericidal solution and must be submerged in one of these solutions between uses.
5. **Forceps** must be rinsed in and wiped with a bactericidal solution to be considered sanitized.

C. Pens

An empty pen which has contained a tortoise exposed to URTD or tortoises euthanized for URTD must be sanitized before any other tortoise is assigned to that pen. Once pens undergo the appropriate protocol, the pens shall be cleaned at least twice per year, preferably in the fall prior to hibernation and mid-summer. Pens are sanitized as follows:

1. **Burrows.** Natural burrows will be collapsed. Burrows made by technicians will be unearthed and the PVC pipe will be laid with the open side up and exposed to the sun. Each burrow will then be sprayed with a 1:10 or greater concentration of bleach and water. The earth surrounding and within the area of the burrow will also be sprayed with the bleach solution. Burrows will be left exposed to direct sunlight for a minimum of 3 days before being reassembled and eligible for tortoise placement. If rain occurs or the burrow gets wet, the burrow shall be exposed to direct

sunlight for a minimum of 3 days after drying out.

2. **Alfalfa and grass.** All alfalfa will be removed from pens and disposed of in trash receptacles during the pen sanitation process. Bermuda grass will be mowed, if necessary, to allow sunlight to penetrate to the soil surface. Grass should be mowed when the burrows are opened and exposed. The sprinkler system for the pen shall be turned off in order to dry out the area and minimize moisture necessary for survival of pathogenic organisms.
3. **Scat.** During the pen sanitation process, technicians will thoroughly examine the pen for tortoise scat. All tortoise scat found will be collected and disposed of in trash receptacles.

D. Person

1. **Hands.** Throughout this document the term "sanitary hands" refers to hands covered by unused latex exam gloves. When a technician or biologist touches any tortoise or other animal while wearing sanitary gloves, those gloves are no longer sanitary and must be disposed of prior to the handling of any other biological material. When gloves tear during use, the biologist or technician whose gloves have torn will thoroughly wash his/her hands with anti-bacterial soap then apply a new pair of sanitary gloves before handling any tortoise.
2. **Footwear.** Sanitizing footwear consists of spraying the bottom of the footwear with a bactericidal solution or a 1:10 or greater concentration of bleach and water.