Designation: E3155 − 19

Standard Guide for Assessing Mammal Health at Chemically Contaminated Terrestrial Sites Using Rodent Sperm Analysis[1]

This standard is issued under the fixed designation E3155; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Contaminated terrestrial properties provide opportunities for determining whether or not toxicological responses of concern have arisen in ecological receptors contacting them. The guide, employing direct health status assessment of rodents captured at contaminated sites, can furnish information to support such determinations, thereby providing a greater degree of realism in health assessments for resident mammals than that offered in generic desktop assessments. This guide's direct health status assessment design involves only wild-type animals in their natural state, with this arrangement circumventing a commonplace species extrapolation element, one that introduces a considerable degree of uncertainty in ecological risk assessments (ERAs). Of note, the guide is not itself a terrestrial ERA method, but rather an additional tool to inform such efforts. In the area of notating significant effects, the guide's biological thresholds-for-effect replace arbitrarily or negotiated differences, or both in response that are only assumed to be biologically meaningful (for example, a 20 % decrement in a measure), and that typically are not confirmed in field studies (**1**).[2] Due to the availability of the thresholds-for-effect, the guide allows for bright-line determinations to be made, creating an opportunity to supply a high level of conservatism to outcomes (see 9.4). Importantly, Rodent Sperm Analysis (RSA) is distinctly different from others in concept and practice because it aims to make yes/no determinations (that is, that reproductive impact has occurred or that it has not) as opposed to generating estimates of the likelihood of certain toxicological outcomes arising in the future. RSA provides a useful line of evidence for ERA, wherein mammal reproduction is a common endpoint, and it may simplify remedial decision-making for contaminated terrestrial sites.

This guide, notably removed from testing with laboratory-reared, commercially available, and chemically naive organisms that are subjected to brief chemical exposures in controlled environments, is predicated on a fundamental underlying premise consistent with the field condition considered (**2, 3**). It is recognized that sufficient time has elapsed at contaminated sites for toxicological effects of concern to have been elicited. Given that typical sites are minimally 30 years old by the time they submit to ERA (**2-8**), the described standard understands that if critical effects (here, reproductive impacts) have not appeared over such a time course, they are unlikely to ever occur. Addressing mammals, one of only two tetrapod classes evaluated in ERA (Class Aves is the other), the guide has three unique features that set it apart from conventional ecological assessment tasks. First, the procedure directly assesses health by evaluating the actual animals that inhabit contaminated sites. (Of note, common to RSA and the desktop-based risk characterizations of many conventional ERAs, is the defining of overall mammal health through the key biological function of reproduction.) Second, the procedure considers the three chemical uptake routes of ingestion, inhalation, and dermal contact, whereas conventional ERAs only report hazards for the first of these. The procedure's third unique feature is its ability to evaluate the effects of chemical mixtures, whereas the conventional hazard quotient (HQ)-reliant ERA process can only review chemicals singly. The procedure is supported by the existence of established sperm-based barometers of reproductive capability/success for the rodent grouping. In contradistinction, for most other current biological measures that can be collected in the laboratory or field (for example, an enzyme level, size of an internal organ), it is not known how much of a contaminated site-mediated sublethal change signifies health compromise (**3, 9**).

1. Scope

1.1 This guide describes the procedures for obtaining and interpreting data associated with a direct health status assessment for mammalian receptors at chemically contaminated terrestrial sites where ERA work is either scheduled or ongoing, and irrespective of the number and type of chemicals that may be present. Through reviewing sperm features, the RSA method reports on the reproductive health of male rodents in their natural environmental settings, with these animals serving as surrogates for other (and larger) site mammals (**4**).

1.2 These procedures are applicable at any terrestrial property that supports small mammals (for example, mice, voles, rats, squirrels) and has contaminated soil. Importantly, chemicals of concern in site soils need not be spermatoxins. Additionally, the RSA method considers that any combination of chemicals or other site stressors might collectively act to compromise reproduction, held to be a sensitive toxicological endpoint for mammals. The anticipated primary application of the method will be at historically contaminated sites (such as Superfund sites). The procedures describe tasks conducted in the field and in a laboratory. For the latter, tasks may be conducted either in an on-site mobile laboratory, or in a more conventional laboratory setting. For certain tasks, a make-shift work space may be suitable as well (see 7.3).

1.3 Initial determinations of compromised or non-compromised reproduction in resident male small rodents are made through a cautious comparative review of sperm parameters. Briefly, for the rodents of a given species collected at both a contaminated site and a habitat-matched (non-contaminated) reference location, arithmetic means are first computed for each of the three sperm parameters of count, motility, and morphology. If one or more of the parameter means of the contaminated site rodents reflect an unfavorable shift (that is, count or motility is less than that of reference location animals; the percentage of abnormally-shaped sperm is greater relative to reference location animals), the percent decrease or increase in each mean is compared to the relevant established sperm parameter benchmark, each in the form of that degree of shift in an unfavorable direction that signifies lesser reproductive success (**2**) (see 9.3).

1.4 Advanced determinations of compromised or non-compromised reproduction in larger site-contacting mammals, the true focus of the RSA method and this guide, are made through an applied spatial movements-based extrapolation scheme. Where established sperm parameter benchmark exceedances are not observed in contaminated-site rodents, other mammals contacting a site are also assumed to have non-compromised reproduction. This follows from the latter all having notably lesser degrees of site exposure due to home ranges that are vastly larger than those of rodents. By way of example, with respective home ranges of 400+ and 640 acres for the red fox and white-tailed deer (**10-14**), these species would spend minimal amounts of their time (for example, 5 %) at prototypical contaminated sites that cover areas of 25 acres or less (**15, 16**). Where one or more sperm parameter benchmarks are exceeded in contaminated-site rodents (certainly indicating that the rodents are reproductively compromised), other site mammals may also be reproductively compromised. The greater the disparity between the home ranges of the target species (that is, the site rodent) and any of the other mammals known to contact the contaminated site in question, the less likely it will be that the latter are reproductively compromised. The RSA method employs the same toxicological extrapolation principles as that used for mammals in conventional desktop-based ERAs. In those ERAs, stressor-mediated responses of rodents (of a laboratory-based study) assist with the interpretation of health effects for an expanded list of mammals that cannot conveniently be evaluated directly for health status (for example, fox, skunk, raccoon, deer, coyote, etc.).

1.5 This guide is arranged as follows:

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1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E1527 Practice for Environmental Site Assessments: Phase I Environmental Site Assessment Process

E1689 Guide for Developing Conceptual Site Models for Contaminated Sites

E1848 Guide for Selecting and Using Ecological Endpoints for Contaminated Sites

E2081 Guide for Risk-Based Corrective Action

E2205 Guide for Risk-Based Corrective Action for Protection of Ecological Resources

E2616 Guide for Remedy Selection Integrating Risk-Based Corrective Action and Non-Risk Considerations

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[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate.

Current edition approved Jan. 1, 2019. Published February 2019. DOI: 10.1520/E3155–19

[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *cauda epididymis, n*—that portion of the male reproductive anatomy in mammals where sperm are stored.

3.1.2 *sperm count, n*—the number of sperm cells in a standard preparation expressed as millions of sperm per gram of cauda epididymis.

3.1.3 *sperm morphology, n—for the purposes of assessing male reproductive health*, the measure of broken, bent, and other damaged sperm cells in a standard count of 200 cells, expressed as a percent.

3.1.4 *sperm motility, n*—the percentage of moving sperm in a properly prepared sample, also known as the "total motile measure".

3.1.5 *target animals, n*—adult male rodents of a species found at both a contaminated terrestrial site of interest, and a nearby, habitat-matched reference location.

4. Summary of Guide

4.1 RSA is a direct health status assessment method for select mammals potentially occurring at contaminated terrestrial sites, that is, it assesses the reproductive health (and thereby, overall health) of animals whose habits do not allow for their moving beyond their site boundaries. It evaluates those mammals that are maximally exposed in the sense of having the greatest degree of direct soil contact. By way of example, many species of small rodents have home ranges of one acre or less (**12**). While some might consider mammals bearing relatively higher chemical body burdens than others to be those who are maximally exposed, there are numerous reasons to not apply such reasoning with regard to this guide. Briefly, body burden information is almost never collected for mammals other than rodents, and there are no databases that relate body burdens in mammals to health effects they might experience. Importantly, there is no way to relate body burden to the sperm parameter thresholds-for-effect that the RSA method utilizes, which unlike other somatic measures, allow for assessment of reproductive system health. Finally, due to the notably reduced densities of larger mammals [for example, red fox at 0.019 - 0.03 animals/acre; (**10-13**)], these species may not be spatially relevant in an ERA context altogether (that is, at best, only individuals as opposed to populations might bear chemical effects, and ERAs are to assess the latter). RSA also assesses reproduction as a toxicological endpoint of great concern in ERA, one which is potentially the most sensitive of endpoints for mammals. RSA capitalizes on the availability of an information type that hardly exists in the toxicological testing realm, namely proven biological thresholds-for-effect, and specifically sperm parameter-based thresholds that are all barometers of reproductive success (**2, 9**) (see 9.3.1). For virtually any biological datum that can be collected (for example, a decrease in an enzyme level), second-order toxicology information is lacking (**2, 8**). Thus, where sublethal effects are an interest, it is not known how much of a somatic change in chemically exposed/treated animals signifies compromised health. In stark contrast to this arrangement, for the three classic sperm parameters monitored in laboratory animal trials (just as they are in human fertility clinics), it is rather precisely known how much of a change (for example, reduction in sperm count) signifies a compromised reproductive state. This guide describes *(a)* necessary planning steps for employing the RSA method, *(b)* details for conducting the method, involving small rodent trapping, recording of field data, and the recording of sperm parameters, and *(c)* analysis and interpretation of the collected data. RSA parallels conventional desktop ecological assessments, in that overall health is defined through a review of reproductive capability. If reproduction is not compromised, any chemical exposure-posed somatic changes that might be occurring (for example, an enlarged internal organ), are deemed inconsequential, for they are not impeding a species from surviving and perpetuating its own (**2, 3**). The RSA method provides a useful line of evidence to assist reproductive capability-based health determinations for terrestrial mammals evaluated in ERAs for contaminated sites.

5. Significance and Use

5.1 The RSA method provides risk and resource managers with an enhanced understanding of the ecological health concerns at the sites they oversee because unlike conventional terrestrial ERAs, actual site mammals are the ones evaluated. Additionally, the HQs of desktop efforts report only on the contaminant exposure route of ingestion, and can only evaluate chemicals singly, whereas RSA findings reflect all three exposure routes as well as the combined effects of multiple chemicals on a highly valued endpoint. Critically, the RSA method incorporates site history considerations that necessarily influence the phenomenon of biological response. If reproductive impacts at contaminated sites were ever to be elicited, such would be apparent today because evaluated sites have, at a minimum, continuously exposed their ecological receptors to chemicals for multiple decades during which time tens and often more than one hundred generations have passed (**5**).

5.2 Application of the subject guide familiarizes remedial decision-makers and risk managers with two concepts. First, rather than attempting to predict health effects arising in site receptors, there may be more value in documenting demonstrated health effects, should such exist in actual site-exposed mammalian receptors. Second, the possibility exists that site receptors never experienced stress or impact over the years since a site first became contaminated.

5.3 Application of the subject guide can allow for substantial cost savings. Often, the outcomes of HQ-based assessments are summarily relied upon to conduct ongoing studies, monitor sites, or implement site cleanups, all of which may be unnecessary. Where RSA applications should demonstrate that maximally site-exposed mammalian receptors (as defined in section 4.1) are not experiencing compromise with regard to the sensitive endpoint of reproductive success, it can become apparent that soil remediation efforts on behalf of mammals are not needed.

5.4 The described RSA method can typically be applied at that point in the ERA process where HQs for one or more mammalian species are found to be greater than 1.0, as in the process's Step 2 (Screening-Level Exposure Estimate and Risk Calculation; where ecological threats are evaluated in a general, as opposed to a specific fashion). Alternatively and particularly at sites that are not governed as rigidly as, for example, Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA; aka Superfund-type) sites, the guide can be applied once it is established that a site has a chemical contamination footprint of interest (that is, that soil concentrations are high enough to potentially be harmful to mammalian site receptors). In light of the propensity for preliminary and refined HQs to suggest mammals are ingesting unhealthful doses of site contaminants, in turn commonly leading to advancing to the field for a verification effort, the application of RSA as a first evaluative effort is intended to be a time- and cost-saving effort.

5.5 The significance of this guide is the method design that reflects an understanding of certain unavoidable ERA process constraints, specifically in the areas of field mammal collection and subsequent tissue analysis. First, the RSA method recognizes that small rodents are the only mammals that can be routinely culled from the field (that is, to be removed and not returned), and further, that this reality is unlikely to ever change. Efforts to regularly harvest larger mammals (for example, fox) may be challenged by local governing agencies and animal care institutions. Additionally, acquiring a sufficiency of larger mammals is time-consuming and labor-intensive, owing to relatively miniscule animal densities. Further, many larger mammals (for example, long-tailed weasel, badger) are not found in all habitats or in all states. In contrast, small rodents occur in virtually every habitat, are relatively easy to collect, and are numerous enough to allow for defensible comparisons between or among sites. In selecting the maximally exposed small rodent to work with (that is, an animal confined to contaminated surroundings throughout its life due to a home range that is almost always of one acre or less), the RSA method features a common basis of comparison (and certainly wherever it should be applied in the United States).

5.6 RSA theory understands that, generally at contaminated terrestrial sites, there is worry that receptors-of-concern might be reproductively compromised. The focus on reproduction as the dominant toxicological endpoint of concern (**6, 7**), recognizes that much method development for reproductive effects in rodents (in support of human health) has occurred (**9, 17**). That reproduction bears this status is evident in the hierarchy of preferred toxicity reference values (TRVs) that ecological risk assessors often select in support of HQ computation. Additional recognition is given to the reality that standardized means for effectively assessing other endpoints of interest in field-collected organisms, such as neurotoxicity or behavior, do not exist. Where established sperm parameter benchmark exceedances are not observed in contaminated site rodents, such can constitute a significant line of evidence in support of a determination that reproduction is proceeding adequately. The RSA method recognizes that impairments to other biological functions (for example, behavior, nerve impulse transmission) of contaminated-site rodents may be occurring despite reproduction proceeding normally (**2, 3**). Where such is the case, the method's supporting theory understands that other endpoints being reached do not necessarily pose a concern for they have not impeded the ability of maximally exposed rodents to survive to the age of reproduction, find mates, and produce viable young (**2, 18**).

5.7 This guide recognizes that an analagous reproductive assessment approach for female rodents, is not available at the present time. Importantly, an absent reproductive assessment approach for females does not constitute a shortcoming of the subject guide. Relevant U.S. EPA guidance, for example, supports evaluating one sex of a species where drug and chemical regulation is concerned, and drawing conclusions based on such information (**19**). In this context several noteworthy points follow. First, over 98 % of all mammalian toxicity studies considered in crafting the U.S. EPA's Soil Screening Levels (SSLs) for ERA (for some 17 inorganic and 4 organic chemical species) are of the single-sex type, with 35 % of the studies being male-only (**20**). Additionally, for 37 % of the universe of chemicals with SSLs, the number of male-only toxicity studies exceeds the number of female-only toxicity studies. Finally, a significant percentage of the most commonly applied toxicological benchmarks for wildlife (**21**) derive from single-sex studies. Critically, with its focus on directly assessing reproduction in male rodents, RSA is notably far less destructive than would be a method involving the culling of female rodents from the field, given that the latter are the ones that bear the young.

5.8 This guide recognizes the value in employing the wild rodent in field-based mammalian receptor assessment. Aside from the reality that rodents may constitute the only mammals that can regularly be culled from sites (discussed above), there are key advantages that accrue to working with these animals. Small rodents occur in nearly all terrestrial habitats, allowing the guide to be broadly applicable in a geographical sense. A second advantage is that the small rodent with perhaps no exception, will likely be the maximally-exposed mammal in terrestrial settings, this again, in terms of having direct contact with contaminated soils. This follows from rodents being non-migratory in nature, having extremely limited home ranges that effectively contain them at contaminated sites, and their spending nearly all of their time directly contacting the ground (that is, contaminated soils; **2, 4, 18**).

5.9 In providing a useful line of evidence in support of ERAs for mammals, this guide employs a straightforward extrapolation approach (**2, 18**), one that is isomorphic to that applied in conventional HQ-based assessments. If site rodents, that have more constant and intimate contact with affected site soils than that of any other site mammal, are not found to have compromised reproduction, larger and wider-ranging mammals, with their considerably lesser degrees of site (that is, contaminated soil) contact, should also be free of reproductive compromise. An appreciation for this extrapolation scheme derives from a review of the principal extrapolation scheme of conventionally-applied desktop-based ERAs. There, a laboratory-based mouse or rat study is routinely used to determine if another mammal (for example, deer, fox, rabbit) is ingesting an unhealthful quantity of a given chemical. With the conventional ERA scheme, there are numerous differences to acknowledge, and even at the level of the rodent. Thus the test animal and the wild form inhabiting the site of interest that is to be assessed, do not match in terms of species, rearing, environment/habitat, or feeding design, and these differences weaken conclusions that can be drawn. In contrast, the subject standard in its initial extrapolation, compares sperm measures, each a proven barometer of reproductive success (**22-25**), in populations of conspecifics living less than a kilometer apart, with one population inhabiting a soil-contaminated area, and the other a contaminant-free one. The RSA method recognizes that small rodents of contaminated sites are integrators of potentially imposing environmental stressors that extend beyond chemicals that may be present in soil and diet items, to include such things as physical habitat disturbances (for example, noise or land vibration). RSA understands that conventional ecological assessments necessarily strive to know of small rodent reproductive capability, as this grouping is held to be a keystone ecosystem element. Where reproductive compromise is not observed in an RSA outcome, there is demonstration that a site's exhaustive list of site stressors, in the actual arrays in which they occur, are not impinging on what is generally held to be the most important toxicological endpoint.

5.10 One limitation of this guide is that the biologically-significant thresholds-for- (reproductive)-effect that are applied, are laboratory-derived. A second limitation of this guide is that shrews generally cannot submit to the RSA method, owing to their exceedingly high metabolism that interferes with their being live-trapped in the field. In the rare case where the only rodents present at a contaminated site of concern should be shrews, the RSA method can probably not be successfully applied. If for any reason a given contaminated site does not offer a small rodent population altogether, or if there is not at least one common small rodent species occurring at both the site of interest and a suitable habitat-matched reference location, or an appropriate reference location cannot be found (see 8.1), the method is not applicable. RSA is intended only to identify if site mammals are reproductively compromised. The method does not concern itself with identifying the chemical(s) or physical site stressors responsible for observed sperm parameter threshold-for-effect exceedances, or the determination of cleanup levels, and such are not method limitations. The situation is analogous to standardized whole effluent toxicity tests conducted with various aquatic test species (for example, *Fundulus sp.*). There, the objective is only to ascertain if the degree of wastewater treatment is adequate to support the aquatic life inhabiting a receiving waterbody's mixing zone. (Standard whole effluent toxicity testing is not designed in the main, to identify the constituent or constituents in effluent that may be responsible for unacceptable test outcomes.)

5.11 This guide is consistent with ERA guidance and guidelines (**26, 27**), where advancing to the field for an environmentally relevant assessment of the health of site receptors (so-called 'field verification') is a recognized formal step. In understanding that sufficient time has elapsed at contaminated sites for reproductive compromise to be evident (if that endpoint was ever to be triggered), this guide is designed to document such demonstrated compromise. Critically, RSA is not a risk assessment method that aims to forecast or predict health effects arising in mammals with ongoing contaminant exposures. The guide then is related to, but distinctly different from other ASTM standards that bear on the toxicological effects prediction aspect of ERA (Guides E1527-13, E1689, E1848-96, E2081, E2205-02, E2616, and E2790). The guide is also consistent with guidelines for reproductive toxicity risk assessment as per the U.S. EPA (**19**). Specifically, assessing the reproductive health of only one sex of a species is deemed adequate for an overall species assessment (**17**). In one key area however, this guide is quite unlike conventional ERAs that are largely restricted to the level of desktop analysis. Whereas conventional assessments rely on either statistically-significant differences in outcome, or on a commonly negotiated difference in biological response (for example, 20 %) when drawing conclusions, this guide primarily avails itself to the utility of a series of established biologically-significant thresholds alluded to previously (**22-25**). Further, a statistical comparison need only be applied for one of two possible RSA outcomes (see 9.3.1 and 9.4).

6. Safety Precautions

6.1 The most serious potential danger to individuals involved with RSA duties is that of becoming infected with rodent-borne disease (for example, Hantavirus or plague). Many rodents in the wild, even at locations far removed from areas where certain rodent-borne diseases are known or expected to occur, may carry the virus. With multiple opportunities to become infected through handling rodents (for example, where field and laboratory personnel have breaks in the skin on the hands), certain safety procedures must be implemented. Importantly, the specter of the presence of infected rodents in the field where RSA is being applied, does not make the implementation of the safety procedures provided below any more burdensome. All individuals checking live animal traps for captures must wear gloves, and must hold the traps down-wind and at arm's length. Traps with adult male rodents to be assessed, must be conveyed to the laboratory by truck (and not by car), with the traps in a separate compartment from the driver and any other passengers. Individuals euthanizing rodents and doing dissections must wear surgical gloves and conduct all such work under a fume hood. Alternatively, a powered air-purifying respirator (PAPR) unit may be worn when conducting such activities.

6.2 Animal carcasses and remains, other than those that are to be preserved for species identification, must be disposed of in red plastic biomedical waste bags at a facility licensed to handle such waste. Animals to be identified to species should be preserved in accordance with the stipulations of the agency or institution overseeing the RSA application (for example, placing in formalin) until they are turned over to the institution where a mammalogist will examine the specimens.

6.3 Disposable scalpel blades must be thrown away in a biomedical waste/sharps container. Surgical tools (forceps, scissors) must be cleaned with alconox or a similar biocide after each use.

7. Apparatus

7.1 *Sperm Analyzer Equipment*—The essential apparatus needed for RSA is a sperm analyzer (**22**). A fully automated sperm analyzer, although not absolutely essential, is highly recommended given the accuracy in reporting that it can provide (following for example, from its ability to discriminate between non-moving or dead sperm and other debris that may appear on a prepared slide) and its rapidity of reporting. An automated sperm analyzer is essential however, where sperm motility is to be monitored. Such equipment has the capability to accurately record the percentage of moving sperm in a microscope slide prepared within 2-3 minutes post-euthanization. A much-recommended but not absolutely required sperm analyzer feature where sperm motion is to be assessed, is an adjustable, heated microscope stage. Ideally this stage is set to 37-38 °C, matching both the temperature at which sperm are maintained in the rodent body, and that of a Petri dish "swim-out" preparation described below (see Section 8, Procedure).

7.2 *Sample Preparation*—A tissue homogenizer is necessary for any preparation made from the cauda epididymis to be used in determining the sperm count, that is, the most important sperm parameter to track with RSA applications (see 8.4.2). One successful method for counting sperm involves placing a 100 µL aliquot of homogenate of the cauda epididymis into a prepared commercially available snap tube that contains a fluorescent dye (for staining the DNA in the sperm heads). An aliquot of the thoroughly reacted sample in the tube is transferred to a microscope slide, which is then placed inside the automated sperm analyzer for the count determination.

7.3 *Animal Housing*—For a multitude of reasons, a mobile laboratory equipped with a fume hood is strongly recommended for euthanizing animals and for harvesting organs for the various measurements and preparations that make up RSA. [It is unlikely that an animal care/handling facility would be conveniently located in close proximity to the contaminated sites and habitat-matched reference locations where RSA applications would proceed. Even if a facility would be located relatively nearby the animal trapping locations, it is unlikely that the facility would be agreeable to sheltering wild rodents, for these might carry diseases and other vectors that can potentially imperil in-house colonies.] A relatively inexpensive but highly serviceable mobile laboratory can be made by retrofitting a utility trailer such that the cargo area has electrical power (to supply fluorescent lighting, air conditioning if needed, and run all method-related equipment), and has a work bench installed to support essential equipment (for example, the sperm analyzer, portable fume hood, analytical scale, vortex). For venting the air above a dissection, an exhaust hose can be directed from the area inside the fume hood to an opening in the trailer's roof. If a mobile laboratory such as is described here is not available, a next choice is a room in a building that ideally has a fume hood to facilitate safe dissections and organ harvesting. In the absence of a fume hood, all technicians handling small rodents must wear PAPR units.

8. Procedure

8.1 *Site Selection*—The RSA method necessitates a preliminary site visit to allow individuals who will be conducting RSA tasks in the field to gain a familiarity with the site environs.

8.1.1 The site visit should accomplish the following:

8.1.1.1 Ascertain the boundaries of the affected site; identify specific areas at which to place animal traps.

8.1.1.2 Identify two or more nearby suitable reference locations, sufficiently distanced from the contaminated site to preclude the possibility of a given small rodent occupying/contacting both a contaminated site and its corresponding reference location. A proper reference location will have multiple ecological aspects matching those of the contaminated site(s) to be investigated. Invariably, reference location features cannot help but match those of contaminated sites, given that they ideally occur as little as 0.40 km away from sites. In that case, the atmospheric and geologic conditions (for example, temperature, humidity, precipitation, soil type) of site and reference will undoubtedly be the same, and such should be field-verified nevertheless. Other ecological aspects to review in the field to further establish a high degree of parity of sites, and to establish overall reference location suitability include vegetative cover (degree, cover type), elevation, drainage, and biota/species lists.

8.1.1.3 Identify a practical and desirable location for deploying a mobile on-site laboratory (if one is to be used) or identify a building room where animal euthanization and method work-up can proceed, or both.

8.1.2 Other related information to assimilate in readiness for RSA application include:

8.1.2.1 Establish the probable small rodent species list for the areas to be animal-trapped.

8.1.2.2 Identify the nearest hospital and the quickest travel route from the study site to the hospital.

8.1.2.3 Locate a nearby vendor for a CO_2 tank and regulator gauge if the agency or institution overseeing the RSA application stipulates that euthanization is to be by asphyxiation with inhaled CO_2.

8.1.2.4 Locate a nearby vendor of oats or horse feed to be used when baiting animal traps.

8.1.2.5 Locate a nearby certified facility at which animal waste (carcasses) and medical waste (sharps) can be disposed of.

8.1.3 It is recommended that the preliminary site visit occur shortly before the actual RSA field work commences, so that site familiarity (for example, recognition of landmarks) is maintained. Of note, an area in spring or summer could look vastly different in the fall or winter.

8.2 *Animal Trapping*—Although animal trapping at a contaminated site of interest and a matched (non-contaminated) reference location can occur concurrently, it is advisable to trap first at the contaminated site. This rule recalls that it is the rodent species that reside(s) at the contaminated site of interest whose reproductive health needs to be assessed, and critically this must be established early on. Pre-selected reference locations therefore will only be demonstrated to have been viable ones for RSA purposes when it is evident that they support the same species that occur at the contaminated site of