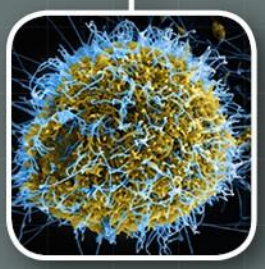

GUIDANCE ON THE INACTIVATION OR REMOVAL OF SELECT AGENTS AND TOXINS FOR FUTURE USE

7 CFR Part 331, 9 CFR Part 121.3, 42 CFR Part 73.3

AUGUST 2017

Centers for Disease Control and Prevention
Division of Select Agents and Toxins

Animal and Plant Health Inspection Service (APHIS)
Agricultural Select Agent Program

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Change/Highlight Section

Revisions: This is a living document subject to ongoing improvement. Feedback or suggestions for improvement from entities registered with the Federal Select Agent Program, as well as the general public, are welcomed. Submit comments directly to the Federal Select Agent Program at:

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Revision History:

March 2017: Revised to accommodate the new regulations.
April 2017: Revised to add further clarification that the new inactivation regulations do not pertain to select toxins, clarification on the level of surrogates that can be used for viruses, bacteria, and regulated nucleic acids, updates to the section dealing with validation, and updates to the section dealing with the inactivation certificate.
June 2017: Revised for clarity and to update the certificate section to align with the regulatory interpretation.
August 2017: Revised for clarity and to update links to other resources.
September 2018: Added additional information about electronic/digital signature.

Introduction

The United States Department of Health and Human Services (HHS) and the United States Department of Agriculture (USDA) have established regulatory requirements for the possession, use, and transfer of biological select agents and toxins (BSAT) that have the potential to pose a severe threat to public health and safety, animal and plant health, and animal and plant products. These requirements can be found at 42 CFR Part 73 (HHS), 7 CFR Part 331 (USDA-Plant Protection and Quarantine), and 9 CFR Part 121 (USDA-Veterinary Services). Collectively, they are referred to as the select agent regulations (SAR).

The following are excluded from the requirements of the SAR:

- A select agent or regulated nucleic acid (that can produce infectious forms of any select agent virus) that has been subjected to a validated inactivation procedure and confirmed through a viability testing protocol.
- A select toxin that has been rendered non-toxic.

This guidance does not apply to:

- Products that are, bear, or contain listed select agents or toxins that are cleared, approved, licensed, or registered under the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et seq.), section 351 of the Public Health Service Act pertaining to biological products (42 U.S.C. § 262), the Virus-Serum-Toxin Act (21 U.S.C. §§ 151-159), or the Federal Insecticide, Fungicide, and Rodenticide Act (7 U.S.C. 136 et seq.) if their use meets the requirements of these laws. See 7 CFR § 331.5(c), 9 CFR §§ 121.5(c), 121.6(c), 42 CFR §§ 73.5(c), 73.6(c).

- Select agents and toxins excluded as an attenuated strain of a select agent or a select toxin modified to be less potent or toxic.[1] See 7 CFR §331.3(e), 9 CFR §§121.3(e), 121.4(e), 42 CFR §§73.3(e), 73.4(e).
- Decontamination for waste disposal. For more detailed information, see the Biosafety/Biocontainment Plan Guidance.
- Exempt clinical or diagnostic laboratories that possess or use a select agent or toxin that is contained in a specimen presented for diagnosis or verification. See 7 CFR §331.5(a), 9 CFR §§121.5(a), 121.6(a), 42 CFR §§73.5(a), 73.6(a).

See 7 CFR §331.3(d)(2); 9 CFR §121.3(d)(2) and 9 CFR §121.4(d)(2); 42 CFR §73.3(d)(2) and 42 CFR §73.4(d)(2) for more information.

[1] As long as the attenuated strain or modified toxin is not subjected to any manipulation that restores or enhances its virulence or toxic activity. Refer to the Exclusion Guidance Document for additional information.

Terminology

The regulatory requirements and this accompanying guidance applies to select agents and regulated nucleic acids that have undergone an inactivation process on or after March 21, 2017 for future use. Inactivated select agents and regulated nucleic acidsare excluded as nonviable.

Glossary of Terms

Term	Definition by the SAR or within this Guidance
Bioburden reduction studies	Studies based on deliberately adding a specific agent ("spiking") and subsequently measuring the removal or inactivation during inactivation steps.
Inactivation	A procedure to render a select agent or regulated nucleic acids as non-viable or a select toxin non-toxic while retaining characteristic(s) of interest for future use.
Infectivity Testing	A protocol to confirm the inactivation procedure by demonstrating the nucleic acids are incapable of producing infectious forms of any of the select agent viruses.
Limit of detection (LOD)/Limit of quantitation (LOQ)	The smallest concentration of a select agent or toxin that can be reliably detected or measured by an analytical procedure.
Kill curve	The results of a dose-response experiment where a select agent is subjected to increasing levels of the inactivating procedure in order to determine the minimum conditions required to render it nonviable.
Margin of error	The amount of error allowed for in case of miscalculation or normal variation in a process.
Non-infectious nucleic acid	Nucleic acids no longer capable of producing infectious forms of a select agent virus (e.g. regulated positive sense RNA virus genomes like FMDV or EEE).
Non-viable select agent	A select agent no longer capable of growing, replicating, infecting, or causing disease.
Non-toxic select toxin	A toxin no longer capable of exerting a toxic effect.
Safety margin	The treatment amount designed into an inactivation procedure beyond that required to reach LOD/LOQ, intended to reduce the probability of inactivation failure.
Sterility assurance level (SAL)	The probability of surviving organisms in material that has been subjected to inactivation.
Toxicity Testing	A protocol to confirm the inactivation procedure by demonstrating the select toxins are non-toxic.

Validated inactivation procedure	A procedure, whose efficacy is confirmed by data generated from a viability testing protocol, to render a select agent non-viable, but allows the select agent to retain characteristics of interest for future use; or to render any nucleic acids that can produce infectious forms of any select agent virus non-infectious for future use.
Verification	Confirmation by an individual or entity that the use by the individual or entity of a validated inactivation or select agent removal procedure will result in an end product that achieves the expected results.
Viability testing protocol	A protocol to confirm the validated inactivation procedure by demonstrating the material is free of all viable select agent.

Inactivation

Historical inactivation failures by registered entities required the Federal Select Agent Program (FSAP) to focus on ways to increase the certainty that inactivated select agents and regulated nucleic acids intended for further use do not contain live agent. This is particularly important when the inactivation procedures are tempered in order to avoid disrupting the physical characteristics of the agent or toxin. FSAP acknowledges that 100% inactivation of material is not possible in both a mathematical and practical sense. However, FSAP expects the risk of live agent or toxin in inactivated material to be as low as possible given the intended use of the material and the consequences of inactivation failures.

Inactivation procedures used to render a select agent nonviable, regulated nucleic acids non-infectious, or select toxins non-toxic may fall into the following categories: physical (e.g. heat or irradiation), enzymatic (e.g. lysozyme), or chemical. Filtration procedures can be used to remove viable select agents from biological fluids, culture supernatant, and other materials.

For a select agent or regulated nucleic acids to be properly characterized as non-viable after treatment, the agent must not have the ability to grow or replicate, and the toxin must not have the ability to cause toxicity. Exposure to the agent, toxin, or nucleic acids must not result in infection or toxicity.

Select Agent or Regulated Nucleic Acid Inactivation

Select agents or regulated nucleic acids are considered non-viable or non-infectious only after being subjected to an in-house validated inactivation procedure that is confirmed through a viability or infectivity testing protocol. The responsibility of assuring non-viability or non-infectivity, remains on the individual or entity possessing the select agent or regulated nucleic acids.

An entity must use a validated inactivation procedure to render a select agent non-viable or regulated nucleic acids non-infectious. This means that the inactivation procedure's efficacy must be confirmed by data generated from a viability testing protocol to render a select agent non-viable for future use. It also means the procedure that renders nucleic acids that can produce infectious forms of select agent virus non-infectious for future use. Validation of an inactivation procedure that renders a select agent non-viable or regulated nucleic acids non-infectious may include:

1) Use of the exact conditions of a commonly accepted procedure (such as autoclaving) whose efficacy is confirmed by data generated in-house by the entity using a viability testing protocol.
2) Use of a published procedure with adherence to the exact published conditions whose efficacy is confirmed by data generated in-house by the entity using a viability testing protocol.
3) Use of entity derived procedure with specific conditions whose efficacy is confirmed by data generated in-house by the entity using a viability testing protocol.

Select Toxin Inactivation

The new inactivation provisions apply to select agents and regulated nucleic acids only. For a select toxin to be considered non-toxic and therefore excluded from the regulations, a validated inactivation procedure should be used but validation does not have to occur in-house and the inactivation provisions found in the records and RO section do not apply. The difference in validation requirements for toxins is due to the non-replicating nature of toxins.

Diagnostic samples

Nonregistered clinical or diagnostic laboratories and other entities that possess, use, or transfer a select agent or toxin that is contained in a specimen presented for diagnosis or verification are exempt from the inactivation requirements, so long as they meet the requirements found in 7 CFR §331.5(a), 9 CFR §§121.5(a), 121.6(a), 42 CFR §§73.5(a), 73.6(a).

Procedure Development

When following an inactivation procedure, the limits of detection of the viability testing procedures and expected run-to-run variation may prevent demonstrating full sterility of inactivated material. These sources of error must be considered to achieve the regulatory requirement of developing a validated inactivation procedure. FSAP expects the entity to determine the risk of live agent in inactivated BSAT material. This risk should take into account the safety and security consequences associated with failure of inactivation as well as the margin of error associated with the inactivation procedure. The variability in the nature of the specific pathogens (including ID50), the use of the inactivated material, and the inactivation procedures prevents establishing a specific national standard for measures of uncertainty associated with inactivation procedures.

If there is any deviation or modification to an inactivation or select agent removal procedure that was previously validated on-site, the entity should revalidate the procedure under the new conditions to ensure the procedure still achieves the appropriate result. For example, if the procedure was validated with Ebola virus and you want to use the procedure to inactivate Chapare virus or if the procedure was validated with a starting concentration of 10^6 organisms and you want to inactivate 10^8 organisms, these modifications would warrant the reevaluation of an existing procedure or redevelopment and revalidation of the procedure.

Entities can either develop their inactivation procedures in-house (see procedure development section below) or they can 1) use exact conditions of a commonly accepted procedure that has been validated in-house, such as autoclaving or 2) use a published procedure and adhere to the exact published conditions that have been validated in-house as applied. For more information on validation of previously developed procedures see the validation and verification sections below.

Procedure development via a kill curve

Entities that develop their inactivation procedures in-house also need to validate the procedures in-house and determine the measures of uncertainty with their particular procedure. This can be accomplished by developing empirical kill curves based on specific conditions that will be used to prepare samples. **Note**: The development of a kill curve is not a regulatory requirement. This information provides guidance for those entities that choose to develop their procedures via kill curves. Kill curves are appropriate for inactivation procedures using heat, chemicals, or irradiation. For a traditional kill-curve, the y-axis represents concentration of organism and the x-axis represents an experimentally-controlled variable, such as time or irradiation dose, for a given treatment. For each kill curve, only one factor, the x-variable, should vary. For example, initial burden and chemical concentration could be held constant while time of treatment is varied. Alternatively, all other factors might be held constant while chemical concentration is varied to produce a kill curve in terms of disinfectant concentration for fixed time.

The efficacy of inactivation treatment can be affected by many variables, so those variables must be held constant. Inherent variability in these factors (like the ability to control temperature precisely) can produce variation in results, and the entity should account for this in the validation process (like more experimental replicates to establish confidence in the results). The following are examples of factors that may influence the inherent variability in an inactivation procedure:

- Exposure or incubation time with inactivation treatment
- Nature of starting material (virus, bacteria, spores, tissue)
- Matrix material (serum, plasma, water, media)
- Dose, temperature, and concentration of inactivation treatment
- Concentration or volume of starting material (virus, bacteria, spores, tissue)
- Incubation temperature
- Process controls
- Container that holds select agent during inactivation (like tubes used during irradiation)

The entity needs sufficient empirical data developed in-house with test microorganisms to determine the inactivation kinetics with a particular procedure, starting with a high concentration of organism to the LOD/LOQ. (For those inactivation procedures where the LOD/LOQ is reached so quickly that multiple data points cannot be collected to adequately determine kinetics, see the Bioburden Reduction section below). Use multiple replicates at each treatment point to assist with fitting the curve that determines the inactivation kinetics. The number of replicates will vary depending on the procedure and agent. It is the entity's responsibility to determine a sufficient number of replicates. For development of the inactivation procedure, use all (100%) of the sample from each treatment point in viability testing to generate data for the curve. If working with large volume cultures 100% of the sample can be filtered and then the filter can be cultured.

Kill Curve Development Steps

1) Decide what the x-variable will be, such as time or irradiation dose, while keeping other conditions, like agent concentration, fixed.
2) Using your knowledge of the organism and inactivation procedure, select initial dosage steps (time duration, intensity of treatment, or what you chose for the x-variable) and number of replicates (typically three) at each dosage. Replicate experiments enough times to demonstrate

reproducibility. For example, select triplicates at 2 minutes, 4 minutes, 6 minutes, 8 minutes, and 10 minutes for a particular organism and treatment. The goal is to produce a wide range of data with measurable, incomplete inactivation to identify (x, y) points on the kill curve.

3) Perform the experiments and collect data.
4) Calculate a safety margin to incorporate into the inactivation procedure (e.g., additional treatment to achieve a 2 log further reduction in bioburden, a target SAL like10^{-6}, etc.).

Procedure development via Bioburden reduction

For some procedures or agents, developing inactivation procedures via a kill curve is not practical and bioburden reduction is preferred. Examples of this include when inactivation is too efficient to allow for the collection of many data points on a kill curve, or for extracts that involve multiple processing steps. Bioburden reduction studies for viable organisms involve the deliberate addition of the specific agent or surrogate (see below for more information on surrogates) and measuring the extent of removal or inactivation during subsequent processing, inactivation, or removal steps. The entity should evaluate critical parameters that influence the effectiveness of inactivation/removal steps (bioburden reduction) such as the factors listed above in the kill curve section. Validation studies should establish the reduction achieved by inactivation and removal steps and be in excess of the greatest possible viable bioburden that may be found in the test materials undergoing inactivation/removal validation.

The procedure of bioburden reduction relies on these steps:

1) Determine inactivation effectiveness of a fully-specified protocol/procedure, e.g. 5 logs.
    a. Create a spiked sample with an organism concentration well above the expected inactivation effectiveness of one application (or one unit of treatment).
    b. Apply the inactivation procedure to the spiked sample and quantitatively measure the effectiveness by counting the remaining viable organisms.
    c. Replicate the experiments enough times to demonstrate reproducibility (this will vary depending on the inherent variability of the inactivation procedure).
2) Apply as many applications as possible to achieve “overkill” in an amount consistent with the observed variability and the acceptable likelihood (presumed to be a very low probability) of survival of the particular organism.
    a. For example, consider a starting concentration of organisms of 10^8 and an inactivation procedure that leads to a 5-log reduction in organisms using one unit of treatment.
        i. The resultant specimen after one unit of treatment should contain a concentration of 10^3 organisms, which can be counted via viability testing.
        ii. Applying another unit of treatment for an additional 5-log reduction in organisms would be considered overkill and provide a safety margin of 10^{-2} or an approximate probability of $\frac{1}{100}$ of a live organism, which can’t be counted via viability testing.
        iii. An additional unit of treatment would provide a safety margin of 10^{-7} and give an approximate probability of $\frac{1}{10000000}$ of a live organism, which also can’t be counted via viability testing.

b. The amount of overkill should be commensurate with the potential consequences for inactivation failure of the particular organism and the need to retain characteristics for future use.

Limitations of the Bioburden reduction procedure

It is important to note that the above bioburden reduction methodology provides only a point-estimate, or the expected value, of the probability of survival. As such, these estimates do not include variance estimate or estimates of assurance. Further, bioburden reduction methodology assumes that increasing the inactivation dose results in the same rate of reduction of viable organism that was seen above LOD/LOQ. Further, this procedure assumes spiked samples represent a naturally infected sample which, for some matrices (tissue), may not be true. As mentioned above, a variety of factors must be considered when developing an inactivation or select agent removal procedure. For example, when inactivating agent in tissues, the spiking of agent at concentrations that are above what would be expected in a natural infection would be performed *in vivo* and the affected organs would be removed and subjected to the inactivation procedure. For initial procedure validation, the tissue would be processed to detect the remaining viable organisms. Once the procedure is validated, one could take an immediately adjacent sample of the affected tissue in subsequent inactivation experiments to verify inactivation of the agent (see verification section below).

Surrogates

Ideally, an inactivation procedure is developed with the exact organism it is intended for. However, there may be occasions when this is not possible. Consider an entity that identifies a select agent in a small diagnostic sample and wants to inactivate the agent for future use. The entity wants to extract nucleic acids from the select agent. Or, consider an entity that wants to fix tissues from a select agent infected animal. In these situations, procedure development studies would leave very little or no sample for experimental purposes. Therefore, surrogate strains that are known to possess equivalent properties with respect to inactivation can be used to develop an inactivation procedure. If there are known strain-to-strain variations in the resistance of a select agent to an inactivation procedure, then an inactivation procedure must be developed using the more resistant strain. If the entity uses surrogates to develop an inactivation procedure, it is recommended that the final inactivation procedure be validated on the select agent it is intended for. However, in some cases, such as with Variola virus, validation with the intended select agent is not possible. In these situations, it is recommended that entities include risk mitigation steps during inactivation procedure development, validation, and verification such as validation of the procedure with all (100%) of the surrogate, inclusion of process controls, a sampling strategy for subsequent inactivation of samples, and/or the incorporation of a safety margin.

Bacteria from the same genus can be suitable surrogates for select agent bacteria, such as *Yersinia pseudotuberculosis* for assay validation for *Yersinia pestis*, but it does depend on the method of inactivation and it cannot be guaranteed that it would work all the time with a similar bacteria. Viruses from the same family can be suitable surrogates for select agent viruses, such as using Ebola as a surrogate for Marburg. For regulated nucleic acids, any positive single stranded RNA can be suitable surrogates for regulated positive single stranded RNA, such as VEE genome as a surrogate for Omsk hemorrhagic fever virus genome. It is the entity's responsibility to research possible surrogates and determine the risk associated with using a surrogate strain.

Procedure Validation

Procedure validation can be accomplished by viability, infectivity, or toxicity testing. An entity must validate their inactivation or select agent removal procedure on-site to determine that the procedure works as intended, including the use of appropriate positive, negative, and process controls. This can be accomplished by using the final inactivation conditions derived from the procedure development step (or from an existing procedure (commonly accepted or published procedure)), and testing for the absence of viable organism, infectivity of regulated nucleic acids, and/or toxicity of regulated toxins. Perform sufficient experimental replicates to determine inherent variability with the procedure. The number of replicates will vary depending on the procedure and agent, and it is the entity's responsibility to determine a sufficient number of replicates. If practical, multiple types of assays for determining viability are recommended.

Once non-viability or non-infectivity have been demonstrated, and the record requirements are met, **the material is no longer subject to the regulations**. It can be moved to lower containment, transferred without approval from FSAP, used in un-registered space, etc.

Examples of items to consider when validating an inactivation or select agent removal procedure are listed below:

- Adherence to standardized extraction kit instructions (manufacturer or in-house derived) for nucleic acids, proteins, polysaccharides, etc.
- Manufacturer performance data for filter used to remove/exclude viable organism.
    - Appropriate pore size of the filter depending on organism size and matrix material (e.g. serum, culture media).
    - Volume of starting material (should not use more volume than manufacturer recommends)
- Appropriate assay for the starting material (virus, vegetative bacteria, or spores).
- LOD/LOQ of the viability testing protocol.
- Concentration of starting material containing select agent and regulated nucleic acids (start with highest concentration expected as a worst case scenario and then set that concentration as the upper limit for subsequent inactivation).
- Matrix materials that could interfere with viability tests.
- Validation with all (100%) of the sample (for large volume cultures you can filter 100% of the sample and then culture the filter)
- Need for neutralization of chemical or antimicrobial treatments.

Viability Testing of Inactivation or Removal Procedures

Validation of inactivation or removal procedures will differ depending on the category of the sample. There are three categories of sample: 1) inactivated agent, 2) extracts, or 3) material (within this section, material refers to sample in which intact select agent has been removed).

Inactivated Organism

To verify an agent as non-viable, viability testing procedures may include, but are not limited to, cell viability assays, growth analysis, *in vivo* exposure, or all of the above. Consider the type of viability test, like broth culture versus agar plates for bacteria. In some instances, extended broth cultures allow for the growth of sub-lethally damaged organisms (REF 1). If any failures in viability testing or inconclusive results occur, reassess procedures. Consider redeveloping the inactivation procedure if you can't identify a variation from the prescribed procedures to explain the failure of viability testing. Inactivation failures when validated procedures are accurately followed is an indication that the procedure is not sufficiently reliable to be used for inactivation.

The LOD/LOQ of the viability testing procedures (related to the detection assay and the sampling of inactivated material) precludes demonstrating full sterility of inactivated material. Consider these limitations when 1) generating validation data, and 2) determining the extent of sampling required for subsequent inactivation (see below).

Extracts

Under ideal conditions, the presence of viable organisms in an extract (for example, nucleic acids, proteins, or polysaccharides) would be a rare event. Treatment conditions are considered extreme due to both destruction of the select agent (like lysis of select agent) and then additional steps (like filtration, precipitation, centrifugation, column capture) to further purify the nucleic acids, proteins, polysaccharides, removing any remaining viable select agents. Because these procedures require the detection of a rare event and require prohibitive sample sizes, statistical methods cannot be practically applied.

For select agent nucleic acid extracts, procedure development via kill curves is not typically performed. This also applies to other types of extracts. Instead, the entity can use a standardized nucleic acid extraction kit (from a manufacturer or derived in-house) for inactivation. Agent concentration, volume, mixing time, inactivation/extraction time, and temperature should be held constant to be considered standardized. Non-viability should be confirmed by initial *in vitro* viability testing using all (100%) of the sample in broth and/or agar for bacteria, plaque or cytopathic effect detection on a permissive cell line for viruses, or other appropriate culture method (e.g. chicken embryo for HPAIV) with suitable positive and negative controls.

If after using the standardized nucleic acid extraction kit, viability is still detected, conduct a separate filtration step using the appropriate pore size filter. Test the filtrate using all of the sample to confirm non-viability during validation. If non-viability is confirmed then this additional step should be added to the inactivation procedure. This should satisfy inactivation and viability testing validation requirements without the need for establishing kill curves. If viability is still detected with the use of a standardized nucleic acid extraction kit and the additional filtration, this procedure should not be used for inactivation.

Removal of viable agent

Within this section, material refers to sample in which intact select agent has been removed. This is not to be confused with extracts which refers to nucleic acids, antigens, lysates, or select agents that have been subjected to lysis before removal. Using a standardized filter with the appropriate pore size for removing a select agent from material, validation by viability testing all (100%) of the sample with an appropriate culture method and positive and negative controls, and verification viability testing on a portion of subsequent samples (see verification section for more information) should satisfy the validation requirements without the need for establishing kill curves.

Infectivity Testing of Inactivation Procedures

An infectivity testing procedure to verify regulated nucleic acids are non-infectious may consist of introduction of the positive (+) strand RNA into permissive cells (like transfection or electroporation) to determine if the + strand RNA is capable of producing infectious virus. Consider the type of permissive cells that permit viral replication for each + strand RNA virus, the procedure of introducing + strand RNA into the cells, and appropriate positive and negative controls.

Toxicity Testing of Inactivation Procedures

Toxicity testing procedures to verify select toxins are rendered non-toxic may include, but are not limited to, functional activity assays (like the ability to cleave SNAP-25 for Botulinum neurotoxin) and *in vivo* exposure (like the mouse bioassay). Appropriate positive and negative controls should be included. A validated procedure, should be used to inactivate a select toxin, however the entity is not required to validate the procedure in-house.

Neutralization of Chemical Treatments Before Viability, Infectivity, or Toxicity Testing

Chemical treatments, such as fixatives and antimicrobials in inactivated samples may interfere with the viability, infectivity, or toxicity testing. Neutralization inactivates residual chemicals or antimicrobials. Therefore, viability tests should be performed once chemical or antimicrobial treatments have been subjected to neutralizing or have been shown not to interfere with the viability test.

The following experiment can be performed to determine if the chemical or antimicrobial treatment is affecting the viability, infectivity, or toxicity test:

- Split the sample in half.
- To one half, add live agent to determine if the chemical or antimicrobial activity interferes with the viability testing protocol.
- If the results demonstrate that the chemical or antimicrobial activity interfere with the viability testing protocol, perform neutralization.

If the experiment demonstrates that the residual chemicals are interfering with the viability, infectivity, or toxicity test, then neutralization should be performed. Two commonly used neutralizing media for chemical disinfectants are Letheen media and D/E Neutralizing media. Letheen media contains lecithin to neutralize quaternaries and polysorbate 80 (Tween 80) to neutralize phenolics, hexachlorophene, formalin, and, with lecithin, ethanol. D/E Neutralizing media will neutralize a broad spectrum of antiseptic and disinfectant chemicals such as aqueous formaldehyde.

The entity can use dialysis, washing of the cells, or dilution of samples to remove chemical treatments (like antibiotics or lysis buffers) that may interfere with viability testing. However, an entity must demonstrate that dialysis, washing, or dilution will neutralize residual chemical activity.

For more information on neutralization, see the Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008.

Verification of a Validated Procedure

Once an entity has validated an inactivation procedure, they may have to verify the validated procedure depending upon the type of sample and risk assessment. This involves the entity determining a sampling strategy for viability or infectivity for subsequent inactivation. Verification of inactivation or removal procedures will differ depending on the category of the sample. There are three categories of sample: 1) inactivated agent, 2) extracts, or 3) material (within this section, material refers to sample in which intact select agent has been removed). The portion of samples subjected to a validated inactivation procedure, (such as 10%) used for verification will depend on the risk assessment. Samples with more inherent variability may require verification viability testing of a portion of all subsequent inactivated samples while samples with low inherent variability may only require verification viability testing of process controls while the risk assessment for fixed tissue samples may allow for no verification viability testing.

Verification of Inactivated Agent

Consider highly pathogenic avian influenza virus (HPAIV) and Newcastle disease virus as an example. When testing a small volume or to achieve a lower detection limit of the assay, increase the number of eggs (i.e. replicates) and passages to increase the probability of detecting a viable organism at the assay limit of detection.

Consider verification of tissue samples containing select agents. This would involve inactivation of the select agent present in the tissues, and then grinding up the tissue for use in a viability test along with appropriate process controls, resulting in no sample for research purposes. In these situations, or when there is a limited amount of the sample, verification is not possible. The validation of the inactivation procedure must provide confidence to allow for the risk associated with not verifying subsequent inactivated samples. If the validation data cannot provide this confidence, then an entity can choose to take an immediately adjacent sample of the affected tissue in subsequent inactivation experiments to verify inactivation of the agent.

Consider verification of chemically inactivated Ebola virus using a validated procedure. Verification may involve viability testing 10% of samples (1 out of every 10 inactivated samples or 10% from every sample) or just the process controls. The sampling strategy will depend on the risk associated with not verifying every subsequent inactivated sample.

Verification of Extract

The term “extract” is commonly used in conjunction with nucleic acids extracted from a select agent. The term “extract” reflects the application of two processing steps: an inactivation step to destroy the select agent (e.g., lysis of select agent) and then another step (such as filtration), to remove any remaining viable select agents. Extracts from a select agent (nucleic acids, antigens, lysates) would be subject to the performance standard for select agents in the new sections 3(d)(4) and 4(d)(4) of the select agent regulations that requires validation of a procedure by confirming its efficacy with data generated in house by the entity using a viability testing protocol

but does not necessarily require verification viability testing on every sample. For nucleic acid extractions (other than *Bacillus anthracis* and *Bacillus cereus* biovar *anthracis*) subsequent to validation, it is not always possible to verify every sample for non-viability. The risk associated with not performing verification viability testing on every subsequent sample can be mitigated by the multi-step procedures used to extract nucleic acids, validation of the procedure with all (100%) of the inactivated sample (extracted nucleic acids), inclusion of process controls, and/or the incorporation of a safety margin. For example, the laboratory could choose to only test the process control, only perform verification viability testing on a portion of the total samples, and/or incorporate a safety margin.

Note: Inactivation of *Bacillus anthracis* and *Bacillus cereus* biovar *anthracis* is subject to the new inactivation requirements and the FSAP policy on Inactivated *Bacillus anthracis* and *Bacillus cereus* biovar *anthracis*. For procedure validation for nucleic acid extracts from *Bacillus anthracis* or *Bacillus cereus* biovar *anthracis,* viability test all (100%) of the sample a sufficient number of times to demonstrate the procedure works. After this validation, verify the procedure by viability testing at least 10% of the sample or production lot of subsequent extracts or production lots to verify the validated procedure.

Verification of Material

Material containing select agents, as opposed to extracts (e.g., nucleic acids, antigens, lysates), that is subjected to a process to remove (e.g. filtration) all viable cells, spores, or virus particles would require verification viability testing on every sample prior to treating it as a non-select agent. The distinguishing feature between "material containing a select agent" and an extract from a select agent is that in the former the select agent will only be removed and in the latter the select agent will be destroyed before removal. The more stringent requirement for viability testing of all material containing a select agent where the select agent was removed is warranted because of the lack of select agent destruction which increases the risk of viable select agent remaining in the material.

For example, consider verification of filtered serum from an animal infected with *Francisella tularensis*. This would require verification of every sample, such as subjecting 10% of every sample to viability testing.

Sample category	Verification required after sample subjected to validated inactivation or select agent removal procedure
Agent (Cell cultures, tissue samples, etc.)	It depends, sampling strategy developed by entity based on risk assessment.
Extracts (nucleic acids, proteins, polysaccharides, etc.)	
Material containing select agents that is subjected to a process to remove (e.g. filtration) all viable cells, spores, or virus particles	Yes, on all samples

Inactivation Failure Reporting Requirements

Reporting requirements regarding inactivation failures require the Responsible Official (RO) to:

> Investigate to determine the reason for any failure of a validated inactivation procedure or any failure to remove viable agent from material. If the investigation does not determine the cause as a deviation from a validated inactivation procedure or a viable agent removal method; or receives a report of any

inactivation failure after the movement of material to another location, the RO must report immediately by telephone or email the inactivation failure or viable agent removal method failure to CDC or APHIS. [Section 9(a)(8)]

The intent of this requirement is to create an entity environment where inactivation or select agent removal failures are investigated to determine the reason for the failure as opposed to merely re-subjecting the material to the inactivation or select agent removal method. Human error is a significant contributor when inactivation or select agent removal methods fail. These situations demand careful attention by the entity to ensure training and reevaluation of the inactivation procedure in order to minimize the likelihood that the situation would reoccur in the future. The regulations only require reporting of inactivation or select agent removal failures to FSAP when the RO cannot establish the reason of the failure of the validated inactivation or select agent removal method. It is recommended that inactivation failures be investigated immediately to prevent the use of a failed procedure. Removal of samples that fail inactivation out of registered space would be a violation of the regulations.

Record keeping requirements

For select agents or material containing select agents or regulated nucleic acids, the registered individual or entity must keep the following records for 3 years:

- A written description of the:
    - Validated inactivation procedure or viable select agent removal method used, including validation data.
    - Viability testing protocol used.
    - Investigation conducted by the entity RO involving an inactivation or viable select agent removal failure and the corrective actions taken.
- The name of each individual performing the validated inactivation or viable select agent removal method.
- The date(s) the validated inactivation or viable select agent removal method was completed.
- The location where the validated inactivation or viable select agent removal method was performed.
- A certificate, signed by the Principal Investigator, that includes the date of inactivation or viable select agent removal, the validated inactivation or viable select agent removal method used, and the name of the Principal Investigator. A copy of the certificate must accompany any transfer of inactivated or select agent removed material.

The information specified in section 17 (a)(8)(vii) must be retained by the entity that performs the inactivation or removal of viable select agent and must accompany the inactivated sample if it is transferred to another entity. The certificate needs to be a written certification of the information but doesn't require any specific format. The required information can be recorded outside of containment. Further, the PI does not have to be present during the performance of the inactivation or select agent removal procedure. . A copy of the certificate must accompany any transfer of the inactivated samples to another entity. However, FSAP recommends a certificate of inactivation accompany all transfers of inactivated select agents (includes intra-entity transfers). The PI who signs the certificate is the one individual who is designated by the entity to direct a project or program and who is responsible to the entity for the scientific and technical direction of that project or program (including all inactivation procedures or removal procedures associated with that project). In the absence of that

PI, an individual designated by that PI and approved by the entity's Responsible Official can sign the certificate but only during the duration of the PI's absence. Each absence of a PI requires a new delegation.

In order for an individual to be the PI's designee to sign the certificate, a person must:

- Be listed on the entity's registration
- Have the knowledge and expertise to provide scientific and technical direction regarding the validated inactivation procedure or the procedure for removal of viable select agent to which the certificate refers. If this requirement causes unintended consequences please contact FSAP.

The signature denotes that the PI or designee that is responsible for the specific agent, has reviewed the inactivation procedure used and the validation or verification data. The certificate should be signed as close to the date of inactivation as possible. There is no regulatory requirement for the receiver to keep the certificate but it is strongly recommended that this information is kept so long as the inactivated sample exists. However, the sender or the PI that performed the inactivation procedure must keep the certificate for 3 years. A PI can review and sign documents electronically. For electronic signature, the method used should:

- Identify and authenticate a person using at least two factors of authentication, including something the person knows (i.e., email password) and something the person has (e.g., a mobile phone with SMS text message access);
- Provide a means to preserve the integrity of the signed record that is (a) portable, (b) independently verifiable, (c) tamper-evident, (d) granular, and (d) verifiable in the long-term; and
- Meet the requirements of Government Paperwork Elimination Act, the Electronic Signatures in Global and National Commerce Act (ESIGN, 15 U.S.C. ch. 96), and the Uniform Electronic Transactions Act.

In addition, the electronic form of signature must be executed or adopted by a person with the intent to sign the electronic record, (e.g., to indicate a person's approval of the information contained in the electronic record). Not only must this intent be captured at the time of each signature, but it must also be captured for each individual signature and be provided as granular evidence within the electronic signature system's audit trail. Each signed document must be backed by an audit trail that captures intent to sign for each individual signature and provides granular, consistent, timestamped evidence as to every step in the entire signature process. The electronic form of signature must be attached to or associated with the electronic record being signed, and each signature should be produced according to established standards. The signature should be independently verifiable, without relying on the electronic signature service or website to be validated (Reference https://www.signix.com/blog/bid/109223/7-Rules-for-E-Signature-Legality-and-Compliance-Part-3).

Once non-viability or non-infectivity, have been demonstrated, and the record requirements are met, **the material is no longer subject to the regulations.** While it is not a regulatory requirement, it is recommended that entities maintain records that identify the recipients of inactivated materials.

Annual review requirements

Inactivation and select agent removal methods must be monitored continuously to ensure adherence to validated methods. Oversight should include investigation of select agent inactivation or removal failures, ensuring that validated procedure are used, training for staff that inactivate the agents, and appropriate record keeping.

The RO is required to review, and revise as necessary, each of the entity's validated inactivation procedures or viable select agent removal methods. The RO must conduct the review annually or after any change in Principal Investigator, change in the validated inactivation procedure or viable select agent removal method, or failure of the validated inactivation procedure or viable select agent removal method. The RO must document the review and ensure training occurs if there are any changes to the validated inactivation procedure, viable select agent removal method, or viability testing protocol. The annual review does not mean the procedures have to be revalidated. For example, the RO could review the inactivation procedures and determine that they are still being used and work as intended. In this situation, revalidation would not be necessary.

Waiver

Entities must inactivate select agents prior to treating them as non-select agents. However, there may be rare situations where there is a need to take material out of containment without following the exact requirements for viability testing. For example, consider a situation where an entity filters serum that contains a select agent but the recovered volume is so low that the requirement to viability test every sample would further deplete the sample volume and not leave enough for experiments. In these situations, an entity may submit a request to FSAP to apply an alternative select agent removal or inactivation procedure and/or an alternative means of validation. To apply for this determination, a registered individual or entity must submit a written request including:

- A justification regarding the alternative procedure including a description of what material is to be waived.
- The inactivation protocol and viability test to be used
- Validation data.
- Any other supporting information/references, such as scientific references.

FSAP will issue a written decision granting or denying the request.

Scenarios

Example 1: A laboratory uses aqueous formaldehyde to inactivate *Francisella tularensis*, and wants to ship these inactivated cells to unregistered entities. They plan on changing the concentration of cells to be inactivated. Should the entity revalidate their inactivation procedure?

It depends. If the concentration of cells is decreased or the incubation time of inactivation is increased, then the inactivation procedure would not need to be revalidated. However, any time there has been a change to an inactivation procedure (e.g. equipment changes, a different strain used with known resistance to inactivation method, or a change in the volume, or increased concentration of cells, or decrease in incubation time) the protocol needs to be revalidated to ensure there are no viable cells present in the sample. Revalidation via a kill curve or bioburden reduction should be performed to determine the conditions needed to inactivate the material. If there are strain-to-strain variations in sensitivity to the inactivation procedure, validate the procedure on the more resistant strain. Also, use a viability testing procedure for subsequent inactivation to verify the effectiveness of the inactivation procedure. In addition, positive controls should be included to ensure that the chemical (formaldehyde) does not interfere with the viability test. If it does interfere, then perform a step to neutralize the formaldehyde.

Example 2: A laboratory in the U.S. has received highly pathogenic avian influenza virus (HPAIV) samples from Vietnam under a valid APHIS/CDC Form 2 Transfer authorization. What can the laboratory do in order to safely perform a real-time reverse transcriptase PCR assay on these samples in its unregistered BSL-2 laboratory to

further analyze the avian influenza A viral RNA?

Work with HPAIV must be conducted in a BSL-3 laboratory with enhancements. Nucleic acids that encode for HPAIV are not subject to the select agent regulations. You could use a validated protocol that extracts nucleic acids and renders the virus nonviable and confirm the protocol by viability testing. Subsequent work could then be conducted in an unregistered BSL-2 laboratory.

Example 3: A registered entity is inactivating 500 milliliters (ml) of *Yersinia pestis* by gamma irradiation and performs viability tests that show no growth of *Yersinia pestis*. During initial development of this inactivation method, the entity validated non-viability using a starting volume of 150 ml of *Yersinia pestis*. Can the 500 ml preparation be worked with as non-select agent material outside of registered space?

No, the entity tested viability on material that did not represent the exact conditions (volume) set by the initial validation of inactivation. The entity would have to generate a kill curve or bioburden reduction to demonstrate that the protocol will inactivate a 500 ml sample.

Example 4: A registered ABSL-3 laboratory extracts nucleic acid from cell cultures infected with Rift Valley fever virus (RVFV) using a commercially available kit. The entity would like to distribute the nucleic acids to several US laboratories to assist them in the development of *in vitro* assays. The laboratory would like to know what it should consider when validating that the kit inactivates the RVFV present in the sample.

The entity should perform viability tests to validate that the kit inactivates the virus and no select agent material remains. When validating the ability of the kit to inactivate RVFV in-house, the entity should consider viability test conditions such as the use of permissive cells to culture material, appropriate controls, and incubation time of the culture. Once the entity has validated that the kit inactivates RVFV, the nucleic acids, which are not subject to the select agent regulations, can be distributed to unregistered laboratories.

References

1) Weller SA, Stokes MGM, and Lukaszewski RA. (2015) "Observations on the inactivation efficacy of a MALDI-TOF MS chemical extraction method on *Bacillus anthracis* vegetative cells and spores". *PLoS One* 10(12): e0143870. doi: 10.1371/journal.pone.0143870.