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Sampling, Laboratory and Data Considerations for Microbial Data Collected in the Field

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Sampling, Laboratory and Data Considerations for Microbial Data Collected in the Field

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Executive Summary

In-order to collect the data necessary to assess the efficacy of remediation efforts following a microbiological contamination incident, a well-defined and thorough sampling and analysis plan (SAP) needs to be developed and implemented. This document summarizes elements that should be considered when planning, developing and implementing a sampling and analysis plan (SAP) for microbiological contamination incidents in which the United States Environmental Protection Agency (EPA) would be responsible for supporting sampling and analysis. It is intended to be an informational companion to users of the EPA's Microbiological Sampling and Analysis Plan (MicroSAP) tool. The MicroSAP is an online tool which steps users through development of SAPs for microbiological contamination incidents for the following sampling stages; site characterization sampling, verification sampling, waste characterization sampling, and post-decontamination sampling. This document includes considerations for: lines of evidence (Section 2.0); field sampling (Section 3.0); laboratory analyses and reporting (Section 4.0); and data quality assessment and interpretation (Section 5.0).

Abbreviations and Acronyms

BGM	Buffalo green monkey [kidney cells]
BSL	biological safety level
CDC	U.S. Centers for Disease Control and Prevention
cDNA	complementary DNA
CFR	Code of Federal Regulations
CFU	colony forming unit
COC	chain of custody
Ct	cycle threshold
DL	detection limit
dPCR	digital PCR
DOT	U.S. Department of Transportation
DQI	data quality indicator
DQO	data quality objective
EIC	external inhibition control
EPA	U.S. Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
ERT	Environmental Response Team
FFA	focus forming assay
FFU	focus forming unit
GC	genomic copies
GPS	Global Positioning System
HASP	health and safety plan
HEPA	high efficiency particulate air
HMR	Hazardous Material Regulations
IAC	internal amplification control
IATA	International Air Transport Association
IFA	immunofluorescent assay
IMS	immunomagnetic separation
L	liter
LOD	limit of detection
LOQ	lower limit of quantitation
LPM	liters per minute
LRN	Laboratory Response Network
MCE	mixed cellulose filters
MDL	method detection limit
MicroSAP	Microbiolobigal Sampling and Analysis Plan tool
mL	milliliter
mm	millimeter
MPN	most probable number
MQO	measurement quality objective
MS	matrix spike
MSD	matrix spike duplicate
OD	optical density

OSHA	Occupational Safety and Health Administration
PAPR	powered air-purifying respirator
PCR	polymerase chain reaction
PFU	plaque forming unit
PNNL	Pacific Northwest National Laboratory
POC	point of contact
PPE	personal protective equipment
QA	quality assurance
QAPP	quality assurance project plan
QC	quality control
qPCR	quantitative PCR
RE	recovery efficiency
RNA	ribonucleic acid
RPD	relative percent difference
RSD	relative standard deviation
RT-PCR	reverse transcription-PCR
RV-PCR	rapid viability-PCR
SAM	*Selected Analytical Methods for Environmental Remediation and Recovery*
SAP	sampling and analysis plan
SCID	sample collection information document
TCVA	total culturable virus assay
µm	micrometer (micron)
VBNC	viable but non-culturable
VSP	visual sampling plan
WebEDR	Web electronic data review
WMP	waste management plan

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1.0 Introduction

Following a wide-area release (intentional or unintentional) of an environmentally persistent pathogen, remediation of contaminated areas might be required in-order to protect human health and the environment. Remediation efforts might include determining the extent and location of contamination (site-characterization) and whether decontamination efforts were efficacious (post-decontamination sampling [1, 2, 3]). In-order to collect the data necessary to assess the efficacy of remediation efforts, a well-defined and thorough sampling and analysis plan (SAP) needs to be developed and implemented. The SAP is important because analytical results can be used by the Incident Command, local health departments, decontamination teams, decision makers and attorneys. The decisions and actions taken will rely on the quality of data generated as defined in the SAP. While the steps of preparing and implementing a SAP might seem straightforward, there is a lack of consensus on how to address uncertainty and variability with microbial field and analytical data, thus making the SAP planning phase and data assessment challenging. The SAP should also include consideration of data quality objectives (DQO), which are used to ensure that collected environmental data are of known and documented quality for the intended use. However, the DQO process can be confusing and difficult to incorporate into the SAP. In addition, in the event a contamination incident occurs, development of SAPs might need to be done quickly, but thorough documentation of how samples are to be collected and analyzed can be time consuming.

This document summarizes elements that should be considered when planning, developing and implementing a SAP for microbial data collection and analysis. It includes lines of evidence (Section 2.0), field sampling considerations (Section 3.0), laboratory analyses and reporting considerations (Section 4.0), and data quality assessment and interpretation considerations (Section 5.0) and is intended as an informational companion to users of the EPA's Microbiological Sampling and Analysis Plan (MicroSAP) tool or its associated SAP template [4]. The MicroSAP is an online tool which guides users in developing SAPs for sampling stages of a microbiological contamination incident in which the U.S. EPA would be responsible for conducting sampling and analysis. Prior to and after the deployment of the EPA MicroSAP tool, the SAP template [4] provides an outline and instructions for the output of the MicroSAP tool. The template can also be used as a "ready to go" outline for creating a SAP and associated DQOs in the event that online access to the MicroSAP tool is not available.

This companion and the MicroSAP tool are applicable for the following phases of a microbiological contamination incident:

- Site characterization – assessment of the extent, location, and magnitude of contamination [2, 5]
- Decontamination verification – monitoring decontamination processes to confirm decontamination has been conducted according to the specified parameters. Examples include using biological indicators during fumigation, monitoring decontaminant concentrations, and documentation of process parameters [2]
- Post decontamination – assessment of the body of data generated to verify that the originally contaminated environment has been sufficiently decontaminated to meet cleanup endpoints [5]

- Waste characterization – assessment of the waste based on all available information [e.g., sampling results] to document that the waste meets regulatory requirements and any additional requirements of waste receivers prior to off-site disposal [6]

While the SAP template does not specifically consider the site clearance phase (clearing an area or building for re-use), it could be adapted to address clearance phase sampling to determine if there is any residual contamination that may pose a hazardous environment and an impediment for re-occupancy. The template could also be used for purposes of establishing a SAP for a biological contamination preparedness exercise.

There are three general phases in preparing a SAP: planning, developing, and implementing. The SAP preparation process can be iterative and might require modification at any time throughout the project. The planning phase includes development of DQOs, which define the criteria for the sampling and analysis activities, as well as supplemental plans, including a quality assurance project plan (QAPP), health and safety plan (HASP) and waste and data management plans. Information and data generated during the initial response is often collected and evaluated to help inform development of the supplemental plans. During the design and development phase, the SAP is developed, reviewed and approved prior to implementation. The implementation phase includes collection and assessment of data, and includes data to assure that the protocols outlined in the SAP and QAPP were followed according to the established criteria [7, 8].

Analyses of samples containing microbial agents have different considerations than chemical agents. These differences include genetic diversity, varying virulence, and host immunity and susceptibility [9]. In addition, pathogens have the ability to grow, to die-off, and to transmit between individual hosts, whereas chemicals can degrade or be transformed, but do not multiply or typically transmit to other individuals once they enter human tissue (although some exceptions exist) [9, 10]. Pathogens can also be endemic in the environment, can present themselves on a seasonal basis, and might respond differently to environmental treatment options than chemicals [9, 10]. Detection of pathogens in environmental matrices can also be complicated because they can interact with other species or debris. For these reasons, ideally, the analytical protocols used for detection are sensitive enough to detect the pathogen of concern, able to distinguish among different pathogens, and capable of determining viability [9].

Since 2003, U.S. Environmental Protection Agency's (EPA's) National Homeland Security Research Center has led the development of *Selected Analytical Methods for Environmental Remediation and Recovery* (SAM), a compendium of selected analytical methods to be used by laboratories when analyzing samples from a contamination event [5]. Use of the same protocols in cases where multiple laboratories are involved in sample analysis reduces confusion; permits sharing of sample load between laboratories; improves data comparability; simplifies outsourcing analytical support to commercial laboratories; and improves interpretation of results, data evaluation, and decision-making. In addition, EPA's SAM companion Sample Collection Information Document (U.S. EPA SCID) for Pathogens [11] provides information on sample collection for many pathogens of concern. However, even with the availability of these compendium documents, there are still some considerations that need to be evaluated when collecting and analyzing field samples.

Figure 1.1 provides an overview of the stages of SAP development as well as some of the critical components and considerations required.

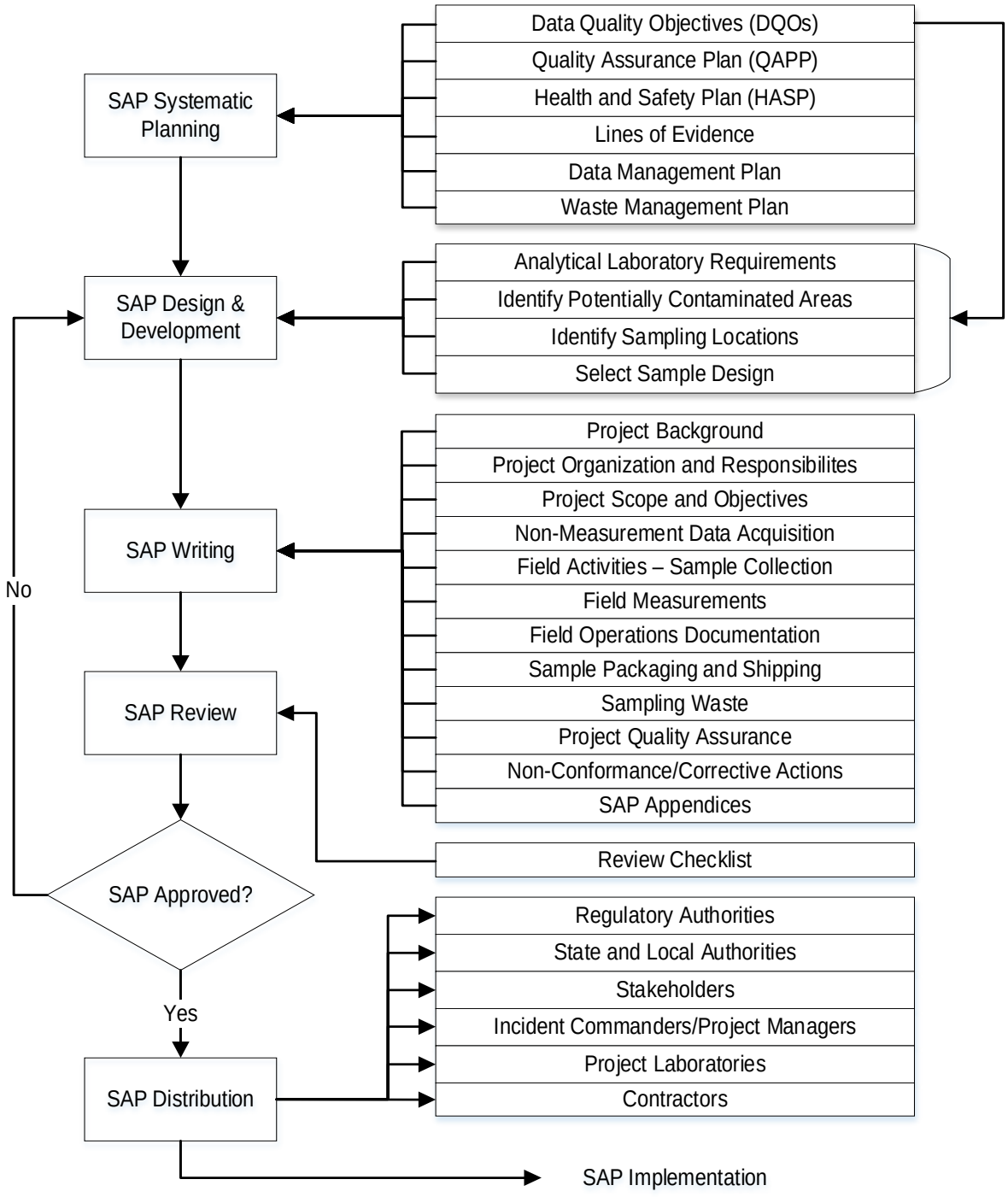

Figure 1.1 Sampling and analysis plan (SAP) development.

2.0 Lines of Evidence

Lines of evidence refer to all sources of information that are pertinent to the contaminant release, its impact, and its mitigation. During the characterization phase of a contamination incident, information is gathered or collected to characterize the specific contaminant, levels (e.g., concentration) of contamination, extent of contamination, potential public health and environmental hazards, mode of dispersion, weather, and site-specific characteristics that may increase or decrease the potential hazard. These lines of evidence include various factors that may influence contamination of a specific area and may affect decisions regarding sampling activities. When developing a SAP, multiple sources of information or lines of evidence should be considered. Such information may be useful in identifying sampling locations as well as the number and type of samples required to meet sampling objectives. Lines of evidence can potentially be used to reduce sampling and analytical demands. For example, if the lines of evidence determine that contamination is present following initial first response efforts, decision makers might decide to go directly to decontamination without taking additional samples [12]. In addition, the number of number of samples needed for waste characterization could potentially be reduced if the lines of evidence presented to waste receivers are deemed acceptable to determine which materials require further treatment prior to disposal at a waste site [6]. If lines of evidence are used to reduce or replace sampling, the DQO process should identify indicators of data quality that must be met prior to use of these data. In addition, site-specific lines of evidence might assist in developing essential QAPP elements (e.g., DQOs, data quality indicators [DQIs] and measurement quality objectives [MQOs]), determining the level of personal protective equipment (PPE) required for samplers which will be included in the HASP, and developing a comprehensive waste management plan (WMP).

All available lines of evidence should be evaluated and used to inform the development of the SAP (**Figure 2.1**).

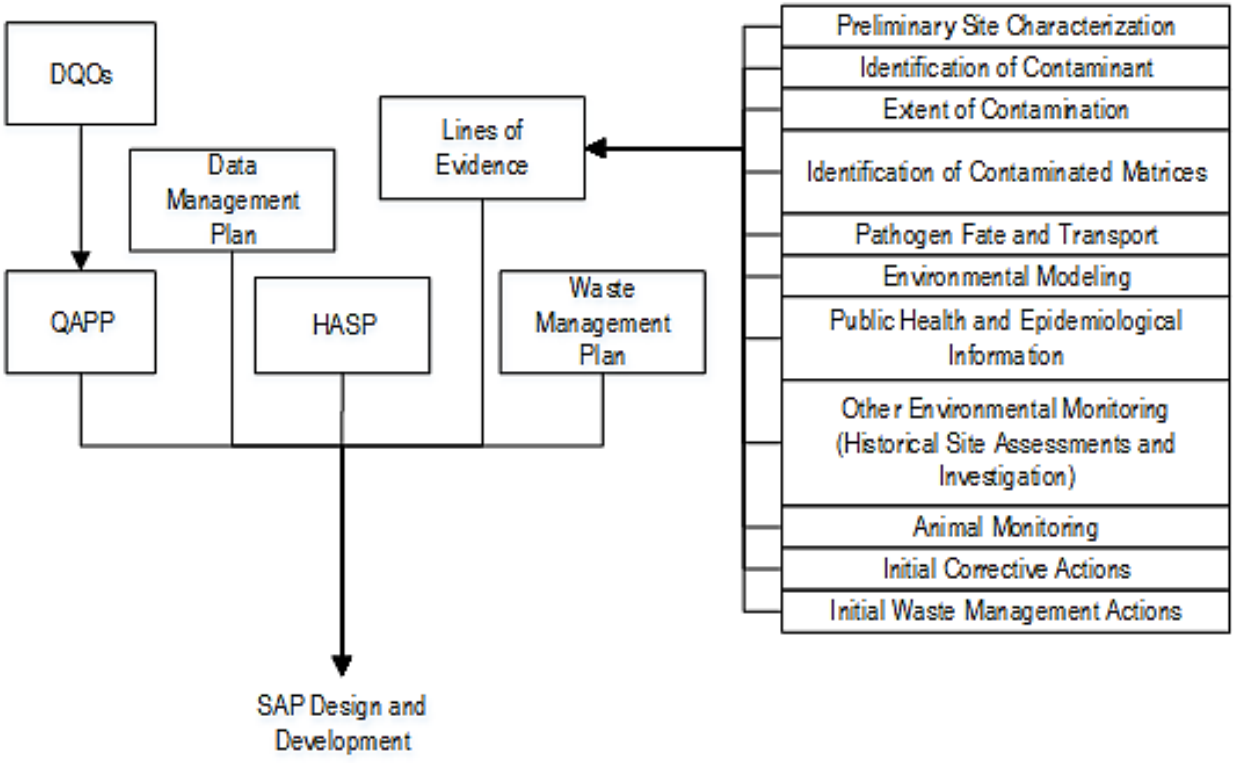

Figure 2.1 Sampling and analysis plan (SAP) systematic planning.

In addition to informing development of the SAP, these lines of evidence support decision making during data collection activities, and can include (but are not limited to):

- First responder reports including law enforcement reports
- Preliminary site characterization – provides pathogen-specific information regarding contaminant levels and distribution at or around site
- Identification of the contaminant – provides insight into appropriate analytical protocols, types of samples, PPE requirements, public health risks and decontamination strategies
- Extent of contamination – used to develop sampling strategy and identify sampling sites
- Identification of contaminated matrices – provides information regarding the types and number of samples, sample collection techniques, analytical protocols and number of samples
- Pathogen fate and transport – provides information regarding environmental persistence, and informs decisions regarding decontamination strategies and decontamination efficacy
- Environmental modeling – provides information regarding potential spread of a contaminant (e.g., direction, area) including weather conditions that may affect pathogen fate and transport
- Public health and epidemiological data – provides information regarding pathogen identification, routes of exposure and frequency/pattern of health-related events
- Environmental monitoring – ongoing monitoring efforts (e.g., water utilities, air monitoring, indoor facilities) provide information to identify the area and extent of contamination
- Animal monitoring – for pathogens, these data may be limited; in some cases, however, the contaminants cause disease in animals which can provide information for pathogen identification, routes of exposure, exposure vectors, and frequency/pattern of health-related events
- Initial corrective actions – provide information regarding appropriate decontamination strategies and efficacy (e.g., flushing, isolation, or containment) and extent of contamination (e.g., have corrective actions mitigated the overall area of contamination?)
- Initial waste management actions – provide information on waste management and issues associated with waste disposal

Once sampling and analyses have been initiated, additional types of information or data might be needed to understand and support decision-making. Examples of additional information that might be needed include information related to quality control (QC) data and considerations, or the determination of the need for additional remediation.

3.0 Field Sampling Considerations

An appropriate and efficient sampling strategy should be identified to ensure that the analytical results are representative of the contamination and are suitable for their intended purpose. Selection of an appropriate sampling strategy would be based on numerous factors, including but not limited to the following:

- DQOs
- Project goals, objectives and requirements
- Nature of the contamination incident
- Characteristics of the contaminant
- Site characteristics
- Health and safety considerations
- Appropriate sampling technique(s)
- Appropriate analytical protocols
- Laboratory capabilities

Sampling strategies are specific for the site being evaluated and should be in place prior to initiating sample collection. The sampling strategies should provide detailed site-specific instructions, including:

- Types of samples (e.g., grab, composite) to be collected or measurements to be performed
- Target pathogens and sample matrices
- Potential interferences, including environmental conditions and weather impacts
- Number of field samples to be collected
- Amount of material to be collected for each sample
- Sample locations and frequencies
- Field QC requirements (e.g., type and frequency)
- Sample preservation and holding time requirements
- Sample packaging and shipping requirements
- Documentation requirements
- Sample collection procedures and techniques
- Required equipment
- Types and sizes of sample containers

Additional information on sample preservation, holding times, and packaging and shipping requirements, are included in EPA's SAM Companion SCID for Pathogens [11] at https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=339261.

This section describes general aspects of sample collection that should be considered during development of a sampling strategy, including supplemental plans and procedures (Section 3.1), general considerations (Section 3.2), sampling techniques (Section 3.3), and sampling approaches (e.g., locations and amounts) (Section 3.4).

3.1 Supplemental Plans and Procedures

The sampling strategy developed in support of sample collection and analysis activities following a contamination incident needs several supplemental documented plans and procedures. These supplemental items are inherent to sample collection and analysis, and are necessary to support the sampling strategy. Supplemental plans and procedures are required to address quality assurance procedures, health and safety aspects, laboratory activities and analytical protocols, waste management and sample integrity.

- **Quality Assurance Project Plan (QAPP)** – The QAPP is a comprehensive document describing in detail the activities that must be implemented to ensure that the results of the data and information collected satisfies the project performance criteria [13]. The elements of a QAPP address aspects of project management, quality assurance (QA), quality control (QC), data collection, production and use [14].

  The overall goal or objectives for a project are defined by data quality objectives (DQOs). To achieve a given DQO, individual components or *data quality indicators* (DQIs) such as precision, accuracy/bias, representativeness, comparability, sensitivity, and completeness associated with the data collection process must be examined and specified. The *measurement quality objective* (MQO) is the acceptance threshold or goal that the DQI must achieve. For example, the MQO for precision could be 10% to 15% for a particular matrix and analyte. Each part of the data collection process has a DQI with an associated MQO.

  Guidance on the technical requirements of a QAPP is provided in *Requirements for Quality Assurance Project Plans* EPA QA/R-5 [14], and *Guidance on Quality Assurance Project Plans* EPA QA/G-5 [15], which presents advice intended to help its users prepare a QAPP. Case studies on how to evaluate data usability is provided in *Guidance on Data Usability Evaluation for Collected and Existing Data*, EPA QA/G-8 [16]. At a minimum, QAPPs should address the following elements:

  - Project Management – key personnel and their roles; organization chart; project description and background; data quality objectives and criteria for measurement data; documentation and records
  - Data Generation and Acquisition – sample design, methods, and handling; analytical methods; quality control; instrument and equipment inspection, maintenance, and calibration; data management
  - Assessment and Oversight – assessments and response actions; reports to management
  - Data Validation and Usability – data review, verification, and validation; verification and validation methods

- **Health and Safety Plan (HASP)** – Safety is a primary consideration for any sampling event, and should be specific to a site and incident. Each pathogen and contamination incident poses specific health hazards, so an incident-specific HASP should be available to samplers. Health and safety plans are dependent on the specific site, sampling phase (site assessment, remediation or post decontamination) and the responsible organization.

The purpose of these plans is to ensure the protection of workers, the environment and surrounding communities in a way that is consistent with requirements needed to perform operational activities. Health and safety plans should follow guidelines provided by U.S. Department of Labor Occupational Safety and Health Administration (OSHA) at: https://www.osha.gov/Publications/OSHA3114/OSHA-3114-hazwoper.pdf [17]. At a minimum, HASPs should include instructions and guidelines regarding:

- Names, positions, and contact information of key personnel and health and safety personnel
- Site- or incident-specific risk assessment (job hazard analyses) addressing sample collection activities
- Training requirements
- PPE on site and usage requirements
- Medical screening requirements (maintain confidential documents properly and securely)
- Site or incident control
- Emergency response plan, containing off-site emergency contact information such as local hazardous materials response teams or additional trained rescue personnel (29 CFR 1910.38 [18])
- Entry and egress procedures
- Spill containment
- Personnel decontamination procedures

> **NOTE:** Decontamination procedures should address personnel monitoring and decontamination during site entry and egress.

Personnel safety requirements and considerations for a particular site may extend beyond concerns related to exposure to pathogens, and can include exposures to physical and chemical hazards. General health and safety considerations that should be considered when implementing the procedures described in this document are provided in Section 3.2.1.

- **Waste Management Plan (WMP)** – A WMP that outlines waste management requirements, procedures, strategies and processes from the point of generation to final deposition should be in place prior to an incident. Ideally, a general WMP will be in place that can be used to prepare an incident-specific WMP. This incident-specific plan should address federal, state and local waste management requirements for the different waste streams, waste characterization and waste acceptance sampling and analysis, identification of waste management facilities, on-site waste management and minimization strategies and tactics, off-site waste management, waste transportation, health and safety, as well as tracking and reporting of waste sampling results. State and local waste management officials should be contacted as early in the development process as possible.

- **Analytical Protocols and Laboratories** – Analytical protocols describe the procedures that will be used in the laboratory to analyze the collected samples. These procedures often include information that can affect the procedures used by individuals collecting

samples (e.g., the types of QC samples required, sample holding times and conditions, the use of dechlorinating or neutralizing agent, and sample sizes). Analytical protocols also often include procedures that might be required to prepare various sample types prior to implementing procedures for pathogen detection and measurement. Further information regarding pathogen-specific protocols can be found in Section 7.0 of EPA's SAM [5], available at https://www.epa.gov/homeland-security-research/sam

The laboratory(ies) that will analyze the samples should be consulted to determine the following:

- Capabilities for handling the suspected pathogen and sample matrix
- Analytical procedures and allowable sample holding times
- Required sample volumes and containers
- Preferred sampling device and collection reagents (e.g., wetting agents, selective media)
- Sample packaging and shipping/delivery requirements
- QC samples
- Sample decontamination procedures
- Sample throughput (number of samples a lab can process per unit time)

- **Sample Documentation** – Careful documentation is needed to ensure data quality and is required during sampling so that all relevant sample information is recorded clearly at the time of sampling. Field sampling forms (paper or electronic) should be included in the sampling plan and should be completed by the person (or people) conducting the sampling. For field measurements and sample collection, documentation should include, but not be limited to [19]:

  - Date and time of measurement or sample collection
  - Location description and/or Global Positioning System (GPS) coordinates
  - Measurement/sample identification
  - Measurement/sample collection protocol
  - Measurement/sample collection equipment used, including identification numbers and the manufacturer name/model number, as appropriate
  - Calibration standards (e.g., pH buffers) including manufacturer, lot numbers and expiration date
  - Initial and continual calibration data and meter end checks including calibration date and initials of person calibrating the equipment
  - Measurement values for non-logging equipment
  - Sample containers (number and type)
  - Sample preservation (e.g., chemical, ice)
  - Physical description of matrix measured or sampled
  - Maps/sketches
  - Conditions that may adversely impact the quality of measurements/samples, if applicable (e.g., rain, wind, smoke, dust, extreme temperatures)
  - Photograph log
  - Sampler's name

- **Chain of Custody (COC) –** The primary objective of the COC is to create an accurate written record that traces samples from the moment of collection through receipt in the laboratory, to the eventual destruction or disposal. The COC helps avoid indefensible evidence in court by documenting samples as they pass from one person to the next. An agency must demonstrate the reliability of its evidence by proving the COC of its samples. A chronological record of who has possessed the sample(s) and of all analyses that were performed on the samples must be maintained. Following COC procedures when handling samples and data helps provide assurance that no tampering has occurred. A sampling technique must include COC considerations and instructions for the lifespan of the sample. In general, the following COC guidelines should be followed:
    - A minimum number of people should collect and handle samples and data
    - The transfer of samples and data from one person to another must be documented on the COC
    - Any unusual appearance, such as the condition of the sample and sample container, should be noted on the COC
    - COC forms must accompany samples and data
    - Samples and data must include identification that is legible and written with permanent ink

3.2 General Considerations

3.2.1 Health and Safety

Samplers must be familiar with microbiological practices used with microorganisms, including those classified as either Biological Safety Level (BSL)-2 or BSL-3 biological contaminants, especially bacteria, as they apply to sample collection, and must comply with all safety requirements included in the HASP. Samplers also should refer to *Biosafety in Microbiological and Biomedical Laboratories* 5th Edition [20] for additional safety information and requirements. Appendix A provides BSL designations for a subset of potential contaminants including bacteria, viruses, protozoa and helminths. This section provides a summary of general considerations when collecting samples to address biological contamination.

3.2.1.1 General

The potential impacts to human health from exposures to a biological agent depend on the agent and the extent of exposure, and can range from mild reactions to death. The primary hazards to sampling personnel are (1) exposure to infectious aerosols, (2) direct and indirect contact of lacerated skin with contaminated air, water and/or surfaces, and (3) accidental inoculation by sharp objects. All sample collection procedures should be performed to minimize the creation of splashes (water and liquids) or aerosols (droplets, dust and other particulates). Access to the work area should be limited or restricted.

In addition to potential harm posed to the individuals involved, unsafe conditions in the field can have an indirect impact on the resulting analytical data. The importance of training, medical monitoring and information included in a HASP should be emphasized. In summary:

- Training – The importance of training (as prescribed in 29 CFR 1910.120 [21] and Biohazards/Biological Agent Awareness training as outlined in 29 CFR 1910.120(e)(2) cannot be overstated, and is critical to ensuring appropriate safety and health conditions for sample collectors.
- Medical Monitoring – Medical examinations should include clearance for work with specific biological agents, and appropriate vaccinations and prophylactic antibiotics. Site workers also should be monitored for fatigue, stress, changes in behavior and general health during sampling events (e.g., site entry and exit), at a minimum.
- Safety and Health Officer – Safety requirements and the HASP are developed and implemented by a designated safety individual (e.g., Safety and Health Officer), who is responsible for:
  - Assessing all site activities for potential safety concerns (job hazard analysis)
  - Ensuring that personnel are informed as to the potential hazards in a sampling area and dictating the requirements for safely working in the area
  - Stopping any job or activity to protect personnel from a dangerous situation
- First Aid – First aid kits are to be available at all times during a sampling event. At least one kit should be carried in any vehicle transporting the sampling team. At least one kit also should be located at the primary sampling site office. Any cut, sore or wound provides a path for contamination to enter the body. All HASPs should require that all injuries be reported and, if required, examined by medical personnel.

3.2.1.2 Personal Protective Equipment (PPE)

Workers conducting environmental sampling for pathogens place themselves at risk for exposure to disease causing agents, and PPE should be used during all sample collection and equipment decontamination activities, as indicated in the HASP. The level of PPE used should provide appropriate personal protection and mobility for the task being performed. Samplers should familiarize themselves with the specific guidance for levels of protection and protective gear developed by OSHA provided in Appendix B of 29 CFR 1910.120 [21] (http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9767). In addition to OSHA guidance, the U.S. Centers for Disease Control and Prevention (CDC) has developed recommendations for PPE based on the BSL of a pathogen. These can be found at http://www.cdc.gov/biosafety/publications/bmbl5/ [20].

A summary of suggested PPE for BSL-2 and BSL-3 is provided below:

- Biosafety Level-2 (BSL-2): Goggles or a face shield should be used for protection against anticipated splashes, spills and sprays. Protective coats, gowns, coveralls or disposable suits should be worn to protect the body and prevent transfer of contaminated dust away from the sample site. After use, protective clothing should be placed in sealed bags for appropriate disposal or decontamination.

- Biosafety Level-3 (BSL-3): An appropriate combination of face protection (goggles or a face shield) and a respirator should be used for protection against anticipated splashes and aerosolized particles and droplets. A powered air-purifying respirator (PAPR) with full face piece and high efficiency particulate air (HEPA) filters provides both face and respiratory protection as a single unit. Wearing a properly functioning and powered air-purifying respirator with a full face-piece that is assigned to the wearer on the basis of quantitative fit testing will reduce inhalation exposures. Protective clothing such as coats, gowns, coveralls or disposable suits should be worn to protect the body and to eliminate transfer of contaminated dust away from the sample site. After use, all protective clothing should be placed in sealed bags for appropriate disposal or decontamination.

3.2.2 Sample Collection Quality Assurance

The quality of the sample and reliability of the resulting data are directly correlated to the integrity and representativeness of the sample.

3.2.2.1 Field and Media Blanks

Sampling controls are used to identify measure and control sources of contamination or error that may be introduced from the time of sample collection through sample analysis. Field and media blanks are collected for data evaluation and authentication, and are transported and analyzed along with field samples. Field blanks are generally sterile (analyte free) collection devices (i.e., swabs, wipes) or reference matrices that are exposed in the field during sample collection (e.g., a sample of contaminant-free water poured into the container in the field or a gauze wipe that is exposed to ambient conditions during sample collection), preserved and shipped to the laboratory with field samples. Field blank results can be used to identify and estimate contamination before and after sampling, during sample shipment and during sample storage prior to laboratory analysis [22]. One field blank is often collected for every ten investigative samples. Media blanks are samples that contain media (e.g., sterile agar plates, impinger collection media) that have not been exposed to the environment and are shipped with the field samples. Media blank samples should be analyzed with field samples to ensure that sample media used for sample collection are not contaminated. The laboratory should be contacted to determine number and type of field and media blank samples that are needed [23].

3.2.2.2 Replicate Samples

Replicate samples (often collected as duplicate samples) should be acquired whenever feasible. Variability in the pathogen concentration between samples is normal. Replicates provide additional QA and allow for averaging results of two or more samples to ensure accurate results and to estimate variability among replicate samples. The cost and time associated with processing additional samples and the value gained from these samples should be considered when determining the number of replicates that should be collected.

3.2.3 Additional Sample Collection Considerations

3.2.3.1 Sampling Equipment and Supplies

Dedicated sampling equipment should be used if possible to prevent cross-contamination. Sampling equipment should be clean – sterile whenever possible – and in good working condition. Sampling containers should be sterile and kept in a clean environment. The sample container's inner portion should not be handled or touched by the sampler. All reusable containers must be cleaned properly and sterilized, and proven free of quantifiable target contaminant before use. Samplers should use the necessary techniques to reduce sample contamination. After collection, samples should be placed in sealed containers to prevent contamination and to contain any sample leakage during storage and/or transport. Storage conditions, holding time (i.e., maximum time between collection and analysis), and transport procedures must also be considered [23].

It is highly recommended that sampling kits be used during sample collection, and that these kits be properly equipped, maintained and organized before deployment of sample collection personnel. Samplers should consult with project managers and the SAP to determine, in advance of sampling, what equipment and materials should be assembled. Sampling kits should contain all required sample containers, materials, supplies, forms and labels needed to conduct sample collection, decontamination, documentation and field packaging activities. Before starting field sampling activities, all necessary equipment and supplies should be identified, available, and organized into individual sampling kits.

3.2.3.2 Training

Samplers must be familiar with microbiological practices used with microorganisms classified as either BSL-2 or -3 as they apply to sample collection, and must comply with all safety requirements (HASP) associated with each specific collection event. All sampling personnel should have training using the sampling equipment and techniques defined in the SAP. Samplers must be trained in collection and handling of samples suspected of containing the contaminants of concern, familiar and recently practiced in the sample collection methods to reduce sample contamination and assure data quality, up to date regarding medical screening requirements, and approved for site entry.

3.2.3.3 Sampling Teams

Any sampling effort requiring the collection of multiple samples should involve sampling team(s) consisting of at least two personnel. Additional personnel may be required for complicated sampling efforts or when site-specific hazards may be encountered. Individual team members should be trained to assume specific activities or duties related to the sampling effort. For example, a two-person sampling team might utilize one person to collect samples while the other coordinates the supply of sampling materials (e.g., sample containers and sampling devices) and is responsible for sample inspection, documentation, packaging and labeling. This team approach can reduce errors, sample contamination, and the time required for sample collection and adds an additional layer of QA to the overall process. Importantly, well organized and trained sampling teams also provide an additional level of safety.

3.2.3.4 Pathogen Recovery

The impact of sample collection techniques and sample matrices on the recovery of pathogens from samples has not been thoroughly evaluated. Importantly, surface and air sampling techniques (**Tables 3.1** and **3.3**) have been evaluated primarily for the collection of *Bacillus anthracis* (*B. anthracis*) spores [24], which may not necessarily represent the recovery that can be achieved when addressing other pathogens. In contrast, recovery of target pathogens using simple grab sampling techniques, such as those used to collect soil (**Table 3.4**) and water (**Table 3.2**) samples is generally efficient and less prone to variability across multiple pathogens.

Because no device/matrix combination is capable of achieving 100% recovery of a pathogen, pathogen loss due to the sample collection technique should be considered to mitigate or understand bias in the analytical results [24]. Several factors that can potentially affect recovery include the level of contamination present, type of sample matrix (e.g., surface, air, water), the size of the area sampled (e.g., surfaces that require the use of multiple swabs or wipes), the type of sampling device used (e.g., the material characteristics of the device, the use of wet versus dry sampling devices), and the characteristics of the organism being sampled. A discussion of factors affecting pathogen recovery is presented in Section 5.2.1.

3.2.4 Sample Shipping and Handling

The integrity of samples collected in the field should be preserved in order to ensure that samples are not compromised prior to sample analysis in the analytical laboratory. Preservation requirements will depend on the pathogen being analyzed, the type of sample or sample matrix, and the analytical protocol that will be used [23].

Biological samples may be subject to U.S. Department of Transportation (DOT) Hazardous Material Regulations (HMR) (see HMR, 49 CFR Parts 171-180) [25, 26] and the CDC's Select Agent Program requirements and rules (42 CFR 72.6) [27]. In addition, air shipments also must comply with International Air Transport Association (IATA) Dangerous Goods Regulations [28]. Personnel with approved DOT/IATA training must perform all shipments of potentially hazardous materials, and such shipments should be packaged, labeled and shipped according to the appropriate ground or air regulations [11].

Some aspects of sample transport are constant across nearly all situations. Samples should be transported to the laboratory as quickly as possible to minimize pathogen degradation. There are important considerations for maintaining sample integrity when shipping samples, including sample preservation and storage, which are briefly mentioned below.

Sample integrity elements to consider include, and are not limited to the following:

- Temperature control (organism specific)
- Holding times and conditions
- Preservation chemicals
- Container compatibility
- Labeling/seal integrity
- Volume (loss)
- Contamination control

More information regarding pathogen- and matrix-specific requirements (including sample preservation) can be found in U.S. EPA SCID for Pathogens, 2017 [11]. The adherence to sample preservation and holding time limits is critical to ensuring valid analytical results. If possible, samples that exceed holding time limits or are improperly preserved should not be analyzed. If additional samples cannot be obtained, analytical results of such compromised samples should be qualified, and the issue documented and understood by data users.

3.3 Sampling Techniques

The tables throughout this section briefly describe potential sampling techniques, their potential uses, and corresponding considerations or potential problems. References that can be consulted for additional information are listed in the last column of each table; full citations for these references are provided in Section 6. Additional information regarding sampling equipment, containers, and preservation requirements to address pathogen contamination is provided in EPA's SAM Companion SCID for Pathogens at https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=339261 [11].

Table 3.1 Particulate Sampling Techniques

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
Swab Sampling	Macrofoam swab	• Commonly used for *Bacillus anthracis* collection (validated for use on stainless steel surfaces) • Used for small (less than 100 cm^2) surfaces and hard to reach locations less than 4 in^2, such as crevices, supply air diffusers, corners, air return grills and difficult to reach locations • Wet swabs perform better than dry swabs • Commonly processed by Laboratory Response Network (LRN) laboratories	• Heavy contamination can easily overwhelm the small surface of the swab; the size of the sampling area should be reduced in these instances • Swabs may be damaged by or have difficulty collecting samples from non-flat or non-porous surfaces	CDC, 2012 [29]; Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]; Hodges *et al*., 2010 [31]; Rose *et al*., 2004 [32]; Silvestri, 2016 [24]; U.S. EPA 2013 [33]
Wipe Sampling	Cellulose Sponge-sticks	• Validated for *B. anthracis* on steel surfaces and a preferred sample collection protocol • Used to sample small, non-porous surfaces, such as walls, floors, table tops • A template (e.g., 10 × 10 inch) can be used for quantitative analysis, to cover an area of known size per the validated method in order to determine recovery, and for statistical purposes.	• Limited use for reaching into porous surfaces, crevices and depressions • The collection medium is dependent on the pathogen and the surface or material to be sampled	CDC, 2012[29]; Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]; U.S. EPA 2013 [33] U.S. EPA, 2017 [34]

Table 3.1 Particulate Sampling Techniques

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
	Gauze wipes	• Can be sensitive to trace levels of biological agents and require little material to be sampled • Used to sample small, non-porous surfaces, such as walls, floors, table tops • A template (e.g., 10 × 10 inch) can be used for quantitative analysis, to cover an area of known size to determine recovery, and for statistical purposes. • Wet wipes perform better than dry wipes	• Limited use for reaching into porous surfaces, crevices and depressions • The collection medium used is dependent on the pathogen and the surface or material to be sampled	CDC, 2012 [29]; Chattopadhyay, 2017 [11]; Silvestri, 2016 [24]; U.S. EPA 2013 [33]
Vacuum Sampling	Vacuum filter and high-efficiency particulate air (HEPA)-filtered vacuum	• Uses HEPA vacuum and collection filter for sampling • Can be used to collect biological material and dust deposited onto porous surfaces such as carpets, fabrics, draperies and other porous surfaces • Can be used for bulk sampling	• Could be difficult to identify the biological agent present in one large bulk sample • Cross contamination can occur from improper sample collection • Samples from the floor should be collected before foot traffic	Calfee *et al*., 2013 [35]; Calfee *et al*., 2014 [36]; Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]; U.S. EPA 2013 [33]
	Micro Vacuum with mixed cellulose filters (MCE)	• 37 mm 0.8µ MCE filter in 3-stage plastic cassette and high flow pump for sampling • Used to collect biological material and dust deposited onto porous surfaces such as carpets, fabrics, draperies and other porous surfaces • Used for bulk sampling	• Could be difficult to identify the biological agent present in one large bulk sample • Cross contamination can occur from improper sample collection • Samples from the floor should be collected before foot traffic • The cassette may become electrostatically charged during collection	Calfee *et al*., 2014 [36]; Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]; U.S. EPA, 2017 [34]

Complete references are in Section 6 of the report.

Table 3.2 Water Sampling Techniques

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
Grab Sampling	Sterile leakproof container, filtration device	• Drinking Water and other water types (e.g., surface waters, ground water, wastewater) • Can be collected as grab or composite samples	• Improper sample collection can result from the use of contaminated equipment, disturbances to the waterbody (e.g., muddy water) and collection of an insufficient sample volume • Large volumes of water may need to be collected (e.g., 100 L) • Samples may need to be dechlorinated prior to analysis	Chattopadhyay, 2017 [11]

Complete references are at the end of the report.

Table 3.3 Aerosol Sampling Techniques

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
Aerosol Sampling	Air filter samplers (designed to collect bacteria and viruses)	• Gelatin filters (3µm pore size) are used to collect pathogens for microscopic, culture, and other assays. High moisture content prevents sample desiccation. Collection times range from 20 to 45 minutes at 2 to 3 liters per minute (LPM). • Mixed cellulose filters (MCE) inert filters are designed to collect pathogens for microscopic and DNA assays. Collection times range from 1 to 8 hours at 2 LPM. Estimated extraction efficiency is 99%. • Teflon® (polytetrafluoroethylene) filters (1µm pore size) designed to collect pathogens for microscopic and DNA assays. Collection times range from 1 to 8 hours at 1 to 2 LPM. Estimated extraction efficiency is 94%.	• Avoid high temperatures and humidity during air sampling • Consult with laboratory for filter selection	Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008[30]
	Impactor samplers	• Airborne particles are drawn through an impactor and collected on solid or adhesive mediums (e.g., agar plates) • Many varieties available • Widely used as a standard for enumerating viable particles in aerosol samples	• Require proper sterilization before use • Require trained personnel to operate • Negatively impacted by high temperatures and humidity • Agent specific medium may have a short shelf life	Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]
	Impinger (wet air) samplers	• Airborne particulates are collected in liquid medium • Many impinger varieties available • Samples can be analyzed using various analytical protocols	• Require proper sterilization before use • Require trained personnel to operate • Negatively impacted by high temperatures and humidity • Pathogen-specific medium may have a short shelf life	Chattopadhyay, 2017 [11]; Emanuel *et al*., 2008 [30]
	Aggressive air samplers	• Can be used to confirm a negative finding of contamination following decontamination • Involves vigorous agitation of surface particulates in a space to aerosolize particles and use of air samplers (filter, impinger, impactor) at specified time intervals	• Rooms to be sampled need to be air tight to contain potential viable contamination • Conducted as the very last step in the clearance process	U.S. EPA, 2013 [33]; U.S. EPA, 2017 [34]

Table 3.4 Soil, Solid and Waste Sampling Techniques

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
Bulk, soils and solids sampling				
Soil Sampling	Sterile sample container such as a 50-mL tube with cap	• Soil samples can be collected directly into container	• Cross contamination can occur from improper sample collection • Contaminants in soil (organic matter, disinfection agents and non-target organisms) can interfere with the analytical process and requires communication with the laboratory prior to analysis • Soil samples may contain rocks or other sharp objects that could compromise the integrity of the sampling container	U.S. EPA/USGS, 2014 [22]
Bulk and Solid Sampling	Sterile sample container varies by matrix type	• Bulk sample material is typically transferred into container using scoop or trowel • Contaminated materials including powders	• Could be difficult to identify the biological agent present in one large bulk sample • Cross contamination can occur from improper sample collection	Emanuel *et al*., 2008 [30]; U.S. EPA, 2017 [34]
Waste sampling				
Post-Decontamination Waste Water/ Liquid Waste	Sterile sample container varies by sample volume (e.g., bottles, carboys)	• Liquids generated during decontamination (e.g., waste bleach solution) • Representative samples of liquid waste stream collected using composite sampling for characterization • Can be collected as grab or composite samples • Large volumes of water may need to be collected (e.g., 100 L) • Sample may need to be neutralized prior to analyses	• Cross contamination can occur from improper sample collection • Sample should be collected throughout the waste stream • Depending on the waste stream, additional parameters may require evaluation • Large volumes of water may need to be collected (e.g., 100 L) • Sample may need to be neutralized prior to analysis	U.S. EPA, 2017 [34]

Technique	Sampling Device	Description and Potential Uses	Potential Problems or Considerations	References
Solid Waste	Bulk materials, bulk materials cut into smaller pieces and placed in jars, solid wastes sampled from containers, surface sampling of solid waste	• Wastes generated during decontamination, waste items not decontaminated, and waste items damaged during decontamination (e.g., PPE, HEPA filters, ceiling tiles, carpets, furniture, materials difficult to decontaminate)	• Need to check with laboratory to determine what samples they will accept • Cross contamination can occur from improper sample collection • May need to collect samples of contamination on waste materials using a sampling technique such as wipes or Sponge-sticks • Depending on the waste stream, additional parameters may require evaluation	U.S. EPA, 2017 [34]

Complete references are in Section 6 of the report. PPE, personal protective equipment

3.4 Sampling Strategies

The following three primary categories of sampling strategies are discussed in this section: judgmental sampling (also referred to as "targeted" sampling), probabilistic sampling, and combined judgmental and probabilistic sampling [2, 37]. While there may be opportunities to implement these sampling strategies, all three require large numbers of samples that could overwhelm available resources.

Table 3.5 provides a brief description of each of the three sampling strategy categories, along with some of the corresponding advantages, disadvantages and considerations. Each category is discussed in more detail in Sections 3.4.1 through 3.4.3. If more information is needed, please refer to Emanuel *et al*., 2008 [30], EPA QA/G-5S [37], EPA QA/G-9R [8], the LLNL 2012 Remediation Guidance for Major Airports after a Bioterrorist Attack [2] and U.S. EPA *Guide for Development of Sample Collection Plans for Radiochemical Analytes in Environmental Matrices Following Homeland Security Events* [38].

Table 3.5 Primary Categories of Sampling Strategies [References 2, 8, 30 and 37]

Sampling Design	Description of Use	Advantages	Disadvantages
Judgmental Sampling	• Selection of sample numbers and locations considered most likely to be contaminated based on professional judgment or knowledge of the feature or condition under investigation • Samples collected next to areas of known contamination, high traffic areas and surfaces likely to be encountered by occupants • Also used for visible (bulk) materials • Used during characterization and clearance phases of a response	• Very efficient with knowledge of the site • Can be less expensive than probabilistic designs • Easy to implement • Can quickly help determine if the area is contaminated • Good way to determine if contamination is widespread or behaves as expected • May require collection of fewer samples than probabilistic sampling • Is incorporated in the Visual Sampling Plan software [41, 42]	• Limited statistically-based conclusions can be made about the target population • Limited quantitative based confidence statements can be made about the population • Conclusions about the target population are limited and depend on professional knowledge • Dependent on professional (subjective) judgment to interpret data relative to study objectives • An optimal design is dependent on an accurate conceptual model
Combined Judgmental and Probabilistic Sampling	• Targeted sampling results help ensure sample collection from locations are the most likely to be contaminated while probabilistic sampling results help protect against the possibility that contamination exists in less likely areas • Results from both sampling protocols are combined using Bayesian statistical protocols to make statistical confidence statements (See also [39, 40]) • Negative sample results during characterization or clearance situation before decontamination using targeted sampling can be augmented with results from probabilistic samples • Following decontamination, targeted samples are collected in locations likely still contaminated (spots contaminated before decontamination) and are augmented with additional probabilistic samples	• Can provide increased confidence in the conclusion that there is no detectable contamination • Combines advantages of probabilistic and targeted sampling approaches • Is incorporated in the Visual Sampling Plan software [41, 42]	• Negative perception of inferring confidence compared to statistically based protocols • Methods do not currently account for situations when the false negative rate exceeds 0

Sampling Design	Description of Use	Advantages	Disadvantages
Probabilistic Sampling	• Apply sampling theory and involve random selection of sampling units and each sample location has a known probability of selection • May be required to achieve an acceptable level of confidence that no detectable contamination exists within the target area • Appropriate for use for quantitative comparisons with risk-based exposure levels and binary detect/no-detect situations	• Can be used when little or nothing is known about where a release occurred • Provides the ability to makes statistical inferences • Uncertainty can be calculated with associated estimates • Can handle decision error criteria • Can supplement judgmental findings to provide greater confidence in findings • Is incorporated in the Visual Sampling Plan software [41, 42]	• More difficult, time consuming and expensive to implement because random locations may be difficult to locate • Number of samples required is dependent on the desired level of confidence • An optimal design is dependent on an accurate conceptual model

Full references are in Section 6 of this report.

3.4.1 Judgmental Sampling

Judgmental sampling involves sampling at locations that have the greatest chance of detecting contamination based on the lines of evidence and best professional judgment [30]. This strategy can help the Incident Command prioritize sampling. Sampling directed by the SAP will be conducted in a stepwise fashion, prioritizing and addressing DQOs as data are received and evaluated. As data becomes available, it may influence decision making and future priorities, which in turn may affect the SAP. Thus, the SAP is a dynamic plan that changes based on new data that is constantly being generated and provided to the Incident Command and decision makers. Judgmental sampling can be the best way to find contamination if based on reliable information and the contaminant behaves as expected. However, making statistical inferences from a non-random sample set is not defensible, unless the DQO specified, for example, is something like "Sample to see if there is any contamination in this area." In this case, if at least one of the judgmental samples comes back positive the inference is that there is contamination somewhere in the area of interest. However, if all the judgmental samples come back negative it is not appropriate to make inferences about the area of interest (e.g., free of contamination) with any quantifiable level of confidence.

3.4.2 Probabilistic Sampling

To be able to make a general statement (inferences) about contamination in an area of interest, samples taken in that area must be randomized. Randomized sampling is one of the underlying assumptions that must hold true in order to reach a valid and defensible inference based on the results of that sample. To the degree that the sample was not random (biased in some way), the inference will be biased and decisions made based on the information will also be biased (flawed).

The area of interest is made up of an almost infinite number of possible sampling locations. The sum of those locations are considered the target population. Samples must be taken in a random fashion from the target population to enable valid inferences to be made about that whole population (in this case the whole area of interest). To characterize the entire area of interest every possible sample location could be sampled; however, that would be costly in both time and resources. A limited number of randomly located samples can be taken to reduce cost and still be representative of the entire population of locations within that area of interest.

In general, probabilistic sampling approaches include (1) simple random sampling, (2) stratified sampling, (3) systematic sampling, (4) ranked-set sampling and (5) adaptive-cluster sampling [33]. A table summarizing these probabilistic approaches is provided in Appendix B. An essential feature of probabilistic sampling is that each potential sampling location has a known probability of selection.

3.4.3 Combined Judgmental and Probabilistic Sampling

Most sampling is really a combination of judgmental and probabilistic sampling. Sampling within an area of interest could be labeled as solely probabilistic; however, the area of interest is selected based on judgment. (i.e., what makes this area worth sampling must be based on some evidence that the area of interest was contaminated). Thus, the probabilistic sampling strategy is really a combination of judgmental and probabilistic sampling. In a similar way, subareas within

the area of interest can be defined based on lines of evidence or decision units. A decision unit is a subarea of the area of interest for which a DQO has been developed. An example of one such DQO might be, "to determine if Subarea A has contamination." When a decision is made from the data collected from that area, it would affect the whole decision unit. For example, if any samples come back positive the decision would be to decontaminate the whole area of interest. If a random sampling method is used in Subarea A, then an inference about all of Subarea A could be made based upon the results. On the other hand, if sample locations were judgmentally determined then a valid inference about all of Subarea A could not be made from those sample results.

The U.S. Centers for Disease Control and Prevention (CDC) and the Department of Homeland Security sponsored the development of an approach [39 and 40] that includes both targeted and probabilistic sampling. The sampling approach ensures that samples are collected from locations perceived as most likely to be contaminated (through targeted samples) while protecting against the possibility that contamination may exist in less likely areas (through probabilistic samples). The approach uses Bayesian statistical methodology to combine results from targeted and probabilistic samples to make statistical confidence statements, and has been incorporated into the Pacific Northwest National Laboratory's (PNNL's) Visual Sampling Plan (VSP) software [41] and VSP Development Team [42]).

This sampling approach might be appropriate in situations where additional confirmatory results might be warranted or requested, such as the following:

- During characterization, targeted sample results that are all negative can be augmented with the results of probabilistic samples.
- During decontamination, targeted samples are collected from locations that are considered more likely to still be contaminated, and the results can be augmented with results from probabilistic samples.

In both situations, the number of probabilistic samples should be selected based on the number of targeted samples so that, if targeted and probabilistic sample results are negative, a statement (an inference) can be made that there is X% confidence that at least Y% of the area does not have detectable contamination (for presence-absence data) or has contamination below an acceptable contamination level.

For the combined approach, the following input parameters affect the required number of probabilistic samples (providing available resources can support the number of samples generated by this model): (1) the percent confidence (X%) desired, (2) the minimum percentage (Y%) of the area that can be stated to not contain detectable contamination, (3) the number of targeted samples collected, (4) how much more likely it is that a targeted sampling location contains detectable contamination than a probabilistic sampling location, and (5) the expected *a priori* probability that a targeted sample result will reveal contamination. Another parameter that affects the required number of probabilistic samples is the false-negative rate, which can vary depending on the (1) sampling protocol, (2) surface material sampled and (3) surface concentration of pathogens. Because the combined approach has not yet been extended to account for situations that result in a false negative rate exceeding 0, the X%/Y% clearance statement that can be made is "X% confidence that at least Y% of the area does not contain

detectable contamination." If the cost in time or resources to process the number of samples recommended is prohibitive, then reducing the confidence level would be indicated.

An important assumption of the mathematical model used in the combined judgmental and probabilistic approach is that the decision area can be divided into areas of high and low probability of contamination (high- and low-probability areas need not be contiguous). The model assumes that all high-probability areas are sampled judgmentally. Consequently, fewer probabilistic samples are necessary when more judgmental samples are collected or when judgmental sampling locations are more likely to contain detectable contamination. Fewer probabilistic samples also are necessary as the probability that a targeted sample result will reveal contamination decreases.

3.4.4 Composite Sampling

In many biological contamination responses, resources are limited, especially regarding laboratory capacity (i.e., the number and availability of laboratories with appropriate analytical capability) and with the ability to accomplish a quick turnaround time for sample results. Based on these limitations, composite sampling can be considered instead of discrete sampling (collection of separate samples from each sampling location) [43]. The decision to collect discrete or composite samples should be based on laboratory throughput, the size of the area requiring sampling, and the applicability of composite sampling to effectively meet the DQOs. During some incidents, both composite and discrete sampling may be appropriate.

In composite sampling, volumes of material from discrete samples are physically combined into a single homogeneous sample or a single collection device is used to collect samples from multiple locations (e.g., wiping or vacuuming more than one location). Composite sampling can be used in conjunction with judgmental, probabilistic, or combined judgmental and probabilistic sampling. It is most cost effective when analytical costs are large relative to sampling costs; it demands, however, that there are no safety hazards or potential biases associated with the compositing process. The main advantage of composite sampling is that it reduces the number of samples that require processing and analysis. The primary disadvantage is that the exact location of a positive result may not be known because the composite sample covers multiple areas. One of the best applications of composite sampling is to restrict the multiple sample locations in each composite sample to the same decision area. If any one of those composite sample locations, or any other sample within that decision area, return a positive result then the whole decision area will be treated collectively. Thus, not being able to determine which, or how many, location(s) were positive will not matter.

One issue with composite sampling that should be addressed in the SAP is the dilution affect that may happen. If the concentration of pathogen within the sample area is uniform, then there is no problem. However, if it turns out that the concentration is not uniform in a way that only one (or other small number) of the composite sample locations has a very low but detectable level, then that one (or small number) sample location(s) will be added to the other sample locations which may move the otherwise detectable level to below the detectable level through dilution. This dilution affect would have to be addressed in the SAP when calculating how the DQO will be reached through composite sampling.

4.0 Laboratory Analyses and Reporting Considerations

EPA's SAM [5] is a repository of analytical methods that have been selected for analyses of environmental samples following a contamination incident. Laboratories, utilities and government staff can use the document to identify protocols for evaluating the nature and extent of pathogen contamination, evaluate the concentration of contaminant at different locations, and assess decontamination efficacy. This repository of methods is organized by contaminant and sample type, and includes protocols that target specific microorganisms and vary with respect to technique and intended use.

To reflect the methods included in SAM [5], the guidance in this chapter focuses on laboratory analysis and reporting considerations associated with culture- and molecular-based protocols. Culture-based protocols are designed to promote growth, which is an indicator of viability, and may support the isolation, identification and quantitation of the contaminant. Molecular-based protocols are used to detect nucleic acids (polymerase chain reaction [PCR] assays) or proteins (e.g., immunological verification, immunomagnetic separation [IMS]) and are usually more rapid than culture-based protocols; most, however, do not confirm the viability of the pathogen. For some microorganisms, both culture and molecular protocols may be available, and variations such as rapid viability-PCR (RV-PCR) may be available to address both detection and viability.

When identifying analytical protocols for use following a contamination incident, the availability of laboratories with capability and capacity to analyze samples for a particular pathogen or using a particular protocol or technique needs to be considered. For example, only a limited number of laboratories have the capability and capacity to analyze large-volume water samples for select agents. Ideally, response planners should identify laboratories based on the pathogen and then work with the laboratories to identify the appropriate analytical protocol(s) to meet the incident-specific requirements. Some laboratories will be familiar with pathogen-specific protocols and may have demonstrated proficiency (e.g., initial and ongoing proficiency testing) with these protocols.

EPA has multiple resources to help identify laboratories, including:

- Compendium of Environmental Testing Laboratories (Lab Compendium) – A searchable database of laboratories that have the capability to analyze environmental samples for chemical, biological or radiochemical contaminants; To register for access to the Lab Compendium, visit https://cfext.epa.gov/cetl/lblogin.cfm?action=None
- EPA Headquarters – Environmental Response Laboratory Network (ERLN) Helpline (703) 461-2400; https://www.epa.gov/emergency-response/environmental-response-laboratory-network
- EPA Regional Laboratories – May be able to provide analytical support or identify appropriate laboratories. https://www.epa.gov/aboutepa/regional-science-and-technology-rst-organizations#branches
- Accessing Laboratory Support – Interactive training that walks the user through the process of identifying laboratories. https://www.epa.gov/waterlabnetwork/accessing-laboratory-support

Other resources include state agencies, and state and local laboratories.

The guidance provided in Sections 4.1 through 4.3 is intended to inform the development of a SAP to meet incident-specific requirements for generating laboratory data.

4.1 Laboratory Sample Handling, Preparation and Reporting

When developing the SAP, the time required from sample collection to completion of analysis includes sample transport, sample handling (receipt and processing), analytical time and data reporting. Protocols for detecting or enumerating microorganisms can vary widely, and the time it takes to complete the analysis is protocol dependent. In general, whereas molecular-based assays are considered rapid, taking a few hours to a day, culture-based assays are more time consuming, and can require up to several days or weeks. Laboratory capacity (i.e., the laboratory's ability to analyze multiple samples simultaneously) will also impact the time required for receipt of analytical results. In general, results can be expected within several days (bacterial culture) of sample delivery to the laboratory (depending on the number of samples), or several hours in special circumstances (e.g., PCR). However, some pathogens (viruses) require lengthy incubation times (e.g., 30 days). Considerations when calculating time to results are discussed in Sections 4.1.1 through 4.1.4.

4.1.1 Sample Receipt

Once samples are received in the laboratory, sample receipt documentation, including COC forms, serve as a record of sample integrity and preservation. On this form, the receiving laboratory should note the date and time received; receiving personnel; and the temperature, quantity and condition of the samples, as well as other pertinent shipping information. The laboratory should notify the point of contact (POC) if samples do not meet storage or shipping requirements (e.g., temperature, pH, holding times) or if the integrity of any sample is compromised (e.g., broken custody seals, leaking or broken sample containers).

4.1.2 Sample Storage and Disposal

Environmental samples should be analyzed for pathogens as soon as possible upon receipt in the laboratory. If analyses cannot begin as soon as the samples are received, samples need to be stored in a manner that will ensure their integrity and not compromise analytical results. The laboratory should have the facilities to store all samples appropriately, and the means to ensure the samples will not exceed their preservation, temperature or holding time requirements. Microbial samples are typically stored at <10°C and not allowed to freeze; however, the analytical protocols that will be used should be consulted for any specific instructions regarding storage conditions, including temperature. For example, samples analyzed for *Vibrio cholerae* should be stored at room temperature.

During development of the SAP, a decision will need to be made regarding whether samples will be archived (i.e., stored in case additional analyses may be needed) and, if so, for how long prior to disposal. The laboratory should be consulted to ensure they have sufficient space to archive samples and an appropriate system for disposal of residual samples and analytical waste. Proper disposal of samples, along with media, filters and other contaminated materials, will be included in the laboratory fee. Accredited laboratories will have appropriate procedures in place for the disposal of all materials they are certified to handle (e.g., if a laboratory is qualified to

process BSL-2 agents, then they will have an appropriate waste stream in place and all handling, including disposal, will be included in their cost estimate). All contaminated and potentially contaminated materials must be disposed of as appropriate for the threat level of the contaminant. If the contaminant requires special precautions (such as a select agent), or if particularly large volumes of contaminated material need to be disposed of, this process can become expensive.

For additional information on disposal of samples and associated analytical waste, refer to EPA's *Laboratory Environmental Sample Disposal Information Document* [44].

4.1.3 Sample Preparation

Sample preparation procedures are generally determined by the sample type and protocol used for analysis. However, in some instances, it may be necessary to use an analytical protocol that has been developed for a specific pathogen and sample type, for the analysis of other sample types, in these cases, different sample preparation procedures may be required. For example, analysis of a particulate sample collected using a vacuum filter may require use of an analytical protocol developed for water samples, coupled with an appropriate sample preparation procedure (e.g., extraction in buffer or growth medium). Importantly, not all of the protocols included in SAM [5] have been evaluated across all sample types, and preliminary testing may be required to determine if the analytical protocol can be used for the sample type of concern. Analytical laboratories, the technical contacts listed in SAM [5], and other technical subject matter experts may provide guidance on protocol applicability and sample preparation requirements.

In some situations, samples might require a unique sample preparation procedure depending on the target pathogen or the level of contamination. Concentration of large-volume water samples using ultrafiltration, for example, can be used to concentrate pathogens in order to improve detection capability for pathogens that may be present in very low concentrations [45]. Virus samples (i.e., filters) also require unique processing and sample preparation procedures including filter elution, virus concentration (flocculation) and preparation of samples for final analysis (e.g., cell culture or nucleic acid extraction/PCR) [46]. These specialized sample preparation procedures will extend the overall analytical time (1-2 days) and, due to their complexity, will likely impact laboratory capacity.

4.1.4 Laboratory Data Reporting

Requirements for laboratory data reporting should be described in the SAP and in the request for proposal for laboratory services. The requirements should consider data format and the procedures for data submission. Statistical support personnel should be consulted to ensure sufficient data are collected (e.g., type and number of samples) and how these data are reported, since these decisions will facilitate timely analyses of the data. If the results are to be shared with another agency, the agency should be contacted or their available guidance consulted to facilitate sharing. Some general considerations in planning for laboratory data reporting procedures include:

- Will quantitative or qualitative data be reported?
- Will a standardized electronic data deliverable be used to report laboratory results?
- Will field log sheets and COC forms be included in data submissions?

- How will the results of QC samples be assessed and reported, and how will they be tagged to the analytical results for the field samples. For example, will the amount of pathogen or surrogate spiked into a matrix spike (MS) sample be reported along with the analytical result or the percent recovery?
- How will results that are non-detect, below the quantitation range, or above the range of quantitation, or that are ambiguous be reported?
    - Results below the limit of detection:
        - Qualitative results − May be reported as present (or absent) or as detect (or non-detect), depending on specific protocol reporting requirements. If protocol sensitivity is known or can be demonstrated, negative results (absence or non-detects) may be reported as less than the limit of detection (LOD) for that protocol (e.g., <1 colony forming units [CFU]/100 mL).
        - Quantitative results − Reporting quantitative results as non-detect (e.g., 0) can be problematic since the actual result may not be zero; when possible these results should be reported as less than the protocol's LOD (e.g., <10 CFU/100 mL or < 1.8 most probable number [MPN]/100 mL). A general assumption for microbiological culture protocols is that the LOD is one viable organism per volume of sample analyzed. ***Note****: Caution should be used when interpreting microbial count data that fall below the established detection limit (DL) or limit of quantitation (LOQ) for a specific protocol particularly in situations where low level contamination is expected (e.g., post-decontamination).*
    - Quantitative results within the analytical range:
        - Actual plate counts should be reported according to protocol-specific requirements. For example, if there are multiple plate counts within the acceptable range (e.g., 20 − 80 CFU), the counts would be averaged and that value would be reported per volume analyzed (e.g., 200 CFU/100 mL). *Note: Interpretation of plate counts that are below the LOQ (e.g., 20 CFU) but are above the LOD, could vary depending on type of data, incident and site-specific objectives, and response phase [24]*.
        - For most probable number (MPN) estimates the value would be taken from the MPN table and reported as MPN/100 mL (e.g., 280 MPN/100 mL). The MPN value may need to be adjusted based on the volume of sample analyzed, if volumes other than 10, 1.0 and 0.1 mL are used. For example, if 0.01, 0.001, and 0.0001 volumes were assayed, the MPN reported would be 2.8×10^3 MPN/100 mL instead of 280 MPN/100 mL.
    - Results above the analytical range: Quantitative results should be reported as greater than the upper limit of the range of quantitation (e.g., >1600 MPN/100 mL).
    - Ambiguity: Some PCR protocols rely on several target gene sequences for pathogen identification. For example, there are three gene markers for *B.*

anthracis. If a sample is not positive for all three markers, a decision regarding interpretation of the results as positive or negative will need to be made.

The following sources may be of use in considering data reporting preferences or requirements:

- *Requirements for Environmental Response Laboratory Network Data Submission* [47]
- *Method Validation of U.S. Environmental Protection Agency Microbiological Methods of Analysis* [48] (pg. 21)
- *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples* [49]
- *Guidance for Quality Assurance Project Plans, EPA QA/G-5* [15]

4.2 Types of Analytical Protocols

Selection of an analytical protocol is driven primarily by the availability of optimized and validated methods for the combination of pathogen and sample type under consideration. The methods identified in EPA's SAM [5] are organized by contaminant and sample type to facilitate this process. Cost, time, laboratory capability (not all laboratories will be qualified to perform all protocols), the number of samples and other factors also contribute to this decision. In most scenarios, it is likely there will be few protocols available that will be appropriate for the specific contaminant and sample type. In some cases, it may be necessary to use a protocol developed for one matrix (e.g., water) for the analyses of another matrix (e.g., soil), which would require additional sample preparation prior to analyses (see Section 4.1.3). It is also important to consider the nature (e.g., quantitative or qualitative) of the results that will be generated by a particular analytical protocol when defining DQOs and developing a SAP.

Some considerations that are pertinent to protocol selection include:

- The purpose of the analyses, and the decisions the analytical data will be used to support
- Whether quantitative or qualitative data will be required
- Whether high-throughput or rapid analysis will be needed (e.g., to determine the extent of contamination)
- Whether an assessment of pathogen viability will be needed (e.g., to determine efficacy of treatment/decontamination)
- Whether complete identification and characterization of the pathogen, or simply detection will be needed
- Potential matrix interferences
- Number of samples (capacity of the laboratories)

For determining the extent of contamination, PCR protocols might be the most appropriate, and time- and cost-effective analytical procedures. Response actions, however, are site- and situation-specific, and other or additional analytical procedures may be appropriate, depending on the specific data needs. In general, bacterial culture analyses can detect lower concentrations of pathogens than PCR analyses, and can determine viability. The use of immunoassays may not be feasible due to their limited availability.

Sections 4.2.1 and 4.2.2 provide information regarding each of these types of protocols, including their advantages and disadvantages. **Table 4.1** provides a summarized comparison of the protocol types, in terms of the type of pathogens (i.e., bacteria, virus, protozoa, and helminth) targeted, the time needed for analysis, and the format of results generated. Decisions regarding which analytical procedures to use will ultimately be made by subject matter experts in conjunction with the Incident Command or Unified Command [2].

4.2.1 Culture-based Protocols

A key feature of culture-based protocols is that they assess the viability of a target organism, in addition to determining its presence. This is particularly important when assessing the efficacy of decontamination efforts. Most microbiology laboratories will be proficient with culture-based protocols for bacterial pathogens. In contrast, there are limited laboratories capable of conducting culture assays for viruses, protozoa and helminths due to the specialized nature (e.g., cell culture); these assays also have increased time-to-results and are more costly.

Culture protocols developed for water samples also can be used for analyses of non-water sample types (i.e. particulates [wipes, vacuum socks], soils) provided the sample (and pathogens) can be suspended in an aqueous medium prior to analysis. For example, the EPA *Yersinia pestis* sample preparation procedures [45] (included in EPA's SAM [3]) can be applied across multiple sample types (matrices) with minor modifications for subsequent analysis of various pathogens. A summary of advantages and disadvantages of culture-based protocols is provided below.

Advantages:

- Assess viability
- Generally do not require expensive equipment or reagents
- Generally available
- Selectivity (pathogen-specific growth media often available)

Disadvantages:

- Slow (multiple days or weeks)
- Require specific technical expertise for positive identification
- Labor intensive
- Propagation of infectious organisms

Common culture-based protocols include: (1) bacterial assays that use either selective growth (preferential growth of a bacteria) or non-selective growth (general or non-specific growth) media and colony formation or turbidity as an indicator of bacterial growth and (2) viral assays that use cultured mammalian host cells (e.g., total culturable virus assay [TCVA]) and either cytopathic effects or plaque formation as an indicator of virus growth or replication. Less common, but important for viability assessment, are culture-based protocols for protozoa and helminths. Culture-based protocols can produce either quantitative information, in the form of target enumeration (e.g., colony forming units [CFUs] for bacteria or plaque forming units [PFUs] for viruses, or direct counts of individual organisms for protozoa and helminth) or qualitative (presence/absence) data.

A quantitative estimate of presence/absence results in some cases can be derived using a culture-based assay that applies a MPN approach. The MPN is a statistically derived estimate of the number of microorganisms based on the presence or absence of growth in replicates of serially diluted samples. The resulting number of positive tubes for each dilution is used to calculate the MPN of the pathogen in the original sample. The MPN approach can be used with most

protocols that generate presence/absence data, including both bacterial (e.g., EPA Method 1682 [50]) and virus (e.g., EPA Method 1615 [46]) methods. The disadvantage of this technique is that it requires a large number of dilutions, multiplied by several replicates at each dilution, and typical incubation periods that range from 48 hours to a month or more for some pathogens.

Bacterial Culture-based Protocols:

Spread and Drop Plates: Spread and drop plates are prepared by adding a bacterial suspension to an agar plate, by either evenly spreading (spread plate) or applying discrete drops (drop plate) of the suspension on to the agar surface. Once plated and incubated to promote bacterial growth, individual colonies can be enumerated, and their morphology evaluated for identification. The type of media used can assist in isolating a particular type of bacteria. These data are generally reported as CFU/mL.

Broth Culture: Broth (or liquid media) culture-based protocols are advantageous for culturing certain types of bacteria, and can be used to disperse non-liquid samples (i.e., soils, particulates [wipes, vacuum socks]) for analysis. Liquid media can be selective or non-selective. Broth cultures are often used to generate MPN estimates of bacterial pathogens. Broth cultures are also commonly used to evaluate bacterial growth by monitoring changes in the optical density (OD) of the culture over time and for the preparation of cell suspensions that can be used for a variety of purposes (e.g., enumeration, spiking, stock cultures, bacterial identification).

Membrane Filtration: The membrane filtration technique can be used to analyze a range of water sample volumes (e.g., 100 mL to 1 L are common). With the appropriate preparation, non-aqueous sample types may also be analyzed using membrane filtration. However, it would only be advisable if the anticipated pathogen levels are high and detection would not be impacted by diluting the sample. When a water sample is passed through the filter, bacteria are captured on the filter's surface. The filter is then placed on an agar plate (selective or non-selective) and incubated. After colonies develop, they are enumerated based on colony morphology and reported as CFU/volume assayed (e.g., 100 mL, 1 L). ***Note***: The presence of dirt, debris, or other suspended particulates in a sample will impact the volume of sample that can be processed by membrane filtration.

Virus Culture-based Protocols:

Total Culturable Virus Assay (TCVA [cell culture]): Viruses that may be present in water are concentrated by passage through electropositive filters. The viruses are then eluted from the filters with a beef extract reagent, and concentrated using organic flocculation. A portion of the concentrated eluate is inoculated onto replicate flasks of Buffalo green monkey kidney cells (BGM cells) to measure infectious viruses. Cultures are examined for the development of cytopathic effects (viral induced cell damage) for two weeks and then subcultured onto fresh BGM cells for confirmation. The virus concentration in each sample is calculated in terms of the MPN of infectious units per liter.

Plaque Assay: The plaque assay is a variation of the TCVA in which a 10-fold dilution of a sample (e.g., water, wastewater, soil) is prepared and an aliquot is inoculated onto susceptible host cell monolayers. The plates or flasks are incubated to allow viruses present in the sample to attach to and infect the host cells. The monolayers are covered with a nutrient medium to form a

gel. The original infected cells release viral progeny following incubation. The spread of the new viruses is restricted to neighboring cells by the gel. Infectious particles produce a plaque, which is a circular zone of infected cells (i.e., cell lysis). Dyes are typically used to enhance the contrast between living cells and plaques. Only viruses that cause visible damage of cells can be assayed using this technique. Results are reported as PFU/mL.

Focus Forming Assay: The focus forming assay (FFA) is a variation of the plaque assay and uses immuno-staining techniques rather than cell lysis to detect plaque formation. During the immuno-staining process, fluorescently labeled antibodies specific for a viral antigen are used to detect infected host cells and infectious virus particles before an actual plaque is formed. This assay is used to quantify classes of viruses that do not lyse the cell membranes. Similar to the plaque assay, host cell monolayers are infected with various dilutions of the sample and covered with a growth medium that restricts the spread of infectious virus and allows clusters of infected cells following an incubation period. Plates are probed with fluorescently labeled antibodies and fluorescence microscopy is used to count and quantify clusters (foci) of infected cells. Results of the FFA are expressed as focus forming units (FFU)/mL.

Protozoa Culture-based Protocols:

Cell culture: Appropriate cell lines or microbiological media are inoculated with the sample that may contain protozoa and, after an incubation period that ranges from 10 hours to 10 days, the cultures are examined microscopically for viable protozoa or examined for plaque formation. Additional confirmation may be conducted to confirm culture results. Results may be expressed as the number of viable protozoa per volume analyzed or PFU per volume analyzed.

4.2.2 Molecular Protocols – Polymerase Chain Reaction (PCR) Assays

PCR assays require sample processing to extract nucleic acid prior to analysis; the target bacterial cell or virus must be disrupted and the genetic material (DNA or RNA) released. This can be accomplished by mechanical (i.e., bead beating for bacteria [45]) or chemical disruption (i.e., one of numerous commercially available products).

PCR assays can be used to analyze both DNA and RNA. DNA represents the genome of most organisms (e.g., bacteria, protozoa) and can be directly amplified using PCR. Conversely, RNA genomes (RNA viruses) must be converted (reverse transcribed) to a complementary DNA (cDNA) copy prior to PCR amplification. PCR assays work on the principle of amplifying (copying many times over) a specific target region of DNA and subsequently analyzing the amplified target DNA. PCR requires specifically designed upstream and downstream primers which bind to complimentary regions of the target DNA of interest and provide sites for initiation of DNA synthesis and hence, define or delimit the region amplified (i.e., amplicon). For conventional PCR protocols, analysis and confirmation of amplicons can be accomplished using various “end-point” determinations (e.g., gel electrophoresis, probe hybridization, DNA melting curves, DNA sequencing). Alternatively, monitoring PCR reactions in real-time as the reaction progresses (real-time PCR or quantitative PCR [qPCR]) can provide a quantitative assessment of initial target DNA by monitoring fluorescent signals generated by a fluorescent dye during the PCR reaction. Results of qPCR assays are generally reported as genomic copies or number of pathogens per unit volume and are based on cycle threshold (Ct) values (i.e., the number of amplification cycles required for the fluorescent signal to exceed a predetermined

signal threshold). Ct values are inversely correlated with target concentration; thus, a low Ct value indicates a relatively high concentration of initial sample target sequences.

A summary of advantages and disadvantages of PCR assays is provided below.

Advantages:

- Provides rapid analysis
- Does not require propagation of a hazardous agent
- Reduces risk of exposure
- Assays generally available

Disadvantages:

- Does not determine viability (unless coupled with a culture protocol – see Rapid Viability PCR in Table 4.1)
- Susceptible to environmental interferences
- Nucleic acid extraction required

Several variations of the PCR assay are described below.

- *Real-time PCR*: Real-time PCR assays monitor DNA amplification in real time, while the assay is progressing, rather than at the end. These assays are also known as qPCR assays.

- *Reverse Transcription PCR*: These assays are referred to as RT-PCR assays (not to be confused with real-time PCR). RT-PCR uses RNA as the initial template instead of DNA. The RNA template is copied (reverse transcription) into cDNA using a reverse transcriptase of viral origin (RNA viruses) prior to PCR amplification of the cDNA. Like DNA-based PCR, RT-PCR can be done as a standard end-point PCR or as real-time PCR.

- *Rapid Viability-PCR*: This assay combines culture and PCR methodologies, integrating high-throughput sample processing, a short incubation period in broth culture, and highly sensitive and specific real-time PCR assays to detect live biological agents in environmental samples. RV-PCR can rapidly detect and identify a low number of viable cells or spores in the presence of high concentrations of dead pathogens as well as in the presence of other non-target biological organisms and complex sample or specimen matrices.

- *Digital PCR (dPCR)*: This assay allows for quantitation of the original target DNA sequences (as opposed to the amplified sequences with qPCR). The PCR reaction is distributed into multiple parallel reactions, and results are reported in terms of sequences per unit volume.

A practical breakdown of commonly used microbial protocol types is provided in Table 4.1, which can assist in narrowing down an appropriate protocol based on incident-specific criteria.

Table 4.1 Commonly Used Protocols for Pathogens

Culture-Based Protocols					
Type	Results Format	Target Pathogens	Time Needed	Assesses Viability	Potential Issues
Plate Culture (Spread, Drop and Membrane Filtration Assays)	Presence/Absence or CFU per mL. Each viable microbial cell grows and forms a colony on solid growth medium. The CFUs per plate should be multiplied by the appropriate dilution factor to determine the number of CFU/mL in the original sample.	Bacteria	Overnight to several days, depending on the target species	Yes	• Presumptive positives (e.g., *Bacillus anthracis*) need to be confirmed with molecular assays such as PCR or biochemical tests • Propagation of infectious pathogens requires biocontainment, suitable for the potential pathogen. • Other non-target microorganisms present in an environmental sample may inhibit the growth of target contaminant • Viable but non-culturable (VBNC) microorganisms (e.g., *Vibrio cholerae*) can produce false-negative results.
Broth Culture	Presence/Absence, OD or cells per mL. Sterile media allows some light to pass through it (translucent), so the growth of bacterial cells (e.g., turbidity) reduces the amount of light transmitted. Other indicators of positive growth may include effervescence (i.e., gas production) and color change due to shift in pH. This can be reported directly or converted to an approximate number of cells per mL (e.g., MPN/mL).				
Cell Culture (Plaque, Focus Forming or Endpoint Dilution Assays)	PFU per mL, FFU per mL or $TCID_{50}$. PFU (for a plaque assay) or FFU (for a focus forming assay) are counted directly, like CFU in a bacterial assay. Alternately, viral activity can be measured by the amount of virus required to produce a cytopathic (cell-killing) effect in 50% of inoculated cell culture, reported as median Tissue Culture Infective Dose ($TCID_{50}$). $TCID_{50}$ can be translated to PFU. This is an endpoint dilution assay.	Viruses Protozoa	Days (approximately 3-16)		• Host cells are not available for many viruses • Propagation of infectious pathogens may require biocontainment

Molecular Protocols						
Type	Target	Results Format	Target Pathogens	Time Needed	Assesses Viability	Potential Issues
Quantitative PCR (qPCR)	DNA	Cycle threshold (Ct) results can be used to calculate DNA sequence copies per volume, which indicates an approximate quantity of the target organism.	All (except RNA viruses)	• 3-5 hours, depending on the number of samples per batch • Typically require up to 60 minutes. Sample preparation time adds up to 2 or more hours • Real-time results are provided with some PCR thermal cyclers	No	• Environmental samples may contain inhibitors that interfere with PCR • Appropriate sample processing is critical • Requires a standard curve to determine approximate copies per volume • Lack of primer specificity may allow for amplification of non-target sequences
Reverse Transcription PCR (RT-qPCR)	RNA	Ct results can be translated into approximate RNA sequence copies per volume, which indicates the approximate quantity of the target organism.	RNA viruses	• 4-6 hours, depending on the number of samples per batch • RT-PCR reactions typically require approximately 100 minutes. Sample preparation adds up to 2 hours	No	• Environmental samples may contain inhibitors that interfere with PCR • Appropriate sample processing is critical • Requires a standard curve to determine approximate copies per volume • Lack of primer specificity may allow for amplification of non-target sequences .
Rapid-Viability PCR (RV-PCR)	DNA	RV-PCR is based on a shift in Ct value in bioagent-specific PCR assay, indicating an increase in DNA due to growth of viable organisms (PCR Ct differential for incubation time point zero hour versus	Bacteria	Confirmed results of first batch within 15 hours. Time needed can vary based on the growth rate of the organism.	Yes	• Environmental samples may contain inhibitors that interfere with PCR • Appropriate sample processing is critical • Lack of primer specificity may allow for amplification of non-target sequences

Molecular Protocols						
		incubation time [final] hour culture).				
Digital PCR (dPCR)	DNA	Sequence per volume. The quantity of sequence in the original reaction is calculated based on the number of positive and negative reactions. A standard curve is not required.	All (including cDNA)	< 1 day	No	• Environmental samples may contain inhibitors that interfere with PCR • Appropriate sample processing is critical • Lack of primer specificity may allow for amplification of non-target sequences • May not be conducive for high throughput sample analysis

Other Assays							
Protocol Type	Target	Target Pathogens	General Information	Results Format	Time Needed	Assesses Viability	Potential Issues
Immunological (including immunomagnetic separation [IMS], immunofluorescent assay [IFA], and target verification)	Surface antigens and proteins of pathogens	Bacteria Protozoa Viruses	Antibodies to detect pathogen-specific antigens.	Quantitative or qualitative	< 1 day	No	• Appropriate sample processing is critical • Sample concentration may be required • Lack of antibody specificity may lead to non-target detection
Microscopy	Whole organisms (e.g., oocysts, cysts)	Bacteria Protozoa Viruses	Organisms may be treated (e.g., immunostaining) to enhance ability to identify specific characteristics	Quantitative or qualitative	Varies based on the volume of the sample and sample processing required prior to examination	No	• Requires microscopy expertise • Can be time-consuming

CFU, colony forming units; cDNA, complementary DNA ; Ct, cycle threshold; FFU, focus forming unit; IFA, immunofluorescent assay; IMS, immunomagnetic separation; MPN, most probable number; OD, optical density; PFU, plaque forming units; TCID50, median tissue culture infectious dose

4.3 QC and Method Performance Considerations

Ongoing implementation of QC in the laboratory is critical to ensuring and understanding the reliability of analytical results. The laboratory should have appropriate QC procedures in place as a routine aspect of their operations, and should adhere to any method-specific QC requirements. Ideally, the laboratory will be certified in the method(s) they use, or in similar procedures. Common QC samples that are used during analysis of environmental samples for microorganisms are described below. These samples are included in analytical batches, and are analyzed using procedures that are identical to those used to analyze the environmental (field) samples. To minimize exposure risks associated with select agents, it may be necessary to use a surrogate (microorganism with properties that are similar to the target) for QC purposes.

Note: Generally, any QC failure would invalidate all associated samples (i.e., sample batch) and re-sampling and re-analysis would be indicated. However, during a contamination incident it may not be feasible to re-sample and in these cases a specific QC failure should be evaluated to determine the probable cause and if some of the data could be qualified and used. The analytical laboratory should be consulted to help determine the cause of any QC failure and the impact on associated sample data.

- **Positive and Negative Controls** – Ideally, a positive control contains a known quantity of the target pathogen or surrogate (in some cases, the concentration is known only to be above the analytical protocol's detection limit); a negative control contains a non-target organism (e.g., does not grow on the medium or produces colonies with different morphology) and does not contain the target pathogen or surrogate. Positive and negative controls can be used throughout the analysis to demonstrate that a method is performing appropriately. Analytical results of a positive control should fall within a specified range of the known quantity or exhibit characteristics typical of the target pathogen. Negative culture controls should produce either no growth or atypical growth (e.g., colony morphology is different from the target pathogen). Negative PCR controls (no template controls) should not exhibit amplification.
- **Matrix Spike (MS)** – MS samples are used to determine the impact of sample components on the ability of the protocol to detect or quantify the target pathogen. To evaluate matrix effects, sample aliquots are spiked with a known quantity of the pathogen or surrogate and analyzed along with the unspiked field samples. Matrix spike duplicates (MSDs) can also be used to evaluate analytical precision in the specific matrix. Analytical results that do not meet method-specific recovery and precision criteria can indicate possible matrix interferences. The results of PCR-based assays, for example, can fall outside the criteria due to enzyme inhibition by compounds commonly found in waters and soils such as humic acids, organic material and metal ions [51]. ***Note***: Not all methods include recovery and precision criteria for MS and MSD samples.
- **Method Blank** – Method blanks consist of a reference matrix sample that is similar to the sample matrix (e.g., buffered water for water samples) and free of the target contaminant. Detection of the target contaminant in a method blank is indicative of contamination during analysis.

- **Duplicate Samples** – Duplicate, replicate or split samples are often used to evaluate method precision. Results are compared and typically used to determine relative percent difference (RPD) or relative standard deviation (RSD).

Specific method or protocol requirements and DQOs may require additional QC samples such as:

- **Sample Collection-Related Controls** − Equipment and field blanks to identify contamination during sample collection.
- **Media Controls** – Media controls are used to verify that the media is performing properly (e.g., positive and negative colony morphology is appropriate).
- **Sterility Checks** – Sterility checks ensure that media, glassware, and laboratory equipment are free of contamination
- **Internal Amplification Control (IAC)** – An IAC is used to demonstrate that PCR reactions are working and to identify matrix inhibition (e.g., amplification is present in control sample extracts but is not evident in the field sample extracts).
- **External Inhibition Control (EIC)** – An EIC is used to demonstrate PCR amplification of a control target template in the presence of a sample extract to identify matrix inhibition (e.g., amplification of EIC in the presence of sample extract is compared to identical positive control in the absence of sample).
- **Staining Controls** – Staining controls ensure that stains are performing appropriately (e.g., incorporation or uptake by cells, or producing the appropriate colors/reactions).

Remediation planners should consult with the laboratories to ensure the number and type of QC samples needed to support data assessments and remediation decisions are analyzed.

5.0 Data Quality Assessment and Interpretation Considerations

Data received from analytical laboratories will need to be assessed to ensure the data are valid. During this assessment, all QC results (Section 4.3) associated with the samples should be reviewed to ensure that there were no apparent issues during analysis that would compromise the data. Once the QC data (and hence sample data) are considered valid or "in control," and/or any limitations associated with the data are understood, the sample data can be used to support decision-making. When interpreting analytical results in support of decision-making, there are multiple things to consider, including whether the data are qualitative or quantitative, as well as any qualifiers associated with the reported values. Although there is precedent for considering some data as semi-quantitative, for the purposes of this document quantitative data refers to any results providing a numerical value, while qualitative data refers to results that are expressed as positive or negative, or as presence or absence. It should be noted, however, that even quantitative data contain some inherent uncertainty. Laboratory reports should indicate any data qualifiers that are associated with the analytical results, including issues pertaining to sample(s) or QC analyses. The analytical laboratory can usually provide information regarding potential data qualifiers that may pertain to a specific protocol and should be considered when developing DQOs.

For some pathogens, both qualitative and quantitative protocols may be available, and a selection of one (or both) should be based on incident-specific objectives and the response phase. During post-decontamination sampling and analysis, for example, the objective may be to monitor decontamination efforts and quantitative analytical results that assess pathogen viability may be appropriate. Conversely, during site characterization efforts, qualitative analytical results may be appropriate for determining the scope of contamination, with or without viability assessment.

Some relevant considerations are discussed below.

5.1 Qualitative and Quantitative Protocols

Qualitative protocols (e.g., PCR, broth culture) include those that do **not** provide direct (e.g., particle counts, CFU) or indirect (e.g., MPN) enumeration of a target pathogen.

Quantitative protocols refer to analytical protocols that provide direct (e.g., colony counts) or indirect (e.g., MPN, qPCR) enumeration of a target pathogen.

A general overview of available pathogen protocols is provided below (**Figure 5.1**).

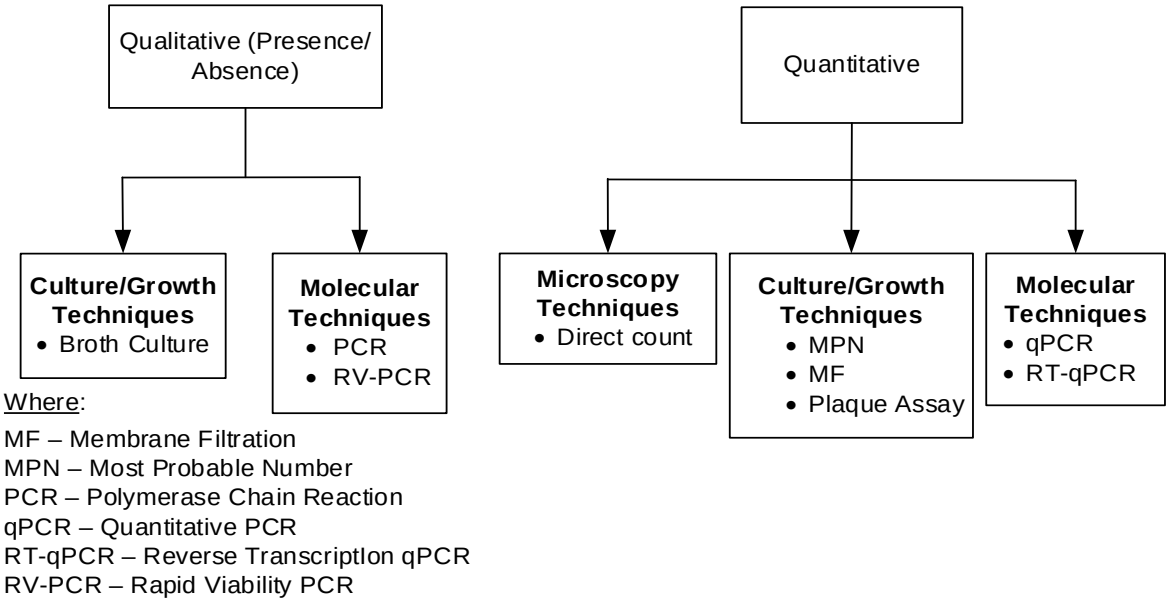

Figure 5.1 Overview of analytical protocols for pathogens.

5.1.1 Qualitative Protocols and Data

As noted above, qualitative data are derived from analytical protocols that provide information regarding the presence or absence (or detection/non-detection) of a target pathogen but do not provide a numerical result. Examples of qualitative protocols include both culture-based protocols (e.g., non-typhoidal *Salmonella* [Method 1200, 52]) and molecular-based protocols (e.g., RV-PCR [*Protocol for Detection of Bacillus anthracis in Environmental Samples During the Remediation Phase of an Anthrax Event*, 53]). Qualitative results may be reported as present (or absent) or as detect (or non-detect) depending on specific protocol reporting requirements. If protocol sensitivity is known or can be demonstrated, negative results (absence or non-detects) may be reported as less than the LOD for that protocol (e.g., <1 CFU/100 mL).

5.1.2 Quantitative Protocols and Data

As noted previously, quantitative data are derived from analytical protocols that are used to provide direct (e.g., colony counts) or indirect (e.g., MPN, qPCR) enumeration of a target pathogen. Quantitative protocols include culture-based protocols such as standard bacterial protocols (e.g., membrane filtration, MPN), direct microscopic enumeration, qPCR and host cell infectivity (viruses and protozoa) assays that rely on pathogen viability/infectivity for target enumeration. Analytical protocols for enumeration are available for most bacteria and protozoa (e.g., immunofluorescence assay for *Cryptosporidium* and *Giardia* [54]), as well as some viruses (e.g., culturable viruses [46]). The direct enumeration of bacterial colonies [45, 53] on spread plates or on filters following sample filtration and subsequent culture provides a quantitative assessment of target bacteria per sample volume (e.g., CFU/100 mL). Most protocols that rely on microbial colony counting also provide guidelines with respect to the acceptable range of colonies that should be present and enumerated on a single filter or culture plate. Reported results may include colony counts from replicate samples or from sample dilutions. In some

cases, results may include colony counts from filters or plates that are not within the ideal acceptable range but can be enumerated accurately.

In contrast to direct enumeration, many microbiological protocols provide an indirect assessment of target pathogen concentration. One common approach is the MPN technique that can be used to enumerate most viable bacterial pathogens (e.g., Method 1200 [52]) as well as some culturable viruses (e.g., Method 1615 [46]). The MPN technique involves culture and analysis of serial sample dilutions in replicate. Estimation of the target concentration is based on the number of positive cultures among replicates at each serial dilution of the sample.

Another common approach for the indirect assessment of pathogen concentrations is qPCR. As previously noted, qPCR uses a fluorescent sequence-specific hybridization probe sequence internal to the two target gene-specific amplification primers to generate a fluorescent signal during DNA amplification. Pathogens containing either DNA genomes (e.g., bacteria, protozoa) or RNA genomes (e.g., enteroviruses and norovirus) can be enumerated using qPCR although RNA must be reverse transcribed (cDNA) prior to amplification (RT-qPCR). For example, EPA Method 1609 [55] uses an arithmetic formula, to calculate the ratio of *Enterococcus* target DNA sequences relative to those in similarly prepared calibrator samples containing a known quantity of *Enterococcus* cells. For analysis of RNA virus genomes by RT-qPCR (e.g., Method 1615 [46]), reverse transcription of the target RNA (i.e., cDNA) is required prior to PCR amplification. RT-qPCR results are generally reported as genomic copies (GC) per sample volume (e.g., GC/L).

5.2 Method Performance Parameters

Method-specific performance criteria (e.g., percent recovery ranges, detection limits) are often included in validated EPA methods and can be used to monitor method performance and facilitate data assessment. These criteria are typically based on results generated by one or more laboratories performing the method for analysis of a specific pathogen in targeted sample types. If method-specific performance parameters are not available, laboratories and remediation planners should work together to develop and define DQOs and appropriate corresponding performance criteria and data quality indicators (DQIs).

If available, method performance parameters can be used to assess the quality of laboratory data. Failure to meet any of the parameters can affect how the results are interpreted, qualified or used. For example, if the laboratory's MS result did not meet the criteria but the ongoing precision and recovery result did, this would be an indication that there may be matrix interferences affecting the recovery of the target pathogen. If the MS recoveries are lower than the criteria, the field sample results could be qualified to indicate that the actual target levels may be higher than the results indicate due potential matrix issues. Although analysis of matrix spikes may indicate matrix interference, there may be no way to eliminate or reduce their impact.

If the results of both the MS and ongoing precision and recovery did not meet their corresponding criteria, there is likely an analytical problem that should be addressed; these data should be considered invalid and, if possible, the problem corrected and the analyses repeated.

5.2.1 Recovery Efficiency

Recovery efficiency (RE) describes how much of a contaminant can be detected in a sample compared to how much is actually present. For analysis of pathogens in environmental samples, recovery efficiency is rarely 100%, and is frequently much lower due to many factors inherent to the protocols used to collect, extract and analyze environmental samples. Pathogen recovery can be significantly impacted by losses associated with the sample matrix, sampling devices and protocols, as well as sample preparation (e.g., nucleic acid extraction) techniques. In addition, when designing a sampling and analysis approach, the impact of sample collection on pathogen viability and infectivity should be considered.

Inefficient recovery of target pathogens during sampling can have significant impacts on the ability of any protocol to detect a target pathogen. Low target concentrations present in the matrix can potentially lead to false negative results, even when using very sensitive analytical protocols due to losses during sampling. The RE of pathogens from various matrices can vary significantly. For example, target recovery using standard sample collection procedures for water and solid matrices (grab samples) can and should be virtually complete (~100%) while target recovery using air and surface sampling procedures can be relatively inefficient (e.g., < 50%). Unfortunately, very little information is available regarding recovery of most pathogens across various matrices and sampling techniques. Such evaluations are difficult due to the various components involved (e.g., target, sample matrix, sample collection, target extraction and analytical procedures) and their inherent complexities.

In addition to the recovery issues noted above for sample collection procedures, sample preparation procedures inherent to some analytical protocols can further impact target pathogen recovery. For example, target loss during nucleic acid extraction procedures prior to PCR analysis is unavoidable as are losses due to other sample preparation procedures such as virus filter elution and concentration (flocculation), and protozoan filter elution and concentration (IMS). For some protocols, recovery losses due to sample preparation are included in the overall determination of protocol sensitivity (Section 5.2.2).

In most cases, analytical recovery should not be a concern when a protocol is used for protocol-specific applications; most protocols have been developed and evaluated to ensure optimal recovery and sensitivity (e.g., detection limit of 1 CFU for bacterial culture techniques) when used for their intended purpose. However, application of a pathogen-specific protocol to sample matrices other than those for which it was designed can potentially result in unanticipated matrix interferences that can impact the ability to accurately assess target presence or concentration.

A review of spore recovery studies using various surface sampling (e.g., swabs, wipes) and air sampling (dry filter sampling) techniques indicates a wide range of reported RE values for differing surface types, spore concentrations and deposition methods [56]. The data were collected under largely controlled conditions and there is the possibility of potential biasing of the RE values due to the individual collecting the samples. Additionally, the identified sampling methods have been characterized only on selected surfaces and mainly focused on indoor surfaces. It is uncertain as to how these surface and air sampling methods perform with respect to non-spore forming pathogens (e.g., bacteria, viruses).

5.2.2 Detection Limits and Quantitation Limits

Note: For the purposes of this document, use of the term method detection limit (MDL) is avoided due the following recent EPA update of the definition, scope and application of this term. "The MDL procedure is not applicable to methods that do not produce results with a continuous distribution, such as, but not limited to, methods for whole effluent toxicity, presence/absence methods, and microbiological methods that involve counting colonies" [57].

Detection Limits – The limit of detection (LOD) or detection limit (DL) is a fixed aspect of an analytical protocol; it is the lowest quantity of a pathogen that can be distinguished from the absence of that pathogen within a stated confidence limit (e.g., 99%). For example, most bacterial membrane filtration methods have a LOD of 1 CFU/volume filtered. As such, the LOD (or DL) is designed to protect against reporting "false positives," and detection of a pathogen at or above the LOD in the absence of that pathogen should be a rare occurrence (less than or equal to 1 percent). LODs (or DLs) are generally first determined based on analysis only, and independently from sample collection and transport/storage procedures. Overall DLs refer to the lowest level of target pathogen that can be reliably detected, including all processes from sample collection through analysis, using defined sample collection, transport/ storage and analytical procedures. Some protocols will provide an LOD (or DL) value and this value is often used to define analytical protocol sensitivity.

Quantitation Limits – In addition to LODs, some protocols specify quantitation limits for the target pathogen. The lower limit of quantitation (LOQ) is the lowest amount of the pathogen that can be measured with acceptable precision and accuracy as required by DQOs. What is considered "acceptable" is determined by the protocol, if it requires it, or by the user. For instrument-based protocols such as qPCR, both the LOQ and the range of quantitation are established from a standard curve of reference sample measurements. Protocols designed to obtain a quantitative analysis may have several required operational limits and performance attributes, one of which is a standard curve [23]. The standard curve defines the relationship between the detector or instrument response and the amount of target. The range of quantitation has an upper bound, as well as a lower bound (LOQ), and measurements above or below those limits, respectively, are not considered valid. For culture plating protocols (e.g., membrane filter or spread plates), instead of a standard curve and range of quantitation, there are countable ranges. Higher variability is expected near the lower end of the reported ranges [58, 59], while accurate counting might also be inhibited by colony overlap when CFU results are above these ranges (too numerous to count)[60]. The LOQ and countable range vary widely (based on the size of the plate, growth medium and colony size) and are thus protocol- and contaminant-dependent. Like LOD and LOQ, a countable range or range of quantitation will be included in some protocols. If not, the analytical laboratory may be able to help define these protocol parameters.

Note: Caution should be used when interpreting microbial count data that fall below the established DL or LOQ for a specific protocol particularly in situations where low level contamination is expected (e.g., post-decontamination). Censoring of positive results that fall below the DL or LOQ, as well as non-detects, can bias results and, importantly, can mask true positive (or negative) results. Any censorship of microbial count data should be justified and detailed in the incident SAP including appropriate DQOs, as necessary. A report evaluating six

options for representing culture-based/microbial count data when no colonies were observed and/or when colonies were observed but were below the limits of quantification of the filter plating or spread plating techniques (i.e., censored data), is available [61] and should be consulted for further information regarding data censorship.

5.3 Specificity and Sensitivity

Specificity and sensitivity are terms that have different definitions for different types or categories of analytical protocols. In a general sense, these terms are used to define the extent to which a protocol responds uniquely to the specified target organism or group of organisms.

Specificity – *Specificity* is the ability to discriminate between the target organism and other non-target organisms. Specificity for microbiology culture protocols (and media) is traditionally demonstrated through the analysis of positive and negative control cultures. In a robust protocol, a single target organism should be discernable in complex matrices containing potentially millions of non-target organisms.

Sensitivity – *Sensitivity* is the proportion of a target organism that can be detected. Data to calculate sensitivity are typically generated by repeated testing of serial dilutions of a "known" spike standard. Protocol sensitivity is generally expressed in terms of an LOD (or DL), as described in 5.2.2, above.

5.4 Data Management and Review

Depending on the scale of the remediation effort, large amounts of data may need to be managed and reviewed. Flat files or spreadsheets can be used to manage smaller amounts of data, but may be too cumbersome to manage larger amounts. For large data sets and multiple data streams (e.g., analytical results, sampling data), it may be more appropriate to use a database to save and query the data. It is EPA Policy to use Scribe[1] wherever practical to collect, store and report sampling and analytical data. Scribe is a database management tool developed by EPA's Environmental Response Team (ERT) for managing environmental data, and was designed to capture sampling data, observational information, monitoring field data and analytical data[62] .

Data security is another consideration when managing large amounts of data. The use of a secure database will enhance security, limiting data access by requiring login privileges and limiting the number of individuals that have the ability to make modifications. Security may be particularly critical when the sampling and analysis effort is in response to a terrorist act or when forensic information is included.

Another consideration when evaluating how data is to be managed is the processes that will be used for data review. To ensure data review is completed in a timely manner that allows decisions to be made, an automated review process may be required. Depending on the amount of data, traditional data review processes can require weeks to months to go through all of the

[1] Scribe is a software tool developed by EPA's Environmental Response Team (ERT) to assist in managing environmental data. For additional information regarding this tool see https://www.epa.gov/ert/environmental-response-team-information-management (last accessed September 2017).

required review cycles to ensure the data are valid and appropriate to support decisions. EPA has developed Web Electronic Data Review (WebEDR) [https://webedr.fedcsc.com/webadr/app/], a Web-based automated data review tool that can be customized by each user to review data using incident and protocol specific data quality objectives. Depending on the amount and type of data, use of WebEDR or a similar tool can reduce the data review time to minutes.

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Appendix A: Biosafety Levels for Potential Pathogen Contaminants

Table A-1 Example Subset of Bacterial Pathogens

Bacteria	Disease	Biosafety Level (BSL) [1]
Bacillus anthracis	Anthrax	BSL-3
Brucella spp.	Brucellosis, Undulant Fever	BSL-3
Burkholderia mallei	Glanders	BSL-3
Burkholderia pseudomallei	Melioidosis	BSL-3
Campylobacter jejuni	Campylobacteriosis	BSL-2
Chlamydophila psittaci (formerly known as *Chlamydia psittaci*)	Parrot Fever	BSL-2; BSL-3 for aerosols and large volumes
Coxiella burnetii	Q-Fever	BSL-3
Escherichia coli O157:H7	Enterohemorrhagic *E. coli* or EHEC	BSL-2
Francisella tularensis	Tularemia, Rabbit Fever	BSL-3
Legionella pneumophila	Legionellosis	BSL-2
Leptospira interrogans.	Leptospirosis	BSL-2
Listeria monocytogenes	Listeriosis	BSL-2
Non-typhoidal Salmonella (Not applicable to *S. Typhi*)	Salmonellosis	BSL-2
Salmonella enterica serovar Typhi *(S.* Typhi*)*	Typhoid Fever	BSL-2; BSL-3 for Aerosol Release
Shigella spp.	Shigellosis	BSL-2
Staphylococcus aureus	Staphylococcal Food Poisoning	BSL-2
Vibrio cholerae	Cholera	BSL-2
Yersinia pestis	Plague	BSL-3

[1] The BSLs listed are those currently determined by the Centers for Disease Control and Prevention's *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition [20] (http://www.cdc.gov/biosafety/publications/bmbl5/) EPA's *Selected Analytical Methods for Environmental Remediation and Recovery* (SAM) (https://www.epa.gov/homeland-security-research/sam) and Canadian governmental agencies (http://laws.justice.gc.ca/eng/acts/H-5.67/page-10.html#docContl)

Table A-2 Example Subset of Viruses, Protozoa and Helminths

Viruses		Biosafety Level (BSL) [1]
Adenoviruses: Enteric and Non-enteric (A-F)		BSL-2
Astroviruses		BSL not specified
Caliciviruses: Noroviruses		BSL-2
Caliciviruses: Sapovirus		BSL-2
Coronaviruses: Severe Acute Respiratory Syndrome (SARS)-associated Human Coronavirus (HCoV)		BSL-2; BSL-3 for propagation
Hepatitis E Virus (HEV)		BSL-2
Influenza H5N1 virus		BSL-3
Picornaviruses: Enteroviruses		BSL-2
Picornaviruses: Hepatitis A Virus (HAV)		BSL-2
Reoviruses: Rotavirus (Group A)		BSL-2
Protozoa	Disease	Biosafety Level (BSL) [1]
Cryptosporidium spp.	Cryptosporidiosis	BSL-2
Entamoeba histolytica	Amebiasis	BSL-2
Giardia spp.	Giardiasis	BSL-2
Naegleria fowleri	Naegleriasis	BSL-2
Toxoplasma gondii	Toxoplasmosis	BSL-2
Helminths	Disease	Biosafety Level (BSL) [1]
Baylisascaris procyonis	Raccoon roundworm infection	BSL-2

[1] The BSLs listed are those currently determined by the U.S. Centers for Disease Control and Prevention's *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition [20] (http://www.cdc.gov/biosafety/publications/bmbl5/) EPA's *Selected Analytical Methods for Environmental Remediation and Recovery* (SAM) (https://www.epa.gov/homeland-security-research/sam) and Canadian governmental agencies (http://laws.justice.gc.ca/eng/acts/H-5.67/page-10.html#docCont)

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Appendix B: Overview of Probabilistic Sampling Approaches

Table B-1 Probabilistic Sampling Approaches [References 2, 8 and 37]

Probabilistic Sampling Approach	Description of Use	Advantages	Disadvantages
Simple Random Sampling	• Samples are collected at random times or locations throughout the sampling period • Appropriate when a quick initial assessment is necessary because little is known about the site • Appropriate for uniform or homogenous populations	• Protects against selection bias so that samples are representative of the population sampled • Provides statistically unbiased estimates of the mean, proportions and variability • Easy to understand and straightforward	• Could result in an uneven distribution of sampling locations, thus is typically used in conjunction with other approaches • Does not consider prior information • Locating and obtaining samples in different geographic locations can potentially make this option difficult to implement and costly
Stratified Sampling	• Sample locations are selected randomly from strata of the sample population (non-overlapping sub-populations thought to be homogenous) • Strata can be chosen based on spatial or temporal proximity, preexisting information or professional judgment • Can be used inside and outside a ventilation system and on different floors of a building	• Separate sampling designs can be developed for each stratum • Can potentially achieve greater precision in the estimates of the mean and variance • Greater precision obtained when the measurement of interest is strongly correlated with the variable used to make the strata • Allows computation of reliable estimates for population subgroups of special interest	• Requires some information on the pathogen, the dispersal method or environment al factors
Systematic Sampling (Grid and Transect)	• Initial sampling unit is selected at random; subsequent samples are taken at regularly spaced intervals over space (grid), time (systematic) or a fixed path (transect). • Used to search for hot spots, to infer means, percentiles or other parameters and for estimating trends over time • Transect sampling can be conducted in previously sampled areas determined to have high concentrations of the pathogen	• Ensures uniform sampling coverage of the contaminated location • Easy approach for designating sampling locations • Systematic grid sampling results in a high probability of finding hotspot of contamination or increases confidence that a large proportion of the surface area is uncontaminated • Transect sampling can be used when location of the contaminant is unknown	• Involves the sampling of large areas to identify area of contamination

Table B-1 Probabilistic Sampling Approaches [References 2, 8 and 37]

Probabilistic Sampling Approach	Description of Use	Advantages	Disadvantages
Ranked-Set Sampling	• Expert judgment or an auxiliary measurement approach is used in combination with simple random sampling to determine with locations should be sampled • The two-phase sampling design identifies sets of field locations using simple random sampling, locations are ranked within each set using professional judgment and then one location from each set is chosen for sampling	• Useful and cost-efficient approach for obtaining estimates of the mean concentration • Useful when the cost of locating and ranking locations is low compared to laboratory measurements • Leads to more representative samples and more precise estimates of the population parameters	• Requires that the ranking approach and analytical protocol be strongly correlated to be effectively used
Adaptive-Cluster Sampling	• Some samples are taken using simple random sampling, and then additional samples taken at locations where measurements exceed a set threshold value or a characteristic of interest • Used for process pathways, air movement pathways and foot-traffic pathways • Helps determine hotspot boundaries • Useful when the characteristic of interest is sparsely distributed by highly aggregated	• Can be used to estimate the contamination characteristics of a larger area based on sampling of a smaller portion of the same area • Tracks the selection probabilities for later phases so that an unbiased estimate of the population mean can be calculated	• May require multiple rounds of sampling and analysis

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