

Guidance Document for Sampling and Lotting of Beef Products and Sample Analysis for Pathogens

Developed by the Beef Industry Food Safety Council

Developed by members of the Beef Industry Food Safety Council

Introduction

This document provides best industry practices for components (lotting, sampling and laboratory analysis) of the pathogen-testing program as a part of an overall food safety system. It is important to recognize that these are just components of the system and their success depends on the proper implementation of the best practices leading to these steps and after these steps.

Why do we test food for microorganisms? How does microbial testing fit into an effective food safety program? Most consumers unfamiliar with microbiology will tell you that if you want to know if food is contaminated, just test it. Unfortunately, as we all know, microbiological sampling of food to detect presence of pathogens is very difficult. Most bacterial pathogens are not homogeneously distributed in our food, so it is difficult to represent the overall level of contamination through the collection of a microbiological sample. In addition, the enteric pathogens like *Salmonella* and *Escherichia coli* O157:H7 are most often present in very low numbers in raw foods of animal origin, when they are there at all. To detect them takes examination of a large number of sample units from a lot, and even then probability works against us in ensuring safety. So are low numbers significant? It depends on the pathogens, but for enteric pathogens, presence at almost any level should be of concern.

Back in the 1980s, a group of food microbiologists was tasked with writing microbiological criteria that would ensure the safety of food. They published their findings in a report called *An Evaluation of the Role of Microbiological Criteria in Foods and Food Ingredients*. After much research and lengthy discussions, the group determined that microbiological criteria were insufficient for ensuring safety, primarily for the reasons discussed above. Assuring the safety of food from production through consumption is a complicated process requiring an organized, deliberate approach to preventing and controlling potential hazards rather than detecting them. The authors of the report realized that process control and prevention was the answer, not microbiological criteria, and recommended that the Hazard Analysis and Critical Control Point (HACCP) System be adopted to ensure food safety. That system is now widely accepted as the most effective and logical way to assure the safety of food. Microbiological testing is an active and important part of a functioning HACCP plan, but it is most likely to be effectively used in verification of the plan. Included in verification activities is validation, defined by the National Advisory Committee on Microbiological Criteria for Food as the element of verification focused on collecting and evaluating scientific and technical information to determine whether the HACCP plan, when properly implemented, will effectively control the defined hazards. Before a HACCP plan can function with assured control, it must be determined that all hazards reasonably likely to occur have been identified and that the plan to control them is scientifically sound and will be effective. Validation, both of individual CCPs as well as the entire HACCP plan, is integral to determining the soundness of a HACCP plan.

Microbiological testing can play a unique role in HACCP plan activities. However, in the production of red meat, because of the non-random distribution, it is generally agreed that

detection of foodborne pathogens is not an effective tool for monitoring CCPs within a slaughter/processing HACCP plan. In addition, pathogens are often absent from a carcass and, when present, their uneven distribution makes it difficult to obtain a truly representative sample. In contrast, microbiological testing can be applied within a HACCP plan to validate and verify the effectiveness of carcass decontamination procedures. It is important to note that verification activities are more accurately conducted to verify the effectiveness of the process that will control hazards rather than to verify the safety of the food product.

In December 2002 the industry began testing all trim destined for use in raw ground product. The difference in the USDA-FSIS data for 2002 and 2003 is likely due to the increase in trim testing. Even with 100% testing, the prevalence of *E. coli* O157 is far from zero. The reasons are many but the principal reason is that *E. coli* distribution is non-random and no amount of testing will eliminate *E. coli* O157 in trim or ground beef. The only way to truly control this and other pathogens is an effective HACCP plan and using testing as a verification of all upstream processes.

An establishment conducts a variety of microbiological testing in their food safety program. Pathogen testing of the trim or ground beef is only one of such tests. It is imperative that the establishment clearly understand the purpose of all testing, how to interpret the results of such testing and most importantly know what to do with the results of the testing.

An establishment may conduct testing to monitor the efficacy of their dressing practices, efficacy of their interventions, environmental monitoring program, trim and ground beef testing and others. The same testing may not be appropriate for the above testing programs, thus the person in charge of the testing decision must be knowledgeable or seek input from others to design a testing program that gives the establishment the maximum information to allow for the control of the process.

Because of the non-random distribution and because of the extreme low frequency (prevalence) of pathogenic microorganisms, pathogen testing cannot be used to verify the effectiveness of the HACCP plan. An indicator organism or indicator organisms that are more widely distributed are better targets for HACCP verification. However some testing of the hazard to be controlled is warranted in a validation concept. Nonetheless testing trim and ground beef for *E. coli* O157:H7 has essentially become the requirement for the product to enter the commerce. The objective of this document deals with recommended best practices for lotting, sampling and laboratory analysis for programs conducting microbial testing for *E. coli* O157:H7 in a variety of products produced in a beef processing plant.

Sampling & Lotting of Beef Products as a Component of a Food Safety System

This document outlines the process for developing a lotting and sampling program. In general the process includes the determination of a lot and then the use of an appropriate sampling program.

The purpose of this document is to identify the expectations and issues that should be considered when developing a lotting and sampling program for pathogen testing.

I. Sampling System Requirements

- Labeling of Sample, Lot and Container must be consistent and performed in a manner to provide complete traceability. It is imperative to ensure that no container or lot ID's can be duplicated in a production day and it is best to not duplicate during a week.
- Sequencing of Production Time and Area produced should be documented in order to allow for sequencing of finished product in the event of a positive pathogen test result or multiple positives test results. This is the primary step to event day and event window management.
- Management of rework has to be performed to maintain identity of time and area of production. The container must not lose the original identity as it may cause product to be inadvertently shipped in an event window or day.
- Incoming Raw Material Lot Management Systems must be in place to accurately track raw material lot and source. It must be documented to ensure all affected product from a production lot of raw material is easily tracked through the system.
- All boxes produced in a box sampling system must have 100% reconciliation and retention to ensure control of tested lot.

II. Carcass

When determining lotting practices for individual carcasses, the following should be considered:

- Lot
 - A minimum of one carcass should comprise the lot. Each carcass is processed through the slaughter floor and is treated with interventions/processing aids as an individual carcass.

- Once lots are sampled/tested, the lot should not be broken down into primal(s)/etc (separated) and should remain intact until negative test results have been received.
- If a test result shows a non-negative result, that individual carcass lot must be kept as an individual lot and no trimming or parts/pieces removed for production.
- Carcass lots are assigned AFTER the carcass has passed USDA-FSIS post mortem inspection and interventions/process aids have been applied.
- Sampling: Use the USDA generic *E. coli* sampling method ([9CFR310.25](#)) or the USDA USMARC carcass sampling method.

III. Trim Sampling (Combo)

- Lot
 - A minimum of one combo but not more than five combos should comprise the lot.
 - Once lots are sampled/tested, the lot cannot be 'split' (separated) and must be sold/shipped intact with adequate traceability records maintained
 - Lots are assigned AFTER all quality testing and audits are completed and the combo is a finished combo ready for shipment.
 - Combo trim may be lotted as produced by time, lean point, source, customer requirements, etc. Regardless of the lotting scheme, it is critical that the tested lot remains intact and clearly identified through to the end user.
- Definition of Robust Verification Sampling
 - Maximize outside surface area of excised sample.
 - Based on best practices of N60 (a minimum of 60-total pieces per lot).
 - All 60 pieces MUST come from DIFFERENT trim pieces.
 - A lot must be represented by a minimum of 60 individual pieces of trim. Other methods that have been validated to be equivalent to or better than N60 best practice (demonstrate that surface material is targeted during sampling or bacteria recovery).
- Define area of the combo to be sampled - ideally the 60 pieces must represent the entire lot. If combos are not sampled from top to bottom, establishments must support randomness of sampling.
- For combos with large primal pieces (e.g. 2-piece chuck), samples must be taken from different pieces. Therefore it is advisable to sample as the combo is filled to ensure different random pieces are selected. However, if the lot is less than 5 combos, it is possible there could be fewer primal pieces in the combo than samples required. In these instances, it is acceptable to sample a large primal no more than twice with at least 12 inches between the samples.

- Define the area of the trim piece to be targeted for sampling. As stated above, bacteria reside on the surface of the carcass and therefore only the external surface of the trim should be sampled. Employee training and routine monitoring is required to ensure samplers take the correct number of pieces and that these pieces are obtained from the external surface of the trim. Methods and verification systems that eliminate sampling error are preferred.
 - The sampling protocol must be verified routinely to ensure that the number of pieces meets or exceeds 60, the size of each sample (surface area) is adequate, target weight of the sample is met and that samplers are targeting external surface tissue.
- The laboratory MUST analyze the entire sample that is taken – subdividing samples is not allowed because the laboratory cannot analyze much more than 375 grams. Therefore the sampling method should be optimized to yield a minimum of 375 grams, but not much more than 375 grams.

IV. Box Trim

- Lot
 - When determining lotting practices for boxed trim, the following should be considered:
 - A minimum box count for a lot can be as small as one box regardless of weight, but the volume of the material in the box should be sufficient to allow for N=60 sampling.
 - The maximum box count should not exceed, by weight, the equivalent of 5 combos which is approximately 10,000 pounds.
 - Lots may be differentiated as produced by time, lean point, source, customer requirements, etc. Regardless of the lotting scheme, it is critical that the tested lot remains intact and can be clearly identified to the end user.
 - Product in the same lot is not produced over more than one production day (all boxes in a lot have the same production date).
 - Lots are assigned AFTER all quality testing and audits are completed and those box(s) are ready for shipment.
- Select a representative sample of all products contained in the lot. Each product type must have an equal chance of being selected, however, where possible, the lot should contain only one product type.
- Sampling should be performed exactly as described above in combo section. This would mean a minimum of 60 pieces with maximum surface area. In the event that more than 60-boxes are produced, more than 60-pieces would be selected. In order to maintain the sample weight for lots greater than 60-boxes, it is permissible to reduce the length of the piece of trim to ensure maximum surface area and not exceed the 375 gram sample standard.

- As with combo sampling, the external surface of the piece must be targeted for sampling (skin surface of the carcass if present).

Frozen product sampling is difficult as it is not easy to target the outside surface of the piece in a frozen state. It is advisable to collect samples for frozen material either prior to freezing or by thawing in a manner to allow for proper surface excision as detailed above. Where possible, the product is sampled and tested as combos prior to boxing and freezing, maintaining lot integrity through the boxing process. If this is not possible, sampling of frozen product should follow USDA Import Procedures, [FSIS Directive 10010.1R3, Attachment 7](#)

V. Ground Beef

- Lot
 - The lot is determined by arbitrary timeframe when packaged in final form. This must be logistically feasible, traceable, and maintainable.
 - Rework must be accounted for in the lotting scheme. Re-work should be returned to the original lot. If an establishment is not able to re-introduce the ground material into the original lot that the material came from, then that ground material may be placed into a separate “rework” lot. This rework lot may contain ground material from numerous lots of tested product that is produced in that day. This rework lot is kept as a separate individual lot and will be considered a part of any non-negative lot that may occur during that production day. Since the rework lot is made up of ground material that has already been subjected to sampling and testing, there is no need for any additional testing to be done unless an establishment adds previously untested or fresh raw material to this lot. No fresh or untested material may be added to the rework lot since the addition of new material will corrupt the lotting integrity. If untested or fresh material is mixed with rework then product should be diverted to cooking.
 - No finished product (ground material) should be carried over into a new production day due to the fact that this will cause a carryover lotting issue and in the event of a positive test result would implicate multiple days of production.
- INITIAL SCREEN
 - Defining point in the process to collect the sample: In order to account for microbiological contamination throughout the process, samples should be collected after a point in the process where no likely additional growth or contamination will occur; thus the point of sampling depends on the ground beef package variable.
 - Samples MUST be collected at a point in the process after the final blender.
 - Every blender or batch must be represented by a sample.

- All components MUST be a part of the blend or batch before samples are taken. (i.e., sample after including, fresh and frozen beef, AMR, seasoning and rework).
 - As published by Barkocy-Gallagher et al., 2002, freezing does not significantly affect the persistence or recovery of *E. coli* O157:H7 in ground beef; therefore, sampling can occur before or after freezing.
 - Define the number of samples to collect during the processing of the lot:
 - A minimum of 65 grams of product must be collected and tested per lot and represent each blender or batch produced.
 - In addition to the initial lot sample, library samples totaling at least 325 grams per lot should be taken and held pending initial screen results. There must be a total of 325 grams per lot of product taken and each blender or batch represented for these library samples. A total of 325 gram sample per lot is required in order to make disposition when a positive occurs.
 - These samples are not analyzed unless there is a positive (detailed in SECONDARY SCREEN below).
 - These samples must be documented using blender ID and time.
- SECONDARY SCREEN
 - If a positive initial screen occurs, submit the library samples described above, to further scope the event. At a minimum, analyze the library sample of the initial negative lot before and after the initial positive lot. (**See Diagrams 1-3**).
 - If the additional lots before or after the positive lot are positive, it will be required to analyze additional lots before and after.
 - If the lots before and after are negative after secondary screen:
 - Hold the affected positive lot and the lots before and after. This product should be diverted away from raw ground/non-intact product and could be sent to a USDA inspected, controlled cooking facility, inedible rendering or landfill.
 - Sublotting of lots before and after the initial positive is only permissible with appropriate data to support these decisions. This cannot be performed without controls for rework, raw material and finished product lot control and system push through (all product processed).

VI. Box Primal

- Lot
 - Each individual package of product should be considered microbiologically independent unless the sampling encompasses more than one package. When determining lotting practices for boxed primals, the following should be considered:
 - Product that is collected, bagged and boxed is considered independent of other bagged product from that line or product source. There has been no documented linkage between individual pieces of product and the presence of *E. coli* O157:H7 and therefore there is no scientific reason to link one primal bag to another. A primal and/or sub-primal lot can be as small as one individual package but must include all product contained within the selected package.
 - In selecting lots, detailed collection of all data available with the primal(s) selected must be maintained in order to clearly identify what was sampled, such as establishment number, pack date, product code, pack time, packaging information, etc.
 - If a sample (n=60) is pulled from one box then the lot would be that box.
 - If a sample is pulled from 4 boxes consisting of 5 bags of a certain primal, the lot would include all primal products in those 4 boxes.
 - If a sample is pulled from boxes out of five pallets, the lot would include the five pallets and all products in those pallets/boxes.
 - Whatever the scheme, it is critical that the tested lot remains intact and can be clearly identified to the end user.
- Select a representative sample of all products contained in the lot. Each product type must have an equal chance of being selected.
- Sampling should be performed exactly as described above in combo section. This would mean a minimum of 60 pieces with maximum surface area. In the event that more than 60 boxes are produced, more than 60 pieces would be selected. In order to maintain the sample weight for lots greater than 60 boxes, it is permissible to reduce the length of the piece of trim to ensure maximum surface area and not exceed the 375 gram sample standard.
- As with combo sampling, the external surface of the piece must be targeted for sampling (skin surface of the carcass if present).
- Primal testing can be conducted in one of two ways:
 - Combo Naked Test – This method follows the combo sampling method as detailed above.
 - Product going directly to package – This method would follow the boxed trim method as detailed above, but each primal piece would require a small piece to be incised from individual primal pieces.

- Testing during production has several components that must be assessed:
 - Assess other like-products being produced as the “tested lot” of primal products may implicate other products.
 - Products that were part of the sampled lot and were subsequently rejected for quality or other reasons, must be tracked and controlled. This includes leakers, damaged boxes, held product for specification review, etc.
- Reconciliation of sampled boxes in inventory must occur. Boxes must be 100% reconciled in inventory before samples are analyzed to ensure all product is under establishment control and no product is shipped prior to receipt of test results.

VII. Bench Trim

- Lot
 - All source material for bench trim must be tracked.
 - The lot should be linked to a production period.
 - If a sub-primal is made into a non-intact product the sub-primal should be held until bench trim results are received unless the sub-primals (post trimming, pre-tenderization, cubing or enhancement) are treated with an antimicrobial after trimming and prior to making non-intact product.
 - When sampling and testing bench trim from non-intact product you must take into account the non-intact sub-primals as part of the lot.
 - Bench trim produced after a sub-primal has been processed into non-intact should NOT be used in raw ground product.
- Sampling:
 - Sampling should be performed exactly as described above in combo section. This would mean a minimum of 60 pieces, selected to maximize external surface area. If more than 60 pieces are sampled, it is permissible to reduce the length of the piece of trim to ensure maximum surface area and not exceed the 375 gram sample standard.

VIII. Box Offal

- Lot
 - Offal products typically used in grinding include hearts, weasands, head meat, cheek meat, oxlips, tongue root trim, etc.
 - When determining lotting of offal an establishment must be able to clearly define and support the microbial independence of the lot, if the lot is not clean up to clean up. This is due to the fact that harvesting and boxing processes typically used in slaughter operations may include multiple areas of product surge, such as holding bins and totes and commingling of product types on a single conveyor, which makes maintenance of lot independence challenging.
 - Depending on the establishment's production and lotting process, the COA's provided for offal product may be a COA letter stating that the product shipped was part of a tested lot but not the entire lot or they may be more traditional COA's if the shipment contained all the product from a tested lot.
 - When designing offal lotting systems, the establishment needs to consider if the production of this type of product can support microbial independence. Does the harvesting and collection of this product leave heavy residue on all equipment it comes into contact with including knives/tables/employee equipment/chutes or collection bins? Is the surge or collection process such that product is not necessarily packaged in time-order? If so, then the most likely lotting scenario would be from clean-up up to clean-up. The separation of these lots most likely will include a clean-up step so that cross contact points are adequately addressed. This is to determine that a true lot separation can be supported as microbiologically independent.
 - There should be a visual verification of this cleaning step. In addition, a microbiological baseline supporting this cleaning step should be conducted and routinely verified. Separation by production days is supportable due to a full clean-up with an inspection between lots. When separating lots into less than a verified cleaning step, the establishment needs to consider how they will support the microbiological independence of those lots. This may be accomplished through:
 - Segregation of collection equipment and packaging
 - No matter what the lot determination/size an establishment must be able to adequately support the lots have clear separation from other lots or product whether the lot is an individual piece or box or by time frame – microbiological independence of the lot is the goal.
 - When collecting samples for a designated lot, a sampling scheme must account for sampling randomness and represent the entire lot. This may be accomplished by taking a sample from every box or isolating the collection of the tested lot in a

method that allows for random sampling such as sampling hearts that do not run down a co-mingled table.

- Select a representative sample of all products contained in the lot. Each product type must have an equal chance of being selected.
- Sampling should be performed exactly as described above in combo section. This would mean a minimum of 60 pieces with maximum surface area. In the event that more than 60-pieces are selected, it is permissible to reduce the length of the piece of trim to ensure maximum surface area and not exceed the 375 gram sample standard.
- As with combo sampling, the outside surface of the piece must be targeted for sampling
- Reconciliation of sampled boxes in inventory must occur. Boxes must be 100% reconciled in inventory before sample is analyzed to ensure proper controls are in place and no product is shipped prior to receipt of test results.

IX. AMR

- Lot
 - All product produced from a clean-up to clean-up must be considered a lot unless an establishment can support alternative lotting and disposition decisions.
- Sampling
 - Select a small sample from each box as it is being produced ensuring that at least 60 random (6-7 grams) samples are taken.
 - Composite at least 60 random samples into one 375 gram composite for the analytical sample.
- All boxes must be accounted for and retained pending sample analysis.

Definitions

- **Lot:** The amount of product which is represented by a sample. This can be determined by time, weight, container (combo or boxes) or number of units, that makes it independent of other lots
- **Sample:** A portion of product that represents the given lot.
- **Lot Management:** The lot should be maintained together and should not expand beyond clean up to clean up. All products in the “Lot” should remain under company control until pathogen test results have been received. Lot integrity should be maintained until negative test results are received (no further processing).

- **Positive:** Any test result that is non-negative. A test result may be suspect, presumptive positive, or confirmed positive.
- **Rework:** Product that is rejected from the process during a single production run.

Diagram 1

A Sample Set Compositing Scheme – 1-hour Lots

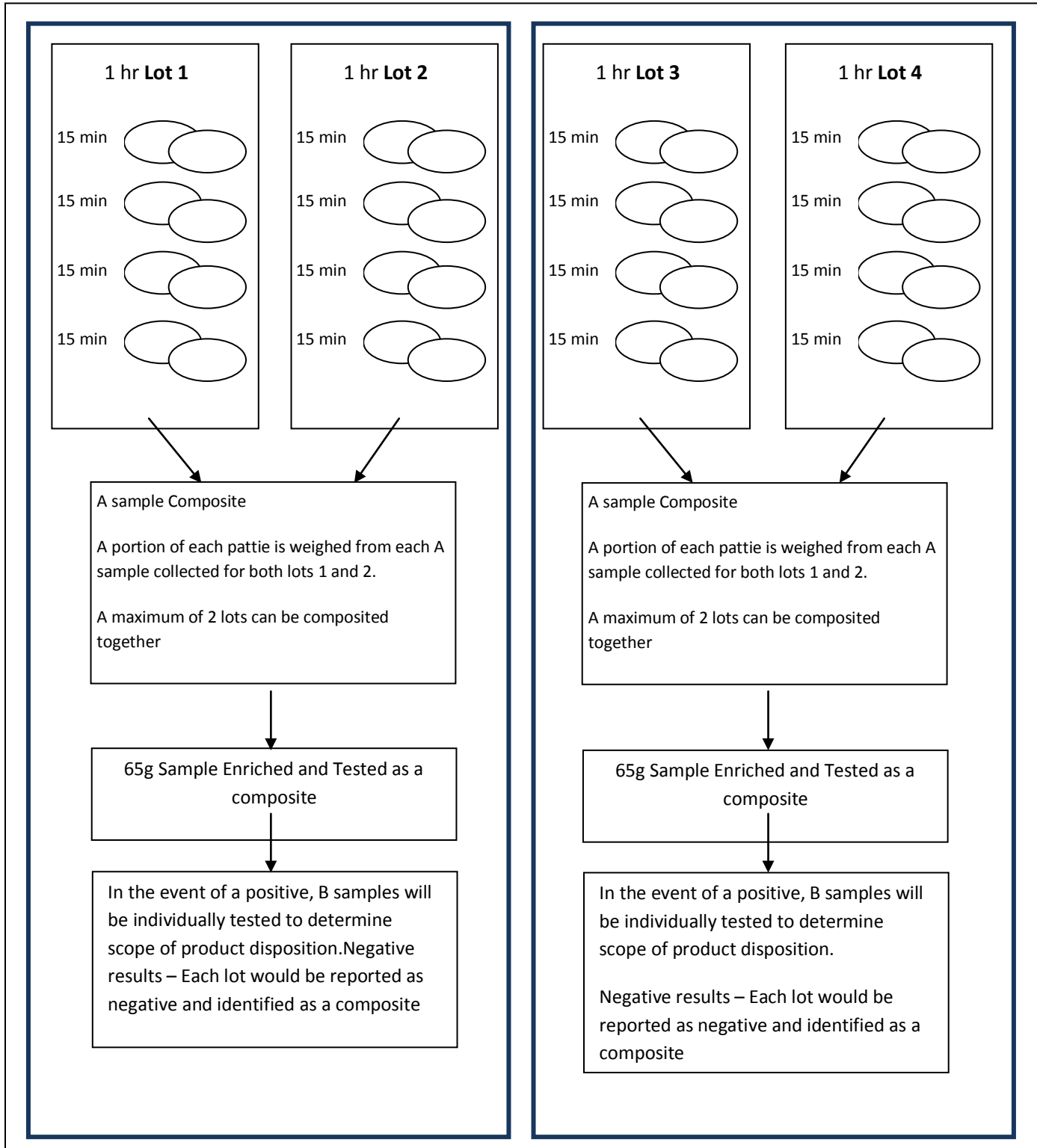


Diagram 2

B Set Testing 1-hour Lotting

Each Lot represents 1 hour of production. 2 lots were composited to obtain A sample Results.

ALL PRODUCT REMAINS ON HOLD UNTIL B SET TESTING IS COMPLETED.

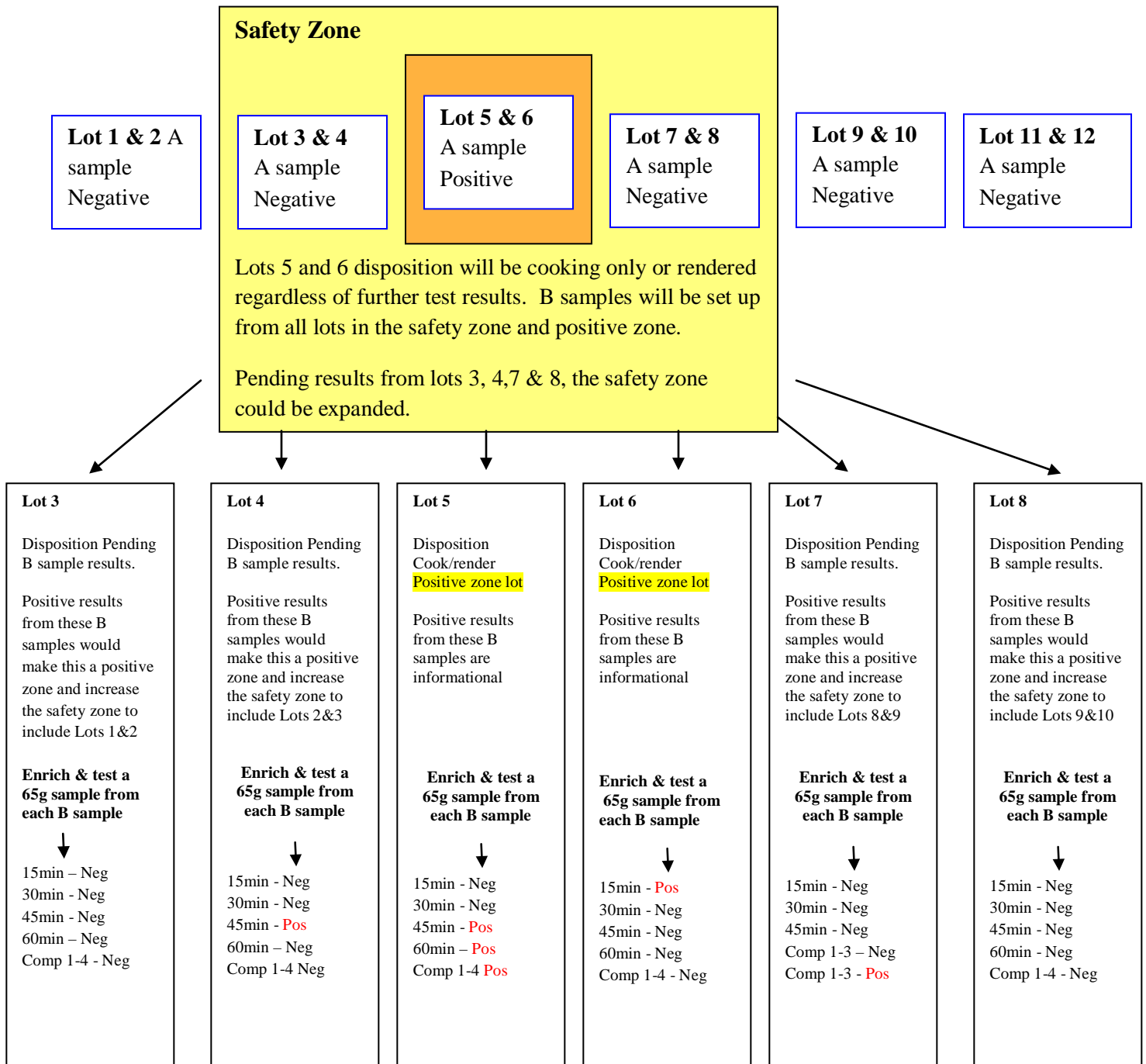
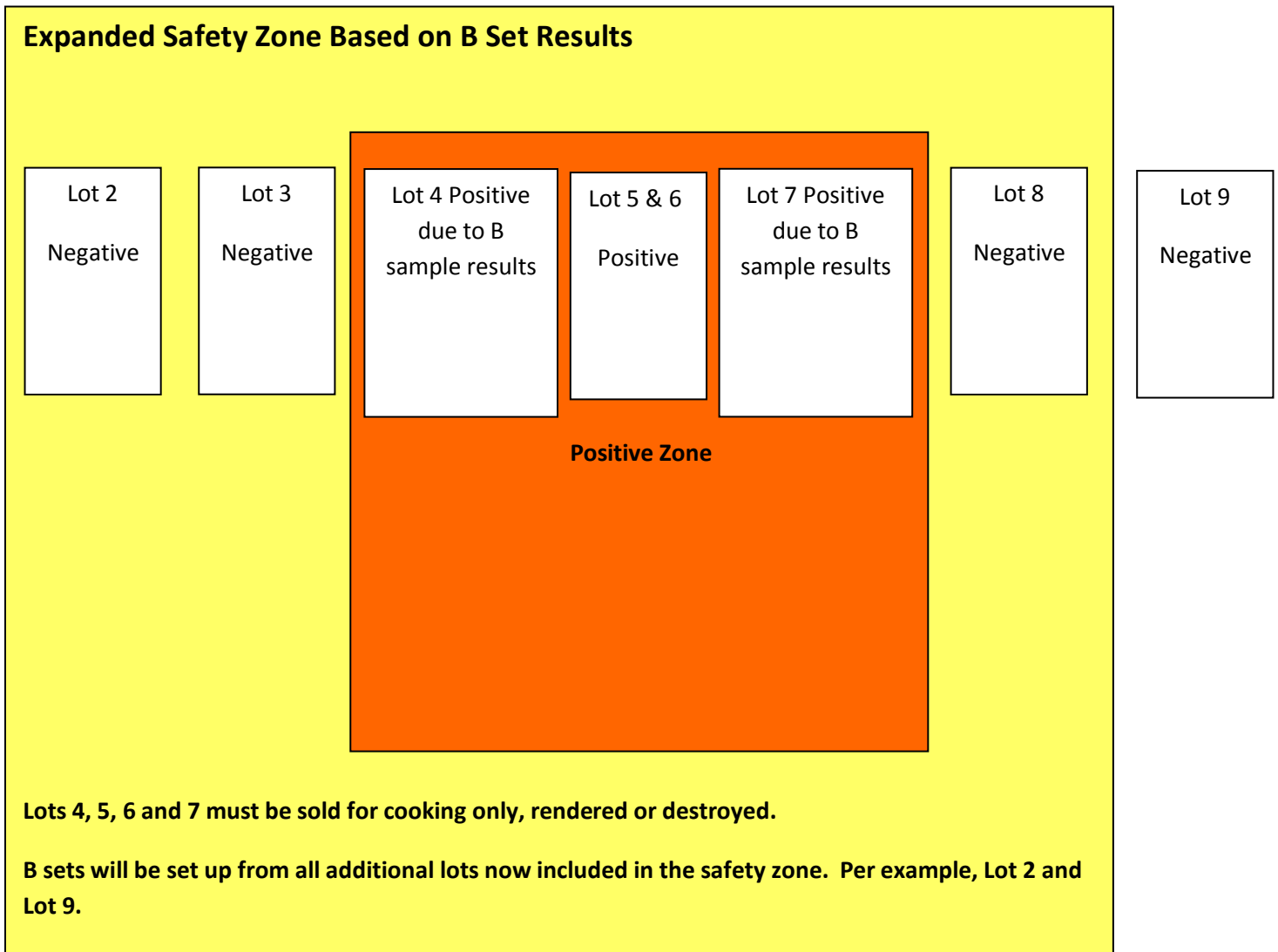


Diagram 3

Additional B Set Testing – 1hr Lot

ALL PRODUCT REMAINS ON HOLD UNTIL B SET TESTING IS COMPLETED.



Sample Analysis

Scope

This document was developed to provide guidelines, recommendations and framework for the development of a program for determining the presence of microbiological targets in samples of beef.

Analysis criteria

Establishments must understand and clearly define the analysis objective(s), potential outcomes and subsequent actions in order to successfully implement and manage a food safety program. Determination of analysis program criteria should be made for each establishment based on complexity of product lines and types, intended use of product, processes, capacity / volume, and contribute added value to the program. **Analysis should only be performed to achieve predetermined objectives of established programs or short term initiatives that involve new analysis technology evaluation or specific process enhancements.**

I. Laboratory selection

The laboratory should be capable of serving as a qualified guide and information resource in presenting options that best fit the technical requirements, business needs and support analysis objectives. A laboratory partner must provide the expertise and credentials required for unequivocal test results. The laboratory partner must also be capable of interpreting and applying the data in an effective manner.

If the laboratory is utilized in the capacity of guidance on method selection, then the laboratory must be capable of supporting the recommended method by ensuring it is fit for use, performed as validated, and results generated and reported are within the scope of the validation for the target organism(s) or tests. The laboratory must have a strong knowledge and understanding of test systems.

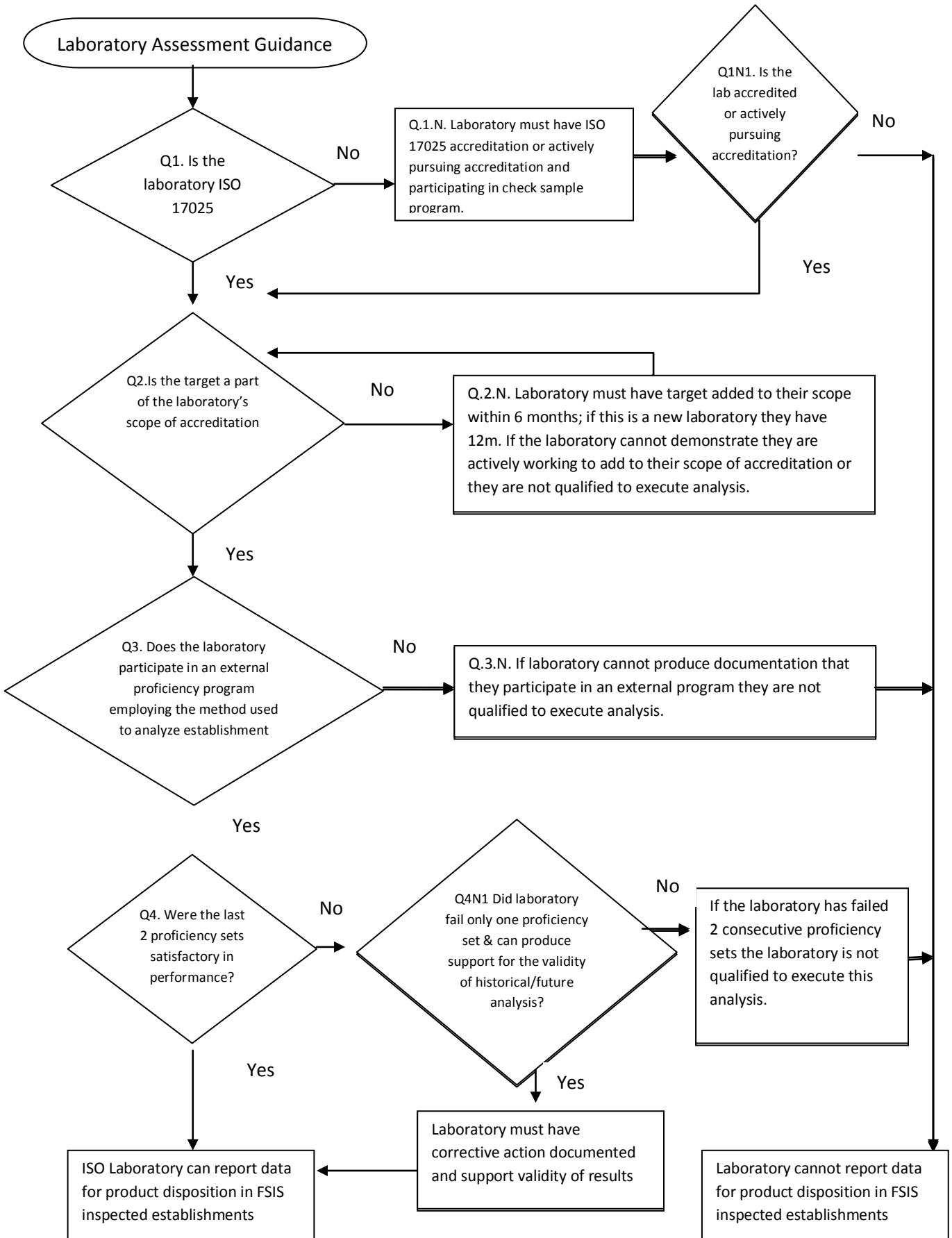
Best practice guidance for accepting a laboratory's qualifications is included below (**Figure 1, Laboratory Assessment Guide**).

Key qualifiers include:

- The laboratory must be accredited to the ISO / IEC 17025 standard for testing laboratories

- The test method of choice must be a part of the laboratory's scope of accreditation. The laboratory should provide an official scope of methods which have been included in the accreditation process. The method you choose for testing should specifically be listed on this scope to include the standard method type.
- The laboratory participates successfully in an external proficiency program which includes the pathogen methodology utilized in the establishment's analysis program.
- Final results are reported in a manner consistent and representative of the validated test method and in accordance with any applicable specifications. (regulatory standards or GLP practices as described by the ISO 17025 Standard)
www.a2la.org/requirements/req17025.pdf

FIGURE 1. Laboratory Assessment Guide



II. Test Method Selection

Method selection should be specific for each analysis program based on factors associated with, but not limited to, product, program goals, time limitations, product intended use, and method performance characteristics relative to specific objectives of established programs. Multiple analysis methods are often warranted due to differences in operational needs.

An establishment must determine methodology for analysis based on factors that include:

1. Test Result Application

Method selection should be performed with a complete understanding of the detection target(s) and confidence that the data generated will support program objectives.

2. Approvals

- A validated method should be the method of choice. Validation, approval, or acceptance by an independent body (i.e. AOAC, AFNOR, USDA-FSIS) is sufficient if all parameters (e.g., sample size, sample type, dilution ratio, incubation time), are validated for the intended purpose of the test. (**see also Appendix 1 on validation**)
- Methods that vary from the approved, validated method should meet the requirements defined in Appendix 1.

3. Fit for Intended Use

- The method must be validated for the specified product matrix. Assay used must demonstrate effectiveness at finding the target organism in the same matrix that is being tested in the field (e.g., an *E. coli* O157:H7 test validated for use with spinach should not be used for meat unless validation data can demonstrate effective detection of target in both matrices).
 - i. Same product type
 - ii. Same amount of sample analyzed and analytical units referenced
- Establishment of an appropriate Limit of Detection (LOD) should: (1) be dependent on the target organism; and, (2) detect low levels of contamination consistent with the regulatory or establishment requirements (see section on “fractional recovery” in validation section below)

- i. Method validation should be consistent with the manner in which the method is implemented in the laboratory. A variation in implementation due to program limitation will require verification by the end user or through collaboration with the kit manufacturer. (e.g., sample size, sample type, incubation time)

4. Business Based Considerations

- Does the method provide added value, required by the business, relative to program cost? *This is an organization / business specific question with organization / business specific answers. The answer to this question may vary between establishments. Consider if a method is better suited for business and/or process(es) than what is currently being used.
- Does the method allow an establishment to fulfill the business / customer service requirements? (e.g., turn-around time)

IV. Building a Laboratory Relationship

When testing is necessary, it is imperative that the establishment build a working relationship with the laboratory. The establishment must work with the laboratory to assure that the information that is being provided is consistent with the defined program expectations. This relationship extends beyond pricing requirements and data integrity discussions, and encompasses fit for use, qualifications and laboratory practices that may impact the information provided to the establishment.

As a part of building the laboratory relationship, it is recommended that a documented method verification checklist is provided to each laboratory service provider for completion and discussion at least annually.

APPENDIX I Sample Analysis Guidelines

Supplemental Validation Parameters

In the event that supplemental test method procedure validation is required, it is important to apply a standardized approach to validation design. Specifically, there are key validation parameters that must be consistently applied in order to demonstrate a method is fit for intended use.

Matrix:

1. Sample matrix must be representative of product to be tested. Assumptions drawn from a more complex matrix could be applied to less complex matrices.

Logic: Properties inherent to the product type (e.g., pH, ingredients and additives) may impact test method performance. Variations on lean point and ingredient components are inherent to all product types, and each lean point or ingredient variation does not require validation, however, a range from high fat to low fat samples could be considered as a part of matrix definition in validations. **Industry experience lend to a focus on high fat as a worst case scenario for most methods.**

Significant differences or changes in matrix must be considered. Validation should occur when appropriate, based on questions that may be raised in the product matrix that cannot be answered by looking to existing science.

Existing science, to understand the potential impact of an individual component on method performance, does not necessarily require validation in the meat matrix. Typically, as a part of the methods approvals multiple product types and matrixes are evaluated,

Validation of a more complex matrix may be applied to a matrix which is considered less complex

The intent of this section is not to require validation for every minor matrix difference that may occur, nor should every minor matrix difference be validated. However, it is intended that as a part of method selection matrix and the impact of matrix components be considered.

2. Sample matrix temperature must be defined within typical product temperature range at the time of analysis, or worst case scenario (frozen). Validation should be completed at lower range temperature for fresh products.

Logic: Initial sample temperature should be a consideration in the validation study design. The sample temperature can impact incubation time requirements and time required to get sample to optimum temperature (fresh 35°F to 40°F; frozen less than 28°F).

3. Test sample portion size used in the validation must be greater than or equal to the typical test portion size analyzed.

Logic: Analytical portion size potentially impacts recovery. Theoretically, the larger the portion size, the more challenging the recovery. Therefore, validation should be completed at greater than or equal to the typical test portion size to be analyzed by the end user.

Inoculation:

1. The organism selected for inoculation during validation should be associated with test product type and historical outbreaks when feasible.
2. If a method has not received a validation approval by an independent body (e.g., AOAC, AFNOR, USDA-FSIS), the method must be validated using at least one challenge strain meeting all the rigors defined in this document. Additionally, a complete inclusivity and exclusivity panel as defined in AOAC Official Methods must be performed. (**This requirement only applies if the method has no level of validation approval. This requirement does not apply if there are procedural variations to an approved method.)
3. Organisms used to inoculate or spike samples should be properly temperature stressed for 24 - 72h relative to actual processing conditions. If validation is completed on a frozen product, the organism should be stressed by freezing; if the validation is completed on fresh product the organism should be stressed by refrigeration.
4. A low level inoculum should be used to achieve fractional recovery per target analytical unit size at the time of enrichment (e.g., 1-5 CFU per 375g). Fractional recovery occurs when 20 – 80% of inoculated samples result in positive detection.
5. Spike level must be verified at time of inoculation and enrichment by plating onto a non-selective medium. Enumeration must be done by standard methods published by a reference standard.

Logic: Method performance is tested at a low level inoculum. A high-level inoculum could result in positive results without any growth required. Low levels are more representative of actual *E. coli* O157:H7 prevalence in meat products.

Enrichment & Incubation:

1. Media formulation: Consistent with use for routine analysis.

Logic: Chemical formulation of the media must remain consistent with the validation.

2. Dilution Ratio: Sample portion to the volume range of media added per test sample portion size.

Logic: Adding an excess media volume or insufficient media volume may impact recovery through modification of enrichment conditions.

3. Incubation Time: Define minimum & maximum incubation time requirements.

Logic: Incubation time requirements impact false negatives due to limitations of growth. Minimum and maximum incubation time must be a consideration in validation study design. The practice of pre-warming media may be critical to achieving incubation times.

4. Incubation Temperature Settings: Define minimum & maximum incubation temperature setting requirements.

Logic: Acceptable incubation temperature setting must be defined. Incubation below validated temperature setting could result in false negatives. Minimum and maximum incubation temperatures settings must be a consideration in validation study design.

Methodology and Design

1. Test methodology for validation should be conducted as it would be in the routine analysis setting for sample analysis (fit for intended use).

Logic: Methodology steps will impact end results and potential for false negatives.

2. Use of a reference method is necessary when validating a new methodology for which no approval currently exists. An unpaired study design is recommended. A reference method may not be necessary for minor modification of an approved, validated method.
3. Reference methods should be cultural methods (i.e., USDA-FSIS MLG, ISO, FDA-BAM).

Replicates & Number of Data Points:

1. Multiple replicates of samples from multiple production days, using a statistically valid sample number (n). *Recommend* at minimum 3 replicates of 20 data points for primary analysis matrix at lowest level inoculum. Study design must also include at least 1 negative control. Alternate replicates and sample numbers may be considered based on the variation to the method.

Logic: The n of a study will greatly impact the % Sensitivity. As an example, 75 data points allows for 1 false negative result and exceeds the USDA requirement of 98% sensitivity. A negative control is necessary to help assure that a positive is not obtained due to possible laboratory cross contamination.

Confirmations

1. Must be completed on all negative results at the longest defined incubation times.

Logic: False negatives resulting from shorter incubation times would impact sensitivity.

2. Confirmations and verifications must be made utilizing a scientifically valid method.

Logic: Confirming negative results as negative at extended incubation times ensures that sensitivity data is not impacted by non-inoculated samples.

Calculations and Assumptions

1. Calculations must be clearly shown
2. Assumptions must be clearly defined

Logic: Assumptions should not be made during the determination and interpretation of a protocol

Data Analysis

When using an unpaired study design, a chi squared analysis should be used to determine whether or not there is equivalency. (reference Pearson here).

1. Fractional recovery, between 20 – 80%, must be achieved.
2. False positive of $\leq 10\%$ with an inoculum of 1-5 CFU in a test portion size

- a. Calculated:

$$\frac{(\text{Total \# of samples analyzed for that treatment} - \text{\#False Negatives})}{\text{Total Number of samples analyzed for that treatment}} \times 100$$

Logic: This calculation is the most simplistic and reduces complexity compared to other means of calculating sensitivity

3. False negative rate of $\leq 2\%$

- a. Calculated: $100 - \text{calculated sensitivity}$

Logic: This calculation is the most simplistic and reduces complexity compared to other means of calculating false negative rates

4. Inclusivity

- a. Original test manufacturer's inclusivity data should be acceptable to address inclusivity concerns when supplemental validation is completed. If a method is identified with major exclusions of organisms that have been associated with a human health event or illness the method should be reviewed with the test manufacturer and use should be re-considered, provided there is a comparable method that meets program objectives.

Logic: This impacts false negatives and increases product risk.

METHODOLOGY REVIEW CHECKLIST

The following checklist will be used as a part of the methodology and data review.

Supplier: _____ Date Method Submitted: _____

Test Method Name: _____ Test Method Manufacturer: _____

<i>Checklist</i>	Method Review	<i>Comments</i>
1. Matrix: Representative of Product tested ? 2. Typical of product temperature range? 3. Test portion size \geq to typical test portion size analyzed?		
Inoculation 1. Organism selection 2. Has method been validated by independent body? 3. Low Level Inoculum 1-5 CFU/target analytical unit size? 4. Fractional recovery 20-80% of inoculated samples positive 5. Spike level verified at time of inoculation & enrichment?		
Enrichment & Incubation 1. Media formulation & preparation 2. Dilution ratio		

3. Incubation time minimum & maximum 4. Incubation temperature range		
Methodology 1. Conducted as it is validated 2. Reference cultural methods used?		
Replicates & Number of Data points 1. Multiple Replicates of Statistically valid # of samples		
Confirmations 1. Completed on negative results at the maximum incubation		
Calculations and Assumptions <ul style="list-style-type: none"> • Clearly shown • Clearly defined 		

This form should be completed and reviewed annually

<i>I. Overview</i>		
1) List the test method name as referenced on the laboratory report or COA:		
2) Briefly describe the test as it is preformed in the laboratory to achieve reported results:		
3) Does this process require the use of multiple validated methods, not including cultural confirmation? <i>YES</i> <i>NO</i> <i>IF yes is answered, the following sections I – IV must be completed for each method completed, note that the validations must be CONSISTENT as the method(s) are applied</i>		
4) Does this process require the use of cultural confirmations? <i>YES</i> <i>NO</i> <i>IF yes Section V must be completed.</i>		
<i>II. Method Overview - Preliminary or Screening Method</i>		
5) Test Method Name as it appears on package insert:		
6) Test Method Manufacturer:		
7) This method claims to detect:		
<i>E. coli</i> O157:H7	YES	NO
<i>E. coli</i> O157	YES	NO
Other (List): _____		
<i>III. Validations - Preliminary or Screening Method</i>		
8) Has this method been validated by an outside source such as AOAC / AFNOR on a product type consistent to the product(s) that our establishment is submitting for testing?		
YES	NO	

<p>If YES answered in 4) above</p> <p>9) List approval body and the identifying number associated with the method:</p>	<p>If NO answered in 4) above</p> <p>10) Has the method been validated in a manner that meets or exceeds BIFSCO best practices and or current regulatory requirements? YES NO</p> <p>11) Validation must be attached to this form.</p>																		
<p><i>IV. Laboratory Processes - Preliminary or Screening Method</i></p>																			
<p>12) Please verify that you are completing this procedure as it has been validated, related to the following elements:</p> <table data-bbox="324 730 1396 1134"> <tr> <td>a) Matrix</td> <td>YES</td> <td>NO</td> </tr> <tr> <td>b) Amount of product weighed and enriched:</td> <td>YES</td> <td>NO</td> </tr> <tr> <td>c) Enrichment type and amount of enrichment added</td> <td>YES</td> <td>NO</td> </tr> <tr> <td>d) Incubation Time</td> <td>YES</td> <td>NO</td> </tr> <tr> <td>e) Incubation Temperature</td> <td>YES</td> <td>NO</td> </tr> <tr> <td>f) The method is being performed as validated</td> <td>YES</td> <td>NO</td> </tr> </table>		a) Matrix	YES	NO	b) Amount of product weighed and enriched:	YES	NO	c) Enrichment type and amount of enrichment added	YES	NO	d) Incubation Time	YES	NO	e) Incubation Temperature	YES	NO	f) The method is being performed as validated	YES	NO
a) Matrix	YES	NO																	
b) Amount of product weighed and enriched:	YES	NO																	
c) Enrichment type and amount of enrichment added	YES	NO																	
d) Incubation Time	YES	NO																	
e) Incubation Temperature	YES	NO																	
f) The method is being performed as validated	YES	NO																	
<p><i>V. Laboratory Processes – Cultural Confirmation</i></p>																			
<p>13) Is this process completed at the same location as the preliminary result? YES NO</p>																			
<p>14) Are the cultural confirmations completed are consistent with the USDA MLG 5.04 http://www.fsis.usda.gov/PDF/MLG_5_04.pdf YES NO</p>																			
<p>If YES answered in 16 above.</p> <p>15) The method is being performed exactly as prescribed in the USDA MLG 5.04?</p> <p>YES NO</p>	<p>If NO answered in 16 above:</p> <p>16) List the differences in method completion</p> <p>17) Attach validation support for the cultural method being utilized.</p>																		

18) We certify that the method is being performed as it has been validated. The laboratory further certifies that the processes and techniques used do not compromise the integrity of results generated

Signed:

Date:

Title:

The following contains expected answers to the questionnaire above and suggested actions if variations occur.

<i>I. Overview</i>		
19) List the test method name as referenced on the laboratory report or COA:	This should be the method name as it appears on your report or COA	
20) Briefly describe the test as it is performed in the laboratory to achieve reported results:	Typically the lab will describe a process similar to below: A (list sample size and other pertinent details) is analyzed following (list test manufacturer name and method). If a presumptive positive result is obtained it is (reported or culturally confirmed) Example: A 375g sample is analyzed following Perfect Test supplied by Perfection Systems. If a presumptive positive result is obtained, according to our customers requirements, samples are culturally confirmed.	
21) Does this process require the use of multiple validated methods, not including cultural confirmation?	YES	NO
<i>IF yes is answered, the following sections I – IV must be completed for each method completed, note that the validations must be CONSISTENT as the method(s) are applied</i> Multiple methods are intended to refer to a series of “screening methods” this could mean a lateral flow type method followed by a PCR based method. If Yes is indicated validations and support must encompass all methods as one system.		
22) Does this process require the use of cultural confirmations?	YES	NO
<i>IF yes Section V must be completed. This question is intended only to indicate if section V is necessary or not.</i>		
<i>VI. Method Overview - Preliminary or Screening Method</i>		
23) Test Method Name as it appears on package insert:	This is necessary to assure that it is clear what method is used, often a test is referenced by manufacturer name. Many manufacturers offer multiple tests.	
24) Test Method Manufacturer:	Another question for clarity only.	
25) This method claims to detect:	Often even sales personnel from manufacturers confuse this – you must understand if the method detects only O157:H7 or O157; typically lateral flows and Enzyme Immunoassays are broader and detect <i>E. coli</i> O157; PCR based methods are typically more specific to <i>E. coli</i> O157:H7	
<i>E. coli</i> O157:H7	YES	NO

<p><i>E. coli</i> O157</p> <p>Other (List): _____</p>	<p>YES</p>	<p>NO</p>
<p><i>VII. Validations - Preliminary or Screening Method</i></p>		
<p>26) Has this method been validated by an outside source such as AOAC / AFNOR on a product type consistent to the product(s) that our establishment is submitting for testing? Refer to Method Selection guidance in this document for more detail</p>		
<p>YES</p>	<p>NO</p>	
<p style="text-align: center;">If YES answered in 4) above</p> <p>27) List approval body and the identifying number associated with the method: As an example AOAC Official Method 2016.08</p>		<p style="text-align: center;">If NO answered in 4) above</p> <p>28) Has the method been validated in a manner that meets or exceeds industry best practices and or current regulatory requirements? YES NO A detailed review of the validation and premise of validation must be completed. If there are questions about the validation refer to BIFSCO Best Practices Guidance Appendix.</p> <p>29) Validation must be attached to this form.</p>
<p><i>VIII. Laboratory Processes - Preliminary or Screening Method</i></p>		
<p>30) Please verify that you are completing this procedure as it has been validated, related to the following elements:</p>		
<p>a) Matrix</p>	<p>YES</p>	<p>NO</p>
<p>b) Amount of product weighed and enriched:</p>	<p>YES</p>	<p>NO</p>
<p>c) Enrichment type and amount of enrichment added</p>	<p>YES</p>	<p>NO</p>
<p>d) Incubation Time</p>	<p>YES</p>	<p>NO</p>
<p>e) Incubation Temperature</p>	<p>YES</p>	<p>NO</p>
<p>f) The method is being performed as validated</p>	<p>YES</p>	<p>NO</p>
<p style="color: red; text-align: center;">If No is the answer to any of these questions there is an issue with the laboratory and or method and it must be addressed immediately.</p>		
<p><i>IX. Laboratory Processes – Cultural Confirmation</i></p>		

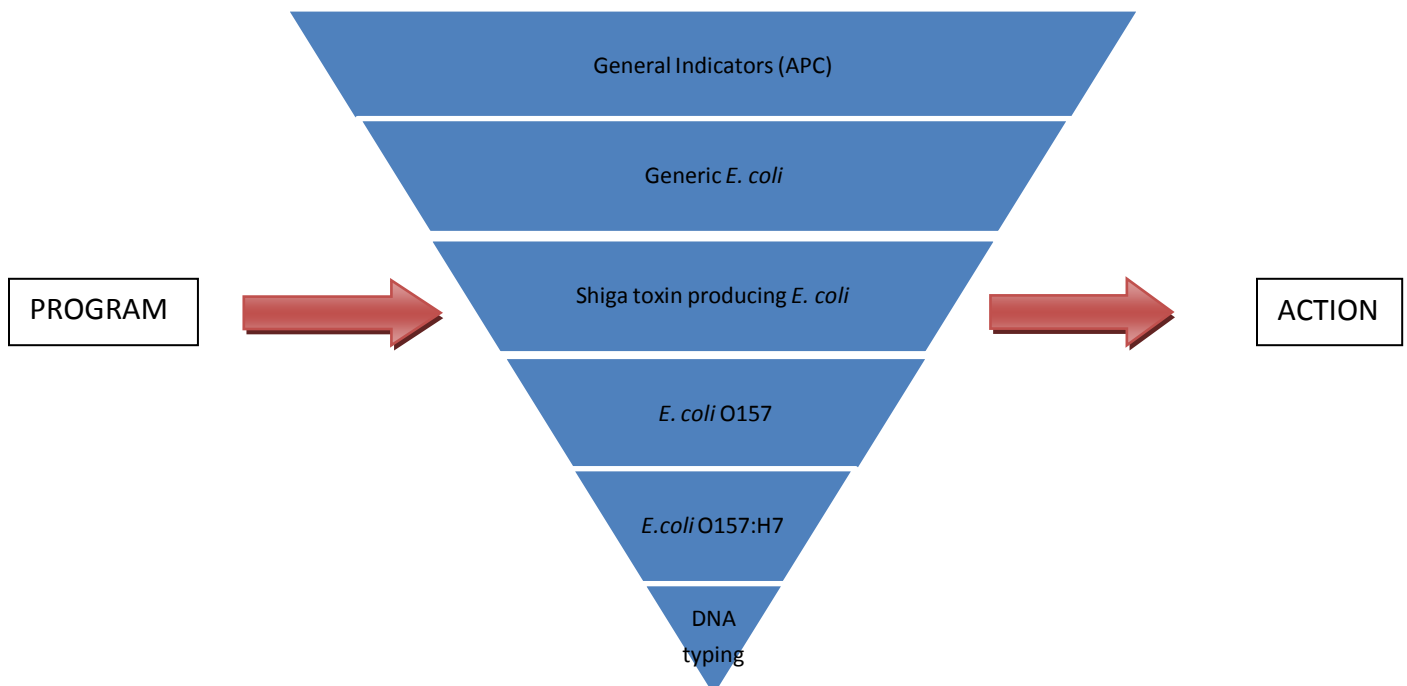
31) Is this process completed at the same location as the preliminary result? YES NO	
32) Are the cultural confirmations completed are consistent with the USDA MLG 5.04 http://www.fsis.usda.gov/PDF/MLG_5_04.pdf YES NO	
<p>If YES answered in 16 above.</p> <p>33) The method is being performed exactly as prescribed in the USDA MLG 5.04?</p> <p>YES NO</p>	<p>If NO answered in 16 above:</p> <p>34) List the differences in method completion</p> <p>Example: A column is defined in the procedure; however, Dynal beads and a bead washer are used.</p> <p>35) Attach validation support for the cultural method being utilized. Dynal support is attached.</p>
36) We certify that the method is being performed as it has been validated. The laboratory further certifies that the processes and techniques used do not compromise the integrity of results generated	
<p>Signed: _____ Date: _____</p> <p>Title: _____</p>	

V. Supplemental Validation Parameters for *E. coli* O157:H7 Test Methods

Validation data must clearly demonstrate that a method is fit for the intended use by the end user. Sections III and IV above are intended to assist processors in gathering information for determination of a test method's fit for the intended use. Typically test method validation is the responsibility of the test manufacturer, and is achieved through recognized independent body approvals (e.g., AOAC, AFNOR). As with any program operating under commercial conditions, minor deviations from standard procedures may occur. An understanding of procedure robustness may prove valuable when determining the adequacy of existing method validation(s). Scientific rationale should be leveraged to determine if variations to original procedure require supplemental validation. Such variations requiring validation may include, but are not be limited to: 1) Product to enrichment ratio; 2) type of product evaluated (often referred to as matrix); 3) test portion size used in the analysis; 4) product temperature, media temperature and/or enrichment temperature at the time of analysis; 5) enrichment media type; 6) duration of enrichment; and, 7) effect of the initial inoculum dose on sensitivity. In order to achieve a

standardized approach to supplemental validation, there are key parameters that must be consistently applied in a validation process in order to consistently demonstrate fit for intended use. Key validation parameters are discussed in detail in Appendix 1.

FIGURE 2. Test target spectrum



**Give examples using multiple approaches across the analysis spectrum and resulting actions.

Definitions

Association of Analytical Communities (AOAC) Founded by USDA in 1884 as the Association of Official Agricultural Chemists, AOAC was renamed AOAC International in 1991. AOAC International is a non-profit scientific association whose technical contributions center on the creation, validation and publication of analytical test methods. ([AOAC INTERNATIONAL Homepage](#))

AOAC PT/RI – AOAC Proficiency Tested / Research Institute: Test kit manufacturers seeking *Performance Tested Methodsm* status are required to produce and submit data to support product performance claims. The AOAC-RI recruits independent experts (known as “**Expert Reviewers**”) and selects a **General Referee** to review the performance data of the method. After the data submission for the method have been reviewed and found to support the product performance claims, by the Expert Reviewers and General Referee, the method performance is verified by an Independent Testing Laboratory. The evaluation is conducted using protocols developed by the Expert Reviewers and General Referee. The data generated by the Independent Testing Laboratory is sent to the Expert Reviewers and General Referee for evaluation to determine whether the independent laboratory data corroborates the data submitted by the manufacturer. If the two sets of data are found to corroborate each other and support the product performance claims, then the Expert Reviewers and General Referee will recommend *Performance Tested Methodsm* status for the method. (<http://www.aoac.org/testkits/programelements.htm>)

AOAC Official Methods of Analysis (OMA) –Independent method validation, of methods by an intercollaboratory collaborative study, in which experienced, competent analysts work independently in different laboratories under the direction of a study director using a specific method to analyze replicated test samples for a particular analyte. <http://www.aoac.org/Official Methods/Food Micro Validation Guidelines.pdf>

AFNOR - Association française de Normalisation (AFNOR) is the French national organization for standardization and its International Organization for Standardization member body. The AFNOR Group develops its international standardization activities, information provision, certification and training through a network of 11 key partners in France who are members of the association.

Enrichment – process of adding necessary nutrients, typically in a broth form to a sample.

Incubation – process of holding enriched samples under defined conditions (typically temperature and time).

Inoculation – act of adding a known organism to a sample.

Fractional recovery - when 20 – 80% of inoculated samples result in positive detection.

False positive - a positive result generated by an organism that is not the target.

Limit of Detection (LOD) - The level at which the lowest concentration of the analyte can be detected in a sample. This is the level that detection is just feasible.

Potential Positive**- a negative sample that causes a positive reaction with the screen test.

Presumptive Positive**- a sample that has typical colonies, observed on Rainbow Agar, and reacts specifically with O157 antiserum.

Confirmed Positive** – a biochemically identified *Escherichia coli* isolate that is serologically or genetically determined to be “O157” that meets at least one of the following criteria:

- 1) Positive for Shiga toxin (ST) production
- 2) Positive for Shiga toxin gene(s) (*stx*)
- 3) Genetically determined to be “H7”

******([reference: MLG – 5.04, Detection, Isolation and Identification of Escherichia coli O157:H7 from Meat Products, Revision: 04](#))

Confirmed Negative - Samples that have been tested with a test method validated as fit for use, and demonstrate an absence of the micro-organism(s) of concern. These results are typically based on an initial screening result.

Unpaired – Inoculated samples randomly assigned to an alternative or a reference method; a proportion of positive (and confirmed) samples using the alternative method compared to the proportion of confirmed positive using the reference method.

Matrix – A substance (gathered or collected) which is the subject of analysis and is considered in terms of specific properties.

Negative control - a test portion with known contents to carry through the method to verify performance.