Understanding Microbiological Sampling and Testing

Office of Public Health Science

Microbiology Division

FSIS 2010 Advanced EIAO Methodology Course

Today's Presentation

- Objective- Cover aspects of microbiological sampling and testing that should be considered for food safety programs.
  - Sample collection- methods and plans
  - Laboratory testing- test portions and methods

Why Do We Test For Bacteria In Foods?

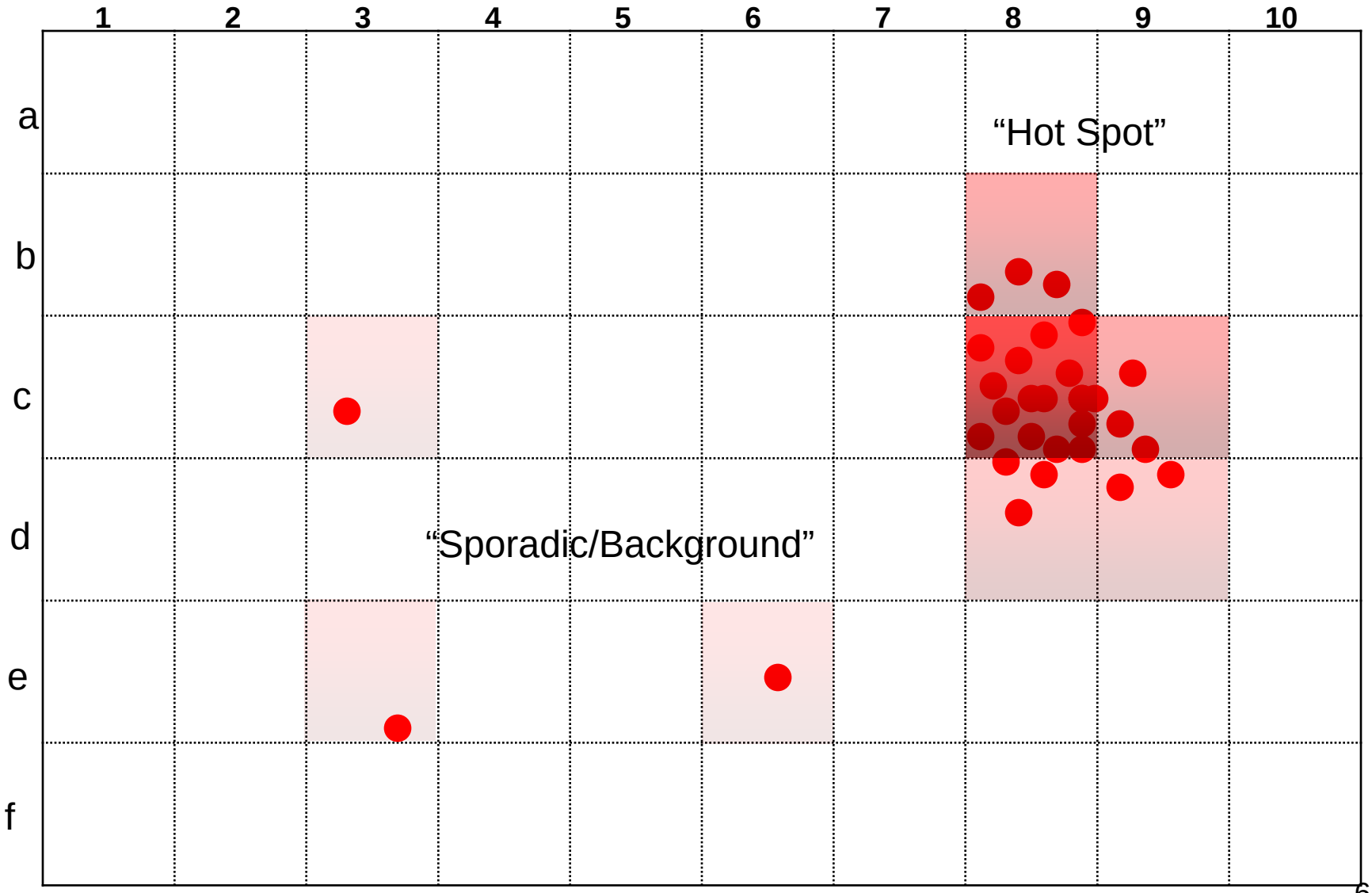
- Foodborne pathogens
  - Why? – Pathogens cause illness!
  - Challenges for pathogen detection:
    - Heterogeneous distribution in a lot or even a sample.
    - Pathogen cells on products are often at low levels and stressed.
- Indicator bacteria
  - Why? - Easier to detect and quantify for process control purposes

Sampling

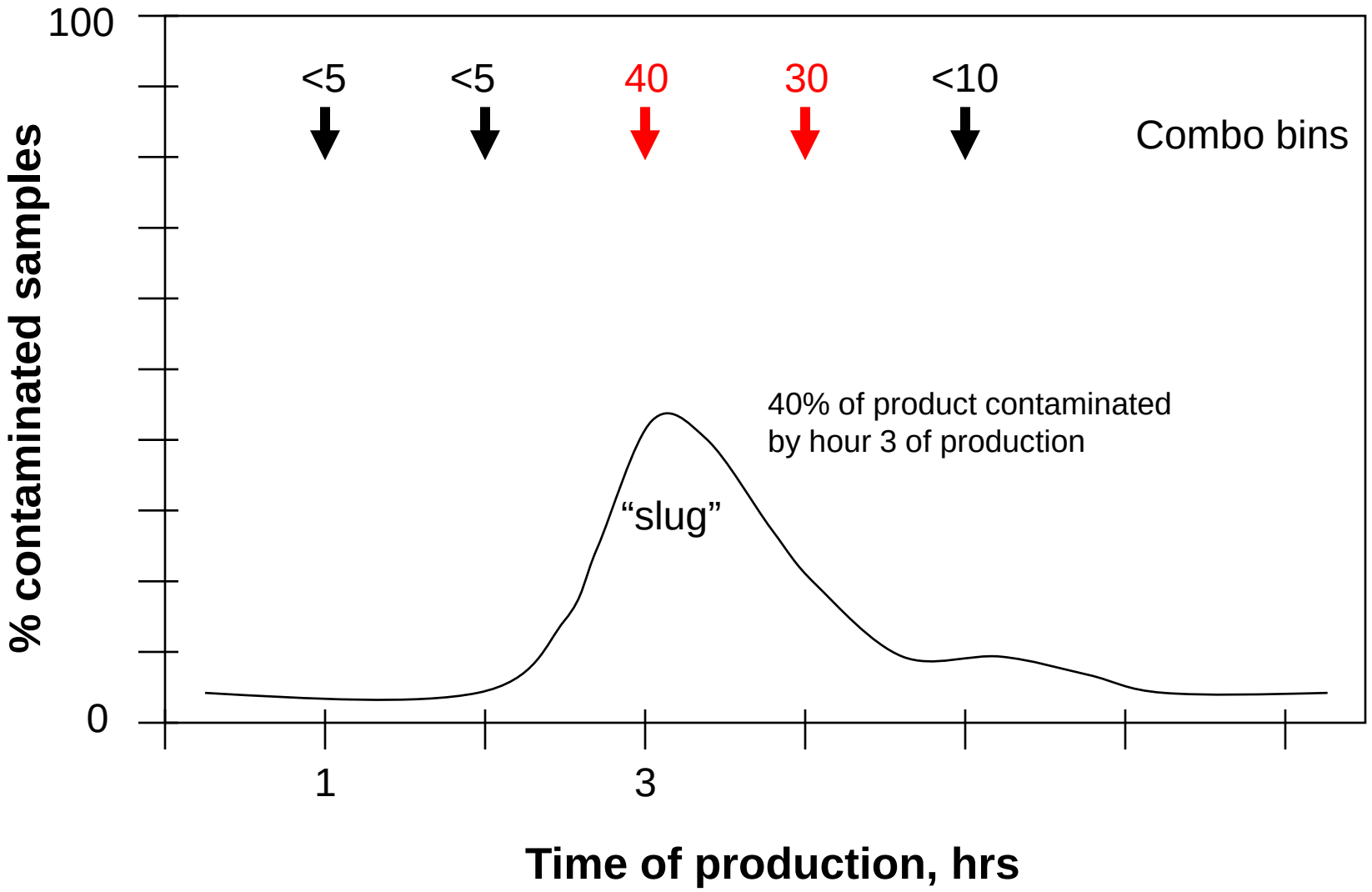
Sampling

- All sampling plans have significant limitations.
  - Therefore, we evaluate relative rigor of the program.
- Best sampling plans provide the opportunity but no guarantee of detection.
  - *i.e.,* scattered contamination is difficult to detect.
- Frequent sampling and sampling multiple sites/time points provides a better opportunity for detection.
  - Examples:
    - Multiple samples per day vs. once per month
    - One “grab” sample per lot vs. “n60” per lot
- Does the type of sampling meet the intended need?
  - Destructive vs. non-destructive sampling

E. coli O157:H7 Contamination in a "n60" Sampled Lot *(illustration)*



E. coli O157:H7 Contamination in Ground Beef (illustration)



What is “n60”?

- “n60” = number of samples (n) = 60
  - Multiple representative samples provides best option for detecting scattered contamination.
  - Provides 95% confidence that no more than 5% of food pieces the size of each “n” in the entire lot are contaminated.
- Keys to success
  - Must ensure that sampling is as representative as possible across the lot
  - Large composite “n60” samples typical need a larger test portion.

Common Sampling Problems

- Small sample or sampling method may not be ideal for detection.
  - Examples- small swab device, small carcass or environmental area sampled
- Sanitizer or excessive intervention might interfere with the test.
  - Insufficient drip time prior to carcass rinse procedure.
- Temperature abuse for the sample prior to testing
  - Holding under refrigeration for long periods allows competing bacteria to grow.
  - Freezing can kill some pathogens (e.g., *Campylobacter*)

Testing

Laboratory Testing

- Two areas of focus
  - The test portion taken from the sample
  - The method used to test that portion
    - Screening methods
    - Confirmatory methods

- Establishments should ensure their lab is aware of FSIS guidance, Directives, and Notices related to testing issues

Establishment Responsibilities For Laboratory Testing

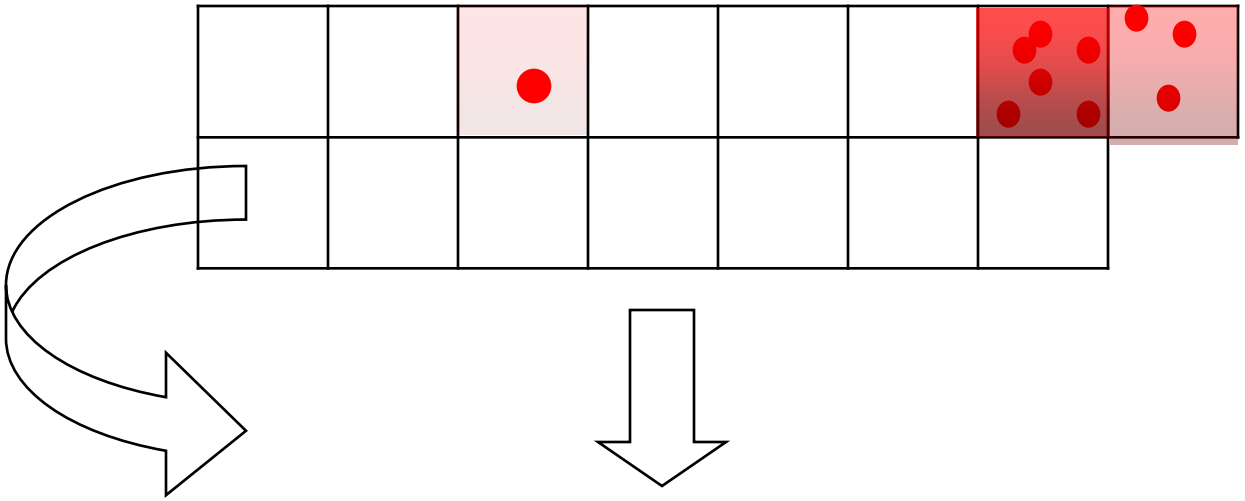
- The establishment is ultimately responsible for the testing they request from private laboratories
- Has the establishment properly conveyed testing needs?
  - *e.g.,* test portion equivalent to FSIS as opposed to the default 25-g in protocols.
- Is the laboratory aware of FSIS expectations?
  - Directives, Notices and guidance (some are pending)
- Establishment should document detailed methodology and validation information for FSIS review.

The “Test Portion”

- Laboratory sample preparation => “test portion”
  - *a.k.a.,* “analytical unit”
  - Definition- the part of the “sample” that is actually tested by the laboratory.
- Test portion determines the theoretical (*i.e.,* best possible) sensitivity of the test
  - *i.e.,* 1 cell/test portion
  - 25-gram- detecting 0.04 cells/gram is possible
  - 325-gram- detecting 0.003 cells/gram is possible

E.Coli O157:H7 Contamination in a N60 Composite Sample *(illustration)*

15 subsamples (25 grams) = 375 grams



Special Considerations for Larger *E. coli* O157:H7 Test Portions?

- Larger test portions (325-375 grams) are most important for “n60” and other composite samples containing many samples.
- Less important for ground beef final product testing when:
  - Trim and components have already been tested using robust sampling and 325-375-gram test portions, and
  - multiple samples are collected throughout the production day.
- Methods must be adapted, optimized and validated for effective use with 325-375 gram test portions.

Pathogen Detection Methods

- Complete multi-step method below can take at least one week:
  - Sample preparation
  - 1-2 stage culture broth enrichment
  - Screening test
  - Selective plating and purification
  - Confirmation using multiple tests

Enrichment

- Test portion is incubated 8-48 hours in a culture broth
  - Why?
    - Contamination levels are too low for detection without enrichment
    - Must grow to high levels so very small volumes have enough for later detection steps.
  - Different pathogens require a different broths
  - One vs two-stage enrichment
    - resuscitation vs selective growth

Considerations for proper enrichment

- Resuscitation (lag phase) can require 2-3 hours before log-phase growth begins.
  - Some samples support slower growth
- Has enrichment broth been tempered to warm temperature prior to incubation?
  - Particularly critical for large test portions or shorter incubation periods.

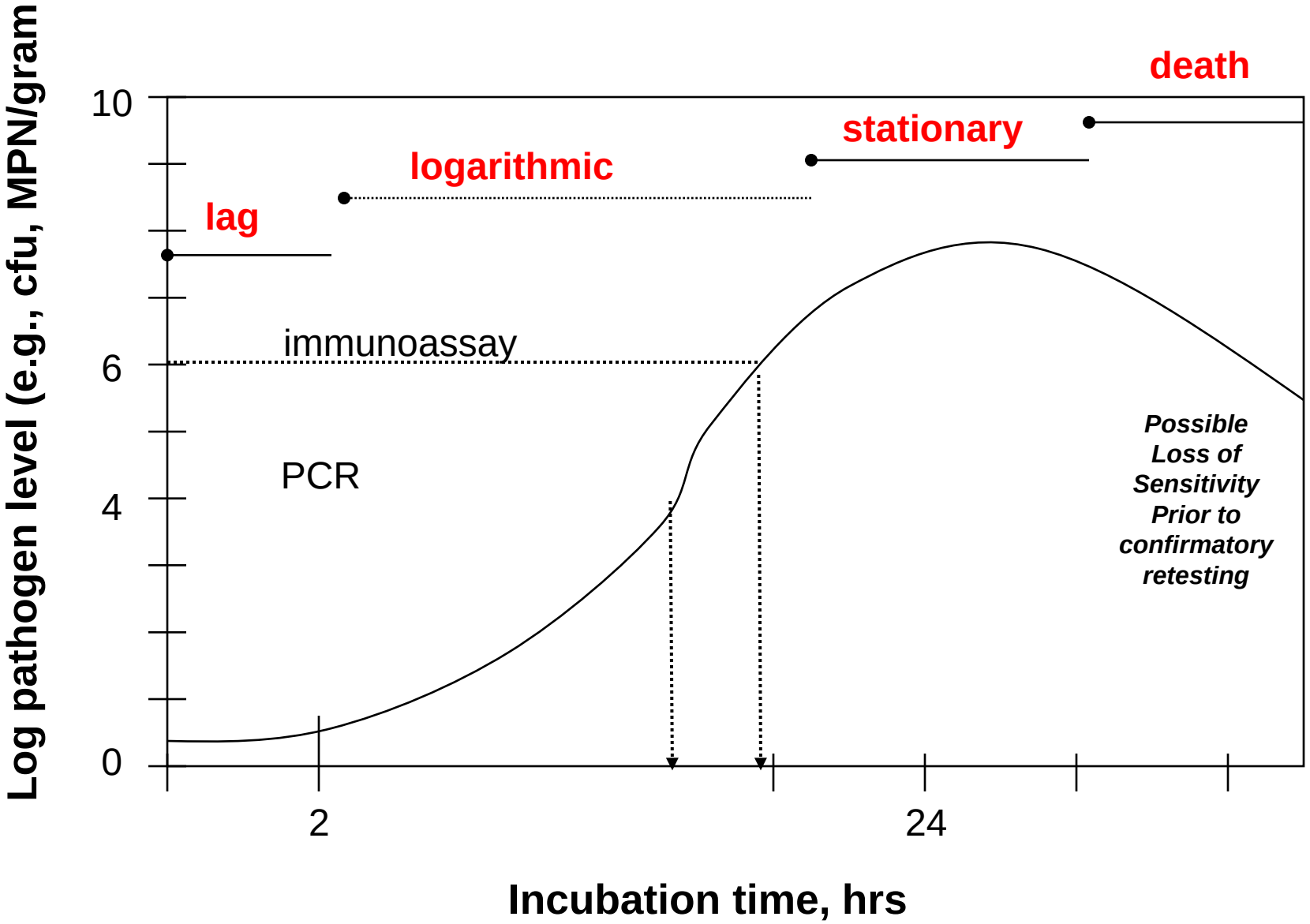
Screening Tests

- Usually commercial testing products
- Most validated screening tests are:
  - Immunoassays (ELISA, ELFA, immunochromatographic devices, etc.)
  - Polymerase Chain Reaction (PCR) assays
- Must be validated for performance with a specific broth and incubation period.

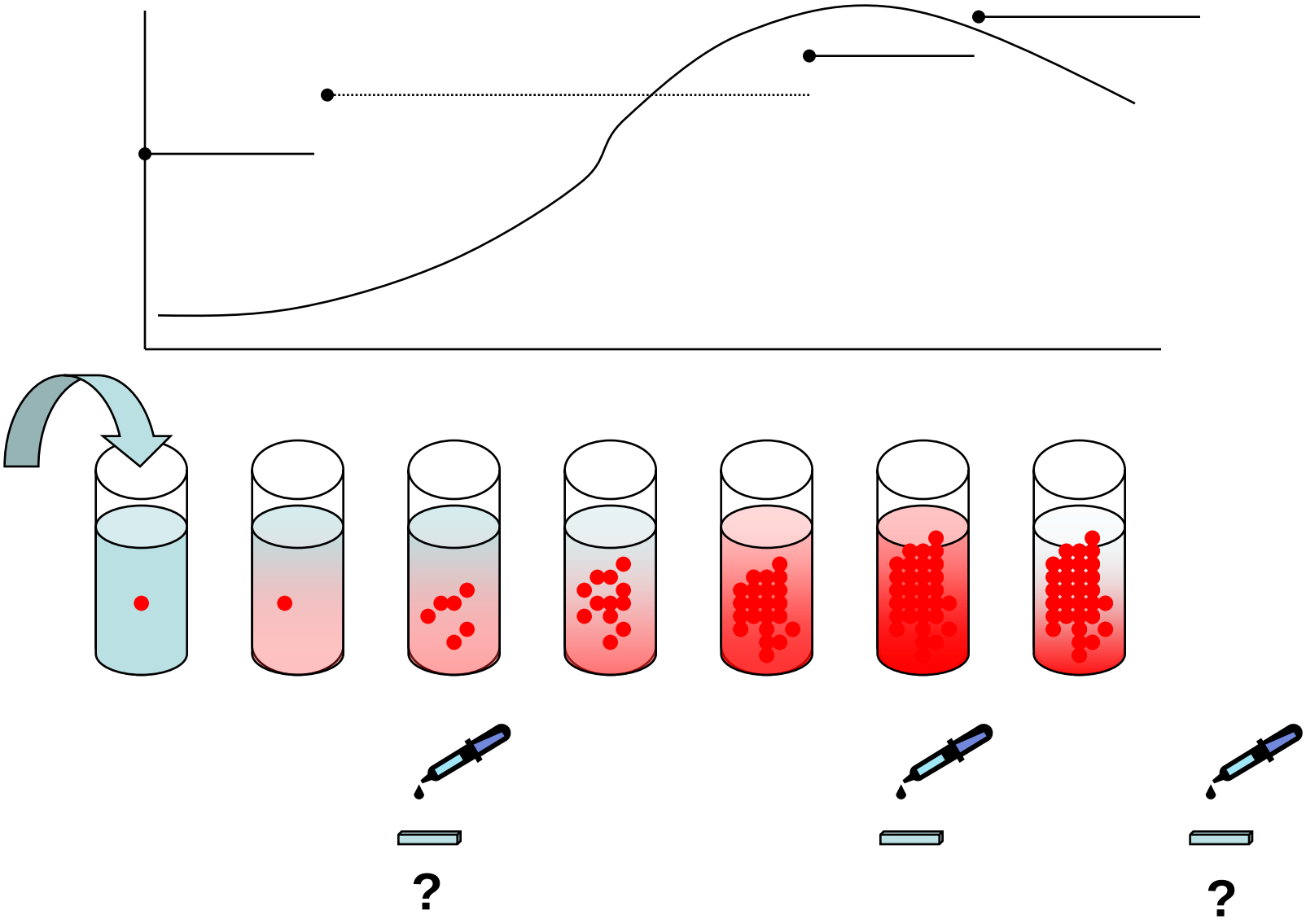
Incubation period

- PCR screens may require less growth than immunoassays.
- Shorter incubation periods (<15 hours) may warrant additional scrutiny of laboratory compliance to the validated protocol.
- Has enrichment/screening combination been validated for a larger test portion?
  - Particular concern for large test portions incubated for shorter periods.
  - *e.g.,* 375-gram test portion incubated for 8 hours
- Proposed incubations < 8 hours may warrant OPHS review.

Pathogen Growth During Enrichment



Role of Enrichment



Considerations for Testing Methods

- Is the method fit for the intended purpose of the analysis?
- Has the method been optimized and experimentally validated for sensitive detection of pathogens?
- Is the laboratory complying to the validated method protocol?

Fitness For Purpose

- Is the method intended for detecting the lowest possible levels of potentially injured pathogen cells in meat/poultry products like the corresponding FSIS method?

- Was this demonstrated by the validation study?

Value of Validation

- Determines performance characteristics of the method in comparison to a gold standard method (*i.e.,* usually FSIS or FDA method).
- Independent evaluation provides credibility
- Rigor varies
- Still must consider fitness for purpose and how the method is applied.
  - *e.g.,* some AOAC-validated methods are not consistent with FSIS goals or Compliance Guidelines.

Method Validation

- Recognized independent method validation organizations:
  - Government- FSIS and FDA
  - AOAC International (U.S.A.)
    - AOAC Official Method (OM) validations
    - AOAC-RI “Performance Tested Method” validations
  - AFNOR (France)- *e.g.,* bioMerieux-Vitek tests
  - Others (ISO, NMKL, etc.)
- However, past validations conducted by these organizations may not be relevant to larger test portions or other testing scenarios.

Testing Method Specifications

- Common specifications determined through experimental validation studies:
  - How well does the method work for low levels of contamination?
    - e.g., sensitivity, false negative rate, limit of detection (LOD)
  - How specific is the test for the target pathogen?
    - e.g., inclusivity, exclusivity
  - How reliable is the method in different hands?
    - e.g., repeatability, reproducibility
- However, these experiments can produce variable results according to experimental design, product, strains, and other factors.
  - Past sensitivity and LOD measurements cited for FSIS MLG methods are not intended as a standard for other methods.

Sensitivity

- Does the method detect low levels of pathogens?
- False negative rate = critical issue
  - Does the method miss positive samples compared to "gold standard" method?
- Limit of detection (LOD)
  - Expressed as CFU/gram or CFU/test portion
  - Rough estimate of performance
    - Variable based on numerous factors (*e.g.,* product, competing flora, pathogen injury, etc.)
    - Note significant differences (*i.e.,* Do not over-interpret)

Specificity

- Bacteria can mutate and evolve into forms that defy the traditional rules.
  - As a result, much diversity within a pathogen species
- False negative potential-
  - Does the test miss some subgroup of the target pathogen?
- False positive potential-
  - Is an unconfirmed result a potential problem?
  - Depends on context (industry vs. FSIS testing)

Method Application

- Can the method accommodate the necessary test portion?
- Does the lab specifically comply to the validated method instructions, or have they altered the method in some way?
- AOAC/AFNOR validations typically apply only to commercial screening methods without regard to any necessary follow-up tests.

Confirmatory Testing

- Non-culture confirmation (*e.g.*,PCR)
- Culture confirmation (e.g., FSIS confirmation)
  - Plating the enrichment on selective and differential agar media
    - Immunomagnetic separation (IMS) necessary prior to plating for *E. coli* O157:H7
  - Suspect colonies = “presumptive positive”
  - Purification and confirmatory identification tests including:
    - Biochemical (e.g., identifies “*E. coli*”)
    - Serological (e.g., identifies “O157” and “H7”)
    - Genetic (e.g., identifies “*stx*” = Shiga toxin genes)

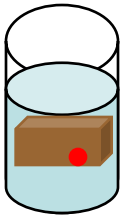
Most Probable Number (MPN) Enumeration Analysis

- Traditional enrichment-based analyses are performed on three or more dilutions, each typically in triplicate, from a single sample homogenate (*i.e.,* MPN = method format, not a specific method per se).
- Advantages:
  - Better sensitivity (lower LOD) than direct plating
- Disadvantages:
  - Very resource intensive/expensive
  - Test portion ≤ 3.3 grams (FSIS method = < 33 grams)
- Application:
  - For quantifying low levels of pathogens (*e.g., Salmonella*, *E. coli* O157:H7, *L. monocytogenes*)

Quantitative Testing

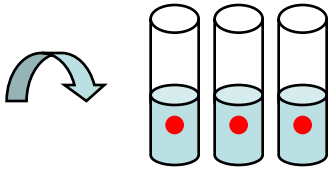
MPN (most probable number)

325 grams
+ 10 fold buffer
= 0.1 grams/mL

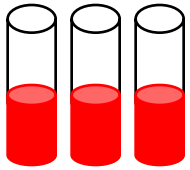


Dilute 1:10, 1:100

10 mL
(1 gram x 3)

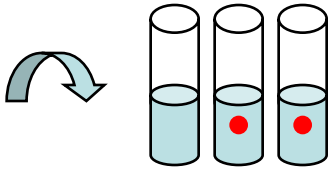


enrich

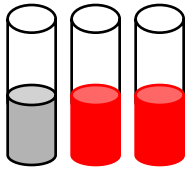


+++

10 mL 1:10
(0.1 gram x 3)

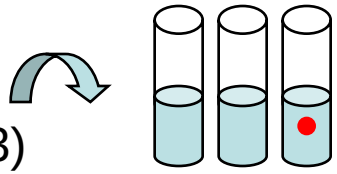


enrich

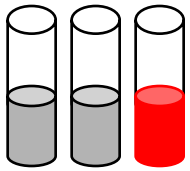


-++

10 mL 1:100
(0.01 gram x 3)



enrich



--+

Example “3-2-1” = Y MPN/g (use MPN table)
Total tested: 3.33 grams (33 grams FSIS method)
Level of Detection = < 0.3 MPN/gram (0-0-0)
= <0.03 MPN/gram FSIS method

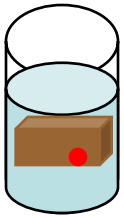
Direct Plating Enumeration Methods

- Product is homogenized in diluent and small volume is directly dispensed onto agar media (*i.e.,* sometimes there is a 1-2 h “resuscitation” step, but enrichment is never used prior to plating)
- Advantages:
  - Allows easy inexpensive quantitative analysis
- Disadvantages:
  - Accommodates only a very small test portion
  - Higher limit of detection (*i.e.,* often 100 CFU/g) not suitable for detecting low levels of pathogens.
- Application:
  - Expedient for higher level analytes (*e.g.,* indicators, *Campylobacter*, *S. aureus*, *C. perfringens*, *B. cereus*)

Quantitative Testing: Direct Plating

CFU (colony forming unit)

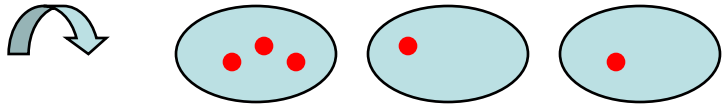
325 grams
+ 10-fold buffer
= 0.1 grams/mL



Dilute 1:10, 1:100

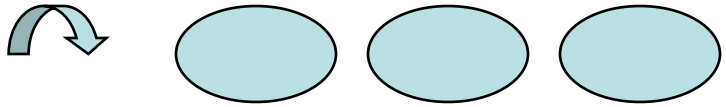
1 mL
(0.1 gram)

No enrichment

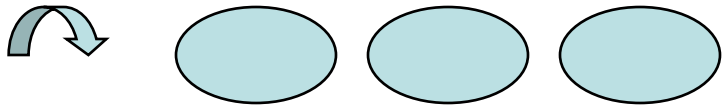


5 cfu/1 mL/0.1 g
= 50 cfu/g

1 mL 1:10
(0.01 gram)



1 mL 1:100
(0.001 gram)



Total tested 0.11 grams
Level of Detection = <10 cfu/gram (0 cfu from homogenate)

Expectations For *Listeria* Environmental Testing Equivalence

- Compliance Guidelines, May 2006, pp. 42-44

- For optimal sensitivity of detection, method for food contact surface testing must:
  - validated by a recognized body (*e.g.,* AOAC, AFNOR)
  - be enrichment-based
  - enrich the entire sponge/swab sample
    - *i.e.,* aliquot from sponge/swab does not provide opportunity to detect bacteria trapped in the sponge.

Analytes for Industry Food Contact or Environmental Surface Testing

- Production establishment laboratories test for one of the following:
  - *Listeria monocytogenes*- Use internationally recognized enrichment-based method that biochemically confirms culture as *L. monocytogenes.*
  - *Listeria* spp.- Use internationally recognized enrichment-based method that uses ELISA, PCR or other screening technology to provide more rapid but less specific *Listeria* spp. result.
  - "*Listeria*-like" indicator bacteria- Use the first part of an internationally recognized enrichment-based method to find suspect *Listeria* colonies (*e.g*., darkened colonies on MOX using the FSIS method).

Issues for Industry Labs

- On-site vs. off-site labs- jurisdiction issues
- Overarching concerns for on-site labs
  - Is testing effective?
  - Is testing safe in that facility?
- Evaluate the following:
  - Are personnel qualified?
  - Does the lab have proper equipment and materials for testing and disposal of contaminated media?
  - Do they follow the validated testing protocol?

ISO 17025 Laboratory Accreditation

- ISO 17025 = protocol for establishing and documenting a microbiology laboratory quality program (*i.e.,* “HACCP” for labs)

- Accrediting bodies = A2LA and others

- Accreditation implies robust quality program but does not necessarily indicate methods meet FSIS expectations.

Questions?