International Association for Food Protection WEBINAR

Multiple Rapid Methods for Identifying Poultry Samples Exceeding a Salmonella Threshold Level

Organized by: Applied Laboratory Methods Professional Development Group (PDG)

Moderator: Amanda Brookhouser-Sisney

Sr. Technical Director, Midwest Laboratories

Sponsored by the IAFP FOUNDATION

Please consider making a contribution

This webinar is being recorded and will be available to IAFP members within one week.

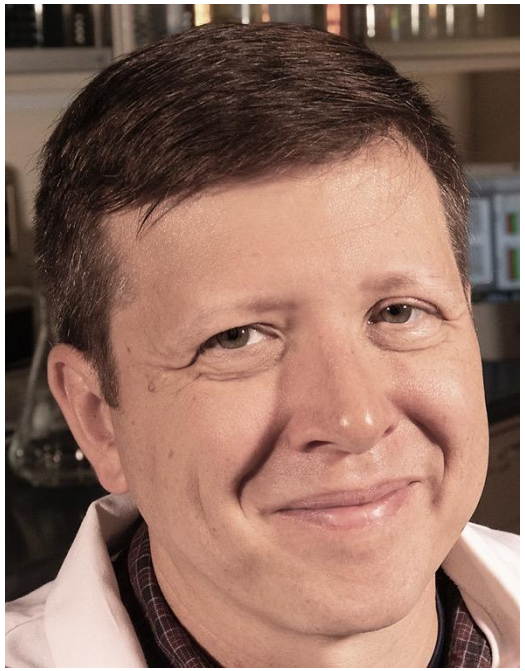
International Association for Food Protection

Webinar Housekeeping

- It is important to note that all opinions and statements are those of the individual making the presentation and not necessarily the opinion or view of IAFP.
- All attendees are muted. Questions should be submitted to the presenters during the presentation via the Questions section at the right of the screen. Questions will be answered at the end of the presentations.
- This webinar is being recorded and will be available for access by IAFP members within one week.
- The recorded version of this webinar will include **closed captioning** for enhanced accessibility.

Presenter

Dr. John Schmidt is a Research Microbiologist in Meat Safety and Quality Research Unit of the USDA-ARS US Meat Animal Research Center in Clay Center, Nebraska. His research encompasses a broad range *Salmonella*, pathogenic *E. coli*, and antimicrobial resistance issues from farm to fork in beef, swine, and poultry systems. Dr. Schmidt serves on the steering committee of the USDA-ARS *Salmonella* Grand Challenge with the goal of a unified ARS strategy in collaboration with key academic researchers to support stakeholders' ability to implement affordable, effective, data-driven interventions.



Multiple Rapid Methods for Identifying Poultry Samples Exceeding a *Salmonella* Threshold Level

John W. Schmidt, Ph. D.

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U. S. Department of Agriculture, Agricultural Research Service,
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IAFP Webinar
4:00 PM Eastern Time, March 4, 2025

DISCLAIMER

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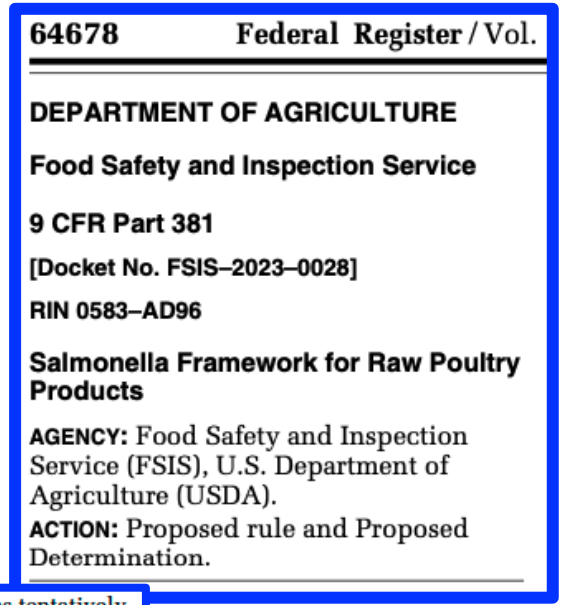
10 CFU/g & PHS Serotypes Adulteration Proposal

- August 7, 2024 *Federal Register* (*FR* 89: 64678-64748 ; FSIS-2023-0028) publication of "Salmonella Framework for Raw Poultry") **proposed rule and determination.**

- **Raw chicken carcasses, chicken parts, comminuted chicken, and comminuted turkey** are adulterated if they contain any *Salmonella* ≥ 10 CFU/g or CFU/mL **AND any detectible level** of a *Salmonella* serotype of **public health significance (PHS)** for that commodity.
  - Proposed Chicken PHS serotypes: Enteritidis, Typhimurium, and I,4,[5],12:i:-.
  - Proposed Turkey PHS serotypes: Hadar, Typhimurium, and Muenchen.



64678 Federal Register / Vol.

DEPARTMENT OF AGRICULTURE

Food Safety and Inspection Service

9 CFR Part 381

[Docket No. FSIS–2023–0028]

RIN 0583–AD96

Salmonella Framework for Raw Poultry Products

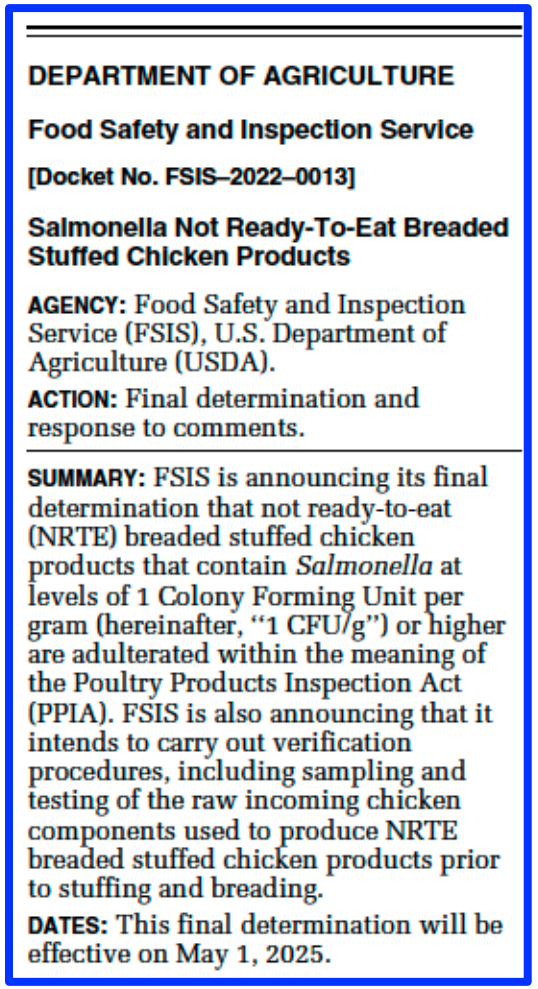
AGENCY: Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture (USDA).

ACTION: Proposed rule and Proposed Determination.

seq.). Specifically, FSIS has tentatively determined that raw chicken carcasses, chicken parts, comminuted chicken, and comminuted turkey are adulterated if they contain any type of *Salmonella* at or above 10 colony forming units/per milliliter or gram (10 cfu/mL(g)) in analytical portion (*i.e.*, mL of rinsate or gram of product) and contain any detectable level of at least one of the *Salmonella* serotypes of public health significance identified for that commodity. The proposed *Salmonella* serotypes of public health significance identified for raw chicken carcasses, chicken parts, and comminuted chicken are Enteritidis, Typhimurium, and I 4,[5],12:i:-, and for raw comminuted turkey are Hadar, Typhimurium, and Muenchen. These are the most highly

Adulteration Threshold: *Salmonella* ≥ 1 CFU/g NRTE Breaded Stuffed Chicken Products

- May 1, 2024 publication in *Federal Register* (*FR* 89: 35033-35053 ; FSIS-2022-0013) of "Salmonella Not Ready-To-Eat Breaded Stuffed Chicken Products" **final determination** active on May 1, 2025.
- Not ready-to-eat breaded stuffed chicken products that contain *Salmonella* at levels of 1 Colony Forming Unit per gram (CFU/g) or higher are adulterated within the meaning of the Poultry Products Inspection Act.
- Page 35050 "FSIS will collect one pound of **incoming chicken component** from the establishment to analyze 325 grams per test for *Salmonella*."



DEPARTMENT OF AGRICULTURE

Food Safety and Inspection Service

[Docket No. FSIS–2022–0013]

Salmonella Not Ready-To-Eat Breaded Stuffed Chicken Products

AGENCY: Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture (USDA).

ACTION: Final determination and response to comments.

SUMMARY: FSIS is announcing its final determination that not ready-to-eat (NRTE) breaded stuffed chicken products that contain *Salmonella* at levels of 1 Colony Forming Unit per gram (hereinafter, "1 CFU/g") or higher are adulterated within the meaning of the Poultry Products Inspection Act (PPIA). FSIS is also announcing that it intends to carry out verification procedures, including sampling and testing of the raw incoming chicken components used to produce NRTE breaded stuffed chicken products prior to stuffing and breading.

DATES: This final determination will be effective on May 1, 2025.

Ac…eaded

Other Rationales for *Salmonella* Quantification

- Risk assessments.
- Process control.
- Performance standards.

- May … 202… Pro…
- Not … *Sal…* are … Act…
- Pag… **component** from the establishment to analyze 325 grams per test for *Salmonella*."

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…nspection Service
…22–0013]
…eady-To-Eat Breaded …roducts
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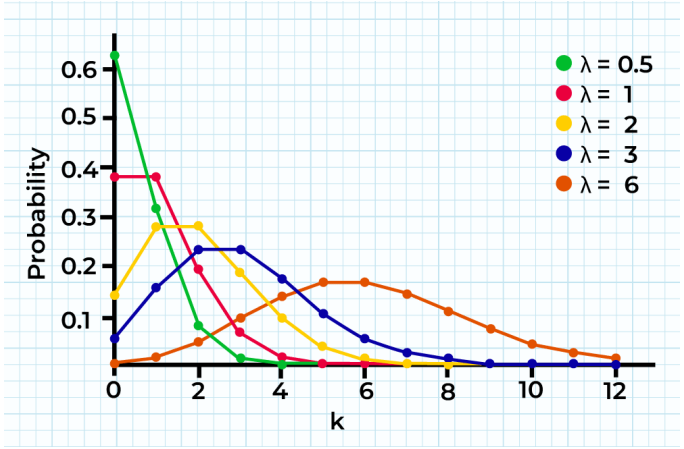
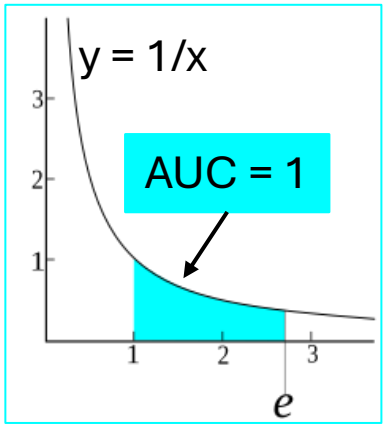
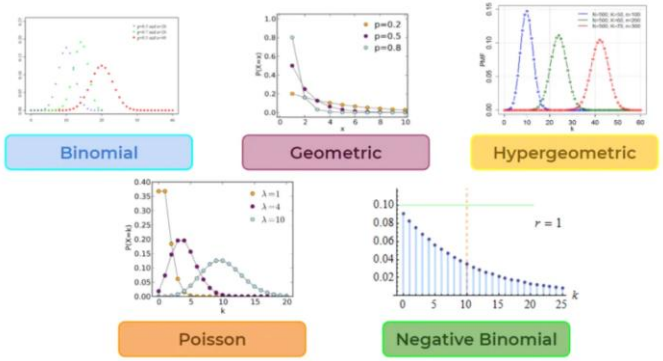
…nnouncing its final … not ready-to-eat …uffed chicken …ain *Salmonella* at …Forming Unit per …"1 CFU/g") or higher …hin the meaning of …ts Inspection Act … announcing that it …t verification …ling sampling and testing of the raw incoming chicken components used to produce NRTE breaded stuffed chicken products prior to stuffing and breading.
DATES: This final determination will be effective on May 1, 2025.

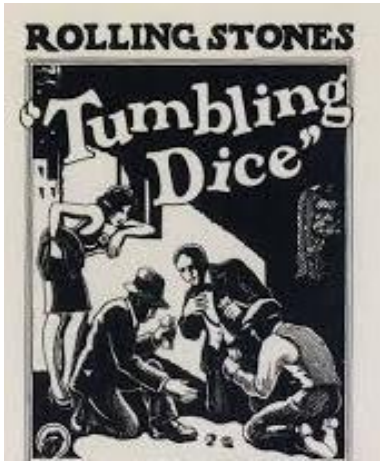
Its All Probability

- How much confidence do I have in the results?
- What are the inherent uncertainties in the results?
- What do the results mean?









A Friend In Need (1903), Cassius Coolidge

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Research Paper

Evaluation of Methods for Identifying Poultry Wing Rinses With *Salmonella* Concentrations Greater Than or Equal to 10 CFU/mL

John W. Schmidt [a,*], Anna Carlson [b], Joseph M. Bosilevac [a], Dayna Harhay [a], Terrance M. Arthur [a,d,1], Ted Brown [b], Tommy L. Wheeler [a], Jessie L. Vipham [c]

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[c] *Kansas State University, Department of Animal Sciences and Industry, 232 Weber Hall, 2900 College Ave, Manhattan, KS 66502, United States*
[d] *Present address: Fremonta Corp., 1945 Kyle Park Ct., San Jose, CA 95125, United states*

Funding:
Food Safety and Inspection Service
U.S. Poultry and Egg Association
Agricultural Research Service National Program 108 — Food Safety

Original Objective

Compare accuracies of 3 methods for quantifying *Salmonella* in inoculated post-chill two-joint turkey wing BPW rinsates

- **MPN quantification.**
  - Gold Standard.
  - No hard Lower Limit of Quantification. For this study 0.11 MPN/mL = –0.96 log MPN/mL.
- **GENE-UP quantification (AOAC International Performance Tested℠ Certificate No. 061801).**
  - Lower Limit of Quantification = 10 CFU/mL = 1.0 log CFU/mL.
- **BAX quantification (AOAC International Performance Tested℠ Certificate No. 081201).**
  - Lower Limit of Quantification = 1 CFU/mL = 0.0 log CFU/mL.

Method	Time to Result	Financial Cost	Technical Burden	Notes
MPN Quant.	2+ Days	Very High	Very High	Gold Standard since 1950s
GENE-UP Quant.	≈ 4 hours	Medium	High	AOAC Certified
BAX Quant.	≈ 10 hours	Medium	Medium	AOAC Certified

Salmonella Inocula

- **Nutrient starved & cold stressed** by incubation for in Phosphate Buffered Saline (PBS) at 4 °C for 18 to 22 hours.

TABLE 1. *Salmonella* strains used to inoculate poultry rinses

Strain label	Serotype	Strain	Isolated from	Used to inoculate
S1	Infantis	0895-1	Turkey	Turkey wing rinses
S2	Senftenberg	0567-1	Turkey	Turkey wing rinses
S3	Schwarzengrund	0841-1	Turkey	Turkey wing rinses
S4	Reading	0567-2	Turkey	Turkey wing rinses
S5	Typhimurium	0105-2	Chicken	Chicken wing rinses
S6	Kentucky	0148-2	Chicken	Chicken wing rinses
S7	Enteritidis	0675-1	Chicken	Chicken wing rinses
S8	Infantis	1159-1	Chicken	Chicken wing rinses

All strains were isolated by Dr. Dayna Harhay from Food Safety and Inspection Service samples collected between 2020 and 2022.

TABLE 4. *Salmonella* stock concentrations used to inoculate turkey rinses.

		log CFU/mL				
Level	N	Mean	STD	Median	Min	Max
VH	12	4.48	0.09	4.49	4.33	4.62
H	12	3.46	0.10	3.47	3.27	3.60
M	12	2.45	0.11	2.45	2.22	2.63
L	12	1.44	0.09	1.43	1.29	1.55

TABLE 5. *Salmonella* stock concentrations used to inoculate chicken wing rinses

		log CFU/mL				
Level	N	Mean	STD	Median	Min	Max
VH	12	4.62	0.15	4.65	4.29	4.79
H	12	3.60	0.14	3.64	3.31	3.76
M	12	2.61	0.14	2.63	2.36	2.82
L	12	1.60	0.17	1.68	1.22	1.79

BAX Quantification Results

- 8 different *Salmonella* strains in Phosphate Buffered Saline at 4 ° C for 18 to 24 h.
- BAX Poultry equation based on Typhimurium ATCC 14028 in Brain Heart Infusion Broth at 37 ° C for 18 h. Applegate et al. *Foods* 12:419 (2023).
- Lag phase duration is significantly impacted by the sampled environment.
- Doubling times vary between serotypes, and even between strains within a serotype.

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General Interest

Response to Questions Posed by the Food Safety and Inspection Service: Enhancing *Salmonella* Control in Poultry Products

The National Advisory Committee on Microbiological Criteria in Foods (NACMCF) [1]

Table B2

Summary of Approved Methods for Molecular Quantitation of Salmonella in Poultry Products (see individual suppler websites for updates)

	bioMérieux	Hygiena
Limitations	Procedure is not the same as the standard qualitative method for presence/absence. Sample prep method requires centrifugation.	Individual curve per matrix requires validation when adding a new matrix (i.e., there are 20 curves today). Culture based bias from the impact of natural microbiota and determination of lag and log phase for each strain.

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Impact of Doubling Time On Population Size

Food Microbiology 93 (2021) 103615

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Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Rapid estimation of *Salmonella enterica* contamination level in ground beef – Application of the time-to-positivity method using a combination of molecular detection and direct plating

Dayna M. Harhay *, Margaret D. Weinroth, James L. Bono, Gregory P. Harhay, Joseph M. Bosilevac

United States Department of Agriculture, Roman L. Hruska U.S. Meat Animal Research Center, Meat Safety and Quality Research Unit, Clay Center, NE, 68933, USA

Table 1

Salmonella strains used and average growth rates observed in GBE and mTSB at 42 °C. DT is doubling time in min; Delta (Δ) DT is the difference in DT between mTSB and GBE. A two-tailed, unpaired *t*-test of statistical significance, with $P < 0.05$ defined as significantly different, was used to evaluate differences in mean DT for fast and slow growing strains. Common superscripts (c - g) indicate values evaluated and outcomes as listed in the footnotes below.

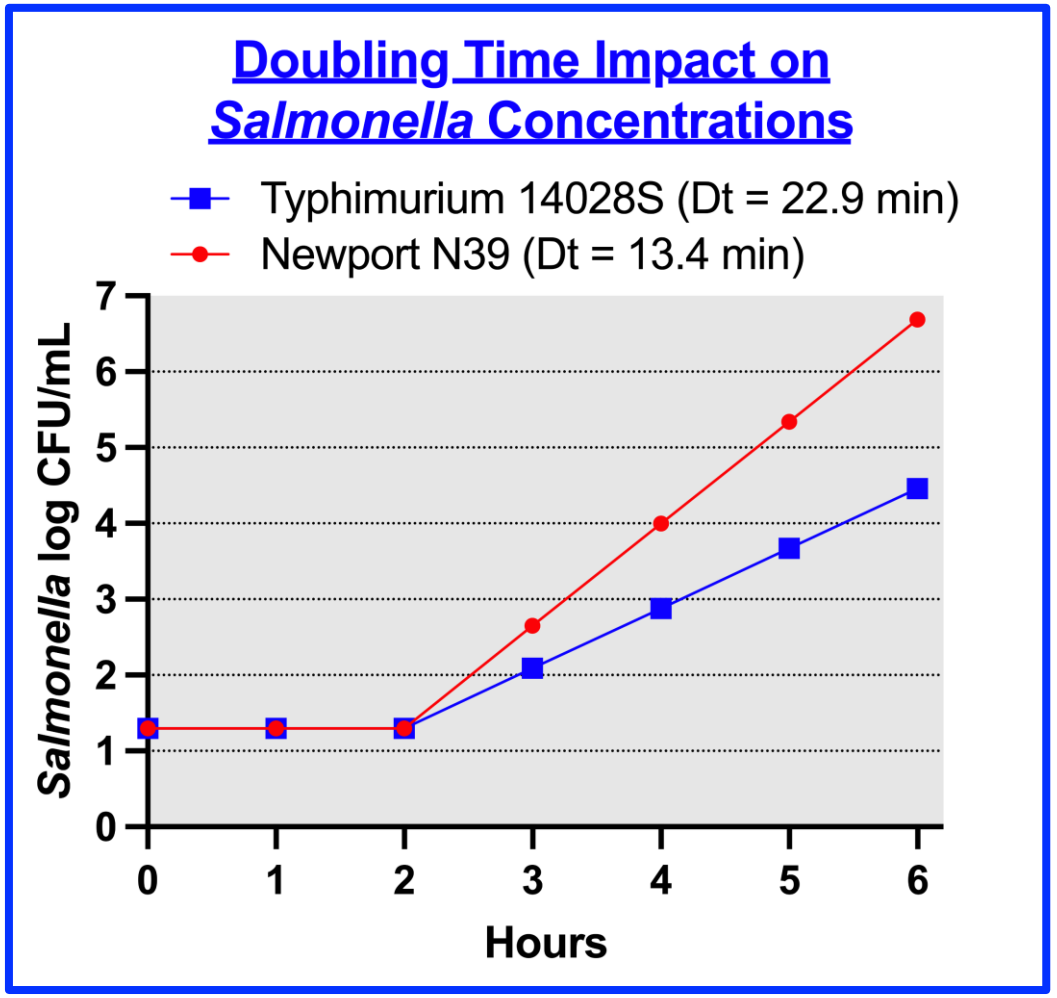
	Serotype	Strain	Isolation Source	DT (min) at 42°C[a] GBE (SD)	mTSB (SD)	□DT
FAST	Newport	N39	Bovine	13.4 (1.04)	17.9 (0.53)	4.5
	Enteritidis	95-14327	Human	14.2 (2.01)	19.8 (0.25)	5.6
	Anatum	A29	Bovine	15.1 (0.99)	18.9 (0.53)	3.8
	Typhimurium (1,4,[5],12:i:-)	3-H79	Bovine	16.2 (0.88)	20.2 (1.18)	4.0
	Typhimurium[b]	T36	Bovine	16.5 (1.17)	20.3 (1.18)	3.8
	Anatum	08-1092	Human	16.6 (0.23)	19.0 (0.56)	2.4
	Montevideo	2012K-1544	Human	16.8 (0.87)	19.8 (0.52)	3.0
	Average			15.5[ce] (1.58)	19.4[cf] (1.06)	3.9[g]
SLOW	Newport	2010K-2159	Human	17.2 (1.31)	19.6 (0.72)	2.4
	Enteritidis	95-2876	Human	18.5 (1.34)	22.4 (2.21)	3.9
	Dublin	SM73-2	Bovine	19.3 (1.00)	21.4 (0.58)	2.1
	Dublin	5-75-E	Bovine	19.3 (0.98)	20.9 (1.34)	1.6
	Newport	N17	Bovine	19.9 (1.09)	19.0 (0.42)	-0.9
	Montevideo[b]	H06	Human	20.6 (1.27)	20.6 (1.85)	0.0
	Typhimurium	14028S	Human	22.9 (2.08)	26.9 (1.96)	4.0
	Average			19.5[de] (1.95)	21.4[df] (2.68)	1.8[g]

[a] Average T0 inoculum was 0.89 CFU/g (95% CI = 0.69 - 1.08)

[b] Salmples incubated at 37°C not 42°C

Two-tailed, unpaired t-test of statistical significance with $P \leq 0.05$ defined as significantly different. Common superscript indicates values evaluated and outcome as follows: [c]Yes, $P < 0.0001$; [d]Yes, $P = 0.0358$; [e]Yes, $P = 0.0004$; [f]No, $P = 0.0632$; [g]Yes, $P = 0.0272$

Impact of Doubling Time On Population Size



- Assumed 2-hour lag time for both strains.
- 20 CFU/mL initial conc.
- 6-hour *Salmonella* concentrations:
  - Newport N39 = 6.69 log CFU/mL
  - Typhimurium = 4.46 log CFU/mL
  - **2.24 log CFU/mL difference**

Poker Sympathy (1903), Cassius Coolidge

A Brief Aside on Units

- CFU/mL = Colony Forming Units per mL. Agar Plating. **1 CFU ≈ 1 cell.**
- MPN/mL = Most Probable Number per mL. Multiple Cultures and Dilutions. **1 MPN ≈ 1 cell.**
- What units should be used with **BAX quantification & GENE-UP quantifications?**
  - Concentrations based on linear regression of Ct/Cp values to CFU values.
  - Linear Regression Estimated Units or LREU/mL.
  - **1 LREU ≈ 1 cell.**

Assumed that 1 *Sal.* CFU/mL = 1 *Sal.* MPN/mL = 1 *Sal.* LREU/mL = 1 *Sal.* cell/mL

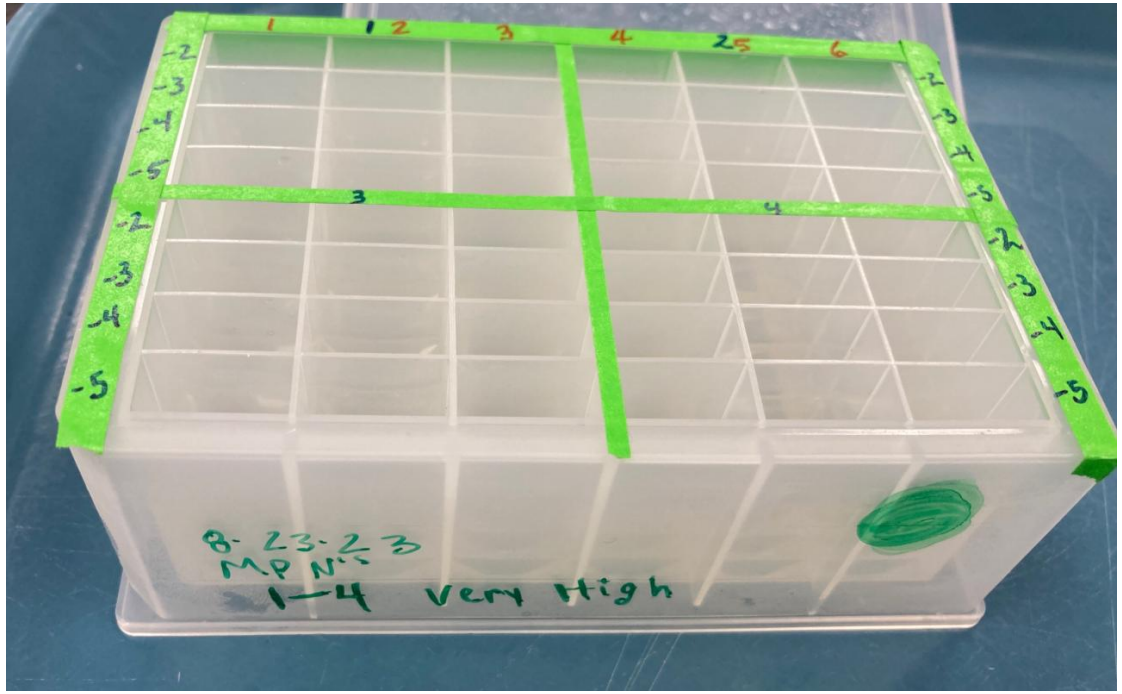
Estimates **(proxies, surrogates, stand-ins, indicators, representatives, etc.) of the number of viable *Salmonella* cells.**

How about "CURe" = "Cell Unit Representative/Replacement"

Most Probable Number Quantification

- 3-tube, 4-dilution MPN.

Sal Inoc. Range	MPN/mL Range	*g* value			
		Dil. 1	Dil. 2	Dil. 3	Dil. 4
Very High	11 to 37,000	0.027	0.0027	0.00027	0.00003
High	1.1 to 3,700	0.27	0.027	0.0027	0.0003
Medium, Low, Very Low	0.11 to 370	2.7	0.27	0.027	0.003



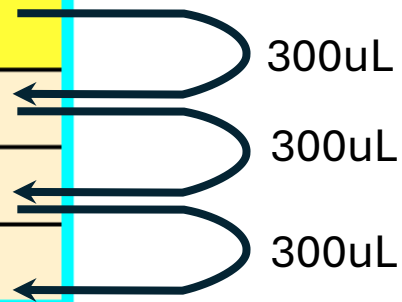
MPN BLOCK (M, L, VL, & NC)						
	1	2	3	4	5	6
A						
B	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
C	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
D	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
E						
F	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
G	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
H	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml

Most Probable Number Quantification

- 3-tube, 4-dilution MPN.

Sample MPN BLOCK

	1	2	3	4	5	6
A	3 mL #1	3 mL #1	3 mL #1	3 mL #2	3 mL #2	3 mL #2
B	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
C	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
D	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
E	3 mL #3	3 mL #3	3 mL #3	3 mL #4	3 mL #4	3 mL #4
F	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
G	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml
H	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml	2.7 ml



Most Probable Number Quantification

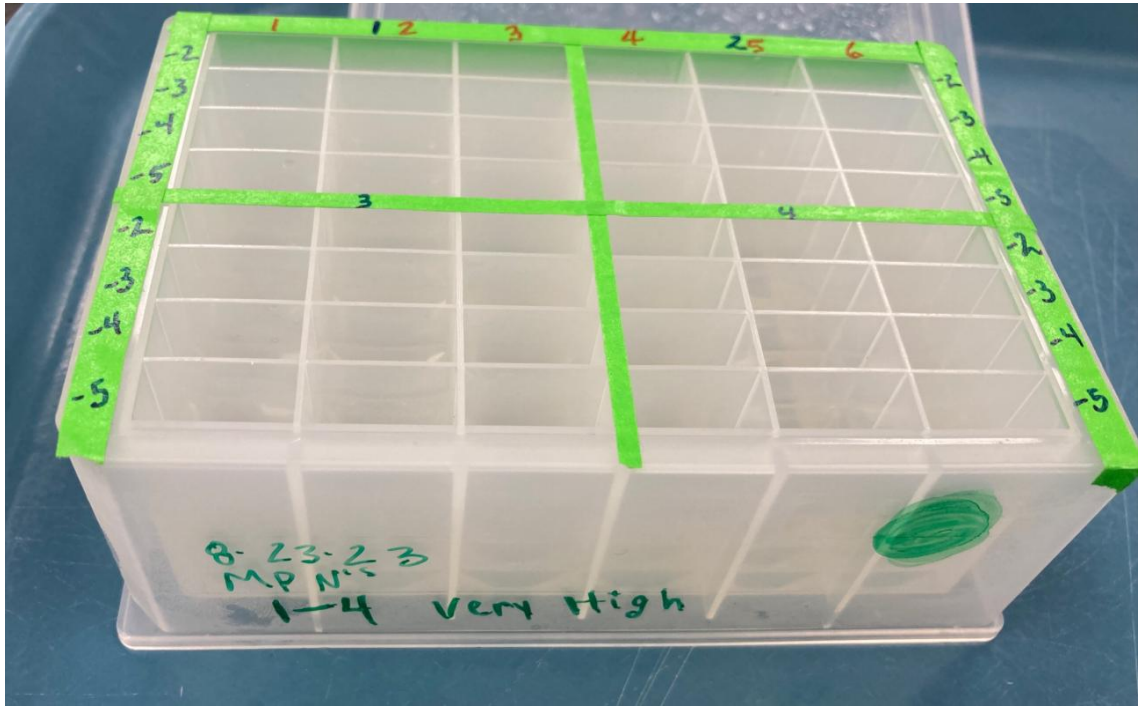
- 3-tube, 4-dilution MPN. Incubate at 42 °C for 18 to 24 hours.

Sample MPN BLOCK

	1	2	3	4	5	6
A	2.7 ml #1	2.7 ml #1	2.7 ml #1	2.7 ml #2	2.7 ml #2	2.7 ml #2
B	0.27 ml #1	0.27 ml #1	0.27 ml #1	0.27 ml #2	0.27 ml #2	0.27 ml #2
C	0.027 ml #1	0.027 ml #1	0.027 ml #1	0.027 ml #2	0.027 ml #2	0.027 ml #2
D	0.003 ml #1	0.003 ml #1	0.003 ml #1	0.003 ml #2	0.003 ml #2	0.003 ml #2
E	2.7 ml #3	2.7 ml #3	2.7 ml #3	2.7 ml #4	2.7 ml #4	2.7 ml #4
F	0.27 ml #3	0.27 ml #3	0.27 ml #3	0.27 ml #4	0.27 ml #4	0.27 ml #4
G	0.027 ml #3	0.027 ml #3	0.027 ml #3	0.027 ml #4	0.027 ml #4	0.027 ml #4
H	0.003 ml #3	0.003 ml #3	0.003 ml #3	0.003 ml #4	0.003 ml #4	0.003 ml #4

Sal Inoc. Range	MPN/mL Range	*g* value			
		Dil. 1	Dil. 2	Dil. 3	Dil. 4
Very High	11 to 37,000	0.027	0.0027	0.00027	0.00003
High	1.1 to 3,700	0.27	0.027	0.0027	0.0003
Medium, Low, Very Low	0.11 to 370	2.7	0.27	0.027	0.003

Most Probable Number Quantification

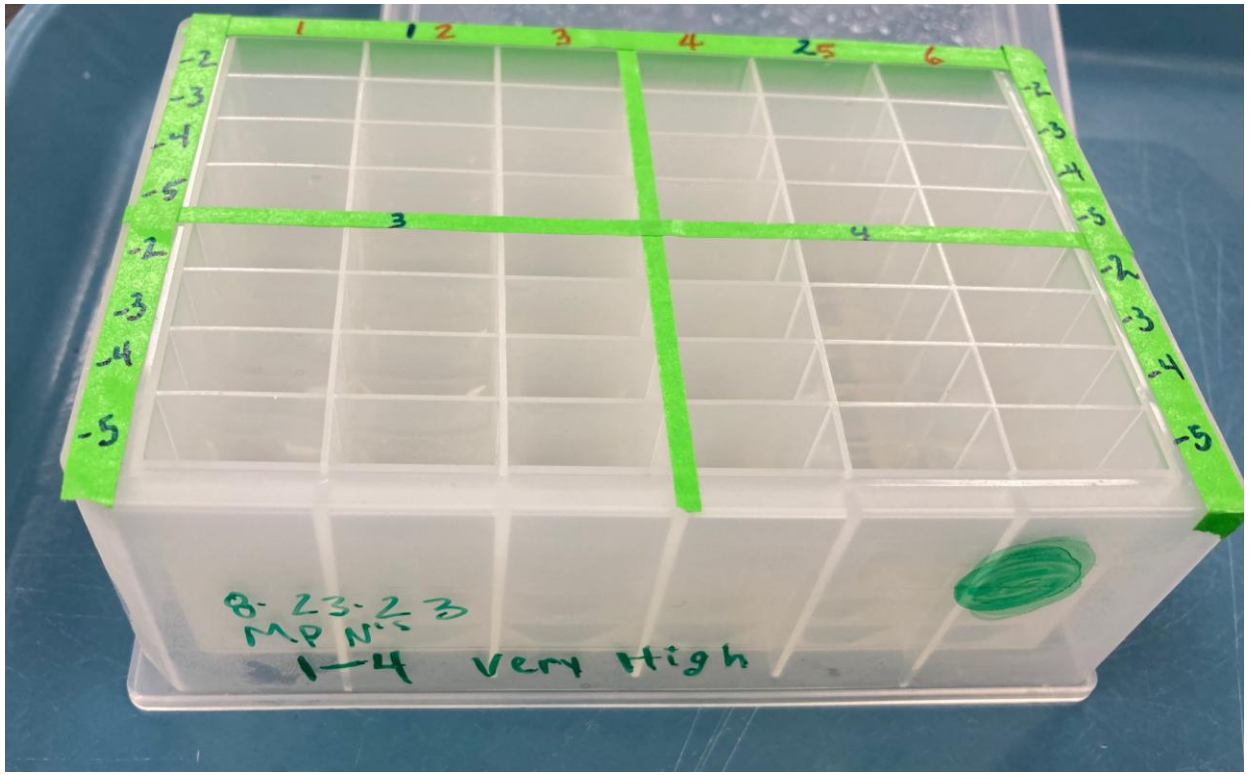


Thank you USMARC Technicians !!

- Julie Dyer
- Frank Reno
- Greg Smith
- Kerry Brader
- Sydney Brodrick

Most Probable Number Quantification

- Hygiena BAX Real-Time PCR Assay *Salmonella* for detection (12 reactions/sample. 8 samples/rack).



hygiena

BAX® System Real-Time PCR Assay

Salmonella

Part KIT2006 (D14306040)

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 031101

KIT CONTENTS

- *Raw comminuted Turkey and Chicken:* Add 325 g portions to 1625 mL BPW. Homogenize by hand until all clumps have been dispersed. Transfer 30 mL of homogenate to a sterile filtered bag. Add 30 mL of prewarmed (45°C) BAX® MP media plus 1 mL/L Quant Solution. Hand mix for 30 seconds and incubate sample at 42°C for 6 hours for raw ground beef and at 7 hours for raw ground pork. Incubate the remaining original homogenate sample (375 g in 1,500 mL BAX MP) at 42°C for 18-24 hours for prevalence testing.
- *Raw Beef Trim and Raw Pork Trim:* Add 375 g portions to 1.5 L prewarmed (45°C) BAX® MP media and hand massage for 30 seconds. Incubate sample at 42°C for 6 hours. After pulling aliquot at the determined timepoint for lysate creation, incubate the remaining original homogenate sample at 42°C for 18-24 hours for prevalence testing.
- *MicroTally on Raw Beef Trim and MicroTally on Raw Pork Trim:* Add one MicroTally cloth to 200 mL prewarmed (45°C) BAX® MP media and hand mix for 30 seconds. Incubate sample at 42°C for 6 hours. After pulling aliquot at the determined timepoint for lysate creation, incubate the remaining original homogenate sample at 42°C for 18-24 hours for prevalence testing.

ENRICHMENT PROTOCOL FOR MPN ESTIMATION

- *Raw comminuted Turkey and Chicken:* Homogenize 65 g samples with 585 mL BPW. Make 3-tube 5-dilution MPN set representing 1 g, 0.1 g, 0.01 g, 0.001 g, and 0.0001 g of sample by setting up the following: For 1 g sample dilution, fill 3 test tubes with 10 mL homogenate. For 0.1 g sample dilution, fill 3 test tubes of 9 mL BPW with 1 mL sample homogenate. For 0.01 g sample dilution, fill 3 test tubes of 9.9 mL of BPW with 0.1 mL of sample homogenate. For 0.001 g sample dilution, add 0.1 mL of sample homogenate to 9.9 mL BPW, then add 1.0 mL from this dilution to 3 tubes containing 9.0 mL BPW. For 0.0001 g sample dilution, add 0.1 mL of homogenate to 99.9 mL BPW, then add 1.0 mL from this dilution to 3 tubes containing 9.0 mL BPW. Incubate tubes at 37°C for 24 hours. Continue with creation of lysates for each incubated tube for prevalence testing.
- *Whole Bird Rinsates:* Make 3-tube 5-dilution MPN set representing 1 g, 0.1 g, 0.01 g, 0.001 g, and 0.0001 g of sample by setting up the following: For 1 mL sample dilution, fill 3 test tubes with 1 mL rinsate and 9 mL BPW. For 0.1 mL sample dilution, fill 3 test tubes of 9 mL BPW with 1 mL from previous sample dilution. For 0.01 mL sample dilution, fill 3 test tubes of 9 mL of BPW with 1 mL from previous sample dilution. For 0.001 ml sample dilution, add 1 mL from previous sample dilution to 9 mL BPW. For 0.0001 mL sample dilution, add 1 mL from previous sample dilution to 9 mL BPW. Incubate tubes at 37°C for 24 hours. Continue with creation of lysates for each incubated tube for prevalence testing.
- *Raw Beef Trim:* Homogenize 65 g samples with 585 mL mTSB and hand mix for 30 seconds. Make 3-tube 5-dilution MPN set representing 1 g, 0.1 g, 0.01 g, 0.001 g, and 0.0001 g of sample by setting up the following: For 1 g sample dilution, fill 3 test tubes with 10 mL homogenate. For 0.1 g sample dilution, fill 3 test tubes of 9 mL mTSB with 1 mL sample homogenate. For 0.01 g sample dilution, fill 3 test tubes of 9.9 mL of mTSB with 0.1 mL of sample homogenate. For 0.001 g sample dilution, add 0.1 mL of sample homogenate to 9.9 mL mTSB, vortex, then add 1.0 mL from this dilution to 3 tubes containing 9.0 mL BPW. For 0.0001 g sample dilution, add 0.1 mL of homogenate to 99.9 mL mTSB, then add 1.0 mL from this dilution to 3 tubes containing 9.0 mL mTSB. Incubate tubes at 42°C for 24 hours. Continue with creation of lysates for each incubated tube for prevalence testing.

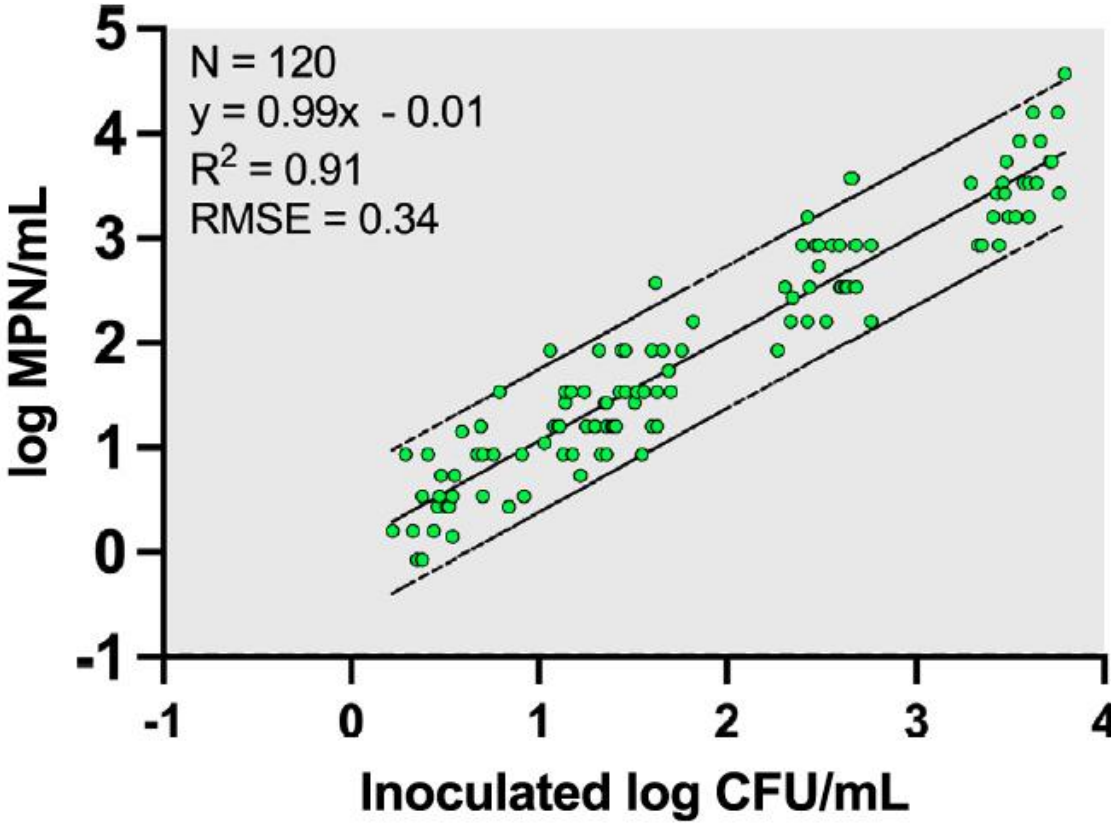
Method Approved by AFNOR Certification

Test portions weighing more than 25 g have not been tested in the context of NF VALIDATION.

For preparation of initial suspensions, follow instructions of EN ISO 6579 and EN ISO 6887 standards.

- *General Protocol for meat products (including meat with spices or herbs), seafood, vegetable, pet food, environmental samples:* Homogenize 25 g sample with 225 mL pre-warmed BPW. Incubate at 37°C for 16-24 hours. Transfer 10 µL enriched sample to 500 µL pre-warmed BHI broth. Incubate at 37°C for 3-4 hours.
- *Egg products:* Homogenize 25 g sample with 225 mL pre-warmed BPW. Incubate at 37°C for 18-24 hours. Transfer 10 µL enriched sample to 500 µL pre-warmed BHI broth. Incubate at 37°C for 3-4 hours.
- *Raw beef (short protocol):* Homogenize 25 g sample with 225 mL pre-warmed BPW. Incubate at 41.5°C for 10-24 hours.
- *Raw Meats and raw seafood:* Homogenize 25 g sample with 225 mL pre-warmed BPW. Incubate at 37°C for 16-20 hours.

MPN Quantification Results



- Slope of 1 is ideal.
- Y-intercept of 0 is ideal.
- $R^2 = 0$ completely useless.
- $R^2 = 1$ linear equation perfectly explains the data.

$$RMSE = \sqrt{\frac{\Sigma(y_i - \hat{y}_i)^2}{N - P}}$$ $P = 2$ (parameter estimates)

MPN Quantification Results

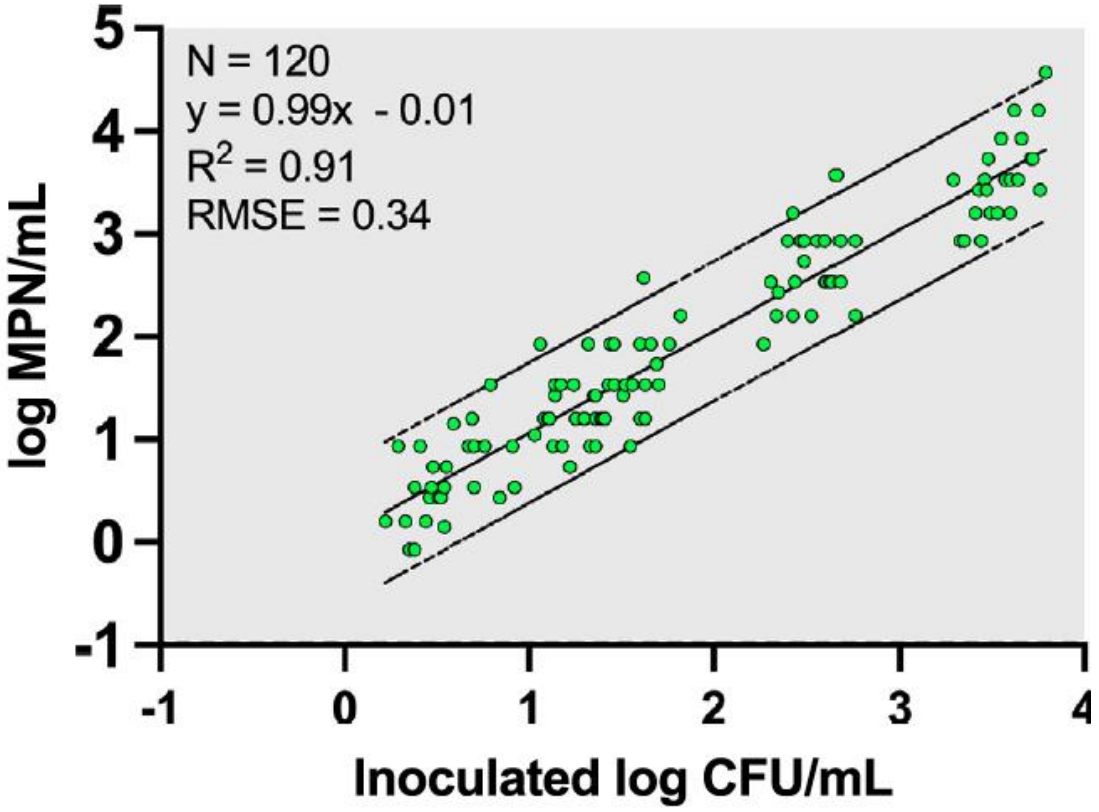


TABLE 6. MPN quantitative discrepancy (QD_{MPN}) of *Salmonella* in poultry wing rinses

Inoculated rinse log CFU/mL range	N	QD_{MPN} (log CFU/mL) Mean	STD	Median	Min	Max
3.00 to 3.99	24	-0.01	0.34	-0.04	-0.51	0.78
2.00 to 2.99	24	0.11	0.36	0.09	-0.56	0.91
1.00 to 1.99	45	0.05	0.34	0.04	-0.62	0.95
0.00 to 0.99	27	0.06	0.34	0.02	-0.45	0.74
0.00 to 3.99	120	0.05	0.34	0.01	-0.62	0.95

QD_{MPN} = MPN log MPN/mL – inoculated rinse log CFU/mL

Pinched With Four Aces (1903), Cassius Coolidge

MPN Quantification Results

Rinse Type	Rinse Name	QD_{MPN}	Inoculated rinse *Salmonella* log CFU/mL	*Salmonella* log MPN/mL	Low 95CI log MPN/mL	High 95CI log MPN/mL	95 CI Range	MPN Result
Chicken	F-11	0.95	1.62	2.57	1.93	3.20	1.27	3-3-3-2
Chicken	F-5	0.91	2.66	3.57	2.93	4.20	1.27	3-3-3-2
Chicken	E-14	0.87	1.06	1.93	1.32	2.54	1.22	3-3-3-0
Chicken	E-1	0.78	3.79	4.57	3.93	5.20	1.27	3-3-3-2
Turkey	C-5	0.77	2.43	3.20	2.56	3.85	1.29	3-3-3-1
Chicken	E-23	0.74	0.79	1.53	0.91	2.15	1.24	3-3-2-0
Turkey	A-15	0.64	0.29	0.93	0.32	1.54	1.22	3-3-0-0
Chicken	F-15	0.61	1.32	1.93	1.32	2.54	1.22	3-3-3-0
Turkey	A-2	0.58	3.62	4.20	3.56	4.85	1.29	3-3-3-1
Chicken	D-24	0.56	0.59	1.15	0.54	1.76	1.22	3-3-0-1
Chicken	D-6	0.53	2.40	2.93	2.32	3.54	1.22	3-3-3-0
Turkey	A-16	0.52	0.41	0.93	0.32	1.54	1.22	3-3-0-0
Chicken	F-21	0.51	0.69	1.20	0.56	1.83	1.27	3-3-1-0
Chicken	D-11	0.49	1.44	1.93	1.32	2.54	1.22	3-3-3-0
Turkey	A-9	0.47	1.46	1.93	1.32	2.54	1.22	3-3-3-0
Chicken	D-13	0.47	1.46	1.93	1.32	2.54	1.22	3-3-3-0
Chicken	D-7	0.46	2.47	2.93	2.32	3.54	1.22	3-3-3-0
Turkey	C-16	-0.45	0.38	-0.07	-0.68	0.54	1.22	3-0-0-0
Turkey	C-11	-0.49	1.22	0.73	0.20	1.26	1.06	3-2-1-0
Turkey	C-4	-0.51	3.44	2.93	2.32	3.54	1.22	3-3-0-0
Chicken	D-5	-0.56	2.76	2.20	1.56	2.83	1.27	3-3-1-0
Chicken	D-12	-0.62	1.55	0.93	0.32	1.54	1.22	3-3-0-0

95% CIs

- N = 120
- Mean 95% CI Range = 1.22 log
- Median 95% CI Range = 1.24 log
- Max 95% CI Range = 1.29 log
- Min 95% CI Range = 0.85 log

MPN Quantification Results

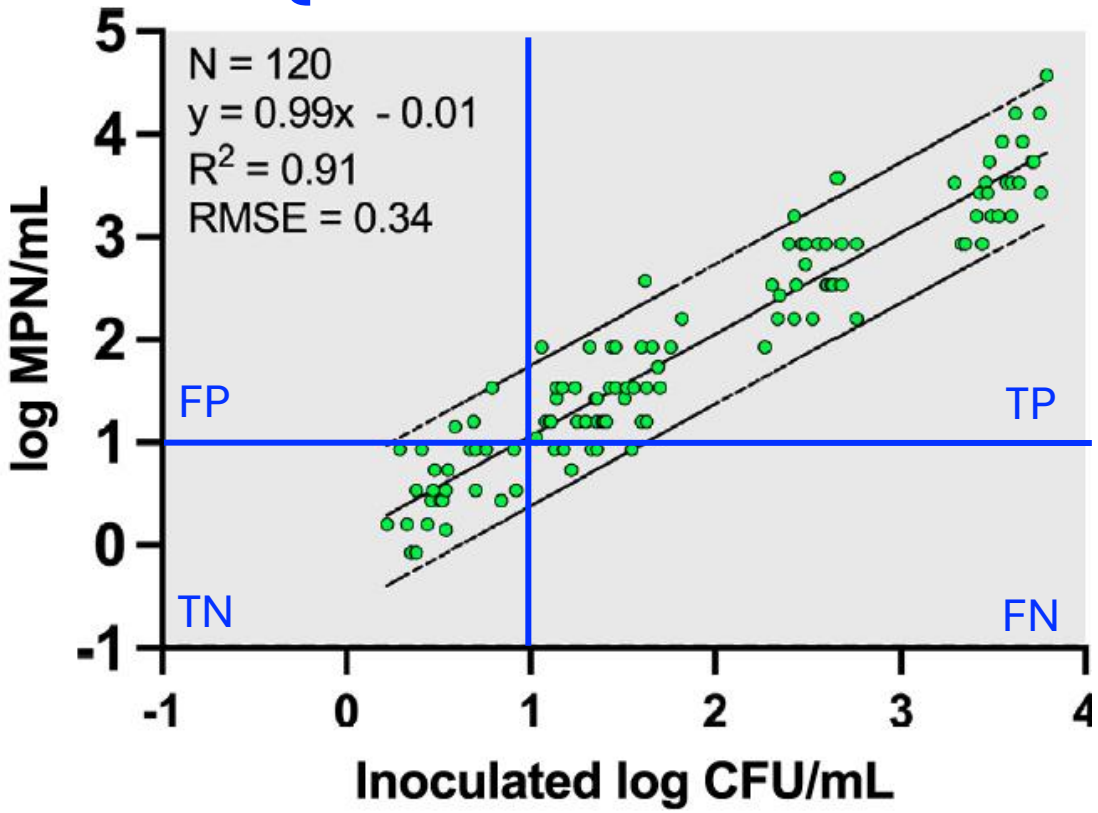
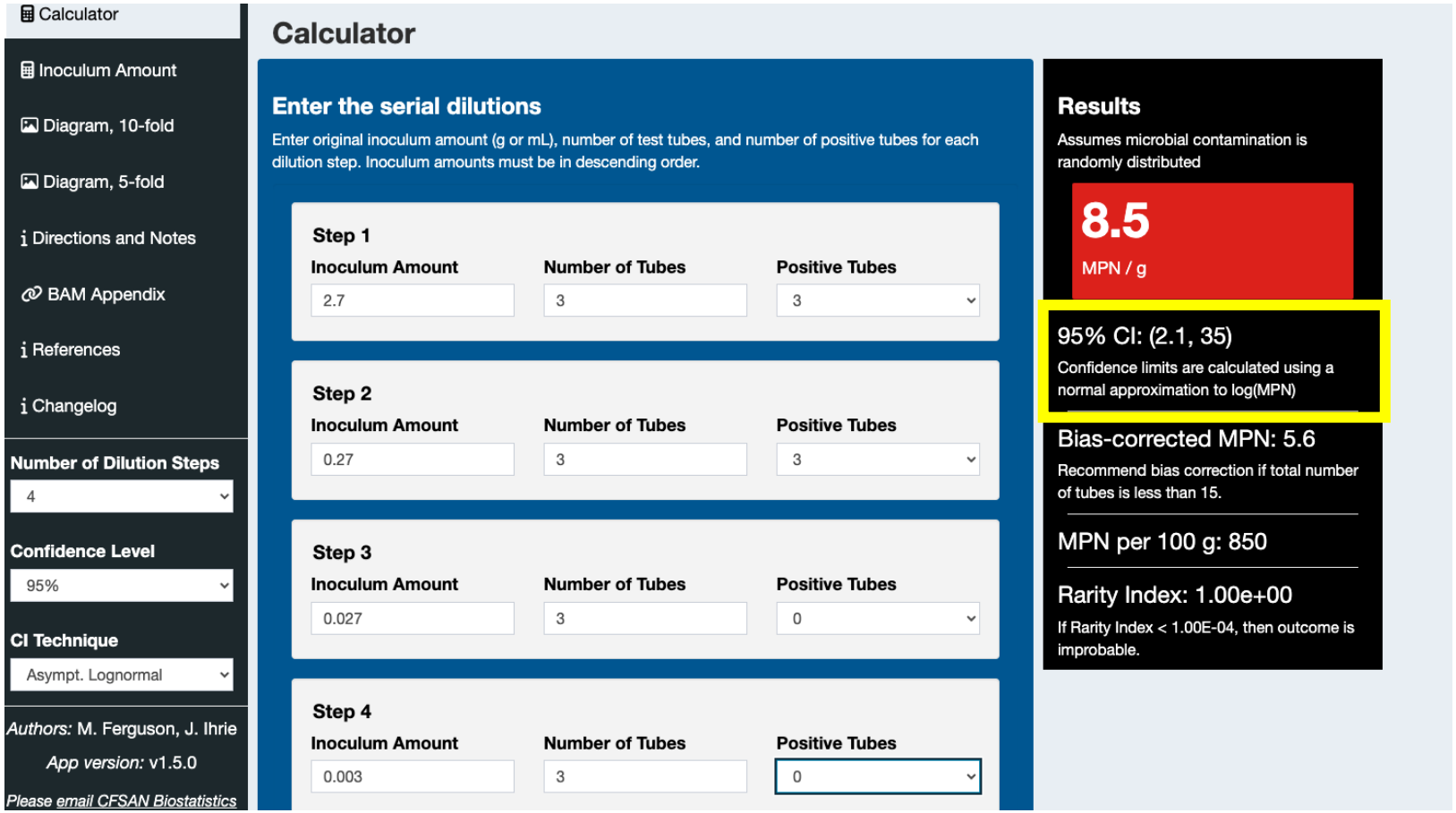


TABLE 11. Proficiencies of quantification and threshold methods for identification of poultry wing rinses with *Salmonella* concentrations ≥ 10 CFU/mL

Method	N	TP	FP	FN	TN	Sens	Spec	PPV	NPV	FNR	FPR	Acc
MPN quantification	120	87	3	6	24	0.935	0.889	0.967	0.800	0.065	0.111	0.925

An Example of Most Probable Number Estimation

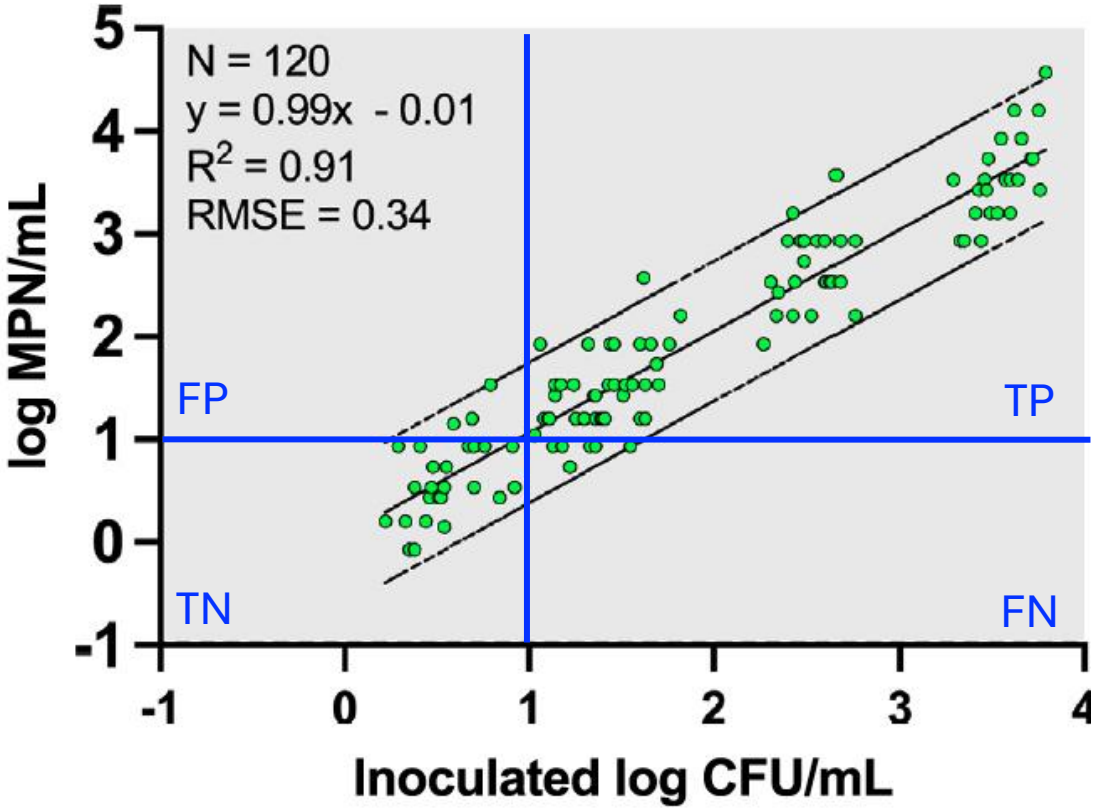
- Turkey Rinse A-11. Inoculated *Sal.* = 23 CFU/mL



- 5% chance that "actual" value is outside the range of 2.1 to 35 CFU/mL.
- Recorded as a "False Negative"
- Result **was** accurate within the 95% CI.

https://pub-connect.foodsafetyrisk.org/microbial/mpncalc/

Most Probable Number Estimations



Rinse Name	Rinse Type	Inoculated rinse CFU/mL	*Salmonella* MPN/mL	Low 95CI MPN/mL	High 95CI MPN/mL	Outcome
D-24	Chicken	3.9	14.1	3.5	57.5	FP
F-21	Chicken	4.9	15.8	3.6	67.6	FP
E-23	Chicken	6.2	33.9	8.1	141.3	FP
D-14	Chicken	13.5	8.5	2.1	34.7	FN
E-20	Chicken	15.1	8.5	2.1	34.7	FN
C-11	Turkey	16.6	5.4	1.6	18.2	FN
F-16	Chicken	21.4	8.5	2.1	34.7	FN
A-11	Turkey	22.9	8.5	2.1	34.7	FN
D-12	Chicken	35.5	8.5	2.1	34.7	FN

Most Probable Number Diminishing Returns

Steps	g values	Tubes per Step	Steps × Tubes	Outcome	MPN/g	Low 95% CI	Hi 95% CI	95% CI Size
4	2.7, 0.27, 0.027, 0.003	3	12	3-3-0-0	**8.5**	2.1	35	32.9
3	0.1, 0.05, 0.01	5	15	4-2-0	**12**	5.1	27	21.9
5	0.1, 0.08, 0.06, 0.04, 0.02	3	15	3-2-1-0-0	**10**	4.4	23	18.6
3	0.1, 0.01, 0.001	10	30	7-1-0	**12**	5.6	24	18.4
3	0.1, 0.05, 0.025	10	30	8-2-0	**10**	5.7	18	12.3

- More Steps = Broader range of estimation.
- More Tubes = Greater resolution.

Calculated at https://pub-connect.foodsafetyrisk.org/microbial/mpncalc/

GENE-UP Quantification

- *Published Lower Limit of Quantification* = *10 CFU/mL (no enrichment).*
- 40 mL of rinsate, centrifugation, customized Promega Wizard genomic DNA isolation.
- DNA pellet resuspended in 50 uL.
- 5 uL of genomic DNA sample added to GENE-UP Salmonella reaction.
- rtPCR is used to determine the concentration of *Salmonella* in the rinsate.
- 4 technical repeats performed on each genomic DNA.

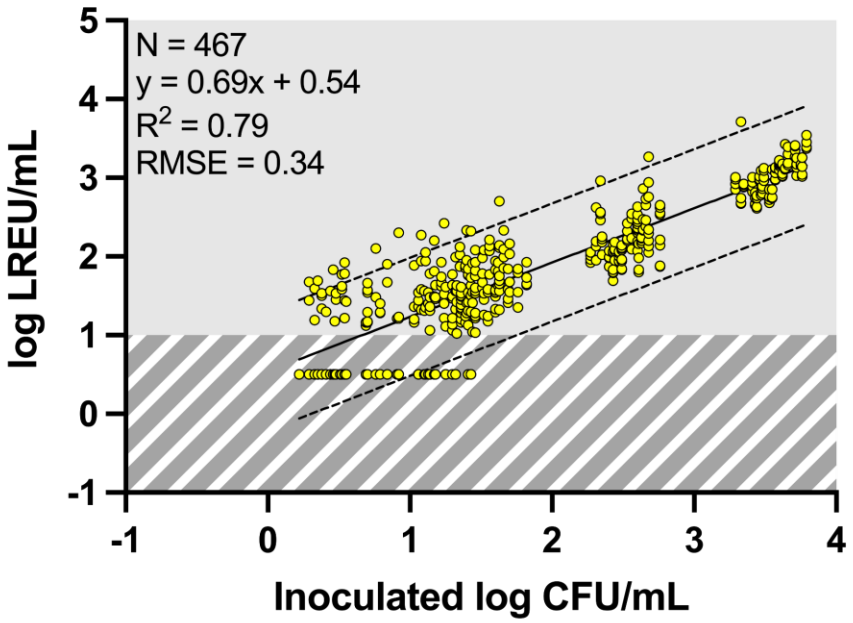
TABLE 7. Technical repeat differences for GENE-UP quantification (TRD_{GENEUP}) of *Salmonella* concentrations in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	N BLQ	TRD_{GENEUP} N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	0	144	0.11	0.16	0.07	0.00	1.04
2.00 to 2.99	24	0	144	0.20	0.21	0.12	0.00	1.14
1.00 to 1.99	45	12	198	0.31	0.25	0.26	0.00	1.38
0.00 to 0.99	24	24	na	na	na	na	na	na

N BLQ, number of rinses with at least one technical repeat with a below limit of quantification or quantification negative result. STD, standard deviation of the mean. Min., minimum. Max., maximum. na, not applicable.

GENE-UP Quantification Results

A



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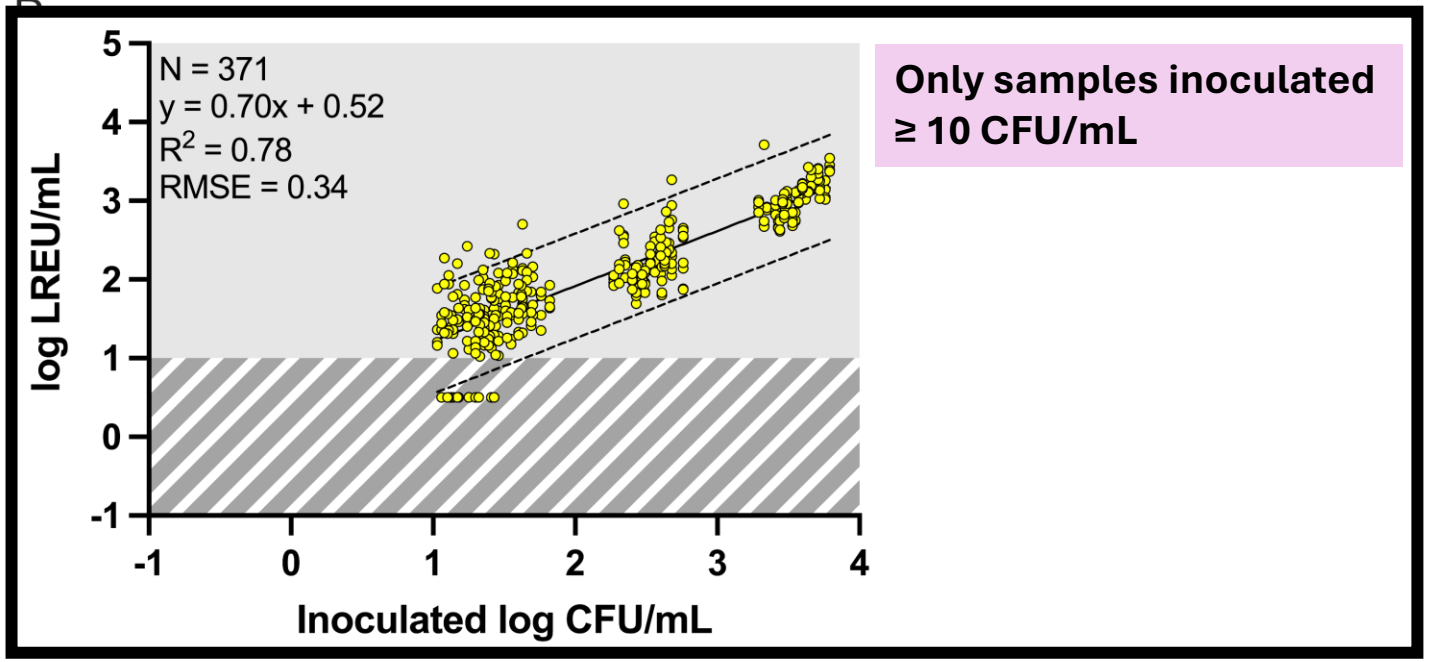


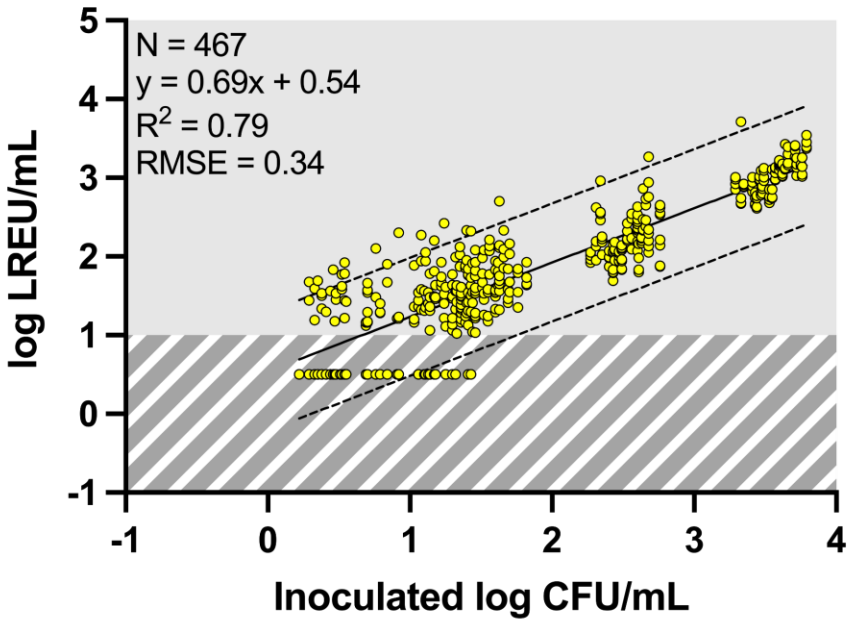
Only samples inoculated ≥ 10 CFU/mL

TABLE 8. GENE-UP quantitative discrepancy (QD_{GENEUP}) in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	QD_{GENEUP}					
		N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	96	-0.52	0.17	-0.52	-0.85	0.38
2.00 to 2.99	24	96	-0.30	0.28	-0.33	-0.89	0.62
1.00 to 1.99	45	179	0.12	0.42	0.13	-0.93	1.19
0.00 to 0.99	24	96	0.39	0.52	0.19	-0.42	1.39
0.00 to 3.99	117	467	-0.05	0.51	-0.11	-0.93	1.39
1.00 to 3.99	93	371	-0.16	0.44	-0.25	-0.93	1.19

GENE-UP Quantification Results

A



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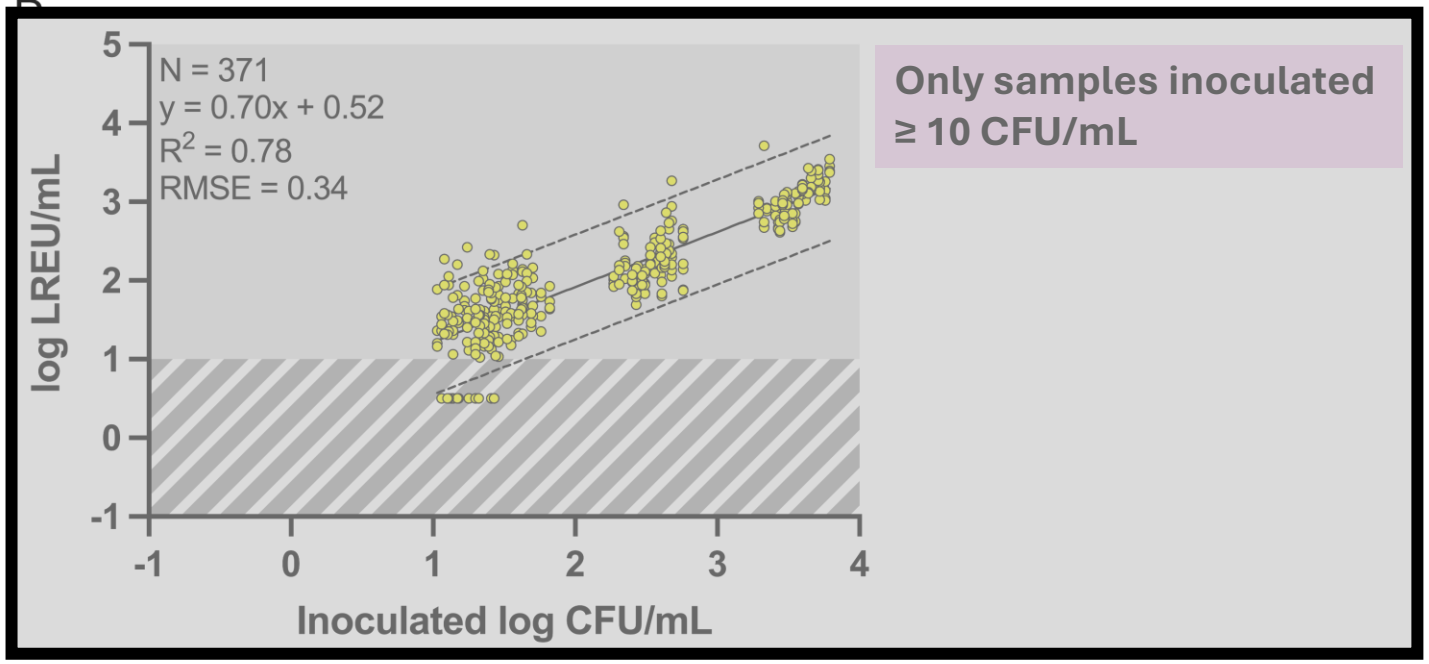
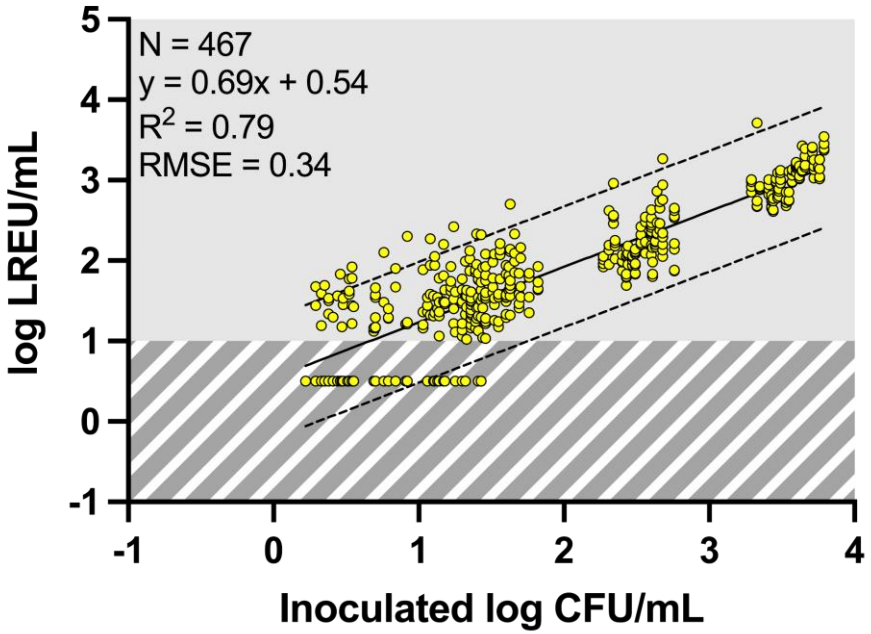


TABLE 8. GENE-UP quantitative discrepancy (QD_{GENEUP}) in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	QD_{GENEUP}					
		N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	96	-0.52	0.17	-0.52	-0.85	0.38
2.00 to 2.99	24	96	-0.30	0.28	-0.33	-0.89	0.62
1.00 to 1.99	45	179	0.12	0.42	0.13	-0.93	1.19
0.00 to 0.99	24	96	0.39	0.52	0.19	-0.42	1.39
0.00 to 3.99	117	467	-0.05	0.51	-0.11	-0.93	1.39
1.00 to 3.99	93	371	-0.16	0.44	-0.25	-0.93	1.19

GENE-UP Quantification Results

A



- Slope of 1 is ideal.
- Y-intercept of 0 is ideal.
- R^2 = 0 completely useless.
- R^2 = 1 linear equation perfectly explains the data.

$$RMSE = \sqrt{\frac{\Sigma(y_i - \hat{y}_i)^2}{N - P}}$$ *P* = 2 (parameter estimates)

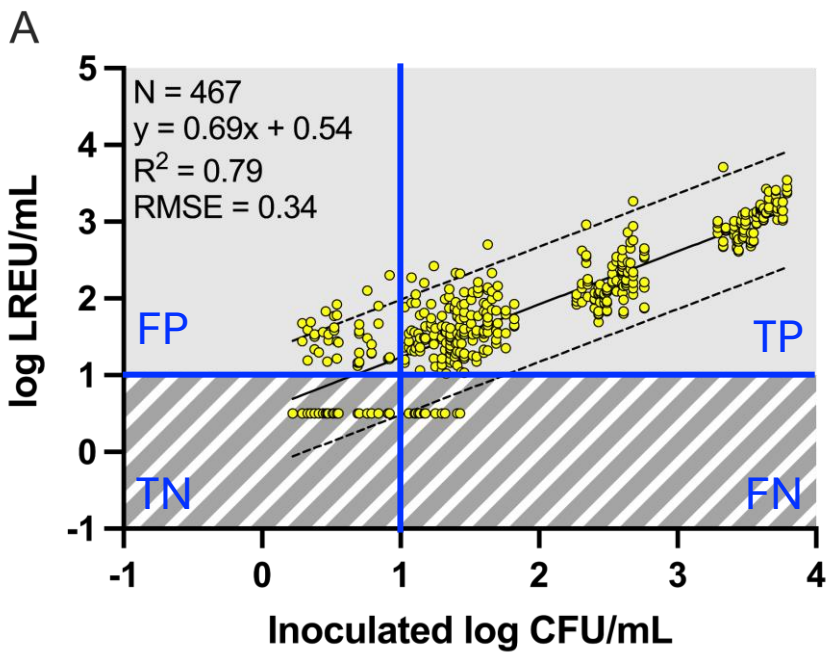
TABLE 8. GENE-UP quantitative discrepancy (QD_{GENEUP}) in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	QD_{GENEUP}					
		N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	96	-0.52	0.17	-0.52	-0.85	0.38
2.00 to 2.99	24	96	-0.30	0.28	-0.33	-0.89	0.62
1.00 to 1.99	45	179	0.12	0.42	0.13	-0.93	1.19
0.00 to 0.99	24	96	0.39	0.52	0.19	-0.42	1.39
0.00 to 3.99	117	467	-0.05	0.51	-0.11	-0.93	1.39
1.00 to 3.99	93	371	-0.16	0.44	-0.25	-0.93	1.19

GENE-UP Quant Estimations

TABLE 11. Proficiencies of quantification and threshold methods for identification of poultry wing rinses with *Salmonella* concentrations ≥ 10 CFU/mL

Method	N	TP	FP	FN	TN	Sens	Spec	PPV	NPV	FNR	FPR	Acc
MPN quantification	120	87	3	6	24	0.935	0.889	0.967	0.800	0.065	0.111	0.925
GENE-UP quantification	467	354	42	17	54	0.954	0.563	0.894	0.761	0.046	0.438	0.874



- Based on this data the GENE-UP estimate should be reporting a "mean estimate with a ± 95 CI of *at least** 0.6 log LREU/mL".
- *lots of caveats.
- Example Result: **10 LREU/mL (95% CI 2.5 – 40 LREU/mL).**

BAX Quantification

- *Published Lower Limit of Quantification = 1 CFU/mL.*
- 30 mL of Turkey Rinsate + 30 mL BAX-MP + Quant Solution.
- Enrich at 42 °C for 6 hours.
- 5 uL of BAX 6-Hour-Enrichment to 200 uL BAX Lysis Buffer + Protease
  → 37 °C for 20 min. → 95 °C for 10 min. → Cool 5 min. = BAX Lysate.
- 30 uL of BAX Lysate used with BAX Sal. rtPCR tablet.
- rtPCR is used to determine the concentration of *Salmonella* in the 6-hour enrichment.

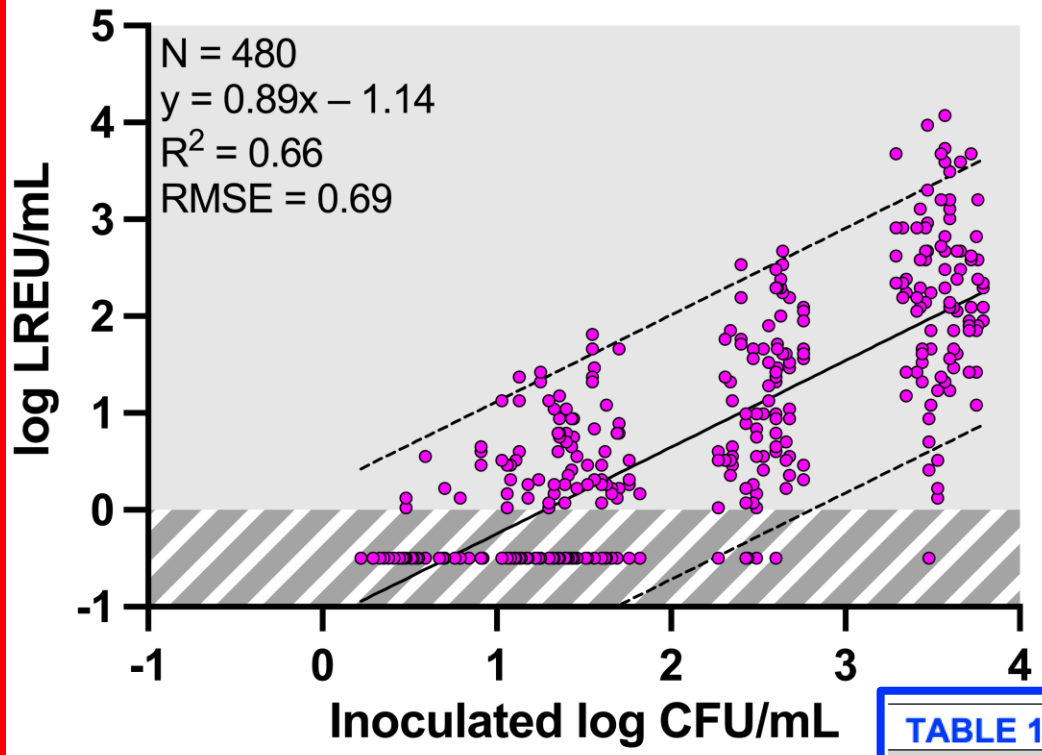
4 Technical Repeats of rtPCR performed with each BAX Lysate.

TABLE 9. Technical repeat differences for BAX quantification (TRD_{BAX}) of *Salmonella* concentrations in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	N BLQ	TRD_{BAX} N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	1	138	0.60	0.45	0.49	0.00	2.31
2.00 to 2.99	24	5	114	0.63	0.46	0.53	0.00	1.74
1.00 to 1.99	45	41	24	0.39	0.32	0.32	0.00	1.44
0.00 to 0.99	27	27	na	na	na	na	na	na

N BLQ, number of rinses with at least one technical repeat with a below limit of quantification or quantification negative result. STD, standard deviation of the mean. Min., minimum. Max., maximum. na, not applicable.

BAX Quant v. 4.22 (published) Estimations



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- Y-intercept of 0 is ideal.
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- R^2 = 1 linear equation perfectly explains the data.

$$RMSE = \sqrt{\frac{\Sigma(y_i - \hat{y}_i)^2}{N - P}}$$ P = 2 (parameter estimates)

TABLE 10. BAX quantitative discrepancy (QD_{BAX}) in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	QD_{BAX} N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	96	-1.35	0.89	-1.27	-3.98	0.50
2.00 to 2.99	24	96	-1.53	0.83	-1.58	-3.10	0.13
1.00 to 1.99	45	180	-1.44	0.64	-1.64	-2.32	0.26
0.00 to 0.99	27	108	-1.00	0.25	-1.02	-1.42	-0.04
0.00 to 3.99	120	480	-1.21	0.78	-1.19	-3.98	0.50

QD_{BAX} = BAX log LREU/mL - inoculated rinse log CFU/mL

BAX Quant v. 4.22 (published) Estimations

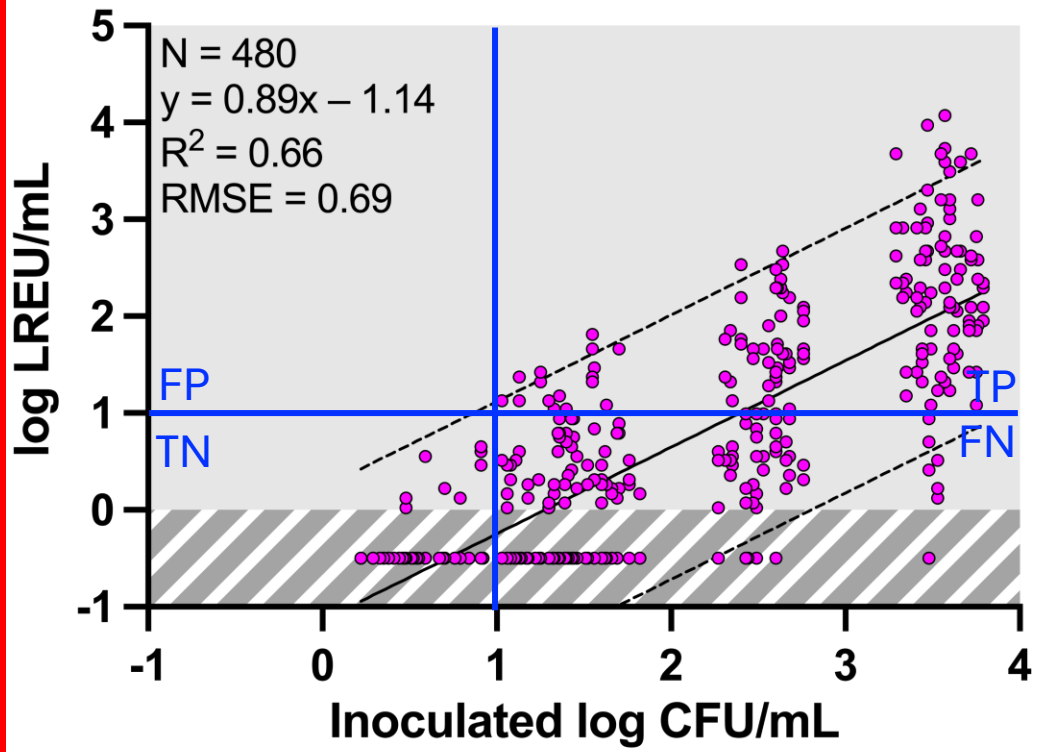


TABLE 10. BAX quantitative discrepancy (QD_{BAX}) in poultry wing rinses

Inoculated rinse log CFU/mL range	N of rinses	QD_{BAX} N	Mean	STD	Median	Min.	Max.
3.00 to 3.99	24	96	-1.35	0.89	-1.27	-3.98	0.50
2.00 to 2.99	24	96	-1.53	0.83	-1.58	-3.10	0.13
1.00 to 1.99	45	180	-1.44	0.64	-1.64	-2.32	0.26
0.00 to 0.99	27	108	-1.00	0.25	-1.02	-1.42	-0.04
0.00 to 3.99	120	480	-1.21	0.78	-1.19	-3.98	0.50

QD_{BAX} = BAX log LREU/mL - inoculated rinse log CFU/mL

- **220 Quantifications below LOQ of 0 log CFU/mL.**

TABLE 7. Proficiencies of quantification and threshold methods for identification of poultry wing rinses with *Salmonella* concentrations ≥ 10 CFU/mL

Method	N	TP	FP	FN	TN	Sens.	Spec.	PPV	NPV	FNR	FPR	Acc.
MPN quantification	132	87	3	6	36	0.935	0.923	0.967	0.857	0.065	0.077	0.932
GENE-UP quantification	515	354	44	17	100	0.954	0.694	0.889	0.855	0.046	0.306	0.882
BAX quantification	528	151	0	221	156	0.406	1.000	1.000	0.414	0.594	0.000	0.581

BAX Quantification

hygiena®

January 6, 2025

Dear Valued Customer,

This letter is to inform you that we recently identified cases where estimates of Salmonella levels in samples are lower than expected when using the BAX® System SalQuant® method, compared to other enumerative methods, such as MPN or plate counts.

The qualitative results (i.e., Presence or Absence) and sensitivity for the BAX Real-Time Salmonella PCR assay are not impacted. Further, results generated from SalLimits™ protocols are not impacted.

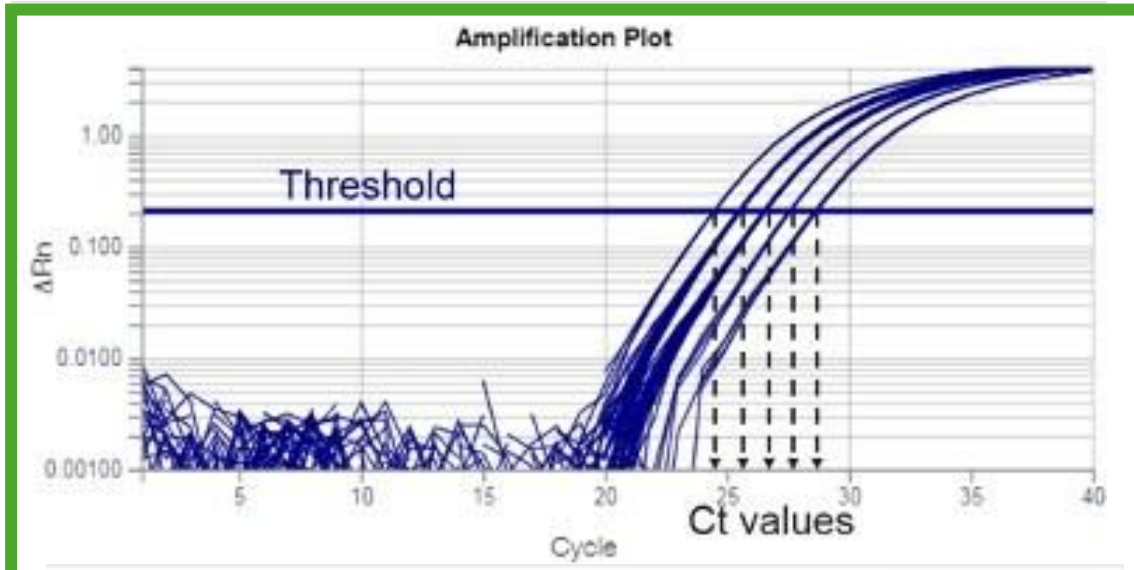
Upon further investigation, it was determined that a change in how the Ct value is generated for the BAX Real-Time *Salmonella* PCR assay in BAX Q7 software versions 3.7 through 5.0 impacts the SalQuant estimation. The Ct value tends to be higher, which leads to an underestimation of the quantification result.

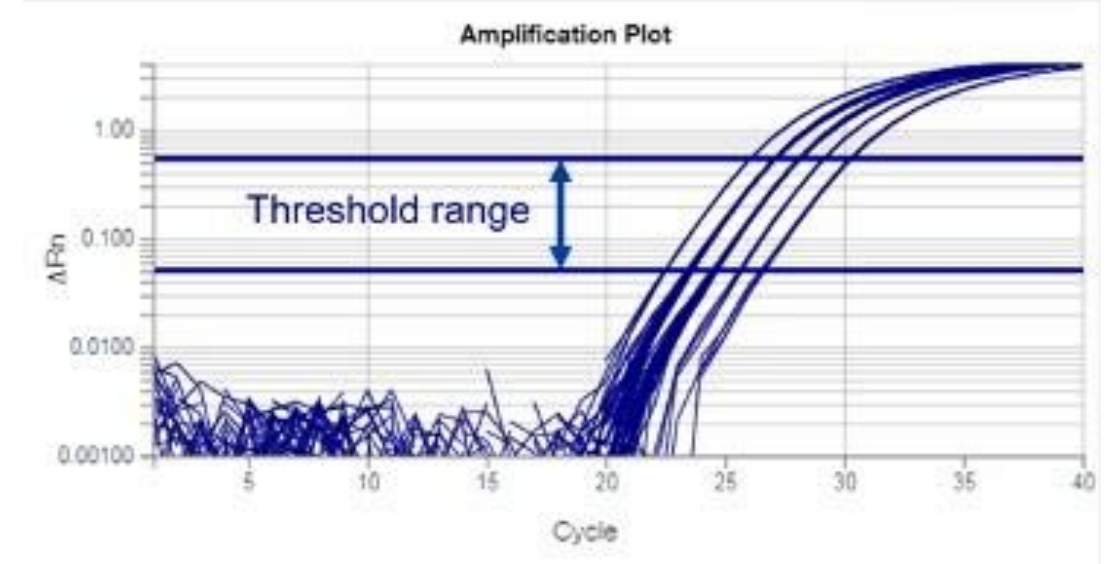
In order to eliminate this underestimation bias, Hygiena recommends reanalyzing .bax files in BAX Q7 software v3.6 and using the Microsoft® Excel® calculator provided by Hygiena for the purposes of quantifying *Salmonella* in samples. Hygiena is available to provide assistance with reanalysis, if needed.

Hygiena is working on a robust solution in BAX software v5.1, expected to be released in February 2025, that eliminates the underestimation bias in the SalQuant results. The solution will introduce a dedicated 'SalQuant' option in the assay dropdown menu of the BAX Q7 software.

- **Version 4.22 used in this study.**
- **Letter asks to re-calc with version 3.7.**

BAX Quant Threshold Issues





Threshold Setting

- Sufficiently above the background fluorescence baseline to be confident of avoiding the amplification plot crossing the threshold prematurely due to background fluorescence.
- In the log phase of the amplification plot where it is unaffected by the plateau phase.
- At a position where the log phases of all amplification plots are parallel.

https://www.sigmaaldrich.com/US/en/technical-documents/technical-article/genomics/qpcr/data-analysis

https://www.thermofisher.com/blog/behindthebench/understanding-ct-values/

BAX QUANT v4.22 versus v3.7

Attribute	Ct Value difference	
	v4.22	v3.7
N	720	720
Mean	1.4	1.1
Std. Dev.	1.1	1.0
Median	1.2	0.8
Min	0.0	0.0
Max	5.8	7.4
% ≥ 1.0 Ct	58.1	44.2
% ≥ 1.5 Ct	41.5	26.7
% ≥ 2.0 Ct	28.2	15.3

% of rinses (N = 120)		
Attribute	v4.22	v3.7
Difference ≥ 1.0 Ct	95.0	85.0
Difference ≥ 1.5 Ct	85.0	66.7
Difference ≥ 2.0 Ct	71.7	43.3

- Each rinse had 4 technical repeats: A, B, C, D.
- Each rinse had six differences: AB, AC, AD, BC, BD, CD.

BAX QUANT v4.22 versus v3.7

Attribute	log difference v4.22	log difference v3.7
N	720	720
Mean	0.4	0.3
Std. Dev.	0.5	0.4
Median	0.2	0.2
Min	0.0	0.0
Max	2.3	2.9
% ≥ 1.0 log	15.4	6.1
% ≥ 1.5 log	3.6	1.4
% ≥ 2.0 log	0.1	0.6

No arbitrary values

Attribute	log difference v4.22	log difference v3.7
N	276	372
Mean	0.6	0.3
Std. Dev.	0.4	0.4
Median	0.5	0.2
Min	0.0	0.0
Max	2.3	2.9
% ≥ 1.0 log	19.6	5.4
% ≥ 1.5 log	4.0	1.9
% ≥ 2.0 log	0.4	0.8

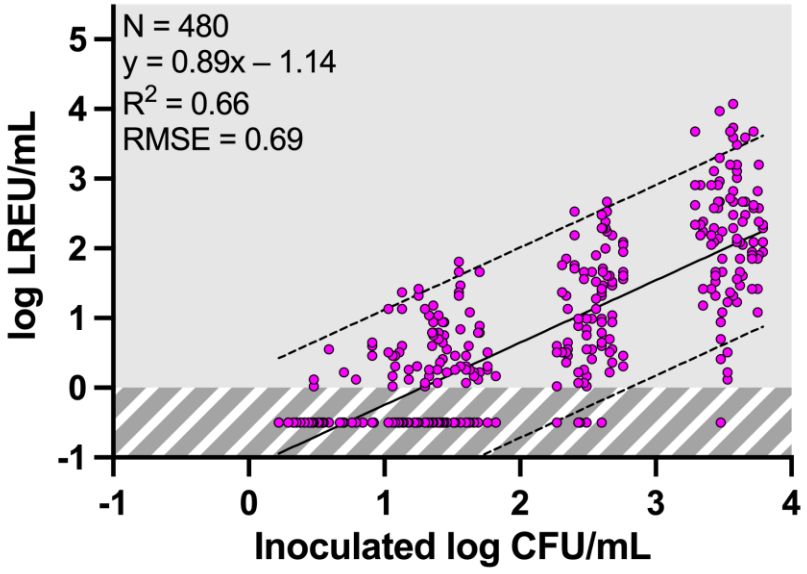
% of rinses (N = 120)		
Attribute	v4.22	v3.7
Difference ≥ 1.0 log	40.0	16.7
Difference ≥ 1.5 log	14.2	3.3
Difference ≥ 2.0 log	0.8	1.7

No arbitrary values

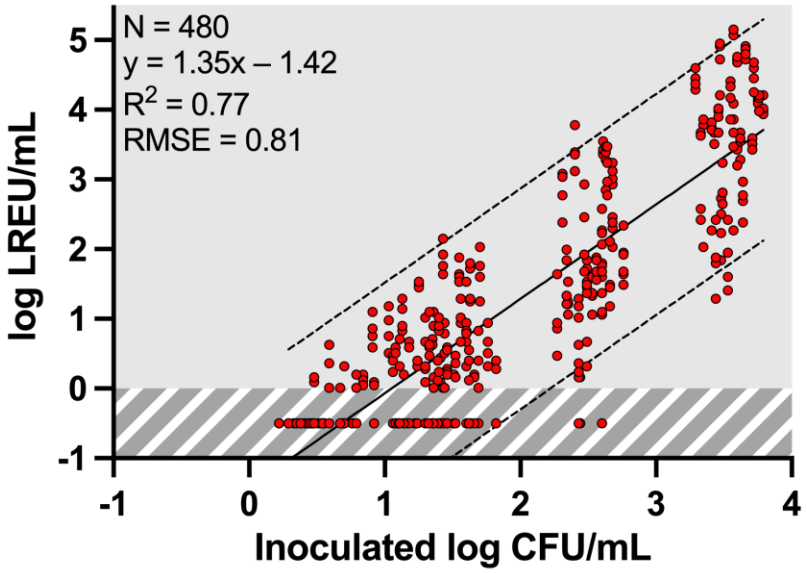
% of rinses		
Attribute	v4.22 (*n* = 46)	v3.7 (*n* = 62)
Difference ≥ 1.0 log	58.7	14.5
Difference ≥ 1.5 log	19.6	4.8
Difference ≥ 2.0 log	2.2	1.6

BAX QUANT v4.22 versus v3.7

Published Results
Software v4.22 b1.12281
Analysis v4.22.0.9784



Hygiena Reanalysis
"Software v3.6"



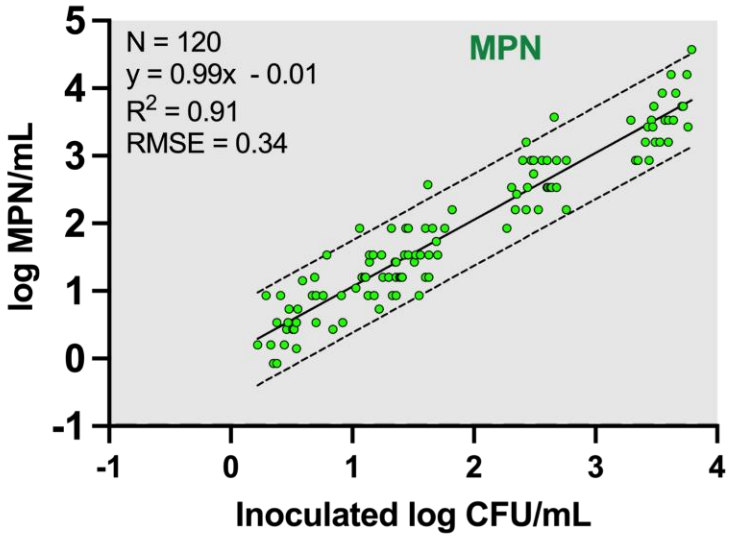
- Slope worse.
- Y-intercept worse.
- R^2 improved.
- RMSE worse.

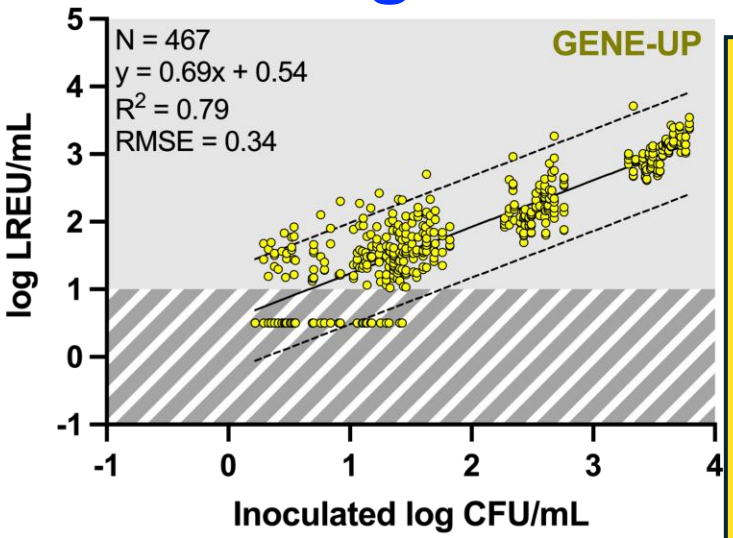
- Slope of 1 is ideal.
- Y-intercept of 0 is ideal.
- R^2 = 0 completely useless.
- R^2 = 1 linear equation perfectly explains the data.

$$RMSE = \sqrt{\frac{\Sigma(y_i - \hat{y}_i)^2}{N - P}}$$

P = 2 (parameter estimates)

Linear Regressions





- Slope of 1 is ideal.
- Y-intercept of 0 is ideal.

- R^2 = 0 completely useless.
- R^2 = 1 linear equation perfectly explains the data.

$$RMSE = \sqrt{\frac{\Sigma(y_i - \hat{y}_i)^2}{N - P}}$$

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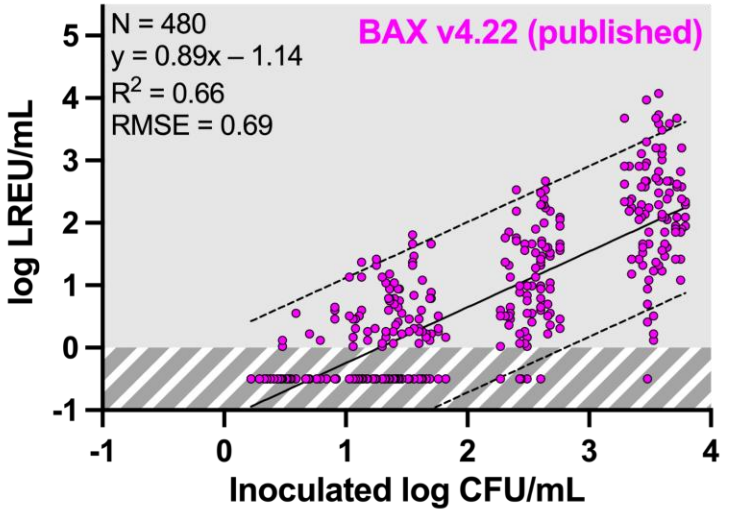
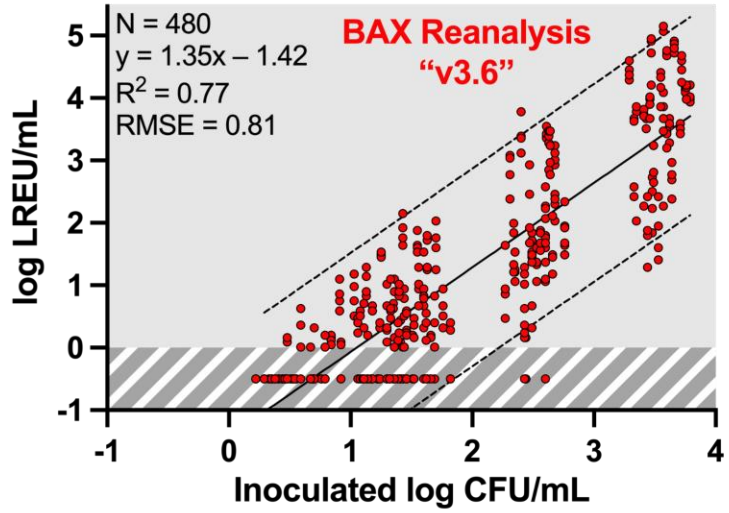




BAX QUANT v4.22 versus v3.7

TABLE X. Proficiencies of quantification methods for identification of poultry wing rinses with *Salmonella* concentrations ≥ 10 CFU/mL

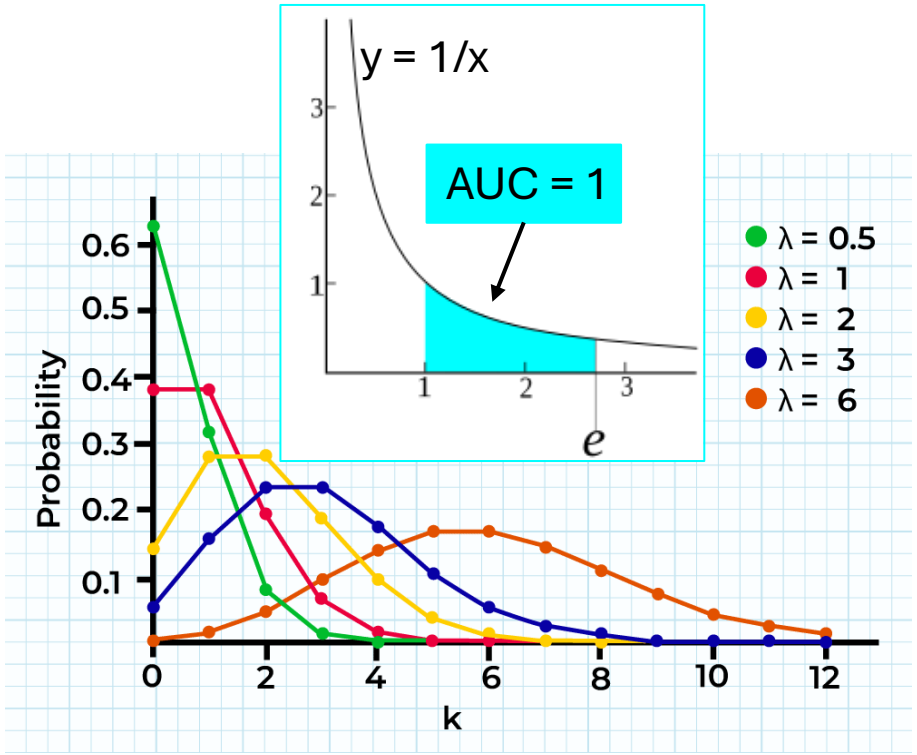
Method	N	TP	FP	FN	TN	Sens	Spec	PPV	NPV	FNR	FPR	Acc
MPN quantification	120	87	3	6	24	0.935	0.889	0.967	0.800	0.065	0.111	**0.925**
GENE-UP quantification	467	354	42	17	54	0.954	0.563	0.894	0.761	0.046	0.438	**0.874**
BAX quantification (Hygiena Reanalysis)	480	208	1	164	107	0.559	0.991	0.995	0.395	0.441	0.009	**0.656**
BAX quantification (published)	480	151	0	221	108	0.406	1.000	1.000	0.328	0.594	0.000	**0.540**

TP, true positive. FP, false positive. FN, false negative. True negative. Sens., sensitivity. Spec., specificity. PPV, positive predictive value. NPV, negative predicative value. FNR, false negative rate. FPR, false positive rate. Acc, accuracy.

$$Acc = \frac{TP + TN}{TP + FP + FN + TN}$$

Salmonella PiLOT (Poisson Limit One Tube) Threshold Test

- Similar in concept to MPN. Both are based on Poisson **Probability** Distribution.
- Poisson applications include customer arrivals for a specific hour (restaurants, websites, etc.), traffic accident frequency, number radioactive decay events during a defined period, number of lottery winners.



Simeon Poisson (1781 – 1840)
Mathematician & Physicist

Poisson Probability Distribution

$$P(k|\lambda) = \frac{(e^{-\lambda})(\lambda^{k})}{k!}$$

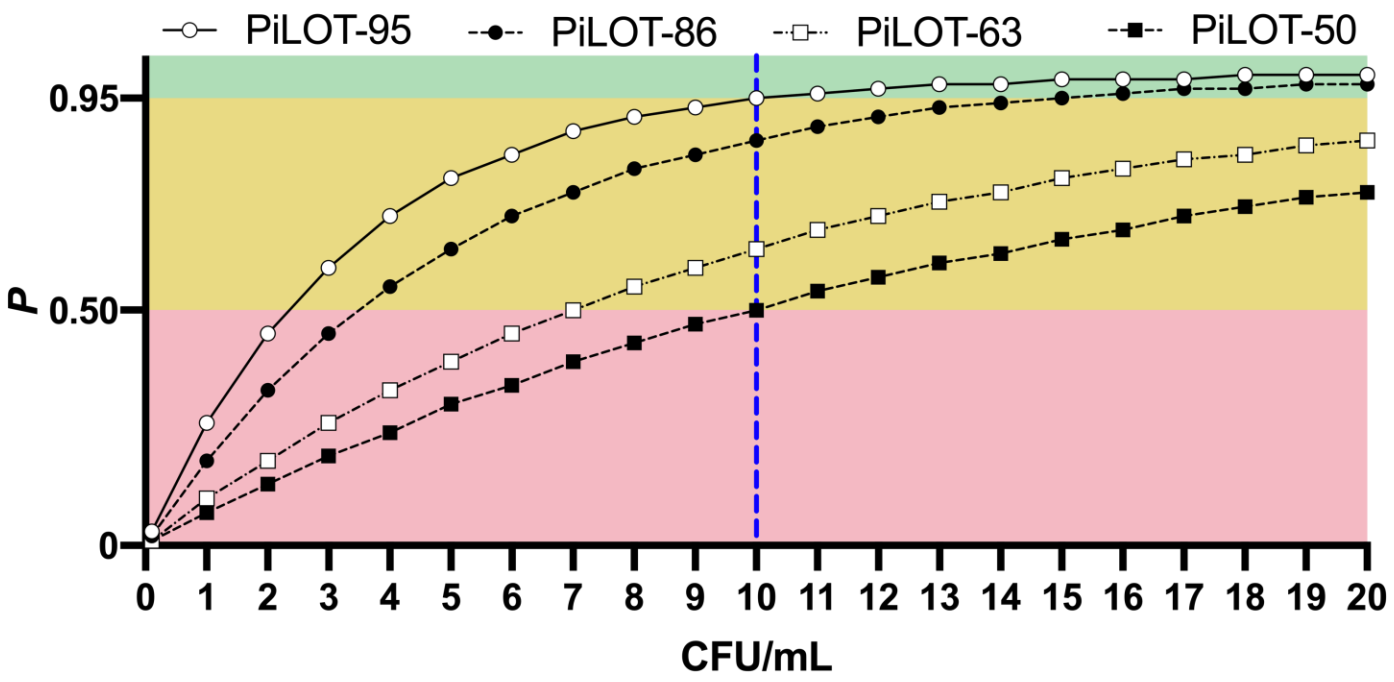
PiLOT Equation for Liquids

$$v = \frac{-1 \cdot \ln(1-P)}{T}$$

Schmidt, *et al*. **Evaluation of methods for identifying poultry wing rinses with *Salmonella* concentrations greater than or equal to 10 CFU/mL.** *J. Food Prot.* 87:100362 (2024)

Prob. of *Salmonella* Positive

$$P = 1 - e^{-vC}$$



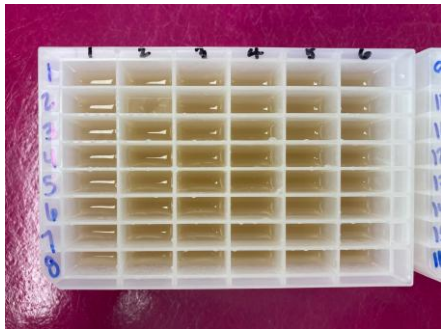
Rinse "C" *Sal. CFU/mL*	"P" Prob. *Salmonella* Positive Test PiLOT-95 *v* = 0.30 mL	PiLOT-86 *v* = 0.20 mL	PiLOT-63 *v* = 0.10 mL	PiLOT-50 *v* = 0.07mL
0.1	0.03	0.02	0.01	0.01
1	0.26	0.18	0.10	0.07
2	0.45	0.33	0.18	0.13
3	0.59	0.45	0.26	0.19
4	0.70	0.55	0.33	0.24
5	0.78	0.63	0.39	0.30
6	0.83	0.70	0.45	0.34
7	0.88	0.75	0.50	0.39
8	0.91	0.80	0.55	0.43
9	0.93	0.83	0.59	0.47
10	0.95	0.86	0.63	0.50
11	0.96	0.89	0.67	0.54
12	0.97	0.91	0.70	0.57
13	0.98	0.93	0.73	0.60
14	0.98	0.94	0.75	0.62
15	0.99	0.95	0.78	0.65
16	0.99	0.96	0.80	0.67
17	0.99	0.97	0.82	0.70
18	1.00	0.97	0.83	0.72
19	1.00	0.98	0.85	0.74
20	1.00	0.98	0.86	0.75
30	1.00	1.00	0.95	0.88
40	1.00	1.00	0.98	0.94
50	1.00	1.00	0.99	0.97

$P < 0.50$ | $0.50 \leq P < 0.95$ | $P \leq 0.95$

Schmidt, *et al*. **Evaluation of methods for identifying poultry wing rinses with *Salmonella* concentrations greater than or equal to 10 CFU/mL.** *J. Food Prot*. 87:100362. doi:10.1016/j.jfp.2024.100362

PiLOT-95 Method For 10 CFU/mL *Salmonella* Threshold

0.3 mL



48 Tests/Block

35 °C

12 h

Commercial *Sal.* Detection (BAX)

+ = 95% Prob. ≥ 10 *Salmonella* cells/mL

− = 5% Prob. ≥ 10 *Salmonella* cells/mL

Schmidt, *et al.* **Evaluation of methods for identifying poultry wing rinses with *Salmonella* concentrations greater than or equal to 10 CFU/mL.** *J. Food Prot.* 87:100362. doi:10.1016/j.jfp.2024.100362

Salmonella PiLOT (Poisson Limit One Tube) Test

TABLE x. Proficiencies of quantification and threshold methods for identification of poultry wing rinses with *Salmonella* concentrations ≥ 10 CFU/mL

Method	N	TP	FP	FN	TN	Sens	Spec	PPV	NPV	FNR	FPR	Acc
MPN quantification	120	87	3	6	24	0.935	0.889	0.967	0.800	0.065	0.111	0.925
PiLOT-86 threshold	104	81	9	0	14	1.000	0.609	0.900	1.000	0.000	0.391	0.913
GENE-UP quantification	467	354	42	17	54	0.954	0.563	0.894	0.761	0.046	0.438	0.874
PiLOT-63 threshold	104	74	8	7	15	0.914	0.652	0.902	0.682	0.086	0.348	0.856
PiLOT-95 threshold	104	81	16	0	7	1.000	0.304	0.835	1.000	0.000	0.696	0.846
PiLOT-50 theshold	104	69	6	12	17	0.852	0.739	0.920	0.586	0.148	0.261	0.827
BAX quantification (reanalysis)	**480**	**208**	**1**	**164**	**107**	**0.559**	**0.991**	**0.995**	**0.395**	**0.441**	**0.009**	**0.656**
BAX quantification (published)	480	151	0	221	108	0.406	1.000	1.000	0.328	0.594	0.000	0.540

TP, true positive. FP, false positive. FN, false negative. True negative. Sens., sensitivity. Spec., specificity. PPV, positive predictive value. NPV, negative predicative value. FNR, false negative rate. FPR, false positive rate. Acc, accuracy.

Method	Time to Result	Financial Cost	Technical Burden	Notes
MPN Quant.	2+ Days	Very High	Very High	Gold Standard since 1950s
GENE-UP Quant.	≈ 4 hours	Medium	High	AOAC Certified
BAX Quant.	≈ 10 hours	Medium	Medium	AOAC Certified
PiLOT Threshold	≈ 12 hours (can be shorter)	Medium	Low	USMARC Developed

CFU/g Thresholds

United States Department of Agriculture

Food Safety and Inspection Service

MLG 4.14

Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg, Siluriformes (Fish) Products and Carcass and Environmental Sponges

MLG 4 Isolation and Identification of *Salmonella* Revision: 14 (Replaces: .13) Effective: 06/05/23

Table 3. Sample Preparation and Enrichment Guide

Product	Sample Preparation		Incubation
	Portion Size	Enrichment Amount determined by volume or weight	Cultural or rapid screen
Ready-to-Eat Meat, Poultry and Siluriformes Products	325 ± 6.5 g	975 ± 19.5 mL BPW	35 ± 2°C for 18 – 24 hr.
Raw Poultry Products	325 ± 32.5 g	1625 ± 32.5 mL BPW	35 ± 2°C for 20 – 24 hr.
Raw Meat and Raw Beef Mixed Products	325 ± 32.5 g	975± 19.5 mL mTSB	42 ± 1°C for 15 – 24 hr.

Equations for *Salmonella* Thresholds

Solids

$$v = \frac{-1 \cdot \ln(1-P) \cdot (g+s)}{T \cdot g}$$

- g = sample size in grams
- s = volume of liquid used to suspend g
- P = Poisson probability of detection
- T = *Salmonella* Threshold in CFU/g

Liquids

$$v = \frac{-1 \cdot \ln(1-P)}{T}$$

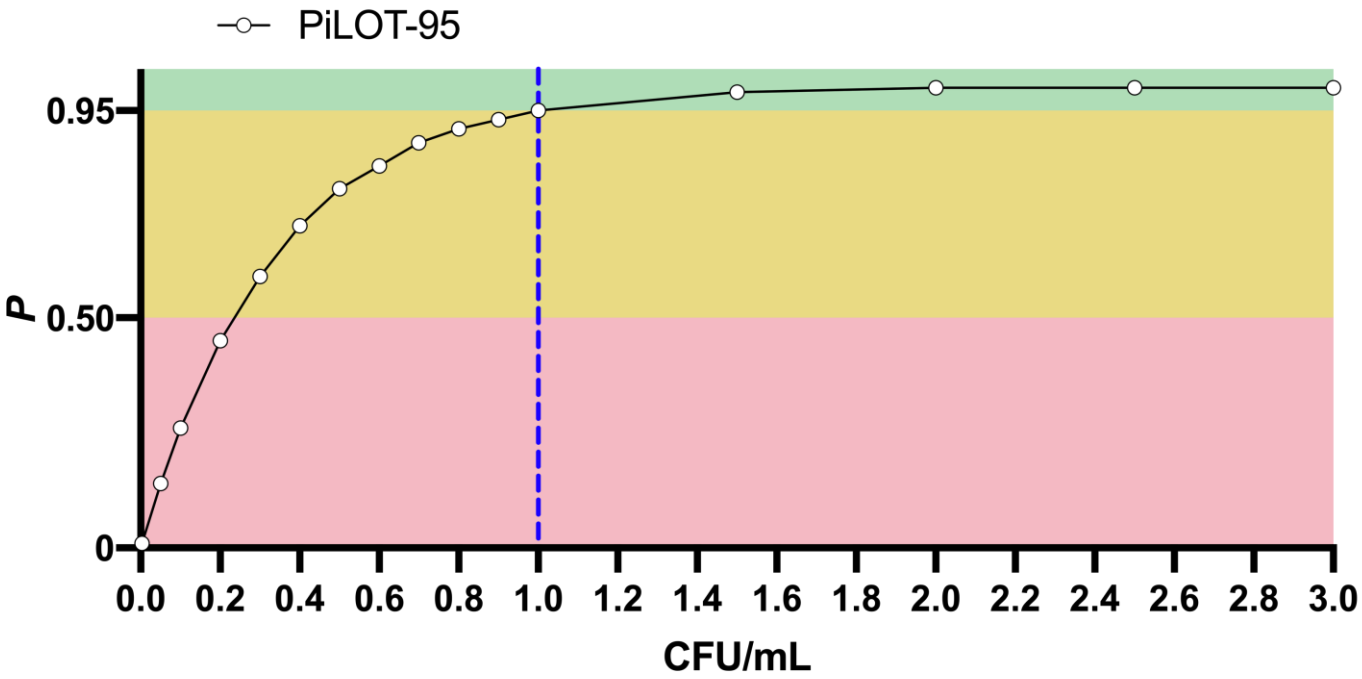
- P = Poisson probability of detection
- T = *Salmonella* Threshold in CFU/mL

FSIS MLG 4.14 Table 3 Product	*g* Sample Size (grams)	*s* Suspension Volume (mL)	*T* Threshold (CFU/g)	*P* Poisson Prob. of Detection	*v* Tested Volume (mL)
RTE Meat, Poultry and Siluiformes	325	975	10	0.95	1.2
Raw Poultry Products	325	1625	10	0.95	1.8
Raw Meat and Raw Beef Mixed Products	375	100	10	0.95	0.4
Pasteurized Liquid, Frozen or Dried Egg Products	100	900	10	0.95	3.0
Fermented Products	325	2936	10	0.95	3.0
Dried Products	325	2925	10	0.95	3.0
RTE Meat, Poultry and Siluiformes	325	975	1	0.95	12.0
Raw Poultry Products	325	1625	1	0.95	18.0
Raw Meat and Raw Beef Mixed Products	325	975	1	0.95	12.0
Pasteurized Liquid, Frozen or Dried Egg Products	100	900	1	0.95	30.0
Fermented Products	325	2936	1	0.95	30.1
Dried Products	325	2925	1	0.95	30.0

Schmidt, *et al*. **Evaluation of methods for identifying poultry wing rinses with *Salmonella* concentrations greater than or equal to 10 CFU/mL.** *J. Food Prot.* 87:100362. doi:10.1016/j.jfp.2024.100362

Potential Issues With PiLOT–95 Test Probabilities

Salmonella CFU/g	*Salmonella* CFU in 325 g Sample	Randomly Distributed *Salmonella* CFU/mL in Sample Suspension	mL of Suspension Transferred to PiLOT Tube	Prob. >0 *Sal.* in PiLOT-95 Test)	*Salmonella* CFU/g
0.003	1	0.001	18	0.01	0.000
0.05	16	0.008	18	0.14	0.05
0.1	33	0.017	18	0.26	0.1
0.2	65	0.033	18	0.45	0.2
0.3	98	0.050	18	0.59	0.3
0.4	130	0.067	18	0.70	0.4
0.5	163	0.083	18	0.78	0.5
0.6	195	0.100	18	0.83	0.6
0.7	228	0.117	18	0.88	0.7
0.8	260	0.133	18	0.91	0.8
0.9	293	0.150	18	0.93	0.9
1	325	0.167	18	0.95	1
1.5	488	0.250	18	0.99	1.5
2	650	0.333	18	1.00	2
2.5	813	0.417	18	1.00	2.5
3	975	0.500	18	1.00	3



- Concerns regarding probabilities of detection > 0.50 for samples with *Salmonella* level from 0.3 to 0.9 CFU/mL will produce too many False Positives.
- In theory, False Positives can be reduced with three threshold tests per sample.

Confidently Above Threshold (CAT) Method

1 **RUNNING HEAD.** Identification of ≥ 1 CFU/g *Salmonella*

2

3 **Identification of chicken component samples containing *Salmonella* concentrations greater than or**

4 **equal to 1 CFU/g†**

5

6 **John W. Schmidt*, Weifan Wu, Dayna M. Harhay, and Tommy L. Wheeler**

7

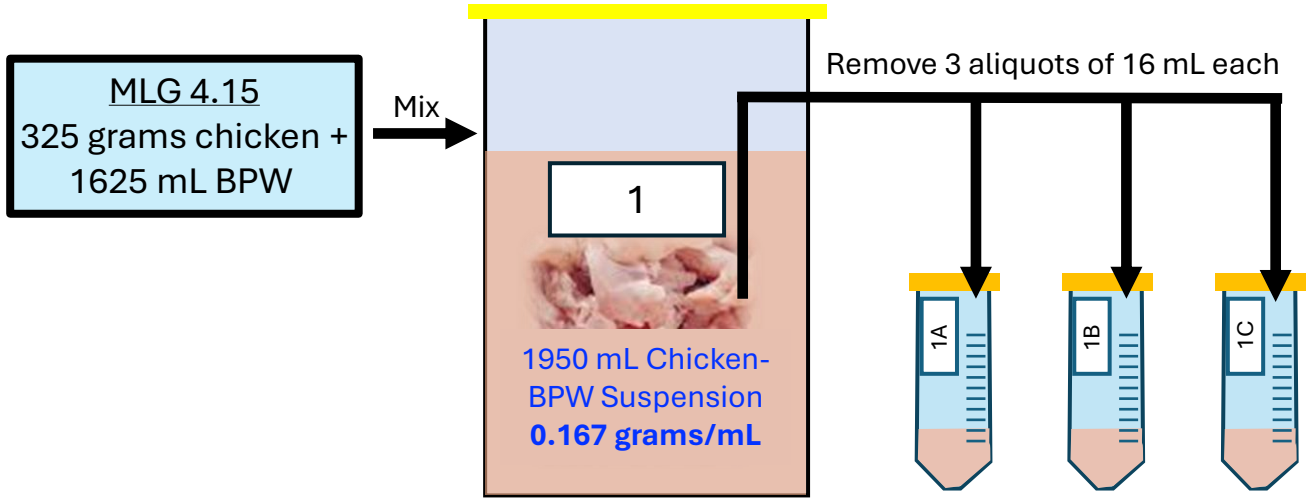
8 United States Department of Agriculture, Agricultural Research Service, U.S. Meat Animal Research Center,

9 PO Box 165, State Spur 18D, Clay Center, NE 68933

Schmidt, *et al*. **Identification of chicken component samples containing *Salmonella* concentrations greater than or equal to 1 CFU/g.** Submitted February 3

Confidently Above Threshold (CAT) Method

Poisson Distribution Probability & Probability of Independent Events



Schmidt, *et al*. **Identification of chicken component samples containing *Salmonella* concentrations greater than or equal to 1 CFU/g.** Submitted February 3

Probability of > 0 *Sal*. CFU in v mL for C concentration

$$P = 1 - e^{-vC}$$

Probability of > 0 *Sal*. CFU in CAT tube "A" for 1 CFU/g

$$P(A) = 1 - 2.718^{-(16)(0.167)}$$

$$P(A) = P(B) = P(C) = 0.931$$

Probability of independent events (MPN assumption)

$$P(ABC) = P(A) \cap P(B) \cap P(C) = P(A) \cdot P(B) \cdot P(C)$$

Probability of > 0 Sal. in All Three CAT Tubes at 1 CFU/g

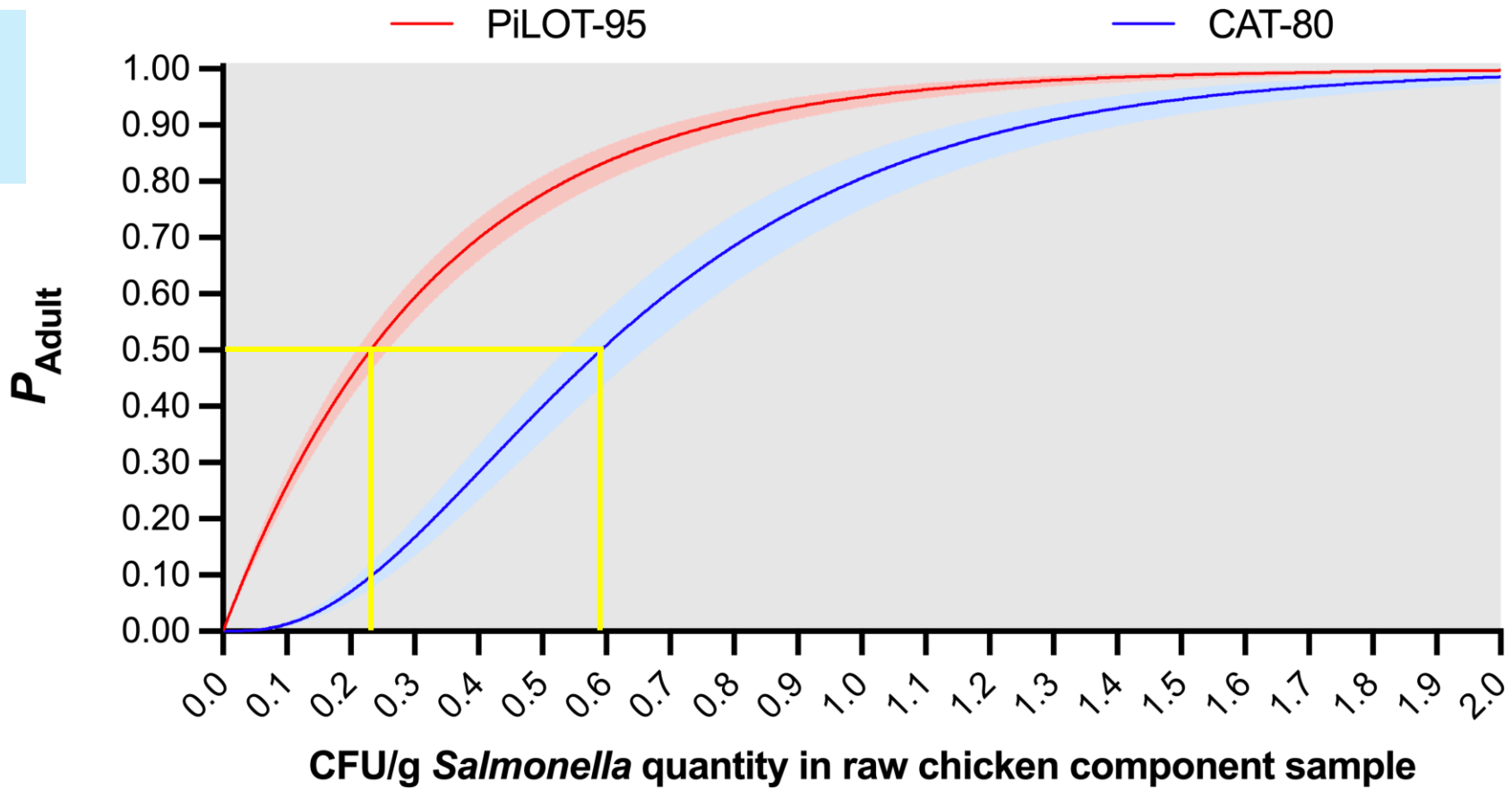
$$P(ABC) = 0.931 \cdot 0.931 \cdot 0.931 = \mathbf{0.80}$$

Probability of > 0 Sal. in All Three CAT Tubes

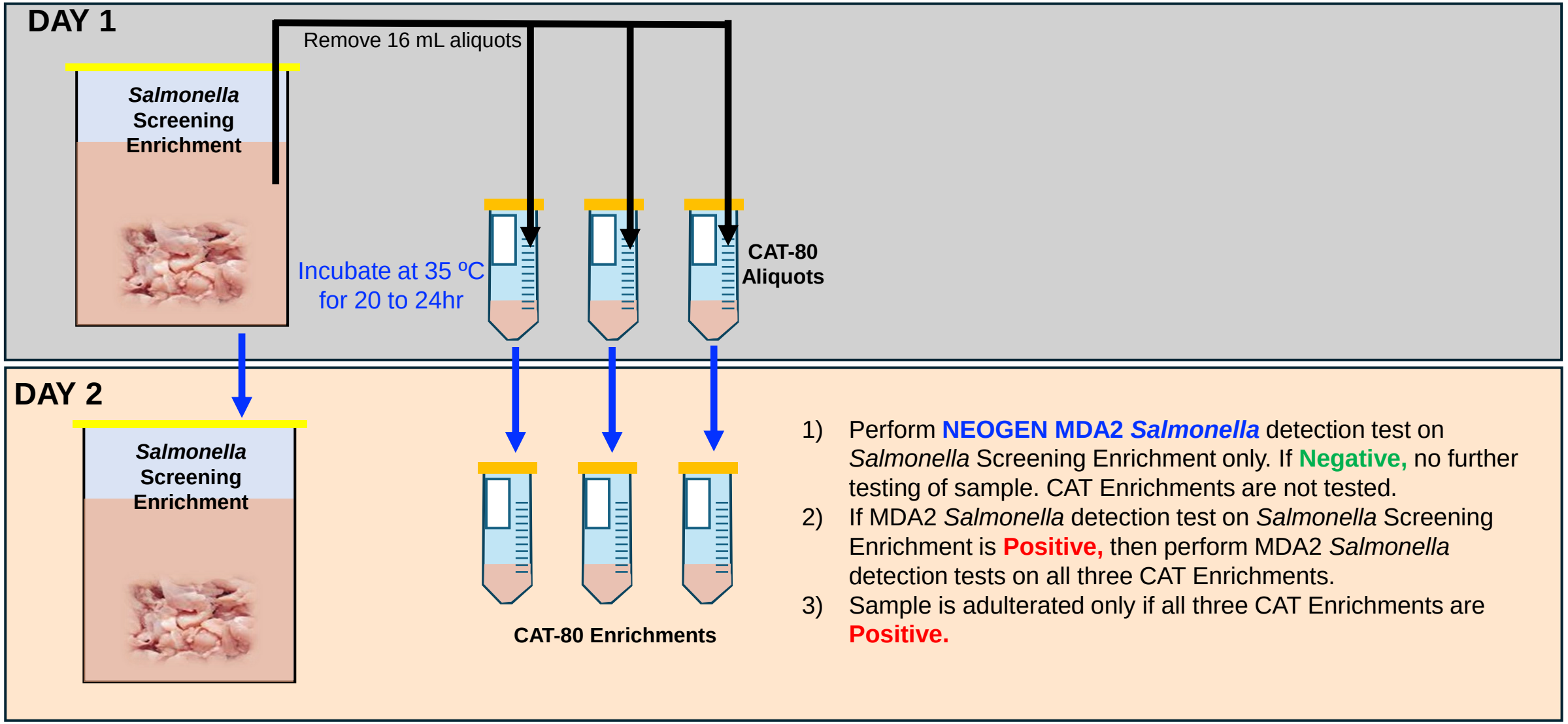
$$P = (1 - e^{-vC})^3$$

PiLOT-95 and CAT-80 Probability Curves

MLG 4.15 Tolerances
Portion Size: 325 ± 32.5 grams
BPW volume: 1625 ± 32.5 mL



NRTE Chicken Component Screen and CAT-80



1) Perform **NEOGEN MDA2 *Salmonella*** detection test on *Salmonella* Screening Enrichment only. If **Negative,** no further testing of sample. CAT Enrichments are not tested.
2) If MDA2 *Salmonella* detection test on *Salmonella* Screening Enrichment is **Positive,** then perform MDA2 *Salmonella* detection tests on all three CAT Enrichments.
3) Sample is adulterated only if all three CAT Enrichments are **Positive.**

PiLOT-95 and CAT-80 Results

TABLE 1. Proficiencies of qualitative threshold methods for identification of raw chicken component samples with *Salmonella* concentrations ≥ 1 CFU/mL

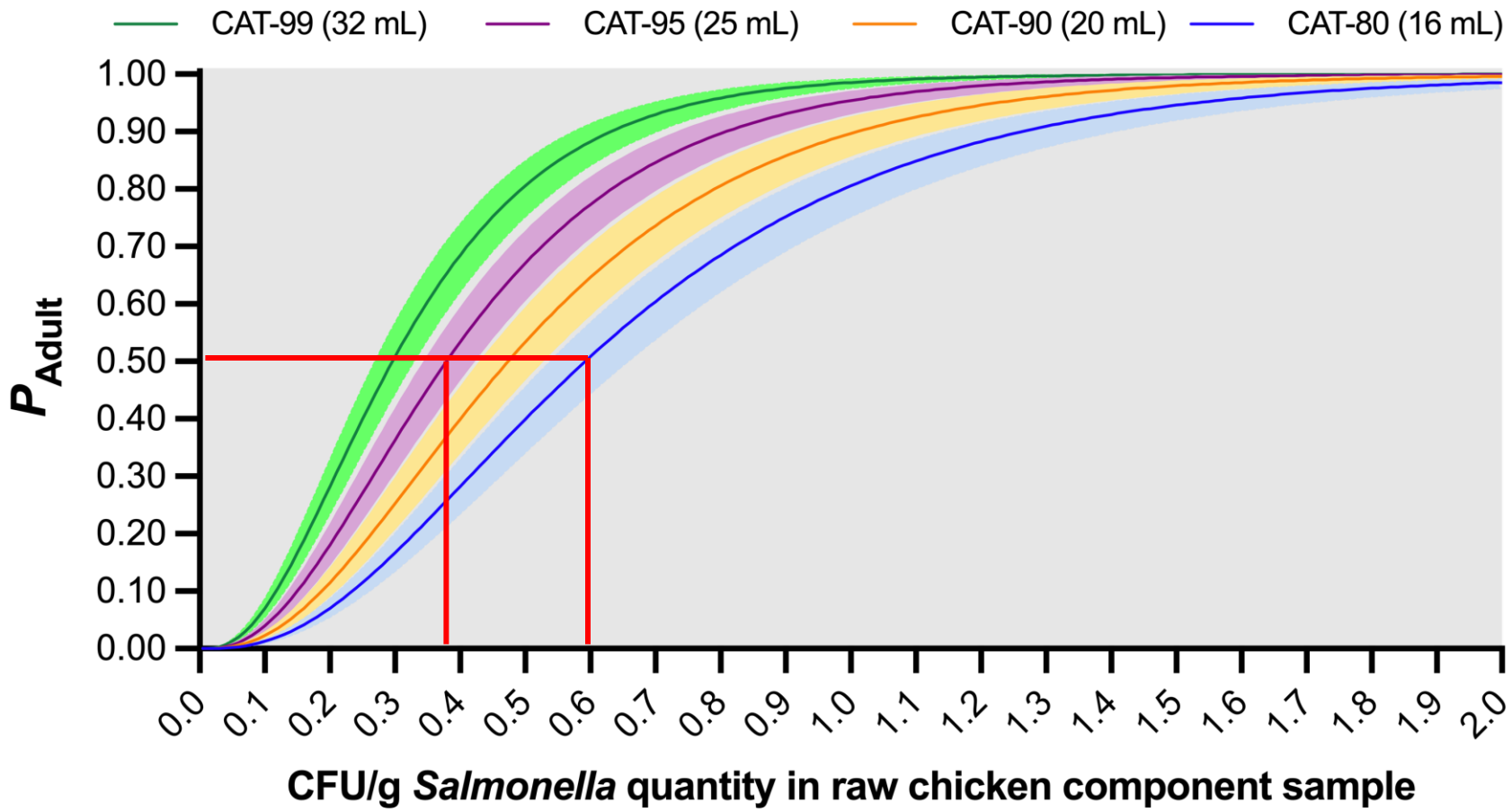
Protocol	N	TP	FP	FN	TN	Sens	Spec	PPV	NPV	FNR	FPR	Acc
PiLOT-95	80	40	20	0	20	1.000	0.500	0.667	1.000	0.000	0.500	0.750
CAT-80	80	40	9	0	31	1.000	0.775	0.816	1.000	0.000	0.225	0.888

TABLE 2. Theoretical probability of presumptive *Salmonella* adulteration (P_{Adult}) and empiric *Salmonella* adulteration rates in raw chicken component

Reference *Salmonella* quantity (Q_{ref})	P_{Adult}		Empiric % adulterated		
	PiLOT-95	CAT-80	N	PiLOT-95	CAT-80
≥ 2 CFU/g	1.00	0.99 – 1.00	16	100.0	100.0
1.5 to 1.9 CFU/g	0.99 – 1.00	0.95 – 0.98	8	100.0	100.0
1.0 to 1.4 CFU/g	0.95 – 0.99	0.81 – 0.93	16	100.0	100.0
0.5 to 0.9 CFU/g	0.78 – 0.93	0.40 – 0.75	12	91.7	66.7
≤ 0.4 CFU/g	0.00 – 0.70	0.00 – 0.28	28	32.1	3.6

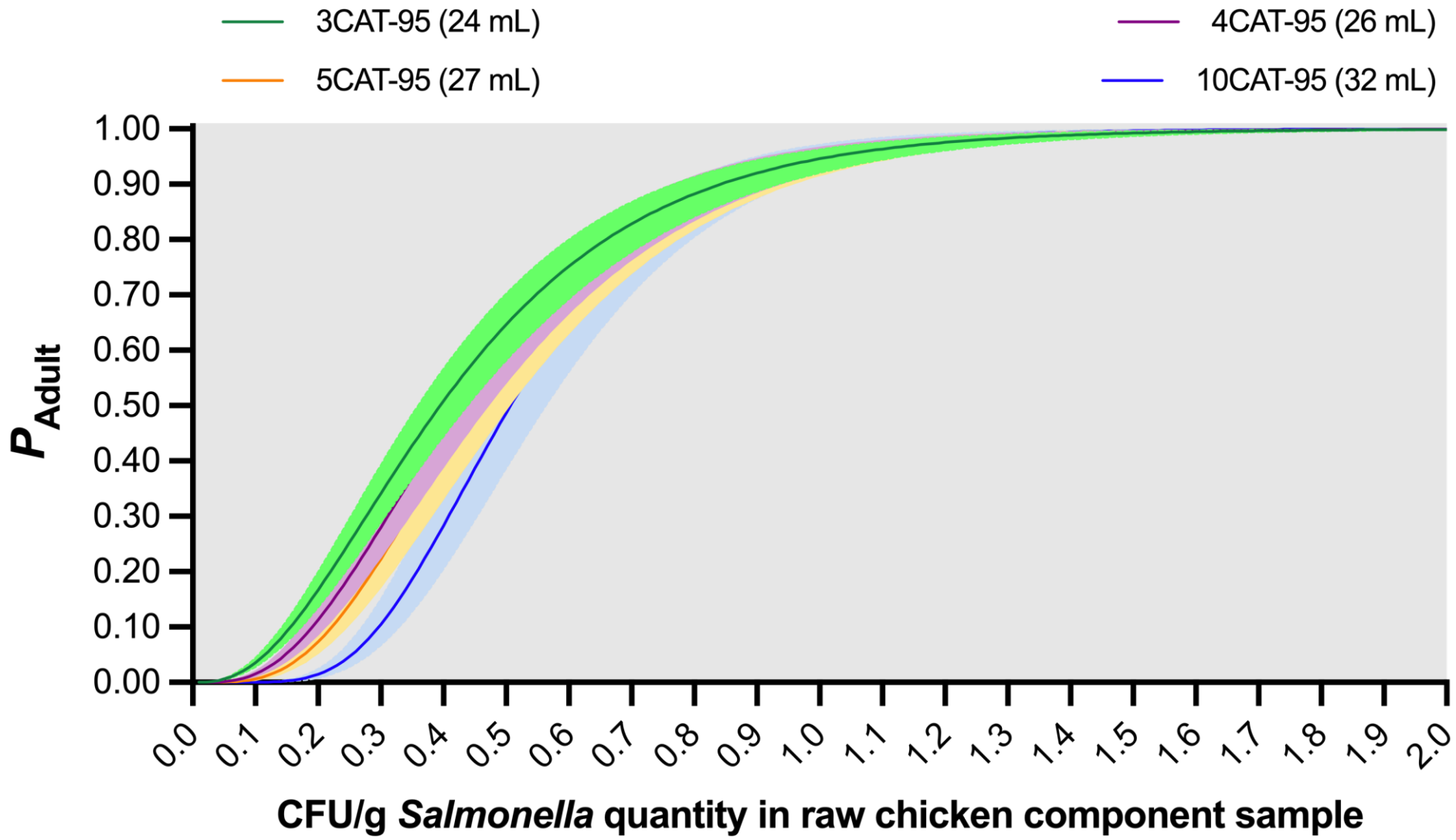
Schmidt, *et al*. **Identification of chicken component samples containing *Salmonella* concentrations greater than or equal to 1 CFU/g.** Submitted February 3

1 mL Threshold 3CAT Options (3 Tube)

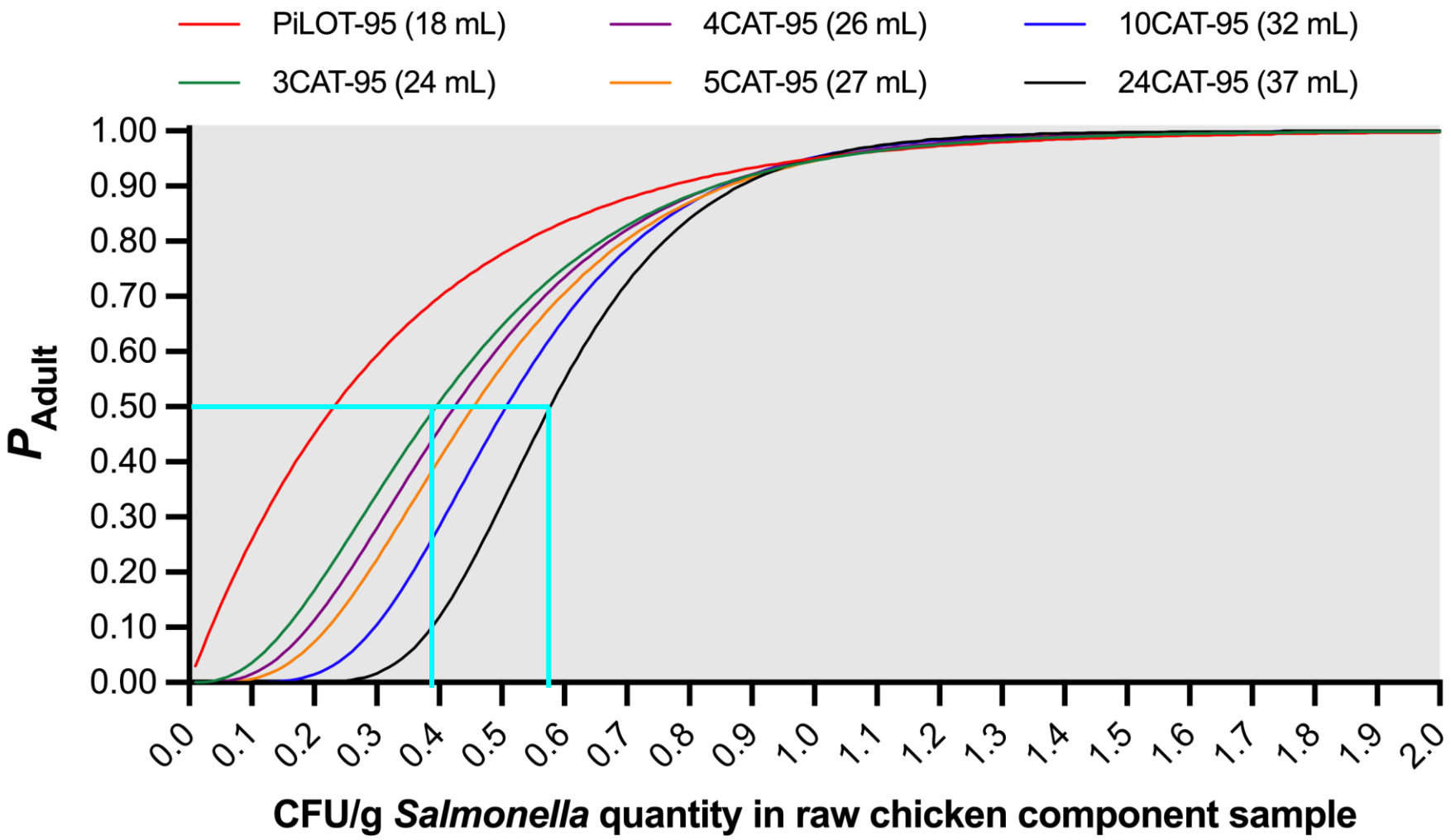


Schmidt, *et al*. **Identification of chicken component samples containing *Salmonella* concentrations greater than or equal to 1 CFU/g.** Submitted February 3

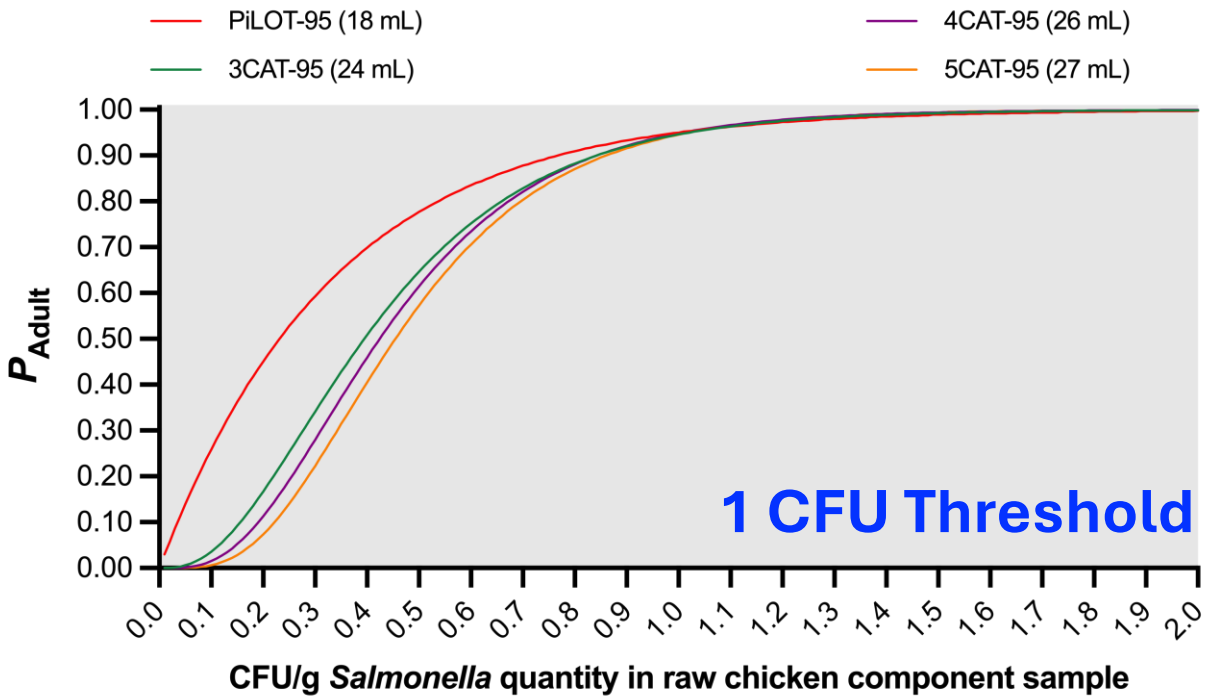
xCAT-95 Options

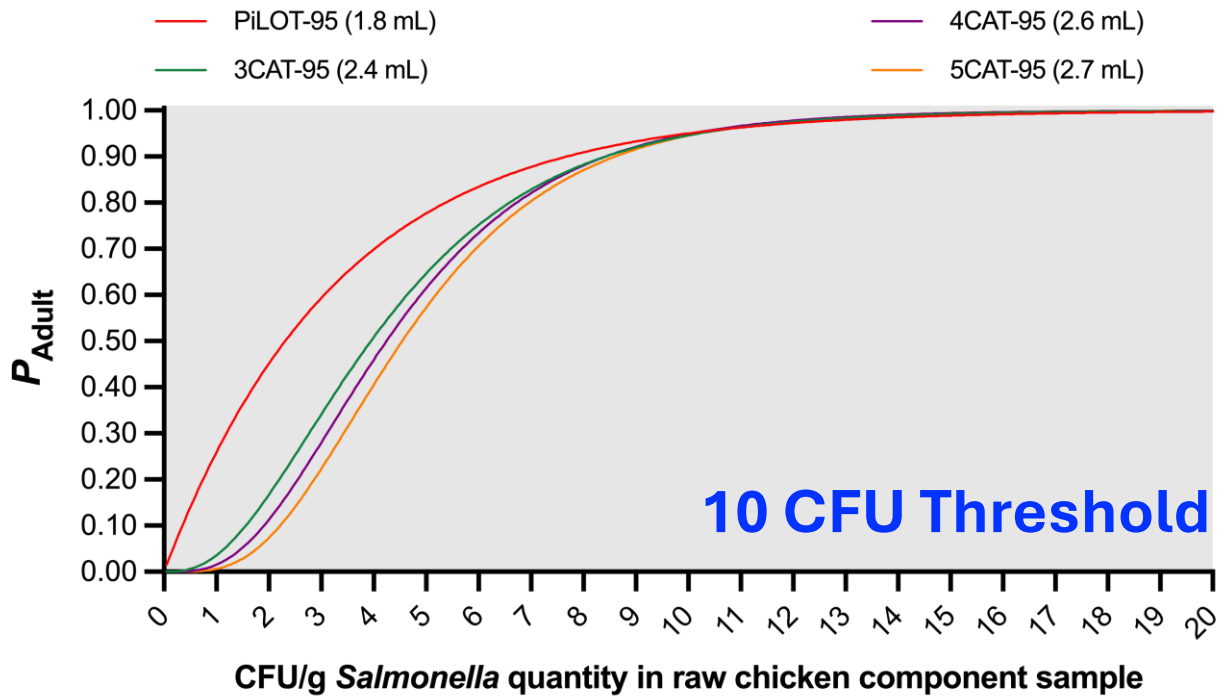


xCAT-95 (Diminishing Marginal Utility of CATs)

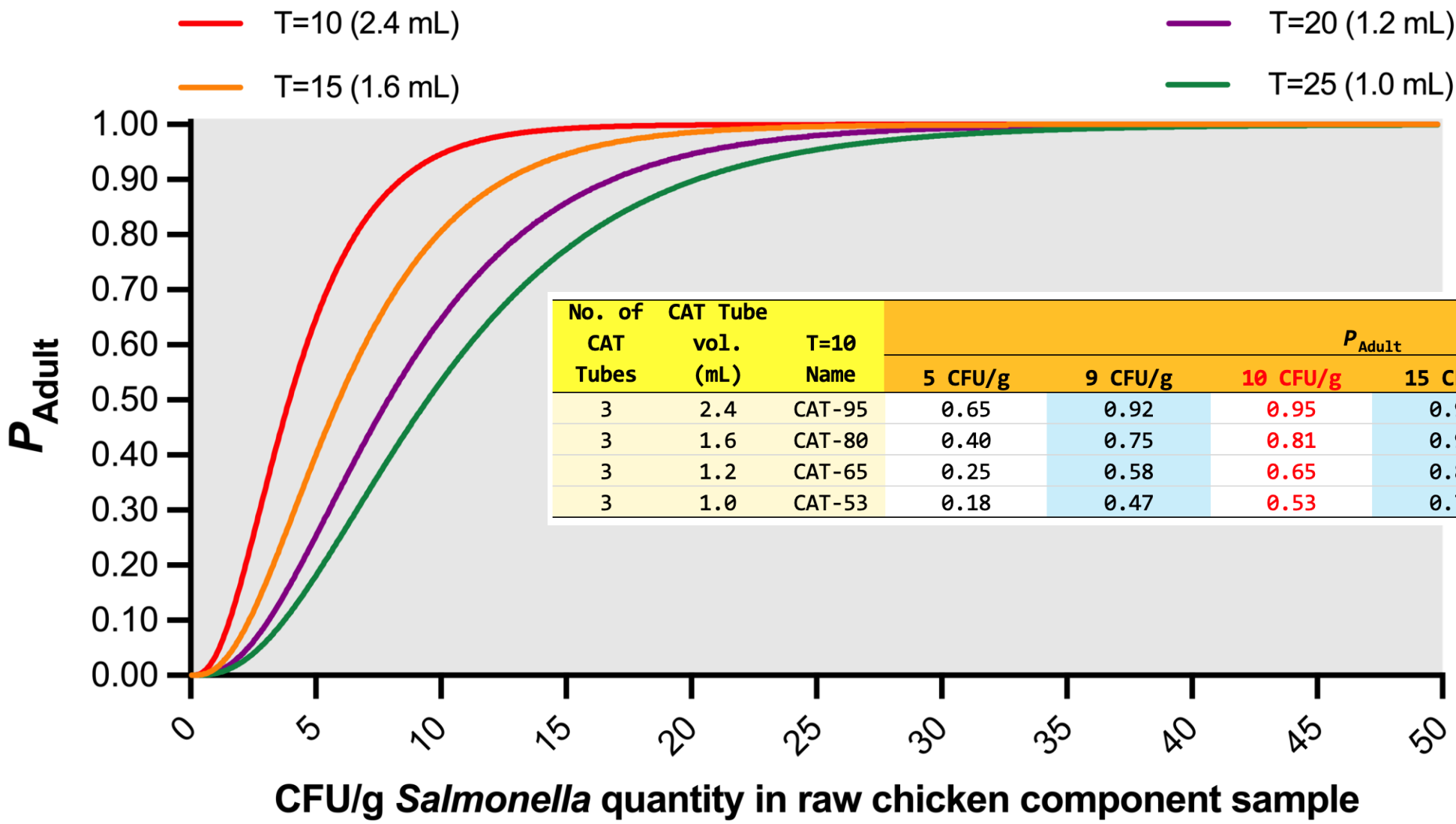


xCAT-95 Threshold 1 and 10 CFU/g



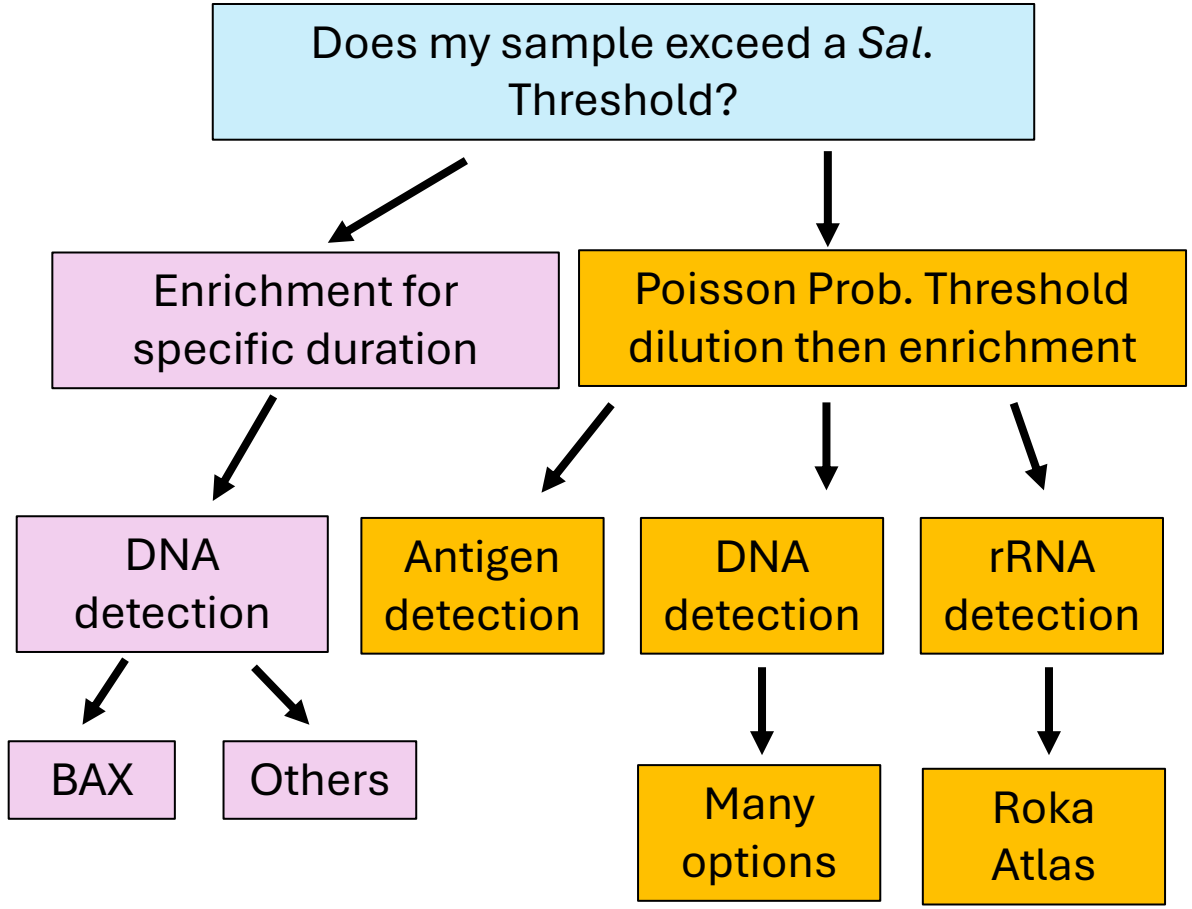


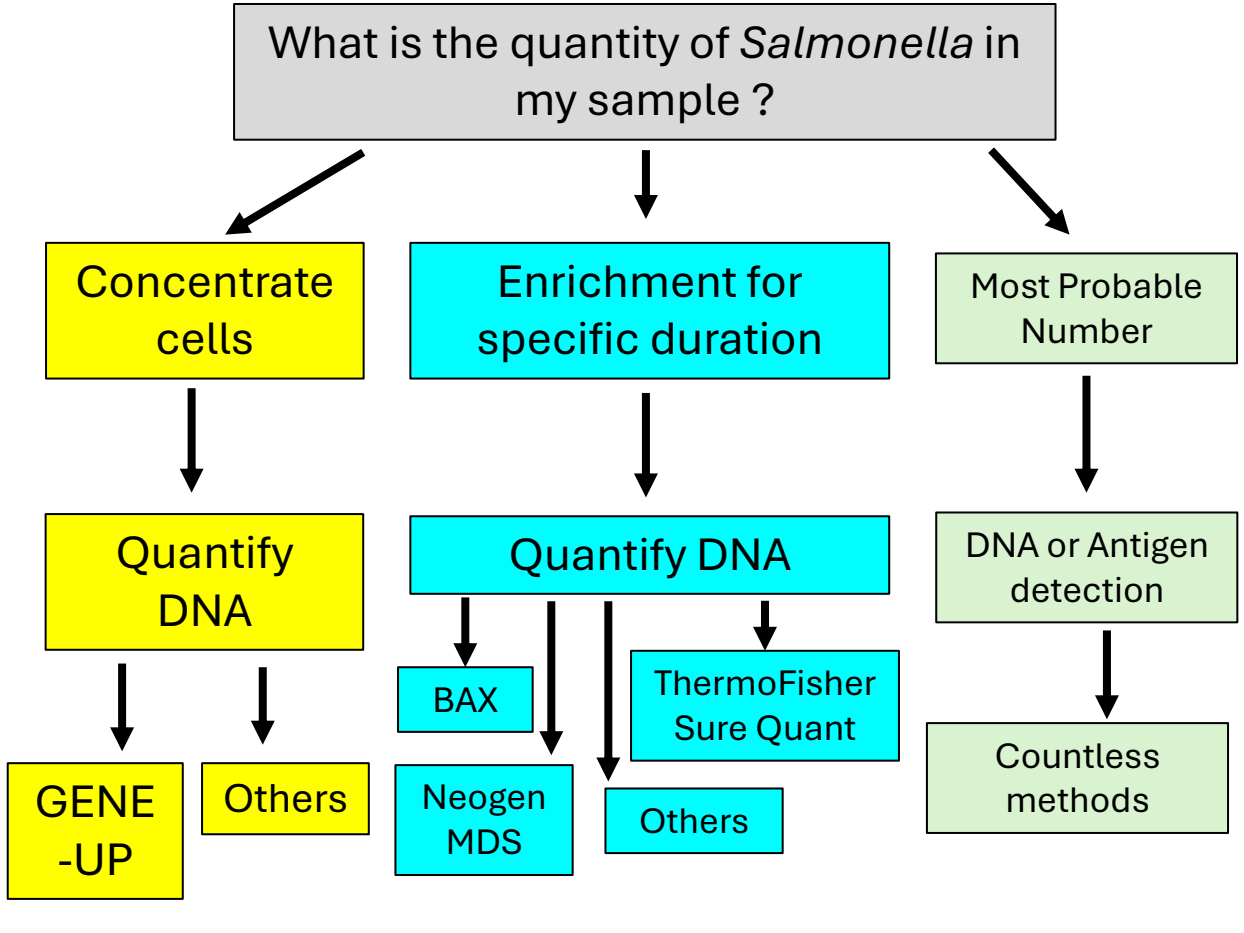
3 Tube CATs



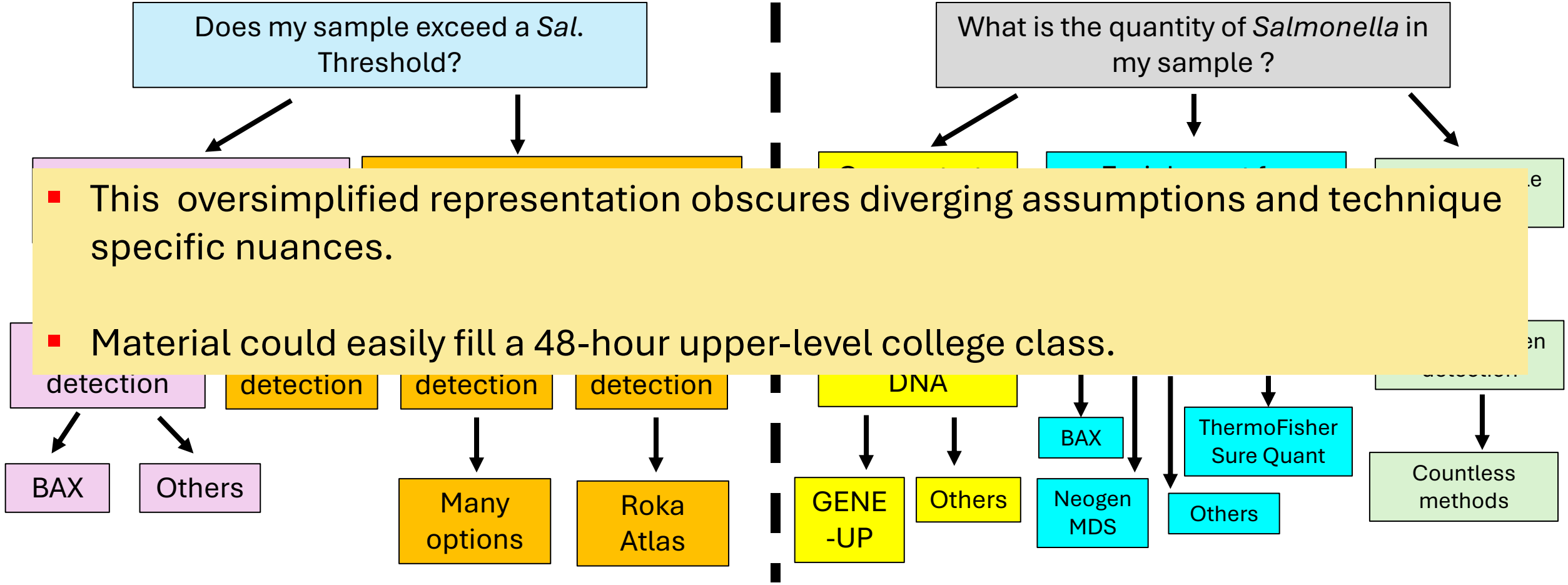
No. of CAT Tubes	CAT Tube vol. (mL)	T=10 Name	P_{Adult} 5 CFU/g	9 CFU/g	10 CFU/g	15 CFU/g	20 CFU/g	25 CFU/g
3	2.4	CAT-95	0.65	0.92	0.95	0.99	1.00	1.00
3	1.6	CAT-80	0.40	0.75	0.81	0.95	0.99	1.00
3	1.2	CAT-65	0.25	0.58	0.65	0.86	0.95	0.98
3	1.0	CAT-53	0.18	0.47	0.53	0.77	0.90	0.95

So, You Want to Want to Know Something About the Quantity of *Salmonella*





So, You Want to Want to Know Something About the Quantity of *Salmonella*



- This oversimplified representation obscures diverging assumptions and technique specific nuances.
- Material could easily fill a 48-hour upper-level college class.

Method Summary

- **Estimated Quantity: Enrichment → qPCR or LAMP**
  - Hygiena BAX Quant (qPCR), ThermoFisher SureCount (qPCR), Neogen MDA2 Quantitative (LAMP).
  - Likely 8 to 14 hours to answer.
  - Issues with previous environment impact on lag/recovery time, strain impact on doubling time, and background flora impact on growth, and quality of genomic DNA preparation.
  - Must report 95% CI for each result.

- **Estimated Quantity: Concentration → qPCR**
  - BioMerieux GENE-UP Quant (Centrifugation → qPCR), Pathotrak (Filtration → qPCR).
  - Likely 3 to 7 hours to answer. Methods more complex; possible processability issues.
  - Issues with sample type, sample preparation, target DNA recovery efficiency.
  - Must report 95% CI for each result.

- **Estimated Quantity: MPN (Multiple dilution levels + Enrichment + *Salmonella* Detection**
  - Too long, too labor intensive.
  - Must report 95% CI for each result.

Method Summary II

- **Binomial Outcome: Probability Based Dilution → Enrichment → *Salmonella* Detection**
  - PiLOT / CAT.
  - Likely 7 to 14 hours to answer.
  - Detection method agonistic (but sensitivities and time to result will vary).
  - Roka Atlas *Salmonella* rRNA detection could shorten to 4 to 6 hours to answer.
  - Must report probability of correct result.

- **Binomial Outcome: Short Enrichment → *Salmonella* Detection:**
  - Hygiena BAX Limit of Detection (eg. LOD10) .
  - Likely 7 to 11 hours to answer.
  - Issues with previous environment impact on lag/recovery time, strain impact on doubling time, and background flora impact on growth, and quality of genomic DNA preparation.
  - Must report probability of correct result.

- **Other methods in development – Pathotrak, Binomial Outcome :**
  - Must report probability of correct result.

- **Other methods in development – digital PCR (dPCR):**
  - Must 95% CIs or probability of correct result.

Conclusions: Life (Biology) is Probability

- AOAC is reconsidering their validation standards.
  - Threshold (PiLOT & CAT) & LOD methods = AOAC "qualitative threshold."

- Future validation protocols should consider the strengths, weaknesses, and assumptions for each step.

- Validations should demonstrate robustness across:
  - *Salmonella* strain
  - Microbial background
  - Prior environmental exposure of sample
  - Sampling buffer
  - Sample condition (*eg*. frozen or fresh)

- **Focus on routine Proficiency Testing (PTs) & repeatability**.
  - Trust your technicians.
  - Ask tech. reps. to perform the PTs that are relevant to your situation.

Future Plans

- Different inoculum stresses:
  - Unstressed: Grown at 37 °C in rich media (*eg.* BHI, TSB) overnight prior to inoculation.
  - Cold stressed: Grown at 35 °C in BPW to specific OD_{600} then held in BPW 4 °C overnight prior to inoculation.
  - Cold + nutrient stressed: Grown at 35 °C in BPW to specific OD_{600} then held in PBS at 4 °C overnight prior to inoculation.

Future Plans

Different inoculation methods

- Inoculate the suspended sample:
  - Advantage: Plate counts of pure inoculum on PetriFilm AC or TSA determine the "inoculation reference level."
  - Disadvantage: Is the inoculation preparation the best representation of actual test conditions?

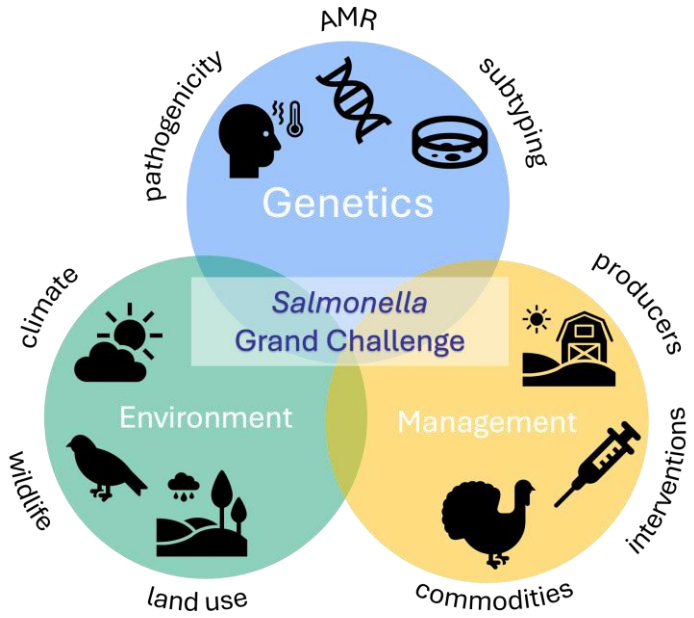
- Inoculate the sample then hold at 4 °C to simulate shipping/holding in lab prior to testing:
  - Advantage: *Likely the best representation of actual test conditions*.
  - Disadvantages:
    - Must perform a quantification of the inoculated sample to "inoculation reference level."
    - Likely MPN. Expensive, time consuming, and will have a broader 95% CI and PetriFilmAC/TSA counts.
    - OK for "equivalency testing", but we need standards.

Closing Thoughts

- More rigorous **Initial** and **On-going Validation** standards.
- Independent experts needed to inform these standards. Will likely need empirical data to support the standards.
- Need to define correct linear regression “fitting”.
- There are enough issues for a full semester upper-level college course.
- More than one person or more than many people working for one week needed to resolve.

USDA-ARS *Salmonella* Grand Challenge

- Bring all ARS *Salmonella* research together for cross talk and to break down silos.
- VISION: Support stakeholders to implement affordable, effective, data-driven strategies to address *Salmonella* food safety goals.
- Aspire to produce decision support tools that incorporate pre-harvest *Salmonella* surveillance, management data, and environmental factors.



Thank You

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Final Thought & Questions

- Go back to the question asked and determine if the limitations of the method employed will allow robust answers.

Bold Bluff or Judge St. Benard Stands Pat on Nothing (1903), Cassius Coolidge

A Waterloo or Judge St. Benard Wins on a Bluff (1903), Cassius Coolidge

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