| | FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM<br>*Standard Operating Procedure* | | |
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| Title: Salmonella die-off on dry food matrices | | | |
| SOP #: see Wiki | Revision: 00 | Revision Date: | Effective Date: 11/02/16 |
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# ***Salmonella*** **die-off on dry food matrices**

## FILE NAME: Salmonella_Die-off_SOP.doc

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# SECTION 1 INTRODUCTION

## 1.1 Purpose

The purpose of this document is to set forth standard guidelines performing an assay for determination of *Salmonella* die-off rate on dry food matrices (e.g., dry per food, dark chocolate) after 14-day incubation at room temperature.

## 1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

The protocol outlined in this document was used in evaluation of methods for detection of *Salmonella* in dry pet food and dark chocolate in 2015-2016 and is recommended for use as a standard for determination of *Salmonella* die-off rates in dry food matrices (e.g., dry pet food, dark chocolate).

## 1.3 Definitions

**DPF:** Dry Pet Food.
**BPW:** Buffered Peptone Water.
**Kibble:** One piece of dry pet food (Pedigree in 2015-2016 experiments) of approximately 1g in size.
**BHI:** Brain Heart Infusion broth.
**BHI agar**: Brain Heart Infusion agar.
**XLD agar**: Xylose Lysin Deoxycholate agar.
**Die-off rate:** log reduction in *Salmonella* CFU, which equals [$\log_{10}$(CFU/g $_{\text{Day 0}}$) – $\log_{10}$ (CFU/g $_{\text{Day 14}}$)].

## 1.4 Safety

*Salmonella enterica* is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *S. enterica*. All waste from these experiments needs to be treated as BSL-2 waste.

# SECTION 2 MATERIALS

- ***Salmonella enterica* cultures**. Location of organisms in freezers is available online in Food Microbe Tracker database.

- **PBS.** Lab stock in media room pH 7.4.

- **BHI agar.** Lab stock in cold room pH 7.4.

- **BPW.** BPW medium must be prepared by following manufacturer's instruction (BD, REF #: 218105; pH 7.2±0.2).

- **NFDM.** NFDM medium must be prepared by following manufacturer's instructions (BEST YET, REF #: N/A).

- **XLD agar.** NFDM medium must be prepared by following manufacturer's instructions (BD, REF #: 278850; pH 7.4±0.2).

IMPORTANT: Record the lot # of each batch of medium used in the experiments in the lab book.

# SECTION 3 PROCEDURES

**The protocol here describes the procedure for determining the *Salmonella* die-off rate on DPF using BPW medium. The procedure on dark chocolate is as described for DPF, with exception of NFDM medium as a replacement for BPW. Parts of the protocol that differ from the DPF protocol will be marked with **(and followed by the details specific to dark chocolate and NFDM medium listed in the parentheses)*.**

## 3.1. Thawing glycerol stocks

(1) Look up the -80°C freezer location of bacterial isolate in online Food Microbe Tracker Streak database, and carry isolate glycerol stocks from freezer to the working bench in a container on ice.
(2) Thaw glycerol stocks only as much as needed to scrape one 10 µl loop of frozen stock, and aseptically streak it for isolation on labeled BHI agar plates and incubate at 37°C overnight (18 – 24 h).
(3) Immediately after streaking, return the glycerol stocks back into -80°C freezer.

## 3.2. Inoculum preparation

(1) Sub-streak (continuously to grow a lawn) 3 individual colonies for three biological replicates on BHI agar and incubate at 37°C for 24 h (culture needs to be in a stationary phase in order to be able to survive unfavorable conditions better).
(2) Pre-warm the BPW ****(NFDM instead of BPW when test is performed on dark chocolate)*** to 35°C prior to use.
(3) Collect with a swab of the culture grown on BHI plate, and re-suspend it in 5 ml of BPW ****(NFDM instead of BPW when test is performed on dark chocolate)***. This should yield ~$10^9$ to $10^{10}$ CFU/ml. Vortex the suspension at speed 7 (Fisher Vortex, Genie 2) for 1 min.
(4) Prepare 2 10-fold dilutions (1 in 9 ml) of a cell suspension and measure and record the $OD_{600}$ value. Read only OD values between 0.2 – 0.4, which are in the linear part of the OD-CFU calibration curve. An OD-CFU calibration curve established for *Salmonella* Typhimurium is used for **estimating** the concentration for all *Salmonella* serotypes and strains. The OD-CFU curve is available in the Appendix 1 of this document.
(5) Prepare inoculum in concentration $10^9$ CFU/ml based on the OD-CFU curve.

## 3.3. Inoculum quantification

(1) Prepare ~1g food samples. DPF kibbles (Pedigree) are already of approximately that size. Dark chocolate must be cut in ~1g pieces (see Troubleshooting section if considering alternative ways of preparing dark chocolate samples, such as melting and aliquoting).

(2) Confirm the inoculum concentration by spiral-plating dilutions $10^{-5}$ and $10^{-6}$ on BHI agar and incubate overnight at 37°. This count is the concentration at $T_{0.}$

### 3.4. Inoculation of dry pet food

(1) Surface inoculate each DPF kibble with three 1µl of prepared culture per kibble, and incubate at 25°C +/- 1°C:

Samples per isolate:

- Biological replicate 1:
  - Technical replicates (2x $T_{24h}$, 2x $T_{7D}$, 2x$T_{14D}$) = 6 kibbles
- Biological replicate 2:
  - Technical replicates (2x $T_{24h}$, 2x $T_{7D}$, 2x$T_{14D}$) = 6 kibbles
- Biological replicate 3:
  - Technical replicates (2x $T_{24h}$, 2x $T_{7D}$, 2x$T_{14D}$) = 6 kibbles

### 3.5. Quantification of *Salmonella* on dry pet food

(1) Sample at time 1 h, 7 days (7D) and 14 days (14D):

- Transfer a single contaminated DPF kibble to 2-oz WhirlPak bag containing 9 ml of sterile and warm BPW (pre-warmed to 35°C), in triplicate ***(use NFDM instead of BPW when testing dark chocolate)***;
- Incubate samples in BPW ***(NFDM when testing dark chocolate)*** at room temperature for 1 h to soften;
- Homogenize samples by stomaching at 230 rpm for 1 min ***(add 18 ul brilliant green to NFDM after homogenization when testing dark chocolate);***
- Measure pH with a pH strip, adjust pH by adding 70-75 µl of 1N NaOH and confirm the target pH of 6.8 (pH paper indicates a range, i.e., 6.5-7.0) by measuring it again;
- Prepare serial ten-fold dilutions (1 in 9 ml) to $10^{-3}$ in BPW (35°C);
- Spiral plate 50 µl of dilutions on XLD and incubate 18 – 24 h at 35°:
  i. $10^{-2}$ and $10^{-3}$ for 1h time point;
  ii. $10^{-1}$ and $10^{-2}$ for 7 days time point;
  iii. $10^{-1}$ and original for 14 days time point.

(2) Count the XLD plates after completed incubation, using Q-count.
(3) Determine the concentration in each sample. **Keep in mind that the food sample in 9 ml of the medium is already a $10^{-1}$ dilution.**
(4) Calculate and record the average CFU/ml of each pair of technical replicates and use these averages to calculate the average of each triplicate of biological replicates.
(5) Calculate and record the standard deviation for each biological triplicate.

### 3.6. Calculation of the die-off rate

(1) Determine the die-off after 14-day incubation by comparing the difference between concentration of cells recovered at 1h (Day 0) and 14 days (Day 14):

$$\text{log reduction} = \log_{10}(\text{CFU/g}_{\text{Day 0}}) - \log_{10}(\text{CFU/g}_{\text{Day 14}})$$

# SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include those described below.

**There are no typical *Salmonella* colonies on XLD agar**

(1) Incubate XLD plates long enough (20 h or longer) in order to observe typical black colored *Salmonella* colonies due to accumulation of hydrogen sulfide.

(2) Confirm that there are no competing microorganisms that are able to grow in *Salmonella* selective media in uninoculated food samples. In case there are natural competing contaminants present in the food samples, the chances of detecting *Salmonella* will decrease. Furthermore, contaminant may completely outcompete *Salmonella*, therefore causing false negative results in downstream assays, such as BAM isolation and BAM MPN. Experience from experiments carried out in 2015-2015, in which contaminant from genus *Cronobacter* was confirmed based on 16S rRNA sequence, demonstrated that presence of competing natural contaminant may result in invalid *Salmonella* MPN assay.

**The die-off rates are unexpectedly high**

(1) Use pre-warmed to 35°, as cold media introduces additional stress that may negatively influence successful recovery and isolation of *Salmonella* after 14-day stabilization on dry food matrix.

(2) Use stationary phase culture for preparing the inoculum, as it is more stress-resistant and will likely survive better in unfavorable conditions (e.g., low aw).

(3) In case of dark chocolate the ~1g sample preparation may play a significant role in success of *Salmonella* recovery. Unstandardized protocols for chocolate melting and aliquoting (not described in this SOP) can result in uneven chocolate fat separation, which may influence *Salmonella* survival on chocolate when surface inoculated. If considering melting and aliquoting chocolate instead of cutting it, keep in mind that heating chocolate above 30-32°C will disrupt tempered chocolate structure and cause fat separation. It is therefore recommended to melt chocolate in water bath at ~30°C and homogenize it well by hand massaging it in a Whirl Pak bag prior aliqoting. It is recommended to double-bag the sample to avoid cross-contamination in water bath. The outer bag must be discarded before homogenization and further sample handling.

## SECTION 5 REFERENCES

This protocol was established based on the following references:

*Example:*

*Ryan et al. (2015). Evaluation of Rapid molecular detection assays for Salmonella in challenging food matrices at low inoculation levels and using difficult-to-detect strains. J Food Prot, 78: 1632-1641.*

*FDA. BAM: Salmonella.*
*http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm*

## APPENDIX 1

### OD-CFU calibration curve for *Salmonella* Typhimurium

| OD 600nm | Ave CFU/ml |
|---|---|
| 0.802 | 2.09E+08 |
| 0.459 | 1.20E+08 |
| 0.283 | 5.52E+07 |
| 0.156 | 4.50E+07 |
| 0.057 | 1.74E+07 |