Rapid Detection of *Salmonella* in Pet Food: Design and Evaluation of Reduced Time-to-Result workflow Using Real-Time PCR

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INTRODUCTION

According to the 2009/2010 National Pet Owners Survey, 62% of U.S. households own a pet, and the estimated sales within the U.S. market for pet food alone is $17.4 billion. Consequently, rapid and robust testing of pet food for food borne pathogens is gaining importance. Food samples create unique challenges, particularly with respect to sample preparation for detection by PCR. This is especially true for high carbohydrate containing matrices, such as pet food. To address these needs, we have designed and evaluated a complete and streamlined workflow to detect *Salmonella* in pet food that includes:

- **Short Pre-enrichment :** Pre-enrichment is conducted under shaking conditions to enhance rapid growth of *Salmonella*.

- **Novel, custom-designed sample preparation:** A novel pre-clarification tray was designed for addressing difficult sample matrices like pet food, which when combined with automated sample preparation, enables high-throughput processing.

- **Lyophilized Real-Time PCR assay:** Highly sensitive and specific real-time PCR assay allows for addition of more sample volume, which creates the possibility of testing pooled samples.

- **Custom Designed Software :** Simplifies data analyses by providing plus/minus results, and differentiating viable *Salmonella* from background.

- **Shortened Time-To-Result:** The streamlined sample-to-answer workflow offers a great advantage over existing culture confirmation methods by reducing the time-to-result to under 2h following pre-enrichment. Traditional culture confirmation typically takes an additional 2-4 days.

MATRICES TESTED

For method comparison, a total of 45 samples for each of the four *Salmonella* strains were analyzed. Each sample set included 5 uninoculated samples, 20 low-level and 20 high-level samples. Target levels of each strain were as follows: 0.2-2 colony forming units (cfu) /25 g for the low level inoculation, 2-10 cfu/25g for the high level inoculation and 0 cfu/25 g for the uninoculated control samples. Each pet kibble was inoculated with a different strain of *Salmonella*. A 24 hour broth culture inoculum was utilized to inoculate a bulk sample of ground pet food and kept for 2 weeks at room temperature (25°C) as per AOAC guidelines. For enumeration a 3-tube MPN analysis of each inoculated food was used to determine the two target inoculum levels. On the day of initiation of analysis, samples of 100 g, 10 g, 1 g and 0.1 g from each inoculum level were prepared for each matrix, enriched with the appropriate medium and confirmed according to the FDA BAM reference method procedures.

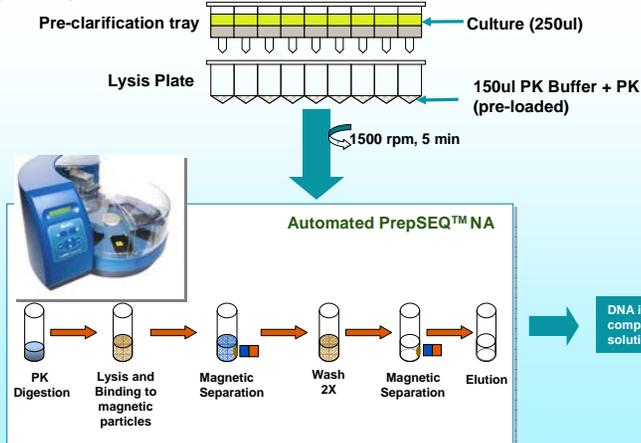| Sample Identification	Inoculating Organism	Sample Identification	Inoculating Organism
1-45	*S. enterica* ser. *Typhimurium* ATCC 14028	101-145	*S. enterica* ser. *Senftenberg* ATCC 43845
201-245	*Salmonella enterica* subspecies Tennessee	301-345	*Salmonella enterica* subspecies Othmarschen

INTEGRATED SYSTEM FOR STREAMLINED SAMPLE-TO-RESULT WORKFLOW

Pre-Enrichment

- 37°C
- 225 rpm
- 12 hours

25g sample
+
225 ml Brain Heart Infusion Broth

Sample Preparation

Pre-clarification tray ← Culture (250ul)

Lysis Plate ← 150ul PK Buffer + PK (pre-loaded)

1500 rpm, 5 min

Automated PrepSEQ™ NA

PK Digestion → Lysis and Binding to magnetic particles → Magnetic Separation → Wash 2X → Magnetic Separation → Elution → DNA in PCR compatible solution

Real-time PCR and Data Analyses

Extracted Nucleic Acids

MicroSEQ® *Salmonella* spp. → 7500 Fast Real-Time PCR → RapidFinder™ Express Software

Key:
- Positive Result
- Negative Result
- Result Warning
- Positive Control
- Negative Control
- Control Warning

Integrated System Performance

Figure 1: Preclarification Improves Signal Intensity by Reducing PCR Inhibitor Load

Lysis Plate
Pre-clarification tray

Preclarification tray
- Filters out PCR inhibitory matrix material.
- Improves assay sensitivity
- Eluate from clarified sample is easier to load into PCR reaction.

Elution Plate
Standard
Pre-clarified

Pre-clarification versus Standard Protocol

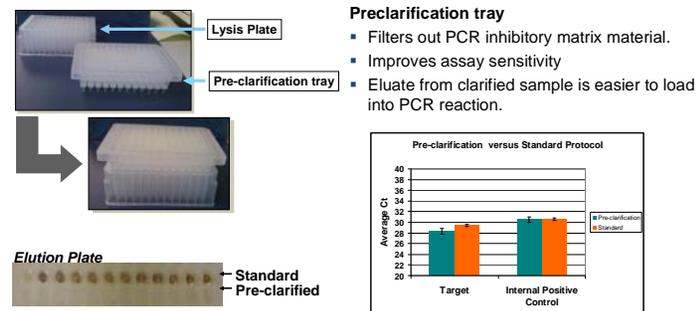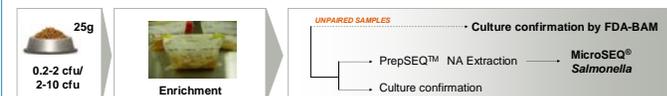**Figure 2: Validation of Complete Workflow Demonstrated Equivalency between the MicroSEQ® *Salmonella* spp. Method and Reference Method**

25g
0.2-2 cfu/
2-10 cfu
Enrichment

UNPAIRED SAMPLES
- Culture confirmation by FDA-BAM
- PrepSEQ™ NA Extraction → MicroSEQ® *Salmonella*
- Culture confirmation

Inoculation Level	MPN/25g	MicroSEQ Presumptive +	MicroSEQ Confirmed +	FDA-BAM Confirmed +	χ2* (MicroSEQ vs. FDA-BAM)
S. enterica ser. Typhimurium ATCC 14028					
Control	0	0/5	0/5	0/5	-
Low	0.1	0/20	0/20	0/20	-
High	2.3	6/20	5/20	6/20	0.12
S. enterica ser. Senftenberg ATCC 43845					
Control	0	0/5	0/5	0/5	-
Low	0.1	4/20	3/20	3/20	-
High	0.4	12/20	13/20	14/20	0.43
S. enterica ser. Tennessee					
Control	0	0/5	0/5	0/5	-
Low	2.3	2/20	2/20	6/20	2.44
High	11.5	18/20	18/20	19/20	0.35
S. enterica ser. Othmarchen					
Control	0	0/5	0/5	0/5	-
Low	0.2	6/20	6/20	7/20	0.11
High	2.3	16/20	16/20	18/20	0.76

* Values > 3.84 are significant at 5%

Conclusions

- Inclusion of pre-clarification during sample preparation addressed problems due to matrix carryover, including PCR inhibition.
- The entire workflow can be completed in a shortened time frame.
- Inclusion of software analyses enabled differentiation of live *Salmonella* from background.
- Validation studies demonstrated equivalency between the presented method and the reference FDA-BAM protocol.