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Validation of low volume enrichment for rapid E. coli O157 screening tests

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Executive summary

Testing of beef destined for use in ground beef products for the presence of *Escherichia coli* O157:H7 has become an important cornerstone of control and verification activities within many meat supply chains. Validation of the ability of methods to detect low levels of *E. coli* O157:H7 is critical to confidence in test systems. Many rapid methods have been validated against standard cultural methods for 25g samples. In this study, a number of previously validated enrichment broths and commercially available test kits were validated for the detection of low numbers of *E. coli* O157:H7 in 375g samples raw ground beef component matrices using 1 liter of enrichment broth (large sample:low volume enrichment protocol). Standard AOAC methods for 25g samples in 225ml of enrichment broth using the same media, incubation conditions and test kits were used as reference methods. No significant differences were detected in the ability of any of the tests to detect low levels of *E. coli* O157:H7 in samples of raw ground beef components when enriched according to standard or large sample:low volume enrichment protocols. This report documents the results of the study according to National Association of Testing Authorities (NATA), Australia validation criteria.

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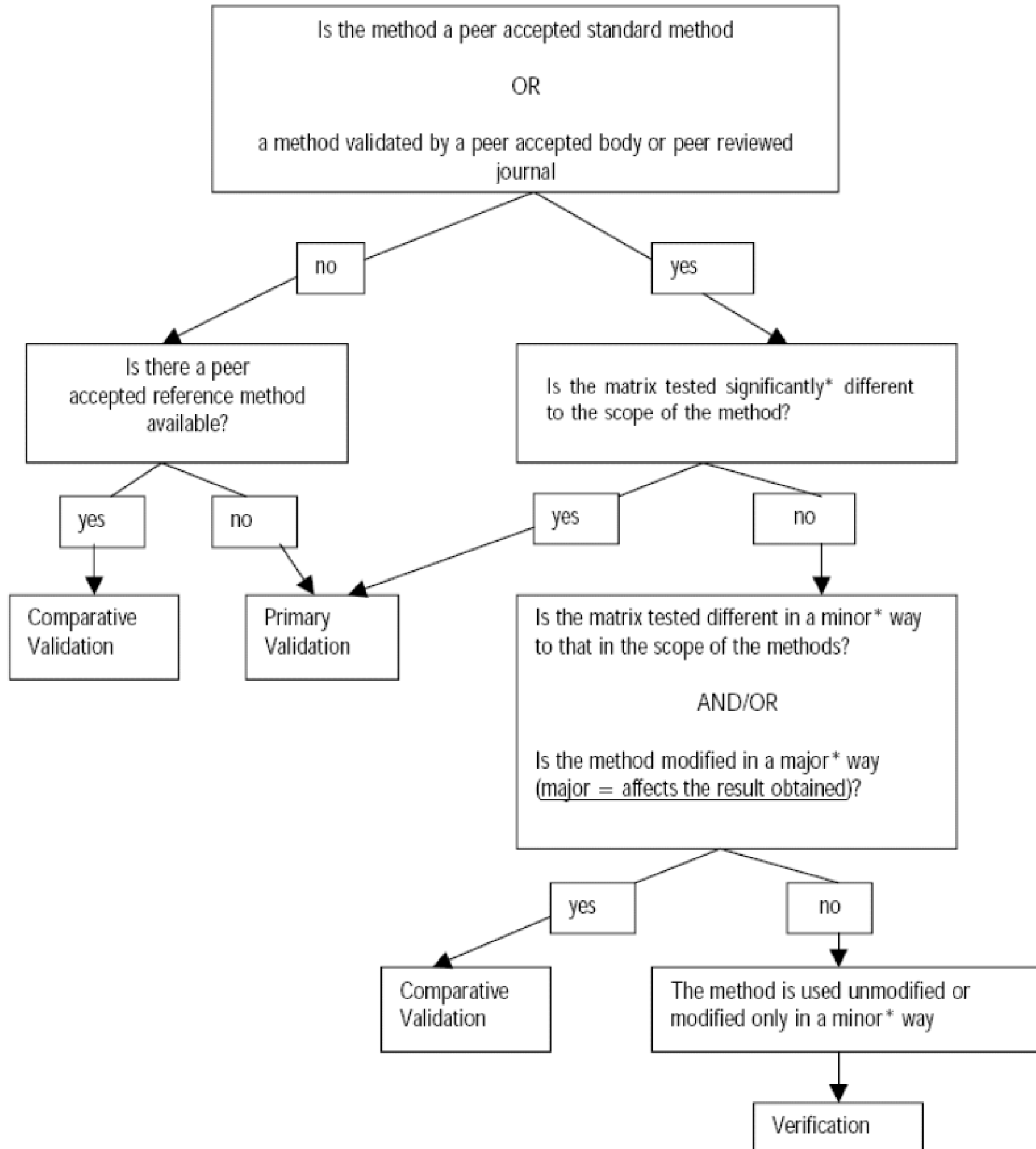
1 Consideration of the method validation decision tree

The National Association of Testing Authorities (NATA) has published[1] a decision tree that determines the requirements for the validation of non-standard methods for which a laboratory seeks accreditation. A number of questions need to be answered.



[1] National Association of Testing Authorities (2007) ISO/IEC 17025 Application Document. Supplementary requirements for accreditation in the field of biological testing.

1.1 Is the method a peer accepted standard method or a method validated by a peeraccepted body of peer-reviewed journal?

The study aims to verify a method modification to the standard methods AOAC Official Method 969.09 VIP Assay, AOAC Official method 996.10 Assurance Assay and AOAC RI performance tested method 050501, BAX MP.
The modification involves replacing 25g sample in 225ml enrichment broth with 375g in 1 Litre enrichment broth. This modification of enrichment ratio to sample has been recommended previously for raw beef based on comparative data generated in peer reviewed articles.[2], [3]

1.2 Is the matrix significantly different from the scope of the method?

Raw beef is included in the scope of all methods and in publications on low volume enrichment.

1.3 Is the matrix tested different in a minor way to the scope of the methods?

Beef for grinding would be expected to have a lower microbial count (less competitors) than raw ground beef.

1.4 Is the method unmodified or modified in a minor way?

The change in the sample: enrichment ratio has been validated in peer review papers. The main aim of the study was to ensure that the modified enrichment procedure was equivalent to the original method for samples with a very low level of contamination (1 target cell/25g or 1 target cell/375g).

2 Use of AOAC International guidelines for validation

The AOAC guidelines were chosen for the following reasons:

The AOAC guidelines incorporate the concept of fractionally positive data. In the AOAC guidelines methods are evaluated at the limit of detection (50% endpoint) of the most sensitive method. If all 20 inoculated samples are positive data is rejected. In the AS 4659 guidelines the inoculum is at a level up to 10 times above the limit of detection. For a significant pathogen such as *E. coli* 0157, particularly in product destined for export to the US, we believe that the AOAC guideline is the method of choice.

The original validation studies were conducted according to AOAC guidelines.
The statistical analysis and acceptance criteria in AS4659 are not applicable to unpaired data. The comparison of 2 different sample sizes and enrichment ratios requires a study design that does not incorporate pairing of duplicate samples for each method. McNemar's test and the AS4659 performance parameter definitions are not valid for unpaired data.

The AS4659 performance parameter definitions are also not valid for unpaired data. AOAC has not usually calculated these parameters, but AOAC has recently addressed the problem of unpaired data and provided a recommended statistical approach for this type of analysis.

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[2] Guerini, M. N., T. M. Arthur, S. D. Shakelford, M. Koohmaraie. 2006. Evaluation of *Escherichia coli* O157:H7 Growth Media for Use in Test-and Hold Procedures for Ground Beef Processing. *J. Food Prot.* 69:1007-1011.
[3] Bosilevac, J. M., and M. Koohmaraie. 2008. Effects of Using Reduced Volumes of Nonselective Enrichment Medium in Methods for the Detection of *Escherichia coli* O157:H7 from raw beef. *J. Food Prot.* 71:1768-1773

3 Validation Report

3.1 Background

The study was in response to new requirement for testing larger sample sizes (375g instead of 25g) of raw beef components destined for grinding in the US. The commercial tests included in this study were the VIP, Assurance (AOAC Official Method 969.09 and 996.10) and BAX MP (AOAC Research Institute Performance Tested Method 050501). These assays have already undergone extensive validation against reference culture methods for standard 25g samples with sample: broth ratio of 1:9 and are acceptable to FSIS for testing product being exported from Australia.

When tests designed for 25g samples are scaled up to 375g then the volume of broth required is usually increased to 3375ml to maintain the 1:9 ratio. However, such large volumes of media are both expensive and inconvenient for routine laboratory use.

Recent studies[4] have shown that a reduction in the volume of enrichment broth giving ratio of approximately 1:3 sample to broth can be used providing valid results. The approach taken in this study[5] was based on AOAC microbiological guidelines.[6] These guidelines while not identical to AS/NZS 4659 guidelines are very similar in their overall approach to validation. However, they provide a more rigorous approach to the detection of low numbers of pathogenic organisms and are consistent with previous validation studies on these methods. In addition, AOAC provides guidelines for statistical analysis and performance criteria for studies where pairing of sample data is not feasible.

3.2 Validation Study Conditions

Target organism: *E. coli* 0157
Matrix: Raw beef components destined for grinding as represented by beef trim (higher fat) and lean cuts.
Alternate method: Modification of the AOAC approved method (AOAC Official Method 969.09 and 996.10) and BAX MP (AOAC Research Institute Performance Tested Method 050501). The modification is to replace 25g in 225 mL of enrichment broth with 375g in 1Litre.
Reference methods: AOAC Official Method 969.09 VIP and 996.10 Assurance and BAX MP (AOAC Research Institute Performance Tested Method 050501).

3.3 Laboratory and testing conditions

The study was conducted at Silliker Australia, Sydney Microbiology Laboratory, Unit C2, Regents Park Estate, 391 Park Rd, Regents Park NSW.
Laboratory is NATA accredited: Accreditation number 2020. The accreditation scope includes testing E.coli 0157 in meat and meat products for export.
As a NATA accredited laboratory appropriate controls on equipment, media quality and records, documentation, environmental monitoring, training etc were observed. Testing was conducted during period February to April 2008 and records are available on request. Batch numbers for kits:
Assurance lot 060807-09 exp.20 June 09, VIP lot no.103007-01 exp. 2 May 09. Bax kits were lot Q7298.

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[4] Guerini et al.2006 loc. cit., Bosilevac and Koohmaraie, 2008 loc. cit.
[5] Ahmed et al., Journal of Food Protection. In Press.
[6] available at www.aoac.org

Personnel: Testing was performed by the Principal Technical Officer, Imtiaz Ahmed, who has a B.Sc (Hons) Food Technology and M.Phil. (Microbiology) from Quaid-i.Azam University in Islamabad Pakistan and 4 years experience in microbiological testing at Silliker, Sydney.
Consultant: Denise Hughes, M.Sc University of Sydney, >20 years experience in food microbiology,8 years as Product Evaluation Manager and AOAC Study Director TECRA Diagnostics, AOAC Study Director of the Year, 1998. Currently runs consulting service, DH Micro Consulting.

3.4 Cultures

For the inoculated food study *E. coli* ACM 5101 (serotype 0157:H7 non toxigenic strain) was used.
This is equivalent to strain 85-170 from the Canadian Centre for Disease Control, Ottawa, Canada.
This strain was recommended by the Australian Collection of Micro-organisms as a non-toxigenic variant of a culture involved in a large Canadian food poisoning outbreak. Culture was supplied in January 08 by Assistant Curator (Jenny Spratley j.spratley@uq.edu.au.). Quality control certificate is available on request. In consideration of safety aspects when working with large volumes of inoculated food enrichments a non-toxin producer was chosen. The strain was unchanged in other aspects and was consistently detected by commercial test kits.
For the inclusivity study a variety of cultures isolated in Australia from meat industry sources, were provided by Narelle Feagan at Food Science Australia. Ten cultures of local origin were considered sufficient because the original AOAC approval studies included extensive data on inclusivity and exclusivity of the test methods.

Table 1: Sources and characteristics of strains used.

Strain ID	Year of isolation	Source	Geographical origin	Serotype	Shiga toxin genes
134	1996	Cattle feces	South East Queensland	O157:H7	2
581	1994	Beef carcass	South Australia	O157:H-	1 & 2
735	1997	Cattle feces	Far North Queensland	O157:H7	2
1668	1999	Beef carcass	Central Queensland	O157:H-	1 & 2
2308	2002	Cattle feces	South East Queensland	O157:H7	-
2340	2002	Cattle feces	South East Queensland	O157:H-	1
2459	2002	Cattle feces	Western Australia	O157:H7	2
2469	2002	Cattle feces	Victoria	O157:H7	1 & 2
2477	2002	Cattle feces	New South Wales	O157:H-	1 & 2
ACM 5101*		Human	Canada	O157:H7	-

* from Australian Collection of Microorganisms, this strain is a Shiga toxin-negative derivative (a spontaneous mutant) of Canadian CDC strain 84-289.

3.5 Matrix selection

The intention of the study was to verify the method modification for one matrix only – raw beef components for grinding. The variation within this matrix is adequately represented by selecting 2 components – beef trim (higher fat) and lean cuts. While 5 matrices are recommended in AS4659 and AOAC guidelines it is understood that this would apply if the approval was for a more general category of food eg. meat: ground beef, salami, sausage, pork, roast beef. According to AOAC guidelines: the number of different food categories depends on applicability of the method. If the method is specific to only one category (eg. enumeration of Listeria in fresh unpasteurised cheese) only one food type needs to be included.

3.6 Preparation of the matrix

For the 20 low inoculum samples, cultures were grown in TSB for 18-24h at 35-37°C. A spread plate count was then performed using 0.1% peptone diluent. Five replicate 0.1mL volumes were plated onto TSA plates incubated at 35-37°C for 24h and dilutions retained at 4°C for 24h. The appropriate aliquot (less than one ml of the diluted culture) was added drop wise to the surface of the thawed pre-weighed sample in the stomacher bag. The inoculum was constant (approximately 1 cell/sample) for both 25g and 375g samples. After inoculation using the appropriate dilution of TSB culture, samples were stored for approximately 36h at 4°C to adapt the inoculum to the temperature of product, as would occur if the product were naturally contaminated. Uninoculated samples and samples for enumerating the inoculum level by the MPN technique were prepared in a similar manner.

For methods with AOAC approval, which, have undergone validation to show equivalence against standard cultural reference methods for detection of pathogens a limit of detection of approximately 1 cell/25g food is normally claimed. This study intended to show there was no loss of sensitivity with the new sample size and enrichment volume. For the inoculated food study the target inoculum was one cell per enrichment regardless of sample size. The MPN is the method choice to determine number of cells present at such as low inoculum level, however the MPN technique still has wide confidence limits. Both the calculated inoculum and MPN results indicate that the inoculum was less than 10 times the detection limit of the test. The inoculated samples complied with AOAC guidelines of between 5-15 positives/ 20 inoculated samples indicating that the comparison was done at the limit of detection (approximately 50% endpoint).

For the inclusivity study cultures were inoculated into enrichment broths appropriate for each test kit with a ratio of meat to enrichment broth of 375g to 1 L. After appropriate enrichment, cultures were diluted 1:100, close to the limit of detection of the kit, were tested in the corresponding test kit to ensure that the kits were sensitive for all ten strains. In this case the limit of detection of the kit refers to the number of target cells, which must be present in the enriched broth to give a positive result in the assay. It was determined that approximately 105 cells/ml were required and the enrichment broths containing 107- 108 cells /ml were diluted 1:100 to represent a level just above the limit of the test detection. This is required by AOAC guidelines however AS4659 guidelines appear to refer only to the limit of detection in terms of inoculum for food sample rather than level in enriched broth.

3.7 Testing

Testing was done in accordance with AOAC guidelines. For beef trim 20 inoculated samples and five uninoculated samples were tested and for lean cuts 20 inoculated samples and five uninoculated samples were tested. Testing of 5 uninoculated samples and 20 samples for one

level of contamination meeting fractional positive requirement is sufficient, the high inoculum level being optional as the only acceptance requirement is fractional recovery at one level.
AS4659 requires 5 inoculated and 5 uninoculated control samples for each matrix. In this study approval for a single matrix only (beef for grinding) was required (see matrix selection). The study therefore exceeds AS4659 exceeds requirements.

3.8 Results

Results have been tabulated according to recent AOAC statistical advice for unpaired samples.
AS4659 guidelines for statistical analysis and acceptance criteria are not relevant to this study for 2 reasons.

1. The data is not paired. It was necessary to compare different sample sizes and different enrichments for each method. For this type of unpaired data the analysis based on McNemar's chi square test is not valid. The Mantel-Haenszel chi-square test is the method of choice.
2. In order to meet AOAC requirements for fractional positive data, the inoculum was sufficiently

low that no target organisms were present in some of the inoculated samples. The statistical analysis recommended in AS4659 assumes that all the inoculated samples contain the target organism. This only becomes a problem when samples are not paired.
The following table is recent AOAC recommendation for statistical analysis of unpaired data from document Study Design supplied by the AOAC RI advisor. unpaired data contingency table

		Confirmed positive	Confirmed negative
Alternative method	Presumptive positive	A	C
	Presumptive negative	C	D
Reference method		E	F

n = A + B + C + D + E + F

$$\text{Mantel=Haenszel } \chi^2 = \frac{(n-1)\,(AF-(B+C+D)E)^2}{(A+B+C+D)(A+E)(B+C+D+F)(E+F)}$$

Relative sensitivity = A/E

False negative rate = C/(A+C)

False positive rate = B/(B+D)

Results summary tables and calculations (footnotes at end of tables)

Inoculated beef trim

Test Beef trim	Inoculum MPN	N	Positives[b] 25g/225ml	Positives[b] 375g/1 liter	Chi-square[c]	Relative sensitivity[d] %	False Negative %[d]
Assurance 8h	2.29	20	7	4	1.10	57	43
Assurance 18-28h	1.75[a]	20	7	9	0.41	128	0
BAX MP	2.29	20	6	8	0.43	133	0
VIP 8h	2.29	20	7	7	0	100	0
VIP 18-28h	1.75[a]	20	7	9	0.41	128	0

Uninoculated beef trim

Test Beef trim	Uninoculated MPN	N	Positives 25g/225ml	Positives 375g/1 L	Chi-square[c]	Relative specificity %	False Positive %
Assurance 8h	0	5	0	0	0	100	0
Assurance 18-28h	0 0	5	0 0	0 0	0 0	100	0
BAX MP	0	5	0	0	0	100	0
VIP 8h	0	5	0	0	0	100	0
VIP 18-28h	0	5	0	0	0	100	0

Inoculated lean cuts

Test Lean cuts	Inoculum MPN	N	Positives[b] 25g/225ml	Positives[b] 375g/1 L	Chi-square[c]	Relative sensitivity[d] %	False Negative %[d]
Assurance 8h	2.29	20	15	13	0.46	86.6	13.4
Assurance 18-28h	2.29	20	9	7	0.41	77.7	22.3
BAX MP	2.29	20	11	7	1.58	63.6	36.4
VIP 8h	2.29	20	15	13	0.46	86.6	13.4
VIP 18-28h	2.29	20	9	7	0.41	77.7	22.3

Uninoculated lean cuts

Test Lean cuts	Uninoculated MPN	N	Positives 25g/225ml	Positives 375g/1 L	Chi-square	Relative specificity %	False Positive %
Assurance 8h	0	5	0	0	0	100	0
Assurance 18-28h	0	5	0	0	0	100	0
BAX MP	0	5	0	0	0	100	0
VIP 8h	0	5	0	0	0	100	0
VIP 18-28h	0	5	0	0	0	100	0

[a] inoculum level was calculated from the initial count
[b] inoculum used for each test kit was constant regardless of sample size
[c] Chi square value >3.84 indicates methods are significantly different at $p<0.05$.
[d] Relative sensitivity, false negative, relative specificity and false positive result are calculated from comparison of number of presumptive positives for alternate method compared to reference method. However samples were not paired and because of low inoculation some samples would contain no target organisms. These parameters are not suitable for acceptance or rejection of the test.

3.9 3.9 Acceptance criteria

According to AOAC guidelines the alternative test is acceptable if it is better than or not significantly different from the reference method using the relevant chi-square test ie. Chi-square is <3.84. On this basis all the modified enrichment methods are acceptable.

4 Conclusion

No significant differences were found in the ability of any of the tests to detect low levels of *E. coli* O157:H7 in samples of raw ground beef components when enriched according to standard (25g to 225ml) or large sample:low volume (375g to 1 liter) enrichment protocols and using the following rapid test methods:

- Assurance (8h) AOAC Official Method 996.10
- Assurance (18-28h) AOAC Official Method 996.10
- BAX MP AOAC Research Institute Performance Tested Method 050501
- VIP (8h) AOAC Official Method 969.09
- VIP (18-28h) AOAC Official Method 969.09

5 Appendices

5.1 Guerini et al., 2006

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Evaluation of *Escherichia coli* O157:H7 Growth Media for Use in Test-and-Hold Procedures for Ground Beef Processing†

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ABSTRACT

Since the mid-1990s, the beef industry has used a process called test and hold, wherein beef trim and ground beef are tested to keep products contaminated with *Escherichia coli* O157:H7 out of commerce. Current O157:H7 detection methods rely on a threshold level of bacterial growth for detection, which is dependent on the growth medium used. Twelve media were examined for growth and doubling time: buffered peptone water (BPW), SOC (which contains tryptone, yeast extract, KCl, $MgCl_2$, and glucose), buffered peptone water plus SOC (BPW-SOC), Bacto-NZYM, RapidChek *E. coli* O157:H7 medium, BioControl EHEC8 culture medium, Neogen Reveal for *E. coli* O157:H7—Eight Hour medium (Neogen Reveal 8), BAX System medium for *E. coli* O157:H7 (BAX), BAX System medium for *E. coli* O157:H7 MP (BAX-MP), modified *E. coli* broth, nutrient medium, and tryptic soy broth (TSB). All media were tested at 37 or 42°C under static or shaking conditions. The eight media with the highest total CFU per milliliter and most rapid doubling times were BPW-SOC, NZYM, RapidChek, EHEC8, Neogen Reveal 8, BAX, BAX-MP, and TSB. The ability of these eight media to enrich *E. coli* O157:H7 in ground beef was further evaluated through time-course experiments using immunomagnetic separation. Of these media, TSB was the easiest to prepare, had a wide application base, and was the least expensive. In the test-and-hold process, the normal ratio of medium to product is 1:10. In this study, a 1:3 ratio worked as well as a 1:10 ratio. Processors using test-and-hold procedures could use 1 liter of TSB to enrich for *E. coli* O157:H7 in a 375-g sample instead of the usual 3.375 liters, thus saving reagents, time, and labor while maintaining accuracy.

The bacterial pathogen *Escherichia coli* O157:H7 has been a concern to the meat processing industry for the last 20 years. In the early 1980s, cases of hemorrhagic colitis caused by *E. coli* O157:H7 were associated with consumption of undercooked ground beef *(15)* and in 1992 and 1993 a ground beef–related O157:H7 outbreak caused hundreds of illnesses and four deaths *(17)*. In response to these events, the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 an adulterant in ground beef *(16)*. In the mid-1990s, the beef industry responded to the safety concerns surrounding *E. coli* O157:H7 contamination in ground beef and implemented a process called test and hold, in which a processor samples the trim (the raw material used to make ground beef) or the ground beef, enriches bacterial cultures for growth in bacterial media, and then tests the enrichment for the presence of *E. coli* O157:H7 *(7, 10)*. The product does not enter into commerce unless the sample contains no detectable *E. coli* O157:H7. This process is extremely expensive and time-consuming for the industry, yet test-and-hold procedures in conjunction with other practices have been extremely effective in enhancing the safety of ground beef products, as noted by dramatic reductions in the number of samples testing positive for *E. coli* O157:H7 in the FSIS verification program *(11)*. These procedures provide insurance for both beef processors and their customers, with the ultimate beneficiary being the consumer.

An effective test-and-hold program depends on highly accurate methods of testing ground beef for *E. coli* O157:H7. Optimization of three testing attributes, i.e., detection time, specificity, and sensitivity, is critical to the success of such programs. Numerous products are available for end-point testing of ground beef bacterial enrichment cultures, and some of those tests were recently studied by our group to determine the efficacy of these methods for detecting *E. coli* O157:H7 in ground beef *(1)*. Because ground beef is a highly perishable product, the testing methodology used must be as rapid as possible (turnaround time of less than 12 h). To achieve rapid results, the bacterial growth medium used in the test-and-hold procedure needs to provide a growth environment that allows for rapid doubling of *E. coli* O157:H7 in ground beef. In work presented here, 12 growth media were evaluated. The optimal growth conditions for *E. coli* O157:H7 were determined for each type of medium based on total CFU per milliliter at 6 h of growth and a doubling time between 3 and 6 h. A 6-h time period for growth was selected to demonstrate that certain media could promote robust growth, thereby allowing a sample to be collected, enriched, and tested for the presence of *E. coli* O157:H7 within an 8-h work shift. In addition, the efficacy of reducing the volume of medium required for testing a 375-g sample of ground beef was examined.

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† Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

MATERIALS AND METHODS

Bacterial strains. Seven strains of *E. coli* O157:H7 were used in these studies. Two phenotypically marked strains were used for experiment protocol 1. Strain 131AC1 *(2, 4)* was made resistant to 50 μg/ml nalidixic acid by serial passage in the presence of increasing concentrations of nalidixic acid. This resistant strain was designated 131AC1(Nal_{50}). The other strain was an *E. coli* O157:H7 bovine isolate transformed with a constitutive plasmid-expressing green fluorescent protein (GFP) *(9)*. Five *E. coli* O157:H7 isolates designated 55AC1, 114AC1, 131AC1, 237AC1, and 299AB3 *(2, 8)* were used for experiments where hourly time points were taken to measure doubling time. These five strains were used in a composite preparation made by combining equal amounts of a 10^9 CFU/ml culture, which was subsequently diluted to 10^4 CFU/ml in buffered peptone water (BPW). Aliquots of this composite preparation were stored in glycerol stocks at −80°C until use.

Bacterial growth media. Twelve bacterial growth media were selected for evaluation. The following media were selective for *E. coli* O157:H7: BAX System medium for *E. coli* O157:H7 (BAX) and BAX System medium for *E. coli* O157:H7 MP (BAX-MP) (DuPont Qualicon, Wilmington, Del.), RapidChek *E. coli* O157:H7 medium (Strategic Diagnostics, Newark, Del.), Neogen Reveal for *E. coli* O157:H7—Eight Hour medium (Neogen Reveal 8, Neogen, Lansing, Mich.), BioControl EHEC8 culture medium (BioControl, Bellevue, Wash.), and a modified *E. coli* broth (mEC broth; Difco, Becton Dickinson, Sparks, Md.) with 20 μg/ml novobiocin. Four nonselective media (Difco, Becton Dickinson) were tryptic soy broth (TSB), BPW, Bacto-NZYM, and nutrient medium. Two media were not commercially available: SOC and buffered peptone water plus SOC (BPW-SOC). SOC was made with 10 g of tryptone, 5 g of yeast extract, and 10 ml of 0.25 M KCl to correct the pH to 7.0. This mixture was autoclaved and cooled to 60°C, and then 5 ml of sterile 2 M $MgCl_2$ and 20 ml of sterile 1 M glucose was added. BPW-SOC was made as described for SOC with the addition of 20 g/liter BPW. With the exception of the SOC and BPW-SOC, all media were made according to the recommendations of the respective manufacturer.

Experiment protocol 1. Two marked strains of *E. coli* O157:H7 were used to determine the optimal growth conditions with regard to temperature (37 versus 42°C) and shaking or static conditions. A large sample of ground beef (80% lean and 20% fat) obtained from a local grocery store 24 to 48 h before beginning the study was inoculated with each marked strain of *E. coli* O157:H7 at 100 CFU/g by mixing the appropriate amount of bacteria with 7% BPW (vol/wt) and thoroughly hand massaging. Ten-gram samples of inoculated ground beef were placed in 90 ml of medium in a Whirl-Pak filter bag (7.5 by 12 in. [19 by 30.5 cm]; Nasco, Fort Atkinson, Wis.), stomached for 1 min at 260 rpm in a Lab Blender 400C (Seward Co., Essex, UK), and then incubated under selected conditions (37°C static, 37°C shaking, 42°C static, or 42°C shaking) for 6 h. Samples were incubated in a Precision Scientific model 818 incubator (Thermo Electron Corp., Milford, Mass.) for static conditions or in an Innova 44 shaker incubator (New Brunswick Scientific, Edison, N.J.) with shaking at 120 rpm. Bacteria were enumerated as described below. The growth in each medium was evaluated in a 12 (medium) × 4 (growth conditions) factorial arrangement with each marked strain for a total of 48 conditions tested.

Experiment protocol 2. The composite *E. coli* O157:H7 mix was used to determine the doubling time and total CFU per milliliter during a 6-h incubation with eight of the most productive media under the optimal condition (42°C static) identified in experiment protocol 1: BPW-SOC, TSB, BAX, BAX-MP, RapidChek, Neogen Reveal 8, Bacto-NZYM, and EHEC8. Ground beef was inoculated with ~2 CFU/g using the same type of ground beef (80% lean and 20% fat) and procedure for inoculation as described above. Ten-gram samples of inoculated ground beef were placed in 90 ml of medium in a Whirl-Pak filter bag measuring 7.5 by 12 in. (19 by 30.5 cm), stomached for 1 min at 260 rpm in a Lab Blender 400C, and then incubated at 42°C static in a Precision Scientific Model 818 incubator. Bacteria were enumerated as described below. The growth in each medium was evaluated in two separate experiments to investigate the total bacterial concentration at 6 h and the doubling time of the composite strains in the six media tested.

Experiment protocol 3. To simulate as closely as possible the conditions of the actual test-and-hold procedure, multiple 600-g samples of ground beef were inoculated with the 5 strain composite of *E. coli* O157:H7 at ~2 CFU/g, stored overnight at 4°C, mixed together, and then divided into 375-g samples. Each sample with medium was placed into a BagFilter bag (38 by 51 cm, 3,500-ml volume; InterScience, St. Nom, France), stomached for 1 min in a Jumbo Mix lab blender (InterScience) at 260 rpm, and incubated under static conditions at 42°C. Subsamples were collected hourly to determine doubling time and total concentration at 6 h using 1, 2, and 3.375 liters of TSB in a 1 (medium) × 3 (volume) factorial design. Bacteria were enumerated as described below.

***E. coli* O157:H7 enumeration.** The procedure for detection of *E. coli* O157:H7 was either direct plating from the different growth media (100-μl samples) or immunomagnetic separation of 1-ml samples plus plating as described previously *(3, 6)*. Direct plating was used for enumeration of the 131AC1(Nal_{50}) and GFP isolates. The 131AC1(Nal_{50}) strain was serially diluted and plated on tryptic soy agar (TSA; Becton Dickinson) with 50 mg/ml nalidixic acid, and colonies were counted visually after incubation at 37°C for 16 h. The GFP strain was likewise serially diluted, plated on TSA, and incubated overnight at 37°C, and colonies were counted with a model UVGL-25 UV light (254 to 365 nM; UVP, Upland, Calif.). In the second and third protocols, the five-strain composite was recovered by immunomagnetic separation and plated onto O157 selective agar (CHROMagar, DRG International, Mountainside, N.J.) supplemented with 5 mg/liter novobiocin (Sigma, St. Louis, Mo.) and 1 mg/liter potassium tellurite. For the immunomagnetic separation, 20 μl of magnetic beads coated with anti–*E. coli* O157 antibody (Dynal, Lake Success, N.Y.) was mixed with the culture for 15 min at room temperature with moderate shaking (~800 rpm) on a microplate shaker. Magnetic beads were captured with a magnetic particle separation device and washed by releasing and recapturing the beads in wells containing 1 ml of washing buffer (phosphate-buffered saline plus Tween 20). This wash step was performed twice, each time in a well with new wash buffer. The washed beads were released in a microplate well containing 50 μl of the washing buffer and then plated on CHROMagar. All plates were incubated at 37°C for 16 h, and bacterial colonies with the correct morphology were counted manually at each hourly time point.

Doubling time calculation. The doubling time (DT) for each medium was determined by the following formula: $DT = t/n$, where t is time in minutes and n is the number of generations. For this work, the doubling time was calculated over the exponential growth phase between hours 3 and 6, which represents a total of 180 min (the assigned value for t). The number of gen-

J. Food Prot., Vol. 69, No. 5 *E. COLI* O157:H7 DOUBLING TIME 1009

TABLE 1. *Estimated number of doublings[a] of two different strains of* E. coli *O157:H7[b] in 12 different media under different growth conditions[c] afte 6 h of incubation*

	O157:H7 GFP				131AC1(Nal$_{50}$)			
	Static		Shaking		Static		Shaking	
Medium[d]	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
BAX	3	5	3	0	4	8	5	7
BAX-MP	1	5	5	5	5	9	6	8
BPW	1	4	2	0	2	6	4	5
BPW-SOC	2	7	5	6	5	9	6	9
EHEC8	6	8	8	8	4	9	6	9
mEC	3	7	7	4	4	7	5	7
Noegen Reveal 8	5	7	5	7	5	8	5	8
Nutrient	0	2	0	0	1	5	3	2
Bacto-NZYM	2	7	5	6	5	9	6	8
RapidChek	3	8	6	7	6	9	7	8
SOC	1	4	2	3	4	8	6	9
TSB	3	7	5	5	6	10	7	10

[a] Values represent the mean number of doublings in a 6-h period rounded to the nearest whole number for triplicate samples.
[b] *E. coli* O157:H7 strain 131AC1 resistant to 50 μg/ml nalidixic acid (131AC1[Nal$_{50}$]) and *E. coli* O157:H7 bovine field isolate transformed with a constitutive GFP-expressing plasmid.
[c] Growth conditions were 37°C static, 37°C shaking, 42°C static, and 42°C shaking. Shaking was at 120 rpm.
[d] BAX System medium for *E. coli* O157:H7 (BAX) and BAX System medium for *E. coli* O157:H7 MP (BAX-MP) (DuPont Qualicon, Wilmington, Del.), RapidChek *E. coli* O157:H7 medium (Strategic Diagnostics, Newark, Del.), Neogen Reveal for *E. coli* O157:H7—Eight Hour medium (Neogen Reveal 8; Neogen, Lansing, Mich.), BioControl EHEC8 culture medium (BioControl, Bellevue, Wash.), modified *E. coli* (mEC) broth with 20 μg/ml novobiocin, tryptic soy broth (TSB), buffered peptone water (BPW), Bacto-NZYM, nutrient medium, and two media not commercially available: SOC and BPW-SOC.

erations was derived from the equation for the expression of growth by binary fission: $b = B \times 2^n$, where b is the number of bacteria at the end of the time interval and B is the number of bacteria at the beginning of the time interval. Solving for n in this equation, $n = 3.3 \times \log$ (number of bacteria at the end of the time interval/number of bacteria at the beginning of the time interval). The entire equation is now DT = 180/3.3 (log hour 6 − log hour 3) and is expressed in minutes per doubling.

Statistical analyses. Doubling time experiments were replicated seven times, and volume ratio experiments for TSB were replicated eight times. Least squares means of each sample were evaluated with the general linear model procedure of SAS statistical software (version 9.1, SAS Institute, Inc., Cary, N.C.).

RESULTS AND DISCUSSION

Growth and doubling time of *E. coli* O157:H7 in ground beef were examined using 12 different media (Table 1). Bacteria are known to respond differently to a variety of growth conditions *(14)*. To evaluate each medium and condition, the growth of two strains of *E. coli* O157:H7, O157:H7 GFP and 131AC1(Nal$_{50}$), was compared, and the results are reported as the estimated number of doublings after 6 h of growth. In the first experiment with the O157:H7 GFP strain, media were assessed based on bacteria achieving ≥5 doublings in a 6-h time period. In a 42°C static culture, bacteria grew well in 9 of the 12 media tested: BPW-SOC, Bacto-NZYM, TSB, BAX, BAX-MP, RapidChek, Neogen Reveal 8, mEC broth, and EHEC8 (Table 1). A few of the 42 and 37°C shaking cultures performed similarly, but in general the total concentrations were highest for bacteria grown under 42°C static conditions (data not shown).

In the second experiment in which the field study strain 131AC1(Nal$_{50}$) was used, a similar trend was observed in which the 42°C static condition was optimal (Table 1). In this experiment, the criterion for selection for further study was any condition under which ≥8 doublings occurred in a 6-h time period. The difference in the number of doublings between this experiment and the first experiment can be attributed to the slower growth of the O157:H7 GFP strain compared with the 131AC1(Nal$_{50}$) strain. Others *(14)* have noted that some bacterial strains that express GFP grow more slowly. The fact that this strain had fewer doublings in 6 h emphasizes the importance of evaluating each medium under the same conditions with more than one bacterial strain (Table 1). In the second experiment, 9 of 12 media performed best under the 42°C static conditions: SOC, BPW-SOC, TSB, BAX, BAX-MP, RapidChek, Neogen Reveal 8, Bacto-NZYM, and EHEC8.

Media selected for further experiments to measure the doubling time and total bacterial growth in a 6-h time period were chosen based on consistent high bacterial numbers in both experiments (Table 1). BPW and SOC alone did not perform well (Table 1), but when combined the performance was superior to that of each individual medium. The optimal growth condition identified in the preliminary experiments was 42°C static, which matches the manufacturer's recommended conditions set forth in the protocols for Neogen Reveal 8, BAX, BAX-MP, EHEC8, and RapidChek. Incubation of samples at 42°C with no shaking

TABLE 2. *Effects of media on total concentration and doubling time of* E. coli *O157:H7 in ground beef after 6 h of incubation*[a]

Medium[b]	Concn (log CFU/ml)[c]	Doubling time (min)[d]	SD (min)[e]
BPW-SOC	4.35 A	17.2 A	1.5
Bacto-NZYM	4.21 AB	18.7 A	3.0
RapidChek	4.17 AB	17.1 A	0.7
TSB	4.16 AB	17.9 A	0.9
BAX-MP	4.12 AB	17.1 A	4.0
EHEC8	4.03 BC	16.8 A	2.5
Neogen Reveal 8	3.77 CD	17.1 A	2.9
BAX	3.37 D	21.1 A	8.6

[a] Within a column, mean values lacking a common letter differ significantly ($P < 0.05$).
[b] BAX System medium for *E. coli* O157:H7 (BAX) and BAX System medium for *E. coli* O157:H7 MP (BAX-MP) (DuPont Qualicon, Wilmington, Del.), RapidChek *E. coli* O157:H7 medium (Strategic Diagnostics, Newark, Del.), Neogen Reveal for *E. coli* O157:H7—Eight Hour medium (Neogen Reveal 8; Neogen, Lansing, Mich.), BioControl EHEC8 culture medium (BioControl, Bellevue, Wash.), tryptic soy broth (TSB), BPW-SOC, and Bacto-NZYM.
[c] Values represent mean log CFU per milliliter of seven replicates as determined by immunomagnetic separation recovery of *E. coli* O157:H7 from 1 ml of sample taken at hour 6.
[d] Doubling time (minutes per doubling) is calculated with the following formula: 180/3.3 × log (hour 6/hour 3).
[e] SD is the average standard deviation of doubling time from seven replicates.

TABLE 3. *Effects of the ratio of sample to growth medium (TBS) on the total concentration and doubling time of* E. coli *O157:H7*[a]

Treatment[b]	Concn (log CFU/ml)[c]	Doubling time (min)[d]	SD (min)[e]
1:3 ratio	4.59 A	15.9 A	2.3
1:5 ratio	4.41 AB	17.6 A	3.3
1:10 ratio	4.51 AB	18.6 A	2.9

[a] A least squares means statistical analysis revealed no significant difference among treatments from eight replicates. Within a column, values with the same letters are not significantly different.
[b] The 1:3 ratio represents 375 g of ground beef in 1 liter of TSB, the 1:5 ratio represents 375 g of ground beef in 2 liters of TSB; and the 1:10 ratio represents 375 g of ground beef in 3.375 liters of TSB.
[c] Values represent mean log CFU per milliliter of eight replicates as determined by immmunomagnetic separation recovery of *E. coli* O157:H7 from 1 ml of sample taken at hour 6.
[d] Doubling time (minutes per doubling) is calculated with the following formula: 180/3.3 × log (hour 6/hour 3).
[e] SD is the average standard deviation of doubling time from eight replicates.

was reported as the preferred growth condition *(5)*. A temperature of 42°C was preferred to 37°C for both TSB and EC media *(5)*.

Once the optimal growth condition (42°C static) and optimal media were identified, experiments to determine the bacterial doubling time in each medium proceeded. To remove any bias a particular medium might exhibit for a single bacterial strain, a mixture of five genetically diverse *E. coli* O157:H7 strains *(2, 3)* was prepared for the inoculum. The concentration (CFU per milliliter) was measured hourly for the eight media, and doubling times were calculated (Table 2). BPW-SOC resulted in the highest (log transformed) concentration, with a 30% higher cell population than that in other media (Table 2). TSB, BAX-MP, RapidChek, and Bacto-NZYM performed comparably. No significant difference was found between the BPW-SOC, TSB, BAX-MP, RapidChek, and Bacto-NZYM total concentrations at 6 h. The bacterial concentration in EHEC8 was slightly lower than that in these five media and was significantly lower ($P < 0.05$) than that in BPW-SOC. The BAX and Neogen Reveal 8 media both performed poorly under the conditions used in this experiment, with much lower ($P < 0.05$) total concentrations at 6 h compared with those in BPW-SOC, Bacto-NZYM, RapidChek, TSB, and BAX-MP. The doubling time for all media, with the exception of BAX, was between 16 and 18 min. The amount of time it took for a bacterial cell to double in BAX was >20 min, which was correlated with the lower concentration (Table 2) of the *E. coli* O157:H7 inoculum. There was no significant difference ($P < 0.05$) in doubling times among the eight media: TSB, BAX-MP, RapidChek, Bacto-NZYM, EHEC8, BPW-SOC, Neogen Reveal 8, and BAX. A larger standard deviation was observed for the data from the BAX medium. Although we did not perform experiments to investigate this result, the possibility exists that lot-to-lot variability in the starting materials may have contributed to this larger deviation. This larger standard deviation did not cause a significant difference in the doubling time in the BAX medium compared with the other seven media.

Five of the eight media performed well, leading to a review of other factors that may influence the selection of a particular growth medium. Factors included were cost, ease of use, and range of applications. BPW-SOC outperformed all media tested (Table 2). Thus, it would seem reasonable to select BPW-SOC as the optimal medium, but when the cost and ease of use are calculated, this selection is less appealing. Preparation of this medium is labor intensive, requiring pH adjustment before autoclaving and the addition of $MgCl_2$ and glucose after autoclaving. The addition of more preparative steps increases the risk that the medium can be made improperly, thereby leading to false-negative results. Of all the media tested, TSB can be used for the broadest range of applications. TSB is used for enrichments of *Salmonella (4)* and other bacteria *(12, 13, 18)*. The manufacturers' recommendations for BAX, BAX-MP, EHEC8, RapidChek, and Neogen Reveal 8 specify that these media are to be used specifically for the enrichment of *E. coli,* and in some cases *E. coli* O157:H7 is specified as the target organism.

TSB is inexpensive and easy to prepare and has a wide application base; it also performed well in doubling time experiments. Therefore, it was selected for further testing to determine whether the ratio of the volume of sample to the volume of medium could be reduced from the standard

1:10 (375 g sample to 3.375 liters of medium) used currently in test-and-hold procedures. Conditions were simulated to match as closely as possible actual test-and-hold procedures. The data indicated that the normal test-and-hold of sample-to-medium ratio of 1:10 can be altered to use 1 liter of medium for each 375-g sample tested (a 1:3 ratio) (Table 3). A least squares means statistical analysis of the eight replicates per treatment revealed no significant difference in total bacterial concentration at 6 h and in bacterial doubling time between any of the three volumes tested. Alteration of the 1:10 ratio to 1:3 would result in a significant cost savings for growth medium, and sample incubation would require less space and would be less cumbersome.

The doubling time for *E. coli* O157:H7 was compared for 12 different media; growth in five of those media was not significantly different (doubling time and total bacterial concentration after 6 h). The superiority of TSB for use in the test-and hold process was documented. Based on this analysis, 1 liter, rather than the conventional 3.375 liters, of medium can be used to enrich 375 g of ground beef.

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Effects of Using Reduced Volumes of Nonselective Enrichment Medium in Methods for the Detection of *Escherichia coli* O157:H7 from Raw Beef†

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ABSTRACT

Recent work from our laboratory revealed that tryptic soy broth (TSB) was a superior enrichment medium for use in test-and-hold *Escherichia coli* O157:H7 methods at levels down to a ratio of three volumes of medium to one volume of sample. Lower ratios were examined for their effect on results obtained from culture isolation, the BAX *E. coli* O157:H7 MP assay, and the Assurance GDS *E. coli* O157:H7 assay. Ground beef and boneless beef trim were inoculated with a high level (170 CFU/65 g of ground beef and 43 CFU/65 g of trim) and a low level (17 CFU/65 g of ground beef and 4 CFU/65 g of trim) of *E. coli* O157:H7 and enriched in 3, 1, 0.5, and 0 volumes of TSB. The volume of TSB used did not affect *E. coli* O157:H7 detection by culture isolation, Assurance GDS detection in ground beef or trim, or the BAX MP assay detection in ground beef. However, BAX MP assay detection of *E. coli* O157:H7 in beef trim was 50, 42, and 33% positive when enrichment volumes of 0.5×, 1×, and 3×, respectively, were used. Optimum results with all methods were obtained using 1 volume of TSB. We concluded that detection test results can be considered valid as long as enrichment medium is used, even when it is less than the specified 3 or 10 volumes.

Escherichia coli O157:H7 is a pathogen that has been associated with disease outbreaks involving meat, produce, and water *(7, 10, 16).* This pathogen was identified as a foodborne threat during outbreaks associated with ground beef in the 1980s and early 1990s *(14, 17).* The U.S. Department of Agriculture Food Safety and Inspection Service implemented several regulations aimed at eliminating this pathogen from red meat *(18).* At the same time, the public and private research sectors have worked to help the beef processing industry implement antimicrobial interventions that reduced *E. coli* O157:H7 contamination *(5, 9, 13).* Unfortunately, interventions have not completely eliminated *E. coli* O157:H7 on beef, and sporadic beef-associated infections continue to occur.

The beef industry responded to the safety concerns surrounding *E. coli* O157:H7 contamination in ground beef and implemented a test-and-hold process, in which a processor samples boneless beef trim (the raw material used to make ground beef) or the ground beef, enriches the sample for growth in bacterial medium, and then tests the enrichment culture for the presence of *E. coli* O157:H7 *(4, 12).* The product does not enter into commerce unless the sample contains no detectable *E. coli* O157:H7. Numerous products are available for endpoint testing of ground beef bacterial enrichment cultures, and some of those tests were recently studied by our group to determine the efficacy of these methods for detecting *E. coli* O157:H7 in ground beef *(2).*

Optimization of three testing attributes (i.e., detection time, specificity, and sensitivity) is critical to the success of test-and-hold programs. Guerini et al. *(6)* evaluated *E. coli* O157:H7 growth media for use in test-and-hold procedures and described the superiority of tryptic soy broth (TSB) as an inexpensive and effective enrichment medium. They also reported that reduced ratios of one part product (trim or ground beef) to three parts TSB, as opposed to the traditional 1:10 dilution of product to medium, improved the economy of enrichment but had no deleterious effects on detection. Thus, decreased enrichment volumes may still provide the proper nutrition and environment for *E. coli* O157:H7 to grow to detectable levels in a rapid fashion while increasing the concentration of the *E. coli* O157:H7 in the medium.

The current *E. coli* O157:H7 test-and-hold system used by the meat industry often relies on samples being shipped to third-party laboratories. In the laboratory, occasional errors can occur that may result in less than the specified amount of enrichment medium or no enrichment medium being added to a sample before incubation. Ratios of product to medium of less than 1:3 have not been evaluated. The studies presented here were conducted to compare the effects of reducing the ratio of product to medium (1:1,

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† Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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1:0.5, and 1:0) on the detection of *E. coli* O157:H7 by standard culture techniques and common molecular tests.

MATERIALS AND METHODS

Study design. Samples ($n = 12$) of beef trim or ground beef (65 g) were inoculated with a low (approximately 10 CFU) or a high (approximately 100 CFU) concentration of *E. coli* O157:H7. One set of samples inoculated at each level were enriched in 3, 1, 0.5, or 0 volumes of TSB. After incubation to enrich for *E. coli* O157:H7, 1 volume of TSB was added to the 0-volume sample and homogenized. Equal volumes of each enrichment were then removed for *E. coli* O157:H7 detection by culture isolation or molecular methods: the BAX *E. coli* O157:H7 MP assay (DuPont, Wilmington, Del.) and the Assurance GDS *E. coli* O157:H7 assay (BioControl, Bellevue, Wash.). Enrichments also were directly spiral plated for assessment of background bacterial growth.

Preparation of inocula. For each day of the experiment, a cocktail of five well-characterized *E. coli* O157:H7 strains (55AC1, 114AC1, 131AC1, 237AC1, and 299AB3) *(3)* was used as the inoculum. The inoculum was prepared by growing one colony of each strain to stationary phase (36 h at 37°C) in 10 ml of TSB and then cold stressing it during dilution steps. Each stationary-phase culture was thoroughly mixed by vortexing, and then equal amounts (1 ml) were combined, mixed, and placed on ice. The starting concentration of cells was assumed to be 10^9 CFU/ml. One milliliter of the 10^9 CFU/ml cocktail was serially diluted 1:10 five times (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) in 9 ml of refrigerator-chilled buffered peptone water (BPW; approximately 10°C) in test tubes that were maintained on ice. Further serial dilutions (10^{-6}, 10^{-7}, and 10^{-8}) were scaled up to provide adequate volumes for multiple inoculations of samples. The 10^{-5} dilution was serially diluted by mixing 2 ml into 18 ml. Twelve milliliters of this solution was serially diluted two times into 108 ml of BPW to provide 108 ml of a 10^{-7} dilution and 120 ml of a 10^{-8} dilution. The 10^{-7} dilution was used as the high inoculum (presumed 100 CFU/ml), and the 10^{-8} dilution was used as the low inoculum (presumed 10 CFU/ml). The actual CFU per milliliter in each day's inoculum were determined by performing colony counts on the 10^{-5}, 10^{-6}, and 10^{-7} dilutions that had been spread plated in quadruplicate onto tryptic soy agar plates and incubated for 16 h at 37°C. The high and low inocula prepared the first day (for beef trim samples) were calculated to be 43 and 4.3 CFU/ml, respectively, and those prepared on the second day (for ground beef samples) were calculated to be 170 and 17 CFU/ml, respectively.

Inoculation and enrichment. Beef trimmings (50:50, fat:trim) and ground beef (80:20, lean:fat) that had never been frozen were obtained from a local abattoir-butcher shop. The trim and ground beef were both fresh cut and ground the day of purchase. Both products were packaged in twist-tie plastic bags and kept refrigerated until use. The beef trim was used 1 day after purchase, and the ground beef was used 2 days after purchase. The outer surfaces of the beef trimmings were cut away with a knife and collected to form a sample equivalent to that used in the "n = 60" method of testing beef trimmings destined for ground beef *(11)*. Sixty-five grams of trimmings and ground beef were weighed out and placed in Whirl-Pak filter bags (Nasco, Fort Atkinson, Wis.). One milliliter of either the low or the high inoculum was added to the trim or ground beef and thoroughly mixed with a Lab Blender (BagMixer 400VW, Interscience Laboratories Inc., Weymouth, Mass.) for 30 s at 420 rpm. The samples were then divided into sets of 12 high inocula and 12 low inocula and enriched by the addition of 0× (0 ml), 0.5× (32 ml), 1× (64 ml), or 3× (194 ml) TSB at room temperature. After TSB was added, each sample was again mixed in the lab blender, sealed, and incubated in a programmable incubator set for 12 h at 42°C followed by a 4°C hold until the samples were processed for *E. coli* O157:H7 detection (approximately 4 to 6 h).

Assessment of background bacterial growth. Fifty microliters of each enrichment was spiral dilution plated using a spiral plater (Autoplater 4000, Spiral Biotech, Norwood, Mass.) onto a ChromeAgar (DRG, Mountainside, N.J.) plate supplemented with 5 mg/liter novobiocin and 1 mg/liter potassium tellurite and incubated at 42°C for 16 h. After incubation, the ratio of suspect *E. coli* O157:H7 colonies (pink) to nonsuspect colonies (white and/or blue) was determined by counting the colonies of each type (suspect and nonsuspect) at the outside edges of the dilution plate. The values for background growth are given as the percentage of nonsuspect colonies in the total number of colonies counted. To ensure suspect colonies were accurately recorded, Oxoid DrySpot O157 latex agglutination tests (Thermo Fisher Scientific, Waltham, Mass.) were used to confirm the suspect phenotype.

Culture detection. One milliliter of each enrichment was removed from the sample bag for culture isolation of *E. coli* O157:H7 by immunomagnetic separation (IMS) as previously described *(1)*.

Molecular detection. Sample enrichments were tested for the presence of *E. coli* O157:H7 using (i) the commercial BAX *E. coli* O157:H7 MP kit with BAX lysis buffer and BAX instruments according to the manufacturer's instructions, (ii) the commercial Assurance GDS *E. coli* O157:H7 kit and the prescribed IMS followed by DNA detection of target in a BioControl GDS instrument, and (iii) a multiplex PCR assay for specific *E. coli* O157:H7 gene markers *(8)*. The multiplex PCR assay was performed using 1 µl of direct sample enrichment in a 25-µl PCR mix and using 20 µl of BAX lysis buffer in a 25-µl PCR mix. Multiplex PCR products were amplified in a MJR PTC 100 thermocycler (Bio-Rad Laboratories, Hercules, Calif.) according to previously described parameters *(1)* and resolved on 2.5% agarose gels stained with ethidium bromide. Multiplex reactions were considered positive for *E. coli* O157:H7 only when both the rfb^{O157} and $fliC^{H7}$ products were present with one additional product (stx_1, stx_2, or *eae*) and were considered negative only when no PCR products were present. When PCR products were present but did not fit the definition of a positive sample, the sample was considered indeterminable.

Statistics. Comparisons of frequencies of *E. coli* O157:H7 detection in each set of samples were made using PROC FREQ and Mantel-Haenszel chi-square analysis in SAS (SAS Institute, Cary, N.C.).

RESULTS AND DISCUSSION

The volume of enrichment medium added to beef trim and ground beef did not affect the culture isolation of *E. coli* O157:H7 from the samples (Table 1); however, significant differences in the levels of background bacterial growth that could interfere with culture isolation were found (Table 2). All but three samples of trim and ground beef contained *E. coli* O157:H7 as determined by culture isolation. Two culture-negative trim samples had been inoculated with low numbers and enriched without medium. The third was a low-inoculum ground beef sample enriched

TABLE 1. *Effects of reduced enrichment volume on the culture isolation of* E. coli *O157:H7 from beef trim and ground beef*[a]

Sample type	No. positive/no. tested (%) at medium-to-sample ratios of[b]:			
	0×[c]	0.5×	1×	3×
Trim				
4.3 CFU/65 g	10/12 A (83)	12/12 A (100)	12/12 A (100)	12/12 A (100)
43 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	12/12 A (100)
Ground beef				
17 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	11/12 A (92)
170 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	12/12 A (100)
All combined	46/48 A (96)	48/48 A (100)	48/48 A (100)	47/48 A (98)

[a] Culture isolation was performed by immunomagnetic separation and plating to selective agar. Values represent the number of culture-positive samples/the total number of samples tested (percentage of positive samples). Within a row, values with common letters are not significantly different ($P > 0.05$).
[b] Sixty-five grams of sample was enriched in 0 ml (0×), 32 ml (0.5×), 64 ml (1×), or 194 ml (3×) of TSB.
[c] The 0× samples were homogenized with 1 volume (64 ml) of TSB after enrichment incubation to provide a 1-ml volume for culture isolation.

with 3 volumes of medium. The two culture-negative trim samples were considered negative because these samples also produced negative results with the other detection methods. These two samples probably received no *E. coli* O157:H7 cells from the inoculum because the cells at this level (4.3 CFU/ml) were not evenly distributed throughout the tube holding the inoculum. Therefore, for the of trim inoculated with low numbers, only the 10 samples determined to contain *E. coli* O157:H7 were used for data analysis and interpretation. Concerning the ground beef sample that was culture negative, it is unlikely that uneven distribution of cells in the low inoculum resulted in a 1-ml aliquot that had no cells because this inoculum was four-fold higher in concentration than that used for the trim. During the IMS steps of culture isolation, bead recovery was variable and was poorer in samples that had a higher fat content. The ground beef sample in question was one in which poor bead recovery was observed, which could explain why the culture isolation did not produce a positive result. This negative ground beef sample produced a positive result when the other methods were used. Therefore, it was included in subsequent data analyses.

TABLE 2. *Effects of reduced enrichment volume on background growth compared with suspect* E. coli *O157:H7 growth in enrichments of inoculated beef samples*[a]

Sample type	% of nonsuspect *E. coli* O157:H7 colonies in cultures with medium-to-sample ratios of[b]:			
	0×[c]	0.5×	1×	3×
Trim				
4.3 CFU/65 g	25 B	99 A	95 A	99 A
43 CFU/65 g	20 C	60 B	65 AB	85 A
Ground beef				
17 CFU/65 g	70 A	40 B	40 B	10 C
170 CFU/65 g	40 B	60 A	40 B	1 C

[a] Background growth in enrichments was determined by enumerating suspect *E. coli* O157:H7 colonies (pink) and nonsuspect colonies (white and/or blue) on spiral dilution plates. Values are the proportion of colonies determined to be background growth, i.e., the percentage of nonsuspect colonies in the total number of colonies counted. Within a row, values with common letters are not significantly different ($P > 0.05$).
[b] Sixty-five grams of sample was enriched in 0 ml (0×), 32 ml (0.5×), 64 ml (1×), or 194 ml (3×) of TSB.
[c] The 0× samples were homogenized with 1 volume (64 ml) of TSB after enrichment incubation to provide sufficient volume for background determination.

The inoculum used in these assays was a cocktail of five strains of *E. coli* O157:H7. These five strains have previously been used individually at levels as low as 1.7 CFU per sample in evaluations of culture and molecular methods *(2)*. In the earlier evaluations, each strain grew to detectable levels, but the levels of competing background bacteria were unknown. In the experiments presented here, we evaluated the amount of background bacterial growth in each enrichment by enumerating the outer regions of a spiral dilution plate. Two opposite effects were observed (Table 2). In beef trim, the ratio of background growth to *E. coli* O157:H7 growth increased with additions of larger volumes of medium, whereas in ground beef enrichments marked decreases in background growth occurred with additions of larger volumes of medium. Generally, samples inoculated at low levels had the highest percentage of background bacterial colonies, whereas samples inoculated at high levels had lower percentages of background colonies. These findings for background bacterial growth in ground beef fit with the fact that we used a 12-h 42°C incubation protocol to select for *E. coli* O157:H7. Under these conditions, it is expected that given enough medium the *E. coli* O157:H7 would be able to outgrow the competing bacteria in the ground beef. However, the contrasting finding in trim is more difficult to explain. Portions of this same 50% fat trim were used to prepare the ground beef used in our studies. The bacteria in the trim were likely diluted out during blending and grinding with lean trim. Therefore, the types of bacteria that made up the background flora in the trim and/or the fact that these bacteria were more concentrated

TABLE 3. *Effects of reduced enrichment volume on the detection of* E. coli *O157:H7 from beef trim and ground beef using the BioControl Assurance GDS* E. coli *O157:H7 assay*[a]

Sample type	No. positive/no. tested (%) at medium-to-sample ratios of[b]: 0×[c]	0.5×	1×	3×
Trim				
4.3 CFU/65 g	9/10 A (90)	12/12 A (100)	12/12 A (100)	8/12 A (67)
43 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	12/12 A (100)
Ground beef				
17 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	12/12 A (100)
170 CFU/65 g	12/12 A (100)	12/12 A (100)	12/12 A (100)	12/12 A (100)
All combined	45/46 A (98)	48/48 A (100)	48/48 A (100)	44/48 A (92)

[a] Values represent the number of Assurance GDS *E. coli* O157:H7–positive samples/the total number of samples tested (percentage of positive samples). Within a row, values with common letters are not significantly different ($P > 0.05$).
[b] Sixty-five grams of sample was enriched in 0 ml (0×), 32 ml (0.5×), 64 ml (1×), or 194 ml (3×) of TSB.
[c] The 0× samples were homogenized with 1 volume (64 ml) of TSB after enrichment incubation to provide a 1-ml volume for GDS detection.

in the trim may be the explanation for these contrary results.

The amount of culture enrichment medium used did not affect the detection of *E. coli* O157:H7 in the ground beef and trim when the Assurance GDS was used (Table 3). The detection of *E. coli* O157:H7 in ground beef with BAX MP assay also was not affected by the different enrichment volumes (Table 4). However, detection in beef trim was more problematic. With the BAX MP assay, only 50% or less of the positive trim samples inoculated at the low level were detected, whereas with the GDS two-thirds or more of these samples were detected. When enrichment medium was left out of the beef trim samples, only 40 and 42% of low and high-inoculum samples, respectively, were detected with the BAX MP assay. When medium-to-sample ratios of 0.5, 1, and 3 were used in the trim inoculated at low levels, only 50, 42, and 33% of *E. coli* O157:H7, respectively, were detected with the BAX MP assay. The reason that the Assurance GDS was more successful than the BAX MP assay for detecting *E. coli* O157:H7 in samples enriched with decreased volumes of medium or low inoculation levels probably is associated with the IMS step in the GDS, which occurs before gene amplification. One milliliter was used for IMS in the Assurance GDS, whereas 5 μl was used in the BAX lysis step that precedes the detection step. The Assurance GDS concentrates *E. coli* O157:H7 by IMS to detectable levels that could be missed in the BAX MP assay.

The multiplex PCR assay used in our laboratory *(8)* was similarly affected by the absence of enrichment medium in trim samples but at both levels of inoculation. In our laboratory, this multiplex PCR assay typically is used to confirm a suspect isolate as *E. coli* O157:H7 rather than as a method to screen for *E. coli* O157:H7 in sample enrichments. We used this multiplex PCR assay to answer the question of whether *E. coli* O157:H7 could be detected in sample enrichment cultures by using 2 μl of the enrichment culture directly in the PCR mix instead of using a DNA preparation made in BAX lysis buffer. Direct testing of the enrichments resulted in no PCR products, probably because

TABLE 4. *Effects of reduced enrichment volume on the detection of* E. coli *O157:H7 from beef trim and ground beef using the DuPont BAX* E. coli *O157:H7-MP assay*[a]

Sample type	No. positive/no. tested (%) at medium-to-sample ratios of[b]: 0×[c]	0.5×	1×	3×
Trim				
4.3 CFU/65 g	4/10 A (40)	6/12 A (50)	5/12 A (42)	4/12 A (33)
43 CFU/65 g	5/12 B (42)	11/12 A (92)	12/12 A (100)	12/12 A (100)
Ground beef				
17 CFU/65 g	11/12 A (92)	11/12 A (92)	12/12 A (100)	11/12 A (92)
170 CFU/65 g	12/12 A (100)	11/12 A (92)	12/12 A (100)	12/12 A (100)
All combined	32/46 A (70)	39/48 A (81)	41/48 A (85)	39/48 A (81)

[a] Values represent the number of BAX *E. coli* O157:H7 MP assay–positive samples/the total number of samples tested (percentage of positive samples). Within a row, values with common letters are not significantly different ($P > 0.05$).
[b] Sixty-five grams of sample was enriched in 0 ml (0×), 32 ml (0.5×), 64 ml (1×), or 194 ml (3×) of TSB.
[c] The 0× samples were homogenized with 1 volume (64 ml) of TSB after enrichment incubation to provide sufficient volume for BAX MP detection.

of various factors such as excessive DNA and cellular debris, possible inhibitors in the enrichment, and the effects of fat in the enrichment. Reactions that were performed with the BAX lysis buffer did work well in the multiplex system, nearly as well as did the BAX MP assay. Seventy-eight percent of the samples detected with the multiplex PCR assay met the criteria for positive interpretation compared with 79% with the BAX MP assay and 97% with the Assurance GDS (data not shown).

The correlation between culture and molecular results was very high. The samples that were negative by culture also were negative by both the BAX MP assay and the GDS. All samples that were considered negative with the GDS also were negative with the BAX assay but not vice versa. The ratio of *E. coli* O157:H7 growth to background bacterial growth probably played a part in the observed results of the commercial detection systems. Samples that were negative with the BAX MP assay but positive after culture and with the Assurance GDS had a much lower concentration of *E. coli* O157:H7 in the enrichments. Because the BAX MP assay is more sensitive to the concentration of *E. coli* O157:H7 than are methods that include IMS, enrichments with low observed levels of *E. coli* O157:H7 probably were not detected.

Both the BAX MP assay and the Assurance GDS specify media other than TSB in their protocols and shorter enrichment times than used in the present study. We used TSB so results could be compared with those of previous studies. If the experiments presented here were repeated using either the BAX MP medium or the GDS mEHEC medium, different results may have been obtained. Both of these media are selective for *E. coli* O157:H7 and have additional proprietary ingredients that reduce background bacteria. Despite the selective nature of these media, our group has previously found that TSB is no less effective an enrichment medium *(7)*, and since that report TSB has become an economical and accepted replacement for these media in test-and-hold programs.

The results obtained with the BAX MP assay and the GDS for trim samples inoculated with low numbers of cells indicated what may be a beneficial effect of using lower volumes of enrichment medium. The *E. coli* O157:H7 cells were concentrated in the reduced volumes so that when 0.5×, 1×, and 3× medium volumes were used, the detection prevalence was 50, 42, and 33% with the BAX MP assay and 100, 100, and 67% with the GDS.

The *E. coli* O157:H7 cells used in our experiments were laboratory grown to stationary phase and cold stressed during dilution steps before use. They were not additionally stressed by acid, heat, or freezing as would occur during beef processing. Therefore, our inoculated strains may not perform like actual process-surviving *E. coli* O157:H7. The methods of inoculum preparation used here are similar to those used previously to determine media efficacy *(6)* and to evaluate differences in detection tests *(2)*.

These studies were conducted to determine the effects of using reduced volumes of TSB for enrichment and detection of *E. coli* O157:H7 inoculated into ground beef and beef trim. Volumes as low as half the sample size can be used with no effect on *E. coli* detection with the commercial molecular systems, but no enrichment medium was required for *E. coli* detection by culture isolation techniques. Optimum results were observed using as little as 1 volume of TSB per sample. Therefore, when less than the specified 3 or 10 volumes of medium are added to an *E. coli* O157:H7 detection test, the result will not necessarily be erroneous. Because inoculated samples were negative for *E. coli* O157:H7 in some of the experimental situations, we recommend that other rapid tests also be evaluated for such realistic variations in enrichment medium.

Note added in proof. Since the completion of these studies, we have become aware of a patent filed that proposes enrichment methods for the detection of pathogens and other microbes in food, water, etc., at ratios of sample-to-medium volumes of 1:0, 1:0.1, and 1:2 (wt/vol) or greater *(15)*.

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E. coli O157 enrichment methods Validation of low volume enrichment protocols for detection of *Escherichia coli* O157 in raw ground beef components using commercial kits

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Validation of low volume enrichment for rapid E.coli O157 screening tests

Abstract
Testing of beef destined for use in ground beef products for the presence of *Escherichia coli* O157:H7 has become an important cornerstone of control and verification activities within many meat supply chains. Validation of the ability of methods to detect low levels of *E. coli* O157:H7 is critical to confidence in test systems. Many rapid methods have been validated against standard cultural methods for 25g samples. In this study, a number of previously validated enrichment broths and commercially available test kits were validated for the detection of low numbers of *E. coli* O157:H7 in 375g samples raw ground beef component matrices using 1 liter of enrichment broth (large sample:low volume enrichment protocol). Standard AOAC methods for 25g samples in 225ml of enrichment broth using the same media, incubation conditions and test kits were used as reference methods. No significant differences were detected in the ability of any of the tests to detect low levels of *E. coli* O157:H7 in samples of raw ground beef components when enriched according to standard or large sample:low volume enrichment protocols. The use of large sample:low volume enrichment protocols provides cost savings for media and logistical benefits when handling and incubating large numbers of samples.

Escherichia coli O157:H7 is a significant human pathogen, often transmitted through foods, including through meat products. Undercooked ground beef products and non-intact whole muscle products such as needle- or blade-tenderized steaks *(1)* have been found to be responsible for a large number of outbreaks and cases, though uncooked vegetables, water and animal contact have also been found to be vehicles for transmission of this organism *(16).*

Infections with *E. coli* O157:H7, as well as with other enterohemorrhagic strains, may cause severe bloody diarrhea, leading in some cases to hemolytic uremic syndrome, thrombotic thrombocytopenic purpura (TTP) and even death *(16).* The infectious dose is considered to be low *(17).*
Slaughter and dressing of cattle is conducted to minimise the likelihood of *E. coli* O157:H7, which may be found in the gut contents and on hides, transferring to the carcase *(8, 9, 12).* Meat processors attempt to achieve this objective through the application of good hygienic practices during slaughter and dressing operations and/or applying antimicrobial interventions *(12).* In either case, there is a chance that *E. coli* O157:H7 may be found on carcases and subsequently on beef trimmings or pieces destined for grinding.

Microbiological testing for *E. coli* O157:H7 has been a significant aspect of the trade in beef destined for grinding for some time. These raw ground beef components include raw esophagus (weasand) meat, head meat, and cheek meat; beef manufacturing trimmings (various fat levels); boneless beef; beef from advanced meat recovery systems, and lean finely textured beef *(2)*. Some consider testing of these products to be the monitoring of a combination of good hygienic practices and/or critical control point(s) *(15).* In 2007, FSIS defined the parameters of sampling and testing that are required for testing to be considered 'robust' and its opinion that robust testing is a best practice essential for controlling *E. coli* O157:H7 *(3).*

FSIS' definition of robust testing requires the testing of 375g of beef per lot *(3).* Most rapid screening kits for *E. coli* O157:H7 have been validated for the testing of 25g samples enriched in 225 ml of broth through Association of Official Analytical Chemists International (AOAC) protocols. FSIS has validated the testing of 65g samples for the methods used in its laboratories (Anonymous, 2008a) and validation studies on 375g samples have also been conducted by US industry *(14)* to demonstrate equivalent sensitivity to 25g samples at a sample:broth ratio of 1:9.
The commercial tests included in this study were the VIP, Assurance (AOAC Official Method 969.09 and 996.10) and BAX MP (AOAC Research Institute Performance Tested Method 050501). These assays have already undergone extensive validation against reference culture

methods for standard 25g samples with sample: broth ratio of 1:9 and are acceptable to FSIS for testing product being exported from Australia.
When tests designed for 25g samples are scaled up to 375g then the volume of broth required is also increased to 3375ml to maintain the 1:9 ratio. One study demonstrated that a relatively low Volume of enrichment broth may be advantageous for testing *(11)* but no studies were conducted to validate this approach by testing multiple samples of product inoculated with low levels of *E. coli* O157:H7. Low volume enrichments have been applied to the detection of *E. coli* O157:H7 in beef trim and ground beef using non-selective media and commercial detection kits *(7)* but these studies did not use an AOAC approved method as a control or use an accepted protocol for validating the low volume enrichment.
In 2007, in response to the FSIS definition of robust testing, many establishments supplying beef for grinding adopted robust testing procedures, if they had not already done so. In Australia, testing according to robust methods became a requirement for export to the USA *(4).* This report is a validation of low volume enrichment for 375g samples of raw ground beef components against the more usual 1:9 sample:broth enrichment ratio for 25g samples in conjunction with a number of commercial screening tests which had been previously determined as acceptable for export purposes using 25g samples and 1:9 enrichment ratio.

Materials and Methods

Study Design. The study was designed with reference to AOAC Guidelines for Validation of Microbiological Methods (10). For each matrix, 5 uninoculated control samples and 20 samples with low level inoculum were tested using the standard 25g AOAC or USDA approved method as the reference method and 375g in 1L enrichment as the alternative method. The target inoculum was 1 cell per enrichment ie. 1 cell/25g for the reference method, and 1 cell/375g for the alternative method.
With the intention that testing the larger sample size should not result in any significant loss of sensitivity, the AOAC design was chosen as appropriate. The AOAC design incorporates a requirement for replication of testing and fractional positive data so that methods are validated for equivalence at the limit of detection of the most sensitive method. If all 20 replicates were positive data would be unacceptable using AOAC criteria. Recent studies of this type require data to fall within the range of 5-15 positives per 20 inoculated samples indicating that the inoculum is sufficiently low to provide a very stringent validation of equivalence. Determination of equivalence between methods relies on a Chi-square analysis of the unpaired data.

Test Organisms. *E. coli* O157:H7 ACM 5101 (Australian Collection of Microorganisms, University of Queensland) was used for inoculation trials in this study. Nine *E. coli* O157 strains isolated from cattle feces or carcases in various parts of Australia were obtained from, Food Science Australia, Cannon Hill for use in inclusivity studies (Table 1). All of the selected strains were known to not ferment sorbitol, and carried genes for the attaching and effacing factor (*eae*) and enterohemolysin (*ehxA*).
Each isolate was maintained at -70°C in 15% glycerol for long-term storage. Working stock cultures were kept on Trypticase Soy Agar (TSA). Cultures for inoculation were grown in Trypticase Soy Broth (TSB) at 35-37°C for 18-24 hr.

Inclusivity. Ten strains were tested. Cultures were inoculated into enrichment broths appropriate for each test kit (Table 2) with a ratio of meat to enrichment broth of 375g to 1 liter. After appropriate enrichment, cultures diluted 1:100, close to the limit of detection of the kit, were tested in the corresponding test kit to ensure that the kits were sensitive for all ten strains *(10).*

Preparation of Samples. Thin surface slices and small pieces of trim from the external surface of beef carcase parts or from beef knuckle were collected from carcasses prior to chilling at an export-registered boning room (fabrication facility) to simulate the N-60 sampling required for

robust testing *(3)*, weighed into sterile bags and immediately frozen. Frozen samples were transported to the laboratory and maintained at -18°C until they were used for analysis. Prior to inoculation samples were thawed overnight at 4°C.

For the 20 low inoculum samples, cultures were grown in TSB for 18-24h at 35-37°C. A spread plate count was then performed using 0.1% peptone diluent. Five replicate 0.1mL volumes were plated onto TSA plates incubated at 35-37°C for 24h and dilutions retained at 4°C for 24h. The appropriate aliquot (less than one ml of the diluted culture) was added dropwise to the surface of the thawed pre-weighed sample in the stomacher bag. The inoculum was constant (approximately 1 cell/sample) for both 25g and 375g samples. After inoculation using the appropriate dilution of TSB culture, samples were stored for approximately 36h at 4°C to adapt the inoculum to the temperature of product, as would occur if the product were naturally contaminated. Uninoculated samples and samples for enumerating the inoculum level by the MPN technique were prepared in a similar manner.

Microbial Analyses. Samples were analyzed for *E. coli* O157:H7 by the AOAC approved enrichment methods appropriate to each kit (Table 2). All methods were performed according to the manufacturer's package insert using mixing by shaking or mastication as described.

For each test kit, 5 uninoculated 375g samples and 20 low inoculum 375g samples (target ~1 cell/375g) were compared with 5 uninoculated 25g samples and 20 low inoculum 25g samples (target ~1 cell/25g).

Each sample was enriched using a sample to broth ratio of 25g to 225ml and 375g to 1 liter according to the appropriate media and incubation conditions listed in Table 2 for each test kit.
An MPN (3x100g, 3x10g, 3x1g,) test was conducted on the day of analysis for each inoculated batch of meat and each uninoculated batch (3 x 3-tube MPN's). Enrichment for MPN was modified EC broth+novobiocin (mEC+n).

All enrichments and MPN tubes were streaked to Cefixime-Tellurite Sorbitol MacConkey (CT-SMAC) agar for typical colonies. Any typical colonies were confirmed serologically for agglutination with "*E .coli O157* Latex Test" (Oxoid DR0620M).
All positive results from the kits were assumed to be true positives regardless of cultural results if the 5 uninoculated control samples gave negative results with the kits.

Statistical Analysis. For comparison of results, the method for 25g was regarded as the reference method and the 375g low volume enrichment method was regarded as the alternative method. A chi square analysis for unpaired samples (Mantel-Haenszel test) was performed to determine if there was a significant difference ($p<0.05$) between the methods.

Results

Inclusivity. The selected strains (Table 1) were grown in the medium and under the incubation conditions (Table 2) appropriate to each test kit in the presence of beef trim. All kits were capable of detecting all strains when present at a concentration close to the limit of detection of the kits. These results satisfy the AOAC requirements *(10)* for demonstrating that the test systems are capable of detecting target strains.

Inoculated Food Study. All uninoculated samples (5 for each combination of sample type and test kit) gave negative results at each sample size. For this reason, all positives detected by the kits were assumed to be true positives.

All beef samples (25g and 375g) to be tested by a particular method were inoculated with the same number of target cells (1.75 – 2.29 MPN, Table 3) which were then held under refrigeration for 36 hours prior to commencing the enrichment procedure. The enrichment conditions for each kit were appropriate for the AOAC-approved method (Table 2). For a valid comparison to be made according to the AOAC protocol *(10)* the inoculum level used must give results in the range of 5-15 positive tests out of 20 for at least one method of each pair being compared *(10).* All test kits gave results within this range (Table 3). Statistical comparisons could therefore be made between the previously approved method (25g sample to 225mL broth) and the alternate method (375g to 1 liter broth). In all cases the Chi-square value was <3.84 which means that no significant difference between the enrichment methods ($p<0.05$) was detected.

Discussion

Tests for *E. coli* O157 are intended to be applied to any beef part that may be used as a raw ground beef component. The vast majority of product exported from Australia to the United States consists of manufacturing trimmings and boneless beef. Knuckle was selected as a lean cut and surface slices of beef trim were selected as a matrix that would have a higher proportion of fat. We believe that there is not sufficient variation in the nature of the other beef products to require the validation of *E. coli* O157 tests with additional matrices.

The Australian Quarantine and Inspection Service (AQIS) approves methods to be used for testing beef destined for grinding in the USA to ensure that FSIS' requirements are met *(6).* AOAC approval of methods is the benchmark set in negotiation between AQIS and FSIS to ensure that test methods are at least as sensitive as those used by FSIS *(3).* Any alteration of approved methods must be supported by validation data that is reviewed by AQIS prior to Australian laboratories being allowed to use these methods for product destined for the US market.

E. coli O157 strains in Australia are noted to be somewhat different to the predominant subtypes in North America *(13).* All test kits were shown to be directed to targets that are found in all of the wide range of Australian isolates.

The validation studies described here have been conducted with reference to AOAC International protocols *(10).* 'Fractional recovery' (in the range of 5-15 positive results per 20 inoculated samples tested) is a requirement of the AOAC validation procedure. All tests methods yielded a satisfactory number of positive results when inoculated with a low number of *E. coli* O157:H7 which had been chilled for 36 hours prior to commencing the test. No significant differences were found in the ability of any of the tests to detect low levels of *E. coli* O157:H7 in samples of raw ground beef components when enriched according to standard (25g to 225ml) or large sample:low volume (375g to 1 liter) enrichment protocols. Bosilevac and Koohmaraie *(7)* also found that low volume enrichments were effective for detecting *E. coli* O157:H7 using commercial kits. However, these studies did not utilize the enrichment broth recommended by the manufacturer and utilized a relatively high inoculum level that did not yield fractional positives in most cases. Since meat is not noted to be inhibitory to *E. coli*, and microbiological media usually contain meat-based peptones, it is not surprising that a higher concentration of meat components did not inhibit the detection of *E. coli* O157:H7.

These data also highlight the sensitivity of these test systems for detection of low levels of *E. coli* O157:H7. Since the US has declared *E. coli* O157:H7 to be an adulterant, there is 'zero tolerance' for its presence in certain meat products. The enrichment method validated in this study is suitable for testing meat to meet the zero tolerance standard for *E. coli* O157:H7.

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Table 1: Sources and characteristics of strains used.

Strain ID	Year of isolation	Source	Geographical origin	Serotype	Shiga toxin genes
134	1996	Cattle feces	South East Queensland	O157:H7	2
581	1994	Beef carcass	South Australia	O157:H-	1 & 2
735	1997	Cattle feces	Far North Queensland	O157:H7	2
1668	1999	Beef carcass	Central Queensland	O157:H-	1 & 2
2308	2002	Cattle feces	South East Queensland	O157:H7	-
2340	2002	Cattle feces	South East Queensland	O157:H-	1
2459	2002	Cattle feces	Western Australia	O157:H7	2
2469	2002	Cattle feces	Victoria	O157:H7	1 & 2
2477	2002	Cattle feces	New South Wales	O157:H-	1 & 2
ACM 5101*		Human	Canada	O157:H7	-

* from Australian Collection of Microorganisms, this strain is a Shiga toxin-negative derivative (a spontaneous mutant) of Canadian CDC strain 84-289.

Table 2: Test kits and incubation conditions validated in this study

Test	Manufacturer	Medium	Incubation time (h)	Incubation temperature (°C)
Assurance (8h)	Biocontrol[c]	EHEC 8 [b]	8-12	42
Assurance (18-28h)	Biocontrol[c]	m TSB + n [a]	18-28	35-37
BAX MP	DuPont Qualicon[d]	BAX [b]	8-24	42
VIP (8h)	Biocontrol[c]	mEHEC [b]	8-12	42
VIP (18-28h)	Biocontrol[c]	m TSB + n [a]	18-28	35-37

[a] modified tryptone soya broth plus novobiocin- formulation of medium given in product documentation
[b] proprietary medium
[c] Bellevue, Washington
[d] Wilmington, Delaware

Table 3: Detection of *E. coli* O157:H7 inoculated into raw ground beef component samples at low levels

	Trim				Knuckle			
Test kit	Inoculum / sample (MPN)[c]	Positives[a] 25g/225ml	Positives[a] 375g/1 liter	Chi-square	Inoculum / sample (MPN)[c]	Positives[a] 25g/225ml	Positives[a] 375g/1 liter	Chi-square
Assurance (8h)	2.29	7	4	1.10	2.29	15	13	0.46
Assurance (18-28h)	1.75[b]	7	9	0.41	2.29	9	7	0.41
BAX MP	2.29	6	8	0.43	2.29	11	7	1.58
VIP (8h)	2.29	7	7	0	2.29	15	13	0.46
VIP (18-28h)	1.75[b]	7	9	0.41	2.29	9	7	0.41

[a] positives out of 20 inoculated samples. All 5 uninoculated control samples tested negative for each method and sample size.
[b] inoculum level was calculated from the initial count on TSA plates.
[c] inoculum used for each test kit was constant regardless of sample size