



U.S. Food & Drug Administration



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Guidance for Industry: Sampling And Microbial Testing Of Spent Irrigation Water During Sprout Production

Contains Nonbinding Recommendations

October 27, 1999

Guidance¹

Introduction

Raw sprouts have been associated with at least eleven foodborne illness outbreaks since 1995. FDA and other public health officials are working with industry to identify and implement production practices that will assure that seed and sprouted seed are produced under safe conditions. While these efforts have improved food safety awareness within the industry and have led to a significantly better understanding of the microbial ecology of sprout-associated foodborne illness, not all industry segments have been reached and outbreaks continue to occur. Consequently, FDA released a guidance document, entitled "Guidance for Industry: Reducing Microbial Food Safety Hazards for Sprouted Seed" (the "sprout guidance"). The sprout guidance identifies a number of areas, from the farm to the sprout facility, where FDA believes immediate steps should be taken to reduce the risk of sprouts serving as a vehicle for foodborne illness and to ensure that sprouts are not adulterated under the Food, Drug, and Cosmetic Act (the act). Specific recommendations in the sprout guidance include: development and implementation of good agricultural practices and good manufacturing practices in the production and handling of seeds and sprouts, seed disinfection treatments, and microbial testing before product enters the food supply.

The agency will closely monitor the safety of sprouts and the adoption of enhanced prevention practices as set out in the sprout guidance. FDA plans to send investigators to sprouting facilities to test water used to grow sprouts (i.e., spent irrigation water) and assess the adoption of preventive controls. Failure to adopt effective preventive controls can be considered insanitary conditions which may render food injurious to health. Food produced under such conditions is adulterated under the act (21 U.S.C. 342(a)(4)). FDA will consider enforcement actions against any party who does not have effective preventive controls in place, in particular, effective microbial testing.

This guidance document, "Sampling and Microbial Testing of Spent Irrigation Water During Sprout Production," is intended to assist sprouters in implementing one of the principal recommendations in the broader sprout guidance, i.e., that producers test spent irrigation water for two pathogens (*Salmonella* spp. and *Escherichia coli* O157:H7) before product enters commerce. Instructions are also provided for the sampling and testing of sprouts for those instances when it is not possible to test spent irrigation water. However, for the reasons discussed below, sprouts should not be tested in lieu of irrigation water.

Why Test

Salmonella and *Escherichia coli* O157:H7 have been the major causes of sprout-associated illness outbreaks. Seeds are the likely source of contamination in most of these outbreaks. Routine use of approved seed disinfection treatments (such as 20,000 parts per million of calcium hypochlorite in water) is likely to reduce the level of contamination if pathogens are present in or on seeds and, in turn, reduce the risk of foodborne illness from the consumption of sprouted seed. However, current approved treatments cannot guarantee total

elimination of pathogens. The same conditions that encourage germination and growth of seeds (e.g., temperature, available moisture, and nutrients), also encourage the growth of bacterial pathogens. Even if only a few pathogens survive a seed disinfection treatment, they can grow to high levels during sprouting and contaminate the entire batch. Therefore, seed disinfection treatments should be combined with microbial testing to ensure that pathogens are not present before sprouts enter the food supply.

As additional food safety controls are identified and implemented, the current recommendation to test irrigation water from every batch of sprouts produced may be changed, e.g., to periodic microbial testing as a tool for validating the effectiveness of food safety systems.

Who Should Perform The Tests

Sample collection

Sample collection should be done by personnel that have been trained to collect representative samples aseptically. Obviously, sample collection should be done on site, either by employees or by contract personnel. Aseptic sampling procedures are described below.

Testing

FDA recommends that all testing for pathogens be conducted in an external, qualified, independent laboratory that should meet several key criteria. First, the lab should be physically separated from the food production facility to prevent cross-contamination from test materials. This is especially important where the materials used in the enrichment step required before testing and the positive controls (described below) can contain pathogens and if not properly handled may contaminate sprouts.

Second, the laboratory should be staffed by personnel with training and experience in analytical microbiology techniques to ensure that tests are performed correctly and that all appropriate safety precautions, including appropriate waste disposal, are followed. Third, the laboratory should have appropriate resources and a demonstrable quality management system.

If testing is done by the sprouter, then the laboratory facilities, personnel, and management system should also meet all these criteria to ensure that testing is reliable and does not create food safety hazards.

Why Sample Irrigation Water

Procedures are provided for testing spent irrigation water and sprouts. Although each has advantages and disadvantages, FDA is recommending testing spent irrigation water.

Spent irrigation water that has flowed over and through sprouts is a good indicator of the types of microorganisms in the sprouts themselves and the microflora in spent irrigation water is fairly uniform. Thus sampling procedures for spent irrigation water are relatively simple. Furthermore, water can be used directly in the test procedures described here. The only potential disadvantage of testing spent irrigation water is that the level of microorganisms recovered in irrigation water is about 1 log less than the level in sprouts. If pathogens are present in sprouts at very low levels, it is possible that they might be missed in water but recovered in sprouts.

Testing the sprouts themselves has several significant disadvantages. First, multiple sprout samples must be taken from different locations in the drum or trays to ensure that the sample collected is representative of the batch. Furthermore, additional preparation (e.g., selecting representative subsamples for analyses, blending or stomaching, and allowing sprout particles to settle out) is required when testing sprouts. Each additional step in any procedure (sampling or testing) introduces a possibility for error.

Consequently, sprouts should not be tested in place of irrigation water unless production methods make it impossible to test spent irrigation water. For example, spent irrigation water may not be available when sprouts are grown in soil. [Note: The recommendation to test irrigation water does not preclude adding the testing of sprouts (either sprouts collected during production or finished product), to a food safety plan that includes testing irrigation water.] Sampling and testing steps specific to sprouts are given in italics and may be disregarded when testing spent irrigation water.

Sampling Plan

Sprouters should have a sampling plan in place to ensure the consistent collection of samples in an appropriate manner. The following factors should be considered in determining when and how to sample.

When to Sample

Pathogens are most likely to be present at detectable levels at or after 48 hours from the start of the sprouting process. Levels will not necessarily increase after 48 hours and may decline slightly. Thus, collecting samples for testing can be done as early as 48 hours after the start of sprouting. If seeds are presoaked (e.g.,

soaked in water for a short time and then transferred to growing units for sprouting), presoak time should be included in the 48 hour minimum.

If you are using rapid test kits, samples may be collected as late as 48 hours prior to shipping and still provide an opportunity for the sprouter to obtain test results before product enters the food supply. However, early results will allow a sprouter to take corrective actions sooner, minimizing the potential for a contaminated batch of sprouts to contaminate other production batches. Earlier testing (i.e., 48 hours after the start of sprouting) will also minimize the time and resources spent on a batch of sprouts if a presumptive positive is found. If a firm's action plan includes running confirmatory tests on a presumptive positive before discarding product, testing earlier rather than later allows more time to run additional tests.

How to Sample

Aseptic procedures are critical to avoid contaminating the sample during sample collection, storing the sample (s), and transporting the sample(s) to the lab. Aseptic sampling procedures, as described below, should be part of a firm's plan for sample collection.

Equipment used to collect samples should be clean and sterile. Sampling tools and sample containers may be purchased pre-sterilized. Alternatively, tools and containers may be sterilized at 121 °C (250 °F) for 30 minutes in an autoclave prior to use. Heat-resistant, dry materials may be sterilized in a dry-heat oven at 140 °C (284 °F) for 3 hours.

The type of sample containers used will depend on the type of samples collected but may include pre-sterilized plastic bags, tubes, cups, and flasks. Containers should be dry, leak-proof, wide mouthed, and of a size suitable for the samples. Sample containers should be properly labeled prior to starting sample collection.

Sample collectors should wear a clean lab coat, sterile gloves, and a hair net to insure they do not contaminate the samples. Hands should be washed immediately before sampling, and prior to putting on sterile gloves. Sterile gloves should be put on in a manner that does not contaminate the outside of the glove. Gloves should be properly disposed of after use.

Hands should be kept away from mouth, nose, eyes, and face while collecting samples.

Sampling instruments should be protected from contamination at all times before and during use. Sampling instruments and samples moving between the sampling site and the sample container should not be passed over the remaining pre-sterilized instruments.

The sterile sample container should be opened only sufficiently to admit the sample, place the sample directly in the container, then immediately closed and sealed. If collecting samples in a container with a lid, the lid and container should be held in one hand while collecting the sample. The lid should NOT be completely removed. (The lid should not be held separately or placed on a counter).

The sample container should be filled no more than 3/4 full to prevent overflow. The air from the container should not be expelled when sealing, particularly for plastic bags. Samples or sampling equipment should not be exposed to unfiltered air currents.

Samples should be delivered to the laboratory promptly. Perishable material should be kept at an appropriate temperature, preferably at 0 to 4.4 °C (32 to 40 °F). Sealed coolant packs should be used to avoid contamination from melting ice.

What to Sample and How Much to Collect

FDA recommends that a sprouter test for pathogens by collecting a sample of spent irrigation water from each production lot or batch. For purposes of this guidance, a production lot or batch is defined as sprouts from a single lot of seed that were started at the same time in a single growing unit (i.e., a single drum or rack of trays). Pooling samples should be avoided as pooling from different production batches may decrease the sensitivity of the tests by diluting the level of pathogens in a contaminated sample with samples that are not contaminated. Pooling samples from different batches also complicates the interpretation of results. If a presumptive positive is found, the sprouter should discard all lots represented by the pooled sample or perform additional tests to determine which batch(s) are contaminated.

1. Sample Collection for Spent Irrigation Water

The volumes given below for spent irrigation water (or sprouts) represent a sufficient sample size to test for both *Salmonella* and *Escherichia coli* O157:H7.

If testing spent irrigation water, 1 liter of water (about 2 pints or one quart) should be aseptically collected as the water leaves a drum or trays during the irrigation cycle.

If sprouts are grown in drums, a single 1 liter sample may be collected.

If sprouts are grown in trays, and all trays in a production lot have a common trough for collecting spent irrigation water, a 1 liter sample may be collected at that point. If there is no common collection point for water from trays, it may be necessary to collect water samples from individual trays and pool these samples. In this case, a sampling plan should be devised to ensure collection of a sample that is representative of the production lot. When 10 or fewer trays make up a production lot, approximately equal volumes of water should be collected from each of the 10 trays to make a total sample volume of 1 liter. For example, collect about 100 ml of water from each of 10 trays to make a 1 liter sample; about 125 ml from each of 8 trays; 167 ml from each of 6 trays, and so on. When more than 10 trays make up a production lot, ten samples should be aseptically collected, approximately 100 ml each from different trays. Again, samples should be collected throughout the entire production lot (e.g., if there are 20 trays in a production lot, collect samples from every other tray in the rack moving from top to bottom, side to side, and front to back). Samples should be placed directly into a clean, sterile, prelabeled container.

2. Sample Collection for Sprouts

If testing sprouts, thirty-two (32) sample units should be aseptically collected, approximately 50 grams each, from different locations in the drum or growing trays. The total sprout sample will be approximately 1,600 g (about 56.48 ounces or 3.53 pounds per production lot or batch). Sample units should be collected throughout the entire production lot (e.g., from top to bottom, side to side, and front to back of the drum or trays). Each 50 gram sample unit should be placed directly into individual clean, sterile, prelabeled containers. (Keeping the thirty-two sample units separate will make it easier for the lab to select representative analytical units for microbial analysis compared to pulling analytical units from a single 1,600 gram mass of sprouts.)

Microbial Testing

Testing Procedures

The testing procedures described in this guidance are screening tests. They were chosen to obtain results as simply and quickly as possible (i.e., to provide answers in 48 hours or less) on the presence or absence of two major pathogenic bacteria, i.e., *Salmonella* and *Escherichia coli* O157:H7. Formal confirmation methods, which take longer than 48 hours, are described in the FDA Bacteriological Analytical Manual (published by AOAC International, Gaithersburg, MD).

The kits identified in this guidance are AOAC approved screening tests and/or FDA has experience with their use. These are also the tests that FDA plans to use as screening tests to monitor spent irrigation water at sprouting facilities. If screening methods, other than those described here are used, they should first be validated either by formal collaborative studies or by comparative studies with standard methods using the specific commodity in question, spent irrigation water or sprouts.

Procedures for determining the presence or absence of *Escherichia coli* O157:H7 and *Salmonella* species using the test kits listed below are provided at the end of this guidance. These procedures should be performed separate from the food production facility by a qualified laboratory, preferably an independent, certified lab.

The rapid test procedures described in this guidance all involve an enrichment step to encourage the selective growth of pathogens, if they are present, in order to make their detection possible. These test kits will NOT detect pathogens in irrigation water or sprouts if the enrichment step is not performed.

In addition, seasonal or regional differences in water quality, type of seed being sprouted, individual sprout production factors, and variations in sampling and analytical conditions may all impact on the effectiveness of the screening tests. Therefore, the lab should periodically run positive controls (i.e., sprout or water samples to which a known quantity of pathogens have been added) to ensure the tests used are capable of detecting pathogens when they are present in the samples being tested.

Test Kits

Escherichia coli O157:H7

1. VIP EHEC, Biocontrol Systems, Inc., Bellview, WA., (AOAC Official method # 996.09)
or
2. Reveal *E. coli* O157:H7, Neogen Corp., Lansing, MI.

Salmonella

1. Assurance Gold *Salmonella* EIA, (AOAC Official method # 999.08)
or
2. Visual Immunoprecipitate (VIP) Assay for *Salmonella*, (AOAC Official method 1B 999.09)

(Both kits are manufactured by BioControl Systems, Inc., 12822 SE 32nd Street, Bellevue, WA 98005).

General Laboratory Instructions

Prepared Media Storage

Unless noted otherwise most media can be made in advance and stored at 20 - 30 °C (68 - 86 °F) in the dark with a shelf life of at least one month. Media should be well wrapped or contained in order to reduce evaporation.

Equipment Sterilization

Safe and proper operation of sterilizing autoclaves requires specially trained personnel. The sterilization time is typically 121 °C (250 °F) for 15 minutes.

Media and Equipment Decontamination

Used culture media and test kits should be decontaminated by autoclaving before disposal. Decontamination should be performed in an area that is totally separated from media preparation and sterilization. Trained personnel should be used to properly decontaminate used media.

Dividing Samples into Subsamples for Analyses

Spent Irrigation Water - A total of 1 L of spent irrigation should be collected for analysis. Two (2) 100 ml subsamples should be analyzed for the presence of *E. coli* O157:H7. Two (2) 375 ml subsamples should be analyzed for the presence of *Salmonella*. Any unused portion of the spent irrigation water should be stored under refrigeration pending completion of the analysis.

Sprouts - Thirty-two (32) 50 g analytical units of sprouts should be collected for analysis. Two (2) of the 50 g analytical units (25 g subsamples from each) should be analyzed for the presence of *E. coli* O157:H7 and thirty (30) of the 50 g sample units (25 g subsamples from each) should be analyzed for the presence of *Salmonella*. Unused portions of the sprout analytical units should be stored under refrigeration pending completion of the analysis.

Sample preparation (stomaching sprouts)

The procedures in this guidance use a blender to prepare sprouts for testing. As an alternative to blending, sprouts may be homogenized in a Stomacher (Model 400). To use a Stomacher, place 25 grams of sprouts in a sterile Stomacher bag, add 225 ml enrichment broth and process on medium speed for 2 minutes.

Interpretation of Results and Subsequent Actions

Interpreting Results

Analyses should be performed in duplicate (two tests for each of the two pathogens). When results are negative for all tests, results are assumed to be correct. When results are positive for one or both tests for either pathogen, the results are considered presumptive and the grower should either:

1. Consider the presumptive positive result(s) to be true and take immediate corrective actions, as described below, OR
2. Ask the testing laboratory to run confirmatory tests and destroy the batch only if the confirmatory tests are also positive for the presence of a pathogen.

In considering the second option, remember that confirmatory testing takes extra time and will lessen the marketable shelf life of the sprouts. (All product should be held until test results are available.) Rapid test kits are for screening ONLY. Confirmatory testing should be done using standard methods in the FDA Bacteriological Analytical Manual (Edition 8, Revision A - 1998).

Corrective Actions

Each facility should have a corrective action plan in place before a positive is found so that, if one does occur, corrective actions can be taken quickly. The following should be included in a corrective action plan.

Seed is the likely source of contamination in sprout-associated foodborne illness outbreaks. Further, recent outbreak investigations have shown that a single contaminated seed lot can result in contamination of multiple production lots of sprouts. Therefore, when a batch of sprouts is considered to be contaminated with a pathogen, the batch of sprouts, the seed lot used to produce the sprouts, and any other sprout production lots that were made from the same seed lot and that are still under control of the sprouter, should be discarded.

In addition, anything in the sprouting facility that has come into contact with the contaminated production lot or its water (e.g., drums, trays, bins, buckets, tools and other sprouting equipment, testing equipment, and

other possible surfaces, such as floors, drains, walls, and tables), should be thoroughly cleaned and then sanitized to avoid contamination of subsequent batches of sprouts. Care must be taken in handling contaminated sprouts or water, equipment, and test materials to avoid accidental exposure to the pathogen (s).

A) Procedure for the Rapid Analysis of *Escherichia coli* O157:H7 in Spent Irrigation Water or Sprouts.

I. Test Kits choose one:

- VIP EHEC⁽²⁾, Biocontrol Systems, Inc., Bellview, WA., or:
- Reveal *E. coli* O157:H7⁽²⁾, Neogen Corp., Lansing, MI.

II. Equipment and Materials

1. Mechanical blender (capable of 10,000 to 12,000 rpm) or Stomacher Model 400 (with required stomacher bags)
2. Sterile blender jars, with cover, resistant to autoclaving for 60 min at 121 °C
3. 1 Balance, with weights (2000 g capacity, sensitivity of 0.1 g)
4. 1 L Erlenmeyer flask
5. 2 Sterile graduated pipettes, 1.0 and 10.0 ml and pipette aids
6. Sterile instruments for use in taking and handling of samples (such as knives, tongs, scissors, spoons, etc.)
7. Sterile culture tubes, 16 x 150mm or 20 x 150mm
8. Incubator/shaker platform, 35 +/- 1 °C
9. pH meter or test strips
10. Fisher or Bunsen burner
11. Magnetic stirrer and stir bars
12. Sterile syringes
13. Sterile 0.2 m filters
14. Distilled water

III. Ingredients

1. Peptone
2. NaCl
3. Na₂HPO₄
4. KH₂PO₄
5. Casamino acid
6. Yeast extract
7. Lactose
8. Acriflavin (antibiotic)
9. Cefsulodin (antibiotic)
10. Vancomycin (antibiotic)

Preparation of antibiotic stock solutions

Prepare a stock solution of each antibiotic (acriflavin, cefsulodin, and vancomycin) by dissolving 1000 mg of each antibiotic in a separate tube containing 10.0 ml of distilled water. Filter-sterilize the solution using a 0.2 m filter and syringe. The stock solution may be stored for several months in foil wrapped tubes at 4 °C (39.2 °C).

Prepare the modified Buffered Peptone Water as described below, autoclave, cool, add antibiotic supplements. Instructions for sprouts are given in italics.

Modified Buffered Peptone Water (mBPW)

Step 1. To make 1000 ml of mBPW, mix the following constituents into distilled water, stirring to dissolve. For spent irrigation water, prepare double strength (2X) mBPW, as follows: (*If testing sprouts, use single strength (1X) enrichment broth base.*)

Modified Buffered Peptone Water(mBPW)

Ingredient	Double strength (2X) (For use with spent irrigation water)	Single strength (1X) (For use with sprouts)
Peptone	20.0 g	10.0 g
NaCl	10.0 g	5.0 g
Na ₂ HPO ₄	7.2 g	3.6 g
KH ₂ PO ₄	3.0 g	1.5 g
Casamino acid	10.0 g	5.0 g
Yeast extract	12.0 g	6.0 g
Lactose	20.0 g	10.0 g
Distilled water*	1000 ml	1000 ml

*pH 7.2 +/- 0.2 (Test pH of distilled water BEFORE adding the ingredients above. If necessary, pH may be adjusted with 1N HCl or 1N NaOH.)

Step 2. Sterilize mBPW by autoclaving at 121 °C (250 °F) for 15 minutes. Remove from autoclave and allow to cool until cool to the touch.

Step 3. Once the medium is cooled and immediately prior to the addition of a subsample, add the following quantity of filter-sterilized antibiotics to 1000 ml of medium. For spent irrigation water, add the quantity of antibiotics listed in the column labeled double strength (2X) to the double strength mBPW. (If testing sprouts, add the quantity of antibiotics listed in the column labeled single strength (1X) to the single strength mBPW.)

Antibiotic supplements for mBPW

Antibiotic Stock Solution	Double strength (2X) (For use with spent irrigation water)	Single strength (1X) (For use with sprouts)
Acriflavin (A)	0.2 ml	0.1 ml
Cefsulodin (C)	0.2 ml	0.1 ml
Vancomycin (V)	0.16 ml	0.08 ml

IV. Testing - The following instructions result in analysis being performed in duplicate: For microbial testing, duplicate sub-samples (analytical units) need to be removed from the sample and placed in enrichment broth. Enrichment broth containing sub-samples are allowed to incubate for a period of time, and a small quantity of the enrichment broth/sample is applied to the test kit device. Specific directions follow:

Step 4.

Water: Two (2) 100 ml subsamples of spent irrigation will be analyzed. From the 1000 ml sample of spent sprout irrigation water, aseptically transfer 100 ml of sample into a sterile 1L flask containing 100 ml of 2X mBPW+ACV. Repeat with second subsample.

Sprouts: Two (2) 50 g analytical units of sprouts will be analyzed. From two of the thirty-two 50 g analytical units collected, aseptically remove and weigh out a 25 g subsample of sprouts. Transfer each of the 25 gram subsamples of sprouts into separate sterile blender jars or sterile stomacher bags. Add 225 ml of single strength enrichment broth with added antibiotic supplements (1X mBPW+ACV) and blend at 10,000 to 12,000 rpm until homogenized (at least 60 seconds) or stomach for 2 minutes on medium setting in a Stomacher Model 400. Transfer sprout homogenate to a 1L Erlenmeyer flask.

Step 5. Incubate the enrichment broth/sample mixtures overnight at 42 °C(107.6 °F) with shaking at 140 RPM.

Step 6. Test each enrichment broth sample for the presence of *E. coli* O157:H7, using either the VIP EHEC device or the Reveal *E. coli* O157:H7 device. Use 0.1 ml from the inoculated and incubated mBPW +ACV to inoculate VIP or 0.12 ml for the Reveal. Follow the manufacturers instructions for the inoculation of test kits.

Step 7. Observe test results within 10 minutes to avoid possible fading of bands which could lead to false negative results. A band in both the test and control chambers is a positive test for contamination. A band in only the control chamber is a negative test. If a band does not appear in the control chamber, the test was not done correctly and must be repeated.

B) Procedure for the *Salmonella* Rapid Analysis of Spent Irrigation Water (or Sprouts)

I. Test kits choose one

- Assurance Gold *Salmonella* EIA, or
- Visual Immunoprecipitate (VIP) Assay for *Salmonella*

Both are manufactured by BioControl Systems, Inc., (12822 SE 32nd Street, Bellevue, WA 98005). For purposes of pre-enrichment and selective enrichment, these test kits provide different instructions for each of three types of foods: (a) processed foods, (b) dried powder processed foods, and (c) raw foods. For the analysis of sprouts and spent irrigation water, use the pre-enrichment/selective enrichment procedures described for (c) raw foods.

II. Equipment and materials.

1. Blender and sterile blender jars OR Stomacher Model 400 with appropriate stomacher bags.
2. Sterile, 16 oz (500 ml) wide-mouth, screw-cap jars, sterile 500 ml Erlenmeyer flasks, sterile 250 ml beakers, sterile glass or paper funnels of appropriate size, and, optionally, containers of appropriate capacity to accommodate composited samples
3. Balance, with weights; (2000 g capacity, sensitivity of 0.1 g)
4. Balance, with weights; (120 g capacity, sensitivity of 5 mg)
5. Incubator, 35 °C (95 °F)
6. Refrigerated incubator or laboratory refrigerator, 4 +/- 1 °C (39 +/- 1 °F)
7. Water bath, 42 +/- 0.2 °C (107.6 +/- 0.2 °F)
8. Sterile spoons or other appropriate instruments for transferring food samples
9. Sterile culture dishes, size 15 x 100 mm, glass or plastic
10. Sterile pipettes, 1 ml, with 0.01 ml graduations; 5 ml with 0.1 ml graduations and 10 ml with 0.1 ml graduations and pipette aids
11. Inoculating needle and inoculating loop (about 3 mm id or 10 l), nichrome, platinum-iridium, chromel wire, or sterile plastic
12. Sterile test or culture tubes, sizes 16 x 150 mm and 20 x 150 mm
13. Test or culture tube racks
14. Vortex mixer
15. Sterile shears, large scissors, scalpel, and forceps
16. Fisher or Bunsen burner
17. pH test paper (pH range 6 - 8) with maximum graduations of 0.4 pH units per color change
18. Sterile syringe
19. Sterile 0.2 m filters

III. Media and reagents

For preparation of media and reagents, refer to sections 967.25 to 967.28 in *Official Methods of Analysis* (published by AOAC International, Gaithersburg, MD USA). Designations within parentheses refer to Appendix 3, Media and Reagents, of the *Bacteriological Analytical Manual* (BAM), Edition 8, Revision A (also published by AOAC International).

1. Buffered peptone water (commercially available-Oxoid, BBL, or Difco)
2. Buffered peptone water + novobiocin
3. Tetrathionate (TT) broth (M145)
4. Rappaport-Vassiliadis (RV) medium (M132)
5. Trypticase soy broth (commercially available)
6. Trypticase soy broth + novobiocin
7. Trypticase soy broth + 2, 4 dinitrophenol + novobiocin
8. 1 N Sodium hydroxide solution (NaOH) (R73)
9. 1 N Hydrochloric acid (HCl) (R36)
10. Novobiocin solution, 0.1%
11. Sterile distilled water

Buffered peptone water (Number 1), Buffered peptone water with novobiocin (Number 2), Trypticase soy broth with novobiocin (Number 6) and Trypticase soy broth with 2,4 dinitrophenol and novobiocin (Number 7), are not included in the BAM. Their preparation is described below.

Buffered Peptone Water (BPW)

(Media & Reagents #1)

Dissolve 20 grams of commercially available buffered peptone water medium in 1 liter distilled water. Mix thoroughly. Dispense 225 ml portions into 500 ml Erlenmeyer flasks. After autoclaving for 15 min at 121 °C, and just before use, aseptically adjust volume to 225 ml with sterile distilled water. Adjust pH, if necessary, to 7.2 +/- 0.2 with sterile 1 N NaOH or 1 N HCl.

Buffered Peptone Water + novobiocin (BPW + n)

(Media & Reagents #2)

Immediately prior to the addition of a 25 g subsample, add 4 ml of 0.1% novobiocin solution to each 225 ml volume of buffered peptone water.

Trypticase soy broth + novobiocin (TSB+n)

(Media & Reagents #6)

Suspend 30 g of commercial available trypticase soy broth medium in 1 L of distilled water. Mix thoroughly. Warm gently on a temperature controlled hot plate until the medium is dissolved. Dispense in 10 ml aliquots in 20 x 150 mm tubes and autoclave 15 min. at 121 °C.

Just prior to sample addition, add 0.1 ml of 0.1% novobiocin solution to each tube containing 10 ml of Trypticase soy broth.

Trypticase soy broth + 2, 4 dinitrophenol + novobiocin (TSB+DNP+n)

(Media & Reagents #7)

Suspend 30 g of commercial available trypticase soy broth medium and 0.1 g of 2, 4 dinitrophenol in 1 L of distilled water. Mix thoroughly. Warm gently on a temperature controlled hot plate until the medium is dissolved. Dispense in 10 ml aliquots in 20 x 150 mm tubes and autoclave 15 min. at 121 °C (250 °F).

Just prior to sample addition, add 0.1 ml of 0.1% novobiocin solution to each tube containing 10 ml of Trypticase soy broth + 2, 4 dinitrophenol.

Novobiocin solution, 0.1%

(Media & Reagents #9)

- Novobiocin, sodium salt 0.1 g
- Distilled water 100 ml

Dissolve novobiocin in distilled water. Do not autoclave. Sterilize by filtering through a 0.2 m filter. Store solution at 4 °C (39.2 °F), protected from light (e.g. wrap container in aluminum foil). Solution can be stored for one week.

IV. Testing

A. Irrigation water-From the 1 L spent irrigation water sample, two (2) 375 ml subsamples will be analyzed for the presence of *Salmonella*.

Step 1. Aseptically transfer a 375 ml subsample directly to a 6 L Erlenmeyer flask containing 3,375 ml BPW + n. Swirl to mix thoroughly. Repeat procedure with second 375 ml subsample of spent irrigation water.

Step 2. Allow flasks to stand for 60 min at room temperature. Mix well and determine pH with test paper. Adjust pH, if necessary, to 6.8 +/- 0.2 with sterile 1 N NaOH or 1 N HCL.

Step 3. Incubate flasks without shaking for 18 - 26 hours at 35 - 37 °C (95 - 98.6 °F). Each flask is considered to contain pre-enrichment broth.

Step 4a. If using the Assurance Gold *Salmonella* Enzyme Immunoassay, transfer 0.1 ml pre-enrichment broth to 10 ml RV medium and transfer another 1.0 ml of pre-enrichment broth to 10 ml TT broth. Incubate in a water bath 5 - 8 hours at 42 °C (107.6 °F). Incubation of the RV medium and TT broth in the water bath is termed the selective enrichment process. Following selective enrichment, transfer and combine 1.0 ml TT broth and 0.5 ml RV medium into a single tube containing 10 ml of prewarmed [42 °C (107.6 °F)] TSB + n broth. Incubate in a water bath 16 - 20 hours at 42 °C (107.6 °F). Continue as described in this kit's instructions for (c) raw foods.

Step 4b. If using the VIP Assay for *Salmonella*, transfer 0.1 ml pre-enrichment broth to 10 ml RV medium

and transfer another 1.0 ml of pre-enrichment broth to 10 ml TT broth. Incubate in a water bath 18 - 24 hours at 42 °C. Incubation of the RV medium and TT broth in the water bath is termed the selective enrichment process. Following selective enrichment, transfer and combine 0.5 ml of TT broth and 0.5 ml RV medium into a single tube containing 10 ml prewarmed [42 °C (107.6 °F)] TSB+DNP+n broth. Incubate in a water bath 5 - 8 hours at 42 °C (107.6 °F). Continue as described in this kit's instructions for (c) raw foods.

B. Sprouts- *Thirty 50 g analytical units of sprouts were collected for Salmonella analysis.*

Step 1. Aseptically weigh out a 25 g subsample from each analytical unit and transfer each subsample to a sterile blending jar (or stomacher bag).

Step 2. Add 225 ml buffered peptone water plus novobiocin (BPW + n).

Step 3. Blend the 25 g sprout subsample with 225 ml BPW + n for 2 min.

Step 4. Repeat procedure for remaining twenty-nine analytical units.

Step 5. *The thirty 25 g sprout subsamples may be analyzed by either of the following two options:*

- Option A:
Each 25 g/225 ml blended sprout homogenate is poured into a 500 ml Erlenmeyer flask, or equivalent container, and analyzed individually.
- Option B:
Fifteen of the thirty 25 g/225 ml blended sprout homogenates are poured into a 6 L Erlenmeyer flask, and analyzed collectively. Repeat with the remaining 15 blended sprout homogenates. Thus, each sample consists of two 375-g composites.

Step 6. *Allow flasks to stand for 60 min at room temperature. Mix well and determine pH with test paper. Adjust pH, if necessary, to 6.8 +/- 0.2 with sterile 1 N NaOH or 1 N HCL.*

Step 7. *Incubate flasks without shaking for 18 - 26 hours at 35 - 37 °C (95 - 98.6 °F). Each flask is considered to contain pre-enrichment broth.*

Step 8a. *If using the Assurance Gold Salmonella Enzyme Immunoassay, transfer 0.1 ml pre-enrichment broth to 10 ml RV medium and transfer another 1.0 ml of pre-enrichment broth to 10 ml TT broth. Incubate in a water bath 5 - 8 hours at 42 °C (107.6 °F). Incubation of the RV medium and TT broth in the water bath is termed the selective enrichment process. Following selective enrichment, transfer and combine 1.0 ml TT broth and 0.5 ml RV medium into a single tube containing 10 ml of prewarmed [42 °C (107.6 °F)] TSB + n broth. Incubate in a water bath 16 - 20 hours at 42 °C (107.6 °F). Continue as described in this kit's instructions for (c) raw foods.*

Step 8b. *If using the VIP Assay for Salmonella, transfer 0.1 ml pre-enrichment broth to 10 ml RV medium and transfer another 1.0 ml of pre-enrichment broth to 10 ml TT broth. Incubate in a water bath 18 24 hours at 42 °C. Incubation of the RV medium and TT broth in the water bath is termed the selective enrichment process. Following selective enrichment, transfer and combine 0.5 ml of TT broth and 0.5 ml RV medium into a single tube containing 10 ml prewarmed [42 °C (107.6 °F)] TSB+DNP+n broth. Incubate in a water bath 5 - 8 hours at 42 °C (107.6 °F). Continue as described in this kit's instructions for (c) raw foods.*

Footnotes:

¹. This guidance has been prepared by the Office of Plant and Dairy Foods and Beverages in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration. This guidance represents the agency's current thinking on reducing microbial food safety hazards for sprouted seeds. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statute and regulations. Following the recommendations in this guidance will not shield any person or any food from appropriate enforcement under the Federal Food, Drug, and Cosmetic Act if adulterated food is distributed in interstate commerce.

². The enrichment procedure described in this guidance for the tests for *Escherichia coli* O157:H7 have been modified by FDA to enhance the ability of the kits to detect *Escherichia coli* O157:H7 in spent irrigation water and sprouts.