AOAC Official Method 992.11
Motile and Nonmotile *Salmonella* in Foods

Polyclonal Enzyme Immunoassay Method
First Action 1992
Final Action 1996
Revised First Action 1999

(Applicable to the detection of motile and nonmotile *Salmonella* in all foods.)

See Table **992.11** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

In polyclonal enzyme immunoassay (EIA) method, proprietary antibodies with high specificity to somatic and flagellar *Salmonella* antigens are bound to microwell plates. Appropriately enriched test portions and positive controls are added to plates; any *Salmonella* antigens present will bind to prepared microwells, forming antibody–antigen complex. Nonreactive test portion material is washed away. Another *Salmonella* specific antibody, which enhances sensitivity of this method by immunochemically linking bound *Salmonella* antigens to enzyme conjugate, is added. An alkaline phosphatase antibody conjugate is added, and, after incubation, unbound conjugate is washed away. The substrate, *p*-nitrophenylphosphate, is added; absorbance of resulting colored complex is read at 405–410 nm.

B. Reagents

(**a**) *Wash solution concentrate.*—2% polyoxyethylene 20 sorbitan monolaurate (Tween 20) in H_2O.

(**b**) *Substrate.*—(*1*) *Substrate tablet.*—5 mg *p*-nitrophenylphosphate per tablet; and (*2*) *Substrate diluent.*—1M diethanolamine in H_2O; or (*3*) *Liquid substrate.*—4.3mM *p*-nitrophenylphosphate.

(**c**) *Positive control.*—Stabilized, inactivated *Salmonella* antigen.

(**d**) *Antibody solution.*—Specific polyclonal flagellar and somatic antibodies to *Salmonella* serotypes (ASSUR-LINK® antibody solution is suitable).

(**e**) *Conjugate solution.*—Specific antibodies to antibody, (**d**), conjugated to alkaline phosphatase.

(**f**) *Stop solution.*—20% ethylenediaminetetraacetic acid (EDTA) in H_2O.

(**g**) *Antibody-coated microtiter wells.*—12-well microtiter well strips, wells coated with polyclonal *Salmonella* antibody, 96-well holder and cover.

(**h**) *M broth.*—Broth containing 5.0 g yeast extract, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g K_2HPO_4, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, and 0.75 g Tween 80. Suspend ingredients in 1 L H_2O and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 ×125 mm screw cap test tubes. Cap tubes loosely and autoclave 15 min at 121°C. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**i**) *Diagnostic reagents.*—For culture confirmation of presumptive positive EIA tests; *see* **967.25B** (*see* 17.9.01).

Items (**a**)–(**i**) are available as Assurance® *Salmonella* Enzyme Immunoassay (EIA) test kit for detection of motile and nonmotile *Salmonella* from BioControl Systems, Inc., 12822 SE 32nd St, Bellevue, WA 98005, USA.

Table 992.11 Interlaboratory study results for motile and nonmotile *Salmonella* in foods

Results	Percent
Agreement[a]	97.2
False negative (EIA)[b]	2.1
False negative (AOAC/BAM)[c]	3.9
False negative (EIA)[d]	1.8
False negative (AOAC/BAM)[e]	3.5

[a] Proportions of test portions which agree between AOAC/BAM culture method and EIA method expressed as percentage.

[b] Proportions of test portions confirmed as positive by AOAC/BAM culture method but negative by EIA method expressed as percentage of total number of correctly classified negative test portions plus number of false negatives by this method.

[c] Proportions of test portions confirmed as positive by EIA method but negative by AOAC/BAM culture method expressed as percentage of total number of correctly classified negative test portions plus number of false negatives by this method.

[d] Proportions of test portions confirmed positive by AOAC/BAM culture method but negative by EIA method expressed as percentage of total number of positive test portions.

[e] Proportion of test portions confirmed as positive by EIA method but negative by AOAC/BAM culture method expressed as percentage of total number of positive test portions.

C. Apparatus

(**a**) *Incubator.*—Capable of maintaining 35–37°C.

(**b**) *Water bath.*—Capable of maintaining 42 ± 0.5°C and 100 ± 2°C (or flowing steam autoclave set at 100°C).

(**c**) *Microplate washer or plastic squeeze bottle.*—For washing 12-well microtiter strips.

(**d**) *Microplate reader.*—Photometer with 405–410 nm filter, capable of reading microtiter plates. May include optional printer.

D. General Instructions

Reagents must be stored at 2–8°C when not in use. Let reagents warm to room temperature before use. Include duplicate positive and one blank test wells with each run of test samples. Use separate pipet for each test portion and reagent to avoid cross-contamination. Kit reagents and components must be used as an integrated unit and may not be mixed with components from other manufacturing batches or sources. Use dedicated trough or glassware for each reagent to avoid cross-contamination. Do not use reagents beyond stated expiration date. Do not reuse microwells.

E. Preparation of Test Portion

(**a**) *Pre-enrichment.*—Prepare test portions according to procedures described in **967.26A** (*see* 17.9.02) or *Bacteriological Analytical Manual,* current edition, AOAC INTERNATIONAL, Gaithersburg, MD, USA, Chapter 5, sec. C, with following exception: *Raw or highly contaminated meat.*—Aseptically weigh 25 g test portion into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (20 000 rpm) and decant blended homogenate into sterile, wide-mouth, screw cap 500 mL jar. Cap jars securely and let stand 60 min at room temperature. Mix well by shaking and determine pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1M NaOH or HCl; cap jar securely and mix well before determining final pH. Loosen jar caps ¼ turn. For all samples, incubate 24 ± 2 h at 35–37°C.

(**b**) *Selective enrichment.*—Following pre-enrichment incubation, transfer 1.0 mL pre-enrichment broth to one 10 mL tube

tetrathionate broth (TT) and 1.0 mL pre-enrichment broth to one 10 mL tube selenite cystine broth (SC) as in **967.26B**(**a**) (*see* 17.9.02). Label tubes. Incubate according to test sample type: *All foods other than raw foods or foods with a high microbial load.*—Incubate selective enrichment broths 6–8 h at 35–37°C. *Raw foods or foods with a high microbial load.*—Incubate tetrathionate enrichment broths 18–24 h in 42 ± 0.5°C water bath and selenite cystine enrichment broths 18–24 h at 35–37°C.

(**c**) *Post-enrichment.*—Following selective enrichment incubation, transfer and combine 0.5 mL of each paired TT and SC broths into single tube containing 10 mL M broth, **B**(**h**). Label tubes. Incubate according to test sample type: *All foods other than raw foods or foods with a high microbial load.*—Incubate M broth enrichments 14–18 h at 35–37°C. *Raw foods or foods with a high microbial load.*—Incubate M broth enrichments 6–8 h in 42 ± 0.5°C water bath.

(**d**) *Preparation of test portion for EIA analysis.*—Following M broth incubation, mix with Vortex mixer, tube contents, and transfer 1.0 mL to tube. Retain M broth test portion tubes for confirmation of presumptive positive results.

Inactivate microorganisms by submersing tube containing 1 mL M broth in boiling water bath for 20 min. Cool tubes to 25–37°C before testing. Boiled test portions can be stored at 2–8°C up to 3 days prior to testing.

F. Enzyme Immunoassay Procedure

Prepare reagent dilutions and let reagents and components reach room temperature before starting assay.

(**a**) *Wash solution preparation*.—Add 1.0 mL wash solution concentrate, **B**(**a**), to 100 mL H_2O (sufficient to wash 48 wells). Label container. Stable for 30 days at 2–8°C.

(**b**) *Substrate solution preparation.*— (*1*) *If using tablet form of substrate*.—Add 1 substrate tablet, **B**(**b**)(*1*), to 5.0 mL substrate diluent, **B**(**b**)(*2*), (sufficient for 48 wells). Label container. Prepare only amount needed for immediate use. Discard unused solution. (*2*) *If using liquid substrate*.—Liquid substrate, **B**(**b**)(*3*), may be used as provided without further preparation.

(**c**) Prepare reader by installing 405 or 410 nm filter in photometer.

(**d**) Fit required number of microwell strips into holder, allowing for 2 positive controls and 1 blank. Reseal unused microwells in foil pouch. Carefully record positive controls, blank, and test portion positions in holder.

(**e**) Vortex mix test portions and positive control before pipetting. Pipet 100 µL of each test portion into test portion wells. Pipet 100 µL positive control, **B**(**c**), into each positive control well. Leave blank well empty.

(**f**) Cover microplate and incubate 30 min at 35–37°C. Do not stack anything on microwell holder during incubation.

(**g**) Wash each well 3× as follows: (*1*) *Washing procedure.*—Completely remove contents of wells with microwell washer. Immediately fill wells with 250 µL wash solution, **F**(**a**). Repeat 2×. Avoid overfilling wells. Avoid underfilling wells (to prevent ineffective washing). Effective washing is critical to obtaining accurate data. (*2*) *Alternative washing procedure.*—Remove contents of well by inverting and vigorously tapping plate. Wash wells 3× using wash bottle (precleaned) containing wash solution and completely filling each well.

(**h**) Immediately after aspiration of third wash, invert antibody solution bottle, **B**(**d**), several times to gently mix. Add 100 µL antibody solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°C.

(**i**) Wash each well 3×, as in (**g**).

(**j**) Immediately after aspiration of third wash, invert conjugate solution bottle, **B**(**e**), several times to gently mix. Add 100 µL conjugate solution to each well, including control and blank wells. Cover and incubate 30 min at 35–37°C.

(**k**) Wash each well 3×, as in (**g**).

(**l**) Immediately after aspiration of third wash, add 100 µL substrate solution, **B**(**b**), to each well, including control and blank wells. Cover and incubate 30 min at 35–37°C.

G. Reading Results

(**a**) Immediately after incubation, **F**(**k**), read absorbance at 405 or 410 nm. Microwell plate reader must be calibrated against blank well before reading test portions and control. Standardize reader by adjusting absorbance of blank well to 0. Next, read 2 positive control wells and then test portion wells. (*Note*: Certain test portions may read <0; this is not uncommon and indicates a negative result.)

(**b**) If reading will be delayed, add 50 µL stop solution, **B**(**f**), to each well. Read within 1 h.

H. Interpretation of Test Results

(**a**) *Control value.*—Positive control reading should be >0.8 absorbance. Readings <0.8 may indicate problems with washing procedure.

(**b**) *Cutoff value.*—Calculate average value of 2 positive control readings (in absorbance units) and multiply by 0.25 to determine cutoff value.

(**c**) *Negative results.*—Test portions with absorbance readings less than cutoff value are negative.

I. Confirmation of Positive EIA Test Portions

Test portions with readings greater than or equal to cutoff value are presumptively positive. Positive test portions must be confirmed using culture methods by streaking from M broth tubes as described in **967.26B** (*see* 17.9.02). Typical or suspicious colonies should be identified as in **967.26C** (*see* 17.9.02), **967.27** (*see* 17.9.03), and **967.28** (*see* 17.9.07).

Reference: *J. AOAC Int.* **75**, 1032(1992).

Revised: June 2000