AOAC Official Method 999.08
Assurance® Gold *Salmonella* EIA for the Visual or Instrumental Identification of Motile and Non-Motile *Salmonella* in All Foods

First Action 1999
Final Action 2002

(Applicable to identification of *Salmonella* in all foods.)

Caution: *See Appendix B*, safety notes on handling microorganisms. Decontaminate all spent media and equipment used in test prior to disposal of media or re-use of equipment.

See Tables **999.08A** and **B** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Assurance Gold *Salmonella* enzyme immunoassay (EIA) contains proprietary antibodies, with a high specificity to *Salmonella* antigens, which are bound to microwell plates. Enriched broths and positive controls are added to microplate test wells. *Salmonella* antigens, if present, bind to antibodies on the microwells. Nonreactive material is washed away. A conjugated antibody is added and binds to *Salmonella* antigens captured on the microwell. After incubation, unbound conjugate is washed away. Substrate is added and the resulting color is read visually or instrumentally, using a microplate reader set at 450 nm.

B. Apparatus

(**a**) *Incubator.*—Maintaining 35–37°C.

(**b**) *Water baths.*—Maintaining 42 ± 0.2°C and 100°C. Alternatively, flowing steam autoclave set at 100°C can be used.

(**c**) *Syringe.*—With 0.2 µm or smaller porosity filter.

(**d**) *Micropipets.*—Accurately dispensing 0.1 mL.

(**e**) *Top loading balance*.—For weighing test portions.

(**f**) *Microplate reader.*—Optional. Photometer with a 450 nm filter, for reading microwell plates. May include printer.

(**g**) *Vortex mixer.*—For mixing contents of tubes.

(**h**) *Microplate washer*.—For washing microwell strips. Plastic squeeze bottle may also be used.

C. Media and Reagents

(**a**) *Extraction reagent.*—Aqueous solution containing 1.1% sodium dodecyl sulfate.

(**b**) *Wash solution concentrate.*—Phosphate buffered saline with Tween 20.

(**c**) *Antibody coated microwells.*—Microwell strips, each well coated with anti-*Salmonella* antibody, 96-well holder, and cover.

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

(**e**) *Conjugate solution.*—Specific antibodies to *Salmonella* spp. conjugated to horseradish peroxidase.

(**f**) *Substrate solution.*—3,3′,5′,5′-tetramethylbenzidine (1.25mM; FW = 240.3).

(**g**) *Stop solution.*—0.75M H_2SO_4. (*Caution*: Avoid contact with skin. If contact occurs, wash area with water.)

(**h**) *Buffered peptone water (BPW).*—Suspend 20 g commercial BPW in 1 L deionized H_2O. Stir until completely dissolved. Autoclave broth at 121°C for 15 min. Final pH is 7.2 ± 0.2.

(**i**) *Buffered peptone water with novobiocin*.—Suspend 20 g dehydrated buffered peptone water in 1 L deionized water. Mix thoroughly. Dispense in 225 mL aliquots for food test portions. Autoclave at 121°C for 15 min. On day of use, add 4 mL 0.1% novobiocin solution, (**r**), to 225 mL BPW. Final pH is 7.2 ± 0.2.

(**j**) *Brilliant green dye water*.—Add 2 mL 1% brilliant green dye solution, (**o**), per 1 L sterilized water.

(**k**) *Reconstituted nonfat dry milk with brilliant green dye*.—Dissolve 100 g nonfat dry milk in 1 L deionized water. Mix thoroughly. Autoclave at 121°C for 15 min. Cool and add 2 mL 1% brilliant greeen dye solution, (**o**), per 1 L nonfat dry milk.

(**l**) *Brain heart infusion broth with enrichment supplement containing Oxyrase® (BHI + O)*.—Suspend 37 g commercial BHI in 1 L deionized water. Mix thoroughly. Autoclave broth at 121°C for 15 min. On day of use, add 1 mL enrichment supplement containing Oxyrase® to each 225 mL bottle after test portion has been added and blending is complete. Final pH is 7.4 ± 0.2.

(**m**) *Rappaport–Vassiliadis R10 medium (RV)*.—Suspend 26.6 g commercial Rappaport–Vassiliadis R10 medium in 1 L deionized water and heat gently to dissolve. Dispense in 10 mL aliquots and sterilize by autoclaving at 116°C for 15 min. Final pH is 5.1 ± 0.2.

(**n**) *5X Rappaport–Vassiliadis R10 medium (5X RV).*—Suspend 133 g commercial Rappaport–Vassiliadis R10 medium, (**m**), in 1 L deionized water and heat gently to dissolve. Dispense in 5 mL aliquots in 25 × 150 mm test tubes and sterilize by autoclaving at 116°C for 15 min.

(**o**) *Tetrathionate broth (TT)*.—Suspend 46 g commercial TT broth into 1 L deionized water. Mix thoroughly. Heat with agitation and boil for 1 min to completely dissolve powder. *Do not autoclave*. Cool to below 45°C. Prepare brilliant green dye solution by dissolving 1 g dye in sterile water and diluting to 100 mL. Add 1 mL 1% brilliant green dye solution to TT broth. Dispense 10 mL aliquots into sterile test tubes and store at 4–8°C. Prepare I–KI solution by dissolving 6 g I and 5 g KI in 20 mL sterile water. On day of use, add 0.2 mL I–KI solution to each 10 mL tube to be used. Final pH is 8.4 ± 0.2.

(**p**) *5X Tetrathionate broth (5X TT).*—Suspend 230 g of commercial TT broth into 1 L deionized water. Mix thoroughly. Heat with agitation and boil 1 min to completely dissolve powder. *Do not autoclave*. Cool to 45°C and add 5 mL 1% brilliant green dye solution, (**o**). Dispense in 5 mL aliquots in 25 × 150 mm sterile test tubes. On day of use, add 0.5 mL I–KI solution, (**o**), to each 5 mL tube to be used.

(**q**) *Trypticase soy broth + 0.1% novobiocin (TSB + n)*.—Suspend 30 g commercial TSB in 1 L deionized water. Mix thoroughly. Warm gently until the media is dissolved. Dispense in 10 mL aliquots and autoclave at 121°C for 15 min. On day of use, add 0.1 mL 0.1% novobiocin solution to each 10 mL tube prior to transfer of selective enrichment. Final pH is 7.3 ± 0.2.

(**r**) *0.1% Novobiocin solution.*—Suspend 0.1 g novobiocin sodium salt in 100 mL purified water. Filter sterilize using a 0.2 µm or smaller porosity filter attached to a syringe. Solution is stable up to 60 days when stored in dark bottle at 2–8°C.

Items (**a**)–(**g**) are available as Assurance Gold *Salmonella* EIA for Detection of *Salmonella* from BioControl Systems, Inc., 12822 SE 32nd St, Bellevue, WA 98005, USA. Item (**l**) is available as Enrichment Supplement Containing Oxyrase® from BioControl Systems, Inc.

Table 999.08A. Interlaboratory study results for detection of motile and non-motile *Salmonella* spp. in foods by Assurance Gold EIA—visual detection

Test sample	Level	MPN/g	Total No. of test portions	Test portions positive			χ^{2b}	Sensitivity rate[c]		Incidence of false negatives among total positive test portions, %[d]		Specificity rate[e]	Incidence of false positives among total negative test portions, %[f]	Agreement between EIA and OMA methods, %[g]
				EIA										
				Pres.[a]	Conf.[a]	OMA		EIA	OMA	EIA	OMA	EIA	EIA	
Ice cream	Low	0.009	75	21	21	16	0.8	73.3	56.7	26.7	43.3	100	0.0	72
	High	0.092	75	73	72	74	0.3	96.0	98.7	4.0	1.3	98.7	1.3	95
	Control	<0.003	75	6	0	0	—[h]	—	—	—	—	—	—	—
Liquid milk	Low	0.009	75	50	50	48	0.0	79.4	76.2	20.6	23.8	100	0.0	63
	High	0.231	75	75	75	75	—	100	100	0.0	0.0	100	0.0	100
	Control	<0.003	75	2	0	0	—	—	—	—	—	—	—	—
Milk chocolate	Low	0.023	75	17	17	13	0.3	62.1	48.3	37.9	51.7	100	0.0	66
	High	0.933	75	75	75	74	0.0	100	98.7	0.0	1.3	100	0.0	99
	Control	<0.003	71	0	0	0	—	—	—	—	—	—	—	—
Dried egg powder	Low	0.023	75	35	35	47	3.2	58.3	78.3	41.7	21.7	100	0.0	49
	High	0.933	75	72	72	75	1.3	96.0	100	4.0	0.0	100	0.0	96
	Control	<0.003	75	0	0	0	—	—	—	—	—	—	—	—
Raw ground chicken	Lot 1	0.933	80	80	79	80	0.0	98.8	100	1.3	0.0	98.8	1.2	99
	Lot 2	0.092	80	55	54	47	1.0	78.3	68.1	21.7	31.9	97.9	2.1	54
Raw ground pork	Low	0.004	80	25	24	22	0.0	59.1	54.5	40.9	45.5	99.4	0.6	54
	High	0.231	80	80	78	79	0.0	97.5	100	2.5	0.0	97.6	2.4	96
	Control	<0.003	80	3	0	0	—	—	—	—	—	—	—	—

[a] Pres. = presumptive data, conf. = culturally confirmed data.

[b] $\chi 2$, as defined by McNemar is $(|a-b|-1)^2/(a+b)$ where a = test portions positive by EIA and negative by OMA and b = test portions negative by EIA and positive by OMA. A χ^2 value > 3.84 indicates significance at $p < 0.05$.

[c] Sensitivity rate is defined as total number of analyzed positive test portions among "known" positive test portions/laboratory divided by total number of "known" positive test portions/laboratory, where "known" positive is defined as test portions confirmed positive by the reference method.

[d] Incidence of false negatives is 100 – sensitivity rate. Low number of total confirmed positives will result in high false negative data.

[e] Specificity rate is defined as total number of analyzed negative test portions among "known" negative test portions/laboratory divided by total number of "known" negative test portions/laboratory, where "known" negative is defined as test portions confirmed negative by the reference method and negative controls.

[f] Incidence of false positives is 100 – specificity rate.

[g] Rate reflects number of confirmed determinations that were equivalent between EIA and OMA.

[h] Statistical analysis not applicable. Methods gave equivalent results.

Table 999.08B Interlaboratory study results for detection of motile and non-motile *Salmonella* spp. in foods by Assurance Gold EIA—instrumental detection

Test sample	Level	MPN/g	Total No. of test portions	Test portions positive			χ^{2b}	Sensitivity rate[c]		Incidence of false negatives among total positive test portions, %[d]		Specificity rate[e]	Incidence of false positives among total negative test portions, %[f]	Agreement between EIA and OMA methods, %[g]
				EIA										
				Pres.[a]	Conf.[a]	OMA		EIA	OMA	EIA	OMA	EIA	EIA	
Ice cream	Low	0.009	75	23	21	16	0.8	73.3	56.7	26.7	43.3	98.6	1.4	72
	High	0.092	75	75	72	74	0.3	96.0	98.7	4.0	1.3	96.2	3.8	95
	Control	<0.003	75	5	0	0	—[h]	—	—	—	—	—	—	—
Liquid milk	Low	0.009	75	50	50	48	0.0	79.4	76.2	20.6	23.8	100	0.0	63
	High	0.231	75	75	75	75	—	100	100	0.0	0.0	100	0.0	100
	Control	<0.003	75	0	0	0	—	—	—	—	—	—	—	—
Milk chocolate	Low	0.023	75	17	17	13	0.3	62.1	48.3	37.9	51.7	100	0.0	66
	High	0.933	75	75	75	74	0.0	100	98.7	0.0	1.3	100	0.0	99
	Control	<0.003	71	1	0	0	—	—	—	—	—	—	—	—
Dried egg powder	Low	0.023	75	35	35	47	3.2	58.3	78.3	41.7	21.7	100	0.0	49
	High	0.933	75	72	72	75	1.3	96.0	100	4.0	0.0	100	0.0	96
	Control	<0.003	75	0	0	0	—	—	—	—	—	—	—	—
Raw ground chicken	Lot 1	0.933	80	80	79	80	0.0	98.8	100	1.3	0.0	98.8	1.2	99
	Lot 2	0.092	80	55	54	47	1.0	78.3	68.1	21.7	31.9	97.9	2.1	54
Raw ground pork	Low	0.004	80	25	24	22	0.0	59.1	54.5	40.9	45.5	99.4	0.6	54
	High	0.231	80	80	78	79	0.0	97.5	100	2.5	0.0	97.6	2.4	96
	Control	<0.003	80	2	0	0	—	—	—	—	—	—	—	—

[a] Pres. = presumptive data, conf. = culturally confirmed data.

[b] $\chi 2$, as defined by McNemar is $(|a-b|-1)^2/(a+b)$ where a = test portions positive by EIA and negative by OMA and b = test portions negative by EIA and positive by OMA. A χ^2 value > 3.84 indicates significance at $p < 0.05$.

[c] Sensitivity rate is defined as total number of analyzed positive test portions among "known" positive test portions/lab divided by total number of "known" positive test portions/lab, where "known" positive is defined as test portions confirmed positive by the reference method.

[d] Incidence of false negatives is 100 – sensitivity rate. Low number of total confirmed positives will result in high false negative data.

[e] Specificity rate is defined as total number of analyzed negative test portions among "known" negative test portions/lab divided by total number of "known" negative test portions/lab, where "known" negative is defined as test portions confirmed negative by the reference method and negative controls.

[f] Incidence of false positives is 100 – specificity rate.

[g] Rate reflects number of confirmed determinations that were equivalent between EIA and OMA.

[h] Statistical analysis not applicable. Methods gave equivalent results.

Table 999.08C

Food type	Pre-enrichment broth
Nonfat dried milk	Brilliant green water
Liquid egg products	Trypticase soy broth (TSB)
Raw foods	Buffered peptone water + novobiocin (BPW + n)
Chocolate based products	Nonfat dried milk + brilliant green
Dried powder processed products	Brain heart infusion broth + enrichment supplement containing Oxyrase® (BHI + O)
All other foods	Buffered peptone water (BPW)

D. General Instructions

Store all reagents at 2–8°C when not in use. Let reagents equilibrate to room temperature before use. Include 2 positive control test wells and one blank test well with each run of tests. Use separate pipet for each test suspension 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 after expiration date. Do not re-use microwells.

E. Enrichment

(**a**) *Pre-enrichment (see Table **999.08C**).*—(*1*) *Foods with a low microbial load (excluding dried powder foods).*—Add 25 g test portion to 225 mL appropriate pre-enrichment broth, pre-warmed to 35–37°C. Mix thoroughly according to BAM/OMA, current edition. Incubate 6–8 h at 35–37°C.

(*2*) *Foods with a low microbial load (dried powder foods).*—Add 25 g test portion to 225 mL prewarmed BHI broth. Mix thoroughly according to BAM/OMA, current edition. Add 1 mL enrichment supplement containing Oxyrase® (BHI + O). Mix well by swirling. Incubate 6–8 h at 35–37°C.

(*3*) *Foods with a high microbial load*.—Add 25 g test portion to 225 mL BPW. Add 4 mL 0.1% novobiocin solution. Mix thoroughly according to BAM/OMA, current edition. Incubate 18–26 h at 35–37°C.

(**b**) *Selective enrichment*.—(*1*) *Foods with a low microbial load (excluding dried powder foods).*—Transfer 25 mL pre-enrichment broth to 5 mL 5X RV medium and transfer another 25 mL to 5 mL 5X TT broth. Vortex mix thoroughly. Incubate in water bath 16–24 h at 42°C.

(*2*) *Foods with a low microbial load (dried powder foods).*—Transfer 25 mL BHI + O broth to 5 mL 5X TT broth. Vortex mix thoroughly. Incubate in water bath 16–24 h at 42°C.

(*3*) *Foods with a high microbial load*.—Transfer 0.1 mL pre-enrichment broth to 10 mL RV medium and transfer another 1.0 mL to 10 mL TT broth. Vortex mix thoroughly. Incubate in water bath 5–8 h at 42°C.

(**c**) *Post enrichment*.—Following selective enrichment, transfer and combine 1.0 mL TT broth and 0.5 mL RV medium into a single tube containing 10 mL pre-warmed TSB + n. For dried powder foods, transfer 1.0 mL TT broth into 10 mL pre-warmed TSB + n. For dried egg products, transfer 0.2 mL TT broth into 10 mL TSB + n. Vortex mix thoroughly. Incubate all foods with a low microbial load 6–8 h at 35–37°C. Incubate foods with a high microbial load in a water bath 16–20 h at 42°C.

(**d**) *Extraction*.—Following TSB + n incubation, vortex mix tube contents and transfer 1.0 mL to a test tube. Retain original TSB + n broth sample tubes for confirmation of presumptive positive results.

Add 0.1 mL extraction reagent, **C**(**a**), to 1.0 mL TSB + n tube and vortex mix. Inactivate microorganisms at 100°C for 10 min. Cool tubes to 25–37°C before testing. Tubes that have been inactivated can be refrigerated at 4–8°C up to 4 days prior to testing.

F. Enzyme Immunoassay

(*1*) Prepare wash solution by adding 5.0 mL wash solution concentrate, **C**(**b**), to 100 mL distilled water. Label container and let equilibrate at room temperature. This volume is sufficient to wash 40 wells. Wash solution is stable 30 days.

(*2*) Install 450 nm filter in microwell plate reader.

(*3*) Fit required number of microwell strips into holder, allowing for 2 positive controls and one blank well. Carefully reseal unused microwells in foil pouch containing desiccant. Carefully record position of positive controls, blanks, and test suspensions in holder.

(*4*) To assure uniformity, mix separately test suspensions, **E**(**d**), and positive control, **C**(**d**), on Vortex mixer before pipetting. Pipet 0.1 mL test suspension into test well; pipet 0.1 mL positive control into each positive well. Leave blank well empty.

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

(*6*) Wash each well 3× using alternative (*a*) or (*b*). (*Note*: Effective washing is critical to obtaining accurate data.) (*a*) Completely remove contents of wells with microwell washer. Immediately fill wells completely with wash solution. Repeat 2×. Avoid overfilling or underfilling wells. (*b*) Remove contents of wells by inverting and vigorously tapping plate. Completely fill each well with wash solution using pre-cleaned wash bottle. Repeat 2×.

(*7*) Immediately after removal of last wash, mix conjugate solution, **C**(**e**), by gently inverting bottle several times. Add 0.1 mL conjugate solution to each well, including control and blank wells. Cover plate with plastic cover and incubate 30 min at 35–37°C.

(*8*) Wash each well 3× as in step (*6*).

(*9*) Immediately after removal of last wash, add 0.1 mL substrate solution to each well, including control and blank wells. Cover plate with plastic cover and incubate at room temperature for 10–15 min.

G. Reading and Interpreting Results

(**a**) *Visual interpretation*.—Use the Color Standard Card. (*Note*: If interpreting the results visually, reading must be taken between 10 and 15 min.) After incubation, place well holder on white background. Looking straight down into well, compare color at center of wells with Color Standard. Edges of wells may reflect color of adjacent wells and appear darker; this should be disregarded. Test wells that are at least as dark as Color II—the positive cutoff—are presumptive positive and should be culturally confirmed. Since color development will continue, reading must be made within 10–15 min.

(**b**) *Instrumental interpretation*.—Use microplate reader. Fit microwell reader with 450 nm filter. Add 0.1 mL stop solution, **C**(**g**), to each well at 15 min. Blue color will turn yellow. After adding stop solution, read and record results. Color is stable 1 h. (*Note*: For valid results, microwell plate reader must be calibrated against blank well before reading tests and control.) (*1*) Standardize reader by reading blank well and adjusting absorbance (A) to zero. (*2*) Read absorbencies of each well, starting with 2 positive controls. When reader is standardized to

blank well, certain tests may read less than zero (a negative reading). This is not uncommon and indicates a negative result.

H. Interpretation of Instrumental Results

(**a**) *Control value*.—Positive control reading should be >0.8 A units. Readings below this value may indicate problems with washing procedure. If readings are below 0.8 A units, repeat test starting from **F**(*1*) or contact kit manufacturer. If readings are above upper limit of microplate reader, use reading of 2.5 to calculate cutoff value.

(**b**) *Cutoff value*.—Calculate average value of 2 positive control readings (in A units) and multiply by 0.25 to determine cutoff value. Test portions with readings ≥ cutoff value are presumptively positive.

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

I. Confirmation of Positive Tests

Presumptive positive tests must be confirmed culturally as described in **967.26** (*see* 17.9.02), **967.27** (*see* 17.9.03), and **967.28** (*see* 17.9.07). Isolate microorganism by streaking refrigerated enrichment broth, **E**(**c**).

Reference: *J. AOAC Int*. **83**, 871(2000).

Revised: March 2002