AOAC Official Method 997.16 LOCATE® Enzyme-Linked Immunosorbent Assay for Identification of *Salmonella* in Foods

First Action 1997

(Applicable to identification of *Salmonella* in all foods. Assays must be confirmed by standard culture methods.)

See Tables **997.16A–C** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Identification of *Salmonella* antigens is based on enzyme immunoassay (EIA) using specific monoclonal antibodies. Test broths to be assayed along with controls are added to wells, and all antigens present will bind onto the well surface. Monoclonal antibody-labeled enzyme (conjugate) is added, and it binds to *Salmonella* antigens if they are present on the surface of the well, thus forming an antibody–antigen complex. Unbound conjugate is removed by washing and substrate is added. A blue product is formed in the presence of fixed antibody-labeled enzyme conjugate. Results can be read visually, or the reaction can be stopped with acid and the absorbance (*A*) at 450 nm can be determined with a microplate reader. Tests with values higher than the recommended cut-off value are considered presumptive positive for *Salmonella* antigens.

B. Reagents

Items (**a**)–(**l**) are available as LOCATE® *Salmonella* assay kit, sufficient for 96 tests, from Rhône-Poulenc, CN7500 Prospect Plains Rd, Cranbury, NJ 08512, USA. Store all materials at 2–8°C.

(**a**) *96-Well microtiter plate.*—Eight 12-well breakable strips.

(**b**) *Microtiter strip well holder.*—Sufficient for securing individual strips.

(**c**) *Positive control.*—One vial containing heat-killed, lyophilized *S. poona* (reacts with antibodies to *Salmonella*) with 0.5% l-Bronidox (Henkel KGaA, D-40191 Düsseldorf, Germany) as preservative.

(**d**) *Negative control.*—One vial containing nonreactive heat-killed *Proteus vulgaris* antigens with 0.5% l-Bronidox (Henkel KGaA) as preservative.

(**e**) *Enzyme conjugate.*—Two vials freeze-dried conjugate containing anti-*Salmonella* antibody conjugated to horse radish peroxidase lyophilized with 0.005 g glycine and 0.028 g sucrose.

(**f**) *Conjugate diluent.*—One vial (20 mL) Tris-NaCl buffer containing 0.01 mL Tween 20 and 1% bovine serum albumin with 0.02% Kathon (Rohm and Haas, Croydon, Surrey CR9-3NB, UK) as preservative.

(**g**) *Wash buffer concentrate (25×).*—One vial (20 mL) Tris–NaCl buffer containing 0.275 mL Tween 20 with 0.2% Kathon (Rohm and Haas) as preservative.

(**h**) *Substrate.*—One vial (15 mL) TMB (3,3,5,5,-tetramethyl benzidine) reagent. Store in dark at 2–8°C.

(**i**) *Stop solution.*—One vial (15 mL) 2% H_2SO_4 solution.

(**j**) *Plate sealer.*

(**k**) *Modified GN broth (GN broth containing 10 μg/mL novobiocin).*—Ingredients per liter of deionized water: 20 g tryptose, 1 g dextrose, 2 g D-mannitol, 5 g sodium citrate, 0.5 g sodium desoxycholate, 4 g K_2HPO_4, 1.5 g KH_2PO_4, and 5 g NaCl. Prepare novobiocin stock solution (1 mg/mL in water) and filter-sterilize. Aseptically add 100 μL novobiocin to each sterile GN broth tube. Novobiocin stock solution may be stored for several weeks in a dark bottle at 4°C.

(**l**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* **967.27** (*see* 17.9.03).

C. Apparatus

(**a**) *Microplate reader.*—Reading absorbance at 450 nm (optional).

(**b**) *Incubators.*—35 ± 1°C.

(**c**) *Water baths.*—42 ± 0.5°C.

(**d**) *Pipets.*—Delivering 50–100 μL.

(**e**) *Plastic squeeze bottle.*—To wash wells.

D. General Instructions

(*1*) Components in kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(*2*) Store all kit components and reagents at 2–8°C when not in use.

(*3*) Bring reagents to room temperature before use.

(*4*) The substrate TMB is light sensitive. Perform the last stage of color development in the dark.

(*5*) Return any remaining strips to the strip holder, cover with the plastic sealer provided, and return to pouch containing dessicant. Fold the pouch and store at 2–8°C.

Table 997.16A Test products, test organisms, and inoculation levels

Product	*Salmonella* serovar	MPN/g
Milk chocolate	—[a]	<0.003
	senftenberg (E_4:G_{comp})	0.093
	senftenberg (E_4:G_{comp})	0.75
Dried egg 1	—[a]	<0.003
	B:r:1 complex	0.043
	B:r:1 complex	0.43
Dried egg 2	—[a]	<0.003
	B:r:1 complex	0.043
	B:r:1 complex	0.460
Nonfat dry milk	—[a]	<0.003
	newport (C_2:eh_{comp})	0.240
	newport (C_2:eh_{comp})	0.75
Black pepper 1	—[a]	<0.003
	cubana (G_2:Z_{29})	1.1
	cubana (G_2:Z_{29})	11.1
Black pepper 2	—[a]	<0.003
	cubana	0.004
	cubana	0.460
Soy flour	—[a]	<0.003
	C_2:Z_{10} en complex	0.043
	C_2:Z_{10} en complex	0.240
Raw ground turkey[b]	—[c]	<0.003
	C_2: en complex[b]	0.093
	G:eh:1 complex[b]	2.4

[a] — = Uninoculated control.

[b] Naturally contaminated products; predominant serovar listed.

[c] — = Low level of naturally occurring *Salmonella*.

Table 997.16B Interlaboratory study results obtained by visual interpretation

Food	Level of *Salmonella* contamination	MPN[a], cfu/g	Total test portions	Test portions positive				χ^{2b}	LOCATE performance rates, %				Agreement between LOCATE and BAM/AOAC methods, %[g]
				LOCATE			BAM/ AOAC culture		Sensitivity[c]	Incidence of false negatives among total positive test portions[d]	Specificity[e]	Incidence of false positives among total negative test portions, %[f]	
				Total	Suspect	Confirmed							
Milk chocolate	Control[h]	<0.003	75	0	1	0	0	—[i]	—	—	99	1	100
	Low	0.093	75	72	72	72	72	—	100	0	100	0	100
	High	0.75	75	74	74	74	73	0.50	100	0	100	0	97
Dried whole egg	Control	<0.003	80	0	1	0	0	—	—	—	99	1	100
	Low	0.043	80	35	35	35	35	—	100	0	100	0	100
	High	0.460	80	79	79	79	79	—	100	0	100	0	100
Nonfat dry milk	Control	<0.003	74	0	5	0	0	—	—	—	93	7	100
	Low	0.240	75	71	69	68	71	1.33	96	4	99	1	96
	High	0.75	75	75	75	75	75	—	100	0	100	0	100
Black pepper	Control	<0.003	90	0	3	0	0	—	—	—	97	3	100
	Low	0.004	90	22	24	21	22	0.00	95	5	98	2	99
	High	0.460	90	83	83	83	83	—	100	0	100	0	100
Soy flour	Control	<0.003	75	0	0	0	0	—	—	—	100	0	100
	Low	0.043	75	42	41	41	41	0.50	98	2	100	0	97
	High	0.240	75	73	73	73	72	0.00	100	0	100	0	99
Raw turkey	Low	<0.003	85	12	12	10	12	0.00	83	17	97	3	96
	Medium	0.093	85	71	73	71	68	1.33	100	0	98	2	96
	High	2.4	85	80	83	78	79	0.00	98	2	94	6	96

[a] Most probable number of colony-forming units (cfu) per gram of food.

[b] χ^2 is defined by McNemar as $(|a - b| - 1)^2/(a + b)$ where a = test portions positive by LOCATE and negative by culture method and b = test portions negative by LOCATE and positive by culture method. A χ^2 value greater than 3.84 indicates significance at $p < 0.05$.

[c] Sensitivity rate is defined as 100 × the total number of analyzed positive test portions among "known" positive test portions ÷ total number of "known" test portions, where "known" positive is defined as test portions confirmed positive by the reference method.

[d] Incidence of false negatives is 100 – sensitivity rate.

[e] Specificity rate is defined as 100 × the total number of analyzed negative test portions among "known" negative test portions ÷ the total number of "known" negative test portions, 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] Reflects the number of confirmed determinations that were equivalent for LOCATE and culture methods.

[h] Uninoculated control samples were by definition known negatives; sensitivity rates were not calculated.

[i] Statistical analysis was not applicable.

Table 997.16C Interlaboratory study results obtained with the automated plate reader

Food	Level of *Salmonella* contamination	MPN[a], cfu/g	Total test samples	Test portions positive					LOCATE performance rates, %				
				LOCATE			BAM/ AOAC culture	χ^{2b}	Sensitivity[c]	Incidence of false negatives among total positive test portions[d]	Specificity[e]	Incidence of false positives among total negative test portions, %[f]	Agreement between LOCATE and BAM/AOAC methods[g], %
				Total	Suspect	Confirmed							
Milk chocolate	Control[h]	<0.003	75	0	1	0	0	—[i]	—	—	99	1	100
	Low	0.093	75	72	72	72	72	—	100	0	100	0	100
	High	0.75	75	74	73	73	73	0.50	99	1	100	0	97
Dried whole egg	Control	<0.003	80	0	1	0	0	—	—	—	99	1	100
	Low	0.043	80	35	32	32	35	1.33	91	9	100	0	96
	High	0.460	80	79	79	79	79	—	100	0	100	0	100
Nonfat dry milk	Control	<0.003	74	0	5	0	0	—	—	—	93	7	100
	Low	0.240	75	71	68	67	71	1.33	94	5	99	1	96
	High	0.75	75	75	71	71	75	2.25	95	5	100	0	95
Black pepper	Control	<0.003	90	0	3	0	0	—	—	—	97	3	100
	Low	0.004	90	22	24	22	22	0.00	100	0	99	1	100
	High	0.460	90	83	83	83	83	—	100	0	100	0	100
Soy flour	Control	<0.003	75	0	0	0	0	—	—	—	100	0	100
	Low	0.043	75	42	42	41	41	0.50	98	2	99	1	97
	High	0.240	75	73	73	73	72	0.00	100	0	100	0	99
Raw turkey	Low	<0.003	85	12	12	10	12	0.00	83	17	97	3	96
	Medium	0.093	85	71	73	71	68	1.33	100	0	98	2	96
	High	2.4	85	80	84	78	79	0.00	98	2	92	8	96

[a] Most probable number of colony-forming units (cfu) per gram of food.

[b] χ^2 is defined by McNemar as $(|a - b| - 1)^2/(a + b)$ where a = test portions positive by LOCATE and negative by culture method and b = test portions negative by LOCATE and positive by culture method. A χ^2 value greater than 3.84 indicates significance at $p < 0.05$.

[c] Sensitivity rate is defined as 100 × the total number of analyzed positive test portions among "known" positive test portions ÷ total number of "known" test portions, where "known" positive is defined as test portions confirmed positive by the reference method.

[d] Incidence of false negatives is 100 – sensitivity rate.

[e] Specificity rate is defined as 100 × the total number of analyzed negative test portions among "known" negative test portions ÷ the total number of "known" negative test portions, where "known" negative is defined as test portions confirmed negative by the reference method and negative controls.

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

[g] Reflects the number of confirmed determinations that were equivalent for LOCATE and culture methods.

[h] Uninoculated control samples were by definition known negatives; sensitivity rates were not calculated.

[i] Statistical analysis was not applicable.

(*6*) Include one positive and one negative control with each group of tests.

(*7*) Reconstituted conjugate should be used within 28 days.

(*8*) Different pipet tips must be used for each test.

(*9*) Incomplete washes will adversely affect test result.

(*10*) Incubation times are crucial. Do not extend or decrease.

(*11*) Treat all materials in contact with bacterial cultures as biohazards and autoclave all materials after use.

E. Enrichment

(**a**) *Pre-enrichment.*—Pre-enrich test portion in nonselective medium to initiate *Salmonella* growth. Procedure will vary with product type and must be performed as indicated in **967.26** (*see* 17.9.02) or in *Bacteriological Analytical Manual*, 8th Ed., AOAC INTERNATIONAL, Gaithersburg, MD 20877, USA.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment culture to a tube containing 10 mL selenite cystine (SC) and 1 mL to a tube containing 10 mL tetrathionate (TT) broth. For raw or highly contaminated foods, RV medium replaces SC broth, in which case 0.1 mL pre-enrichment culture is added to 10 mL RV. Incubate according to product type: (*1*) *Processed foods*.—Incubate SC and TT broths for 6 h at 35 and 42°C, respectively. (*2*) *Raw and highly contaminated products*.—Incubate TT broth and RV medium for 18 ± 2 h at 42°C.

(**c**) *Post-enrichment.*—After selective enrichment incubation, transfer 1 mL TT broth culture to tube containing 10 mL modified GN broth. Transfer 1 mL SC broth culture to separate tube containing 10 mL modified GN broth. Incubate according to product type: (*1*) *Processed foods* .—Incubate both GN broths for 18 h at 42°C. (*2*) *Raw and highly contaminated products*.—Incubate GN broths for 6 h at 42°C. Reincubate TT broth, SC broth, and RV medium at their respective temperatures for incubation up to total of 24 ± 2 h.

(**d**) *Preparation for enzyme immunoassay.*—After incubation, transfer 1 mL of each modified GN broth into 10 mL test tube, mix combined GN broths, and autoclave or boil for 20 min. Cool broth to room temperature and perform enzyme immunoassay.

F. Assay Procedure

(*1*) Remove the number of wells required to perform assay: one well per test portion, one positive control, and one negative control well. Press these firmly into the tray provided.

(*2*) Add 100 µL controls/well and 100 µL autoclaved or boiled incubated broths, **E**(**d**)/well to the microtiter plate. Record test positions on the record sheet. Incubate microtiter plates for 30 min at room temperature.

(*3*) Wash each well with a working solution of wash buffer (ca 250 µL/well) and quickly invert plate to empty contents into a container. Repeat this washing process 3 more times with working wash buffer and once with distilled water. Strike the plate face down several times on a paper towel placed on a flat surface.

(*4*) Add 100 µL working conjugate to each test and control well. Incubate for 30 min at 22–25°C.

(*5*) Wash each well as in step *3*.

(*6*) Add 100 µL TMB substrate/well. Incubate in the dark at room temperature (22–25°C) for 30 min.

(*7*) For visual interpretation, read results immediately after incubation. Do not stop reaction with stopping solution.

(*8*) For automated interpretation, stop reaction with 100 µL stopping solution/well. Read within 5 min. Measure plate absorbance with a microplate reader at 450 nm and record absorbance values on record sheet.

G. Assay Results

(*1*) *Visual assessment.*—Must be done prior to addition of stop solution. Positive control should give strong blue color and negative control should be clear or very pale blue. A test is considered positive when it is bluer in color than the negative control. It is considered negative when its color intensity is equal to or less than that of the negative control.

(*2*) *Automated interpretation.*—Place plate in reader and read at 450 nm. The negative control should always give $A_{450} < 0.3$ and the positive control, $A_{450} > 1.0$. $A_{450} > 0.3$ for negative control indicates insufficient washing. For the test, $A_{450} \geq 0.3$ indicates a positive test, and <0.3 indicates a negative test.

For both visual and automated interpretation, negative and positive tests can be interpreted only if the expected results are produced with controls. If controls are not within limits, the test must be repeated.

H. Confirmation

Enrichment broths and GN broths must be streaked on selective media as described in **967.28B** (*see* 17.9.07), and typical or suspect colonies must be identified as described in **967.26C** (*see* 17.9.02), **967.27** (*see* 17.9.03), and **967.28** (*see* 17.9.07). As an alternative to conventional tube system [**967.27** (*see* 17.9.03)] for *Salmonella,* any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in **978.24** (*see* 17.9.04), **989.12** (*see* 17.9.05), and **991.13** (*see* 17.9.06).

Reference: *J. AOAC Int.* **81**, 419(1998).