AOAC Official Method 993.06
Staphylococcal Enterotoxins in Selected Foods

Polyvalent Enzyme Immunoassay Method (TECRA SET)
First Action 1993
Final Action 2000

(Applicable to detection of 1.3–3.3 ng/mL staphylococcal enterotoxin in extracts prepared from selected foods [*see* Table **993.06**] containing 4–10 ng/mL staphylococcal enterotoxin. Specific toxin serotypes A to E are not differentiated.)

See Table **993.06** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Detection of staphylococcal enterotoxins (SETs) is based on enzyme immunoassay (EIA) using mixture of high-affinity capture antibodies to each toxin (A–E). SETs present in test sample extract will bind to antibody mixture that has been adsorbed onto surface of microtiter wells. Other materials in test extract are washed away. Enzyme-labeled antibodies (conjugate) specific for SETs are added, and assay results are identified by conversion of colorless substrate to green product.

B. Apparatus

(**a**) *Strip holder.*—For securing individual wells or strips of 12 wells.

(**b**) *Strip cover.*—To cover microtiter plate, use plastic film wrap or aluminum foil with plastic microtiter plate top.

(**c**) *Package insert.*—Color comparison chart.

(**d**) *Blender.*—High speed.

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

(**f**) *Centrifuge.*—Capable of 1000–3000 *g*.

(**g**) *Syringes.*—Disposable, plastic, ca 25 mL.

(**h**) *Tubes.*—10 mL, polypropylene.

(**i**) *Plastic squeeze bottle.*—500 mL.

(**j**) *Pipets.*—(*1*) 50–240 µL, adjustable; (*2*) 5–50 µL, adjustable; with suitable plastic tips.

(**k**) *Cotton.*—Absorbent.

(**l**) *pH Paper.*—0–14 range.

(**m**) *Microtiter plate shaker.*—Optional.

(**n**) *Microtiter plate reader.*—Optional, but recommended; 414 ± 10 nm, single-wavelength reader; 405 and 490 nm (414 and 492 nm), dual-wavelength reader.

C. Reagents

(**a**) *Anti-SET antibody-coated microtiter strips.*—Strips of plastic wells coated with antibodies to SET (serotypes A–E) produced in sheep.

(**b**) *Wash solution.*—Contains 1.5 g tris(hydroxymethyl)aminomethane (Tris), 6 g NaCl, 2.0 g polyoxyethylenesorbitan monolaurate (Tween 20), and 0.001 g thimerosal in 25.0 mL H_2O.

(**c**) *Test suspension additive solution.*—Contains 2 g Tween 20 and 0.001 g thimerosal in 6.0 mL H_2O.

Table 993.06 Interlaboratory study results for detection of *Staphylococcus aureus* enterotoxins in foods by visual polyvalent enzyme immunoassay method

Food	Enterotoxin, ng/g	Reader[a]	X[b]	s_r	s_R	RSD_r, %	RSD_R, %
Beef/pasta	0	D	0.077	0.012	0.015	15.8	19.5
Beef/pasta	0	S	0.074	0.011	0.051	14.5	69.2
Beef/pasta	6	D	1.488	0.094	0.346	6.3	23.2
Beef/pasta	6	S	1.501	0.068	0.176	4.5	11.7
Chicken	0	D	0.075	0.009	0.019	11.5	24.9
Chicken	0	S	0.078	0.011	0.043	13.7	54.6
Chicken	4	D	0.470	0.038	0.128	8.2	27.2
Chicken	4	S	0.466	0.036	0.085	7.7	18.1
Lobster bisque	0	D	0.075	0.007	0.018	9.2	23.7
Lobster bisque	0	S	0.077	0.012	0.052	15.1	68.2
Lobster bisque	8	D	0.410	0.025	0.087	6.2	21.3
Lobster bisque	8	S	0.389	0.019	0.066	4.8	16.9
Mushrooms	0	D	0.095	0.008	0.015	8.6	16.2
Mushrooms	0	S	0.104	0.020	0.046	18.9	44.0
Mushrooms	10	D	0.435	0.031	0.099	7.0	22.7
Mushrooms	10	S	0.464	0.040	0.054	8.6	11.6
Nonfat milk	0	D	0.067	0.004	0.017	5.5	25.4
Nonfat milk	0	S	0.070	0.011	0.043	15.3	61.5
Nonfat milk	5	D	1.161	0.185	0.420	15.9	36.2
Nonfat milk	5	S	1.234	0.085	0.174	6.9	14.1

[a] D = Dual-wavelength microtiter plate reader; S = single-wavelength microtiter plate reader.

[b] X = Mean absorbance value.

(**d**) *Positive control solution.*—Prepare by diluting 25 µL positive control concentrate (containing SET serotype B, 0.1 g $Na_2B_4O_7$, 0.1 g NaCl, and 0.001 g thimerosal in 4.0 mL H_2O) in 2.5 mL wash solution, (**b**).

(**e**) *Negative control solution.*—Contains 0.0072 g Tris, 0.1 g NaCl, 0.001 g thimerosal, and 0.01 g Tween 20 in 6.0 mL H_2O.

(**f**) *Conjugate diluent.*—Contains 0.2 g $Na_2B_4O_7$, 0.1 g NaCl, 0.1 g gelatin, and 0.001 g thimerosal in 13.5 mL H_2O.

(**g**) *Conjugate.*—Vial contains lyophilized anti-SET (A–E) antibodies conjugated to horseradish peroxidase, 0.003 g $Na_2B_4O_7$, 0.002 g $CaCl_2$, and 0.0001 g thimerosal. Before use, reconstitute vial in 13 mL (vial) conjugate diluent, (**f**).

(**h**) *Substrate diluent.*—26.0 mL contains 0.2 g acetic acid and 0.01 g H_2O_2 in 26 mL H_2O.

(**i**) *Substrate.*—Vial of lyophilized preparation contains 0.01 g 2,2′-azino-di(3-ethylbenzthiazoline sulfonate), 0.01 g EDTA, and 0.1 g NaH_2PO_4. Before use, reconstitute vial in 26 mL (vial) substrate diluent, (**h**).

(**j**) *Stop solution.*—6.0 mL contains 0.15 g NaF in 6.0 mL H_2O.

(**k**) *Tris buffer.*—0.25M, pH 8. Add 30.28 g tris(hydroxymethyl)-
aminomethane to 700 mL H_2O. Stir on magnetic stirrer to dissolve. Adjust solution to pH 8.0 by adding HCl. Dilute to 1 L with H_2O and mix.

(**l**) *Sodium hydroxide solution.*—1M NaOH.

(**m**) *Hydrochloric acid solution.*—Dilute HCl solution, ca 0.1M.

(**n**) *Sodium hypochlorite solution.*—2%.

Items **B**(**c**) and **C**(**a**)–(**j**) are available as TECRA SET kit (TECRA International Pty. Ltd., PO Box 788, Willoughby, NSW, Australia, or International BioProducts, Inc., PO Box 0746, Bothell, WA 98041, USA).

D. General Instructions

It is not necessary to perform immunoassay under sterile conditions.

Do not mix components from different kit lots or use materials after expiration date. Bring reagents and test samples to room temperature (20–25°C) before testing begins. Store reagents at 2–8°C before and after use. Store wells not needed in reusable foil pouch with silica gel bag at 2–8°C. Before testing begins, ensure that all wells are securely in holder. Take care not to dislodge strips during testing. Well holders may be reused. *Do not reuse microtiter well*s.

To avoid chemical contamination, do not touch top or edge of wells with fingers or pipet tips. Use new pipet tip for each test suspension. Do not cross-contaminate wells. If plastic troughs are used to dispense conjugate and substrate, keep them separate. After use, rinse troughs thoroughly with water and dry; incomplete washing will adversely affect test outcome.

Mix all reagents and test suspensions well before use.

E. Preparation of Test Suspensions

Prepare test suspensions following (*1*) and (*2*), except for milk, omit (*1*).

(*1*) Add 50 mL 0.25M Tris buffer, pH 8.0, **C**(**k**), to each 25 g test portion. Blend 3 min at high speed. Transfer slurry to centrifuge bottle and centrifuge 10 min ≥ 3000 *g*.

(*2*) Prepare 25 mL disposable plastic syringe by inserting cotton plug ca 0.5 cm thick. Pump ca 5 mL water through plug to ensure plug is tightly packed. Remove plunger and pour in test suspension extract supernate. Insert plunger and carefully pump extract through, collecting in polypropylene tube. Check pH with pH paper and adjust, if necessary, to pH 7.0–8.0 with dilute NaOH or HCl.

Add 50 µL test suspension additive, **C**(**c**), to test sample and mix thoroughly. Use 200 µL filtered test sample for EIA analysis, **F**.

For raw or fermented food or for processed food with obvious can defects that might result in growth of organisms that produce peroxidase, check test suspension extract for presence of peroxidase which could interfere with proper interpretation of test results. To determine peroxidase presence, add 50 µL test suspension extract to 50 µL substrate, **C**(**i**), in empty microtiter well (no antibody to SET) and let stand 10 min. If test remains colorless (or original color), no peroxidase is present. If color changes to blue (or bluish-green), test extract contains intrinsic peroxidase which must be inactivated before EIA analysis. To inactivate intrinsic peroxidase, add 1 mL 30% (w/v) sodium azide solution to 4 mL test sample (final sodium azide concentration, 6%). Mix and let stand 1–2 min at room temperature. Retest test for peroxidase presence, as above. If reaction is colorless (or original color), proceed with EIA analysis.

F. EIA Determination

Secure desired number of anti-SET-coated wells in holder, allowing 1 well for each extract, 1 well for negative control, and 1 well for positive control. Fill each well with wash solution; let stand 10 min at room temperature (20–25°C). Empty wells by quickly inverting holder; remove any residual liquid by firmly striking holder facedown on paper towel several times.

Transfer 200 µL of test extracts and controls into separate wells and record position of each. Cover wells with plastic wrap or aluminum foil with plate top to prevent evaporation. Incubate wells 2 h at 35–37°C.

Wash wells using squeeze bottle with wash solution as follows: (*1*) ensure that wells are pressed firmly into holder; (*2*) quickly invert holder, emptying contents into trough containing 2% sodium hypochlorite; (*3*) remove any residual liquid by firmly striking holder facedown on paper towel several times; and (*4*) completely fill each well with wash solution from squeeze bottle, washing wells thoroughly (wash solution running into other wells during this procedure does not cause cross-contamination). Repeat steps (*1*)–(*4*) 3 , and empty wells as in steps (*2*) and (*3*).

Add 200 µL reconstituted conjugate, **C**(**g**), to each well. Cover wells and incubate 1 h at room temperature.

Empty wells and wash thoroughly 5 using steps (*1*) and (*2*). Empty wells and remove residual liquid as in steps (*2*) and (*3*).

Add 200 µL reconstituted substrate, **C**(**i**), to each well. Incubate 30 min at room temperature. Tap sides of plate gently to disperse color throughout wells (color development concentrates around well edge). Place wells on white background. Observe positive control well by looking directly down into well. Compare color in positive control well with color comparison chart. Continue incubating until positive control well reaches color ca equivalent to panel 4 on color comparison chart, reading against white background. Typical incubation time is 30–45 min.

Add 20 µL stop solution, **C**(**j**), to each well. Gently tap sides of plate to mix contents. Determine assay results visually or using microtiter plate reader.

G. Interpretation of Results

(**a**) *Visual interpretation using color comparison chart.*—Place wells on white background. Compare color of individual test wells with color comparison chart by looking directly down into well.

Positive control is calibrated internal standard. Obtaining correct color for positive control ensures that reaction time allows maximum sensitivity and reproducibility. If color of positive control is negative, repeat test with fresh reagents.

If color of negative control is darker than negative range on color comparison chart (panels 1 and 2), it is likely that wells were washed inadequately. Assay must be repeated.

Test sample is considered positive for SET when color of negative control is within negative range on color comparison chart and color of test sample is as (or more) intense than panel 3 on color comparison chart.

Test sample is considered negative for SET when colors of *both* negative control and test sample are within negative range on color comparison chart (panels 1 and 2).

(**b**) *Absorbance measurement using microtiter plate reader.*—Adjust microtiter wells to be flush in holder.

For single-wavelength microtiter plate reader, read absorbance at 414 ± 10 nm. Standardize reader to zero with wash solution.

For dual-wavelength readers, set absorbance at 405 and 490 nm (or 414 and 492 nm) for measuring and reference wavelengths, respectively.

Absorbance of positive control should be ≥ 1.0 to indicate that all reagents are functional.

Absorbance of negative control should be ≤ 0.200. If >0.200, well was not washed adequately, and assay must be repeated. Refer to troubleshooting guide in package insert.

Test sample is considered positive for SET if absorbance is >0.200 and is considered negative if absorbance is ≤ 0.200.

Reference: *J. AOAC Int.* **77**, 357(1994).

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