

Addendum I

DRY REHYDRATED FILM METHOD FOR STANDARD COLONY COUNT

(Petrifilm 3M)

(Method according to Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29)

- (1) Mix milk thoroughly before sampling from bulk milk
 - (i) Laboratory work must be performed at room temperature.
 - (ii) Prepare a 1:10 dilution by adding 1 ml of milk to 9 ml of sterile phosphate buffer. Mix well. Prepare a 1:100 dilution by adding 1 ml of the 1:10 dilution to 9 ml of sterile phosphate buffer. Mix well. Prepare a 1: 1000 dilution by adding 1 ml of the 1:100 dilution to 9 ml of sterile phosphate buffer. The final pH should be between 6.6 and 7.4.
- (2) Place the films for aerobic bacterial counting on a flat surface and label them. Lift the top film and carefully transfer 1 ml of the 1:1000 dilution to the center of the bottom film by holding the pipette perpendicular to the film. Release the top film to drop onto the sample. Repeat the process with the 1:100 dilution of the sample.
- (3) Distribute the sample evenly on the film by applying gentle downward pressure with a spreader. Remove the spreader and leave the film undisturbed for one minute to solidify.
- (4) Stack the films in piles of not more than 20 and incubate the films, with the clear sides up, at $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 48 ± 2 hours.
- (5) Remove the films from the incubator at the end of the incubation period and count the colony forming units (CFU) with the aid of magnification under uniform artificial illumination.

- (i) All the red colonies, regardless of their size and intensity, should be counted. Films with 25 - 250 CFU should be counted. Calculate the number of viable bacteria per ml milk.
- (ii) An estimated count can be made on films where the CFU exceeds 250, by counting at least 4 squares or 20 percent of the growth area. Calculate the number of viable bacteria per ml milk and record as “estimated” count.
- (iii) The presence of very high concentrations of colonies cause the entire growth area of the film to become red or pink in colour and/or numerous bacteria are growing on the edges of the growth zone. Report these as too numerous to count (TNTC).

Phosphate buffer

Potassium dihydrogen orthophosphate5.08 g
 Disodium hydrogen orthophosphate in 2 l distilled water 13.63 g
 Sterilize for 15 minutes at 121° C

Dry rehydrated film for standard colony count

	% solids on film
Cold water soluble gel	1 - 10%
Tetrazolium indicator dye	< 1%
Standard method nutrients.....	1 - 5 %

Addendum II

DRY REHYDRATED FILM METHOD FOR COLIFORM AND *ESCHERICHIA COLI* COUNT (Petrifilm 3M)

(Method according to the Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29)

- (1) Mix raw or pasteurized milk thoroughly before sampling from bulk. The pH should be between 6.6 and 7.4.
- (2) Place the films for *E. coli* and coliform counting on a flat surface and label them. Lift the top film and carefully transfer 1 ml of the milk to the center of the bottom film by holding the pipette perpendicular to the film.
- (3) Slowly roll the top film onto the sample to prevent air bubbles being trapped under the top film.
- (4) Distribute the sample evenly on the film by applying gentle downward pressure with a spreader. Remove the spreader and leave the film undisturbed for one minute.
- (5) Stack up to 20 films and incubate the films, with the clear side up, at 32°C for 24 (±2) hours.
- (6) At the end of the incubation period remove the films from the incubator and count the colonies with the aid of magnification under uniform artificial illumination.

Re-incubate films for an additional 24 (±2) hours to detect any additional *E coli* growth.

- (i) Blue colonies associated with gas are *E. coli* and red colonies associated with gas are coliform colonies. Colonies that are not associated with gas are not counted as coliforms colonies. All the red and blue colonies associated with gas represent the coliform colony count. Films with 15 - 150 colonies should be counted.
- (ii) An estimated count can be made on films where the colonies exceed 150, by counting at least four squares and multiplying the obtained number of colonies by 5. Record as “estimated” coliform colony count.
- (iii) The presence of very high concentrations of colonies cause the entire growth area on the film to become purple blue (*E. coli*) or reddish (coliforms) and /or many small colonies and/ or small gas bubbles are present. This must be recorded as too numerous to count (TNTC).

Dry rehydrated film for coliform and E.coli counts

	% of solid on plate
Violet red bile nutrients	1 - 5%
Cold water soluble gel	1 - 10%
Tetrazolium indicator dye	<1%
Glucuronidase indicator	<1%

Addendum III

ASCHAFFENBURG AND MULLEN PHOSPHATASE TEST

(Method according to the Foodstuffs, Cosmetics and Disinfectants Act, No 54 of 1972: Regulations relating to milk and milk products, No R.1555. *Government Gazette* No 18439, 21 November 1997, 4-29)

1. All glassware and media must be sterile.
2. Milk samples to be examined can be stored at 4°C, but must not be frozen. Heat the samples to 20°C - 25°C before examination.
3. **Precautions:**
 - 3.1 Do not test a milk sample that shows signs of sourness.
 - 3.2 Pipettes must not be contaminated with saliva.
 - 3.3 The substrate (di-Sodium-p-nitrophenyl-phosphate) must be stored in a fridge (4°C).
 - 3.4 Do not perform the test in direct sunlight.
 - 3.5 Use only distilled water for the test.
 - 3.6 Glassware must be cleaned in a specific manner and stored separately from other glassware.
4. **Cleaning of glassware:**
 - 4.1 Rinse with water.
 - 4.2 Wash thoroughly with warm water containing soda.
 - 4.3 Rinse with distilled water and air dry.
 - 4.4 Sterilize and store for future use.

- 4.5 Glassware for the test shall not be used for any other purpose and shall be kept separate from all other operations in the laboratory.

5. Preparation of chemicals:

5.1 Buffer solution:

Weigh 0.175 g anhydrous sodium carbonate and 0.075 g sodium bicarbonate and place into a 50 ml volumetric flask. Fill with distilled water to the 50 ml mark. Dissolve the contents by gentle mixing. If the meniscus falls below the 50 ml mark, refill with buffer solution and again mix gently. Return the stopper and mark contents clearly.

5.2 Buffer substrate solution:

Weigh 0.03 g p-nitrophenyl phosphate and place into a 20 ml volumetric flask. Add above buffer substrate up to the 20 ml mark and mix gently. If the meniscus falls below the 20 ml mark, more buffer solution must be added and mixed gently. Stopper and store the solution in a fridge (4°C). Protect the solution against sunlight by storing in a brown bottle or wrapping the flask in foil to exclude all light. Label the flask clearly-date, the solution and initial. Under correct storing conditions the solution has a storage life of 7 days after which it must be disposed of.

- 5.3 Put 5 ml of the buffer substrate solution into a test tube (that will fit into the "Lovibond" apparatus) and stopper. Heat contents to 37°C in a water bath.
- 5.4 Add 1 ml of the milk sample, return the stopper and mix by shaking.
- 5.5 Incubate for exactly 2 hours at 37°C in a water bath.
- 5.6 Prepare a control sample consisting of 5 ml buffer substrate solution and 1 ml boiled milk of the same milk sample. Mix by shaking and incubate for exactly 2 hours in a water bath at 37°C.
- 5.7 Remove the milk sample and control and read the result with the "Lovibond". Use the Lovibond disk displaying A.P.T.W.5 or A.P.T.W.7.
- 5.8 Record the result.

Addendum IV

COUNTING OF SOMATIC CELLS IN MILK BY MEANS OF THE FOSSOMATIC (FSCC)

(Method as per the instruction manual of the Fossomatic)

1. General

Milk samples:

- Unpreserved milk samples should be stored in a refrigerator between 0°C and 5°C for not longer than 60 hours.
- Samples preserved with potassium dichromate can be stored for 7 days at room temperature.

Fossomatic:

- An approximate standardization is carried out by the manufacturers.

2. Equipment and materials

- Fossomatic;
- Water bath operating at 40°C.
- Pressure supply of at least 6 bar.
- Fixative: Potassium Dichromate.
- Reagents:
 - * Ethidium Bromide;
 - * Triton X-100;
 - * Potassium Hydrogen Phthalate;
 - * Potassium Hydroxide;
 - * Ammonia solution (25%);
 - * Deionized water;

3. Preparation of solutions

A) Basic solutions:

3.1 Ethidium Bromide - **DANGER CARCINOGENIC**

3.1.1 Weigh off 1.0 g Ethidium Bromide

3.1.2 Dissolve the 1.0 g Ethidium Bromide in 1 000 ml deionized water.
The process can be speeded up by heating to 40-60°C.

3.1.3 Store in a lightproof and airtight bottle no longer than 60 days (1 litre will be enough for \pm 10 000 samples).

3.2 Triton X - 100

3.2.1 Dissolve 10 ml Triton X - 100 in 1 000 ml deionized water heated to \pm 60°C.

3.2.2 The solution can be stored airtight for maximum 25 days (1 litre will be enough for \pm 5 000 samples).

3.3 Buffer

3.3.1 Dissolve 51.0 g Potassium Hydrogen Phthalate and 13.75 g Potassium Hydroxide in 10 litres of deionized water. Process can be speeded up by heating to 50°C.

3.3.2 Add 150 ml of the Triton X - 100 solution.

3.3.3 Store airtight no longer than 7 days (10 litres will be enough for \pm 1000 samples).

B) Working solutions:

4. Dye solution

4.1 Add 26 ml Ethidium Bromide solution (3.1.3) to 2.5 litres of buffer (enough for \pm 250 samples).

5. **Rinsing liquid**
 - 5.1 Add 10 ml of Triton X-100 solution (3.2.2) and 25 ml of a 25 % ammonia solution to 10 litres of deionized water (enough for \pm 350 samples).
6. Working solutions should not be used if more than 7 days old.
7. Procedure for fixing and counting of samples.
 - 7.1 Milk samples must be taken in a sterile, particle-free container with a leak proof cap.
 - 7.2 Add from 5mg to 10mg of potassium dichromate to a 5 ml sample and mix to a uniform colour.
 - 7.3 Store for at least 18-24h at room temperature. If they have to be counted within 24 hours, they should be treated with potassium dichromate for at least 3 hours.
 - 7.4 Prepare samples by warming it to 40°C in a water bath for 5 minutes.
 - 7.5 Remove the sample from the bath and keep at room temperature until counted (within 15 minutes).
 - 7.6 Use a clean pipette tip for each sample. Press the tip firmly on the pipette.
 - 7.7 Mix sample prior to counting by inverting it gently at least 5 times.
 - 7.8 Open the cap.
 - 7.9 Draw up 0.2 ml sample by pressing the button down to the first stopping position only. Release the button of the pipette slowly while taking care that no air bubbles are drawn up.
 - 7.10 Keep the pipette in a vertical position.
 - 7.11 Wipe tip on the outside gently with a tissue to remove excess milk. Take care not to touch the bottom end of the tip with the tissue.
 - 7.12 Inject the sample into the intake chamber of the Fossomatic by pressing the button on the pipette down firmly and completely and once only.
 - 7.13 Results are displayed and printed and must be multiplied by 1000 to give the somatic cell count per ml milk. Identify the result with the sample number.

Addendum V

THE BRILLIANT BLACK REDUCTION TEST FOR DETECTING RESIDUES OF ALL ANTIBIOTICS AND SULFONAMIDES (BR TEST AS)

(Method according to Bishop *et al.* (1994) *In:* Marshall R.T. (ed.) Standard methods for the examination of dairy products. American Public Health Association, Washington, D.C.: 389-391)

The BR TEST AS combines the methods of agar diffusion and colour reduction. The BR test system contains endospores of *Bacillus stearothermophilus* var. *calidolactis*, strain C953, nutrients, the redox indicator "brilliant black," the antifolate "Tetroxoprim (TXP)," and agar-agar. The BR TEST AS detects the residues of all antibiotics and sulfonamides in one cycle. The detection limits of the various antibiotics and sulfonamides are compiled in Table 19. Drug residues exceeding the detection limits inhibit the metabolism of the indicator test organisms during incubation. Thus, reduction of the indicator is prevented and the blue colour of the BR test system is maintained. During incubation of inhibitor-free milk samples, the blue oxidation stage of the redox indicator is irreversibly converted to the yellow reduction stage. The test is useful with raw or pasteurised fluid milk products.

A. *Equipment and supplies:*

1. BR TEST AS 12x8 test strip kit: Contains three BR test plates consisting of 12 strips for 8 samples each and 36 adhesive tapes for sealing the test strips. The test plates, which are welded with an aluminium foil film, have a shelf life of 3 months when stored at 2° to 4°C.
2. Microliter pipet: 0.1 ml, with pipet tips.
3. Water bath: Without a mechanical agitator; alternatively a thermostat-controlled heating block (64° ± 1°C).
4. Floating stands made of Styropor: As supports for the BR test tubes in the water bath.

5. Inhibitor-free milk: When the negative result is available, this milk can be frozen in small portions and stored as a negative standard.

6. Positive milk standard:

a. Lyophilised penicillin standard

b. Lyophilised sulphonamide standard

Dissolve each in 5 ml of MS (microbiologically suitable) water, producing positive milk standards containing 0.005 IU of penicillin G sodium (a) and 0.5 μg of sulfamethazine per millilitre (b).

B. Controls:

Prepare a negative control to check the proper function and to determine the reduction time. Prepare one positive control from each of the penicillin and sulphonamide standards to check the test sensitivity.

1. Negative control: 0.1 ml of inhibitor-free bulk milk.

2. Positive control: Lyophilised positive standards dissolved in 5 ml of MS water.

Reduction time is over when the redox indicator in the negative control has changed from blue to yellow. Read the two positive controls at the same time.

Table 19: Minimum inhibitor concentrations (MIC)^a of antibiotic and sulphonamide substances detectable by the BR TEST AS

Substance	Minimum Inhibitor Concentrations
Penicillin-G-Na ^b	0.0025-0.003
Procaine penicillin G	0.0020
Cloxacillin	0.0200-0.040
Ampicillin	0.0015
Cephapirin	0.0020
Dihydrostreptomycin	3.000-5.000
Neomycin	0.200-0.450
Gentamycin	0.350-0.400
Erythromycin	0.030-0.075
Tylosin	0.009-0.030
Chlortetracycline hydrochloride	0.350-0.650
Oxytetracycline hydrochloride	0.200-0.300
Tetracycline hydrochloride	0.200
Chloramphenicol	1.800-3.000
Bacitracin ^b	0.040-0.050
Virginiamycin	0.120-0.200
Sulfadimidine-Na (sulfamethazine)	0.075-0.300
Sulfanilamide	2.000
Sulfadoxine	0.030-0.100
Sulfaguanide	0.200
Sulfathiazole	0.005-0.010
Sulfadiazine	0.010-0.080
Sulfaphenazole	0.007-0.010
Dapsone	0.003-0.006

^aBecause the BR TEST AS functions with milk only, all MIC values have been determined in raw milk. The added inhibitors bind with the milk proteins. Only the free, not the protein-bound, portion has a direct antibacterial effect. Thus, the absolute test sensitivity for some of the drugs is considerably higher than that shown in the Table. The MIC values have been determined in the residue laboratory of the Tierärztliche Hochschule in Hanover, Germany, by Prof. Dr. Wenzel and Dr A Ebrecht.

^bValues are in international units per millilitre; all other values are in micrograms per millilitre (ppm) of milk.

C. Procedure, screening test:

1. Indelibly and clearly mark each tube, strip, or plate to identify the samples.
2. Remove the cap(s) from the tube(s), or the cover(s) from the strip(s) or plate(s).
3. Add 0.2 ml of the well-mixed sample to each tube or well, changing the pipet or pipet tip with each new sample.
4. Cover the tube(s) with the cap(s), or the strip(s) or plate(s) with adhesive foil.
5. Incubate at $64^{\circ} \pm 1^{\circ}\text{C}$ for approximately 2.75 hours. Incubation time with a heating block is more variable than with a water bath because heat transfer is less efficient in the former. Tubes are placed in the Styropor floater for incubation in the water bath; strips and plates are floated in the bath without a support.
6. Incubate a negative control with each set of tests and each new lot of tubes, strips, or plates within a set. Stop the incubation when the colour in this control changes from blue to yellow. Check for the change in colour at 10-minute intervals after 2.5 hours of incubation.
7. Interpretation: Samples containing inhibitor residues above concentrations in Table 19 show no colour change; that is, they retain their blue to the end of incubation. They are considered positive.

Addendum VI

BRUCELLA RING TEST

(Method according to the standards written up by the South African Institute of Medical Research (S.A.I.M.R.) Code 0615)

A. Equipment and materials

Brucella abortus antigen (obtained from the Veterinary Research Institute, Onderstepoort).

4 x 30 μl pipette tips

Pipettes

3 x 1 ml plastic pipettes

3 x sterile soda glass tubes (15x125 mm)

37°C \pm 0.5°C incubator

10 ml boiled milk (negative control)

30 μl Brucella positive control (used for TMX controls)

B. Milk sampling

1. Milk must be thoroughly mixed before sampling.
2. The sample should be taken well below the milk surface.
3. Milk from 2-3 cans may be pooled. In the case of bulk tanks a single sample is satisfactory provided the test is done with a double volume of milk but a single volume of antigen.
4. Refrigerate the milk sample at 4°C for at least 12 hours before testing.
5. Milk may be stored for up to 2 weeks at 4°C without loss of titre but souring may be a problem.
6. Avoid excessive shaking of milk sample.
7. Excessive heating (45°C for more than 5 minutes) leads to destruction of Brucella antibody.
8. The test cannot be carried out on homogenized pasteurised milk.

9. The milk must have sufficient cream but too much cream can interfere with test readings.
10. Sour milk makes reading of the test impossible.

C. Method

1. Mix antigen thoroughly and pour sufficient for the day's testing into another bottle and keep at room temperature together with rest of samples for at least 1 hour before testing. Any antigen left after a day's testing must be discarded.
2. Shake antigen thoroughly and dispense 0.03 ml (30 μ l) amounts into three tubes labeled "test" "negative control" and "positive control".
3. Mix the milk samples thoroughly but gently and dispense 1 ml into the tube labeled "test" and the "positive control" tube. Add 1 ml of boiled milk sample into the tube labeled "negative control".
4. Add 30 μ l Brucella positive control sera to the positive control tube.
5. Shake well to mix thoroughly within 1 minute after the milk has been added to the antigen.
6. Incubate at 37°C \pm 0.5°C for 1 hour.

D. Results:

Positive: Cream layer darker blue or same shade blue as milk column.

Negative: Cream layer white or lighter shade of blue than the milk column.

Negative control will have no blue ring.

Positive control will have a blue ring.

Information

Individual cows may be tested but tend to give more false positives. This may be overcome by serial dilutions of the milk with known negative milk. A reaction of 1:10 or higher is indicative of infection.

Addendum VII

BAIRD-PARKER AGAR BASE

(Method according to the manufacturer's instructions: Biolab Code C 41)

A highly selective medium for the isolation of coagulase-positive *Staphylococci*

COMPOSITION

Tryptone	10.0
Meat extract	5.0
Glycine	12.0
Yeast extract	1.0
Lithium chloride	5.0
Sodium pyruvate	10.0
Agar	20.0
pH = 6.8 (± 0.2)	

PREPARATION

Suspend 63 g in 1 litre of distilled water. Bring to the boil whilst stirring until dissolved. Dispense 90 ml volumes into bottles and sterilize in the autoclave at 121°C for 15 minutes. This medium can be stored for 1 month at 4°C.

To each 90 ml of the basal medium aseptically add at 50°C, 1 ml of 1% sterile Potassium Tellurite solution (BX15) and 5 ml of a 50% Egg Yolk Emulsion (BX14). Pour 15 ml volumes into petri dishes.

METHOD

Plates should be used within 24 hours and should be dry when inoculated.

Spread 0.1 to 1 ml of the dilutions on the surface of plates. Incubate at 35°C (or 37°C) for 24-26 hours and for a further 24 hours if no *Staphylococcus aureus* are seen.

EVALUATION

Staphylococcus aureus colonies are black, glossy and convex with a diameter of 1 to 1.5mm with a white margin surrounded by a clear zone 2 to 5mm broad. After 48 hours they may give an opaque zone extending into the clear medium. The medium tends to inhibit growth of coagulase-negative organisms, colonies which appear are irregular and usually have broad opaque zones.

Micrococci occasionally grow to form very small black or brown colonies. Yeasts may grow as white colonies, and *Bacillus* species as brown, matt colonies.

Addendum VIII

THE STAPHYLASE TEST

(Method according to the manufacturer's (Oxoid) instructions)

1. General

- 1.1 Principle: To determine the presence of coagulase producing staphylococci through clumping of fibrinogen-sensitized sheep red blood cells. (*Staphylococcus aureus*, *Staphylococcus intermedius* and *Staphylococcus hyicus* subsp. *hyicus* (11-89%).
- 1.2 Detection of coagulase ("clumping factor"). Easy reading because of colour reagents.

2. Materials

The Staphylase Test Kit (Oxoid) consisting of:

- Staphylase Test Reagent.
- Staphylase Control Reagent.
- Disposable Reaction Cards.

Note: **BOTH REAGENTS CONTAIN 0.1% SODIUM AZIDE AS PRESERVATIVE.**

3. Procedure

- 3.1 Store Staphylase Test kits at 2°C - 8°C in an upright position.
- 3.2 Perform this test on all KOH(-), catalase(+) cultures.
- 3.3 Shake the Test and Control Reagents vigorously to obtain a homogenous suspension. Reagent cells in the dispensing pipettes should be mixed in the suspension.
- 3.4 Cut the reaction cards into strips, one strip with test and control circle for each culture.

- 3.5 Using a 1 μ l plastic loop, smear colonies on the test and control strip of a reaction card for that colony.
- 3.6 Add one drop of test reagent to the test circle and one drop of control reagent to the control circle.
- 3.7 Mix the contents of the test circle with a 1 μ l plastic inoculation loop. Observe for agglutination while mixing. Repeat the process with a new 1 μ l plastic inoculation loop for the control circle. The control circle should show no agglutination. If the control circle shows a positive reaction, contact your supervisor.
- 3.8 Do the test with the positive control culture available.
Note: *Staphylococcus sciuri* may show false positive results.
The use of high-salt media may show a weaker reaction than usual. The staphylase test has an extremely high correlation with the tube coagulation test.
- 3.9 Record results.
- 3.10 Clean up workplace and store materials.

Addendum IX

DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN

(Method according to the manufacturer's (Oxoid) instructions)

PRINCIPLE OF ASSAY

Polystyrene latex particles are sensitized with purified antiserum taken from rabbits, immunized individually with purified staphylococcal enterotoxins A, B, C and D. These latex particles will agglutinate in the presence of the corresponding enterotoxin. A control reagent is provided which consists of latex particles sensitised with non-immune rabbit globulins. The test is performed in V-well micro titre plates. Dilutions of the food extract or culture filtrate are made in five rows of wells, a volume of the appropriate latex suspension is added to each well and the contents mixed. If staphylococcal enterotoxins A, B, C and D are present, agglutination occurs, which results in the formation of a lattice structure. Upon settling, this forms a diffuse layer on the base of the well. If staphylococcal enterotoxins are absent or at a concentration below the assay detection level, no such lattice structure can be formed and, therefore, a tight button will be observed.

The diluent provided contains sodium hexametaphosphate, which has been shown to reduce the incidence of non-specific reactions with components of food matrices.

PRECAUTIONS

This product is for *in vitro* diagnostic use only.

Do not freeze.

Reagents with different lot numbers should not be interchanged.

Reagents and diluent contain 0.1% sodium azide as a preservative. Sodium azide may react with lead or copper plumbing to produce metal azides which are explosive by contact

detonation. To prevent azide accumulation in plumbing, flush with copious amounts of water immediately after waste disposal.

STORAGE

The SET-RPLA Kit must be stored at 2°C to 8°C. Under these conditions the reagents will retain their reactivity until the date shown on the kit box. After reconstitution, the enterotoxin controls should be stored at 2°C to 8°C. Under these conditions, the reconstituted enterotoxin controls will retain their reactivity for 3 months, or until the date shown on the kit box, whichever is the sooner.

METHOD OF USE

1. **Materials required but not provided.**

Blender or homogeniser

Micro titre plates (V-well) and lids

Fixed or variable pipette and tips (25µl)

Centrifuge capable of generating 900g (typically 3 000rpm in a small bench top centrifuge)

Membrane filtration unit using low protein-binding disposable filters with a porosity of 0.2µm-0.45µm (such as Millipore SLGV)

Sodium chloride solution (0.85%)

Sodium hypochlorite solution (>1.3% w/w)

25µl dropper (optional)

25µl diluter (optional)

Micro mixer (optional)

Moisture box (optional)

2. Components of the kit

- TD901** **Latex sensitised with anti-enterotoxin A.** Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin A.
- TD902** **Latex sensitised with anti-enterotoxin B.** Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin B.
- TD903** **Latex sensitised with anti-enterotoxin C.** Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin C.
- TD904** **Latex sensitised with anti-enterotoxin D.** Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin D.
- TD905** **Latex control.** Latex suspension sensitised with non-immune rabbit globulins.
- TD906** **Staphylococcal enterotoxin A control.**
- TD907** **Staphylococcal enterotoxin B control.**
- TD908** **Staphylococcal enterotoxin C control.**
- TD909** **Staphylococcal enterotoxin D control.**
- TD910** **Diluent.** Phosphate buffered saline containing bovine serum albumin and sodium hexametaphosphate.

3. Toxin Extraction or Production

3.1 Blend 10g of sample with 10 ml of sodium chloride solution (0.85%) in a blender or homogeniser.

3.2 Centrifuge the blended sample at 900g at 4°C for 30 minutes.

NOTE: If refrigerated centrifuge is not available, cool the sample to 4°C before centrifugation.

- 3.3 Filter the supernatant through a $0.2\mu\text{m}$ - $0.45\mu\text{m}$ low protein-binding membrane filter.
Retain the filtrate for assay of toxin content.

4. **Control**

Each reconstituted toxin control will cause agglutination with its respective sensitised latex. The use of the toxin controls will provide references for the positive patterns illustrated below (see Interpretation of Test Results). The controls should be used from time to time only to confirm the correct working of the test latex. The toxin controls are not provided at a specified level and therefore must not be used as a means of quantifying the level of toxin detected in the test sample.

5. **Assay method**

5.1 *Working reagents*

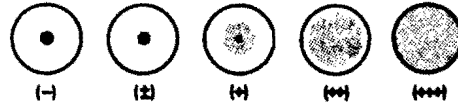
The latex reagents and diluent are ready for use. The latex reagents should be thoroughly shaken before use to ensure a homogeneous suspension. To reconstitute the control reagents, add 0.5 ml of diluent (TD910) to each vial. Shake gently until the contents are dissolved.

- 5.2 Arrange the plate so that each row consists of 8 wells. Each sample needs the use of 5 such rows.
- 5.3 Using a pipette or dropper, dispense $25\mu\text{l}$ of diluent in each well of the 5 rows.
- 5.4 Add $25\mu\text{l}$ of test sample to the first well of each of the 5 rows.
- 5.5 Using a pipette or diluter and starting at the **first well** of each row, pick up $25\mu\text{l}$ and perform doubling dilutions along each of the 5 rows. **Stop at the 7th well** to leave the last well containing diluent only.
- 5.6 To each well in the first row, add $25\mu\text{l}$ of latex sensitised with anti-enterotoxin A.
- 5.7 To each well in the second row, add $25\mu\text{l}$ of latex sensitised with anti-enterotoxin B.
- 5.8 To each well in the third row, add $25\mu\text{l}$ of latex sensitised with anti-enterotoxin C.
- 5.9 To each well in the fourth row, add $25\mu\text{l}$ of latex sensitised with anti-enterotoxin D.
- 5.10 To each well in the fifth row, add $25\mu\text{l}$ of latex control.

- 5.11 To mix the contents of each well, rotate the plate by micro mixer or agitate by hand. Take care that no spillage occurs from the wells.
- 5.12 To avoid evaporation, cover the plate with a lid. Placing the plate in a moisture box is an acceptable alternative. **Leave the plate undisturbed** on a vibration-free surface at room temperature for 20 to 24 hours. It will help subsequent reading of the test if the plate is placed on black paper for the duration of this incubation.
- 5.13 Examine each well in each row for agglutination, against a black background.
- 5.14 Centrifuge tubes, membrane filters, micro titre plates, lids and pipette tips should be sterilized by autoclaving at 121°C or disinfected before disposal in hypochlorite solutions (>1.3% w/w).
- 5.15 Dispose of culture extracts, food extracts, samples and toxin controls in hypochlorite solutions (>1.3% w/w).

INTERPRETATION OF TEST RESULTS

The agglutination pattern should be judged by comparison with the following illustration:



Results classified as (+), (++) and (+++) are considered to be positive

Results in the row of wells containing latex control should be negative. In some cases, non-specific agglutination may be observed. In such cases the results should be interpreted as positive, provided that the reaction with sensitised latex is positive to a higher dilution of test samples than that seen with the latex control. The last well in all rows should be negative. If positive patterns are observed in some of these wells, the reaction should be regarded as invalid.

NOTE: Certain staphylococcal strains are known to produce more than one enterotoxin.

LIMITATIONS OF THE TEST

The sensitivity of this test in detecting the enterotoxins has been reported to be 0.5ng/ml in the test extract. When a food extract is made with a dilution ration of 1:1 with diluent, the sensitivity is, therefore, 1ng/g of food matrix. The detection limit will vary according to any extra dilution conditions dictated by the type of food matrix. Concentrations of the enterotoxin in the food extract can be effected by a variety of methods, such as ultrafiltration. Production in culture of SETs depends on the growth conditions. A positive result obtained by the culture demonstrates the production of one or more SET under those circumstances; it does not imply the *in vivo* production of toxins to those levels.

Addendum X

Daily information sheet regarding the purchasing of milk

Date:

Day:

Week:

Sample no	Place of purchase	Time of purchase	Price	Temp	Pasteurised ?	Expiry date

Time at beginning of Sampling:

