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EUROPEAN UNION-REFERENCE LABORATORY FOR COAGULASE POSITIVE STAPHYLOCOCCI

Maisons-Alfort Laboratory
for Food Safety

Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices

European screening method of the EU-RL for
"COAGULASE POSITIVE STAPHYLOCOCCI,
INCLUDING *STAPHYLOCOCCUS AUREUS*"

Version 5, September 2010

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REPUBLIQUE
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This document is the fifth version of the European Screening Method of the European Union - Reference Laboratory for Coagulase Positive Staphylococci, including *Staphylococcus aureus* (EU-RL for CPS). This version updates the fourth version (dated on April 2010).

It comprises the detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices (milk and milk products and other food matrices).
It is a temporary document until the fully interlaboratory validation, planned in January 2011, of Vidas SET2 and Ridascreen SET Total detection kits for the detection of SE types A to E in all type of food matrices.

National Reference Laboratories (NRLs) for CPS are informed that a free-of-charge confirmation can be performed by the EU-RL for CPS in case of a positive result only if obtained strictly according to this document.

In this case and with prior agreement of the EU-RL, the extracts or the samples may be sent to the EU-RL with:
- the document entitled: Application form for confirmatory quantitative analysis « Staphylococcal enterotoxins in all types of food matrices » **(ANNEX I)**
- five CPS strains (in storage agar tube) isolated from each sample to be tested, the transport being conducted in accordance with the international transport regulation
- a minimum of 75 g test portion (in case of SFPO, a minimal size of the test portion to analysis is equal to **12.5 g**), which is requested to perform analysis.

The parcel should be addressed under refrigerated conditions at the address below:

ANSES
Maisons-Alfort Laboratory for Food Safety
CAT-BAC
Pôle HQSA
22, rue Pierre Curie
94700 Maisons-Alfort
FRANCE

List of the annex:
Application form for confirmatory quantitative analysis «Staphylococcal enterotoxins in all types of food matrices »

Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices

Foreword

The Commission Regulation (EC) N° 2073/2005 of 15 November 2005 [1] on microbiological criteria for foodstuffs prescribes in its Annex I a food safety criterion 1.1.21 the detection of staphylococcal enterotoxins in cheeses made from raw milk or thermized milk, when the criteria M for *S. aureus,* applied at the time during the manufacturing process when the number of staphylococci is expected to be the highest, is higher than 10^5 cfu/g. The criterion 1.1.21 refers to the European Screening Method of the European Union Reference Laboratory for Coagulase Positive Staphylococci, including *Staphylococcus aureus* (EU-RL CPS), as the analytical reference method for the screening of staphylococcal enterotoxins in the cheeses concerned.
Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases resulting from the ingestion of staphylococcal enterotoxins (SEs) preformed in foods by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *Staphylococcus aureus.* These toxins are heat and pH-resistant. The European Food Safety Authority reported that *Staphylococcus spp.* was involved in 5.5% of Food Borne Outbreaks (FBO) reported in 2008 [4].

1. Scope

This document describes the European Screening Method of the EU-RL for CPS in all types of food matrices.
Only one screening method will be used to detect staphylococcal enterotoxins (SEA to SEE) in milk and milk products in the frame of Regulation (EC) 2073/2005 and in all types of food matrices (containing milk or not) in case of Staphylococcal Food Poisoning Outbreak (SFPO) and controls.

This method comprises the extraction step by dialysis-concentration and the detection step using an immuno-enzymatic detection kit:

-Vidas SET2 (bioMérieux) or
-Ridascreen SET Total (R-biopharm)

2. References

[1] Anonymous (2005), Commission Regulation (EC) N° 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, modified by Commission Regulation (EC) N° 1441/2007 of 5 December 2007. Official Journal of European Union, L 338, 22.12.2005 pp. 01 – 26.

[2] Hennekinne J-A., Ostyn, A., Guillier F., Gohier, M., Messio, S., Dragacci S., Krys S, Lombard B. (2007). Interlaboratory validation of the Vidas SET2 detection kit for an use in official controls of staphylococcal enterotoxins detection in milk products. *J. AOAC Int*, 90, 3, 756-764.

[3] Hennekinne, J-A., Guillier, F., Perelle, S . De Buyser, M-L., Dragacci, S., Krys.S, Lombard, B. (2007). Intralaboratory validation according to the EN ISO 16140 standard of the Vidas SET2 detection kit for a use in official controls of staphylococcal enterotoxins in milk products. *J. Appl. Microbiol.* 102 , 1261–1272.

[4] The Community Summary Report on food borne outbreaks in the European Union in 2008. The EFSA Journal (2010), 1496.

3. Definitions

DC: Dialysis-concentration
ELFA: Enzyme Linked Fluorescent Assay
ELISA: Enzyme Linked ImmunoSorbent Assay
RFV: Relative Fluorescence Value
TV: Test value

4. Principle

The detection of staphylococcal enterotoxins consists in two steps:

1. Extraction/concentration

The sample is mixed and homogenised with distilled water. The toxins diffuse in water and are recovered, after two centrifugations, in the supernatant. This aqueous phase is concentrated overnight by dialysis.

2. Immuno-enzymatic detection (Vidas SET2 or Ridascreen SET Total)

The Vidas SET2 detection is based on an Enzyme Linked Fluorescent Assay (ELFA) test. It is a rapid and fully automated kit detection without differentiation of the staphylococcal enterotoxins types A to E, using a cone coated with antibodies specific for SEA, SEB, SECs, SED and SEE. An immune complex is formed between the coated antibodies, the toxins in the concentrated extract and the anti-SE antibodies conjugated with alkaline phosphatase. All reagents are included in the wells of the strip used.
Briefly, the concentrated protein extract is distributed in the strips and incubated in the automate Vidas. Two fluorescence measurements (sample, standard) are performed for each test by the automate and give relative fluorescence values (RFV). The ratio between these two measurements (test value or TV) is interpreted to declare or not a sample as positive.

The Ridascreen SET Total is a sandwich type enzyme immunoassay (ELISA) for combined detection of the Staphylococcus enterotoxins (SET) types A, B, C, D and E. The surface of the microtiter plate is coated with specific, purified antibodies which can bind the enterotoxins contained in a food sample. Sample components not bound by the antibodies are then removed in a washing step. By adding specific antibodies against the toxins as well as enzyme marked detector molecules, the sandwich complex is formed (antibody-antigen-antibody-complex). The presence of enterotoxins is revealed after adding an enzyme substrate/chromogen solution. The blue colour indicates the presence of staphylococcal enterotoxins in the sample. After addition of the stop solution which leads to a colour change from blue to yellow, the presence of SEs is determined by a colorimetric measurement at a double wavelength of 450/630 nm.

5. Reagents

5.1. Distilled or osmosed water
5.2. Hydrochloric acid (5N and 1N)
5.3. Sodium hydroxide (5N and 1N)
5.4. Polyethylene glycol 20 000 (PEG), quality for synthesis
5.5. Sodium chloride, quality for analysis ACS ISO
5.6. Di-sodium hydrogen phosphate (dodecahydrate), GR ISO
5.7. Phosphate Buffered Saline solution (PBS solution) ($NaCl/Na_2HPO_4$: 145 mM/10 mM), pH 7.3 $\pm$ 0.2. For example, to prepare 1L of PBS solution: dissolve 9 g of sodium chloride and 3.58 g of di-sodium hydrogen phosphate dodecahydrate in 1L of distilled water. Adjust pH at 7.3 ± 0.2 using HCl.

5.8. Vidas SET2 detection kit. bioMérieux, Marcy l'Etoile, France (ref: 30 705)
5.9. Ridascreen SET Total detection kit. R-Biopharm AG, Darmstadt, Germany (Art. N°: R4105)

6. Materials and equipment

6.1. Mixer
6.2. Analytical balance and weighing vessels
6.3. Homogenisation material (e.g. Turrax, better than blender or stomacher). For whey powder and all types of food difficult to mix, a turrax is highly recommended in order to have an homogeneous sample.
6.4. Shaker for beakers at room temperature
6.5. pH-meter
6.6. Spinner, 3130 to 10 000 g, capable of being refrigerated at 4°C and centrifuge tubes
6.7. Dialysis membrane, MWCO: 6 000 - 8 000 Daltons, flat width: 23 ± 2 mm (e.g. Spectra/Por®1, ref: 132 650, Spectrum)
6.8. Closures, l = 35 mm (e.g. Spectra/Por®, ref: 132 736, Spectrum)
6.9. Incubator/shaker for microplates, capable of being heated at 35 °C - 37 °C
6.10. Laboratory glass-ware or polypropylene-ware to avoid the adsorption of toxins (funnel, beaker, vial)
6.11. Funnel
6.12. Glass-wool
6.13. Tray
6.14. Fridge (5 °C ± 3 °C) and freezer (≤ -18 °C)
6.15. Vortex
6.16. Automated Vidas instrument. bioMérieux, Marcy l'Etoile, France
6.17. Microplates reader (450/630 nm)
6.18. Micropipettes

7. Procedure

7.1. Preparation

Before beginning the extraction, store the samples at 5 °C ± 3 °C (6.14). The sample should be completely defrosted before the extraction step.

As staphylococcal enterotoxins can be heterogeneously dispatched in the sample, mix the whole sample if possible, or a representative part of it, with a mixer (6.1).

Weight 25 g ± 0.1 g (5.2) of the mixed sample and transfer this test portion into a beaker (6.10).

NOTE 1: In case of cheese with rind, take about 10% of rind for 90% of cheese.

NOTE 2: In case of powder samples, reconstitute the sample by weighting 12.5 g of sample and 12.5 g of distilled water or follow the manufacture's instructions (ex: milk powder at 10%).

NOTE 3: In the case of a suspected SPFO, the minimal size of the test portion to perform the analysis is equal to 12.5 g.

7.2. Extraction step

Add 40 mL of warm distilled or osmosed water (5.1) (38 °C ± 2 °C) to the test portion (7.1) and homogenise the mixture by using a turrax, a blender or a stomacher (6.3). Rinse the system with distilled water.

NOTE 1: In case of liquid product, don't add 40 mL of distilled water.

Allow the toxin to diffuse by shaking the sample (6.4), at room temperature for at least 30 min.

Acidification step: Acidify the mixture with a few drops of hydrochloric acid (5.2) in order to obtain a **pH between 3.5 and 4.0.**

NOTE 2: During the acidification of the sample in order to keep enterotoxins in a good shape, a specific attention has to be given to: i) a pH-meter (6.5) must be used and ii) ***pH between 3.5 and 4.0*** *before centrifugation has to be respected.*
Be careful not to have a pH < 3.0 using hydrochloric acid. If the pH < 3.0, take another 25g test portion and proceed as described on 7.1.

Centrifuge the mixture at least at 3130 g for 15 min at **4 °C or room temperature** (6.6). Transfer the supernatant in a beaker.

NOTE 3: Do not hesitate to rinse with distilled water at each step to recover a maximum of toxin.

NOTE 4: If the supernatant is not clear enough, centrifuge again as described above.

NOTE 5: The pH of the supernatant after the first centrifugation has to be < 4.5. If it is not the case, acidify until obtaining a ***pH between 3.5 and 4.0*** *and centrifuge again as described above.*

Neutralisation step: Neutralise the mixture using sodium hydroxide (5.3) in order to obtain a **pH between 7.4 and 7.6**. Centrifuge again (6.6) as described above. Recover the whole neutralised aqueous phase.

NOTE 5: During the neutralisation of the sample in order to keep enterotoxins in a good shape, a specific attention has to be given to: i) a pH-meter (6.5) must be used and ii) ***pH between 7.4 and 7.6*** *after neutralisation has to be respected.*
Be careful not to rise above pH 9.0. If the pH is > 9.0, take another 25g test portion and proceed as described on 7.1.

7.3. Dialysis-concentration step

For each sample:

Prepare a 30% (w/v) PEG solution (30 g PEG (5.4) / 100 mL distilled water (5.1)).

Cut about 50 to 60 cm of a dialysis membrane (6.7).

Soak the membrane into distilled water following the instructions of the manufacturer (at room temperature during at least 30 minutes).

Rinse the membrane with distilled water (outside and inside) (5.1).

Lock one end of the membrane with a closure (6.8), fill it up with the neutralised aqueous phase as prepared on 7.2 by using a funnel (6.11) and a small piece of glass-wool (6.12) to discard suspended particles. Lock the other end of the membrane with a second closure.

NOTE 1: If the sample to analyse is very salty or very sweet, conduct a dialysis under agitation with 2 L of distilled water, two times during one hour.

Lay down the filled dialysis membrane in a tray (6.13) containing the 30% (w/v) PEG solution.
Allow the extracts to concentrate overnight at 5 °C ± 3 °C (6.14).

NOTE 2: If the extract is not concentrated enough, lay down it in the PEG solution for more time or add some powder of PEG.

Take the dialysis membrane out of the PEG solution and rinse the outer-part of the membrane with distilled water to remove all traces of PEG.

Recover the concentrated extract using:
- **the PBS solution (5.7) in the case of extract containing milk or milk products**
- **osmosis water in the case of extract containing no milk nor milk products**

Rinse well the inner-part of the dialysis membrane to obtain a final concentrated extract mass ranging from 5.0 g to 5.5 g (maximum 5.8 g for the stick extracts).

During this step, it is recommended:
- To rub the inner-parts of the dialysis membrane (one against another inner-part) with the fingers in order to take off and to recover the maximum of enterotoxins.
- To pour small drops of PBS or osmosis water (several additions) and to rinse in several times the inside membrane in order to recover all enterotoxins.

Transfer carefully the concentrated extract into a glass vial (6.10).

NOTE 3: If the initial weight of the sample to be tested is lower than 25 g (7.1, note 3, in case of SFPO), take care to obtain a final ratio equal to 5 between the weight of the concentrated extract and the weight of the test portion.

- In case of SFPO or studies, if the test portion mass (TPM) is such as:
  - 17.5 g ≤ TPM < 25.0 g, take care to obtain a final mass (FN) equal to 3.5 g ≤ FN < 5.0 g (respect the ratio equal to 5 between test portion and extract mass)
  - 12.5 g < TPM < 17.5 g, take care to obtain a final mass equal to 3.5 g (accepted until 3.9 g)

NOTE 4: If the concentrated extract is analysed within 48 h, store it at 5 °C ± 3 °C (6.14), otherwise store it at ≤ -18 °C (6.14). The extract should be completely defrosted and homogenised before testing.

In case of Vidas SET2 detection, the assay must be performed immediately after extraction. It is not recommended to store the extracts before the detection.

7.4. Detection

Perform the detection using Vidas SET2 or Ridascreen SET Total detection kits as following procedures:

7.4.1. Detection using Vidas SET2 kit

Perform the Vidas SET2 test on 500 µL of concentrated extract according to the manufacturer's instruction.

Results are analysed automatically by the computer. The calculation of the test value appears on the result sheet.

7.4.2. Detection using Ridascreen SET Total kit

Perform the Ridascreen SET Total test on 100 µL of concentrated extract according to the manufacturer's instruction with a **dual** wavelength measurement (450/630 nm).

7.5. Interpretation of results

7.5.1. Vidas SET2

- Determination of the Test Value (TV):

The test value (TV) of the sample is calculated by the automated Vidas instrument as:
TV **=** sample RFV/standard RFV

- Interpretation:

Consider that staphylococcal enterotoxins SEA to SEE are detected in the 25 g test portion if the TV of the extract is higher or equal to 0.13.

Consider that staphylococcal enterotoxins SEA to SEE are not detected in the 25 g test portion if the TV of the extract is lower than 0.13.

If obtaining a positive result, this screening result shall be confirmed. Samples related to screening positive results may be sent to the EU-RL for confirmatory analysis according to the instructions on page 2.

7.5.2. Ridascreen SET Total kit

- Quality control:

The absorbance of the positive control shall be higher than or equal to 1.0 when reading at 450/630 nm.
The absorbance of the negative control shall be lower than or equal to 0.10 when reading at 450/630 nm.
If one of these controls (positive and negative) do not meet these requirements, the results are invalidated and another test shall be performed.

- Determination of the cut-off value:

The cut-off value for evaluation of results as positive or negative is calculated by adding 0.15 to the OD-value of the negative control:

Cut-off value = absorbance value of negative control + 0.15

- Interpretation:

Consider that staphylococcal enterotoxins SEA to SEE are detected in the 25 g test portion when the test is valid meaning that the absorbance of the sample is higher or equal to the cut-off value.

Consider that staphylococcal enterotoxins SEA to SEE are not detected in the 25 g test portion when the test is valid meaning that the absorbance of the sample is lower than the cut-off value.

If obtaining a positive result, this screening result shall be confirmed. Samples related to screening positive results may be sent to the EU-RL for confirmatory analysis according to the instructions on page 2.

8. Analytical problems due to matrix

It can happen that the Vidas instrument can not take off very stick extract obtained after dialysis-concentration step through the Solid Phase Receptacle (SPR). In this case, the EU-RL advices the laboratories to better use the Ridascreen SET Total kit.

Some difficulties can be encountered during the analysis of specific matrices, in particular during the extraction step by dialysis concentration especially in case of raw meat products. In all cases, please strictly respect the ESM extraction step, because a very small modification of the method could lead to false results.
If analytical difficulties are observed, please contact the EU-RL for CPS in order to find the most suitable solution.
Comments: Due to possible analytical difficulties encountered during the SEs detection analytical steps, the EU-RL for CPS could give out some results such as "analysis not performed due to the complex type of the matrix" or "results not interpretable".

Information will be given to the requester such as:
- Analysis not performed because not judicious (for example: in case of raw matrices in which the CPS were not found and/or environmental conditions of matrices can prevent the growth of CPS). The EU-RL will reject sample for example in case of raw vegetable.
- Analysis successfully completed. In this case, a report will be edited and will specify why the analysis has been stopped.

9. Interferences

It is well known that the immunological detection of staphylococcal enterotoxins in food matrices has several drawbacks. Non-specific SEs reactions with some of the commercially available kits were previously reported with various food types or with food contaminated by other micro-organisms than *Staphylococcus* spp.

Some interferences can be attributed to endogenous enzymes such as lactoperoxidase or alkaline phosphatase coming from raw milk:

- **The alkaline phosphatase** is usually present in cheeses made from raw milk.

In case of positive result by Vidas SET2 and if interferences due to alkaline phosphatase are suspected:
1. Place 600 µL of treated concentrated extract in a tube,
2. Perform a heat-treatment at 80 °C during two minutes (to destroy alkaline phosphatase),
3. After cooling, perform a new detection using Vidas SET2 detection kit

As this heat-treatment can lead to a loss of serological activity of the enterotoxins present in the concentrated extract, it is not performed before the detection step due to possible false negative results.
However, in case of a positive result obtained by the Vidas SET2 detection kit and if interferences are suspected, such a procedure can be applied.
In case of suspected interferences with Vidas SET2 kit, perform if possible the detection with Ridascreen SET Total kit.

- **The lactoperoxidase** is resistant to pH 3 and its molecular weight could explain the fact that it is not destroyed or inactivated during the extraction step.

In case of a positive result obtained by the **Ridascreen SET Total detection kit** and if interferences due to endogenous lactoperoxidase are suspected, the following test can be performed:

1. Place 100 µL of treated concentrated extract in a tube and add 50 µL of substrate and 50 µL of chromogenic solutions from the **Ridascreen SET Total** detection kit,
2. If a blue-green colour appears, endogenous lactoperoxidase is present in the concentrated extract and can explain a false positive result obtained with the screening method.

If interferences with **Ridascreen SET Total detection kit** are suspected, perform if possible the detection with the Vidas SET2 kit.

In case of suspected interferences, the related samples may be sent to the EU-RL for confirmatory analysis according to the instructions on page 2.

10. Analysis report

The analysis report will specify the type of the detection kit used: Vidas SET2 or Ridascreen SET Total.

11. Handling precautions and decontamination

Staphylococcal enterotoxins are harmful if swallowed, inhaled or absorbed trough skin (wear gloves).
Contaminated single use reagents are eliminated in a clinical waste which will be incinerated.
Other contaminated materials can be decontaminated during at least 2 hours in a 1.8° sodium hypochlorite solution.

ANNEX I

Application form for confirmatory quantitative analysis « Staphylococcal enterotoxins in all types of food matrices » (1/2)

Fill one application form for each sample to analyse

FROM	TO
Name & address: Phone number: Fax number/E-mail: File number: Date & signature:	ANSES Maisons-Alfort Laboratory for Food Safety EU-RL for CPS Unit: CAT, team: BAC <u>Delivery address:</u> Pôle HQSA 22, rue Pierre Curie 94700 Maisons-Alfort FRANCE <u>Postal address:</u> 23, avenue du Général de Gaulle 94706 Maisons-Alfort cedex FRANCE

Sample: ❑ Extract ❑ Volume (specify :……...)
❑ Sample ❑ Weight (specify :……..)
❑ CPS strains ❑ Number (specify :……..)

Nature of sample:
❑ Milk ❑ Cheese ❑ Matrix containing milk product (specify: ……...…...……)
❑ Meat ❑ Seafood product ❑ Ready to eat meal ❑ Other matrix (specify: ……..)

Sample number					
Origin of the sample (laboratory, school, restaurant,…)					
Number of CPS / g					
Strain of CPS (your references)	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
NRL results using Vidas SET 2 detection	❑ Vidas SET2 kit Batch number: ……..…….....			Test value (TV): ……...….	
NRL results using Ridascreen SET Total detection	❑ Ridascreen SET Total kit batch number: …….			Negative control absorbance: ……..……...…… Cut off value: ……..……...…… Test absorbance value:…….	

Context of the analysis:
❑ Confirmation after: ❑ official control ❑ SPFO ❑ own check ❑ other, specify:

❑ Alert: ❑ no ❑ yes (specify :……..) ❑ other, specify:

Send the original results to:

Application form for confirmatory quantitative analysis « Staphylococcal enterotoxins in all types of food matrices» (2/2)

In the case of dairy product or sample containing milk or milk products:

Sample number	
Type of milk	❑ Cow ❑ Goat ❑ Sheep ❑ Other (specify :……)
Milk	❑ Raw ❑ Other (specify :……)
Type of cheese	❑ Uncooked pressed cheese ❑ Soft cheese ❑ Other (specify :……)
Stage during the manufacturing process	❑ 24 hours ❑ 48 hours ❑ 72 hours ❑ Other (specify :……)

To fill when the food is suspected to be involved in SPFO:

Number of patients / number of meals served	
Symptoms	❑ Nausea ❑ Abdominal pain ❑ Vomiting ❑ Diarrhoea ❑ Fever ❑ Unknown ❑ Other, specify: ……..……
Symptom's delay	Date and hour of the suspected meal: Date and hour of the symptoms:
Approx: quantity of food ingested	

Comments:.....
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