



RAY 32/02 – 06/08
ALTERNATIVE ANALYTICAL METHODS FOR AGRIBUSINESS
Certified by AFNOR Certification
www.afnor-validation.com

RayAl Salmonella OPTIMA 40 hours Enrichment

This method is certified by AFNOR Certification for the detection of motile and non motile *Salmonella* in food and feed products and environmental samples (breeding samples are excluded) (validation ref n° RAY 32/02 - 06/08 valid until 30/06/2012).

This method is adapted to the detection of all *Salmonella* both motile and non-motile.

This method is also approved by:

- AOAC Research Institute - all food, feed, HACCP and water items
- NMLK
- DNVL (Danish National Veterinary Laboratory)
- DVFA
- NordVal - all types of food and feed of animal origin.

1. Introduction

The rapid detection of pathogens in foods is essential for ensuring the safety of consumers. *Salmonella* detection in samples is difficult because of the low number of bacteria among a large competing flora. Traditional methods for the detection of food borne bacteria use time consuming growth in culture media followed by isolation, biochemical identification, and serology. Results are obtained within 3 to 5 days.

RayAl *Salmonella* Optima gives a negative or a presumptive positive result in 36 to 44 hours, after 2 enrichment steps.

The ELISA can be performed in less than 2 hours; the presumptive results may be obtained after 36 hours.

2. Intended Use

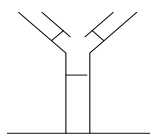
Our enrichment procedure is remarkable for its simplicity however it requires laboratory facilities and qualified and trained personnel. Basic training is recommended to first time users and is given by RayAl Ltd.

The detection limit of the kit is in the range of 10^5 - 10^6 cells per ml in the enrichment broth.

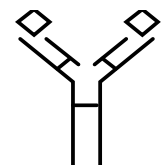
3. Principle of the Assay

Typically 25 gram of sample is enriched in pre-enrichment buffer followed by selective enrichment according to the chosen protocol. An aliquot of the selectively enriched sample is withdrawn and boiled. After cooling the samples they are investigated for the presence of somatic and flagella Salmonella antigens by a sandwich ELISA (Enzyme Linked Immuno-Sorbent Assay) method.

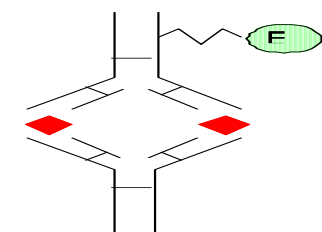
3.1 Affinity-purified rabbit antibody specific to Salmonella somatic and flagella proteins has been attached to the wells of microtitre strips.



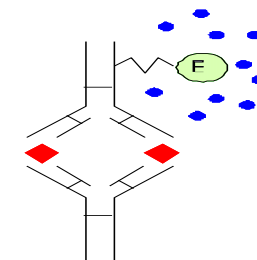
3.2 The heat treated samples, containing the bacterial protein antigens, are cooled to room temperature and added to the antibody coated wells. Salmonella protein antigens present in the samples are bound immunologically by the antibody.



3.3 After washing to remove unbound material enzyme-conjugated affinity purified goat antibody specific to Salmonella proteins is added to the wells. If Salmonella proteins are present in the samples, the enzyme-conjugated antibody will bind to the proteins and thus to the well.



3.4 After a second washing step where any unbound enzyme-conjugated antibody is removed, enzyme substrate is added. A blue colour is formed by the action of bound enzyme on the substrate in those wells where Salmonella proteins are present.



The reaction with the substrate is stopped after 15 minutes (possible to extend to 30 minutes) with diluted sulphuric acid which changes the colour in the wells to yellow.

The ELISA test kit contains 5 x 96 microwells and liquid reagents which are stabilised and supplied ready to use at working concentration. Only the Washing Buffer has to be diluted 25 times in distilled (or deionised) water. The plate is in 8 well Break-Apart™ strip format. Reagents bound to the wells of the microtitre strips are dry, stabilised and ready for use.

4. Reagents Provided

Each kit contains sufficient material for 5 x 93 determinations, including controls.

The kit expiry date is 1 year after the production release and is indicated on each label.

- 4.1 Five microtitre plates consisting of microtitre wells coated with antibodies against *Salmonella* spp.
- 4.2 Negative Control. (Green colour code). 5 ml in working dilution. Contains diluent with stabiliser.
- 4.3 Positive Control. (Red Colour Code). 5 ml in working dilution. Contains heat-killed *Salmonella typhimurium* (10^6 /ml) in diluent with stabiliser.

- 4.4 Conjugate. (Orange Colour Code). 60 ml in working dilution. Contains horseradish peroxidase-antibody conjugate in diluent with stabilisers.
- 4.5 Substrate. (Blue Colour Code). 60 ml in working dilution. Contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 0.03% hydrogen peroxide. Clear or slightly faint blue solution.
- 4.6 Stop Solution. (Yellow Colour Code). 60 ml in working dilution. Contains 0.2 M sulphuric acid.
- 4.7 Washing Buffer Concentrate. 5 x 60 ml. Contains 0.075 M Tris-HCl/2.5 M NaCl with 5% Tween 20, pH 7.2. Each bottle should be diluted 25 times by addition of 1440 ml of deionised or distilled water before use.

The kit and unused kit components should be stored at 2-8°C.

Do not freeze.

Unused diluted Washing Buffer may be stored for 1 month at 2 – 8°C.

5. Reagents and Equipment required but not supplied with the kit.

- 5.1 Waterbath 85°C-100°C for heat treatment of the samples
- 5.2 Deionised or distilled water.
- 5.3 Micropipette for dispensing 100µl and tips
- 5.4 Microtitre Plate Washer.
- 5.5 Air incubator for 37°C accuracy ±1°C and Air incubator or waterbath for 41.5°C accuracy ±1°C. It is important that incubator and waterbath have an efficient air/water circulation.
- 5.6 Microwell Plate Reader with 450 nm filter.
- 5.7 Stopwatch.
- 5.8 Measuring cylinder for 250 ml or 1L
- 5.9 Sterile flasks or stomacher bags suitable for enrichment culture
- 5.10 Sterile 10 ml test tubes suitable for selective enrichment culture.
- 5.11 Sterile transfer pipettes approximately 1 ml for transfer of aliquots to boiling tubes.

- 5.12 Heat resistant tubes or microtitre plate heat resistant tray.
- 5.13 Autoclave for decontamination of samples.
- 5.14 Stomacher for homogenisation of samples
- 5.15 Vortex
- 5.16 Buffered Peptone Water (for example, Oxoid CM509) according to ISO 6579.
- 5.17 RVS broth, for example RayAL RVS. RVS broths from other manufacturers may also be used.

6. Precautions.

Not for diagnostic use of medical specimens

6.1 Technical Precautions

- 6.1.1 Components must not be used after the expiry date printed on the label.
- 6.1.2 Reagents are provided at fixed working concentration. Optimum sensitivity and specificity will be reduced if reagents are modified or not stored under the recommended conditions.
- 6.1.3 Do not mix different lots of reagents.
- 6.1.4 Avoid microbial contamination of opened reagent bottles.
- 6.1.5 Ensure that no cross contamination occurs between wells. It is essential for proper performance of the test that the enzyme-conjugated antibody is not allowed to contaminate other reagents and equipment.
- 6.1.6 Ensure that kit components are not exposed to temperature more than 40° C.

6.2 Safety Precautions

- 6.2.1 The Stop Solution contains sulphuric acid which is corrosive. Wash immediately with large quantities of water if the solution comes into contact with skin or mucous membranes.
- 6.2.2 The *Salmonella typhimurium* in the positive control has been tested and shown to be non-viable.
- 6.2.3 Do not eat, drink or apply cosmetics in the work area where the test is performed.

6.2.4 Do not pipette by mouth. Avoid contact of kit components with injured skin.

7. Preparation of broths and samples

7.1 Preparation of the enrichment media.

Dissolve Buffered Peptone Water with magnetic stirring into pure water. Autoclave for 15 minutes at 121°C. Allow to cool to room temperature before applying to samples. Dissolve Rappaport-Vassiliadis Soy broth from RayAl into pure water with magnetic stirring (should another RVS be used, follow manufacturer instructions). Dispense 10 ml into appropriate tubes. Autoclave at 115°C for 15 minutes. Ensure a precision of 1% of the preparation. Allow to cool before applying sample.

7.2 Preparation of samples

Standard enrichment protocol

Pre-enrichment.

25 ± 1 grams of the sample to be tested is homogenised, if necessary by stomacher, and incubate for 16-20 hours in 225 ml of buffered peptone water at 37 ± 1°C.

In the context of AFNOR validation, test portions weighing more than 25 g have not been tested.

Selective enrichment.

0.1 ± 0.01 ml of the enriched sample is transferred to 10 ml of Rappaport-Vassiliadis Soy broth and incubated for 24 hours at 41.5°C ± 1°C in an incubator or in a waterbath. For transfers use pipettes of required precision. Ensure that the bench time of RVS inoculated samples is kept at minimum. When processing a large number of samples, transfer the samples to tubes in individual racks, when each rack is completed transfer immediately to the incubator. This is important to avoid extensive growth of competing non-salmonellas.

When the incubation period in RVS is completed, agitate the tubes gently and transfer 1ml aliquot to a glass or polypropylene test tube.

The un-boiled RVS samples should be kept for verification until ELISA results are obtained. They should be kept at 41.5°C if the ELISA test is to be performed within 2 hours or at 2°C-8°C if the ELISA test is to be performed within longer period of time. It is possible to keep RVS broths for 48 hours at 2°C-8°C prior to the ELISA test.

Heat the aliquot to 85-100°C for 15 - 20 minutes in the test tube. After heating, the sample is cooled to room temperature. This may conveniently be done by placing the test tube in cold tap water for 5 minutes.

8. Procedure of the ELISA test

Take the test kit from storage at 2-8°C one hour before use to allow the components to reach room temperature.

8.1 Determine the number of wells required for the test. Take the required number of strips from the pouch and fit them to the frame provided. Unused strips should be returned to the pouch and stored at 2-8°C.

STEP 1

8.2	The first well in the strip is reserved as a 'blank' for measuring the absorbance of the substrate. Pipette 0.1 ml of Negative Control (Green colour code) into the second well.
8.3	Pipette 0.1 ml of Positive Control (Red colour code) into the third well.
8.4	Pipette 0.1 ml of each boiled and cooled sample separately into consecutive wells in the strip. If there are wells left over at the end of a test strip the Positive or Negative Controls may be repeated.

- 8.5 The frame containing the strips is incubated for 30 minutes at $37 \pm 1^\circ\text{C}$. An "overtime" of 5 minutes is tolerated.
Go immediately to the next step before the incubation is completed.
- 8.6 Prepare Washing Buffer solution by mixing 60 ml of Concentrate with 1440 ml of water.
- 8.7 After 30 minutes sample incubation, the contents of the wells should be aspirated, removing as much of the liquid as possible. Use preferably a washing device. Wash the wells 5-7 times with diluted washing buffer. If you are washing with multichannel pipettes wash the wells at least 7 times to ensure sufficient washing. The washing technique is critical to the performance of the test. Ensure complete filling and emptying of the wells through all the steps of each wash cycle.

STEP 2

- 8.8 Pipette 0.1 ml of Conjugate (Orange colour code) into all wells except the 'blank'.
- 8.9 Incubate for 30 minutes at $37 \pm 1^\circ\text{C}$. An "overtime" of 5 minutes is tolerated.
- 8.10 Wash the wells at least 5-7 times with diluted washing buffer as described in section 8.7.

STEP 3

- 8.11 Pipette 0.1 ml of TMB Substrate (Blue colour code) into all wells, including the 'blank' well.
- 8.12 The wells are incubated with substrate for 15 or 30 minutes at room temperature. (Start stopwatch at well A1)

STEP 4

- 8.13 After 15 or 30 minutes the colour reaction is stopped by addition of 0.1 ml of Stop Solution (Yellow colour code) to all wells including the 'blank' well. The stop solution will cause the blue colour in wells with positive samples to change to yellow.
- 8.14 The optical densities are read within 10 minutes in a Microwell Reader with a 450nm filter. Before the reading inspect the wells for air bubbles and if present remove by puncturing with a needle. The photometer should be zeroed against the 'blank' well before the other wells are read. Do not use reference filter.

(When the user is familiar with the test, the short procedure description on the back of this booklet may be followed).

Automatic ELISA equipment is allowed if the prescribed ELISA protocol is followed.

9. Interpretation of results.

Results are expressed precisely as optical density (OD) measurements using a Microwell Reader
For proper functioning of the test the OD reading of the Negative Control should be less than 0.100 and the OD reading of the Positive Control should be above 0.500. Should the value of Negative or Positive controls not meet those criteria, the test is considered as not valid and must be performed again.
Sample from wells with OD values above 0.200 are considered as positive. Typical values for the negative control is 0.030 and for the positive control 0.900-1.700. (The value of the blanking well (usually A1) should always be subtracted)

The presence of Salmonella in samples which are positive is verified using a recognised method of cultivation.

Confirmation of positive results.

Samples with OD's above 0.200 are considered positive for salmonella. In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed in one of the following ways. Using the conventional tests described in the standardised methods by CEN or ISO. The confirmation step must start from the (none heated) RVS broth stored at 41.5°C or 2-8°C including the purification step.

Purification: purify on 2 agar plates (XLD and a second agar of preferred choice). Incubate agars as specified by standard Salmonella cultural protocols then, perform confirmation tests. Alternatively, it is possible to perform confirmation tests directly if the colonies are well isolated.

In case of discrepant results (positive with ELISA but non-confirmed by the standard confirmation method), lab should implement all the necessary means in order to ensure the quality of the result.

10. Performance limitations.

Proper performance of the enrichment phase is important to the assay. Use the recommended media for optimal performance. Other media may cause less than optimal performance. Test performance has been optimised using the culture reagents specified in section 5.9 and 5.11 and it is strongly recommended that only these media be used.

In case of problem, ensure correct temperature setting of the incubator used for selective enrichment using a thermometer calibrated to a certified standard.

Washing of the wells is a critical step. It is not recommended to use squeeze bottles or other uncontrolled devices. Use multichannel pipettes or preferably multichannel washing devices designed for

microplates. Automatic washing devices provide optimal results.

The enzyme peroxidase used in the kit will be inactivated in the presence of sodium azide. Therefore ensure that residues of sodium azide are not present in washing devices, buffer reservoir, tubes and the immuno-washer. Some kit manufacturers use sodium azide as stabiliser in the kit reagents when other enzymes than peroxidase are used. Possible sources of error in performance leading to invalid results are insufficient washing or contamination of equipment and reagents.

Specific applications

Should an automated system be used to perform the ELISA test, it is important to follow recommendations from the instrument manufacturer.

11. Warranty

Good results depend on the proper utilisation of the kit by carefully following the instructions for use.

If the kit fails to perform according to the specification please immediately contact:

RayAl Ltd

mansfield i-centre
Oakham Business Park
Hamilton Way
Mansfield
NG185BR

Telephone: 01623 600680

Facsimile: 01623 600681

E-mail: info@rayal.com

Performance data will be mailed on request



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Telephone +44 1623 00680
Facsimile +44 1623 600681
e-mail: info@rayal.com
Website: www.rayal.com

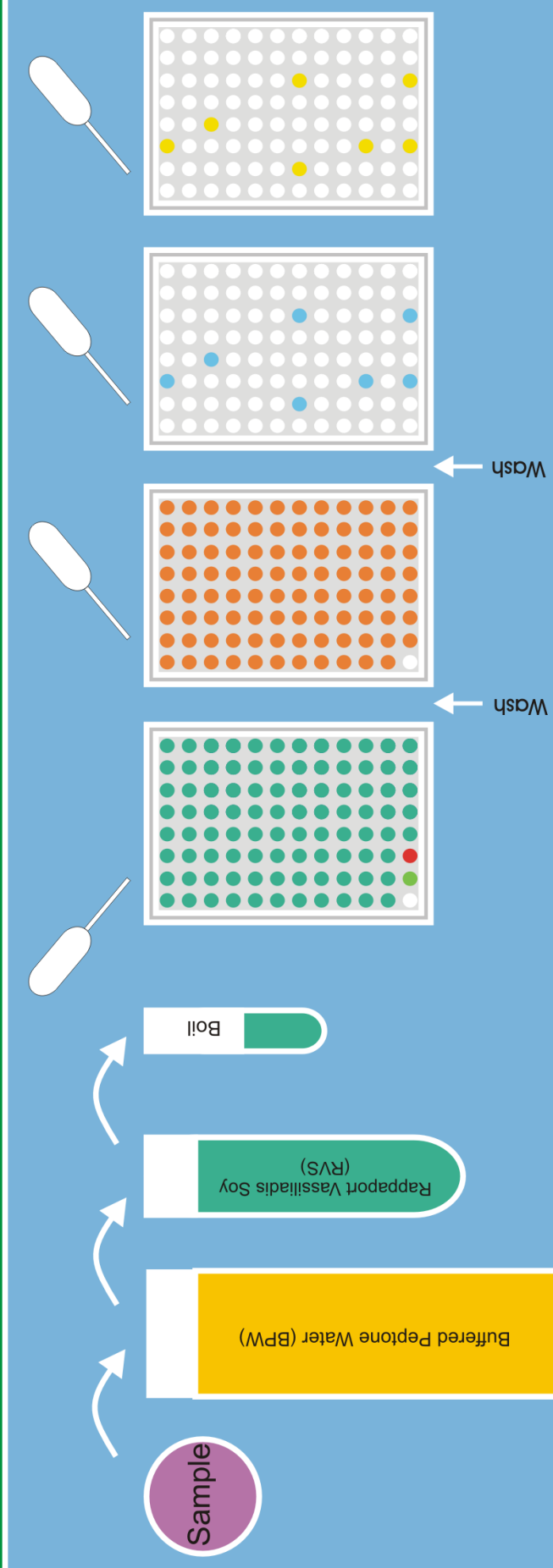
Certified by:
AFNOR Certification
Approved by:
AOAC RI, NordVal
NMKL, DNVL, DFVA

Summary of the Procedure

Step 1	Pipette 0.1 ml Negative Control Pipette 0.1 ml Positive Control Pipette 0.1 ml of each boiled sample
30 Minutes	Incubate at 37°C Wash 5-7 times with diluted Washing buffer
Step 2	Pipette 0.1 ml of Conjugate excl. blank
30 Minutes	Incubate at 37°C Wash 5-7 times with diluted Washing buffer
Step 3	Pipette 0.1 ml Substrate TMB + blank
15 or 30 Minutes	Incubate at room temperature
Step 4	Pipette 0.1 ml of Stopping Solution + blank
	Read results immediately on a Reader at 450nm

Salmonella ELISA Test OPTIMA - 40 hours of enrichment

Food, Feed Products and Environmental Samples



	25g in 225 mL BPW	16-20h 37°C						
Incubation	0.1 mL in 10 mL RVS	18-24 h 41.5°C	1-2 mL aliquot	20 min. 85-100°C	Pipette samples 0.1 mL	30 min. 37°C	Conjugate 0.1 mL	30 min. 37°C
							Substrate 0.1 mL	15 or 30 min. 20-25°C
							Stop solution 0.1 mL	Read 450nm