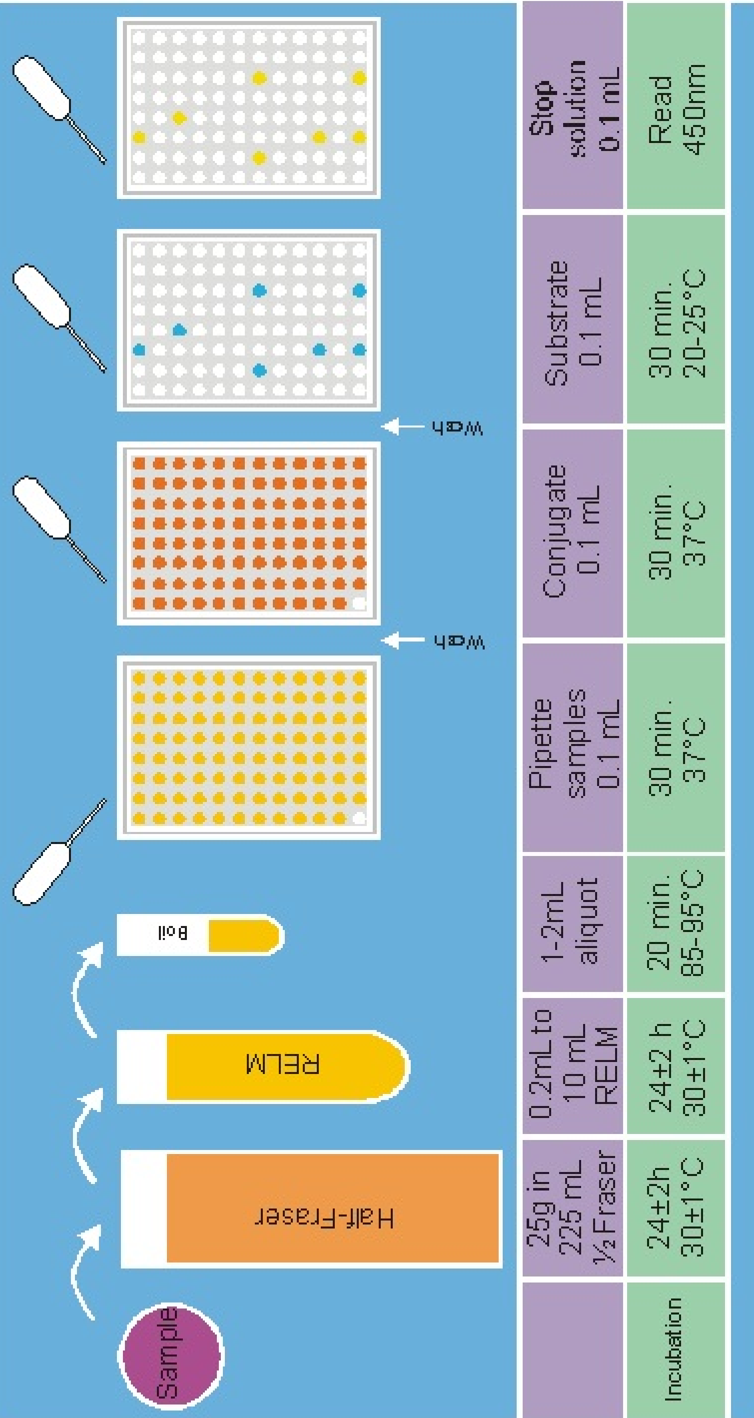


RAY 32/03 07/10
ALTERNATIVE METHODS FOR AGRIBUSINESS
Certified by AFNOR Certification
www.afnor-validation.org

RayAl Listeria

ELISA test for rapid detection of *Listeria* in food and environmental samples.
The certificate RAY 32/03- 07/10 can be obtained from RayAl Ltd or from AFNOR Certification. The expiry date of the AFNOR VALIDATION is specified on the certificate.

1. Introduction

The rapid detection of pathogens in foods is essential for ensuring the safety of consumers. 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 Listeria gives a negative or a presumptive positive result in 44 hours, after 2 enrichment steps.
The ELISA can be performed in less than 2 hours; the presumptive results may be obtained after 46 hours.

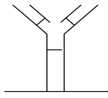
2. Intended Use

Basic training is recommended to first time users and is given by RayAl Ltd.
It is important to follow Good Laboratory Practices (ISO 7218 for example).
The detection limit of the kit is in the range of 10^5 - 10^6 cells per ml in the enrichment broth.

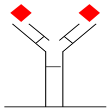
3. Principle of the Assay

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 An aliquot of the selectively enriched sample is withdrawn and boiled.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

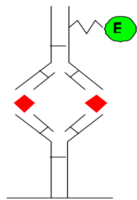
3.1 Affinity-purified antibody specific to *Listeria* flagella protein has been attached to the wells of microtitre strips.



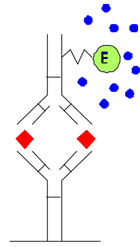
3.2 The heat treated samples, containing the bacterial protein antigens, are cooled to room temperature and added to the antibody coated wells. Listeria protein antigens present in the samples are bound immunologically by the antibody.



3.3 After washing to remove unbound material enzyme-conjugated affinity purified antibody specific to Listeria proteins is added to the wells. If Listeria 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 *Listeria* proteins are present.



The reaction with the substrate is stopped with diluted sulphuric acid which changes the colour in the wells to yellow.

The ELISA test kit contains 5 plates each with 96 microwell, bottles with 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 plates are in 8 well dividable 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 93 determinations, including controls.
The kit expiry date is indicated on each label.

4.1 Five microtitre plate consisting of microtitre wells coated with antibodies against *Listeria* (breakable strips).
4.2 Negative Control. (Green colour code). 5ml at working dilution. Contains diluent with stabiliser.
4.3 Positive Control. (Red colour code). 5ml at working dilution. Contains heat-killed *Listeria* (2 x 10^6/ml) in diluent with stabiliser.

4.4 Conjugate. (Orange colour code). 60ml at working dilution. Contains horseradish peroxidase-antibody conjugate in diluent with stabilisers.

4.5 Substrate. (Blue colour code). 60ml at working dilution. Contains 3, 3’, 5, 5’-tetramethylbenzidine (TMB) and 0.03% hydrogen peroxide.
4.6 Stop Solution. (Yellow Colour Code). 60ml at working dilution. Contains 0.2 M sulphuric acid.
4.7 Washing Buffer Concentrate. 5 x 60ml. Contains 0.075 M Tris-HCl/2.5 M NaCl with 5% Tween 20, pH 7.2. Each bottle should be diluted by addition of 1440ml 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 sample heat treatment.
5.2 Deionised or distilled water.
5.3 Micropipette for dispensing 100µl and tips
5.4 Microtitre Plate Washer.
5.5 Incubator for 30˚C accuracy ±1˚C.
5.6 Microwell Plate Reader with 450 nm filter.
5.7 Stopwatch.
5.8 Measuring cylinder for 250ml or 1L.
5.9 Sterile flasks or stomacher bags suitable for enrichment culture.
5.10 Sterile 10ml test tubes suitable for selective enrichment culture.
5.11 Sterile transfer pipettes approximately 1ml for transfer of aliquots to boiling tubes.
5.12 Heat resistant tubes (glass or polypropylene).
5.13 Autoclave for decontamination of samples.
5.14 Stomacher for homogenisation of samples.
5.15 Vortex.
5.16 Half-Fraser broth.
5.17 Selective broth RayAl Enrichment for Listeria Medium (RELM).

6. Precautions

6.1 Not for diagnostic use of medical specimens.
6.2 Components must not be used after the expiry date printed on the label.
6.3 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.
6.4 Do not mix different lots of reagents.
6.5 Avoid microbial contamination of opened reagent bottles.
6.6 Ensure that no cross contamination occurs between wells.
6.7 Ensure that kit components are not exposed to temperature more than 40˚C.
6.8 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.
6.9 The *Listeria* strain in the positive control has been tested and shown to be non-viable.
6.10 Do not eat, drink or apply cosmetics in the work area where the test is performed.
6.11 Do not pipette by mouth. Avoid contact of kit components with damaged skin.

7. Preparation of broths and samples

7.1 Preparation of the enrichment media

Dissolve Half-Fraser with magnetic stirring into pure water. Autoclave for 15 minutes at 121˚C. Allow to cool to room temperature before applying to samples.
Dissolve RELM broth from RayAl into pure water with magnetic stirring.

Dispense 10ml into appropriate tubes.
Autoclave at 121˚C for 15 minutes. Allow to cool before applying sample.

7.2 Preparation of samples

Standard enrichment protocol

Pre-enrichment.
Typically, x grams of the sample to be tested are homogenised in 9x ml of Half-Fraser broth, if necessary by stomacher, and incubated for 24±2 hours at 30 ±1˚C.
In the context of AFNOR validation, test portions weighing more than 25 g have not been tested.
Selective enrichment.
0.2 ml of the enriched sample is transferred to 10ml of RELM broth and incubated for 24±2 hours at 30˚C ±1˚C. 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-Listeria.
When the incubation period in RELM is completed, agitate the tubes gently and transfer 1ml aliquot to a glass or polypropylene test tube.
The un-boiled RELM-samples should be kept for verification until ELISA results are obtained. They should be kept at 30˚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 RELM broths for 72 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.
8.3	Pipette 0.1ml of Negative Control (Green colour code) into the second well.
8.4	Pipette 0.1ml of Positive Control (Red colour code) into the third well.
8.5	Pipette 0.1ml 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.
8.6	The frame containing the strips is incubated for 30 minutes (± 5 min) at 37˚±1C. Go immediately to the next step before the incubation is completed.
8.7	The Washing Buffer Concentrate is diluted 25 times with distilled or deionised water. Each vial contains 60ml of Concentrate and should be diluted with 1440ml of water.
8.8	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. The washing technique is important to the performance of the test. Ensure complete filling and emptying of the wells through all the steps of each wash cycle.

STEP 2	
8.9	Pipette 0.1ml of Conjugate (Orange colour code) into all wells except the 'blank'.
8.10	Incubate for 30 minutes (± 5 min) at 37˚±1C.
8.11	Wash the wells at least 5-7 times with diluted washing buffer as described in section 8.7.

STEP 3	
8.12	Pipette 0.1ml of TMB Substrate (Blue colour code) into all wells, including the 'blank' well.
8.13	The wells are incubated with substrate for 30 minutes (± 5 min) at room temperature. (Start stopwatch at well A1).

STEP 4	
8.14	After 30 minutes the colour reaction is stopped by addition of 0.1ml 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.
8.15	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.

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 0.150 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 1.000-2.500. (The value of the blanking well (usually A1) should always be subtracted).
The presence of *Listeria* in samples which are positive has to be confirmed.

Confirmation of positive results

Samples with OD's above 0.200 are considered positive for *Listeria*.
In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed in one of the following ways.
Isolate (from unheated RELM stored at 30˚C or 2-8˚C) on *Listeria* selective agar plate, then use conventional tests described in the standardised methods by CENorISO including the purification step.
Isolate (from unheated RELM stored at 30˚C or 2-8˚C) on Ottavioni Agosti agar or Rapid Lmono, then perform genus confirmation tests (Gram and catalase). It is possible to perform confirmation tests directly (without prior purification) if the colonies are well isolated.

Identification of the species can be performed out of the AFNOR VALIDATION certification.

In case of discrepant results (positive with ELISA but non-confirmed by the standard

confirmation method), the 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.
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.
It is not recommended to use solution containing sodium azide for cleaning the washing devices (the enzyme peroxidase used in the kit will be inactivated in the presence of sodium azide).
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:

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website: www.rayal.com

Summary of the Procedure

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