Centre for Environment Fisheries & Aquaculture Science

Cefas

European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

# Use of proprietary *Salmonella* detection kits for detection and identification of *Salmonella* spp. in bivalve molluscs, live echinoderms, gastropods and tunicates.

This advice note provides guidance on the use of proprietary methods for the detection of *Salmonella* spp. in live bivalve molluscs (LBM) and other mollusc species in the context of Official Controls. This guidance was produced in response to a question from the NRL network for monitoring bacteriological and viral contamination of bivalve molluscs at the NRLs workshop in 2016 (Berlin) on the use of molecular based detection systems. However, the principles described here can be applied to all alternative (proprietary) methodologies irrespective of the basis of the test. This note has been produced in consultation with the European Union Reference Laboratory (EURL) *Salmonella* with reference in Annex I to ISO/TC34/SC9/WG3 Method Validation – protocol for the verification of reference and validated alternative methods implemented by a single laboratory.

## Official Controls for *Salmonella* spp. in Bivalve Molluscs, live echinoderms, gastropods and tunicates

The reference method for detection of *Salmonella* in LBM is ISO/EN 6579 [1] (Anon 2005). The application of this control is performed as a food safety criterion to demonstrate absence in 25g (sampling plan n=5, c=0) for products placed on the market during their shelf-life. The NRL network have previously noted Commission Opinion that *Salmonella* spp. monitoring in production areas is not foreseen [Resolution 4, 10th NRLs workshop https://eurlcefas.org/media/7686/workshop_weymouth_2011_resolutions.pdf]. For the most part, Official Control testing (OCT) in the EU for *Salmonella* in LBM is focused upon End Product Testing (EPT).

## EU Reference Method

EN/ISO 6579 and the revised EN/ISO 6579-1 is a conventional bacteriological qualitative method which requires primary enrichment of shellfish homogenate in a non-selective liquid media followed by a secondary semi-selective enrichment, colony isolation on differentiating media and downstream confirmation by sub culture purified multiple colony identification. Colony identification is by application of a suite of biochemical tests. Positive results are confirmed within 5 days of sample collection.

## Use of Alternative Methods for Detection of *Salmonella* spp. in Bivalve Molluscs

### Provision in the EU reference method for *Salmonella*

Note 2, section 9.5.1 of EN/ISO 6579-1 states that alternative procedures can be used for isolate confirmation, provided the suitability of the alternative procedure is verified. Suitably verified proprietary identification kits can therefore be used to confirm identification of colonies as an alternative to the application of the suite of traditional biochemical tests set out in ISO 6579-1. Where laboratories choose

[1] Note ISO 6579-1 now published as part of the M/381 CEN mandate will replace ISO 6579 in subsequent revisions of Commission Regulation (EC) No. 2073/2005.

to use alternative methods to those prescribed in ISO 6579-1 for isolate identification, a secondary validation or laboratory verification should be carried out. Advice on scope and design of such verification studies can be obtained from the EURL. Laboratories choosing to use alternative identification methods, should be able to provide National accreditation bodies, National or EU Reference Laboratories or Competent Authorities, details of secondary validation and supporting data for audit on request.

There is no specific provision in EN/ISO 6579 or EN/ISO 6579-1 for use of alternative methods for identification of *Salmonella spp.* for screening from enrichment broths.

### Provision in the microbiological criteria

Article 5 of Commission Regulation (EC) No. 2073/2005 on microbiological criteria states that the use of alternative analytical method is acceptable when the method is validated against the designated reference method, and if a proprietary method is certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols. Accordingly, if the alternative (proprietary) methods have been validated against the stated reference method in accordance with EN/ISO 16140 (part 2) and certified by a recognised third party (AFNOR, MicroVal etc), the provision exists for use for Official Controls. Certification of validated alternative methods must include relevant food categories and food types (e.g. raw bivalve molluscs). Generally, it is the case that the alternative method replaces the full reference method (e.g. ISO 6579 for detection of *Salmonella* in LBM).

Any laboratory wishing to use an alternative (proprietary) method as an alternative to application of the reference method should ensure that,

- The validation has been undertaken for the correct reference method, in this case EN/ISO 6579.
- That the validation has been certified by a recognised validating certification body e.g. AFNOR, MicroVal.
- That the certification includes details on the food categories, food types and food items[2], and that those items include raw bivalve molluscs.

**Where laboratories choose to use alternative methods to EN/ISO 6579 secondary validation sometimes known as laboratory verification shall be carried out[2].**

### Accreditation

All laboratories undertaking testing of LBM under a Competent Authority Official Control programme must be accredited to EN ISO/IEC 17025 for the specific test method used.

### Approval by Competent Authority

Member State Competent Authorities are responsible for OCT, consequently for all OCT the testing laboratory should seek formal approval for the use of alternative methods by the relevant Competent Authority prior to use in Official Control programmes.

### Supervision by the NRL and Proficiency Testing

Official laboratories undertaking OCT do so under the supervision of the relevant National Reference Laboratory (NRL), this should include use of the alternative method in NRL/EURL proficiency testing (PT). Laboratories using alternative methods in PT should notify the scheme organisers and provide details of the method in use.



[2] For definitions see ISO 16140-1

## Annex I - Example secondary validation technical protocol

### Principle

Comparative $LOD_{50}$ determination is used for secondary validation of qualitative methods (e.g. *Salmonella* spp.). The estimated $LOD_{50}$ determined for the alternative method is compared to the $LOD_{50}$ reference value or the estimated $LOD_{50}$ determined from a validated reference method. Where validation data for a specific food type is not available (e.g. raw bivalve molluscs) the recommended $LOD_{50}$ determined should be equal or lower than 3 cfu/test portion on each separate test occasion.

Note. This secondary validation protocol only applies where the reference method is to be replaced in its entirety with an alternative method.

### Selection of food items

The user laboratory should carry out this technical protocol in its entirety on separate food items (i.e. oysters, mussels, or clams, etc) from the relevant food category under test (i.e. raw, unprocessed bivalve molluscs). Further details and definition of food categories and food items are given in ISO 16140 parts 1 and 2. This verification procedure should be undertaken on the food items most commonly tested in the laboratory and claimed in the scope of the application of the user laboratory.

### Experimental Design

Prepare *Salmonella* spp. cultures for inoculating the food items (e.g. oyster homogenate). Cultures for inocula can be either,

- Characterised laboratory strains isolated from the food category under test
- Reference materials
- Culture collection isolates

Inoculation levels:

- The high-level inoculum shall be ≤30 cfu/test portion
- The intermediate-level inoculum shall be a 1/2 dilution of the high-level inoculum to give an estimated intermediate-level of 5 – 15 cfu/test portion
- The low-level inoculum shall be a 1/10 dilution of the high-level inoculum to give an estimated low level of 1 - 3 cfu/test portion

As a minimum, prepare 8 identical test portions of the food item. The food item must be demonstrated not to be naturally contaminated with *Salmonella* spp.

Inoculate 2 portions at a high inoculum level (e.g. 20 cfu/test portion)

Inoculate 2 portions at an intermediate inoculum level (e.g. 10 cfu/test portion)

Inoculate 2 portions at a low inoculum level (e.g. 2 cfu/test portion)

Retain 2 portions as uninoculated blanks.

At the same time as inoculation of the test portion determine the concentration in the inoculate by plating the high-level inoculum, at least in duplicate, onto a non-selective medium (e.g. plate count agar, nutrient agar).

The inoculated test portions shall be tested according to the full protocol of the alternative method being verified. Estimation of the $LOD_{50}$ is calculated by reference to the number of positive and negative results.

$LOD_{50}$ can be calculated by reference to Table 1 and 2. The estimated $LOD_{50}$ should be adjusted according to the level of the high-level inocula and dilution factor for subsequent inocula levels.

Repeat the procedure on at least 2 further occasions separated by at least 7 days.

### Acceptance criteria

All high-level inoculated test portions should return positive results, if negative results are obtained in the test portions inoculated with high-levels, the experimental protocol should be repeated.

All uninoculated blank test portions should return negative results, if positive results are obtained in the blank test portions, the experimental protocol should be repeated.

Raw, unprocessed bivalve molluscs validation data are not included in Annex C of ISO/IEC 6579-1- validation data and performance criteria. Therefore, it is recommended that the $LOD_{50}$ determined using this technical protocol **should be equal or lower than 3 cfu/test portion on each separate test occasion.**

**Table 1: Example calculation of estimated $LOD_{50}$ based on the number of positive results per level of contamination**

| **Example No.** | **High-level inoculation** (*ca*. 10 x $LOD_{50}$/ test portion) | **Intermediate-level inoculation** (*ca*. 5 x $LOD_{50}$/ test portion) | **Low-level inoculation** (*ca*. 1 x $LOD_{50}$/ test portion) | **Blank control** | **Estimated $LOD_{50}$** (cfu/test portion) |
|---|---|---|---|---|---|
| 1 | 2/2 | 2/2 | 2/2 | 0/2 | <1 x $LOD_{50}$ |
| 2 | 2/2 | 2/2 | 1/2 | 0/2 | 1 x $LOD_{50}$ |
| 3 | 2/2 | 2/2 | 0/2 | 0/2 | 2 x $LOD_{50}$ |
| 4 | 2/2 | 1/2 | 2/2 | 0/2 | 2 x $LOD_{50}$ |
| 5 | 2/2 | 1/2 | 1/2 | 0/2 | 3 x $LOD_{50}$ |
| 6 | 2/2 | 1/2 | 0/2 | 0/2 | 4 x $LOD_{50}$ |
| 7 | 2/2 | 0/2 | 2/2 | 0/2 | Unreliable result |
| 8 | 2/2 | 0/2 | 1/2 | 0/2 | 5 x $LOD_{50}$ |
| 9 | 2/2 | 0/2 | 0/2 | 0/2 | 8 x $LOD_{50}$ |

**Table 2: Example calculation of estimated $LOD_{50}$ using a known inocula concentration**

| Example No. | High-level inoculation (20 cfu /test portion) | Intermediate-level inoculation (10 cfu /test portion) | Low-level inoculation (2 cfu /test portion) | Blank control | Estimated $LOD_{50}$ |
|---|---|---|---|---|---|
| 1 | 2/2 | 2/2 | 2/2 | 0/2 | <2.0 |
| 2 | 2/2 | 2/2 | 1/2 | 0/2 | 2.0 |
| 3 | 2/2 | 2/2 | 0/2 | 0/2 | 4.0 |
| 4 | 2/2 | 1/2 | 2/2 | 0/2 | 4.0 |
| 5 | 2/2 | 1/2 | 1/2 | 0/2 | 6.0 |
| 6 | 2/2 | 1/2 | 0/2 | 0/2 | 8 |
| 7 | 2/2 | 0/2 | 2/2 | 0/2 | Unreliable result |
| 8 | 2/2 | 0/2 | 1/2 | 0/2 | 10 |
| 9 | 2/2 | 0/2 | 0/2 | 0/2 | 16 |