# European Union Reference Laboratory (EURL)

# Proficiency Testing Schemes

## Enumeration of *Escherichia coli* and the detection of *Salmonella* spp. in Common Mussels (*Mytilus edulis*)

**EURL ring trial reference number: RT 37**

**Sample numbers: RT 37A and RT 37B**

**Contents** **Page number**



This scheme is intended to provide proficiency testing samples for laboratories undertaking examination of live bivalve molluscs from production areas in accordance with Regulation (EC) No. 854/2004 and from throughout the production chain in accordance with Regulation (EC) No. 2073/2005.

The scheme is organised by the European Union Reference laboratory (EURL) for monitoring bacteriological and viral contamination of bivalve molluscs. The NRL is designated by the European Commission in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the EURL/HPA Shellfish Scheme (www.hpa.org.uk) through examination of aspects of the methods not covered under the Shellfish Scheme (initial sample preparation and preparation of initial dilutions).

The EU stipulated reference method for enumeration of *E. coli* in live bivalve molluscs in ISO TS 16649-3, Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Anon 2005). The EU stipulated reference method for detection of *Salmonella* spp. in live bivalve molluscs is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (Anon 2002).

A scoring system is used to help assess participants' performance. Details of this system are included as Appendix II of this report. The purpose of scoring is to help the EURL, member state NRLs and other participating laboratories identify incorrect or outlying results. Further information on the use of scoring in proficiency testing and on recommended procedures for following up poor performance can be accessed via the EURL website (www.crlcefas.co.uk) or obtained by contacting the EURL. The European Commission has produced a protocol for management of underperformance in comparative testing and/or lack of collaboration of NRLs with EURLs activities.

If you are experiencing problems with any aspects of these distributions please contact the EURL (contact details below), or alternately refer to the troubleshooting guide included as Appendix III of this report.

Further advice on microbiological testing of bivalve mollluscan shellfish can be obtained via the EURL website (www.crlcefas.co.uk)

Due to the nature of this scheme repeat samples are not available.

| | |
|---|---|
| **Distribution date:** | **22nd November 2010** |
| **Report date:** | **25th January 2010** |
| **Report compiled by:** | **Louise Stockley** |
| **Authorisation by:** | **Rachel Rangdale** |

## Samples

### Sample preparation

One batch consisting of approximately 2600 (38kg) common mussels (*Mytilus edulis*) was collected from a UK commercial harvesting area. Around 30kg of mussels were placed in a recirculation tank in preparation for bio-accumulation and the remaining mussels (8kg) were shucked and homogenised.

### RT 37A

Two thousand mussels were evenly spread across 2 trays and immersed in a small scale depuration unit that had been partially filled with 500 litres of filtered (50 micron filter) seawater and maintained at a temperature of 12°C (approximate temperature of seawater in harvesting area). Seawater was re-circulated at 28 litres per min (with UV) for 24 hours to allow the shellfish to acclimatize. Screened raw sewage was collected from a local sewage treatment works and analysed to determine the *E. coli* levels using a membrane filtration method (Anon 2000). The mussel trays were removed from the tank and 1 litre of screened raw sewage ($\approx 7 \times 10^6$ cfu/100ml) was added and thoroughly mixed. The mussels were re-immersed in the tank and the temperature of the seawater was increased to 17°C with constant re-circulation (without UV). After 3 hours of exposure the mussels were removed. Each sample of RT 37A comprised 35 randomly selected mussels.

### RT 37B

Approximately 600 (8kg) mussels were shucked and homogenised. This was then pooled to form one homogeneous sample and spiked with an overnight culture of *Salmonella* Bristol (NCTC 9853). Each sample of RT 37B comprised a single aliquot of 100ml of homogenised shellfish flesh and intravalvular fluid.

**Note:** Repeat samples were not available for this scheme

### Sample distribution and examination

Samples were packed in accordance with Cefas protocol for packaging shellfish for transportation and distributed on 22nd November 2010 to 32 participating laboratories. Participants were required to analyse the material in duplicate immediately on receipt using their routine laboratory procedures. Supplementary advice on sample acceptance, receipt and processing is available via the EURL website (www.crlcefas.org).

### Sample temperature

Temperature recorders (Thermotrack, Progress Plus) were included in each consignment. Participants were required to record the internal air and sample temperature on arrival and to return the recorder. Temperatures recorded by participants are shown in Appendix I.

## Results

### Reference results

**Sample RT 37A:** Ten randomly selected sub-samples were analysed in duplicate for *E. coli* using EURL SOP No. 1175 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_E_coli_04_04_08.pdf (Table 1).

**Table 1: Reference results – Sample RT 37A**

| Sample description | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median ±3*$SD_T$ |
| RT 37A | $1.3 \times 10^3 – 1.7 \times 10^4$ | $7.0 \times 10^3$ | $6.4 \times 10^3$ | $1.2 \times 10^3$ - $4.2 \times 10^4$ |

GM- geometric mean, $SD_T$ – theoretical standard deviation

**Sample RT 37B:** Ten randomly selected sub-samples were analysed in duplicate for *Salmonella* spp. using EURL SOP 1176 http://www.crlcefas.org/InformationCentre/docs/CRL_SOP_SALMONELLA_17_11_07.pdf applied in a 3 x 3 MPN format (Table 2).

**Note:** Regulation (EC) No. 2073/2005 requires presence/absence testing for *Salmonella* spp. in live bivalve molluscs. Quantitative data is provided for information only.

**Table 2: Reference results – Sample RT 37B**

| Sample description | *Salmonella spp.* | No. of replicates giving positive results | Mean MPN *Salmonella* spp. per g |
|---|---|---|---|
| **RT 37B** | *Salmonella* spp. detected in 25g | 12 | $4.9 \times 10^2$ |

**Participants' results**

Performance assessment was performed according to the procedures described in the EURL/HPA EQA shellfish scheme for a single distribution, with minor modifications (Appendix II).

**Sample RT 37A**

Participants' results and scores allocated for sample RT 37A are shown in Tables 3, 4 and Figure 1.

**Note:** The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' results. SD calculations were based on the inherent variability of the 5 x 3 MPN method (0.26 $\log_{10}$). Reference values were excluded from calculation of participants' median.

**Summary statistics – Sample RT 37A**

| | |
|---|---|
| Total participants reporting duplicate results for *E. coli* MPN | 32 |
| Participants reporting MPN results within the expected range[1] | 31 |
| Participants reporting MPN results outside the expected range for one replicate | 0 |
| Participants reporting MPN results outside the expected range for both replicate | 1 |
| Participants reporting MPN results inconsistent with ISO 7218 (Anon 2007)[2] | 2 |

[1]expected range = participants' median ± theoretical 3SD

[2] on this occasion points were not deducted from participants returning results inconsistent with ISO 7218, laboratories were reminded that 5 x 3 MPN tables from ISO 7218 or those provided by the EURL should be used for MPN determination.

**Sample RT 37B**

Participants' results and scores allocated for sample RT 37B are shown in Table 3.

**Summary statistics – Sample RT 37A**

| | |
|---|---|
| Total participants returning results | 32 |
| Participants reporting expected result | 31 |

**Table 3: Participants results and allocated scores**

| Lab ID | RT 37A *E. coli* MPN/100g | | | RT 37B *Salmonella* spp. in 25g | |
|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Score | Result | Score |
| 3* | 3500 | 1700 | 12 | Present | 2 |
| 7* | 5400 | 5400 | 12 | Present | 2 |
| 9* | 3500 | 2400 | 12 | Present | 2 |
| 10* | 16000 | 5400 | 12 | Present | 2 |
| 13* | 2400 | 2400 | 12 | Present | 2 |
| 21 | 7500 | 17000 | 7 | Present | 2 |
| 22* | 5400 | 9200 | 12 | Present | 2 |
| 27* | 5400 | 5400 | 12 | Present | 2 |
| 32* | 7000 | 4600 | 12 | Present | 2 |
| 33* | 24000 | 24000 | 12 | Present | 2 |
| 35* | 4600 | 4900 | 12 | Present | 2 |
| 41* | 3500 | 3500 | 12 | Present | 2 |
| 43* | 3500 | 4600 | 12 | Present | 2 |
| 44* | 7900 | 940 | 12 | Present | 2 |
| 47* | 16000 | 16000 | 12 | Present | 2 |
| 58 | 3500 | 3500 | 12 | Present | 2 |
| 68* | 3500 | 3500 | 12 | Present | 2 |
| 69 | 13000 | 11000 | 12 | Present | 2 |
| 76 | 16260 | 16260 | 7 | Present | 2 |
| 84 | 1700 | 1300 | 12 | Present | 2 |
| 86* | 17000 | 3300 | 12 | Present | 2 |
| 90 | 9200 | 24000 | 12 | Present | 2 |
| 98 | 13000 | 7900 | 12 | Present | 2 |
| 106 | 5400 | 3500 | 12 | Present | 2 |
| 111 | 7900 | 4900 | 12 | Not detected | 0 |
| 121 | 1700 | 2400 | 12 | Present | 2 |
| 122 | 4300 | 2200 | 12 | Present | 2 |
| 127 | 35000 | 54000 | 2 | Present | 2 |
| 147* | 9200 | 5400 | 12 | Present | 2 |
| 159 | 3500 | 5400 | 12 | Present | 2 |
| 176 | 3500 | 2400 | 12 | Present | 2 |
| 194 | 4600 | 3300 | 12 | Present | 2 |

* Designated NRL's

**Table 4: Participants results RT 37 A**

| | *E. coli* MPN/100g | | | |
|---|---|---|---|---|
| | Range | Median | GM | Median±3*$SD_T$ |
| **Participants results** | $9.4 \times 10^2 – 5.4 \times 10^4$ | $5.2 \times 10^3$ | $5.7 \times 10^3$ | $8.6 \times 10^2$ - $3.1 \times 10^4$ |

GM - geometric mean, $SD_T$ - theoretical standard deviation

**General comments**
Thirty-two laboratories (17 NRL and 15 other laboratories) returned results for this distribution. Fifty-nine percent of samples arrived within 24 hour of dispatch. Two samples arrived outside of 48 hours. Sixteen laboratories analysed the samples on the day of arrival. The remaining laboratories analysed the samples the following day with the exception of Laboratory 106 which analysed the sample 2 days after receiving the material. It is recommended in the good practice guide that samples are analysed within 36 hours of collection (Anon 2010). Temperature loggers stored in each consignment showed an in transit temperature range of 0.5 - 7.5°C with the exception of Laboratory 122 who received the sample 8 days after dispatch and recorded a sample temperature of 18.9°C. Temperature data recorded by participants and temperature buttons returned are given in Appendix I.

**Sample RT 37A**
Thirty-one laboratories returned both replicate *E. coli* MPN/100g results between ±3 SD of the participants' median for RT 37A. Laboratory 127 returned two replicate results above +3 SD but below +5 SD of the participants' median.

Laboratories 21 and 76 reported one or both MPN value(s) that were not consistent with 5 x 3 MPN tables in ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations (Anon 2007) or 5 x 3 MPN tables derived from this standard and previously supplied to NRLs by the EURL. Laboratories are reminded that the 5 x 3 MPN tables from ISO 7218 should be used for MPN determination. Further advice can be obtained from the EURL on request.

Thirty laboratories cited ISO TS 16649-3 (Anon 2005) and 1 laboratory cited Donovan *et al* (1998) as their laboratory method for enumeration of *E. coli*. Laboratories are reminded that 5 x 3 MPN tables in Donovan *et al* (1998) and those contained in ISO 7251 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* – Most probable number technique, differ slightly from those contained in ISO 7218.

**Sample RT 37B**
Thirty-one laboratories returned expected results for RT 37B with the exception of laboratory 111 who did not detect *Salmonella* spp. in the sample.

Twenty-five laboratories used the EU specified reference method for detection of *Salmonella* spp (ISO 6579). Six laboratories referenced NMKL 71: *Salmonella* detection in foods (NMKL 71, 1999). **Laboratories are reminded that for official control testing of live bivalve molluscs for *Salmonella* spp. the EU reference method is ISO 6579, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. (Anon 2002).**

**References**
Anon 2000. Microbiology of recreational and environmental waters 2000. Detection and enumeration of coliform organisms and faecal coliform organisms by membrane filtration.

Anon 2007 ISO 7218:2007 Microbiology of food and animal feeding stuffs - General recommendations and guidance for microbiological examinations. Geneva, Switzerland.

Anon 2010. Microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application. EURL publication, Issue 4, August 2010.

Anon 2005 ISO TS 16649-3:2005. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.

Anon. 2002. ISO 6579:2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.

Donovan TJ, Gallacher S, Andrews NJ, Greenwood MH, Graham J, Russel JE, Roberts D, Lee R. (1998). 'Modification of the standard method used in the united kingdom for counting *Escherichia coli* in live bivalve molluscs'. Communicable disease and public health 1: 188-96.

NMKL Method No. 71, 5 ed, 1999: *Salmonella* detection in foods.

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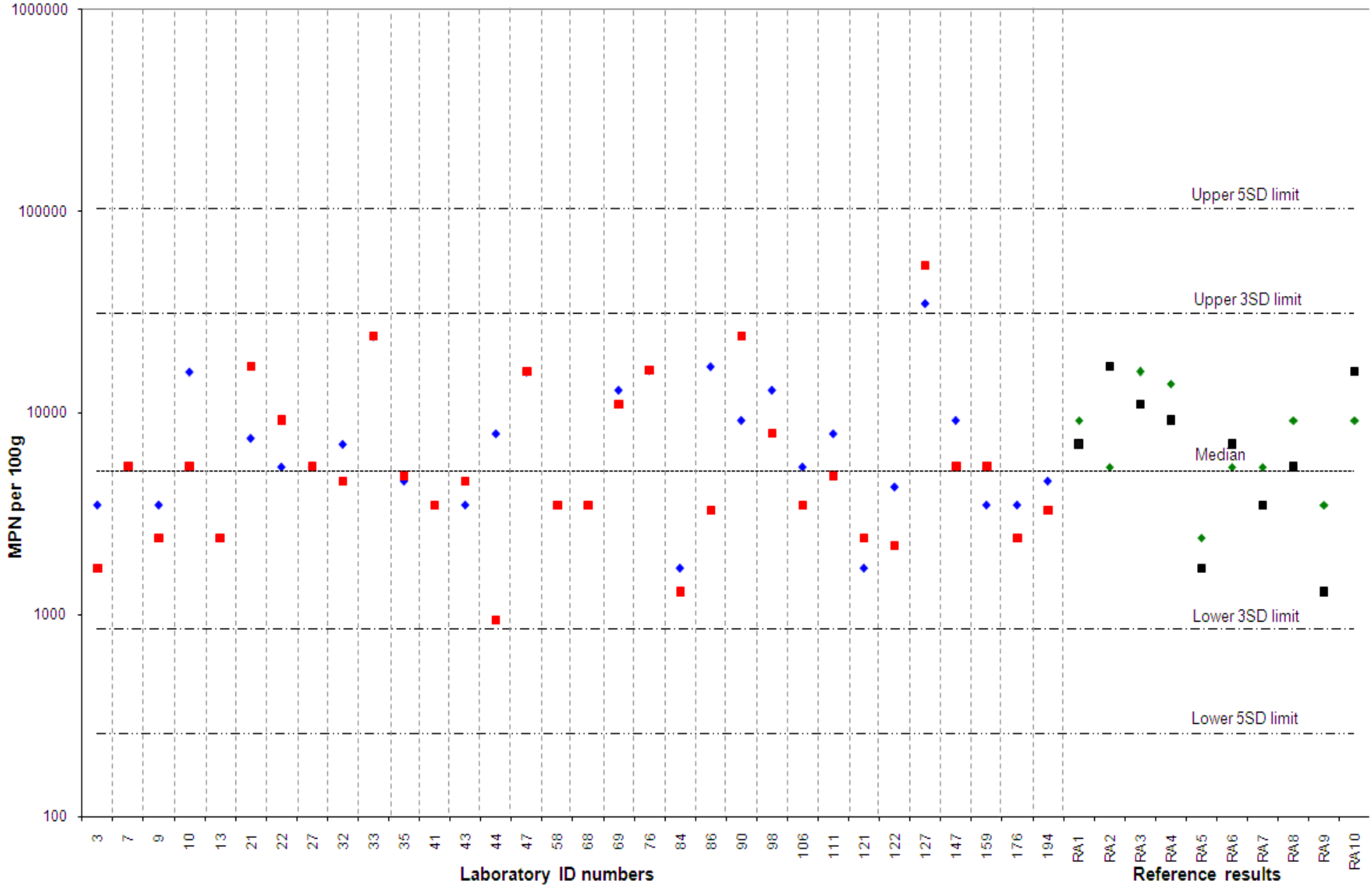
**Results chart - Sample RT 37A**



**Appendix I:**

**Sample arrival and temperature**

| Lab ID | Date arrived | Time of arrival | Temp. logger (°C) | Sample (°C) | Storage (°C) | Date analysed |
|---|---|---|---|---|---|---|
| 3 | 23.11.10 | 10:20 | 2 - 5 | 4 | 5 | 23.11.10 |
| 7 | 23.11.10 | 14:00 | 2 - 6 | 5 | 1 - 5 | 24.11.10 |
| 9 | 23.11.10 | 11:00 | - | 7 | 3.7 | 23.11.10 |
| 10 | 23.11.10 | 11:00 | 2 - 5.5 | 5.1 | - | 23.11.10 |
| 13 | 23.11.10 | 11:50 | 1.5 - 5.5 | 5 | 3.5 | 24.11.10 |
| 21 | 23.11.10 | 14:20 | 1 - 7.5 | 2.6 | - | 24.11.10 |
| 22 | 24.11.10 | 07:30 | 2 - 5.5 | 2.2 | 2.2 | 24.11.10 |
| 27 | 23.11.10 | 12:15 | 1 - 4.5 | 3 | 2 - 6 | 24.11.10 |
| 32 | 23.11.10 | 12:00 | 2 - 4.5 | 3.6 | 4 | 23.11.10 |
| 33 | 23.11.10 | 13:10 | 3.5 - 6 | 3.1 | 1 | 23.11.10 |
| 35 | 24.11.10 | 10:30 | 1 - 2.5 | - | 6.5 | 24.11.10 |
| 41 | 23.11.10 | 11:00 | 2.5 - 3.5 | 3.4 | 2 - 6 | 23.11.10 |
| 43 | 23.11.10 | 15:30 | 3 - 6 | 4 | 6 | 24.11.10 |
| 44 | 23.11.10 | 15:05 | 2.5 - 4 | 2.8 | 3 | 24.11.10 |
| 47 | 23.11.10 | 15:00 | 2.5 - 4.5 | 4.4 | 4 | 23.11.10 |
| 58 | 24.11.10 | 13;30 | 1 - 4 | - | 7.4 | 25.11.10 |
| 68 | 23.11.10 | 13:30 | 1.5 - 4 | 5.5 | 3 | 24.11.10 |
| 69 | 24.11.10 | 10:30 | 0.5 - 5.5 | 4.6 | - | 24.11.10 |
| 76 | 23.11.10 | 10:00 | 2.5 - 4 | - | 4.1 | 24.11.10 |
| 84 | 24.11.10 | 16:00 | 1.5 - 6 | - | 0 - 4 | 25.11.10 |
| 86 | 24.11.10 | 16:50 | 1 - 2.5 | 3.8 | 4.4 | 25.11.10 |
| 90 | 25.11.10 | 09:55 | 1 - 4 | 4 | 4 | 25.11.10 |
| 98 | 23.11.10 | 16:00 | 1.5 - 4.5 | 4.5 | 4 | 24.11.10 |
| 106 | 23.11.10 | - | - | - | 4 | 25.11.10 |
| 111 | 24.11.10 | - | 1 - 4 | - | - | 25.11.10 |
| 121 | 24.11.10 | 11:30 | 2.5 - 4 | 4 | - | 24.11.10 |
| 122 | 30.11.10 | 14:45 | 3 - 18.5 | 18.9 | 4 | 01.12.10 |
| 127 | 23.11.10 | 13:30 | - | - | 2 - 6 | 24.11.10 |
| 147 | 23.11.10 | - | 2 - 4.5 | 4 | 3 | 23.11.10 |
| 159 | 24.11.10 | 13:00 | 2.5 - 5.5 | 3 | - | 24.11.10 |
| 176 | 24.11.10 | 11:00 | 3 - 4.5 | 4.8 | 1 - 5 | 25.11.10 |
| 194 | 24.11.10 | 09:30 | 2.5 - 6 | 1.3 | - | 24.11.10 |

**Appendix II**

***E.coli* MPN scoring**

| Result | Score allocated |
|---|---|
| Return of results | 2 |
| All replicate MPN results within the expected range | 10 |
| Or | |
| One replicate MPN result reported is outside the expected range and falls between the median ±3SD and median ±5SD value | 7 |
| Or | |
| Both replicate MPN results are outside the expected range and fall between the median ±3SD and median ±5SD value | 4 |
| Or | |
| One replicate MPN result reported is outside the median ±5SD value | 5 |
| Or | |
| Both replicate MPN results reported is outside the median ±5SD value | 0 |
| Or | |
| Single MPN result reported only | 5 |
| Or | |
| Tube combination inconsistent with MPN reported (one replicate) | 7 |
| Or | |
| Tube combination inconsistent with MPN reported (both replicates) | 5 |
| Or | |
| Sample not examined or results returned late - no explanation received | 0 |
| Or | |
| High censored result (e.g. MPN = >18000 per 100g) | Score not allocated |

***Salmonella* spp scoring**

| Result | Score allocated |
|---|---|
| Fully correct results | 2 |
| Misleading result, e.g. failure to isolate *Salmonella* | 0 |

**Appendix III:**

**Troubleshooting advice**

1. Methods – Ensure that the method used is appropriate for the examination of the sample.

    a. Ensure that any dilutions have been calculated correctly.
    b. Ensure that MPN tables (if used) are interpreted correctly.

2. Culture Medium - Check the quality control data for media to ensure that they are within specifications and performing adequately.

3. Equipment - Check that the equipment used for the procedures (incubators, refrigerators, measuring instruments) are calibrated and performing adequately.

4. Staff Training - Check that the staff performing the tests are fully trained and familiar with all the procedural steps.

5. Clerical Procedures - Check that the sample labeling, laboratory numbering and clerical procedures are adequate have you procedures for ensuring that test results are reported accurately and on time.

6. Accreditation- Check that quality procedures are documented and adhered to at all times.

7. Internal quality controls (IQC) – Ensure that adequate controls are in place and that and documentation for dealing with IQC failures is appropriate.

Further advice can be obtained from the EURL on request.

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