European Union Reference Laboratory (EURL) Proficiency Testing Scheme

Enumeration of *Escherichia coli* and the detection of *Salmonella* spp. in bivalve molluscan shellfish

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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 EURL is designated by the European Union in accordance with Regulation (EC) No. 882/2004. The scheme is intended to compliment the EURL/PHE Shellfish Scheme through examination of aspects of the methods not covered under the Shellfish Scheme (initial sample preparation and preparation of initial dilutions) (http://www.hpa.org.uk/ProductsServices/MicrobiologyPathology/ExternalQualityAssessmentProficiencyTesting/EQAPTForFoodWaterAndEnvironmentalMicrobiology/ShellfishScheme/ and to provide additional data for laboratories for ISO 17025 accreditation purposes.

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 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 I of this report. The purpose of scoring is to help the EURL, 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.eurlcefas.org) or obtained by contacting the EURL. The European Union 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 II of this report.

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

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

Sample preparation

Samples 1 and 2

A single batch of approximately 1500 Pacific oysters (*Crassostrea gigas*) was collected from a UK commercial harvesting area at 8:30am on the 21st November 2016 and was placed in a large sterile container before being thoroughly mixed. Sample 1 and sample 2 provided to participating laboratories comprised of 15 randomly selected Pacific oysters from this bulk material.

Sample 3

A single batch of approximately 400 Pacific oysters (*Crassostrea gigas*) was collected from a UK commercial harvesting area on the 17th November 2016. On arrival, the Pacific oysters were shucked and homogenised before being pooled together to form one homogenate. Sample 3 was aliquoted in 100 ml volumes on the 20th November. Prior to distribution sample 3 was spiked with estimated levels of *E. coli* and *Salmonella* spp..

Sample distribution and examination

Each individual sample was packed in accordance with the Cefas protocol for packaging shellfish for transportation. Samples were collected at 11:00am on the 21st November 2016 to 39 participating laboratories. Participants were requested to analyse the samples immediately on receipt using their routine methods. Sample 1 and 3 were examined for *E. coli* and samples 2 and 3 were examined for *Salmonella* spp.

Sample temperature

Participants were requested to record the internal sample temperature on arrival. Temperatures recorded by participants are shown in Appendix I.

Results

Reference results - *E. coli*

Ten randomly selected samples were analysed in duplicate on 2 consecutive days (22.12.16 and 23.12.16) for *E. coli* using EURL SOP No. 1175 (Table 1). Sample homogeneity was assessed following the procedure described in ISO 22117. The sample material was considered sufficiently homogenous.

Table 1: *E. coli* MPN/100g reference results

Sample and type	Date of Analysis	Range	Median	GM	Median ±3*SD_T
Sample 1 - Oysters	22.12.16	$2.0 \times 10^1 – 7.8 \times 10^2$	2.3×10^2	2.1×10^2	$4.4 \times 10^1 – 1.2 \times 10^3$
	23.12.16	$7.8 \times 10^1 – 4.9 \times 10^2$	1.7×10^2	1.8×10^2	$3.2 \times 10^1 – 8.9 \times 10^2$
Sample 3 - Homogenate	22.12.16	$7.8 \times 10^2 – 7.9 \times 10^3$	2.3×10^3	2.2×10^3	$4.3 \times 10^2 – 1.2 \times 10^4$
	23.12.16	$3.3 \times 10^2 – 1.1 \times 10^4$	1.7×10^3	1.8×10^3	$3.2 \times 10^2 – 8.9 \times 10^3$

GM - geometric mean, SD_T - theoretical standard deviation (0.24 $\log_{10}$)

Reference results – *Salmonella* spp.

Ten randomly selected samples were analysed on 2 consecutive days (22.12.16 and 23.12.16) for *Salmonella spp.* using EURL SOP No. 1176 (Table 2).

Note: Regulation (EC) No. 2073/2005 requires presence/absence testing for *Salmonella* spp. in live bivalve molluscs

Table 2: Reference results

Sample and type	Date of Analysis	*Salmonella spp.*	No. of replicates giving the expected result
Sample 2 - Oysters	22.12.16	Absent in 25g	9 [1]
	23.12.16	Absent in 25g	7 [1]
Sample 3 - Homogenate	22.12.16	Present in 25g	10
	23.12.16	Present in 25g	10

[1] *Salmonella* spp. was detected in a proportion of the reference samples examined.

Participants' results

Performance assessment was according to the procedures described in the EURL/PHE EQA shellfish scheme for a single distribution, with minor modifications (Appendix II). Participants' results and scores allocated for PT 64 are shown in Tables 3, 4, 5 and Figure 1.

Note: The median and upper and lower limits (±3 SD and ±5 SD) were calculated from participants' results. SD_T calculations were based on the inherent variability of the 5 x 3 MPN method (0.24 log_{10}). Reference values were excluded from the calculation of the participants' median.

Table 3: Participants' results

GM - geometric mean, SD_T – theoretical standard deviation (0.24)

Sample and type	*E. coli* MPN/100g			
	Range	Median	GM	Median±3*SD_T
Sample 1 - Oysters	<2.0 x 10^1 – 1.3 x 10^3	1.7 x 10^2	1.7 x 10^2	3.2 x 10^1 – 8.9 x 10^2
Sample 3 - Homogenate	1.7 x 10^2 – 7.9 x 10^3	2.2 x 10^3	2.1 x 10^3	4.2 x 10^2 – 1.2 x 10^4

Table 4: Summary statistics of participants' results

E. coli	Sample 1 - Oysters	Sample 3 - Homogenate
Participants reporting duplicate results for *E. coli* MPN	39	38
Participants reporting a single MPN result	0	0
Participants reporting MPN results within the expected range for both replicates [1]	34	37
Participants reporting MPN results outside the expected range for one replicate	3	0
Participants reporting MPN results outside the expected range for both replicates	2	1
Participants reporting MPN results as censored results for one replicate	1	0
Participants reporting MPN results as censored results for both replicates	1	0
Participants reporting tube combination and / or MPN results inconsistent with ISO 7218 (Anon 2007) [2]	6	6

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

[2] points deducted from participants returning results inconsistent with ISO 7218.

Table 5: Participants' results and allocated scores for whole animal samples

Lab ID	Sample 1			Sample 2 [a]	
	E. coli MPN/100g			*Sal.* spp. in 25g	
	Rep 1	Rep 2	Score	Rep 1	Score
3 *	330	230	12	Not Detected	-
7 *	490	1300	9	Not Detected	-
9 *	130	130	12	Not Detected	-
10 *	490	490	12	Not Detected	-
13 *	130	330	12	Not Detected	-
17 *	45	78	12	Not Detected	-
19 *	130	78	12	Not Detected	-
22 *	780	690	12	Not Detected	-
23 *	78	130	12	Not Detected	-
27 *	170	230	12	Not Detected	-
32 * [b]	490	45	8	Not Detected	-
33 *	78	130	12	Present	-
35 *	330	170	12	Not Detected	-
39 *	230	170	12	Not Detected	-
41 *	690	230	12	Not Detected	-
42 *	68	20	9	Not Detected	-
43 *	230	490	12	Not Detected	-
44 * [b]	130	78	8	Not Detected	-
47 *	78	45	12	Not Detected	-
48	450	330	12	Not Detected	-

Lab ID	Sample 1			Sample 2 [a]	
	E. coli MPN/100g			*Sal.* spp. in 25g	
	Rep 1	Rep 2	Score	Rep 1	Score
54 [b]	1300	1100	2	Not Detected	-
68 *	210	130	12	Present	-
69 *	330	490	12	Not Detected	-
72	330	310	12	Not Detected	-
83 *	230	230	12	Not Detected	-
90 *	490	330	12	Not Detected	-
96	78	78	12	Not Detected	-
98 [b]	80	330	8	Not Detected	-
100	170	170	12	Not Detected	-
102 * [b]	13	4.5	0	Not Detected	-
131	330	330	12	Not Detected	-
147 *	140	110	12	Not Detected	-
170 * [d]	<200	<200	8	NE	-
187	78	110	12	Not Detected	-
203 *	45	78	12	Not Detected	-
209	210	330	12	Not Detected	-
212 * [d]	230	440	8	NE	-
235 [b c]	130	<20	3	Not Detected	-
245 *	130	330	12	Not Detected	-

* Designated NRL's,

[a] No scores allocated as *Salmonella* spp. was detected 4/20 replicate reference samples.

[b] Scores deducted as tube combination inconsistent with rules specified in ISO 7218.

[c] Score deducted as incorrect MPN value given for recorded tube combination.

[d] MPN tube combination is not required for this method, the maximum overall score is reduced to reflect this (8).

NE – Not examined.

Table 6: Participants' results and allocated scores for homogenate sample

Lab ID	Sample 3				
	E. coli MPN/100g			*Sal.* spp. in 25g	
	Rep 1	Rep 2	Score	Rep 1	Score
3 *	3300	2300	12	Present	2
7 *	3300	1300	12	Present	2
9 *	2300	1700	12	Present	2
10 *	3300	2200	12	Present	2
13 *	7900	4900	12	Present	2
17 *	1300	1700	12	Present	2
19 *	3300	2300	12	Present	2
22 *	1400	1400	12	Present	2
23 *	1100	780	12	Present	2
27 *	2100	4900	12	Present	2
32 *	1300	2200	12	Present	2
33 *	3300	1300	12	Present	2
35 *	3300	1300	12	Present	2
39 *	1300	3300	12	Present	2
41 *	3300	1700	12	Present	2
42 *	1300	690	12	Present	2
43 *	1300	1300	12	Present	2
44 * [a]	1300	3300	8	Present	2
47 *	2200	2200	12	Present	2
48	3300	3300	12	Present	2

Lab ID	Sample 3				
	E. coli MPN/100g			*Sal.* spp. in 25g	
	Rep 1	Rep 2	Score	Rep 1	Score
54 [a]	2400	1700	8	Present	2
68 *	3300	4600	12	Present	2
69 *	3500	2400	12	Not Detected	0
72	1400	1300	12	Present	2
83 *	3300	4900	12	Present	2
90 *	2300	4900	12	Present	2
96 [b]	3500	5400	8	Present	2
98 [a]	1300	1300	8	Present	2
100 [d]	NE	NE	-	NE	-
102 * [a b]	330	170	2	Present	2
131	4900	4900	12	Present	2
147 *	2300	4900	12	Present	2
170 * [c]	1620	1800	8	NE	-
187	2300	3300	12	Present	2
203 *	1100	1100	12	Present	2
209	2300	1100	12	Present	2
212 * [c]	1300	1500	8	NE	-
235 [a]	5400	1700	8	Present	2
245 *	1700	2200	12	Present	2

* Designated NRL's,

[a] Scores deducted as tube combination inconsistent with rules specified in ISO 7218.

[b] Score deducted as incorrect MPN value given for recorded tube combination.

[c] MPN tube combination is not required for this method, the overall maximum score is reduced to reflect this (8).

[d] On receipt of the laboratory results, it was noted that no results were recorded for Sample 3. The EURL contacted the laboratory and was informed that sample 3 was not included in the box.

NE – Not examined.

General comments

Thirty-nine laboratories (29 NRLs and 10 other laboratories) were sent material with all laboratories returning results. Information provided by laboratories on the arrival time of the material showed that 90% (35) of laboratories received the material the day after dispatch (22.11.16), with 51% (18) of these laboratories analysing the material on arrival. All laboratories received material within 48 hours of dispatch. In total 56% (22) of laboratories analysed the material on the same day as receiving the material, with 44% (17) analysing the material one day after receipt.

Arrival temperatures were recorded in the range of 0.7 – 9.6°C. All temperature data, arrival and analysis dates and times recorded by participants are shown in Appendix I.

Sample analyses

Thirty-nine laboratories returned the report form for this PT distribution. Laboratory 100 did not examine sample 3 for *E. coli* or *Salmonella* spp. because the sample was not included in the dispatched box. For this reason, no score was given for this sample. The EURL recommends that all laboratories read the accompanying note before performing any analysis to ensure the correct samples and paperwork have been included in the dispatched box.

Sample 1 – Oysters (*E. coli* only)

Thirty-four laboratories returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median with 31 laboratories (91%) obtaining full marks. Laboratories 7 and 42 reported 1 replicate and Laboratory 54 reported both replicate results between ±3 and ±5 SD of the participants' median. Laboratory 235 reported 1 replicate result outside ±5 SD of the participants' median. Laboratory 102 reported 1 replicate falling between ±3 and ±5 SD and the other falling outside ±5 SD of the participants' median.

Six laboratories (laboratories 32, 44, 54, 98, 102 and 235) were deducted points for reporting tube combinations inconsistent with the guidance given in ISO 7218 for interpretation of 5 x 3 MPN tables or for the reporting of an incorrect MPN value for the reported tube combination. Participants are reminded that for official control testing of live bivalve molluscs in the EU, the 5 x 3 MPN tables or MPN calculator in ISO7218:2007/Amd1:2013 and the EURL generic protocol for enumeration of *E. coli* in bivalve molluscs (Issue 13) should be used.

Sample 2 – Oysters (*Salmonella* spp. only)

Ten replicate samples were analysed on consecutive days during the testing period. It was noted that presumptive colonies of *Salmonella* spp. were detected in 4 of the 20 replicate samples. Further identification using whole genome sequencing and multi locus sequence typing was carried out on the isolates and it was confirmed that the *Salmonella* strains isolated had originated naturally in the sample. Previous studies at the UK NRL estimated that the limit of detection of ISO 6579 in seeded bivalve shellfish matrix is 4 CFU/25g. However, these data were not established using naturally contaminated samples and the potential effects of sample transit were not considered. For this reason, participants' Salmonella results were not allocated a score for this sample.

Sample 3 – Homogenate (*E. coli* and *Salmonella* spp. only)

Thirty-seven laboratories returned duplicate *E. coli* MPN/100g results falling between ±3 SD of the participants' median with 32 laboratories (86%) obtaining full marks. Laboratory 102 reported both replicate results between ±3 and ±5 SD of the participants' median.

Six laboratories (laboratories 44, 54, 96, 98, 102 and 235) were deducted points for reporting tube combinations inconsistent with the guidance given in ISO 7218 for interpretation of 5 x 3 MPN tables and the reporting of

incorrect MPN value for the reported tube combination. Participants are reminded that for official control testing of live bivalve molluscs in the EU, the 5 x 3 MPN tables or MPN calculator in ISO7218:2007/Amd1:2013 and the EURL generic protocol for enumeration of *E. coli* in bivalve molluscs (Issue 13) should be used.

Thirty-six laboratories returned results for *Salmonella* spp. with 35 correctly reporting the presence of *Salmonella* spp. in sample 3 and received a score of 2. Laboratory 69 did not detect *Salmonella* spp. in the sample and received a score of 0.

Summary

Twenty-nine laboratories (74%) achieved full marks for both samples tested for the enumeration of *E. coli*. For this distribution the EURL recommended participants to analyse the sample with 4 dilutions. Laboratories who did not follow the advice given in ISO7218:2007/Amd1:2013 and/or the EURL generic protocol for calculating the MPN value for *E. coli* incurred a deduction (7 laboratories). Laboratories are requested to note from ISO ISO7218:2007/Amd1:2013 that '*In any circumstance when more than three dilutions are made, it is essential that all measured data values be used. It is not scientifically correct to "select" any combination of values on the premise that these values are more "correct" than other combinations. The results from all possible combinations of positive tubes should be recorded and the MPN calculator (http://standards.iso.org/iso/7218/) used to derive MPN values*'.

Those laboratories who achieved <40% of the maximum possible score in this distribution for *E. coli* enumeration (<5 out of the maximum 12 score) and / or *Salmonella* spp. detection (score of 0) should review their laboratory procedures. In the first instance refer to the troubleshooting guide included as Appendix III. However, further guidance is available from the EURL.

References

Anon 2015. ISO TS 16649-3. Microbiology of food and animal feeding chain - 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. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva, Switzerland.

Anon 2013. ISO 7218:2007/FDAM 1:2013. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Organization for Standardization, Geneva.

Anon 2001. ISO TS 16649-2. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 2: Colony-count technique at 44˚C using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Geneva, Switzerland.

Anon 2010. ISO 22117:2010. Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by interlaboratory comparison. Geneva, Switzerland.

Figure 1: Results chart sample 1 – Pacific oysters

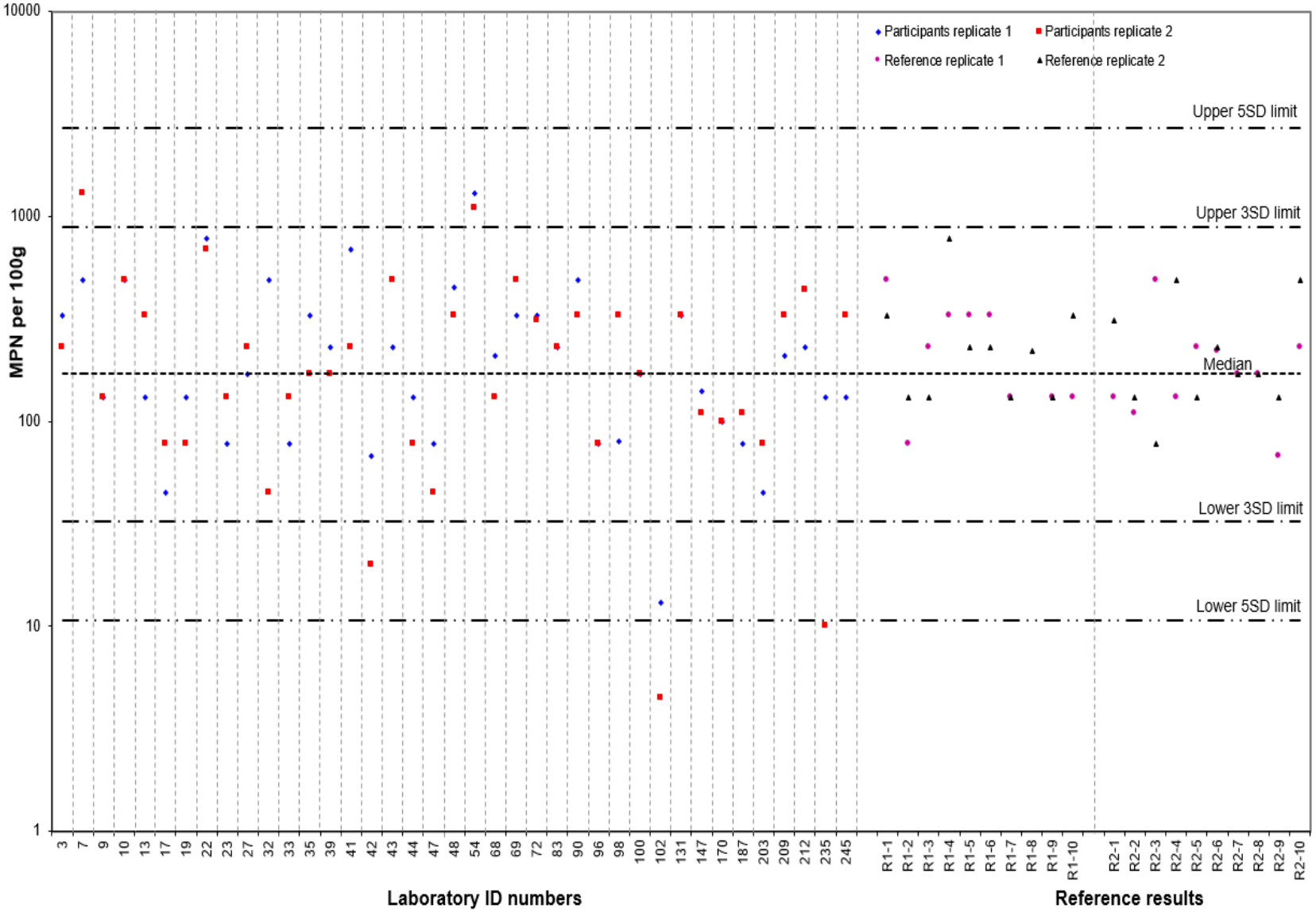
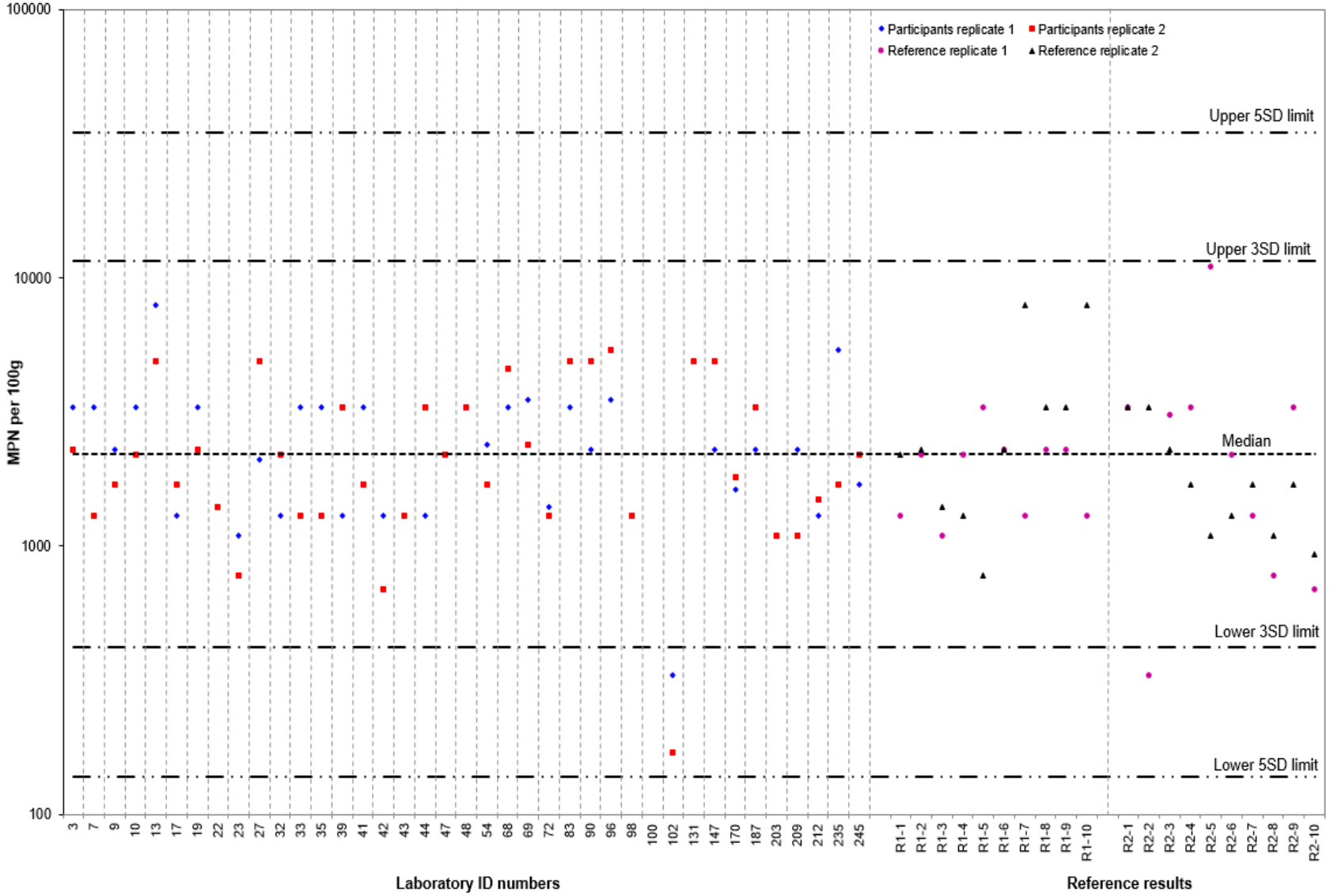


Figure 2: Results chart sample 3 – shellfish homogenate



Appendix I

Sample arrival and temperature

Lab ID	Date arrived	Time of arrival	Sample (°C)	Storage (°C)	Date analysed
3 *	23/11/2016	14:30	1.9		23/11/2016
7 *	22/11/2016	13:30	3	3 ± 2	23/11/2016
9 *	22/11/2016	11:00	6	3	22/11/2016
10 *	22/11/2016	12:00	5.8		22/11/2016
13 *	22/11/2016	09:30	1.8	1.9	22/11/2016
17 *	22/11/2016	14:30	4.5		22/11/2016
19 *	22/11/2016	13:25	9.6	4	23/11/2016
22 *	22/11/2016	11:42	8.2	3	22/11/2016
23 *	22/11/2016	11:45	1.6	4	23/11/2016
27 *	22/11/2016	12:10	5		22/11/2016
32 *	22/11/2016	09:30	4.9		22/11/2016
33 *	22/11/2016	14:50	3.1	3 ± 2	23/11/2016
35 *	22/11/2016	09:30	4.3		22/11/2016
39 *	22/11/2016	12:00	3.7		22/11/2016
41 *	22/11/2016	11:30	5.2	4 ± 2	22/11/2016
42 *	22/11/2016	15:45	4	3	23/11/2016
43 *	22/11/2016	16:00	2	4	23/11/2016
44 *	22/11/2016	16:00	2	3 ± 2	23/11/2016
47 *	22/11/2016	08:30	1.9 - 2.2		22/11/2016
48	22/11/2016	11:30	4.2	2 - 4	22/11/2016
54	23/11/2016	10:00	3		23/11/2016
68 *	22/11/2016	10:35	2.8		22/11/2016
69 *	22/11/2016	12:20	2.1	5	22/11/2016
72	22/11/2016	12:00	2.3	2.5	23/11/2016
83 *	23/11/2016	14:25	2	3	23/11/2016
90 *	23/11/2016	09:10	4.2		23/11/2016
96	22/11/2016	12:05	3.2	3 ± 2	23/11/2016
98	22/11/2016	09:30	4.7	4	22/11/2016
100	22/11/2016	13:15	4.1	3 ± 2	23/11/2016
102 *	22/11/2016	11:20	1	3	23/11/2016
131	22/11/2016	14:05	2.4	3	23/11/2016
147 *	22/11/2016	13:15	4	4	23/11/2016
170 *	22/11/2016	13:25	9.6	4	23/11/2016
187	22/11/2016	14:00	4.5		22/11/2016
203 *	22/11/2016	12:25	2 ± 3	2 ± 3	23/11/2016
209	22/11/2016	11:30	6.7	4	22/11/2016
212 *	22/11/2016	11:30	5.2		22/11/2016
235	22/11/2016	12:30	4.6	3	23/11/2016
245 *	22/11/2016	11:30	0.7	4	23/11/2016

* Designated NRLs

Appendix II:

***E. coli* MPN scores allocated to participants returning 2 replicate results**

Result	Returning of results	Score allocated		Total score
		Replicate 1	Replicate 2	
Both replicate MPN results are within the expected range	2	5	5	12
One replicate MPN result is outside the expected range and falls between the median ±3SD and median ±5SD values	2	5	2	9
Both replicates MPN results are outside the expected range and fall between the median ±3SD and median ±5SD values	2	2	2	6
One replicate MPN result is outside the median ±5SD value	2	5	0	7
Both replicates MPN results are outside the expected range. The first falls between the median ±3SD and median ±5SD value and the second falls outside the median ±5SD values.	2	2	0	4
Both replicates MPN results reported is outside the median ±5SD value	2	0	0	2

***E. coli* MPN scores allocated to participants returning 1 single replicate result**

Result	Returning of results	Score allocated	Total score
Single replicate MPN result is within the expected range	2	5	7
Single replicate MPN result is outside the expected range and falls between the median ±3SD and median ±5SD values	2	2	4
Single replicate MPN result reported is outside the median ±5SD value	2	0	2

***E. coli* score deductions**

Result	Score deducted	
	Replicate 1	Replicate 2
Tube combination inconsistent with MPN reported and / or tube combination selected not consistent with rules given in ISO 7218:2007/Amd 1:2013 or MPN tables provided by the NRL	2	2
High censored result (e.g. MPN = >18000 per 100g)	2	2
Sample not examined or results returned late - no explanation received	12	

***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 the dilutions analysed are as specified on the report form.
    c. Ensure that MPN tables (if used) are interpreted correctly.

    Interpretation of MPN tables

    Where three dilutions have been tested for a sample, record the number of TBGA/TBX positives for each dilution to give a three figure tube combination number. Use the MPN tables included in ISO 7218 and the EURL generic *E. coli* protocol. Only category 1 or 2 tube combinations are included in the tables and should be reported.

    Where more than three dilutions have been tested for a sample, use the Excel spreadsheet MPN calculator (http://standards.iso.org/iso/7218/) to determine the MPN from all the dilutions tested. Combinations that do not appear in the tables or obtained from the Excel calculator as category 3 are not acceptable and should not be used.

    If the tube combination result is an unacceptable combination, the result is reported as 'void'.

2. **Culture media** - 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 and that you have 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 adequate controls are in place and follow-up procedures are in place to deal with IQC failures.

Further advice can be obtained from the EURL on request.

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