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Guidance note

Determination of limit of detection (LOD_{95}) and limit of quantification (LOQ) characteristics for the method for quantification of viruses in bivalve molluscan shellfish

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History of Procedure

Issue	Date	Section	Changes
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Contents

1 INTRODUCTION

This document comprises guidance for laboratories wishing to determine limit of detection (LOD_{95}) and limit of quantification (LOQ) characteristics for the method for quantification of norovirus and hepatitis A virus (HAV) in bivalve molluscan shellfish as applied in their laboratory.

Many different approaches to determination of LOD_{95} and LOQ are available; this document describes a method using a log_2 dilution series of norovirus GI and GII and HAV prepared in proteinase K supernatant from shellfish digestive gland. Multiple subsamples at each level of the dilution series are tested, and the results used to determine LOD_{95} using a probability of detection function recommended by ISO/TC 34/SC 9/WG2 Statistics. The LOQ is determined as the lowest level above the LOD_{95} where the standard deviation of the log-transformed results is <0.33.

This guidance is suitable for characterisation of methods compliant with ISO 15216-1:2017; Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR -- Part 1: Method for quantification.

This guidance is appropriate for use with any species of bivalve mollusc, however application of LOD_{95} and LOQ values determined with one species of shellfish to other species is not best practise. Laboratories should therefore either determine LOD_{95} and LOQ separately for all species tested, or ensure that the species used for this exercise is the most commonly tested. In all cases, where results are reported in relation to the LOD_{95} and LOQ values, the shellfish species used to determine these values should be noted.

2 REPLICATION

A minimum of 10 replicate extractions at each contamination level should be used to generate LOD_{95} and LOQ characteristics using the methods described below. This allows for a small number of missing data points due to unacceptable RT-PCR inhibition or extraction efficiency results. The use of fewer replicates, or the presence of large numbers of missing data points due to unacceptable RT-PCR inhibition or extraction efficiency results will result in a less robust estimate of LOD_{95} and LOQ values.

3 NEGATIVE SUPERNATANT

Dissect sufficient norovirus and HAV-negative shellfish to produce at least 60g digestive tissues.

NOTE: the virus-negativity of the shellfish used as matrix in this procedure should be established through a suitable testing procedure, e.g. testing of multiple subsamples of shellfish with increased numbers of real-time RT-PCR replicates. In addition, determination of typical extraction efficiency and RT-PCR inhibition levels for the shellfish used to produce the supernatant should be determined, to ensure that use of supernatant from these shellfish is unlikely to result in large numbers of unacceptable results (see above).

Split the digestive tissues into aliquots of no more than 10g. Carry out virus extraction on all aliquots by proteinase K digestion as described in the method protocol undergoing characterisation, but modified such that the amount of proteinase K solution and process control virus material is increased proportionately to the quantity of digestive tissues used (e.g. with 10g digestive tissues use 10ml proteinase K solution and 50µl process control virus material). After virus extraction pool together supernatant from all extractions. Record the weight in g of digestive tissues processed and volume of supernatant recovered. Remove a 1.5ml portion and store both this and the remaining supernatant at -20°C.

NOTE: an absolute minimum of 55ml supernatant must be produced by this procedure; if less than this amount is produced the process should be repeated with additional shellfish and the supernatants pooled together.

4 INITIAL CALIBRATION EXTRACTIONS

NOTE: the results generated by the calibration extractions do not form part of the method characterisation data. This part of the process is designed to help each lab optimize contamination levels for the method characterisation.

Defrost the 1.5ml portion of digestive tissue supernatant. Use GI and GII norovirus and HAV stocks to artificially contaminate the supernatant [where prior information on stock concentrations are available aim to add approximately 10,000 copies per ml of supernatant]. Split into 3 x 500µl aliquots and subject to RNA extraction

and real-time RT-PCR according to the method protocol undergoing characterisation. Test for GI, GII, HAV and the process control virus. Discount any results where the RT-PCR inhibition or extraction efficiency results are below the acceptable threshold.

5 PREPARATION OF DILUTION SERIES

Prepare a $\log_2$ dilution series of norovirus GI and GII and HAV in digestive tissue supernatant as follows:-

- Defrost the digestive tissue supernatant. Transfer 10.4ml to one tube (labelled e.g. "neat") and 5.2ml to each of 8 tubes (labelled e.g. "1:2", "1:4", "1:8", "1:16", "1:32", "1:64", "1:128", "1:256").
- Taking into account the results of the calibration extractions, use GI, GII and HAV stocks to artificially contaminate the supernatant in the "neat" tube to levels that will provide results of 1000-2000 copies/g. Mix well.
- Transfer 5.2ml of contaminated supernatant to the tube labelled "1:2" to create a 1:2 dilution. Mix well.
- Transfer 5.2ml of contaminated supernatant from the tube labelled "1:2" to the tube labelled "1:4" to create a 1:4 dilution. Mix well.
- Repeat previous steps until a $\log_2$ dilution series down to 1:256 has been produced.
- Label 10 x 1.5ml tubes for each dilution ("neat" to "1:256"). Transfer 500µl of the relevant dilution to each tube and store these subsamples at -20°C.

6 TESTING/GENERATION OF DATA

Defrost one subsample from each dilution (9 tubes in total). Subject to RNA extraction (alongside 500µl water as a negative control) and real-time RT-PCR according to the method protocol undergoing characterisation. Test for GI, GII, HAV and the process control virus and calculate the levels recorded in each subsample. Discount any results where the RT-PCR inhibition or extraction efficiency results are below the acceptable threshold.

Repeat until all stored subsamples have been extracted and analysed by real-time RT-PCR.

NOTE: this will entail 10 extractions of 9 samples (plus a negative control) a total of 90 individual extractions (or 100 including negative controls).

7 ANTICIPATED VALUES

After all real-time RT-PCR analysis is complete generate anticipated results for each dilution as follows:-

> geometric mean of obtained results for all subsamples where the RT-PCR inhibition and extraction efficiency results are acceptable (n=10 where all results are acceptable) at the "neat" dilution multiplied by the dilution factor.

EXAMPLE:-

Where the 10 "neat" subsamples give results of 804, 968, 1129, 1186, 1182, 1067, 1167, 1124, 1099 and 862 copies/g respectively (geometric mean = 1050.28 copies/g), the anticipated results for the different dilutions are as follows:-

DILUTION	ANTICIPATED VALUE
neat	1050.28 x 1 = **1050.28 copies/g**
1:2	1050.28 x 1/2 = **525.14 copies/g**
1:4	1050.28 x 1/4 = **262.57 copies/g**
1:8	1050.28 x 1/8 = **131.28 copies/g**
1:16	1050.28 x 1/16 = **65.64 copies/g**
1:32	1050.28 x 1/32 = **32.82 copies/g**
1:64	1050.28 x 1/64 = **16.41 copies/g**
1:128	1050.28 x 1/128 = **8.21 copies/g**
1:256	1050.28 x 1/256 = **4.10 copies/g**

8 DATA ANALYSIS

An example data set is given in Annex 1; annotated spreadsheets demonstrating the analysis of this data set according to the following instructions are available from the FAORC website. Note the examples and the text below describe analysis for one virus. The process must be repeated for GI, GII and HAV separately.

Determination of LOD_{95}

Determine the LOD_{95} for the data using the approach given in: Wilrich C, Wilrich PT. 2009. Estimation of the POD function and the LOD_{95} of a qualitative microbiological measurement method. J AOAC Int. 92(6):1763-72.

Calculator PODLOD_ver9.xls available at

http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html

- Ensure that macros are enabled.
- In the spreadsheet enter:
  - Sample size A_0 as 2
  - No. of matrices as 1
  - No. of contamination levels as the number used (9 for the example data given in Annex 1)

This will generate a data input table.

- Enter the calculated anticipated values in the left hand column.
- Enter the total number of valid results at each anticipated value in the second column.
- Enter the number of positive results at each anticipated value in the third column.
- Press control b to start the calculation.

A box containing the results of the calculation will appear. Record the LOD_{95} value (shown as "Detection limit $d_{0.95,i}$"). The upper and lower confidence limits may be recorded but are not required for the determination of the LOQ).

Sub-selection of data to use for determining linearity and the LOQ

Discard the data points where the anticipated values are lower than the determined LOD_{95} value.

Determination of linearity

- Use the data points remaining after discarding those below the LOD_{95}.

- Determine the slope of the linear regression line for the log_{10}-transformed obtained values (y axis) plotted against log_{10}-transformed anticipated values (x axis). [In Excel, use either the function LINEST or the function SLOPE: if the Analysis Toolpak add-in has been activated, the Regression function can be used. Do not constrain the regression line to pass through the origin: i.e. if using the LINEST function, ensure that argument "Const" is set to 1 or omitted; if using the Regression function, ensure that the "Constant is Zero" box is not checked].
- If the value of the slope lies between 0.9 and 1.1, accept that the response is linear. In this case all of the data points retained after determination of the LOD_{95} are used to determine the LOQ.
- If the value for the slope lies outside that range, repeat the calculation of the slope parameter after excluding the data points corresponding to the lowest remaining anticipated value.
    - If the value of the slope is now between 0.9 and 1.1, the data points used for this estimation are used to determine LOQ.
    - If the value still lies outside the range 0.9 to 1.1, seek advice from the FAORC.

Determination of the LOQ

- Using all data retained after determination of linearity, separately determine the standard deviation (SD) of the log_{10}-transformed obtained results for each anticipated value (if there are no QC failures, this will be the SD of 10 results for each anticipated value).
- The LOQ is the lowest anticipated level where the SD is <0.33 and where the SDs of all higher anticipated levels are also <0.33. If all of the SD values are <0.33, then the LOQ equals the lowest anticipated value in the data set retained after determination of linearity.
- Round the LOQ value to a whole number and report to a maximum of three significant figures (i.e. 54.7 becomes 55; 1141.3 becomes 1140).
- The assessment may be presented visually by plotting the SD against the anticipated level with a reference line at 0.33 SD.

ANNEX 1. EXAMPLE DATA SET

Dilution	Anticipated value (copies/g)	Obtained result (copies/g)	Log_{10} anticipated result	Log_{10} obtained result
neat	1050.28	804	3.02	2.91
neat	1050.28	968	3.02	2.99
neat	1050.28	1129	3.02	3.05
neat	1050.28	1186	3.02	3.07
neat	1050.28	1182	3.02	3.07
neat	1050.28	1067	3.02	3.03
neat	1050.28	1167	3.02	3.07
neat	1050.28	1124	3.02	3.05
neat	1050.28	1099	3.02	3.04
neat	1050.28	862	3.02	2.94
1:2	525.14	313	2.72	2.50
1:2	525.14	629	2.72	2.80
1:2	525.14	739	2.72	2.87
1:2	525.14	426	2.72	2.63
1:2	525.14	476	2.72	2.68
1:2	525.14	312	2.72	2.49
1:2	525.14	427	2.72	2.63
1:2	525.14	435	2.72	2.64
1:2	525.14	491	2.72	2.69
1:2	525.14	679	2.72	2.83
1:4	262.57	331	2.42	2.52
1:4	262.57	217	2.42	2.34
1:4	262.57	314	2.42	2.50
1:4	262.57	203	2.42	2.31
1:4	262.57	253	2.42	2.40
1:4	262.57	169	2.42	2.23
1:4	262.57	240	2.42	2.38
1:4	262.57	228	2.42	2.36
1:4	262.57	217	2.42	2.34
1:4	262.57	276	2.42	2.44
1:8	131.28	145	2.12	2.26
1:8	131.28	95	2.12	2.08
1:8	131.28	166	2.12	2.32
1:8	131.28	127	2.12	2.21
1:8	131.28	171	2.12	2.33
1:8	131.28	120	2.12	2.18
1:8	131.28	90	2.12	2.06
1:8	131.28	112	2.12	2.15
1:8	131.28	111	2.12	2.15
1:8	131.28	153	2.12	2.29

Dilution	Anticipated value (copies/g)	Obtained result (copies/g)	Log_{10} anticipated result	Log_{10} obtained result
1:16	65.64	148	1.82	2.17
1:16	65.64	95	1.82	1.98
1:16	65.64	22	1.82	1.34
1:16	65.64	54	1.82	1.73
1:16	65.64	132	1.82	2.12
1:16	65.64	25	1.82	1.40
1:16	65.64	139	1.82	2.14
1:16	65.64	167	1.82	2.22
1:16	65.64	132	1.82	2.12
1:16	65.64	35	1.82	1.54
1:32	32.82	48	1.52	1.68
1:32	32.82	67	1.52	1.83
1:32	32.82	82	1.52	1.91
1:32	32.82	28	1.52	1.45
1:32	32.82	56	1.52	1.75
1:32	32.82	91	1.52	1.96
1:32	32.82	-	1.52	0.00
1:32	32.82	51	1.52	1.71
1:32	32.82	29	1.52	1.46
1:32	32.82	61	1.52	1.79
1:64	16.41	-	1.22	0.00
1:64	16.41	66	1.22	1.82
1:64	16.41	-	1.22	0.00
1:64	16.41	35	1.22	1.54
1:64	16.41	27	1.22	1.43
1:64	16.41	25	1.22	1.40
1:64	16.41	-	1.22	0.00
1:64	16.41	42	1.22	1.62
1:64	16.41	-	1.22	0.00
1:64	16.41	44	1.22	1.64
1:128	8.21	-	0.91	0.00
1:128	8.21	-	0.91	0.00
1:128	8.21	-	0.91	0.00
1:128	8.21	21	0.91	1.32
1:128	8.21	-	0.91	0.00
1:128	8.21	-	0.91	0.00
1:128	8.21	26	0.91	1.41
1:128	8.21	-	0.91	0.00
1:128	8.21	-	0.91	0.00
1:128	8.21	-	0.91	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	30	0.61	1.48
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00
1:256	4.10	-	0.61	0.00