Centre for Environment Fisheries & Aquaculture Science

Cefas

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

Guidance for troubleshooting problematic results in virus proficiency testing

INTRODUCTION

Evidence from Proficiency Testing (PT) suggests that different methods can give different results in the detection and quantification of viruses in bivalve shellfish samples. It is therefore as a first principle recommended that laboratories experiencing problems in PT adopt a method based on ISO 15216-1:2017. Comparison of the ISO with an in-house method can help to isolate the root causes of problems. ISO 15216-1:2017 provides flexibility in the choices of reagents for RNA extraction and RT-PCR, however specific reagents and protocols are provided in the informative annexes; these methods were tested by 13 labs during the validation of ISO 15216-1. The specific methods in the informative annexes are also included in the EURL generic protocol (available at https://eurlcefas.org/public-documents/methods.aspx). Alternative in-house methods should be tested against the ISO method (including the informative annexes) on a range of naturally contaminated positive and negative samples to ensure they are comparable before adoption for routine testing.

Problematic results in PT can be broadly classified into 3 types

- False positives (positive results in samples intended as negative)
- False negatives (negative results in samples intended as positive)
- Results outside the acceptable quantification limits

Further details for troubleshooting results of these types are given below.

FALSE POSITIVES (Positive results in samples intended as negative)

False positive results are most often caused by contamination, either between samples (cross-contamination) or through contamination of samples with the products of PCR amplification.

Prevention of contamination

It is imperative that laboratories separate areas used for sample preparation and PCR/post-PCR activities (including preparation of positive control materials) in accordance with ISO 22174. Failure to understand the important of strict separation, particularly in laboratories unfamiliar with the use of PCR for diagnostic detection in low titre samples, is the root cause of many contamination events. In addition to strict separation, the use of separate workspaces for testing unknown food samples and potentially highly contaminated clinical or environmental samples (e.g. wastewater) is desirable to avoid cross-contamination. Where laboratory separation is not currently implemented, it is advisable that the laboratory prioritises this, otherwise repeat problems with false positive results can be anticipated.

To identify contamination rapidly it is vital that laboratories follow the guidance in the EURL generic protocol and ISO 15216 on the use of negative controls.

Removal of contamination

The severity of contamination can be approximated by assessing the equivalent concentrations in the affected samples. Where these are low, close to the limit of detection of the assay, and where there is a mixture of positive and negative PCR replicates from a sample or set of samples, the contamination issue can normally be rectified taking simple steps as described below. Where positive results equivalent to high concentrations are present across all samples intended as negative this is indicative of a severe contamination problem that will require more radical attention. The physical source of contamination must be identified and either thoroughly cleaned with a product that can degrade nucleic acid, or removed and replaced. Swabbing and testing using PCR of multiple different areas within the laboratory can aid identification of the contamination source. It may also be useful to determine whether the contamination is caused by RNA (e.g. from highly contaminated clinical/environmental samples) or DNA (from positive controls/PCR products); this can be achieved through testing in parallel using mastermixes that can amplify DNA and RNA, or DNA only. Where contamination is severe it is likely that even after removal of the source of contamination residual contaminating nucleic acid will be widespread in the laboratory environment. The laboratory must be thoroughly cleaned and potentially contaminated reagents replaced. Following these steps, absence of contamination should be confirmed by testing multiple aliquots of negative material (e.g. water) and checking for complete absence of amplification.

Where levels of contamination are low it is likely that thorough cleaning of the laboratory followed by replacement of reagents will be sufficient to remove contamination. This should be confirmed by testing multiple aliquots of negative material as above however.

FALSE NEGATIVES (Negative results in samples intended as positive)

False negative results can arise due to a variety of different issues with sample processing, virus extraction, RNA extraction or PCR.

If quantification standards are provided by the EURL, poor sensitivity (negative or high Cq values [e.g. over 25] with the undiluted controls) may indicate an issue with the PCR, for example poor quality reagents. Note however, that the quantification controls used by the EURL are designed to react with the primers in the informative annexes of ISO 15216, and the controls may not work with all primer/probe combinations. False positive results for one target virus but not for the others may also be indicative of a problem with the PCR primers and probes for the affected target virus.

Where results with unextracted positive material are as intended but all/most extracted samples intended as positive (including non-matrix samples e.g. Lenticules if provided) produce negative results, this may indicate a problem with RNA extraction.

Where results are as intended with non-matrix samples (e.g. Lenticules if provided), but false negative results are obtained with matrix samples, then this indicates a problem with the matrix-specific parts of the procedure e.g. sample processing or virus extraction. It should be noted that PCR is particularly sensitive to ineffectively extracted shellfish matrix which is known to cause inhibition.

In all cases affected laboratories should ensure that the method specifications provided in the normative part of ISO 15216 should be followed, and to ensure best practise, the specific methods and reagents for samples processing, virus extraction, RNA extraction and PCR provided in the informative annexes of the ISO and also the EURL generic protocol should be used where possible. Proper storage of reagents is also important and appropriate recommendations should be followed.

Where results are indicative of a serious failure of the detection method, it may be possible for the EURL to provide training.

RESULTS OUTSIDE THE ACCEPTABLE QUANTIFICATION LIMITS

Where results reported are above the upper acceptable quantification limit (determined through analysis of all participants' results) this is likely to be caused by either an incorrect concentration ascribed to the laboratories quantification standard, or by mistakes in the quantity calculations. Experience suggests it is unlikely that results of this type are caused by "super-efficient" extraction of the samples. Where results are below the lower acceptable limit, in addition to an incorrect concentration ascribed to the laboratories quantification standard, or mistakes in the quantity calculations as described above, problems with incorrect or inefficient sample processing, virus or RNA extraction, or PCR can contribute as with false negative results (see above).

Comparison of the laboratory's Cq values for the affected sample with the reference values in the PT, and also those of other laboratories, can help with the identification of the problem; where these are similar it is most likely that problems with the quantification of the standard or with the quantification calculations are major factors in the unacceptable results. However, this is only an indication since different PCR reagents and platforms can produce widely different Cq values from equivalent starting material.

For many PT distributions the EURL will provide ready-to-use quantification standards. If possible the results for the laboratory's own standards should be compared with these. If the results are significantly different this is indicative of a problem with the laboratory's own standards. Laboratories should ensure that they are using quantification standards consistent with the instructions in ISO 15216-1 i.e. linear dsDNA quantified diluted to working concentrations using an appropriate buffer (e.g. TE). A method for generation of such a standard is provided in the EURL generic protocol at https://eurlcefas.org/public-documents/methods.aspx. Quantification of the standards should use spectrophotometry, fluorimetry or digital PCR; ideally, independent confirmation of the concentration by more than one method is valuable. Care should also be taken to ensure that dilution of the standards to working concentration is carried out correctly and should be double checked during troubleshooting.

Errors in quantity calculations can also result in results outside the acceptable range. Laboratories should check their calculations carefully against the formulae given in ISO 15216-1 and the EURL generic protocol, again double checking during troubleshooting. In addition a ready-to-use quantification spreadsheet for use with the ISO method is provided on the EURL website at https://eurlcefas.org/public-documents/methods.aspx.

Where problems with quantification standards or quantity calculations can be ruled out, and where Cq values are higher, and quantities lower, for the affected samples, the root cause of the problem may be inefficient extraction of virus RNA from the samples. The rectification approach should be as for false negative results (see above).