Use of the EURL-AP reference material to check the 1% level threshold of horse meat in beef

1. Scope

The protocol describes how to use the set of reference materials provided by the EURL-AP to check through a semi-quantitative PCR approach if the 1% of horse meat in mass fraction of meat is or not exceeded.
The set of reference materials consists of three levels : 0.5%, 1% and 2% (in mass fraction of horse meat in beef). The 1% level is the most important one as it is with this one that the semi-quantification is performed. The 2% level has interest if one wants to check (what the EURL-AP strongly advises) that the combination of DNA extraction and PCR methods chosen by the laboratory are fit for the purpose of semi-quantification.
Finally the 0.5% level is provided in order to be able to perform the screening step but its use at that level is not at all mandatory while use of the 1% calibrator for the confirmatory test is mandatory.

2. Definitions

C_t : threshold cycle
CTAB : cetyltrimethylammonium bromide; the CTAB method is a DNA extraction method in which this compound is used
DeltaC_t or ΔC_t : difference between two measured C_t values
DNA : deoxynucleic acid
PCR : polymerase chain reaction
SD : standard deviation

3. Description of the meat mixes

The reference material set send to a laboratory consists of three levels of horse meat in beef:

1) 3 x 20 g of a meat mix consisting of 0.5% (mass fraction) of horse meat in beef
2) 50 g or 100 g * of a meat mix consisting of 1% (mass fraction) of horse meat in beef
3) 50 g or 100 g * of a meat mix consisting of 2 % (mass fraction) of horse meat in beef

* size is depending on the production batch

The mixes have been homogenized and it was checked that homogeneity was sufficient when performing a DNA extraction on test portions of 200 mg.
The meat mixes were frozen and sent to the laboratory in frozen state.

4. Reception of the set of reference materials at laboratory level

As the samples are sent in frozen state, you can store them immediately at reception at -20℃. Should the samples for any reason be defr osted then it is advised that at reception you immediately proceed as described in step 5.1.

5. Step by step operations

5.1. Handling of the reference materials before DNA extraction

Before doing any DNA extraction on one of the reference materials, you must thaw it completely and then mix it thoroughly in a blender. Once this has been done, you can isolate the aliquots that are required for the DNA extraction but we advise you to keep the rest of the mix in aliquots that have the size of the test portions for later DNA extractions and to freeze this material (-20℃) in the meantime. By doing so, you avoid frequent freeze-thawing cycles that affect the amount of targets contained in the reference material.

5.2. Important remark linked to the use of the reference materials

It was observed by the EURL-AP that the DNA extracts obtained from the reference materials might be dependent on the operator who prepared them. We therefore strongly advise that, when analyzing samples, their DNA extracts should be compared to that of reference materials extracted by the same person as the one having extracted the DNA of the samples under analysis. This is linked to the fact that the method relies on absolute copy numbers extracted from the samples and not on a ratio of targets.

5.3. Choice of a DNA extraction method and of a PCR method

The DNA extraction method to be used is left to the choice of the laboratory. The same is valid for the PCR method to be used during the confirmatory test, except that the PCR target to be used has to be a mitochondrial one with a target size that is not too large (ideally below 100-150bp).

Before starting analysis on samples, a laboratory can check if its combination of DNA extraction and PCR methods is suitable for a semi-quantitative analysis by applying the method both to the 1% and 2% reference materials. The reason for adding a 2% reference material in the set is limited to that purpose (see item 5.5).

Just as an indication the DNA method advised by the EURL-AP is the CTAB-method (based on Annex A.3.1. of ISO 21571:2005) applied to test portions of 200 mg with a final recovery of the DNA in 200 µl. The PCR protocol advised by the EURL-AP is the one that was recommended in 2013 (EU recommendation 91/2013 with the protocol available on the website of the EURL-AP : http://eurl.craw.eu/img/page/sops/Protocol%20for%20detection%20of%20horse%20DNA%20using%20real-time%20PCR.pdf).

5.4. Use of the 1% reference material

This reference material is the main one of the set as it provides the standard to which results have to be compared; its use is mandatory for the confirmatory tests as it provides a way to get harmonized results throughout the EU.

It is strongly advised to use this calibrator for a semi-quantitative determination in the way that is described hereafter.

Weigh out five test portions (size to be adapted to the extraction protocol in use in each laboratory). Perform the DNA extraction method on all five test portions by the same operator. With the DNA extracted from the 1% reference material, set up a real-time PCR run for detection of horse DNA in which for the DNA extracts of each test portion, there are three PCR replicates. Concerning the amount of DNA to be used, try out several dilutions of the extracts in order to check at which one inhibition is absent. If you already have some idea what dilution might be convenient, the

number of dilutions can be limited. Apply the DeltaC_t method to successive dilutions to find out if inhibition is present (e.g. for tenfold dilutions the difference of C_t between these dilutions should be close to 3.3 cycles). On those dilutions for which there is no inhibition and that are also workable on samples, determine the mean C_t (normally derived from 15 data : 5 extracts x 3 replicates) as well as the standard deviation on the C_t (SD_{Ct}).
The C_t will of course depend on how the fluorescence threshold is set. With a CTAB DNA extraction method yielding 200 µl of DNA extract when starting from 200 mg of reference material at 1%, the C_t obtained on 5 µl of a 30 fold diluted extract is about 25 cycles. Try also to get results in a range of C_t in between 22 and 28 cycles. In that case, the standard deviation on the C_t should not exceed 0.25 cycle. While the cut-off value to determine if a sample is or not above the 1% level should be calculated as follows:

$$Cut-off\ (1\%) = \overline{C_t} - 0.5\ cycle \quad \textbf{(formula 1)}$$

Check also if you have no outlier before doing this calculation. Suppress therefore C_t values that are below the mean C_t minus three times the standard deviation or above the mean C_t plus three times the standard deviation.
See in item 6 how to exactly use this cut-off value to define if a sample is or not above the 1% threshold level.

5.5. Use of the 2% reference material to check the fitness for purpose of the combination of DNA extraction and PCR methods chosen by the laboratory

Before starting analyzing samples, we strongly advise that the laboratory checks if the combination of DNA extraction and PCR methods that were chosen is suitable for the purpose of a semi-quantitative analysis. Therefore, it is important to extract the DNA not only from the 1% reference material but also from the 2% reference material. Proceed in the same way as for the 1% reference material by using five test portions that are each analyzed in triplicate during the PCR at same dilution rate as the one that appeared valid for the 1% reference material. By doing so you will get fifteen C_t values for the 2% extracts. Ideally all of them should have a C_t value that is below the cut-off value defined with the 1% reference material. If on these 15 C_t values obtained for the 2% reference material, up to two of them are equal or greater than the cut-off, then the method may still be acceptable but if it is more, then the method has a problem. Moreover none of the mean C_t values of the five test portions from the 2% reference material should be equal or larger than the cut-off.

5.6. Use of the 0.5% reference material for screening

Use of the 0.5% reference material is not mandatory but may be helpful for the screening step. To use it, proceed like for the 1% or the 2% reference materials by doing DNA extracts on five test portions and analyze them in PCR triplicates at the dilution rate that appeared convenient to avoid inhibition. With the 15 C_t figures that can be obtained you can calculate a mean C_t ($\overline{C_t}$) value as well as a standard deviation (SD_{Ct}) and so derive a cut-off value for the screening step :

$$Cut-off\ (0.5\%) = \overline{C_t} - SD_{Ct} \quad \textbf{(formula 2)}$$

Ideally the mean C_t should be in a range from 23 to 29 cycles and in this case the standard deviation should not exceed 0.6 cycle.

6. Interpretation of results

To decide whether the 1% level in mass fraction of horse meat in another meat is exceeded or not, compare the C_t of a sample with the cut-off determined with the 1% reference material (mind that the DNA of samples and of calibrators used should have been extracted by the same operator).

If the $C_t \geq$ cut-off value, then it has to be considered that the 1% level in mass fraction is not exceeded.

If the $C_t <$ cut-off value, then it has to be considered that the 1% level in mass fraction is exceeded.

We advise to check the C_t per test portion (mean value if there are PCR replicates) with the cut-off and to analyze two test portions per sample. If both test portions provide a consistent result, this will correspond to the statement given in a report. If results are conflicting it is advised to perform the PCR again on extracts of both test portions. Either this will lead to consistent results between both test portions which is then the final result for the sample or there is still a discrepancy between the results of the test portions and then it has to be considered that the sample does not exceed the 1% level.

With this cut-off most types of meat mixes that were analyzed at 1% (mass fraction) of horse meat in beef will be considered as not exceeding 1% in mass fraction.

Should PCR inhibition occur on a sample with a dilution rate that was used for the reference material, then the easiest way to make a statement on that sample is to find out a dilution at which there is no longer inhibition and to compare it to the cut-off determined in terms of C_t according to formula n° 1 but to which a correctiv e ΔCt has to be added to take into account the extra dilution of the sample compared to the reference (e.g. if the sample is diluted two times more than the reference then one unit of C_t has to be added to the cut-off value of the reference to get the cut-off at the same dilution rate as that of the sample). In a more general way the formula then becomes :

$$Cut-off\ (1\%) = \overline{C_t} - 0.5 + \frac{\log \delta}{\log 2}$$ (**formula 3**)

With δ representing the additional dilution rate of the sample compared to that of the reference material at 1%.

7. Remarks

Once a cut-off is determined, it can be applied on several runs. As long as the reaction is done in the same conditions (same batches of master mix, oligonucleotide, probe…) it should be valid. Nevertheless the calibration can be repeated on each plate but take care to use reference material extracted by the same operator as the one having performed extractions on the samples.

Mind that strictly the protocol as applied is valid for raw meat mixes. If other more complex products are considered, the analysis should focus as much as possible on the meat contained in the product. In absence of a better alternative, the cut-off determined for meat mixes has then to be applied for PCR results on these products too but in fact this will result in a somewhat less stringent threshold.