European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval Building, Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 62 03 50 32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Addendum to the EURL-AP protocol :

Cut-off to check the 1% level threshold of horse meat in another meat

1. Scope

The protocol describes the determination of a cut-off by which in a semi-quantitative way it can be established if the content of horse meat in another meat exceeds or not 1% (in mass fraction).

2. Validation status and performance characteristics

It is an in-house validated protocol of the EURL-AP, no inter-laboratory study was done to validate it. Several mixes of meat (five different types) were analysed and it was observed with two different extraction protocols ('Promega Wizard® Magnetic DNA Purification System for Food' and 'CTAB') that the fat content in the mix may influence the yield of targets extracted. This effect was more pronounced with the Promega extraction kit. That is why to have a harmonized approach it is asked to use as mix a blend of horse steak and of beef steak that were each ground separately to minced meat and then mixed to get a 1% content of mass fraction of horse meat in the mix. The cut-off established with different runs (up to 3 tested) on the same DNA extracts of the in-house reference material comes up with a comparable figure calculated on 12 PCR measurements as detailed in 6.3.

3. Definitions

Ct : 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

4. Equipment

- Real-time PCR instrument for plastic vials or wells (glass capillaries are not recommended for the buffer composition of the PCR protocol)
- Plastic reaction vessels suitable for real-time PCR instruments (enabling undisturbed fluorescence detection)
- Software for evaluating the real-time PCR data
- Microcentrifuge
- Micropipettes and filter tips
- Vortex
- Rack for reaction tubes
- Grinder or blender
- Balance
- 1.5/2.0 ml microcentrifuge tubes or vials
- Additional appropriate equipment related to the type of DNA extraction method used

5. Reagents

5.1. PCR reagents

See the recommended EURL-AP protocol for detection of horse DNA

5.2. Material for determination of the cut-off

- Horse steak
- Beef steak
- Reagents linked to the DNA extraction method chosen by the laboratory

6. Step by step operations

6.1. In-house reference mix of meat at 1% in mass fraction

Prepare separately minced meat of horse and minced beef by mixing respectively the horse steak and the beef steak in a grinder or a “Waring®-blender-like” blender. The in-house reference at 1% of minced horse meat in minced beef was performed at EURL-AP with a total amount of 50 g of mix (i.e. 0.5 g of minced horse meat with 49.5 g of minced meat). This amount is indicative but it is important not to use a too high total amount as it should be homogenized in a blender.

6.2. Preparation the DNA extract from the in-house reference material at 1%

From the homogenized in-house reference material (see 6.1), weigh four test portions (size to be adapted to the extraction protocol that is used by the laboratory). At the EURL-AP, the test portion used has a size of 100 mg and is extracted with the Promega Wizard® Magnetic DNA Purification System for Food kit. To check the present protocol with other DNA extraction methods, it had also been tested with a CTAB extraction protocol (test

portion size : 200 mg). Other protocols valid for extraction of DNA from meat could be applied at this step as long as it is the same extraction method that is used on the samples to be compared to the reference.
Aliquot the DNA extracts of the in-house reference material in order to limit the thawing-freezing cycles on these samples as this might affect the copy number of the PCR targets.

6.3. Cut-off determination

Set up a PCR run in which three replicates of each of the four test portions are tested at the dilution that seems convenient with the extraction protocol that was chosen. In parallel use also a more diluted extract (e.g. tenfold diluted) to check by the ΔCt between the two dilutions if there is or not an inhibition (for a tenfold dilution, the ΔCt should be ~ 3.3 cycles). With the dilutions used at the EURL-AP the measured ΔCt never exceeded 0.8 units in Ct which was considered as acceptable. In such a situation where it is considered that no inhibition of the PCR is interfering, the mean Ct ($\bar{C}_t$) of the in-house reference mix at 1% can be determined with the 12 available data (4 extracts X 3 replicate PCR's per extract) as well as the Standard deviation (SD_{C_t}).
With these figures the cut-off is the defined as :
Cut-off = $\bar{C}_t - (2 \times SD_{C_t})$ (formula n°1)
If at the dilutions used, inhibition is observed, then more diluted samples have to be considered (once again at two levels to check the ΔCt). As soon as by this way an appropriate dilution level for the measurements has been found, this level can be used to determine the cut-off according to formula n°1. Min d that as much as possible samples have then to be diluted in the same way as what is done to measure the cut-off (if there is a difference this can be corrected, see 7).

7. Interpretation of results

To decide whether the 1% level in mass fraction of horse meat in another meat is exceeded or not compare the Ct of a sample with the cut-off.
If the Ct ≥ cut-off value, then it may be considered that the 1% level in mass fraction is not exceeded.
If the Ct < cut-off value, then it may be considered that the 1% level in mass fraction is exceeded.
We advise to check the Ct per test portion with the cut-off and to analyse 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 a consistent result between both test portions that 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 analysed at 1% of horse meat in mass fraction will be considered as not exceeding 1% in mass fraction.
Should the inhibition occur on a sample with a dilution rate that was used for the in-house reference, 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

Ct according to formula n° 1 but to which a corrective 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 Ct 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).

8. Remarks

Once a cut-off is determined it can be applied on several runs and as long as the reaction is done in the same conditions (same batches of master mix, oligonucleotide, probe…) it should be valid. For safety it is always possible to analyse on the same run, the DNA extracts of the in-house reference.
Mind that strictly the protocol as applied is valid for 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.
The protocol was primarily intended for the PCR assay recommended by the EURL-AP for detection of horse DNA. However, other real-time PCR assays that are valid for detection of horse DNA should most probably also be fit for determination of a cut-off as outlined in this protocol.