Standard Operating Procedure for Qualitative Detection of Oat and Cereals (barley, rye, wheat)

This SOP describes the analytical procedure for the qualitative detection of oat and cereal DNA in raw materials and several processed food stuffs. The method is based on a standard PCR, followed by either detection of oat-specific amplicons on an agarose gel, or by detection of cereal-specific amplicons by means of a melting curve analysis.

The validated detection limit is 1mg oat in 1kg soya or corn. For other type of samples, the LOD needs to be determined on a case-by-case basis.

Method performance may vary according to the matrix under investigation. False positive or negative results cannot be excluded, due to matrix effects or genetic variability of the target genes.

1. DNA EXTRACTION

a) Material & consumables :

- Air-oven (Memmert)
- Centrifuge (Jouan BR4i or Sigma 2K15)
- Micro-centrifuge
- Rotative agitator for 1,5ml and 2ml tubes, as well as for 15ml and 50ml tubes
- Vortex agitator
- Vacman system (Promega, cat n°A7231)
- Laminar flow clean bench
- Micropipets :P1000/ P200/P20
- Sterile H20 molecular biology grade

- Wizard resin (Promega cat n°A7701)
- Wizard mini columns (Promega cat n°A721C)
- Sterile microtubes 1.5 ml and 2,2 ml
- Sterile plastic 2ml syringes (cat n°300185 / Batai ller)
- Sterile 50ml tubes (cat n°300186 / Batailler)
- Sterile filter tips for micropipets p20/p200/p1000

b) Solutions and buffers :

- CTAB lysis buffer (20g/l)
- TE buffer
- Isopropanol 70%
- Proteinase K (20mg/ml)

c) DNA extraction :

- All samples need to be ground and perfectly homogenized prior to DNA extraction.
- After grinding, transfer a 1g test portion into a sterile 50ml tube and add 5ml CTAB lysis buffer and 20µl of the Proteinase K solution. Mix by vortexing. Some matrices may require more CTAB buffer due to absorption effects. In these cases, adjust the volumes of CTAB buffer and Prot K accordingly. For routine purposes all samples are analysed in duplicates.
- Incubate for 2 hours at 65°C on a rotative agitato r.
- Centrifuge for 10min at 10000 rpm. The solid and liquid phases need to be clearly separated
- Transfer around 1ml of the supernatant into a new sterile 2.2ml tube. Add 800 µl chloroform and vortex briefly.
- Centrifuge for 10 min at 14000 rpm.
- Recover around 500µl of the aqueous phase and transfer into a new sterile 2.2ml tube
- Add 1ml Wizard resin. Gently mix by inverting the tube for about 1 min.
- Place the minicolumns on top of the VacMan system and place a 2ml syringe on each minicolumn
- Deposit the mixture containing the resin and the aqueous phase into the syringe, and pass the liquid through the column by pressure
- Wash the minicolumn with 2ml isopropanol 70%
- Place the minicolumn onto a new sterile 2.2ml tube and centrifuge for 2min at 10000 rpm until any residual traces of isopropanol have been removed
- Place the dried minicolumn onto a new sterile 1.5ml tube and add 60µL of sterile TE buffer. Incubate for 1 min at room temperature
- For elution of the sample DNA, centrifuge for 1min at 10000 rpm. The DNA can be stored at +4°C prior to use.

2. DETERMINATION AND ADJUSTMENT OF THE DNA CONCENTRATION

For some samples, it may be required to determine the exact amount of total DNA subjected to the PCR. In this case, the determination of the DNA concentration can be carried out in a fluorescence microplate reader. The DNA extract can then be adjusted to the desired amount of DNA per PCR, prior to the amplification step. The technique described here quantifies DNA by intercalation of a fluorochrome into the DNA molecule. After excitation at a specific wavelength, the fluorescence emission can be measured, and the intensity of the emission is directly proportional to the amount of DNA in the sample. The fluorochrome Hoechst 33258 is used here. Excitation is carried out at 360nm, the emission is measured at 460nm. The determination of the DNA concentration of the sample is done by means of a calf thymus DNA standard curve.

a) **Material & consumables :**

- Microplate Reader GENios FL (TECAN)
- 96 well microplates (Dutscher, cat n°655076)
- Computer
- DNA quantitation kit - Fluorescence Assay (Sigma)
- Micropipets P1000, P200, P20, P2
- Sterile filter tips 1000µl, 200µl, 30µl
- Sterile 1.5ml tubes
- Sterile H2O molecular biology grade

The calculation of the DNA content is carried out according to the indication of the kit manufacturer. For oat and other cereals, the size of the haploid genome is available in the literature.

3. MASTERMIX SETUP AND PCR :

a) Material & consumables

- Thermocycler 9700 Perkin Elmer
- Sterile H20 PCR grade
- Buffer Takara Ex HS (cat n° RR006A Takara biomedica ls)
- $MgCl_2$ (cat n° N808-0241, Perkin Elmer)
- BSA (cat n° B8894, Sigma)
- dATP, dCTP, dGTP, dTTP (Takara Ex HS cat n° RR006A Takara biomedicals)
- Primer : CER 1F and CER 2R
- Taq Takara Ex Hs Polymerase (cat n° RR006A Takara b iomedicals)

- Sterile PCR tubes 200µl and sterile tube caps
- Sterile microtubes 2,2 ml and 1.5ml
- Sterile filter tips
- Primer sequences specific for oat, barley, rye and wheat:

Cer1F	5' AATCCTGAGCCAAATCCGTG 3'
Cer2R	5' CATGTAGAATGGGACTCTCTCTTTG 3'

b) Setup of the PCR mastermix

Reagent	**Volume/tube**
H2O PCR grade	26.5 µL
MgCl2 (25mM)	3 µL
Takara (10X) buffer	5 µL
BSA (0.02µg/µl)	5 µL
dNTP Takara (2.5mM)	5 µL
Primer Cer1F (20µM)	1 µL
Primert Cer2R (20µM)	1 µL
Takara Taq Polymerase (5 unités/µl)	0.5 µL
DNA	3 µL
Volume total	50 µL

PCR cycling program :

Denaturation	*PCR (45 cycles)*	*Hold*
95℃ : 10 min	95℃ : 30 sec 65℃ : 30 sec 72℃ : 60 sec	72℃ : 5 min

C. DETECTION OF AMPLIFICATION PRODUCTS ON AN AGAROSE GEL

a) Material & consumables

- Electrophoresis chamber and power supply
- Microwave oven
- High precision balance
- Micropipets p20 et p200
- Multi channel micropipet
- Video system : ref Ip115sd, philips
- UV table
- Detection and documentation system

b) Solutions and buffers

- TBE buffer 0.5x
- Loading buffer
- EDTA 0,5 M (pH8)
- Ethidium bromide

Detection of an oat-specific amplification products on an agarose gel :

Prepare a 2% agarose gel according to standard laboratory procedures decribed in the literature (e.g. Maniatis). The PCR product is loaded onto the gel. The expected fragment size for the oat-specific amplification product is 217 bp.

Detection of oat-specific amplification products using the melting curve technology :

Alternatively to the detection of oat amplicons on an agarose gel, the detection of the amplification product can be carried out by means of a specific FRET hybridisation probe. However, this technique does not differentiate between oat and other cereals such as rye, barley, oat and wheat. After the PCR, an aliquot of the amplification product is mixed up with the FRET probe and subjected to a melting curve analysis using the LightCycler.

The PCR conditions, including the composition of the mastermix, primer sequences, and the PCR cycling program, are the same as described above. The following FRET hybridisation probes are used for detection of the amplification products :

FluoCer1 5'-TAACTCgATTCgTTAgAACAgCTTCCATTgAgTCTCT-3'FL
RedCer1 5'-LC Red640-CCTATCCTTTTCCTTTgTATTCTAgTTCgAgA-PH

After amplification, an aliquot of the PCR reaction is mixed with the FRET probes :

- Probe FluoCer1 : 0.2µl of the stock solution (20µM)
- Probe RedCer1 : 0.2µl of the stock solution (20µM)
- H2O PCR grade : 4.6µl per reaction
- PCR amplification product : 15µl

The total volume of 20µL is transferred into a LightCycler capillary and analysed as follows :

- close the capillaries and briefly spin down the liquid in a microcentrifuge using the LightCycler capillary adaptors. Verify that no air bubbles are present
- Place the capillaries, together with a positive and a negative control, into the LightCycler caroussel

- Program the following LightCycler melting curve program, or open the respective program in "*open experiment file*".

PCR (1 cycle)	*Hold*
95℃ : 30 sec	40℃ : 30 sec
42℃ : 2 min	
85℃ (à 0.1℃/sec)	

- Enter the sample list in "*Edit sample*" before start of the run.
- At the end of the run, open the "LightCycler Data Analysis" window. Select channel F2.
- The melting curve temperature for the cereal-specific amplicon is around 65.2℃ (same temperature for oat, barley, rye, and wheat). The temperature may slightly vary from run to run. A comparison of the melting curve temperature with the positive control DNA is recommended in order to avoid false positive or false negative detection due to misinterpretation of the melting curves.
- The specific melting curve temperature may vary, depending on factors like machine-to-machine variability, or different software versions used for the melting curve analysis. The respective characteristic melting curve peak needs to be validated in each laboratory prior to application in routine.