

Standard Operating Procedure for the Quantitative Detection of Oat DNA in Spiked Samples by RealTime-PCR

This SOP describes the analytical procedure for quantitative detection of oat DNA in raw materials (model systems = soya, corn) and processed food stuffs (model system = yoghurt). The method is based on Realtime-PCR carried out on a LightCycler instrument.

Method performance may vary according to the matrix under investigation. The practical LOD for analytical samples needs to be determined on a case-by-case basis. False positive or negative results, as well as under- and/or overestimation of quantification results cannot be excluded, due to matrix effects or genetic variability of the target genes.

1. DNA EXTRACTION

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 H2O 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

Solutions and buffers :

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

DNA extraction :

- Solid 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 20 μ l 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 agitator.
- 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 μ l chloroform and vortex briefly.
- Centrifuge for 10 min at 14000 rpm.
- Recover around 500 μ l 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 60 μ L of sterile TE buffer. Incubate for 1 to 5 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

The determination of the DNA concentration is necessary for the preparation of the oat DNA standard curve. Further, 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.

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 REALTIME-PCR :

Material & consumables :

- LightCycler (LC) instrument (Roche Diagnostics)
- Caroussel for LC capillaries (Roche Diagnostics)
- Computer for LC (Hewlett Packard; supplied with LC instrument)
- Centrifuge for 96 well plates
- Capillaries for LC instrument (Roche Diagnostics, cat n° 1909339)
- Micropipets (Gilson or equivalent supplier): 2-20µl, 100-1000µl, P2, P20, P200, P1000
- Multichannel micropipets (Eppendorf or equivalent supplier) : 0.5-10µl, 30-300µl
- Centrifugeuses : Jouan BR4, Sigma Qiagen 4-15C or equivalent supplier
- Taq hot start (5U/µl), 10x buffer, dNTP (2.5mM) : (TaKaRa cat n°: RR006A)
- BSA (Roche Diagnostics cat n°711454 or equivalent supplier)
- Sterile filter tips for micropipets P2, P20, P200, P1000)
- PCR primer: CER 1F and CER 2R (Proligo, MWG or equivalent supplier)
- Hybridisation probes: fluoCER1 and redCER1_APD (Tib MolBiol or equivalent supplier)

Primer and probe sequences specific for oat, barley, rye and wheat :

Cer1F	5' AATCCTGAGCCAAATCCGTG 3'
Cer2R	5' CATGTAGAATGGGACTCTCTCTTTG 3'
fluoCER1	5' TAACTCgATTcGTTAgAACAgCTTCCATTgAgTCTCT--FL
redCER1_APD :	5' LC Red640-CACCTATCCTTTTCCTTTgTATTCTAgTTCgAgA—PH

Setup of the PCR mastermix :

Reagents	Vol/PCR (µL)
H ₂ O	7.15
PCR Buffer Takara (10X)	2
MgCl ₂ (25mM)	1.2
BSA (0.02µg/µL)	2
dNTP (2.5mM)	2
Primer Cer1 F (20µM)	0.9
Primer Cer2R (20µM)	0.9
Probe fluoCer1 (20µM)	0.8
Probe redCer1-APD (20µM)	0.8
TaqPol Takara (5U/µl)	0.25
Volume total	18 µL
DNA :	2 µL

4. PREPARATION OF GENOMIC AND PLASMID DNA STANDARD CURVES FOR QUANTIFICATION OF OAT

Standard curve prepared from genomic oat DNA :

Oat : 13.23 pg per haploid genome (ge)

- Genomic DNA from oat is extracted according to the protocol described in chapter 1. DNA EXTRACTION.
- The concentration of the DNA extract is determined as described in chapter 2. DETERMINATION AND ADJUSTMENT OF THE DNA CONCENTRATION.
- DNA extracts are adjusted to 15ng/ μ L
- Prepare the standard curve point containing 1000ge/ μ L by adding the appropriate amount of 1/10 TE buffer to the oat DNA extract adjusted to 15ng/ μ L
- Prepare the standard curve point containing 100ge/ μ L by a 10-fold dilution of the 1st standard curve point (1000ge/ μ L) in 1/10 TE buffer
- Prepare the standard curve point containing 10ge/ μ L by a 10-fold dilution of the 2nd standard curve point (100ge/ μ L) in 1/10 TE buffer
- Prepare the standard curve point containing 1ge/ μ L by a 10-fold dilution of the 3rd standard curve point (10ge/ μ L) in 1/10 TE buffer

In routine analysis, the following standard curve points are analysed in duplicates :

- 1000 ge / μ L
- 100 ge / μ L
- 10 ge / μ L
- 1 ge / μ L

Standard curve prepared from the recombinant plasmid containing the oat PCR product :

- Plasmid pIRMM 0040 (pUC18 containing the oat sequence)
- Plasmid size : 2919pb
- Stock solution 10ng/ μ L, containing 3114391604 plasmid copies

All dilutions are carried out in 1/10 TE buffer

- Prepare a solution containing 5×10^6 plasmid copies/ μ L from the 10ng/ μ L stock solution
- Prepare a solution containing 5×10^4 plasmid copies/ μ L from the solution containing 5×10^6 plasmid copies/ μ L
- Prepare the first standard curve point containing 25000 plasmid copies / μ L from the solution containing 5×10^4 plasmid copies/ μ L
- Prepare the second standard curve point containing 5000 plasmid copies / μ L from the solution containing 25000 plasmid copies/ μ L
- Prepare in intermediate concentration containing 1000 plasmid copies / μ L from the solution containing 5000 plasmid copies/ μ L
- Prepare the 3rd standard curve point containing 250 plasmid copies / μ L from the solution containing 1000 plasmid copies/ μ L
- Prepare the 4th standard curve point containing 25 plasmid copies / μ L from the solution containing 250 plasmid copies/ μ L

In routine analysis, the following standard curve points are analysed in duplicates :

- 25000 copies / μ L
- 5000 copies / μ L
- 250 copies / μ L
- 25 copies / μ L

5. LIGHTCYCLER QUANTIFICATION RUN

For details with regard to the LightCycler instrument, refer to LightCycler Operator's Manual version 3.0

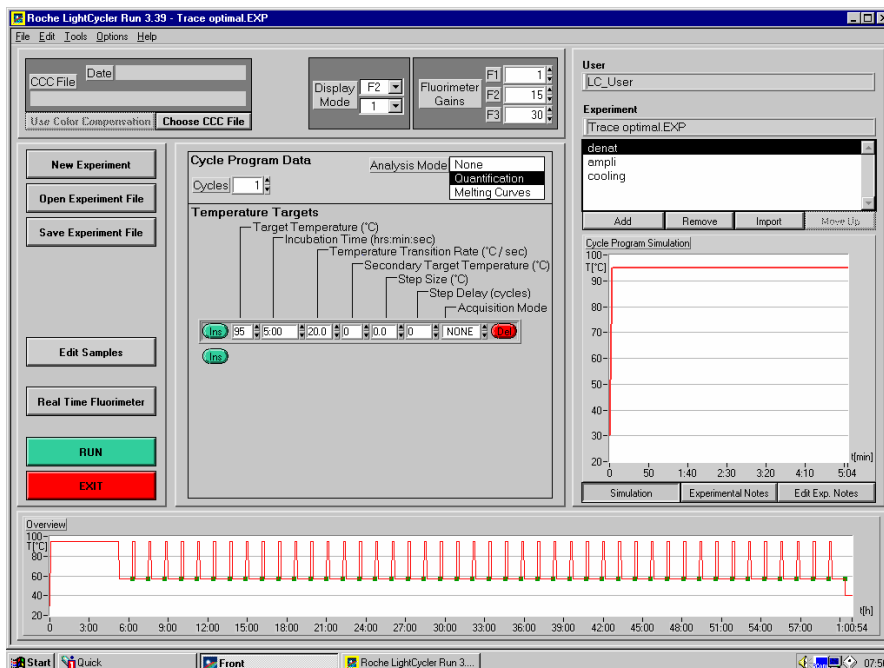
Starting the Lightcycler Instrument :

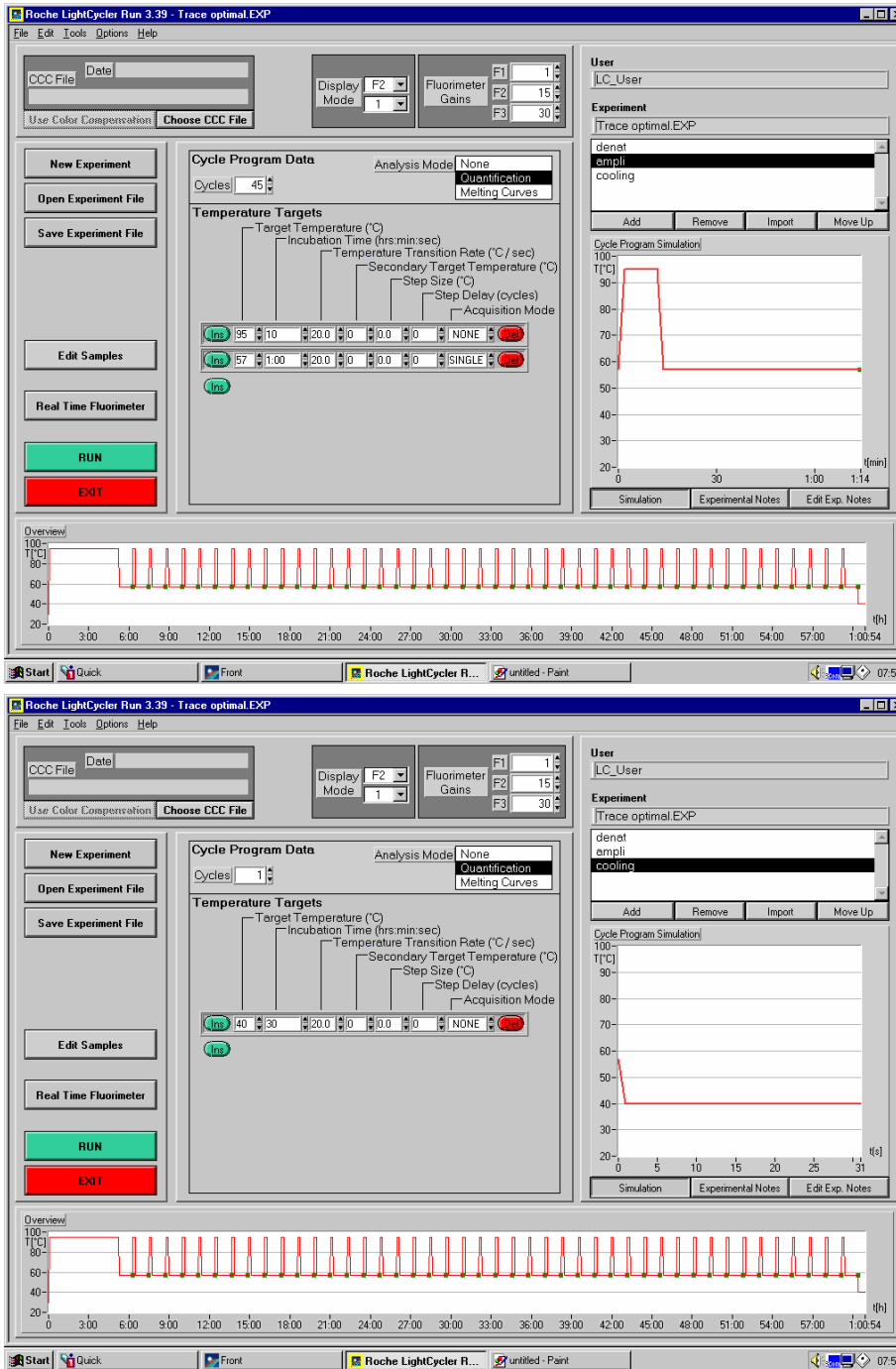
- Switch on the LightCycler instrument
- Switch on the computer; when the window " Begin Logon box " appears, press buttons CTRL + ALT + DEL and enter password
- Start the LightCycler Software v3.0

Cycling program :

- Click on "Run"
- To start a experiment, click on "Open Experiment File", and select a folder. The run parameters appear.
- program the following experiment file by clicking on "New experiment", as illustrated in the figures below :

1 X	95°C	5 min
45 X	95°C	10 sec ; acquisition mode "none"
	57°C	60 sec ; acquisition mode "single"
	40°C	30 sec





Create a sample sheet :

For details with regard to the LightCycler instrument, refer to LightCycler Operator's Manual version 3.0

Analytical samples :

- Enter the sample name in "Edit samples" : each sample should be analysed in duplicates

Standard curve points :

- Enter the standard curve points and the concentration in terms of copies/ μ L in "Edit samples" : each standard curve point should be analysed in duplicates

Negative controls :

- Enter the controls in "Edit samples". Each run should include at least one reagent negative control and one extraction blank.

→ Start the run

Data analysis :

For details with regard to the LightCycler instrument, refer to LightCycler Operator's Manual version 3.0

- For data analysis select channel F2/F1
- Use color compensation
- Analysis mode : fit points
- Baseline adjustment : arithmetic
- Number of points : 2

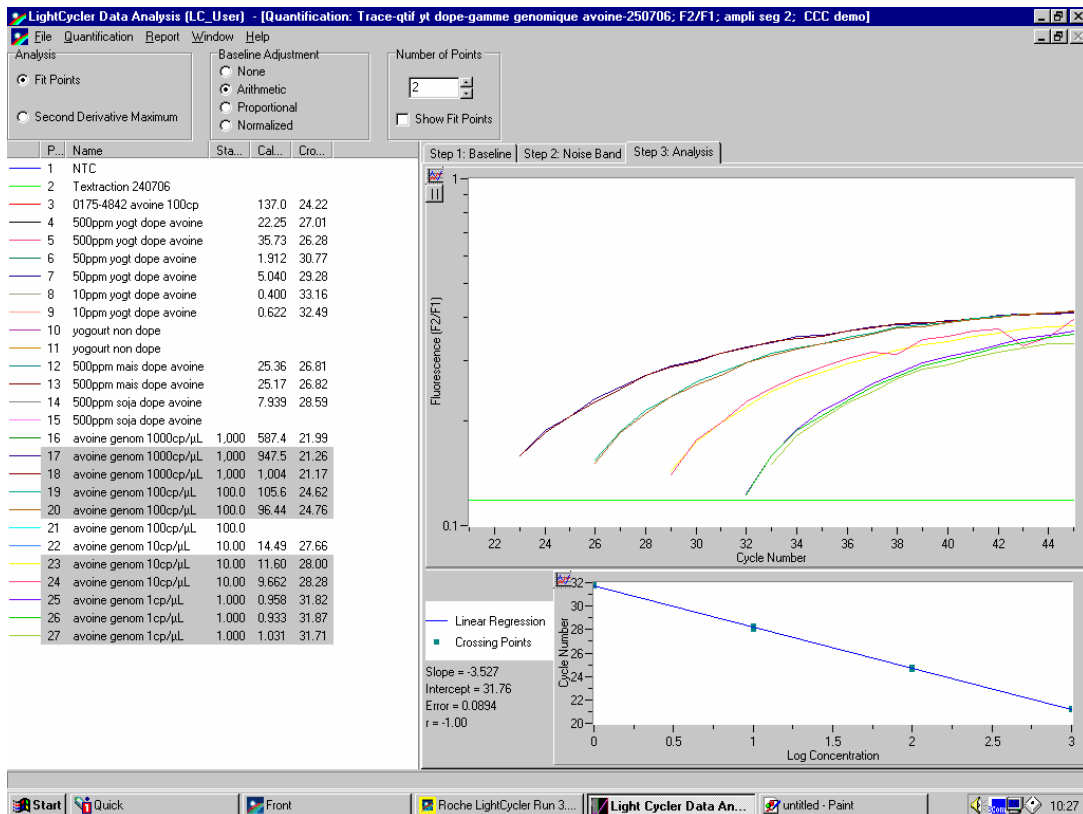
Place the noise band above the negative control. The position of the noise band can be adjusted in order to fulfill the following acceptance criteria :

- slope < -2.8
- $r = >0.98$

Acceptance criteria :

- all negative controls must be negative (absence of signal)

Example :



Data analysis :

For each analytical sample replicate, the LightCycler software calculates the Ct (crossing point) value and the respective amount of oat DNA copies, according to the standard curve.

Acceptance criteria for analytical samples :

- positive result for both sample replicates
- OR
- negative result for both sample replicates

A "+/-" result cannot be exploited and requires re-analysis of the sample

When using the plasmid DNA standard curve :

- For each analytical sample calculate the mean value in terms of DNA copies, from the two repeat measurements

When using the genomic oat DNA standard curve :

- For each analytical sample calculate the mean value in terms of oat DNA genome equivalents, from the two repeat measurements