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JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of maize line GA21 using real-time PCR

Protocol

Method development:
Monsanto Biotechnology Regulatory Sciences
(only for the real-time PCR part)

Method validation:
Joint Research Centre – European Commission
Biotechnology & GMOs Unit

Contents

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event GA21 DNA to total maize DNA in a sample. The procedure includes the following three modules:

a) DNA extraction: CTAB DNA extraction and purification protocol

b) Spectrophotometric quantitation of the amount of total DNA

c) Quantitative real-time PCR methodology specific for the GA21 event

The PCR assay has been optimised for use in an ABI Prism® 7700 sequence detection system. Other systems may be used, but thermal cycling conditions must be verified. The use of 200 ng of template DNA per reaction well is recommended.

DNA is extracted by means of a CTAB DNA extraction and purification protocol. For references, see Murray and Thompson (1980), Wagner *et al*. (1987) and Zimmermann *et al*. (1998). The protocol has been validated for soybeans (Anon, 1998), potato (Anon, 1996) and tomato (Anon, 1999). It has been tested for maize in a multi-laboratory pre-validation. The method was adopted from: Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Nucleic Acid Extraction. CEN/TC 275/WG11N0031. Draft November 2002.

Subsequently, purified DNA is quantified by means of spectrophotometry in order to determine the amount of DNA to be analysed by means of real-time PCR. The procedure "Basic ultraviolet spectrometric method" has been adopted from the Annex B "Methods for the quantification of the extracted DNA" of the prEN ISO 21571:2002. The method has been widely used and ring-tested (Anon. 2002).

For specific detection of event GA21 genomic DNA, a 112-bp fragment of the region that spans the 5′ insert-to-plant junction in maize event GA21 is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantitation of event GA21 DNA, a maize-specific reference system amplifies a 70-bp fragment of *adh*1, a maize endogenous gene, using a pair of *adh*1 gene-specific primers and an *adh*1 gene-specific probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantitation of the amount of

event GA21 DNA in a test sample, event GA21 and *adh*1 Ct values are determined for the sample. Standard curves are then used to calculate the relative content of event GA21 DNA to total maize DNA.

2. Validation status and performance characteristics

2.1 General

The method has been optimised for maize seeds, grain and flour containing mixtures of genetically modified GA21 and conventional maize.

The reproducibility and trueness of the method was tested through collaborative trial using samples of the CRM IRMM-414 series.

2.2 Collaborative trial

The method was validated in a collaborative trial by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with 15 laboratories.

Each participant received 12 unknown samples. The samples consist of six reference materials (CRM IRMM-414) of dried maize powder containing mixtures of genetically modified GA21 maize in conventional maize (w/w) between 0.1 % and 4.26 %.

For each unknown sample one DNA extraction has been carried out. Each test sample was analyzed by PCR in four repetitions. The study was designed as a blind duplicate collaborative trial. Each laboratory received each level of GM GA21 in two unknown samples, and the two replicates for each GM level were analyzed in two PCR plates.

A detailed validation report can be found under http://gmo-crl.jrc.it/statusofdoss.htm

2.3 Limit of detection

According the method developer, the relative LOD of the method is at least 0.05%. The relative LOD was not assessed in a collaborative trail. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.4 Limit of quantitation

According the method developer, the relative LOQ of the method is 0.1%. The lowest relative concentration of the target sequence included in collaborative trail was 0.1%.

2.5 Molecular specificity

The method utilizes the unique DNA sequence at the junction of the insert and the genomic DNA flanking the insert. The sequence is specific to GA21 and thus imparts specificity to the detection method.

The specificity was experimentally tested against DNA extracted from plant materials containing the specific targets of NK603, MON863, MON810 maize, and from conventional corn, Roundup Ready® soybean, conventional soybean, Roundup Ready® canola, conventional canola and Roundup Ready® wheat. None of the materials yielded detectable amplification.

The target sequence is a single copy sequence in the haploid GA21 genome.

3. Procedures

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Maintain strictly separate working areas for DNA extraction, PCR set-up and amplification.
- All the equipment used must be sterilized prior to use and any residue of DNA has to be removed.
- In order to avoid contamination, filter pipette tips protected against aerosol should be used.
- Use only powder-free gloves and change them frequently.
- Clean lab-benches and equipment periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.

3.2 DNA extraction

a. Moisten 200 mg of sample with 300 µl of sterile deionised water in a 1.5 ml tube.
b. Mix with a sterile loop until homogeneity is reached.
c. Add 700 µl of CTAB-buffer pre -warmed to 65°C; mix with a loop or a clean spatula
d. Add 10 µl of RNase solution; shake.
e. Incubate at 65° C for 30 min.
f. Add 10 µl of Proteinase K solution; mix smoothly.
g. Incubate at 65° C for 30 min.
h. Centrifuge for 10 min at 12000 g
i. Transfer supernatant to a 1.5 ml tube, containing 500 µl chloroform; shake for 30 sec.
j. Centrifuge for 15 min at 12000 g until phase separation occurs.
k. Transfer the aqueous upper phase into a new 1.5 ml tube containing 500 µl chloroform; shake.
l. Centrifuge for 5 min at 12000 g.
m. Transfer upper layer to a new 1.5 ml tube.
n. Add 2 volumes of CTAB precipitation solution, mix by pipetting.
o. Incubate for 60 min at room temperature.
p. Centrifuge for 5 min at 13000 rpm; discard the supernatant.
q. Dissolve precipitate in 350 µl NaCl (1.2 M).
r. Add 350 µl chloroform and shake for 30 sec.
s. Centrifuge for 10 min at 12000 g until phase separation occurs.
t. Transfer upper layer to a new reaction tube.
u. Add 0.6 volumes of isopropanol, mix smoothly by inversion. Incubate for 20 min at room temperature.
v. Centrifuge for 10 min at 12000 g. Discard the supernatant.
w. Add 500µl of 70% ethanol solution and shake carefully.
x. Centrifuge for 10 min at 12000 g. Discard the supernatant.
ATTENTION: drain the supernatant carefully. DNA pellets may detach from the bottom of the tube at this stage.
y. Dry pellets and dissolve DNA in 100 µl sterile, TE buffer.

z. **NOTE**: for thorough homogenisation of the DNA solution, it is recommended to re-suspend the sample by gentle agitation at +4°C for 24 h.

The DNA solution may be stored at ~ 4°C for a maximum of one week, or at -20°C for long-term storage.

3.3 Spectrophotometric measurement of DNA concentration

3.3.1 Measurement of a reference DNA solution

The correct calibration of the spectrometer can be verified as follows, with the use of a reference DNA solution:

a) For blank measurement only dilution buffer is used to fill the measurement vessel.

b) The reference DNA solution (Calf Thymus or Herring Testes DNA or Lambda DNA) is filled into the measurement vessel.

Absorption is measured for both blank and reference DNA solutions at λ= 260 nm and λ= 320 nm.

3.3.2 Measurement of a test DNA solution of unknown concentration

a) Blank measurement: mix the dilution buffer with a 2M sodium hydroxide solution, at the final NaOH concentration of 0.2M. This solution is used for the blank measurement.
b) Mix the DNA solutions with a 2M sodium hydroxide solution and, if needed, with dilution buffer, at the final NaOH concentration of 0.2M.
c) Measure the absorption after 1 min incubation time for both blank and reference DNA solution at λ = 260 nm and λ = 320 nm. The reading is stable for at least 1 h.

Example for blank measurement: Mix 90 µl dilution buffer and 10 µl of 2M sodium hydroxide solution and transfer to a 100 µl measurement vessel.

Example for the test DNA solution: Mix 80 µl of dilution buffer or water, 10 µl of 2M sodium hydroxide solution, 10 µl of DNA solution of unknown concentration and transfer to a 100 µl measurement vessel.

3.3.3 Evaluation

The absorption (OD) at 320 nm (background) is subtracted from the absorption at 260 nm resulting in the corrected absorption at 260 nm. If the corrected OD at 260 nm equals to 1, then the estimated DNA concentration is 38 µg/ml for single stranded DNA (denatured with sodium hydroxide).

Reliable measurements require OD values at λ=260 nm greater than 0.05.
The concentration of the double stranded test DNA solution is finally calculated taking into consideration the denaturation and the dilution factor applied.

3.4 Real-time PCR for quantitative analysis of GA21 maize

3.4.1 General

The PCR set-up for the taxon specific target sequence (*Adh*1) and for the GMO (GA21) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µl per reaction mixture with the reagents as listed in Table 1.

3.4.2 Calibration

Separate calibration curves with each primer/probe system are generated in the same analytical amplification run.

The calibration curves consist of five dilutions of DNA extracted from the 4.26 % CRM IRMM-414. A series of one to three dilution intervals at a starting concentration of 110,092 maize genome copies may be used (corresponding to 300 ng of DNA with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA) (Arumuganathan & Earle, 1991).

A calibration curve is produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by use of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy numbers measured for the unknown sample DNA is obtained by interpolation from the standard curves.

3.4.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. **Keep thawed reagents at 1-4°C on ice**.

2. In two reaction tubes (one for GA21 system and one for the *adh1* system) on ice, add the following components (Table 1) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixtures in the final volume/concentration per reaction well, for GA21/*adh*1 specific systems.

Component	Final concentration	µl/reaction
TaqMan® Universal PCR Master Mix (2X)	1x	25 µl
Primer GA21-F/*adh*1-F	150 nM	-
Primer GA21-R/*adh*1-R	150 nM	-
Probe GA21/*adh*1	50 nM	-
Nuclease free water		up to 50 µl
Template DNA (maximum 300 ng, see 3.4.1 and 3.4.2)		5 µl
Total reaction volume:		50 µl

3. Mix gently and centrifuge briefly.
4. Prepare two 1.5 ml reaction tubes (one for the GA21 and one for the *adh*1 master mix) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of master mix (e.g. 45 x 3 = 135 µl master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 5 x 3 = 15 µl DNA for three PCR repetitions). Low-speed vortex each tubes at least three times for approx 30 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a micro-centrifuge. Aliquot 50 µl in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Table 2:

Table 2. Reaction conditions.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG pre-PCR decontamination		50 °C	120″	No	1x
2	Activation of DNA polymerase and denaturation		95 °C	600″	No	1x
3	Amplification	Denaturation	95 °C	15″	No	45x
4		Annealing & Extension	60 °C	60″	Measure	

3.5 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. *adh*1) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the update button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y-axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. GA21 system).

e) Save the settings and export all the data into an Excel file for further calculations.

3.6 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instruments software calculated the Ct-values for each reaction.

The standard curves are generated both for the *adh*1 and GA21 specific system by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of GA21 DNA in the unknown sample, the GA21 copy number is divided by the copy number of the maize reference gene (*adh*1) and multiplied by 100 to obtain the percentage value (GM% = GA21/*adh*1 * 100).

4. Materials

4.1 Equipment (equivalents may be substituted)

DNA extraction:

- Water bath or heating block
- Microcentrifuge
- Micropipettes
- Vortexer
- 1.5/2.0 ml tubes
- Tips and filter tips for micropipettes
- Rack for reaction tubes
- Vinyl or latex gloves
- Optional: vacuum dryer apt to dry DNA pellets

Spectrophotometry:

- UV spectrophotometer. Single beam, double beam or photodiode array instruments are suitable.
- Vortexer
- Measurement vessels. e.g. quartz cuvettes or plastic cuvettes suitable for UV detection at a wavelength of 260 nm. The size of the measurement vessels used determines the volume for measurement. This should be one of the following: half

micro cuvettes (1000 μl), micro cuvettes (400 μl), ultra micro cuvettes (100 μl) and quartz capillaries (3 μl to 5 μl). The optical path of standard cuvettes is usually 1 cm.

Real-time PCR:

- ABI Prism® 7700 Sequence Detection System. Applied Biosystems Part No 7700-01-200/208
- ABI Prism® 7900HT Sequence Detection System. Applied Biosystems Part No 4329002 or 4329004
- Software: Sequence Detection System version 1.7 (Applied Biosystems Part No 4311876) or equivalent versions
- MicroAmp® optical 96-Well reaction plates. Applied Biosystems Part No N801-0560)
- MicroAmp® optical adhesive covers. Applied Biosystems Part No 4311971
- MicroAmp Optical caps. Applied Biosystems Part No. No 801-0935
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml tubes

4.2 Reagents

DNA extraction:

- CTAB: Cetyltrimethylammonium Bromide (Ultrapure grade)
- TRIS: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade)
- EDTA: Ethylenediaminetetraacetic acid, disodium salt (titration 99.9%)
- Ethanol (96% at least)
- Isopropanol (99.7% at least)
- Chloroform (99% at least)
- NaCl (99% at least)
- NaOH (98% at least, anhydrous)
- Distilled sterile water
- RNase A solution 10 mg/ml
- Proteinase K solution 20 mg/ml

Spectrophotometry:

- Dilution buffer: TRIS: Tris[hydroxymethyl] aminomethane hydrochloride (Molecular Biology grade). 10 mM, pH 9.0.
- NaOH (98% at least, anhydrous)

- Hydrochloric acid (HCl), φ (HCl) = 37 %
- Herring Testes DNA, Calf Thymus DNA, or Lambda DNA

Real-time PCR:

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
GMO target sequence	
GA21 primer F	CTTATCGTTATGCTATTTGCAACTTTAGA
GA21 primer R	TGGCTCGCGATCCTCCT
GA21 probe	6-FAM-CATATACTAACTCATATCTCTTTCTCAA CAGCAGGTGGGT-TAMRA
Reference gene target sequence	
*Adh*1 primer F	CCAGCCTCATGGCCAAAG
*Adh*1 primer R	CCTTCTTGGCGGCTTATCTG
*Adh*1 probe	6-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT-TAMRA

5. Buffers and Solutions

The following describes the preparation, storage and stability of the buffers used in this procedure. Volume may be scaled as needed. Equivalent reagent may be substituted.

DNA extraction:

- **CTAB buffer (1 litre)**

  Weight and mix in an appropriate cylinder:

20 g/l CTAB	20 g
1.4 M NaCl	82 g
0.1 M Tris-HCl	15.75 g
20 mM Na_2EDTA	7.5 g

  a. Add 500 ml of sterile distilled water.
  b. Adjust pH to a value of 8.0 with 1M NaOH.
  c. Fill up to 1000 ml and autoclave.

  Store at 4° C for up to 6 months.

- **CTAB-precipitation solution (200 ml)**
  Weight and mix in an appropriate cylinder:

5 g/l CTAB	1 g
0.04 M NaCl	0.5 g

  a. Add 100 ml of distilled water.
  b. Adjust pH to a value of 8.0 with 1 M NaOH.
  c. Fill up to 200 ml and autoclave.

  Store at 4° C for up to 6 months.

- **NaCl 1.2 M (100 ml)**
  a. Dissolve 7 g of NaCl in 100 ml sterile distilled water in a cylinder.
  b. Autoclave

  Store at room temperature for up to 5 years

- **Ethanol-solution ~70 % (v/v) (100 ml)**
  a. Mix 70 ml of pure ethanol with 30 ml of sterile distilled water

  Store at room temperature or at -20° C for up to 5 years

- **NaOH 1M (50 ml)**
  a. Dissolve 2 g of NaOH in 50 ml of sterile water in a cylinder or a 50 ml conical tube.

  Store at room temperature for up to 6 months

- **TE buffer, pH 7.0 (Tris/HCl 10 mM, EDTA 1 mM, pH 7.0) (250 ml)**
  a. Mix 100 ml of nuclease-free water, 2.5 ml of 1M Tris, pH 8.0 and 0.5 ml of 0.5M EDTA
  b. Adjust pH to 7.0 with HCl
  c. Adjust final volume to 250 ml with nuclease-free water
  d. Filter sterilise

  Store at room temperature for up to 5 years

- **RNAse A 10 mg/ml**
  a. Dissolve the RNase A at a final concentration of 10 mg/ml in sterile water.
  b. If indicated by supplier: boil the RNase A solution at 95°C for 15’ to remove any residual nuclease activity.

c. Aliquot solution as appropriate (thawing and re-freezing should be avoided)

Store aliquots at -20° C for up to 6 months

- **Proteinase K 20 mg/ml**
  a. Dissolve the Proteinase K at a final concentration of 20 mg/ml in sterile distilled water according to the supplier specifications.
  b. Aliquot solution as appropriate (thawing and re-freezing should be avoided)

  Store aliquots at -20° C for up to 6 months

Spectrophotometry:

- **Reference DNA solution**
  A DNA 10 mg/ml stock solution is prepared by dissolving 100 mg DNA (from Herring Testes or from Calf Thymus or Lambda DNA) in 10 ml dilution buffer (TRIS/HCl 10 mM, pH 9.0). At this concentrations DNA dissolves and homogenises slowly and the resulting solution is very viscous. The stock solution is further diluted with dilution buffer up to the desired working concentration (e.g. 25 μg/ml).

- **NaOH 2M (50 ml)**
  a. Dissolve 4 g of NaOH in 50 ml of sterile water in a cylinder or a 50 ml conical tube.

  Store at room temperature for up to 5 years

6. References

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