ASEAN GUIDELINES ON GENETICALLY MODIFIED ORGANISM ANALYSIS

1. SCOPE

These guidelines provide the requirements for laboratories to perform Genetically Modified Organism (GMO) analysis using DNA-based detection methods for determination of the presence of GMOs in food and feed samples.

This document does not cover health and safety matters. Laboratories are to exercise the appropriate precautions when handling chemicals and conduct risk assessment. Laboratory practices should conform to national health and safety regulations.

2. DEFINITIONS

a) **Certified reference material**: reference material accompanied by a certificate, one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body

b) **Environment control**: control used to determine that there is no nucleic acid contamination from, for example, the air in the laboratory

c) **Extraction blank control**: control generated by performing all steps of the extraction procedure except the addition of the test portion, for example, by substitution of water for the test portion

d) **Forward flow**: principle of material/sample handling applied to ensure that the laboratory sample, raw and processed test portion (including amplified DNA) remain physically segregated during the whole procedure

e) **GMO content**: identity and quantity of GMO or GMO-derived material in the product

f) **Integration border region**: junction region where one element originates from the host organism and the other originates from the DNA introduced during transformation

g) **Laboratory sample**: sample as prepared for sending to the laboratory and intended for inspection or testing

h) **Limit of Detection (LOD)**: The practical LOD is the lowest relative quantity of the target DNA that can be detected, given a known number of target taxon genome copies. For

the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

i) **Limit of Quantitation (LOQ)**: The practical LOQ is the lowest relative quantity of the target DNA that can reliably be quantified, given a known number of target taxon genome copies. For the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

j) **Measurement uncertainty**: parameter associated with the result of a measurement, which characterizes the dispersion of the values that could reasonably be attributed to the analyte

k) **Negative DNA target control**: reference DNA, or DNA extracted from a certified reference material, or known negative sample not containing sequence under study

l) **Positive DNA target control**: reference DNA, or DNA extracted from a certified reference material, or known positive sample representative of the sequence or organism under study

m) **Positive extraction control**: control used to demonstrate that the DNA extraction procedure has been performed in a way that will allow for extraction of a target DNA

n) **Reference material**: material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials

o) **Screening method**: method that will rapidly and reliably eliminate (screen) a large number of negative (or positive) test samples and restrict the number of test samples requiring the application of a rigorous method

p) **Specificity**: property of a method to respond exclusively to the characteristic or analyte under investigation

q) **Target taxon**: taxon to which the GMO belongs

r) **Test sample**: test portion sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time

3. OBJECTIVES

The development of these guidelines is intended for aiding ASEAN Member States to establish GMO detection methods for laboratories and to ensure harmonisation amongst ASEAN Member States.

4. GUIDANCE ON LABORATORY SET-UP AND OPERATION

DNA-based methods for the analysis of foods derived from biotechnology apply techniques that require specific apparatus and handling techniques that differ from most chemical

analytical methods. It is therefore necessary to provide information and instructions on the laboratory set-up and handling techniques.

It is important to ensure segregation of key areas and a forward flow to prevent contamination. Refer to Section 7 for details on laboratory design and requirements for conducting tests using DNA-based methods.

5. SAMPLING

Generally, DNA-based technologies test a sub-sample from a larger sample because the lot can be very large (e.g. tonnes) and the test can only accommodate small samples (e.g. grams). Developing appropriate sampling plans can help minimize errors attributable to sampling, and ensure that the sample is an accurate representation of the lot. Accepted standards for sampling methods include ISO 24333 (Cereals and cereal products – Sampling) and ISO 21294 (Oilseeds – Manual or automatic discontinuous sampling), and Codex CAC/GL 50-2004 (General Guidelines on Sampling).

6. GUIDANCE ON METHOD SELECTION

The specificity of particular target analytes and detection methods may vary considerably. It is therefore important to ensure that the chosen method(s) provide the desired specificity.

6.1 Methods using DNA as the analytical target

The specificity of analytical methods using DNA as the target to determine the presence of GMO-derived material depends on the specific properties of the targeted DNA-sequence. The different applications and classifications of specificity are as follows:

a) Taxon-specific methods target DNA sequences found in a single taxon, usually a species but possibly of lower or higher taxonomic rank.

b) Screening methods target DNA sequences found in several but not necessarily all transformation events. These sequences may also be found in non-GM material, for example due to the presence of natural viruses or bacteria. Screening methods may be useful to assess whether or not a product is likely to contain GMO-derived material. An example of a screening method is a qualitative PCR targeting the CaMV 35S promoter.

c) Construct-specific methods target DNA sequences that are only found in GMO-derived material, i.e. genetically engineered combinations of DNA sequence elements.

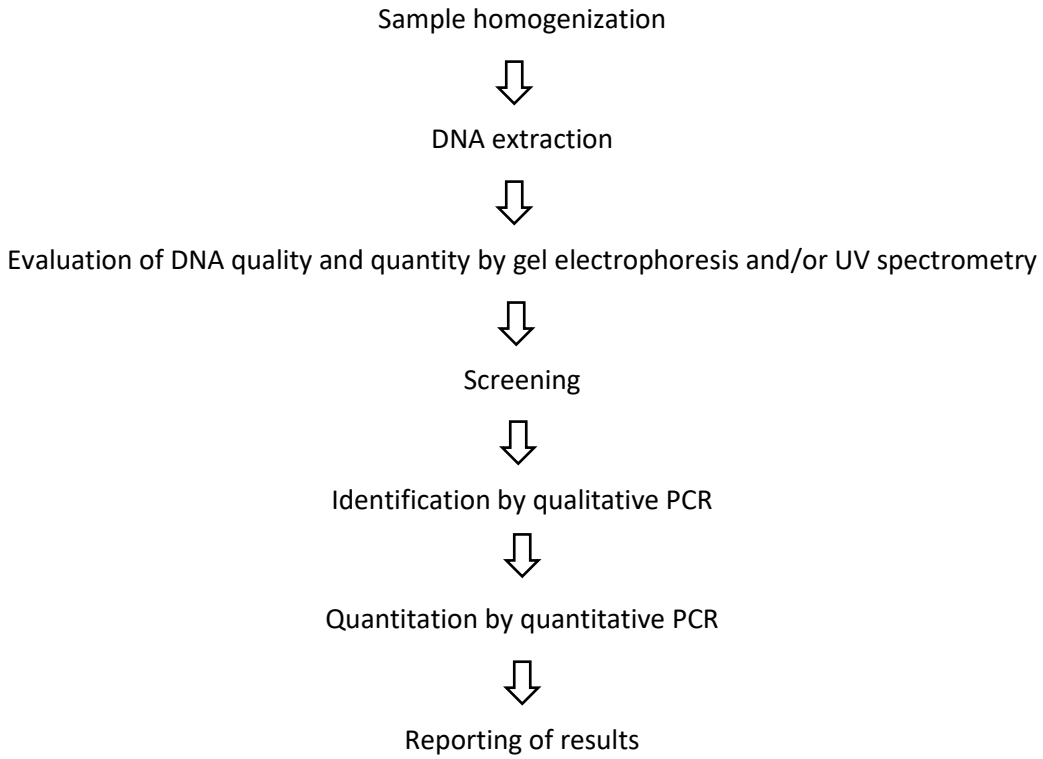
d) Event-specific methods target DNA sequences that are only found in material derived from a single transformation event, usually a DNA sequence spanning the junction between the inserted DNA and the host genome (the integration border region). The number of copies of the event-specific DNA sequence is always one per haploid GM-genome.

7. GENERAL LABORATORY AND PROCEDURAL REQUIREMENTS

The procedure includes the following steps:

- obtain a representative sample (Laboratory sample)
- homogenize the laboratory sample
- reduce the laboratory sample to the test sample (or test portion)
- prepare and grind the test sample (or test portion) and homogenise
- weigh the homogenised sample to extract the analyte
- screening test, interpret and report the results.

The workflow for a GMO testing laboratory may be as follows:



7.1 Laboratory Design

For DNA-based methods, the following laboratory design applies.

A minimum of four separately designated contained/dedicated work areas with their own apparatus are required for:

a) sample receipt, preparation, grinding and homogenization
b) extraction of the nucleic acid from the test material
c) set up of PCR/amplification reactions
d) subsequent processing, including analysis and characterization of the amplified DNA sequences, if applicable.

Physical separation through the use of different rooms is the most effective and preferable way of ensuring separate work areas, but other methods may be used to prevent contamination.

7.2 Personnel

Staff shall wear different laboratory coats in different work areas, such as the grinding area or post-PCR area. They shall also wear disposable gloves. All PCR procedures shall be carried out under non-contaminating conditions as far as is possible.

All personnel who perform steps in the testing procedure should be trained and competent to work with the techniques as appropriate.

7.3 Apparatus and Equipment

The laboratory should use properly maintained equipment suitable for the methods employed. Apparatus and equipment shall be maintained according to manufacturers' instructions.

All equipment used must be kept clean at all times. Any spills on apparatus, equipment or work surfaces must be cleaned immediately with suitable procedures to avoid any cross contamination when handling samples.

7.4 Materials and Reagents

For the analysis, unless otherwise stated, use only analytical grade reagents suitable for molecular biology, free from DNA and DNases. PCR reagents should be stored in small aliquots to minimize the risk of contamination.

In order to avoid contamination, sterile technique should be adopted in the PCR set-up area; i.e. powder-free gloves, sterilized plastic ware, autoclaved reagents, disposable plastic ware, aerosol-protected pipette tips should be used.

8. PREPARATION OF THE TEST PORTION

8.1 General

Take appropriate measures to ensure that the test portion is representative of the laboratory sample.

The test portion shall be of sufficient size and shall contain a sufficient number of particles to be representative of the laboratory sample [e.g. 3,000 particles at a Limit of Detection (LOD) of 0.1 %] to allow a statistically valid conclusion to be made.

However, for certain matrices containing very low amounts or degraded DNA, insufficient DNA suitable for analysis can be extracted. In these cases, the test portion may be increased.

DNA extractions shall be carried out at least on two test portions.

8.2 Samples

Samples shall be properly labelled to ensure that the results are associated with the correct samples used. Labels should contain sufficient information to clearly differentiate between different samples.

Laboratory samples shall be sufficiently homogeneous before reducing the laboratory sample and taking the test portion.

For liquid samples, shake the vessel containing the sample to improve the homogenization of the product. For solid matrices that cannot easily be suspended, the matrix shall be ground to reduce the particle size and/or facilitate the extractability of DNA.

9. DNA EXTRACTION/PURIFICATION

The objective of nucleic acid extraction methods is to provide nucleic acids suitable for subsequent analysis. The basic principle of DNA extraction consists of releasing the DNA present in the matrix and further, concurrently or subsequently, purifying the DNA from polymerase chain reaction (PCR) inhibitors.

9.1 General

The quality and yield of nucleic acid extracted using a given method on a given matrix should be both repeatable and reproducible in terms of analysis, provided sufficient nucleic acid is present in the matrix from which it has been extracted.

In order to obtain a good quality DNA, it is advisable, where relevant, to remove polysaccharides, RNA and/or proteins, lipid fractions, dyes, and salts.

DNA purification can be performed by different means such as fractionated precipitation, using solvents like phenol, chloroform, ethanol, isopropanol, and/or by adsorption on solid matrices (anion exchange resin, silica or glass gel, diatomaceous earth, membranes, etc.).

Re-suspend the DNA in a buffer solution that prevents DNA from degradation.

DNA may also be extracted and purified with suitable commercial kits.

9.2 Controls

The minimum control to be included is an extraction blank control and may also include a positive extraction control, and an environment control.

9.3 Preparation of PCR-quality DNA using basic cetyl trimethylammonium bromide (CTAB) method

9.3.1 General

The method is applicable to the extraction of DNA from plants and plant-derived matrices, in particular because of its ability to remove polysaccharides and polyphenolic compounds that would otherwise affect the DNA quality. It is also useful for some other matrices (see 9.3.6.5).

9.3.2 Principle

The method consists of a lysis step (thermal lysis in the presence of CTAB), followed by several purification steps in order to remove contaminants, such as polysaccharides and proteins.

9.3.3 Safety Precautions

All organic chemicals must be handled in a fume hood.

9.3.4 Chemicals and Reagents

- α-Amylase (optional), type IIa from Bacillus species, 1500 to 3000 units/mg of protein
- α-Amylase solution (optional), 10 mg/ml
- Chloroform ($CHCl_3$)
- Ethanol, $\phi(C_2H_5OH)$ = 96 %
- Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$)
- Hexadecyl-trimethyl-ammonium-bromide (CTAB) ($C_{19}H_{42}BrN$)
- Hydrochloric acid, ϕ(HCl) = 37%
- Isopropanol [$CH_3CH(OH)CH_3$]
- Proteinase K (optional), approximately 20 Units/mg of lyophilisate
- Proteinase-K solution (optional), 20 mg/ml, dissolved in sterile water
- RNase A, DNase-free, (optional) from bovine pancreas, approximately 50 Units/mg of lyophilisate
- RNase-A solution (optional), 10 mg/ml
- Sodium chloride (NaCl)
- Sodium chloride solution, c(NaCl) = 1.2 mol/l
- Sodium hydroxide (NaOH)
- Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$)
- CTAB extraction buffer, ρ(CTAB) = 20 g/l, c(NaCl) = 1.4 mol/l, c(Tris) = 0.1 mol/l, $c(Na_2EDTA)$ = 0.02 mol/l. Adjust the pH to 8.0 with HCl or NaOH.
- CTAB-precipitation buffer, ρ(CTAB) = 5g/l, c(NaCl) = 0.04 mol/l
- Ethanol solution, $\phi(C_2H_5OH)$ = 70%
- TE buffer, c(Tris) = 0.01 mol/l, $c(Na_2$-EDTA) = 0.001 mol/l. Adjust the pH to 8,0 with HCl or NaOH.

Legend:
Φ: volume fraction (volume of a constituent divided by the volume of all constituents of a mixture)
c: concentration (amount of substance dissolved per unit volume)
ρ: mass concentration (mass of solute per unit volume of solvent)

9.3.5 Apparatus and equipment

- Pipettes
- Fume hood
- Oven, waterbath or incubator, preferably with a shaker.
- Centrifuge, e.g. microcentrifuge, capable of achieving an acceleration of up to 12,000 g
- Mixer, e.g. Vortex
- -20°C freezer
- Refrigerator
- Analytical balance
- pH meter

9.3.6 Procedure

9.3.6.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. The volume of reagents used may be scaled up accordingly if more starting materials are used.

9.3.6.2 Sample extraction

Weigh 200 mg to 300 mg of the test sample into a tube.

Add 1.5 ml of pre-warmed (65°C) CTAB extraction buffer and mix. (In some cases a higher amount of buffer may be required to suspend the matrix.) Add 10 µl of α-amylase solution (optional), 10 µl of RNase A solution (optional) and mix gently. Incubate for 30 min at 65°C, under agitation. Add 10 µl of proteinase K solution (optional), smoothly mix the tubes and incubate for 30 min at 65°C, under agitation (optional). Centrifuge for 10 min at approximately 12,000 g. Transfer the supernatant to a new tube, add 0.7 to 1 volume of chloroform and mix thoroughly.

Centrifuge for 15 min at approximately 12,000 g. Transfer the upper phase (aqueous) to a new tube.

9.3.6.3 CTAB precipitation

Add 2 volumes of the CTAB precipitation buffer. Incubate for 60 min at room temperature without agitation. Centrifuge for 15 min at 12 000 g. Discard the supernatant. Dissolve the precipitated DNA by adding 350 µl of NaCl solution. Add 350 µl of chloroform and mix thoroughly. Centrifuge for 10 min at 12,000 g. Transfer the upper aqueous phase into a new tube.

9.3.6.4 DNA precipitation

Add 0.6 volume of isopropanol, mix smoothly by inverting the tube and keep the tube at room temperature for 20 min. Centrifuge for 15 min at 12,000 g. Discard the supernatant. Add 500 µl of ethanol solution to the tube and invert several times. This is the critical step ensuring the complete removal of CTAB. Centrifuge for 10 min at 12,000 g. Discard the supernatant. Dry the DNA pellet

and redissolve it into 100 µl of water or an appropriate buffer, e.g. TE buffer. This is the DNA master stock.

9.3.6.5 List of applicable matrices

The method has been successfully applied to extract DNA from the following matrices: baby food, baking mix, biscuits, candies, canned corn, cakes, cereal grains, chocolate, cornflakes, pastries, gravy, maize flour, maize starch, maize seeds/grains, maize semolina, milk powder, pet food, popcorn, potato chips, potato starch, rape seeds, rape leaves, soya lecithin, soya flour, soya drinks, soya beans, tofu, sugar beet leaves, sugar beet seeds, taco shells, tobacco, tomatoes, tortilla chips, wheat starch. This method may not be suitable for highly processed food such as oil.

10. QUANTITATION OF THE EXTRACTED DNA BY ULTRAVIOLET SPECTROMETRIC METHOD

10.1 General

This section describes a method to determine the concentration of DNA in solutions.

10.2 Principle

Nucleic acids in solution absorb ultraviolet (UV) light in the range from 210 nm to 300 nm with an absorption maximum at 260 nm. Since DNA, RNA and nucleotides have their absorption maximum at 260 nm, RNA and nucleotide contamination of DNA solutions cannot be determined by UV spectrometry. Therefore, RNA, oligonucleotides and nucleotides must be removed before DNA determination. Moreover, double-stranded DNA absorbs less UV light than single-stranded DNA. Since the proportion of single-stranded DNA in the solution is unknown, to avoid overestimation of the DNA content, all the DNA in the test sample is converted to its single-stranded form by using the denaturing agent sodium hydroxide. Since nucleic acids do not absorb at 320 nm, reading at 320 nm is informative for the determination of background absorption due to light scattering and UV-active compounds.

10.3 Application range

The method is applicable to DNA concentrations in the range from 2 µg/ml to 50 µg/ml. Before quantitation, suitable dilutions of the extracted DNA to be quantified should be made, in order to be in the linear range of the spectrometric measurement (optical density between 0.05 to 1).

10.4 Reagents

- Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$)
- Sodium hydroxide (NaOH)
- Sodium hydroxide solution, *c*(NaOH) = 2 mol/l

- Hydrochloric acid, ϕ(HCl) = 37%
- Carrier DNA, e.g. Herring Sperm DNA, or Calf Thymus DNA
- Dilution buffer, c(Tris) = 0.01 mol/l. Adjust the pH to 9.0 with HCl.
- DNA reference solution:
  Prepare a DNA stock solution with 10 mg/ml by dissolving 100 mg carrier DNA in 10 ml of dilution buffer. DNA dissolves at this concentrations only slowly and the resulting solution is very viscous. Afterwards dilute this prepared stock reference DNA-solution further with dilution buffer up to the desired working concentration (e.g. 25 µg/ml).

10.5 Apparatus and equipment

- UV-spectrometer, single-beam, double-beam or photodiode array instruments
- Mixer/shaker, e.g. Vortex
- Measurement vessels, for example quartz cells/cuvettes or plastic cells/cuvettes suitable for UV detection at a wavelength of 260 nm. The size of the measurement vessels used determines the volume for measurement: half-micro cells (1000 µl), micro cells (400 µl), ultra-micro cells (100 µl) and quartz capillaries (3 µl to 5 µl). The optical path of standard cell is usually 1 cm.

10.6 Procedure

10.6.1 Measurement of a DNA reference solution

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

- for blank measurement only dilution buffer is used to fill the measurement vessel
- for reference DNA measurement the vessel is filled with DNA reference solution

Absorption is measured for both the blank and reference DNA solutions at wavelengths of 260 nm and 320 nm.

10.6.2 Measurement of a test DNA solution of unknown concentration

For the blank solution, mix dilution buffer plus sodium hydroxide solution, so that a final NaOH substance concentration of 0.2 mol/l is reached. This mixture is used to fill the measurement vessel.

For the measurement of test DNA, mix the test DNA solution with sodium hydroxide solution and, if needed, with dilution buffer, to obtain a final NaOH substance concentration of 0.2 mol/l. This mix is used to fill the measurement vessel.

Measure the absorption after 1 min incubation time for both the blank and test DNA solutions at wavelengths of 260 nm and 320 nm. The reading is stable for at least 1 h.

10.7 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 1, then the estimated DNA concentration is 50 µg/ml for double stranded DNA, or 37 µg/ml for single-stranded DNA (i.e. denatured with sodium hydroxide), respectively.

Reliable measurements require OD values at a wavelength of 260 nm to be greater than 0.05.

Finally, calculate the mass concentration, ρ, of the double-stranded test DNA solution, taking into consideration the denaturation and the dilution factor applied according to Equation (1): $\rho DNA = F \times (OD_{260} - OD_{320}) \times 37$ where

F is the dilution factor;
OD_{260} is the absorbance at 260 nm;
OD_{320} is the absorbance at 320 nm;
37 is the conversion factor, in micrograms per millilitre.

11. EVALUATION OF DNA QUALITY BY GEL ELECTROPHORESIS

11.1 General

This section describes a routine method to determine the quality of DNA in solutions. Agarose gel electrophoresis of DNA and ethidium bromide (EtBr) staining provide a way of estimating the quantity of DNA and to analyse at the same time its physical state (e.g. degree of degradation, presence of residual RNA and of some contaminants).

11.2 Principle

DNA separates electrophoretically, on the basis of its charge and molecular mass, when loaded onto a molecular sieve (agarose gel) and subjected to an electric field in the presence of a buffer solution.

EtBr intercalates into the DNA and, when excited by ultraviolet light, emits orange fluorescence. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescence produced by the unknown sample with that of a series of quantity standards.

11.3 Safety Precautions

EtBr is a powerful mutagen and a carcinogen and must be handled with care. The use of gloves is compulsory. All solutions and gels containing EtBr should be decontaminated before disposal.

Ultraviolet light (UV-C) is dangerous especially for eye retina. Always wear UV protection devices (facial mask and/or goggles with UV protection in particular) when using it.

11.4 Reagents

The agarose gel electrophoresis may be carried out as TAE buffer electrophoresis or as TBE buffer electrophoresis.

- Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules
- Boric acid (H_3BO_3), for the TBE buffer system only
- Bromophenol blue ($C_{19}H_9Br_4O_5SNa$) and/or xylene cyanole FF ($C_{25}H_{27}N_2O_6S_2Na$)
- DNA quantity standard, of suitable molecular mass
- DNA molecular mass standard
- Glacial acetic acid (CH_3COOH), for the TAE buffer system only
- Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$)
- Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$)
- Ethidium bromide solution, c(EtBr) = 0.5 mg/l
- Glycerol ($C_3H_8O_3$)
- Sodium acetate ($C_2H_3O_2Na$), for the TAE buffer system only
- Hydrochloric acid, ϕ(HCl) = 37%
- Sodium hydroxide (NaOH)
- Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$)
- Sample loading buffer solution (5x), ϕ(glycerol) = 50%, ρ(bromophenol blue) = 2.5 g/l and/or ρ(xylene cyanol) = 2.5 g/l, dissolved in electrophoresis buffer solution

  It is advisable to store the EtBr solution as a concentrate (e.g. 10mg/ml) at 5°C in the dark (EtBr is light-sensitive).

  As EtBr is a mutagen, safer alternatives to visualize DNA may be used.

11.4.1 TBE Buffer

- Tris/borate (TBE) buffer solution (0.5x), c(Tris) = 0.055 mol/l, c(boric acid) = 0.055 mol/l, c(Na_2EDTA) = 0.001 mol/l

  Adjust the pH to 8.0 with HCl or NaOH. It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionised water.

11.4.2 TAE Buffer

- TAE buffer solution (1x), c(Tris) = 0.050 mol/l, c($C_2H_3O_2Na$) = 20 mmol/l, c(Na_2-EDTA) = 0.001 mol/l

  Adjust the pH to 8.0 with glacial acetic acid or NaOH. It is advisable to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold

concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffers can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionised water.

11.5 Apparatus and equipment

- Microwave oven or boiling water bath
- Equipment for agarose gel electrophoresis, with accessories and power supply
- Ultraviolet (UV) trans-illuminator or lamp, preferably with wavelength of 312 nm
- Recording instrument, for example a photo documentation system with 3000 American Standards Association (ASA) films and UV filter adequate for EtBr-emitted fluorescence. As an alternative, a video-documentation system with charge-coupled device (CCD) camera, adequate UV filter and (optional) quantitative analysis software may be used.

11.6 Procedure

11.6.1 General

The agarose gel electrophoresis may be carried out as TAE buffer electrophoresis or as TBE buffer electrophoresis. Use the same buffer to dissolve the agarose and to fill the electrophoresis tank.

11.6.2 Agarose gel preparation

Gels should not be thicker than 1 cm.

The agarose concentration and quality determines the resolution capacity of the gel. For high molecular mass DNA quantitation, agarose concentrations between 8 g/l and 10 g/l are used. For low molecular mass DNA (e.g. degraded or restricted) higher agarose concentrations are used (up to 40 g/l).

Weigh an appropriate amount of agarose and add it to the electrophoresis buffer solution. Allow the solution to boil in a microwave oven or in a water bath, until the agarose is completely dissolved. Replace the volume lost by evaporation with an equivalent amount of water, mix by swirling (avoid air bubbles trapping), cool down the solution to about 60° C and keep it at this temperature until usage. Prepare a gel support (gel tray) with a suitable sample comb placed in right position. Pour the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

Carefully remove the samples comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells reside closer to the cathode (negative electrode). Fill the cell with the electrophoresis buffer. Overlay the gel with approximately 2 mm of the same buffer.

11.6.3 DNA sample preparation

Mix the sample DNA solutions (e.g. 5 µl to 10 µl) with approximately 20% (with respect to the final sample volume) of loading buffer (e.g. add 2.5 µl of loading buffer to 10 µl of DNA sample), mix and apply the mixture to the sample slots (wells) with a micropipette. If the unknown samples are suspected to be too concentrated, also provide some dilutions of them to be loaded onto the gel.

To determine the size of the extracted DNA fragments, add the sample loading buffer in the proportion of 20% with respect to the sample volume to a suitable amount of the DNA molecular mass standard and carry out electrophoresis in parallel.

11.6.4 Submarine electrophoresis

Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm, with respect to the distance between the electrodes, is recommended). Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration of the agarose in the gel.

11.6.5 Staining

After completing the electrophoresis, incubate the gel for 15 min to 50 min in the EtBr solution at room temperature, possibly in the dark (and/or in a stainless steel tank with a cover) with gentle shaking.

If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 mins.

As an alternative to post-electrophoresis staining, EtBr can be added to the gel before pouring it. In this case, EtBr is added to the gel to a final concentration of 0.01 mg per millilitre of gel when the gel has been cooled to a temperature of 60°C.

If the gel is cast with EtBr, load the unknown sample and the DNA quantity standard into separate slots produced with the same comb on the same gel. Otherwise the quantity of EtBr will be different for the two, so yielding erroneous quantitation results. To minimize the problems of EtBr movement in the gel, some EtBr can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required.

11.6.6 Gel recording

Transfer the gel to the trans-illuminator surface, switch on the UV light and record the DNA fluorescence using an appropriate recording instrument.

11.7 Evaluation and interpretation

The DNA content of the sample is estimated by comparing the unknown samples with the DNA quantity standard samples that underwent electrophoresis in parallel. This evaluation can be carried out visually or with the aid of a quantitation software able to calculate an adequate calibration curve.

Genomic DNA extracted from seeds should be intact and seen as a high molecular weight band on agarose gel. Genomic DNA extracted from processed foods are most likely fragmented and appears as a smear on agarose gel.

12. QUALITATIVE DETECTION OF GMOs BY NUCLEIC ACID-BASED METHODS

12.1 Principle of the method

12.1.1 General

Qualitative analysis consists of specific detection of target nucleic acid sequences in the test samples. Each method shall specify the target sequence.

A qualitative result shall clearly demonstrate the presence or absence of the genetic element under study, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

12.1.2 PCR amplification

Amplification of the target sequence occurs in vitro through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers and deoxyribonucleoside triphosphates in a defined reaction buffer.

12.1.3 Detection and confirmation of PCR products

PCR products are detected by gel electrophoresis or an appropriate alternative, if necessary, after isolation by means of a suitable separation procedure.

The identity of any detected target sequence can be verified by an appropriate technique (e.g. by restriction enzyme analysis, by hybridization or by DNA sequence analysis).

In the case of real-time PCR analysis, amplification and detection occur simultaneously.

12.2 Reagents

It is advisable to store the reaction solutions required for the analytical method at approximately –20°C if not specified otherwise.

It may also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid subjecting them to repeated freeze-thaw cycles, and/or to reduce chances of cross contamination.

- Target DNA/control
- Water
- Deoxyribonucleoside triphosphate (dNTP) solution, containing dATP, dCTP, dGTP, and dTTP or dUTP
- PCR buffer solution
- Thermostable DNA polymerase
- Forward and reverse primers

12.3 Apparatus and equipment

- Thermal cycler
- Electrophoresis chamber, with power supply
- Pipettors covering range of 1 - 1000µl
- Laminar flow PCR cabinet with UV light for master mix preparation
- Biosafety cabinet (Class II) for addition of DNA template

12.4 Procedure

12.4.1 Quality, integrity and amplifiability of nucleic acid extracts

The nucleic acid solution shall be pure enough for subsequent analysis. The quality and amount of nucleic acid extracted using a given method on a given matrix shall be both repeatable and reproducible. Nucleic acids for use in PCR should be substantially free of PCR inhibitors.

12.4.2 Performance criteria

12.4.2.1 Limit of detection (LOD)

The practical LOD is the lowest relative quantity of the target DNA that can be detected, given a known number of target taxon genome copies. For the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

The optimized PCR should be able to amplify in 40 - 50 PCR cycles from a pure reference sample of 100 copies of template DNA enough copies of the PCR product to be detectable. The characteristic temperature/time profile for each primer system and the reaction mixture appropriate for the apparatus used and the number of cycles should be adhered to.

In general, the specificity of the reaction should be enhanced as much as possible. Hot-start PCR is recommended as a means of reducing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerization.

12.4.3 Aspects of PCR design

12.4.3.1 General

Because the performance of each specific PCR should be comparable with other specific PCRs, the following aspects of PCR design (12.4.3.2 to 12.4.6) shall be taken into account.

12.4.3.2 Size of PCR products

The size of the target sequence shall be selected to match the range of molecular mass available in the nucleic acid extract being analysed.

12.4.3.3 Primers

A. Primer design

The primer sequences should preferably have the following characteristics wherever practicable:

- length of each primer: 18 to 30 nucleotides
- optimal annealing temperature $\approx$ 60°C (should be established experimentally), i.e. estimated melting temperature ≤ 65°C
- GC:AT ratio = 50:50 if possible, or else as close to this ratio as possible
- high internal stability (avoid concentration of Gs and Cs in short segments of primers)
- minimal 3' end complementarity to avoid primer-dimer formation
- minimal secondary structure
- minimal dimer formation with specific detection probe(s) designed for the PCR

B. Validation of primers

i. General

The ability of the primers to detect the target sequence shall be validated.

Primer validation should be carried out in two steps: a first theoretical evaluation, and a second experimental evaluation.

ii. Theoretical evaluation of the specificity

Theoretical evaluation shall as a minimum be carried out by performing a sequence similarity search (e.g. FastA, Blast®) against one of the major nucleic acid sequence databases (e.g.

EMBL, GenBank®). Homologous gene sequences may be retrieved from the sequence databases and aligned to obtain an estimate of the chance of finding similar sequences in the target taxon or other organisms.

iii. Experimental evaluation of the specificity

The specificity of primers shall always be experimentally evaluated to confirm the primers' ability to discriminate between the target and closely related non-target sequences.

Primers designed to detect taxon-specific target sequences should be shown to detect these sequences reliably in a representative number of different members of the taxon.

12.4.4 PCR target descriptions

For the qualitative detection and identification of GMOs, various PCR tests may be performed, depending on the type of matrices under study and/or the requirements of the analysis. These analyses may be directed at sequences specific for target taxa, genetic constructs and transformation events, as well as elements suitable for screening purposes.

12.4.5 Controls

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each diagnostic PCR assay. If available, certified reference materials should be used as positive and negative controls.

12.4.6 PCR set-up, detection and confirmation of PCR products

It may be desirable in some cases to confirm a positive or negative result for a particular genetic modification. This may be achieved by employing primers to an alternative target sequence, which is suitable for confirmation of screening test results.

A positive identification of the specific target DNA sequence may be confirmed by an appropriate method other than size determination of the PCR product, by hybridization of the PCR product with specific probes, or carrying out restriction analyses of the PCR product.

12.5 Interpretation

12.5.1 General

Interpretation of the PCR results are as follows:

a) positive if a specific PCR product has been detected, and all the controls give results as expected
b) negative if a specific PCR product has not been detected, and all the controls give results as expected

If the results are ambiguous, the procedure shall be repeated.

12.5.2 Interpretation of controls

Each control has a valid value and, if the observed result for any control is different from the valid value, the analysis shall be repeated. The valid values for the controls are as follows:

- positive extraction controls shall always be positive
- extraction blank controls shall always be negative
- positive DNA target controls shall always be positive
- negative DNA target controls shall always be negative
- amplification reagent controls shall always be negative.

12.5.3 Verification

Verification of positive or negative results for target sequences may be achieved as described in section 12.4.6.

12.6 Expression of results and quality assurance

12.6.1 General

A negative result shall not be expressed as “GMO not present”.

As a minimum, the LOD should be provided with a reference material, and a relative value based on a specified matrix (preferably a given amount of genomic DNA solution, e.g. 100 ng of 0.01 % GTS 40-3-2 DNA).

12.6.2 Expression of a negative result

The following text may appear in the test report:

“For sample X, target sequence Y was not detected.

The LOD of the method is x % determined with Z (identify the reference material).”

If it cannot be demonstrated that the amount of target DNA included in the PCR is sufficient for the LOD to be applicable, then the following sentence shall be added:

“However, the amount of the target DNA extracted from species X may be/was insufficient for the LOD to be applicable for this sample.”

12.6.3 Expression of a positive result

The following text may appear in the test report:

"For sample X, target sequence Y was detected."

The identity of the GMO may be included, if available.

12.6.4 Quality assurance requirements

Results from both test portions shall be consistent. If one test portion gives a positive result and the other gives a negative result, then the analysis shall be repeated, if possible by increasing the quantity of template nucleic acid in the reaction so as to obtain consistent results for both test portions.

13. QUANTITATIVE DETECTION OF GMOS BY NUCLEIC ACID-BASED METHODS

13.1 Principle

13.1.1 General

Quantitative analysis consists of the quantitation of target DNA sequences in the test samples. Each method specifies the target sequences(s).

Quantitation may be performed using competitive or real-time PCR.

A quantitative analysis should clearly express the quantity of the target genetic element, relative to the quantity of a specific reference, appropriate calibrants and controls, and be within the dynamic range of the analytical method used and the test portion analysed.

The analysis generally consists of

- amplification of one or more specific target sequences,
- detection and confirmation of the specificity of the PCR product(s), and
- quantification of the amplified fragments relative to calibrants.

13.1.2 Quantitation of PCR products

The principle of quantitation is usually to determine the ratio (expressed as a percent) of two DNA target sequences; i.e. a sequence representing the GMO of interest and an (endogenous) target taxon-specific sequence.

Calibrants (calibration materials) used for quantitation should be traceable to certified reference materials (CRMs), if available. If not available, other suitable reference material should be used.

13.2 Reagents

13.2.1 All reagents and materials used in the analysis should be identical, or equivalent, to those specified in the method. Otherwise, all reagents and materials should be of molecular biology grade. These reagents shall be stored and used as recommended by the supplier or according to the laboratory quality assurance specifications.

13.3 Apparatus and equipment

- Real-time thermal cycler
- Pipettors covering range of 1 - 1000µl
- Laminar flow PCR cabinet with UV light for master mix preparation
- Biosafety cabinet (Class II) for addition of DNA template

13.4 Guidelines concerning the procedures

13.4.1 General

The concentration of the DNA sequence of interest should be within the dynamic range of the method.

The DNA extracted from each test portion should be analysed at least in duplicate. Appropriate controls should be included.

13.4.2 Calibration of the analysis

An appropriate number of calibration points and replicates covering the range of quantitation shall be applied [e.g. four calibration points with two replicates (total 4 × 2 values) or six calibration points with one measurement at each point (total of 6 values)].

As an alternative to genomic DNA calibration reference materials, for example, a dilution series of a plasmid or synthetic dsDNA containing the target sequence may be used.

13.4.3 Quantitation considerations

PCR methods should be appropriately designed to minimize the variability. Calculation of the GMO content based on copy numbers of target sequences per haploid genome is influenced by the homo- and heterozygosity of the species under investigation.

Use of the $\Delta\Delta C_t$ (cycle of threshold) method is only valid if the amplification efficiencies of the target taxon-specific assay and the GMO-specific assay are very similar.

13.4.4 Quality assurance requirements

Consistency between measurements is desirable to obtain reliable estimates of target sequence quantities. If a specified GMO-derived DNA is to be reported (in percent), the following may be considered:

a) within test portion consistency:

- through rejection of measurements < LOQ, and
- through maximum deviation observed between dilutions and individual measurements equals the value expected from the corresponding dilution factor ± 33 %;

b) between test portion consistency:
- estimated relative GMO-derived DNA concentrations obtained under 13.4.4a) for each test portion should not differ by values greater than −50 % to +100 % of the estimated quantity value (equal to a ΔCt of 1 in real-time PCR).

In order to guarantee accuracy of the measurements, a reference material (RM), preferably certified (CRM), for the quantity of the event concerned, with an appropriate level of metrological reliability and with reasonable similarity of matrix shall be selected and analysed. In the absence of a CRM, in-house RM may be prepared by a procedure demonstrating stability, homogeneity and traceability, and ensuring the absence of bias.

13.4.5 Performance criteria

13.4.5.1 Limit of quantification

The practical LOQ is the lowest relative quantity of the target DNA that can reliably be quantified, given a known number of target taxon genome copies. For the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

13.5 Interpretation of results

The two possible outcomes of the PCR results will be:

a) fit for quantitation of the target sequence if:

- the result is positive according to Section 12.5
- the analysis produces an unambiguous measurement value
- the target sequence content is within the dynamic range of the method
- the analysis is calibrated in an acceptable way (see Section 13.4.2)

b) not fit for quantitation of the target sequence if any of the conditions listed above are not fulfilled.

If the GM target sequence content or the taxon-specific target sequence content is below the limit of quantitation, the result shall only be expressed qualitatively.

13.6 Expression of results

The results should clearly state the quantity of the GM target sequence relative to the target taxon-specific sequence. The results may also provide values for the measurement uncertainty. Furthermore, the LOD and LOQ of the method should be reported.

The target sequences may or may not be detected, or the quantity of at least one of them may be below the limit of quantitation. Table 1 describes the four scenarios and the corresponding expression of the result to be included in the test report.

Table 1 – Expression of results

Results	**Expression of the results**
Target taxon-specific sequence is not detected.	"For species x, DNA was not detected."
Target taxon-specific sequence is detected but GM target sequence is not detected.	"For species x, GMO-derived DNA was not detected."
The target taxon-specific sequence and the GM target sequence are both detected but the quantity is below the LOQ of at least one of the target sequences.	For each GMO, state: "GMO (specify the GMO) derived DNA as determined by detection of [specify target sequence] derived from [specify species] was detected."
The target taxon-specific sequence and the GM target sequence are both detected and the quantity is above the LOQ for both target sequences.	For each GMO, state: "The content of GMO (specify the GMO) derived DNA as determined by detection of [specify target sequence] derived from [specify species] is X ± uncertainty % (specify the unit used)."

14. TEST REPORT

14.1 The test report should contain at least the following information:

a) all information for identification of the laboratory sample;
b) any other information relating to the sample (e.g. insufficient size, degraded state);
c) date and type of sampling procedure(s) used, if applicable;
d) date of receipt;
e) storage conditions, if applicable;
f) analysis start and end date, if applicable;
g) person(s) responsible for the analysis;
h) size of the laboratory sample and test sample;
i) DNA extraction method used
j) reference materials used

k) results expressed according to Section 12.6 and/or Section 13.6 and to the requirements of the specific method along with the units used to report the results and the calculation method used;
l) any particular observations made during testing;
m) any deviations, additions to, or exclusions from, the test specification;
n) LOD and/or quantification.

The measurement uncertainty and its level of confidence shall, on request, be made available to the requestor of the results.

ANNEX A – Detection of CaMV 35S promoter by PCR (adapted from ISO 21569)

1 General

1.1 This method describes the detection of a variable copy number DNA sequence from the cauliflower mosaic virus (CaMV) 35S promoter. As this promoter is present in many genetically modified plants, this method may be used to screen for the presence of GM-plant-derived DNA.

2 Principle

2.1 A 195 bp DNA fragment from the CaMV 35S promoter sequence is amplified by PCR and can be visualised by agarose gel electrophoresis. For identification of the PCR product, a verification step should be performed.

2.2 Promoters are recognition or binding sequences for RNA-polymerases, which are responsible for gene expression. The 35S promoter from CaMV is often used in genetically modified plants.

3 Molecular specificity

3.1 The method has been designed to target a sequence described in GenBank® database accession No. V00141.

3.2 A false positive result can occur since the amplified sequence is derived from cauliflower mosaic virus which infects cauliflower and other members of the family Brassicaceae (Cruciferae) as well as Resedaceae and Solanaceae.

3.3 Positive results derived from samples of Brassicaceae, Resedaceae and Solanaceae should therefore be treated carefully. Positive results may indicate the presence of a GM-plant-derived product but should not be interpreted as proof of the presence of GM-plant-derived products without additional confirmation.

3.4 Methods for the detection of cauliflower mosaic virus can be used to distinguish between a viral infection and GM material.

4 Limit of detection (LOD)

4.1 The absolute LOD has not been determined. A relative LOD of 0.1% genetically modified soya beans in soya bean flour IRMM-410 and of 0.1% genetically modified maize Event 176 (Bt 176) IRMM-411 in maize flour (mass fraction) certified reference materials (CRMs) has been demonstrated.

5 Preparation of the test portion

5.1 Refer to Section 8 of the Guidelines on preparation of the test portion.

6 DNA extraction/purification

6.1 Refer to Section 9 of the Guidelines on DNA extraction and purification of the test portion.

7 Quantitation of the extracted DNA by ultraviolet spectrometric method

7.1 Refer to Section 10 of the Guidelines on quantifying the extracted DNA.

8 Evaluation of DNA quality by gel electrophoresis

8.1 Refer to Section 11 of the Guidelines on evaluating DNA quality by gel electrophoresis.

9 Reagents for PCR

9.1 Water

9.2 PCR buffer (without MgCl2), 10×

9.3 25 mmol/l MgCl2 solution

9.4 dNTP solution, 2.5 mmol/l (each)

9.5 Oligonucleotides

9.5.1 Forward primer

35s-1: 5'- gCT CCT ACA AAT gCC ATC A -3'

9.5.2 Reverse primer

35s-2: 5'- gAT AgT ggg ATT gTg CgT CA -3'

9.6 Thermostable DNA polymerase for hot-start PCR, 5 IU/µl

9.7 Xmn I (Asp 700) restriction enzyme, 10 U/µl

10 Procedures for PCR

10.1 PCR set-up

10.1.1 Prepare the PCR master mix with the reagents listed in Table A1 below.

Table A1: Reagents for preparation of CaMV 35S promoter PCR master mix

Reagents	Volume per sample (µl)	Final concentration
DNA template	1	10 – 50ng
Water	15.9	-
10 × PCR buffer (without $MgCl_2$)	2.5	1 ×
25 mmol/l $MgCl_2$ solution	1.5	1.5 mmol/l
10 mmol/l dNTP solution	2	0.8 mmol/l
5 µmol/l primer 35s-1	1	0.2 µmol/l
5 µmol/l primer 35s-2	1	0.2 µmol/l
5 IU/µl Taq DNA polymerase	0.1	0.5 IU
Total volume	25µl	

10.2 PCR controls

10.2.1 0.1% GTS 40-3-2 certified reference materials (IRMM-410) may be used as a positive control.

10.2.2 Refer to Section 9.2 in the Guidelines for other appropriate controls.

10.3 Thermal cycling programme

10.3.1 The thermal cycling programme in Table A2 has been optimized for GeneAmp® PCR-systems 2400 or GeneAmp® 9600 thermal cyclers and AmpliTaq Gold® DNA polymerase. Optimisation may be necessary if other thermal cyclers are used. The time for activation/initial denaturation depends on the polymerase used. If a hot-start polymerase is used, manufacturer recommendations should be followed.

Table A2: Thermal cycling programme

Activation/initial denaturation	10 min at 95°C

Amplification	20 s at 94°C 40 s at 54°C 60 s at 72°C	40 cycles
Final extension	3 min at 72°C	

11 Identification

11.1 The identity of the PCR product may be verified by restriction analysis of the PCR product with Xmn I, which is expected to yield two fragments (115 bp and 80 bp).

12 Restriction analysis

12.1 Add 1 µl of Xmn I restriction enzyme to 15 µl of PCR product and incubate at 37°C for at least 90 minutes in a thermomixer or an incubator.

12.2 Separate the DNA fragments by gel electrophoresis on a 4% agarose gel.

13 Interpretation of results

13.1 The target sequence is presumed to have been detected if the size of the PCR product corresponds to 195bp, determined by comparison with certified reference material such as GTS 40-3-2 soya (IRMM-410).

13.2 The detection of fragments with a size of 195 bp indicates that the sample contains amplifiable DNA of CaMV or GM origin within the assessed limitations of specificity described in Section 3 of this Annex.

ANNEX B – Detection of NOS-terminator by PCR (adapted from ISO 21569)

1 General

1.1 This method describes the detection of a variable copy number DNA sequence from the Agrobacterium tumefaciens nopaline synthase (NOS) terminator. As the NOS-terminator is present in many genetically modified plants, this method may be used to screen for the presence of GM-plant-derived DNA.

2 Principle

2.1 A 118 bp DNA fragment from the NOS-terminator sequence is amplified by PCR and can be visualized by gel electrophoresis. The identity of the PCR product can be verified, for example by DNA sequencing. However, no verification procedure has been validated.

3 Molecular specificity

3.1 The method has been designed to target the Agrobacterium tumefaciens nopaline synthase terminator sequence described in GenBank® database accession No. V00087.

3.2 A false positive result can occur since the amplified sequence is derived from Agrobacterium, which is a soil bacterium present in nature. Positive results may indicate the presence of a GM-plant-derived product but shall not be interpreted without additional confirmation. The potential contamination of the material with Agrobacterium or related bacteria should be considered.

4 Limit of detection (LOD)

4.1 The absolute limit of detection has not been determined, but this method has been demonstrated to detect 50 copies of GTS 40-3-2 soya DNA.

5 Preparation of the test portion

5.1 Refer to Section 8 of the Guidelines on preparation of the test portion.

6 DNA extraction/purification

6.1 Refer to Section 9 of the Guidelines on DNA extraction and purification of the test portion.

7 Quantitation of the extracted DNA by ultraviolet spectrometric method

7.1 Refer to Section 10 of the Guidelines on quantifying the extracted DNA.

8 Evaluation of DNA quality by gel electrophoresis

8.1 Refer to Section 11 of the Guidelines on evaluating DNA quality by gel electrophoresis.

9 Reagents for PCR

9.1 Water

9.2 PCR buffer containing 15 mmol/l $MgCl_2$, 10×

9.3 dNTP solution, 4 mmol/l (each)

9.4 Oligonucleotides

9.5 Forward primer

HA-nos118f: 5'- gCA TgA CgT TAT TTA TgA gAT ggg-3'

9.6 Reverse primer

HA-nos118r: 5'-gAC ACC gCg CgC gAT AAT TTA TCC-3'

9.7 Thermostable DNA polymerase for hot-start PCR, 5 IU/µl

10 Procedures for PCR

10.1 PCR set-up

10.1.1 Prepare the PCR master mix with the reagents listed in Table B1 below.

Table B1: Reagents for preparation of NOS-terminator PCR master mix

Reagents	Volume per sample (µl)	Final concentration
DNA template	5	
Water	14.84	-
10 × PCR buffer (with 15 mmol/l $MgCl_2$)	2.5	1 ×
16 mmol/l dNTP solution	1	0.64 mmol/l
20 µmol/l primer HA-nos118f	0.75	0.6 µmol/l
5 µmol/l primer HA-nos118r	0.75	0.6 µmol/l
5 IU/µl Taq DNA polymerase	0.16	0.8 IU
Total volume	25µl	

10.2 PCR controls

10.2.1 0.1% GTS 40-3-2 certified reference materials (IRMM-410) may be used as a positive control.

10.2.2 Refer to Section 9.2 in the Guidelines for other appropriate controls.

10.3 Thermal cycling programme

10.3.1 The thermal cycling programme in Table B2 has been optimized for Perkin Elmer 2400/9600/9700 thermal cyclers and AmpliTaq Gold® DNA polymerase. Optimisation may be necessary if other thermal cyclers are used. The time for activation/initial denaturation depends on the polymerase used. If a hot-start polymerase is used, manufacturer recommendations should be followed.

Table B2: Thermal cycling programme

Activation/initial denaturation	10 min at 95°C	
Amplification	25 s at 95°C 30 s at 62°C 45 s at 72°C	50 cycles
Final extension	7 min at 72°C	

11 Identification

11.1 It is recommended to verify the identity of the PCR product derived from the unknown sample by restriction, DNA sequencing or DNA hybridization.

12 Interpretation of results

12.1 The target sequence is presumed to have been detected if the size of the PCR product corresponds to 118bp, determined by comparison with certified reference material such as GTS 40-3-2 soya (IRMM-410).

12.2 The detection of fragments with a size of 118 bp indicates that the sample contains amplifiable DNA of NOS-terminator origin within the assessed limitations of specificity described in Section 3 of this Annex.

REFERENCES

1) ISO/IEC17025:2017 General requirements for the competence of testing and calibration laboratories
2) ISO 21569:2005/AMD 1:2013: Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods
3) ISO 21570:2005/AMD 1:2013: Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods
4) ISO 21571:2005/AMD 1:2013: Foodstuffs - Methods of analysis for the detection of genetically Modified organisms and derived products - Nucleic Acid Extraction
5) ISO 24276:2006/AMD 1:2013: Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions

Further suggested documents and websites related to GMO detection

1) EU reference methods for GMO Analysis: https://gmo-crl.jrc.ec.europa.eu/gmomethods/
2) Compendium of reference methods for GMO analysis: https://publications.jrc.ec.europa.eu/repository/bitstream/JRC64876/gmo-jrc_reference%20report_2011_publ.pdf
3) Hougs L, Gatto F, Goerlich O, Grohmann L, Lieske K, Mazzara M, Narendja F, Ovesna J, Papazova N, Scholtens I, Žel J. 2017. Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. EUR 29015 EN, Publication Office of the European Union, Luxembourg, 2017, ISBN 978-92-79-77310-5, doi:10.2760/645114, JRC 109940.
4) Berben G, Charels D, Demšar T, Hochegger R, Nardini E, Onori R, Schulze M, Philipp P, Weber T. Guidelines for sample preparation procedures in GMO analysis. EUR 27021. Luxembourg (Luxembourg): Publications Office of the European Union; 2014. JRC94042
5) Waiblinger, H. U., Grohmann, L. and Mankertz, J. 2010. A practical approach to screen for authorized and unauthorized genetically modified plants. Anal Bioanal Chem. 396: 2065-2072