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Horizontal methods for molecular biomarker analysis — Methods of analysis for the detection of genetically modified organisms and derived products —

Part 4: Real-time PCR based screening methods for the detection of the *P-nos* and *P-nos-nptII* DNA sequences

Méthodes horizontales d'analyse moléculaire de biomarqueurs — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés —

Partie 4: Méthodes de dépistage PCR en temps réel pour la détection des séquences ADN P-nos *et* P-nos-nptII

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

A list of all the parts in the ISO/TS 21569 series can be found on the ISO website.

Horizontal methods for molecular biomarker analysis — Methods of analysis for the detection of genetically modified organisms and derived products —

Part 4: Real-time PCR based screening methods for the detection of the *P-nos* and *P-nos-nptII* DNA sequences

1 Scope

This document specifies a procedure for the detection of a DNA sequence of the promoter region of the nopaline synthase gene (*P-nos*) from *Agrobacterium tumefaciens* and a procedure for the detection of the DNA transition sequence between *P-nos* and the neomycin-phosphotransferase gene (*nptII)* from the Tn5 transposon of *Escherichia coli* K12. The *nos*-promoter and the *P-nos-nptII*-construct are frequently found in genetically modified plants. The *P-nos* and *P-nos-nptII* specific methods are based on real-time PCR and can be used for qualitative screening purposes. For identification and quantification of a specific genetically modified plant (event) a follow-up analysis has to be carried out.

The methods described are applicable for the analysis of DNA extracted from foodstuffs. They may also be suitable for the analysis of DNA extracted from other products such as feedstuffs and seeds. The application of these methods requires the extraction of an adequate amount of amplifiable DNA from the relevant matrix.

The DNA sequence amplified by the *P-nos* element-specific method can be detected in samples which contain DNA of the naturally occurring Ti-plasmid of *A. tumefaciens*. For this reason, it is necessary to confirm a positive screening result. Further analyses are required using construct-specific or event specific methods.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 21569, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21570, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods*

ISO 21571:2005, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

4 Principle

DNA is extracted from the test sample applying a suitable method (see ISO 21571). The DNA analysis consists of two parts:

a) verification of the amount and amplifiability of the extracted DNA, e.g. by means of a target taxon specific real-time PCR (according to ISO 21570), see also Reference [1];

b) detection of the *P-nos* and/or *P-nos-nptII* sequence in a real-time PCR, see References [2], [3] and [4].

5 Reagents and materials

5.1 General

For the purpose of this document, only chemicals and water of recognized analytical grade, appropriate for molecular biology shall be used. Unless stated otherwise, solutions should be prepared by dissolving the corresponding reagents in water and be autoclaved. For all operations in which gloves are used, it should be ensured that these are powder-free. The use of aerosol-protected pipette tips as protection against cross contamination is recommended.

5.2 PCR reagents

5.2.1 Thermostable DNA polymerase (for hot-start PCR).

5.2.2 PCR buffer solution (containing magnesium chloride and deoxyribonucleoside triphosphates, dNTPs).

Ready-to-use reagent mixtures or mixes of individual components can be used. Reagents and polymerases which lead to equal or better results may also be used.

5.2.3 Oligonucleotides (see Tables 1 and 2).

Table 1 — Oligonucleotides for detection of *P-nos* element

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
P-nos as target sequence[4]:		
Primer p-nos-F1	5'-AAg CAC ATA CgT CAg AAA CCA TTA TT-3'	400 nmol/l
Primer p-nos-R	5'-TCA gTg gAg CAT TTT TgA CAA gAA-3'	400 nmol/l
Probe p-nos-Tm	5'-(FAM)-CgC gTT CAA AAg TCg CCT AAg gTC AC-(BBQ)-3'[a]	100 nmol/l

[a] FAM: 6-Carboxyfluorescein, BBQ: BlackBerry® Quencher. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results.

Table 2 — Oligonucleotides for detection of *P-nos-nptII* construct

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
P-nos-nptII as target sequence[4]:		
Primer p-nos-F2	5'-TTC CCC TCg gTA TCC AAT TAg Ag-3'	400 nmol/l
Primer NPTII-R	5'-gAT TgT CTg TTg TgC CCA gTC A-3'	400 nmol/l
Probe NPTII-Tm2	5'-(FAM)-AgC CgA ATA gCC TCT CCA CCC AAg C-(BBQ)-3'[a]	100 nmol/l

[a] FAM: 6-Carboxyfluorescein, BBQ: BlackBerry® Quencher. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results.

6 Apparatus

Requirements concerning apparatus and materials shall be according to ISO 21569. In addition to the usual laboratory equipment, the following equipment is required.

6.1 Real-time PCR device, suitable for the excitation of fluorescent molecules and the detection of fluorescence signals generated during PCR.

7 Procedure

7.1 Preparation of test sample

It should be ensured that the test sample used for DNA extraction is representative of the laboratory sample, e.g. by grinding or homogenizing of the samples. Measures and operational steps to be taken into consideration are described in ISO 21571 and ISO 24276.

7.2 Preparation of DNA extracts

Concerning the preparation of DNA from the test sample, the general instructions and measures described in ISO 21571 shall be followed. It is recommended to choose one of the DNA extraction methods described in ISO 21571:2005, Annex A.

7.3 PCR setup

The method is described for a total volume of 25 µl per PCR. The reaction setup is given in Table 3.

Reagents are completely thawed at room temperature. Each reagent should be carefully mixed and briefly centrifuged immediately before pipetting. A PCR reagent mixture is prepared which contains all components except for the sample DNA. The required amount of the PCR reagent mixture depends on the number of reactions to be performed, including at least one additional reaction as a pipetting reserve. Add 5 µl of sample DNA to each reaction.

Mix the PCR reagent mixture, centrifuge briefly and pipette 20 µl into each reaction vial. For the amplification reagent control, add 5 µl of water into the respective reaction set-up. Pipette either 5 µl of sample DNA or 5 µl of the respective control solution (extraction blank control, positive DNA target control). If necessary, prepare a PCR inhibition control as described in ISO 24276.

Transfer the reaction set-ups into the thermal cycler and start the temperature-time programme.

Table 3 — Components of the PCR reaction

Total reaction volume	25 µl
Sample DNA (up to 200 ng) or controls	5 µl
PCR buffer solution[a] (including $MgCl_2$, dNTPs and hot-start DNA polymerase)	12,5 µl
Primers	see Table 1 or 2
Probe	see Table 1 or 2
Water	add to obtain 25 µl

[a] In the collaborative trial TaqMan® Universal PCR Mastermix (Life Technologies) was used as PCR buffer solution. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products from other manufacturers may be used if they can be shown to give equivalent or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

7.4 Temperature-time programme

The temperature-time programme as outlined in Table 4 has been used in the validation study. The use of different reaction conditions and real-time PCR cyclers may require specific optimization. The time for initial denaturation depends on the master mix used.

Table 4 — Temperature-time programme

Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95 °C	10 min	no	1
2	Amplification	Denaturation	94 °C	15 s	no	45
		Annealing and elongation	60 °C	60 s	yes	

8 Accept/reject criteria

8.1 General

A corresponding real-time PCR device-specific data analysis programme is used for the identification of PCR products. The amplification results may be expressed in a different manner, depending on the device used. In the absence of detectable PCR products (e.g. negative controls) the result can be expressed as “undetermined”, “no amp”, or the maximum number of reaction cycles performed. If the amplification of the DNA target sequence in a sample (e.g. positive controls) occurred, a sigmoid shaped amplification curve should be observed. The cycle number at the crossing point of the amplification curve and the fluorescence threshold is calculated (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic interpretation does not provide a meaningful result, it may be required to set the baseline and the threshold manually prior to interpreting the data. In this case, the device-specific instructions given in the manual regarding the use of the interpretation software shall be applied.

8.2 Identification

The *P-nos* or *P-nos-nptII* target sequence is considered as detected, if

— by using the *P-nos* specific primers p-nos-F1 and p-nos-R and the probe p-nos-Tm or the *P-nos-nptII* specific primers p-nos-F2 and NPTIIR and the probe NPTII-Tm2, a sigmoid shaped amplification curve is observed and a C_t value or C_p value is calculated,

— in the PCR control reactions with no added DNA (PCR reagent control, extraction blank control), no amplification has occurred, and

— in the reactions for the amplification control (positive DNA target control, PCR inhibition control) the expected C_t values or C_p values are achieved.

9 Validation status and performance criteria

9.1 General

Validation followed a two part process:

a) in-house validation followed by a pilot interlaboratory study;

b) a collaborative trial validation.

9.2 Robustness of the method

The robustness of the *P-nos* and *P-nos-nptII* methods was tested in a pilot interlaboratory study with four participating laboratories with regard to changes in the conditions of the following factors: real-time PCR machines[1)] (ABI7900, ABI7900HT, ABI 7500 and LC480), PCR master mix kits and volumes (Qiagen Quantitect Master Mix, TaqMan® Universal Master Mix and Roche Probes Master Mix, 19 µl or 21 µl of master mix plus 5 µl of sample DNA), annealing temperature (59 °C and 61 °C), and primer or probe concentration (both lowered by 30 %). For each tested factor 3 PCR replicates were analysed with 20 copies each of the target sequence. Under these changed conditions all PCR reactions gave positive results as expected, hence the method can be considered robust.

9.3 Collaborative trial

The reliability of the methods was tested in an interlaboratory trial with 12 participants according to ISO 5725 (all parts), which was organized by the German Federal Office for Consumer Protection and Food Safety (BVL). The participants received for analysis 24 DNA samples containing different concentrations of the *P-nos-nptII* sequence and 12 DNA samples not containing this sequence. All samples were labelled with randomized codes.

The *P-nos* and *P-nos-nptII* sequences are used for genetic modifications in different plant species. The interlaboratory test employed genetically modified rapeseed (Topas19/2), genetically modified potato (EH92-527-1) and the corresponding non-GM plant material as test samples. Taking into account their genetic constitution, 20 copies or 50 copies of the *P-nos-nptII* sequence were used against a background of 20 ng/µl of species DNA in 0,2x TE-buffer (see Table 5). The background DNA had been obtained from the batch of material that was used for the negative samples.

Table 5 — DNA-samples used in the collaborative trial

Species	GM-event	Copies per µl	Number of replicates
Rapeseed	Non-GM rapeseed	0	6
	Topas19/2	4	6
	Topas19/2	10	6
Potato	Non-GM potato	0	6
	EH92-527-1	4	6
	EH92-527-1	10	6

Each sample submitted to the participants was analysed in a single PCR with 5 µl of the DNA using the *P-nos* and *P-nos-nptII* PCR systems under conditions as given in Tables 3 and 4.

1) This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the products named. Equivalent products may be used if they can be shown to lead to the same results.