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

Part 6:

Real-time PCR based screening methods for the detection of *cry1Ab/Ac* and *Pubi-cry* DNA sequences

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

Partie 6: Méthodes de dépistage PCR en temps réel pour la détection de séquences ADN cry1Ab/Ac *et* Pubi-cry

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Foreword

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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 6: Real-time PCR based screening methods for the detection of *cry1Ab/Ac* and *Pubi-cry* DNA sequences

1 Scope

This document specifies a procedure for the detection of a DNA sequence of the modified *cry1Ab/Ac* gene and a procedure for the detection of the DNA transition sequence between the maize ubiquitin promoter (*Pubi)* and the *cry1Ab/Ac* gene. The modified *cry1Ab/Ac* gene and the *Pubi-cry* construct are frequently found in genetically modified Bt plants. Both detection 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.

This document is applicable for the analysis of DNA extracted from foodstuffs. It 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.

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 portion 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 *cry1Ab/Ac* and/or the *Pubi-cry* DNA sequence in a real-time PCR, see References [2] and [3].

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 *cry1Ab/Ac*

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
cry1Ab/Ac as target sequence[2]:		
Bt-F1(mod)	5'-gAg gAA ATg CgT ATT CAA TTC AAC-3'	400 nmol/l
Bt-R	5'-TTC Tgg ACT gCg AAC AAT gg-3'	400 nmol/l
Bt-P	5'-(FAM)-ACA TgA ACA gCg CCT TgA CCA CAg C-(NFQ)-3'[a]	100 nmol/l
[a] FAM: 6-Carboxyfluorescein, NFQ: non-fluorescent quencher (dark quencher).		

Table 2 — Oligonucleotides for detection of *Pubi-cry*

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
Pubi-cry as target sequence[2]:		
Pubi-F2	5'-ATT TgC TTg gTA CTg TTT CTT TTg TC-3'	300 nmol/l
Cry-rr-R	5'-TTg TTg TCC ATg gAT CCT CTA gAg T-3'	600 nmol/l
Pubi-T2	5'- (FAM)- ACC CTg TTg TTT ggT gTT ACT TCT gCA-(NFQ)-3'[a,b]	100 nmol/l
[a] FAM: 6-Carboxyfluorescein, NFQ: non-fluorescent quencher (dark quencher). [b] The Pubi-T2 probe is three bases shorter than the probe described in Reference [3] but identical specificity and sensitivity is achieved.		

Equivalent reporter dyes and/or quencher dyes may be used for the probe if they can be shown to yield similar 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 samples

It should be ensured that the test portion 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 should be as described in ISO 21571 and ISO 24276.

7.2 Preparation of DNA extracts

Concerning the preparation of DNA from the test portion, 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.

Completely thaw reagents 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.

Table 3 — Reaction setup for the amplification

Total reaction volume	25 µl
Sample DNA (up to 200 ng) or controls	5 µl
PCR buffer solution[a] (including $MgCl_2$, dNTP's and hot-start DNA polymerase)	12,5 µl
Primers Bt-F1(mod) and Bt-R or primers Pubi-F2 and Cry-rr-R	see Table 1 or 2
Probe Bt-P or probe Pubi-T2	see Table 1 or 2
Water	add to obtain 25 µl

[a] In the interlaboratory 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 names. 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.

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 setup. 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 setups into the thermal cycler and start 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 should be applied.

8.2 Identification

The *cry1Ab/Ac* or *Pubi-cry* target sequence is considered as detected, if

— by using the specific primers Bt-F1(mod) and Bt-R and the probe Bt-P or the specific primers Pubi-F2 and Cry-rr-R and the probe Pubi-T2, 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 controls (positive DNA target control, PCR inhibition control) the expected C_t values or C_p values are achieved.

NOTE In accordance with the requirements specified in ISO 24276, DNA extracted from Bt11 certified reference material (ERM-BF412f) can be used as positive target control for the cry1Ab/Ac method, while for the Pubi-cry method a synthetic plasmid containing the target sequence can be used as positive target control.

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

The robustness of the method was tested by in-house validation using two different PCR master mixes (TaqMan® Universal master mix and LC480 Probes master)[1)], different annealing temperatures (59 °C and 61 °C) and different primer and probe concentrations (both lowered by 30 %), which were tested for each condition in three PCR replicates using 60 target copies in each replicate. These deviations of the experimental conditions did not cause any difficulties in detecting the target sequences. In the collaborative trial the robustness of the method was checked with regard to six different real-time PCR machines. No influence on the performance or difficulties of the method was observed. Hence, the method can be considered robust.

9.3 Collaborative trial

The reliability of the methods was tested in a collaborative trial with 17 participants[2], which was organized by the German Federal Office for Consumer Protection and Food Safety (BVL) according to ISO 5725-2. The participants received for analysis 10 DNA samples containing different concentrations of the *cry1Ab/Ac* and/or *Pubi-cry* DNA sequence and 6 DNA samples not containing this DNA sequence (see Table 5). All samples where labelled with randomized codes.

Table 5 — DNA samples and standard DNAs used in the collaborative trial

Material used for preparation of sample DNA	*gos9*[1]		*cry1Ab/Ac*		*Pubi-cry*	
	PCR	copies/ µl	PCR	copies/ µl	PCR	copies/ µl
Mass fraction of 0,05 % Bt63 rice	+[a]	75x10^3	+	n.d.[b]	-[a]	0
KeFeng6 rice	+	20x10^3	+	1x10^1	+	1x10^1
Mass fraction of 4,89 % Bt11 maize (ERM-BF412f) mixed with non-GM rice	+	20x10^3	+	1x10^1	-	0
KeFeng6-positive rice noodle RASFF 2009.0717 (C_t = 33,2 for *cry1Ab/Ac* and *Pubi-cry*)	+	20x10^3	+	n.d.	+	n.d.
GM-positive basmati rice RASFF 2011.1646 (C_t = 33,0 for *cry1Ab/Ac* and *Pubi-cry*)	+	20x10^3	+	n.d.	+	n.d.
Non-GM Basmati rice	+	78x10^3	-	0	-	0
Non-GM Thai rice	+	74x10^3	-	0	-	0
Non-GM rice noodle	+	26x10^3	-	0	-	0
Mass fraction of 4,89 % Bt11 maize (ERM-BF412f) (standard DNA)	-	0	+	1x10^2	-	0
Plasmid RR1 (standard DNA)	+	5x10^2	+	5x10^2	+	5x10^2

[a] The expected PCR result is indicated by '+' (positive) and '-' (negative).

[b] n.d. = not determined.

Each sample submitted to the participants was analysed in a single PCR with 5 µl of the DNA using the *cry1Ab/Ac* or *Pubi-cry* PCR systems under conditions as given in Tables 3 and 4.

An overview of the results of the collaborative trial specifying the false-positive/false-negative rates for the *cry1Ab/Ac* or *Pubi-cry* methods is provided in Table 6.

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.