TECHNICAL SPECIFICATION

ISO/TS 21569-3

Second edition

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

Part 3: Construct-specific real-time PCR method for detection of P35S-pat-sequence for screening for genetically modified organisms

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

Partie 3: Méthode PCR en temps réel construit-spécifique pour la détection de la séquence P35S-pat pour criblage des organismes génétiquement modifiés

PROOF/ÉPREUVE

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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 of the voluntary nature of standards, 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

This second edition cancels and replaces the first edition (ISO/TS 21569-3:2015), which has been technically revised. The main changes compared with the previous edition are as follows:

— the section on in silico search has been updated;

— minor typographical changes have been made throughout.

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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

Part 3:
Construct-specific real-time PCR method for detection of P35S-pat-sequence for screening for genetically modified organisms

1 Scope

This document describes a procedure for the detection of the DNA transition sequence between the 35S promotor (*P35S*) from *Cauliflower mosaic virus* and a modified phoshinothricin-acetyltransferase gene (*pat*) from *Streptomyces viridochromogenes*. The *P35S-pat* construct is frequently found in genetically modified plants with tolerance for phosphinothricin-containing herbicides. The *P35S-pat* construct specific method is based on a real-time PCR and can be used for qualitative and quantitative screening purposes. For identification and quantification of a specific event, a follow-up analysis can be carried out.

This document is applicable to the analysis of DNA extracted from foodstuffs. It can also be suitable for the analysis of DNA extracted from other products such as feedstuffs and seeds. The application of this method requires the extraction of an adequate quantity and quality 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 16577, *Molecular biomarker analysis — Terms and definitions*

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

ISO 21571, *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:

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

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

4 Principle

DNA is extracted from the test portion by applying a suitable method. 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 tax on specific real-time PCR (see ISO 21570[1]):

b) detection of the P35S-pat construct in a real-time PCR[2][3].

5 Reagents and materials

5.1 General

Chemicals of recognized analytical grade, appropriate for molecular biology shall be used, as a rule. The water used shall be double distilled or PCR grade water (i.e. nuclease and nucleic acid free). For all operations in which gloves, the gloves should be 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 (dATP, dCTP, dGTP and dUTP).

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

5.2.3 Oligonucleotides (see Table 1).

Table 1 — Oligonucleotides

Name	DNA sequence of the oligonucleotide	Final concentration in the PCR
P35S-pat construct as the target sequence[2][3]:		
Primer 35SP03.f	5′-AAg TTC ATT TCA TTT ggA gAg gAC A-3′	200 nmol/l
Primer pat-7.r	5′-Cgg CCA TAT CAg CTg CTg TAg-3′	200 nmol/l
Probe GSS01.s	5′-(FAM)-CCg gAg Agg AgA CCA gTT gAg ATT Agg C-(TAMRA)-3′[a]	100 nmol/l
[a] FAM: 6-Carboxyfluorescein, TAMRA: 6-Carboxytetramethylrhodamine.		

NOTE Equivalent reporter dyes and/or quencher dyes can be used for the probe if they can be shown to yield similar or better results.

5.2.4 Standard DNA for calibration

A standard DNA solution of a known concentration (ng/µl) can be used to calculate the copy number of the P35S-pat target sequence.

When using genomic plant DNA as the standard DNA, the number of haploid genome equivalents should be calculated on the basis of the molecular mass of the plant haploid genome by applying Formula (1):

$$n_g = \frac{c_{DNA} \times 1\ 000}{m_{hg}} \qquad (1)$$

where

n_g is number of genome equivalents per microlitre;

m_{hg} is the haploid genome mass in picograms;

c_{DNA} is the DNA concentration in nanograms per millilitres.

The respective copy number for the P35S-pat sequence can be calculated based on the genome equivalents. In doing so, the number of integrations into the plant genome as well as the degree of zygosity of the plant material used shall be taken into consideration.

6 Apparatus

For apparatus and materials, follow 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 Test sample procedure

The testing plan for P35S-pat screening assumes that the test samples are a representative sample drawn from the laboratory sample. Simple representative sampling implies that each test sample has both an equal and an independent chance of being drawn from the laboratory sample. Measures and operational steps to be taken into consideration shall be as described in ISO 21571 and ISO 24276.

7.2 Preparation of the DNA extracts

Concerning the preparation of DNA from the test portion, the general instructions and measures described in ISO 21571 should 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 set-up is given in Table 2.

Completely thaw reagents at room temperature. Each reagent should be carefully mixed and briefly centrifuged immediately before pipetting. Prepare a PCR reagent mixture that contains all the 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 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 2 — Reaction set-up for the amplification

Element	Set-up
Overall 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
Primer 35SP03.f and pat-7.r	see Table 1
Probe GSS01.s	see Table 1
Water	to 25 µl

[a] In the collaborative study, depending on the real-time PCR devices, different PCR buffer solutions were used [TaqMan Universal PCR Mastermix (Life Technologies, Darmstadt), QuantiTect Multiplex PCR NoROX or QuantiTect Probe PCR Mastermix (Qiagen GmbH, Hilden)]. 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. 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 3 was 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 3 — Temperature-time programme

Step	Parameter		Temperature °C	Time	Fluorescence measurement	Cycles
1	UNG activation (optional)		50	2 min	no	1
2	Initial denaturation		95	10 min	no	1
3	Amplification	Denaturation	95	15 s	no	45
		Annealing and elongation	60	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 control), the result can be expressed as “undetermined”, “no amp” or the maximum number of possible cycles. If the amplification of the DNA target sequence occurs in a sample (e.g. positive control), a sigmoid-shaped amplification curve can be observed, and the cycle number is calculated at which a predetermined fluorescence threshold value is exceeded (C_t value or C_p value).

If, due to atypical fluorescence measurement data, the automatic interpretation does not provide a meaningful result, it might be necessary 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 target sequence is considered as detected, if:

— by using the P35S-pat specific primers 35SP03.f and pat-7.r and the probe GSS01.s, a sigmoid-shaped amplification curve can be observed and a predetermined fluorescence threshold value is exceeded;

— in the PCR control set-ups with no added DNA (PCR reagent control, negative extraction control), no sigmoid shaped amplification curve can be observed and a predetermined fluorescence threshold value is not exceeded;

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

8.3 Calculation of P35S-pat copy numbers

With DNA standards containing defined amounts of P35S-pat copy numbers (see 5.2.4 and 9.3), a calibration curve can be generated and used for calculation of P35S-pat copy numbers in unknown samples.

9 Validation status and performance criteria

9.1 Robustness of the method

The robustness of the method has not been tested with respect to small modifications of factors such as reagent concentrations (e.g. primers, probe) or reaction conditions (e.g. annealing temperature).

NOTE In the collaborative trial, the robustness of the method has been checked with regard to different real-time PCR machines and PCR buffer solutions. The real-time PCR machines and the PCR buffer solutions had no influence on the performance of the method.

9.2 Collaborative trial for determination of LOD

The limit of detection (LOD) of the method was tested in a collaborative study coordinated in 2011 by the German Federal Office of Consumer Protection and Food Safety (BVL) with a total of 10 participants. The participants received four DNA samples from GM-positive plants containing the P35S-pat target sequence.

To prepare the samples, certified reference materials produced from the American Oil Chemists' Society (AOCS, Urbana USA) and the Institute for Reference Materials and Measurements (IRMM, Geel Belgium) were used. The materials used were DNA from the leaves of T45 canola (AOCS, 0208-A2), of A2704-12 soybeans (AOCS, 0707-B2), of T25 maize (AOCS, 0306-H) and DNA extracted from TC 1507 maize powder (IRMM, ERM-BF418d). DNA concentrations were determined spectrophotometrically. Numbers of genome equivalents per µl were calculated by applying Formula (1). On the basis of the number of genome equivalents, the respective copy number for the P35S-pat sequence was calculated, considering the number of integrations of the P35S-pat sequence into the plant genome as well as the degree of zygosity of the plant material used (see Table 4). Copy numbers of sample DNAs were adjusted to approximately 100 copies of the P35S-pat sequence per µl.

Table 4 — Characteristics of reference materials for determination of LOD

GM event	Source of reference material	Material/ % (mass fraction) GM	Haploid genome mass (pg)[4]	Zygosity	*P35S-pat* copy numbers per haploid genome	Length of the *P35S-pat* PCR product (bp)
T45 canola	AOCS	DNA / 99,99 %	1,3	homozygous	1[5]	111
A2704-12 soybean	AOCS	DNA / 99,99 %	1,13	homozygous	2[6]	102
T25 maize	AOCS	DNA / 99,99 %	2,7	homozygous	1[7]	111
TC1507 maize	IRMM	powder / 10 %	2,7	heterozygous	1[8]	102

On the basis of these four standard DNA solutions, dilution series were prepared by the participants in order to obtain DNA solutions with copy numbers of 50 to 0,1 per 25 µl PCR reaction. The participants analysed each diluted DNA solution with the P35S-pat real-time PCR method in a sixfold determination

under the conditions described in Tables 1 to 3. The results of this collaborative trial study are listed in Table 5.

Table 5 — Results of the collaborative trial for determination of LOD

P35S-pat copy numbers per PCR	Number of positive results (C_t < 45) out of 60 results			
	T45 canola	TC1507 maize	T25 maize	A2704-12 soybean
50	60	60	60	60
20	60	60	60	60
10	59	60	60	60
5	46	59	56	55
2	26	52	41	36
1	11	42	21	24
0,1	3	8	3	2

9.3 Collaborative trial for quantification of the P35S-pat construct in rapeseed

The method was validated in a collaborative study coordinated in 2004 by the Sub-committee for Method Development of the German National and Federal Joint Committee on Genetic Engineering (LAG) with a total of 14 participants. The participants received five seed samples and five DNA samples.

For preparation of the seed samples, non-GM rapeseed was mixed with genetically modified GS40/90 rapeseed in ratios of 980 g/20 g (2 % GM), 990 g/10 g (1 % GM), 995 g/5 g (0,5 % GM), 999 g/1 g (0,1 % GM) and 1 000 g/0 g (0 % GM), homogenized and subdivided into samples of 30 g using a sample divider. Participants had to grind the seed samples and isolate the DNA thereof. For the preparation of the DNA samples provided to the participants, genomic DNA isolated from a plant of GS40/90 oilseed rape was mixed with genomic DNA isolated from a plant of non-GM oilseed rape to obtain solutions containing 2 %, 1 %, 0,5 %, 0,1 % and 0 % of GS40/90 oilseed rape DNA. Concentrations of the DNA samples were adjusted to 20 ng/µl.

Each sample was analysed by the participants in a threefold determination with the P35S-pat real-time PCR method under the conditions described in Tables 1 to 3. For calibration, six DNA standards (100, 300, 900, 2 700, 8 100, 100 000 copies; provided) were measured twofold in the same PCR analysis run. In addition, the same number of measurements was carried out using a taxon-specific real-time PCR method targeting the pepC gene of oilseed rape[2]. The calibration curves were created by plotting the C_t values against the logarithm of the copy numbers of the target sequence provided for the calibration solutions. The respective copy numbers for the samples were calculated by interpolation from the calibration curve.

A summary of the results is presented in Table 6. The quantitative results are given in Table 7. Before the calculation of the mean GM contents and of precision data using statistical tests in accordance with ISO 5725-2[9], Grubbs and Cochran outliers were identified and eliminated if applicable. Since the number of pepC alleles in different varieties of oilseed rape is not known, it is not possible to use the results of the taxon-specific PCR to calculate the oilseed rape genome copy number. Therefore, the trueness of the method could not be determined. In addition, it is assumed that the true GM content in the seed samples could deviate from the expected content. The degree of deviation cannot be estimated.

