

**SLOVENSKI STANDARD
SIST-TS CEN/TS 15634-3:2016**

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**Živila - Odkrivanje prisotnosti alergenov v živilih z metodami molekularne biologije
- 3. del: Lešnik (Corylus avellana) - Kvalitativno odkrivanje specifičnega niza DNK
v čokoladi s PCR v realnem času**

Foodstuffs - Detection of food allergens by molecular biological methods - Part 3:
Hazelnut (Corylus avellana) - Qualitative detection of a specific DNA sequence in
chocolate by real-time PCR

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Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen
Verfahren - Teil 3: Haselnuss (Corylus avellana) Qualitativer Nachweis einer
spezifischen DNA-Sequenz in Schokolade mittels Real-time PCR

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Produits alimentaires - Détection d'allergènes alimentaires par des méthodes de biologie
moléculaire - Partie 3 : Noisette (Corylus avellana) – Détection qualitative d'une
séquence d'ADN spécifique dans du chocolat, par PCR en temps réel

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67.190	Čokolada	Chocolate

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en

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Foodstuffs - Detection of food allergens by molecular biological methods - Part 3: Hazelnut (*Corylus avellana*) - Qualitative detection of a specific DNA sequence in chocolate by real-time PCR

Produits alimentaires - Détection d'allergènes alimentaires par des méthodes de biologie moléculaire - Partie 3: Noisette (*Corylus avellana*) - Détection qualitative d'une séquence d'ADN spécifique dans du chocolat, par PCR en temps réel

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 3: Haselnuss (*Corylus avellana*) - Qualitativer Nachweis einer spezifischen DNA-Sequenz in Schokolade mittels Real-time PCR

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European foreword

This document (CEN/TS 15634-3:2016) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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EN 15634, Foodstuffs — Detection of food allergens by molecular biological methods, is currently composed with the following parts:

- Part 1: General considerations;
- Part 2: Celery (*Apium graveolens*) — Qualitative determination of a specific DNA sequence in cooked sausages by real-time PCR [Technical Specification];
- Part 3: Hazelnut (*Corylus avellana*) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR [Technical Specification];
- Part 4: Peanut (*Arachis hypogaea*) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR [Technical Specification];
- Part 5: Mustard (*Sinapis alba*) and soya (*Glycine max*) — Qualitative detection of a specific DNA sequence in cooked sausages by real-time PCR [Technical Specification].

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1 Scope

This Technical Specification describes a procedure for the qualitative detection of hazelnut (*Corylus avellana*) in chocolate. DNA is extracted from the chocolate and a specific DNA sequence for hazelnut detected from the gene for corA 1 [4], [5].

2 Principle

The total DNA is extracted from the sample and the DNA content estimated. A 152 bp long sequence from the gene for corA 1 is multiplied using real-time PCR. The amplicon formed in this way is detected by annealing a sequence-specific probe and generating a fluorescence signal [4], [5].

3 Reagents

As a rule, analytical grade chemical reagents suitable for molecular biology shall be used. The water used shall be double distilled or equivalent quality. Solutions should be prepared by dissolving the appropriate reagents in water and autoclaving, unless indicated differently.

3.1 DNA extraction with CTAB

3.1.1 Chloroform.

3.1.2 Ethanol, volume fraction $\varphi = 96\%$.

3.1.3 Ethylenediaminetetraacetic acid disodium salt (Na₂EDTA). *iTeh STANDARD PREVIEW (standards.iteh.ai)*

3.1.4 Cetyltrimethylammoniumbromide (CTAB).

3.1.5 Hydrochloric acid, mass fraction $w = 37\%$. *SIST-TS CEN/TS 15634-3:2016 https://standards.iteh.ai/catalog/standards/sist/bab8aa17-f92e-45dd-8b51-30eaa45f8e76/sist-ts-cen-ts-15634-3-2016*

3.1.6 Isoamyl alcohol.

3.1.7 Isopropanol.

3.1.8 Proteinase K.

3.1.9 Sodium chloride.

3.1.10 Sodium hydroxide.

3.1.11 Tris(hydroxymethyl)aminomethane (TRIS).

3.1.12 Chloroform isoamyl alcohol mixture.

Mix 24 parts by volume of chloroform (3.1.1) with one part by volume of isoamyl alcohol (3.1.6).

Commercially available and comparable mixtures can be used.

3.1.13 CTAB extraction buffer solution, containing CTAB (mass concentration $\rho = 20\text{ g/l}$), sodium chloride (substance concentration $c = 1,4\text{ mol/l}$), TRIS ($c = 0,1\text{ mol/l}$), Na₂EDTA ($c = 0,02\text{ mol/l}$). Adjust the pH value with hydrochloric acid to pH = 8,0.

3.1.14 Ethanol solution, $\varphi = 70\%$.

3.1.15 Proteinase K solution, $\rho = 20\text{ mg/ml}$.

The freshly produced Proteinase K solution should be stored in the form of aliquots at -20 °C.

3.1.16 TE buffer solution, containing TRIS ($c = 0,001 \text{ mol/l}$) and Na₂-EDTA ($c = 0,0001 \text{ mol/l}$). Adjust the pH value with hydrochloric acid or sodium hydroxide solution to pH = 8,0.

3.2 DNA purification by means of solid phase extraction

For the DNA purification, different methods may be used.

Various systems are commercially available for DNA purification by means of solid phase extraction, including spin filter columns or plates or also with vacuum operated systems. Commercially available kits can also be used. Observe the manufacturer's data for this (see also 6.3.1).

3.3 Real-time PCR reagents

3.3.1 PCR master mix¹⁾, containing reaction buffer, dNTPs, MgCl₂ and Hotstart Taq polymerase.

3.3.2 Oligonucleotides, 5 µmol each.

3.3.2.1 Hazelnut iF, 5'- TAC AgC ATC ATC gAg ggA ggT C - 3'.

3.3.2.2 Hazelnut iR, 5'- CTC CTC ATT gAT TgA AgC gTT g - 3'.

3.3.2.3 Hazelnut probe, 5'- FAM - AgA Tgg Cgg CAg CCC CTC AT - TAMRA - 3'²⁾

3.3.3 Negative PCR control, conducted with DNA-free water instead of the DNA extract from the sample and without PCR inhibitors. **iTech STANDARD REVIEW** (standards.itech.ai)

3.3.4 Negative extraction control, performing all steps of the DNA extraction procedure, except addition of the test portion, e.g. by substitution of a corresponding amount of water for the test portion. <https://standards.itech.ai/catalog/standards/sist/bab8aa17-b92e-45dd-8b51-30aa458e71cistsopen-15634-3-2016>

3.3.5 Negative process control, sample of the food matrix without target sequence, which passes through all steps of the analytical process.

3.3.6 Positive PCR control³⁾, reaction containing the target DNA in a specified quantity or number of copies.

3.3.7 Positive process control, sample of the food matrix with known quantity of hazelnut, which passes through all steps of the analytical process.

3.3.8 External amplification control (inhibition control), control DNA that is added to an aliquot of the extracted nucleic acid in a specified quantity or number of copies and used in a separate reaction to check the influence of co-extracted substances from the sample matrix on the amplification.

-
- 1) Ready-to-use reagents or single components may be used as a PCR master mix, insofar as they provide comparable or better results.
 - 2) FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine; equivalent reporter dyes and/or quencher dyes may be used if they are shown to give comparable or better results.
 - 3) DNA for the positive PCR control is extracted from phenotypically identified pure hazelnuts as described in 5.3 and 5.4. DNA mass concentration is determined as described in 5.5.

4 Apparatus and equipment

General aspects are described in EN ISO 24276 [3].

Plastic and glass materials shall be sterilized and free of DNA before use. In addition, the use of aerosol protected filter tips is obligatory due to the high sensitivity of the PCR analytics and the resultant risk of DNA contamination. In addition to the usual laboratory facilities, the following equipment is required.

4.1 DNA extraction

- 4.1.1 Suitable reaction vials**, 1,5 ml and 2 ml, DNA-free.
- 4.1.2 50 ml centrifuge tubes**, sterile.
- 4.1.3 Thermostat or water bath**, preferably with shaker function.
- 4.1.4 Centrifuge**, suitable for centrifuging 50 ml centrifuge tubes at 8 000 g^4 .
- 4.1.5 Centrifuge**, suitable for centrifuging 1,5 ml and 2 ml reaction vials at 14 500 g .
- 4.1.6 Apparatus and/or material for grinding the sample**, e.g. blender or mill.
- 4.1.7 UV spectrometer or other detection instruments**, suitable for estimating the amount of DNA.

4.2 PCR

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- 4.2.1 Suitable PCR tubes**. [SIST-TS CEN/TS 15634-3:2016](#)
- 4.2.2 Microcentrifuge for PCR tubes**. <https://standards.iteh.ai/catalog/standards/sist/bab8aa17-f92e-45dd-8b51-30caa4218c76/sist-ts-cen/TS-15634-3-2016>
- 4.2.3 Real-time PCR equipment**, suitable for excitation and for emission measurement of fluorescence-marked oligonucleotides.

NOTE Laboratories participating in the interlaboratory study used the following real-time PCR equipment: Rotor Gene 2000™ or 3000™, Stratagene Mx 3005P, ABI PRISM® 7000 or 7500, ABI PRISM® 7700 or 7900HT and Roche LightCycler®.⁵⁾

5 Procedure

5.1 General

General aspects are described in EN ISO 24276 [3].

5.2 Sample preparation

Ensure, e.g. by milling or homogenizing, that the test sample is representative of the laboratory sample.

4) $g = 9,81 \text{ m} \cdot \text{s}^{-2}$

5) Rotor Gene 2000™ and 3000™, Stratagene Mx 3005P, ABI PRISM® 7000 and 7500, ABI PRISM® 7700 and 7900HT and Roche LightCycler® are examples of suitable products available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

5.3 DNA extraction with CTAB

Measures and work steps to be considered for the DNA extraction are described in EN ISO 21571 [2].

It is acceptable to use a commercially available kit instead of the DNA extraction procedure described below, if it is ensured that comparable or better results are obtained.

In parallel to the test samples, carry out the controls listed in 3.3.4, 3.3.5 and 3.3.7 adequately.

Prepare every sample **twice** in accordance with the following scheme:

- Weigh 2 g of the sample into 50 ml centrifuge tubes;
- Add 10 ml of CTAB extraction buffer solution (3.1.13);
- Add 30 µl of Proteinase K solution (3.1.15) and mix;
- Incubate and shake for 90 min at 65 °C;
- Centrifuge for 5 min at 6 000 *g* to 8 000 *g*;
- Place 500 µl of chloroform isoamyl alcohol mixture (3.1.12) in a 2 ml reaction vial;
- Add 700 µl of supernatant and mix thoroughly for 30 s;
- Centrifuge for 15 min at about 14 500 *g*;
- Place 500 µl of cold isopropanol (3.1.7) in a 1.5 ml reaction vial;
- Add 500 µl of supernatant (aqueous phase) and mix carefully;
- Incubate for 30 min at room temperature;
<https://standards.itech.ai/catalog/standards/sist/bab8aa17-f92e-45dd-8b51-30caa4518c70/sist-ts-cen-ts-15634-3-2016>
- Centrifuge for 15 min at about 14 500 *g*;
- Carefully remove and discard the supernatant;
- Fill the reaction vial with 500 µl of ethanol (3.1.2) and swirl the reaction vial several times;
- Centrifuge for 5 min at about 14 500 *g*;
- Carefully remove and discard the supernatant;
- Dry the extracted DNA;
- Dissolve the dried DNA extract in 100 µl of TE buffer solution (3.1.16).

5.4 DNA purification by means of solid phase extraction

Purify DNA extract according to the instructions given by the respective kit manufacturer.

The DNA extract can be stored at 4 °C for a short period. If storage times exceed more than one week, the DNA extracts should be stored at temperatures of -18 °C.