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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction

Produits alimentaires — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés — Extraction des acides nucléiques

Reference number
ISO 21571:2005(E)

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

ISO 21571 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with Technical Committee ISO/TC 34, *Food products*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

The search for genetically modified origin of ingredients is performed by means of the following successive (or simultaneous) steps. After sample collection, nucleic acids are extracted from the test portion. Extracted nucleic acids can be further purified, simultaneously or after the extraction process. Afterwards, they are quantified (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR). These steps are detailed in this and the following International Standards:

ISO 21568, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling.*

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.*

Further information about definitions and general items involving the steps cited above are collected in:

ISO 24276, *Foodstuffs — Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions.*

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of a patent concerning the silica-based extraction method (No. EP 0389063/USP 5,234,809) given in Clause A.4.

ISO takes no position concerning the evidence, validity and scope of this patent right.

The holder of this patent right has assured the ISO that he/she is willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statement of the holder of this patent right is registered with ISO. Information may be obtained from:

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights other than those identified above. ISO shall not be held responsible for identifying any or all such patent rights.

Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction

1 Scope

This International Standard provides general requirements and specific methods for DNA extraction/purification and quantitation. These methods are described in Annexes A and B.

This International Standard has been established for food matrices, but could also be applicable to other matrices, such as grains and feed.

It has been designed as an integral part of nucleic-acid-based analytical methods, in particular ISO 21569 on qualitative analytical methods, and ISO 21570 on quantitative analytical methods.

2 Normative references

The following referenced documents are indispensable for the application 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 24276:—[1)], *Foodstuffs — Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

ISO 21568, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Sampling*

3 Principle

3.1 General

The objective of nucleic acid extraction methods is to provide nucleic acids suitable for subsequent analysis (see ISO 24276).

NOTE The “quality” of DNA depends on the average length of the extracted DNA molecules, the chemical purity and the structural integrity of the DNA sequence and of the double helix (e.g. intra-, inter-strand linking between DNA bases, single-strand gaps, cross-linking with polyols, haemin, etc). Moreover, such alterations are often sequence-specific and consequently not randomly distributed all over the genome (see References [1], [2], [3] and [4]).

Users of this International Standard should note that some methods (e.g. all silica-based methods), might be covered by patent rights.

1) To be published.

3.2 DNA extraction

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.

DNA extraction/purification methods are described in Annex A. Method-selection is an experience-based choice of the user, taking into account the scope and examples of matrices as given in each method.

Alternative protocols are suitable provided that the method has been validated on the respective matrix under investigation.

3.3 DNA quantitation

Quantitation of extracted DNA could be useful for subsequent PCR analysis.

It may be performed by either physical (e.g. measure of absorbance at a specific wavelength), chemical-physical (e.g. use of intercalating or binding agents able to emit fluorescence), enzymatic (e.g. bioluminescence detection) methods or by quantitative PCR. The latter method is especially suitable for composite matrices or for samples with a low DNA content or whose DNA is degraded.

There are several methods available to quantify the DNA present in a solution, as described in Annex B. It is for the user to choose the most appropriate one to be applied, depending on the amount and quality of DNA to be quantified and, consequently, on the matrix from which the DNA has been extracted.

Alternative protocols are suitable, provided that the method has been validated on the respective matrix under investigation.

4 General laboratory requirements

Accidental contamination of DNA can originate from dust and spreading aerosols. As a consequence, the organization of the work area in the laboratory is logically based on

— the systematic containment of the methodological steps involved in the production of the results, and

— a “forward flow” principle for sample handling.

The latter ensures that the DNA to be analysed and the amplified DNA generated by PCR remain physically segregated.

Further details can be found in ISO 24276.

5 Procedure

5.1 Preparation of the test portion

5.1.1 General

Commodity-specific variables (e.g. humidity) and processing can impact the amount and quality of DNA extracted from the material under investigation. Therefore the performance characteristics of a given DNA extraction method depend on the matrix.

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 an LOD of 0,1 %) to allow a statistically valid conclusion to be made (see ISO 21568).

For practical/technical reasons, it is not recommended to exceed a size of 2 g.

Any restrictions that arise from the size of the test portion which prevent it from being representative shall be reported and taken into consideration in the interpretation of the analytical results. The methods for DNA extraction in Annex A describe test portions from 200 mg to 500 mg, which are usually adequate for DNA-rich raw materials (e.g. ground grains, flour). 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.

Storage of standards, samples and test portions shall comply with ISO 24276 and shall be organized in such a way as to preserve the biochemical parameters to be analysed (for details, see ISO/IEC 17025).

5.1.2 Samples

All operations for the preparation of test samples (e.g. grinding, homogenization, division, drying) shall be carried out in accordance with the procedures described in ISO 24276, taking care to prevent all contamination of the sample or modification of its composition.

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. In the case of non-homogenous products like raw oils, check that the sediments have been completely removed from the walls of the vessel.

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. In such a case, attention shall be paid to the particle size. The test portion subjected to extraction shall contain a minimum number of particles as specified in ISO 21568. Milling/grinding devices should be capable of being thoroughly cleaned and shall be selected in order to achieve the expected particle number and particle size distribution within the test portion as defined in ISO 21568.

If components of the laboratory sample have been removed prior to extraction, then such procedures shall be reported.

Final food products that are solid or paste and have high lipid contents are often not easy to grind to the desired particle size in a single step. Several procedures may therefore be added, such as lipid removal using hexane after intermediate grinding, freezing or freeze-drying before grinding.

In order to facilitate the grinding of paste or viscous products, it is possible to apply one of the following treatments to certain matrices:

— heating to a maximum temperature of 40 °C;

— dissolving in an appropriate liquid such as water;

— freezing at a temperature below or equal to −20 °C.

Homogenize the whole laboratory sample. Sample the two test portions, taking into account possible dilutions or concentrations.

During milling/grinding, precautions should be taken to ensure that the heating of the sample is kept to a minimum since heating can have a negative impact on the quality of the extracted DNA.

Milling/grinding techniques with a high risk of cross-contamination (such as the combined use of liquid nitrogen and mortar) shall be avoided as far as possible. As a rule of good practice, any dust-producing methodological step should be contained from all other analytical steps.

If salts, spices, powdered sugars and/or other substances that could potentially interfere with the extraction or analytical method are present, appropriate purification steps should be considered according to the selected method (see Annex A).

For example, in samples from composite matrices, the target matrix (e.g. the breading layer of fish sticks) can be isolated for DNA extraction.

5.2 DNA extraction/purification

5.2.1 General

The following considerations apply for the design of extraction methods.

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 the following:

— polysaccharides (pectin, cellulose, hemi-cellulose, starch, thickeners, etc.) using appropriate enzyme treatments (e.g. pectinase, cellulase, hemi-cellulase, α-amylase) or organic extraction (e.g. CTAB/chloroform);

— RNA and/or proteins using an appropriate treatment, such as enzymatic treatment by RNase and proteinase, respectively;

— the lipid fractions using for example enzyme treatments, or solvents (e.g. *n*-hexane);

— salts (e.g. from the extraction/lysis buffer, from the precipitation step) able to interfere with the subsequent analysis.

In particular for solid or dried samples, the volume of lysis/extraction buffer should be adapted to guarantee the DNA is dissolved.

NOTE 1 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.). Several DNA purification principles may be combined. If appropriate, extraction and purification can be performed within the same step.

Should a DNA co-precipitant such as glycogen, PEG or t-RNA be used to improve the DNA recovery during the precipitation steps, it should neither contain any detectable level of nuclease activity or PCR inhibitors/competitors, nor bear any sequence similarity with the potential PCR target under study. For genetically modified plants, a carrier DNA may be used (e.g. salmon or herring sperm DNA).

When using vacuum freeze dryers to dry the DNA pellets obtained after a precipitation step, the risk of cross contamination should be taken into account.

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

When setting up a new type of DNA extraction, or when applying one of the methods described in Annex A to a new matrix, the potential quality and integrity of the extracted DNA using the chosen protocol should be estimated by the following approach. A known quantity of a tracer DNA is added to the lysis buffer plus sample used for DNA extraction. When the chosen tracer is a predetermined amount of DNA or represents a predetermined number of copies of a particular DNA-sequence mixed to a matrix at start of DNA extraction, attention shall be paid to ascertain the lack of DNA sequence similarity between the tracer DNA and the target DNA sequence under study.

NOTE 2 The use of a tracer DNA is a good approximation to a real situation where DNA of a given matrix, complexed to other components (e.g. proteins) is expected. Such a method may also be used to estimate the presence of soluble and *trans*-acting PCR inhibitors in the extracted DNA (see ISO 24276, ISO 21569 and ISO 21570).

However, tracer DNA may give a misleading impression of recovery, since tracer DNA may be much easier to separate from matrix than the target DNA.

5.2.2 Controls

The controls to be included are described in Table 1 of ISO 24276:—. These should as a minimum include an extraction blank control and a positive extraction control, but may also include an environment control.

5.2.3 Control of DNA purity: Internal PCR control

When setting up a new type of extraction, the presence of PCR inhibitors in the extracted DNA may be estimated using DNA spikes (see ISO 24276, ISO 21569 and ISO 21570). The amount of added DNA shall not exceed the maximum level supported by PCR and shall contain a definite number of target sequence copies. This number should be determined individually for each target sequence and indicated as a multiple of the existing lower limit of detection. Ideally, the target concentration of the positive control PCR should correspond to the sensitivity needed in the analysis. Care shall be taken when using highly concentrated cloned target DNA. As far as possible, the positive controls shall conform to the conditions of the test material with regard to the nucleic acids they contain.

5.3 Quantitation of the extracted DNA

5.3.1 General

The quality, integrity and amount of the nucleic acid template influences the performance of the analytical method, and hence the analytical results obtained. The limit of detection of a specific method may therefore depend on the amount of nucleic acids used.

Quantitation of DNA is helpful

— to compare the efficiency of different DNA extraction protocols for a given matrix (repeatability), and

— to measure the concentration of nucleic acids prior to analysis.

5.3.2 Range of application

Each method of quantitation shall be applied within its dynamic range, also considering its level of precision.

5.3.3 Quantity standards

The accuracy of the quantitation methods depends on the nucleic acid standards used to calibrate the method.

If using a method that is sensitive to the size and/or quality of the nucleic acid fragments, then the nucleic acid standards that match the size and/or quality of the expected nucleic acid as extracted from the sample shall be used.

The reference material used should ensure traceability to stated references, usually national or international Standards, through an unbroken chain of comparison [see ISO Guide 30].

When a method using intercalating agents is employed, high molecular mass DNA standard should be used when high molecular mass DNA is to be quantified. Low molecular mass DNA should be used when low molecular mass DNA is to be quantified. High molecular mass nucleic acid usually also contains a certain amount of lower molecular mass fragments. This means that many methods for DNA quantitation suffer from a certain degree of inaccuracy, which should be taken into account.

NOTE Additionally, depending on the matrix and type of extraction method, a certain portion of the extracted DNA may be recovered as single-stranded DNA (with much poorer intercalation capacity), leading to an underestimation of the overall DNA content. In contrast, single-stranded DNA is equally well detected by physical measurements.

At least three points (preferably replicated) are required for the construction of a good calibration curve. The amount of standard DNA used for each calibration point depends on the sensitivity of the method and on the dynamic range under consideration.

5.4 Stability of extracted DNA

The DNA extracted shall be stored under such conditions that the stability is ensured to perform the subsequent analyses.

Repeated freezing and thawing of DNA solutions should be avoided.

6 Interpretation

The DNA extraction method employed shall be appropriate to obtain the quality and quantity of nucleic acid required for the subsequent analysis.

The quality of the extracted nucleic acid should be assessed using an analytical method that is influenced by the same quality parameters as the analysis to be performed on the extracted nucleic acid (e.g. if the analysis to be performed is PCR, then an additional PCR should be used for the assessment of the quality of the extracted DNA).

Further parameters for method compatibility can be found in ISO 21569, ISO 21570 and ISO 24276.

7 Test report

When issuing the final test report in accordance with ISO 24276, the following additional information to document the activity of the laboratory shall be included:

— a statement describing the derivation of the test portions, and any preliminary processing of the sample before nucleic acid extraction;

— the size of the test portions used for the nucleic acid extraction;

— the nucleic acid extraction method used;

— any special observations made during testing;

— any operation not specified in the method or considered to be optional but that can have an effect on the results;

— the interpretation of the results;

— the experimentor's name.

Handling and storage of raw data are described in ISO/IEC 17025 and related quality assurance schemes. Consistency should be achieved.

Annex A
(informative)

Methods for DNA extraction

A.1 Preparation of PCR-quality DNA using phenol/chloroform-based DNA extraction methods

A.1.1 Basic phenol/chloroform method

A.1.1.1 General

This routine method (see Reference [5]) is suitable for the extraction of DNA from a wide variety of matrices (see A.1.1.8).

Phenol is usually very suitable for nuclease destruction and protein denaturation.

When foliar or green material (e.g. chicory leaves, dried alfalfa) is investigated, many PCR inhibitors may also be co-precipitated together with DNA. For this reason, difficulties may be encountered in obtaining PCR-amplifiable DNA reproducibly.

The corrosive hazardous properties of phenol must be taken into consideration, thus the use of DNA extraction methods based on CTAB and/or PVP and/or silica adsorption are favoured as primary alternatives.

A.1.1.2 Validation status

The method has been widely applied in all areas of biology, agronomy and medicine, over the past 40 years, but has never been evaluated through interlaboratory studies for GMO detection in foodstuffs.

A.1.1.3 Principle

The method consists of a lysis step (thermal lysis in presence of sodium dodecyl sulfate and a high EDTA content) followed by the removal of contaminants (such as lipophylic molecules, polysaccharides and proteins) and nucleases from the DNA-containing aqueous phase using phenol and chloroform. A final DNA precipitation with ethanol concentrates the DNA and eliminates salts and residual chloroform. The critical step of the method is the lysis step [5].

A.1.1.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.1.1.5 Reagents

A.1.1.5.1 Ethanol, volume fraction ϕ (C_2H_5OH) = 96 %

Store and use at −20 °C.

A.1.1.5.2 Glacial acetic acid (CH_3COOH).

A.1.1.5.3 Potassium acetate ($C_2H_3O_2K$).

A.1.1.5.4 Hydrochloric acid, ϕ(HCl) = 37 %.

A.1.1.5.5 Isoamyl alcohol [$(CH_3)_2CHCH_2CH_2OH$].

A.1.1.5.6 Phenol (C_6H_5OH).

A.1.1.5.7 Chloroform ($CHCl_3$).

A.1.1.5.8 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.1.1.5.9 Ethylenediaminetetraacetic acid dipotassium salt (K_2EDTA) ($C_{10}H_{14}N_2O_8K_2$).

A.1.1.5.10 Potassium hydroxide (KOH).

A.1.1.5.11 Potassium chloride (KCl).

A.1.1.5.12 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.1.1.5.13 Proteinase K, approximately 20 Units/mg lyophilisate.

A.1.1.5.14 RNase-A, DNase-free, from bovine pancreas, approximately 50 Kunitz Units/mg of lyophilisate.

A.1.1.5.15 Equilibrated phenol, pH $>$ 7,8.

Use phenol equilibrated against extraction buffer (A.1.1.5.18) without SDS, or prepared according to Reference [5], or according to the manufacturers recommendations.

A.1.1.5.16 Chloroform-isoamyl alcohol

Mix 24 volume parts of chloroform (A.1.1.5.7) with 1 volume part of isoamyl alcohol (A.1.1.5.5).

A.1.1.5.17 Phenol-chloroform-isoamyl alcohol

Mix 1 volume part of equilibrated phenol (A.1.1.5.15) with 1 volume part of the chloroform-isoamyl alcohol solution (A.1.1.5.16).

A.1.1.5.18 Extraction/lysis buffer, substance concentration c(Tris) = 0,050 mol/l, c(K_2EDTA) = 0,050 mol/l, mass concentration ρ(SDS) = 30 g/l.

Adjust the pH to 8,0 with HCl or KOH.

A.1.1.5.19 TE buffer, c(Tris) = 0,010 mol/l, c(K_2EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or KOH.

A.1.1.5.20 Proteinase-K solution, ρ = 20 mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.1.1.5.21 RNase-A solution, ρ = 10 mg/ml lyophylisate.

Store at −20 °C, but avoid repeated freezing and thawing.

A.1.1.5.22 Ethanol solution, ϕ (C_2H_5OH) = 70 %.

Store and use at −20 °C.

A.1.1.5.23 Potassium acetate solution, c($C_2H_3O_2K$) = 3 mol/l.

Adjust the pH to 5,2 with glacial acetic acid. Do not autoclave. If necessary, filter through a 0,22 µm filter.

A.1.1.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.1.1.6.1 Centrifuge, capable of achieving a minimum acceleration of 10 000 g.

In some steps a refrigerated centrifuge is required.

A.1.1.6.2 Water bath or incubator, working in a temperature range from 60 °C to 70 °C.

A.1.1.6.3 Vacuum dryer (optional).

A.1.1.6.4 Freeze dryer (optional).

A.1.1.6.5 Mixer, e.g. Vortex®[2)]

A.1.1.6.6 Reaction vessels, resistant to freezing in liquid nitrogen.

A.1.1.7 Procedure

A.1.1.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.1.1.7.2 Extraction procedure

Weigh 0,25 g of the test sample into a microtube.

Add 1,6 ml of extraction buffer (A.1.1.5.18) and, when necessary (e.g. in protein-rich matrices), 50 µl of proteinase K solution (A.1.1.5.20.) Incubate at 60 °C to 70 °C, usually for between 30 min to 2 h (overnight incubation is also possible). Add RNase A (A.1.1.5.21) up to a final concentration of 0,1 µg/ml. Centrifuge at 5 000 g for 30 min and recover the supernatant in a fresh tube. Add 1 volume of equilibrated phenol (A.1.1.5.15) to the supernatant, then mix gently and thoroughly. Centrifuge at 5 000 g for 15 min and recover the upper aqueous phase in a fresh tube. Add 1 volume of phenol-chloroform isoamyl alcohol (A.1.1.5.17) to the supernatant, then mix gently and thoroughly. Centrifuge at 5 000 g for 15 min and recover the aqueous phase in a fresh tube. Repeat this step once or more times (depending on the matrix) until the interface between the phases is clean.

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2) Vortex is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Add 1 volume of chloroform/isoamyl alcohol (A.1.1.5.16) to the supernatant, then mix gently and thoroughly. Centrifuge at 5 000 g for 10 min and recover the upper aqueous phase in a fresh tube. Repeat, if necessary, until the interface between the phases is clear. Mix the supernatant with 0,1 volume of potassium acetate solution (A.1.1.5.23.) and 2,5 volumes of 96 % ethanol (A.1.1.5.1), then mix thoroughly by inversion. Incubate for at least 5 min in liquid nitrogen, or 1 h at −80 °C, or overnight at −20 °C. Centrifuge at 10 000 g (or up to 13 000 g) at 4 °C for at least 15 min, then carefully discard the supernatant.

Carefully wash the DNA pellet with 2 volumes of 70 % ethanol solution (A.1.1.5.22). Centrifuge at 10 000 g to 13 000 g at 4 °C for 15 min, then discard carefully the supernatant. This step is essential for the removal of the precipitating salts that could interfere with the subsequent analysis (e.g. PCR).

Dry the pellet and re-dissolve it in 100 µl of water or appropriate buffer, e.g. TE buffer (A.1.1.5.19). This is the DNA master stock. Add RNase-A (A.1.1.5.21) up to a final concentration of 0,1 µg/ml.

A.1.1.8 List of examples

The method has been successfully applied to extract DNA[3)] from the following matrices:

acidified soya beans[3)], dehydrated alfalfa, baby biscuits[3)], baby milk[3)], bacteria and spores thereof, barley seeds, beef/pork paté[3)], beer[3)], blue cheese, brownies[3)], canned corn, carrot seeds, cereal bars[3)], cheese, chicken nuggets, chicory leaves, chicory roots, chocolate cookies[3)], chocolate paste[3)], cinnamon cookies[3)], compotes, cornflakes[3)], cracked rice, dessert cream[3)], dried pea seeds, maize biscuits[3)], maize feeding oil cakes, maize flour, maize gluten feed, maize seeds, manioc hard pellets for feed, manioc tapioca meat, fresh and cooked[3)] meats (beef, pork, chicken and turkey), melon fruit pulp, melon seeds, minced meat, muesli ingredients[3)], muesli[3)], mung bean sprouts[3)], oat seeds, potato tuber, rapeseed feeding oil cake, glupacolza, rapeseed seeds, sausages (slicing[3)] and cocktail[3)] (see A.1.2 for an improved extraction method), schnitzel, sooie hoi sin[3)], soup balls, soya protein in meat preparations[3)], soya lecithin (raw brown and yellow refined[3)]), soya sprouts[3)], soya drinks, soya beans, soya bean cream, soya bean feeding oil cake, soya bean tofu, spaghetti sauce[3)], spelt seeds, sugar beet (dried pulp), sugar beet (fresh root), sugar beet seeds, sunflower seeds, tofu, tomato fresh fruit, tomato purée[3)], tomato seeds, vegetarian hamburger, waffles (with[3)] and without[3)] chocolate), wheat bran, wheat flour, wheat gluten feed, wheat seeds, wheat semolina, yoghurt[3)] (see A.1.3 for an improved extraction method).

A.1.2 Phenol/chloroform method: Protocol for starter cultures of fermented sausages

A.1.2.1 General

This method is designed to isolate the total DNA, including bacterial genomic DNA, from sausages. The applicability of the method to obtain DNA of high quality suitable for the specific detection of recombinant DNA by PCR has been demonstrated for fermented sausages[6] as well as for thermally treated fermented sausages, so-called summer sausages[7]. In addition, the extraction method was shown to be suitable to isolate total DNA from cream to detect specifically *Staphylococcus aureus* in this food matrix[8]. (For a summary of the matrices for which the method is suitable, see A.1.2.8.)

A.1.2.2 Validation status

This method has been validated by an interlaboratory study on a test portion of 0,4 g (see A.1.2.9).

3) Repeatability may depend on the batch of the matrix and/or to its production technology. In some cases, DNA could not be found or was degraded in such a way that PCR results were below the limit of detection of the method, irrespective of the PCR primers/protocols used. This may be a source of low reproducibility between laboratories.

A.1.2.3 Principle

The method consists of the recovery of bacterial cells from the food matrix by homogenization of the sausage samples, followed by a centrifugation step. The sediment contains not only bacterial cells but also meat particles. For specific lysis of the bacterial cells, the cell walls are degraded by addition of lysozyme. To improve the degradation of cell walls of meat lactobacilli, mutanolysin can be added. Complete lysis of the cells is performed by addition of the detergent SDS (sodium dodecyl sulfate) and proteinase-K, followed by several extractions of the aqueous phase with phenol and/or chloroform. The phenol/chloroform extraction step is important to eliminate any nuclease activities and PCR-inhibiting substances including those arising from the food matrix (e.g. haematin). A final precipitation of DNA by ethanol is performed.

A.1.2.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.1.2.5 Reagents

A.1.2.5.1 Isopropanol [$CH_3CH(OH)CH_3$].

A.1.2.5.2 Ethanol, $\varphi(C_2H_5OH)$ = 96 %.

Store and use at −20 °C.

A.1.2.5.3 Glacial acetic acid (CH_3COOH).

A.1.2.5.4 Hydrochloric acid, ϕ(HCl) = 37 %.

A.1.2.5.5 Sodium hydroxide (NaOH).

A.1.2.5.6 Isoamyl alcohol [$(CH_3)_2CHCH_2CH_2OH$)].

A.1.2.5.7 Phenol (C_6H_5OH).

A.1.2.5.8 Chloroform ($CHCl_3$).

A.1.2.5.9 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.1.2.5.10 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.1.2.5.11 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.1.2.5.12 Lysozyme

50 000 U/mg protein (1 U will produce a ΔA_{450} of 0,001 per minute at pH 6,24 and 25 °C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2,6 ml reaction mixture using a 1 cm light path).

A.1.2.5.13 Sucrose ($C_{12}H_{22}O_{11}$).

A.1.2.5.14 Proteinase-K, approximately 20 Units/mg lyophilisate.

A.1.2.5.15 Sodium acetate ($C_2H_3O_2Na$).

A.1.2.5.16 Equilibrated phenol, saturated with Tris/HCl (pH > 7,8) buffer, or prepared according to Reference [5], or according to the manufacturer's recommendations.

A.1.2.5.17 Chloroform-isoamyl alcohol

Mix 24 volume parts of chloroform (A.1.2.5.8) with 1 volume part of isoamyl alcohol (A.1.2.5.6).

A.1.2.5.18 Phenol-chloroform-isoamyl alcohol

Prepare by mixing 25 volume parts of equilibrated phenol (A.1.2.5.16) with 24 volume parts of chloroform (A.1.2.5.8) and 1 volume part of isoamyl alcohol (A.1.2.5.6).

A.1.2.5.19 Mutanolysin-solution, containing 500 U/ml or 5 000 U/ml mutanolysin, dissolved in sterile water.

Do not autoclave. Store at –20 °C, but avoid repeated freezing and thawing.

A.1.2.5.20 Lysozyme solution, containing 10 mg/ml, dissolved in sterile water.

Do not autoclave. Store at –20 °C, but avoid freezing and thawing.

A.1.2.5.21 Sucrose solution, $\rho(C_{12}H_{22}O_{11})$ = 400 g/l.

A.1.2.5.22 Buffer solution A, c(Tris) = 0,020 mol/l, $c(Na_2EDTA)$ = 0,020 mol/l, c(NaCl) = 0,1 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.1.2.5.23 Extraction/lysis buffer, containing one volume part of buffer solution A (A.1.2.5.22) and one volume part of sucrose solution (A.1.2.5.21).

A.1.2.5.24 SDS solution, ρ(SDS) = 250 g/l.

A.1.2.5.25 Proteinase-K solution, containing 20 mg/ml, stored at –20 °C, dissolved in sterile water.

Do not autoclave. Store at – 20 °C, but avoid repeated freezing and thawing.

A.1.2.5.26 Ethanol solution, $\phi(C_2H_5OH)$ = 70 %.

Store and use at –20 °C.

A.1.2.5.27 Sodium acetate solution, $c(C_2H_3O_2Na)$ = 3 mol/l.

Adjust the pH to 5,2 with glacial acetic acid.

A.1.2.5.28 TE buffer, c(Tris) = 0,010 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.1.2.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.1.2.6.1 Instrument and/or material for chopping tissue (e.g. scalpel).

A.1.2.6.2 Centrifuge, capable of achieving an acceleration of 12 000 g.

In some steps a refrigerated centrifuge is required.

A.1.2.6.3 Water bath or incubator.

A.1.2.6.4 Vacuum dryer (optional).

A.1.2.6.5 Mixer, e.g. Vortex®[2)].

A.1.2.7 Procedure

A.1.2.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.1.2.7.2 Sample preparation

Chop the meat sausage, homogenize it and add 200 mg to 500 mg to 3 volumes of water (up to 1,5 ml). Keep at room temperature for about 10 min.

A.1.2.7.3 Extraction procedure

Carefully transfer 500 µl of the aqueous phase (suspension) to a new reaction vessel. Centrifuge for 10 min at 12 000 *g*.

Discard the supernatant and re-suspend the pellet in 500 µl of extraction/lysis buffer (A.1.2.5.23).

Add 50 µl of lysozyme solution (A.1.2.5.20). Incubate at 37 °C for 1 h. If the results are not satisfactory, lysozyme may be combined with 10 U of mutanolysin (A.1.2.5.19), but the matrix-specific effect of this should be tested prior to routine application.

Add 25 µl of SDS solution (A.1.2.5.24) and 25 µl of proteinase-K solution (A.1.2.5.25), then incubate for 10 min at 60 °C.

Add 1 volume of phenol/chloroform/isoamyl alcohol (A.1.2.5.18) and mix.

Centrifuge the mixture for 3 min at about 12 000 *g*. Transfer the upper aqueous phase to a new vial.

Add 1 volume of chloroform-isoamyl alcohol (A.1.2.5.17) and mix.

Centrifuge for 3 min at about 12 000 *g*. Transfer the upper phase to a new reaction vessel.

Add 0,1 volume of sodium acetate solution (A.1.2.5.27) and 1 volume of isopropanol (A.1.2.5.1). Mix gently several times by inversion.

Keep at room temperature for at least 30 min. Centrifuge for 15 min at about 12 000 *g*. Decant the supernatant and discard it.

Wash the pellet carefully with at least 500 µl of ethanol solution (A.1.2.5.26), shaking gently. Centrifuge the mixture for 10 min at about 12 000 *g*. This step is essential for the removal of the precipitating salts that could interfere with the subsequent analysis (e.g. PCR).

Discard the supernatant.

Dry the pellet and re-dissolve it in 100 µl of water or appropriate buffer, e.g. TE buffer (A.1.2.5.28). This is the DNA master stock.

A.1.2.8 List of examples

See Table A.1.

Table A.1 — List of matrices for which the method has been successfully applied

Matrices successfully analysed	Microorganism	Reference
Fermented sausage	*Lactobacillus curvatus*	[6]
Summer sausage (thermally treated)	*Lactobacillus curvatus*	[7]
Cream	*Staphylococcus aureus*	[8]

A.1.2.9 Validation

The validation data in Table A.2 have been elaborated in a collaborative study carried out by the working group “Development of methods for identifying foodstuffs produced by genetic engineering techniques” of the Commission of the former German Federal Health Board for the implementation of methods according to article 35 of the German Foodstuffs Act[6].

In this collaborative study, two samples were false positive, probably caused by incorrect packing. Mutanolysin was not used for this study.

Table A.2 — Validation data

Number of laboratories participating	Number of sausage samples per laboratory	Number of total samples	Number of samples correctly identified
15	10	150	148 (control samples and GMO samples)

A.1.3 Phenol/chloroform method: Protocol for starter cultures of yoghurt

A.1.3.1 General

This method describes a procedure for the extraction of bulk DNA from starter cultures used for the fermentation of the milk product yoghurt. This procedure was successfully carried out with plain yoghurts, yoghurts containing different ingredients such as fruits, additives and stabilizers and with products of different fat content (see A.1.3.8 and References [9], [10], [11]). DNA of PCR quality was also extracted from yoghurts that were thermally treated [12].

A.1.3.2 Validation status

This method has been validated in an interlaboratory study; see A.1.3.9.

A.1.3.3 Principle

This method is basically a phenol-chloroform extraction procedure that is adapted for a special food matrix. The Gram-positive starters of the yoghurts are harvested after resolving the coagulated casein at alkaline pH. The recovered cells are re-suspended in a buffered aqueous solution and treated with lysozyme (and mutanolysin) in order to damage the cell walls. Cell lysis is carried out by adding an ionic detergent such as sodium dodecylsulfate (SDS). Proteins are removed by proteinase-K treatment and several subsequent phenol/chloroform and chloroform steps. DNA is finally precipitated with alcohol.

A.1.3.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.1.3.5 Reagents

A.1.3.5.1 Isopropanol [$CH_3CH(OH)CH_3$].

A.1.3.5.2 Ethanol, $\phi(C_2H_5OH)$ = 96 %.

Store and use at −20 °C.

A.1.3.5.3 Glacial acetic acid (CH_3COOH).

A.1.3.5.4 Sodium chloride (NaCl).

A.1.3.5.5 Sodium citrate ($C_6H_5Na_3O_7$).

A.1.3.5.6 Hydrochloric acid, ϕ(HCl) = 37 %.

A.1.3.5.7 Sodium hydroxide (NaOH).

A.1.3.5.8 Isoamyl alcohol [$(CH_3)_2CHCH_2CH_2(OH)$].

A.1.3.5.9 Phenol (C_6H_5OH).

A.1.3.5.10 Chloroform ($CHCl_3$).

A.1.3.5.11 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.1.3.5.12 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.1.3.5.13 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.1.3.5.14 Lysozyme, 50 000 U/mg protein (1 U will produce a ΔA_{450} of 0,001 per minute at pH 6,24 and 25 °C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2,6 ml reaction mixture with 1 cm light path).

A.1.3.5.15 Sucrose ($C_{12}H_{22}O_{11}$).

A.1.3.5.16 Proteinase-K, approximately 20 Units/mg lyophilisate.

A.1.3.5.17 Sodium acetate ($C_2H_3O_2Na$).

A.1.3.5.18 Sodium citrate solution, $\rho(C_6H_5Na_3O_7)$ = 400 g/l.

A.1.3.5.19 Sodium hydroxide solution, c(NaOH) = 0,4 mol/l.

Dissolve in sterile water, but do not autoclave. Prepare freshly before use.

A.1.3.5.20 Sodium chloride/sodium citrate solution (SSC, concentrated 5×), c(NaCl) = 0,75 mol/l, $c(C_6H_5Na_3O_7)$ = 0,075 mol/l.

It is advisable to prepare the SSC solution as a concentrated stock solution (e.g. SSC 20×), because solutions with a high salt concentration are usually more stable. Dilute before use.

A.1.3.5.21 Equilibrated phenol, adjusted to pH 8, saturated with Tris/HCl (pH > 7,8) buffer, or for example, prepared according to Reference [5], or according to the manufacturer's recommendations.

A.1.3.5.22 Chloroform-isoamyl alcohol

Mix 24 volume parts of chloroform (A.1.3.5.10) with 1 volume part of isoamyl alcohol (A.1.3.5.8).

A.1.3.5.23 Phenol-chloroform-isoamyl alcohol

Prepare by mixing 25 volume parts of equilibrated phenol (A.1.3.5.21) with 24 volume parts of chloroform (A.1.3.5.10) and 1 volume part of isoamyl alcohol (A.1.3.5.8).

A.1.3.5.24 Mutanolysin solution, containing 500 U/ml or 5 000 U/ml mutanolysin, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.1.3.5.25 Lysozyme solution, containing 10 mg/ml of lysozyme, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid freezing and thawing.

A.1.3.5.26 Sucrose solution, $c(C_{12}H_{22}O_{11})$ = 400 g/l.

A.1.3.5.27 Buffer solution A, c(Tris) = 0,020 mol/l, $c(Na_2EDTA)$ = 0,020 mol/l, c(NaCl) = 0,100 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.1.3.5.28 Extraction/lysis buffer, containing 1 volume part of buffer solution A (A.1.3.5.27) and 1 volume part of sucrose solution (A.1.3.5.26).

A.1.3.5.29 SDS Solution, ρ(SDS) = 250g/l.

A.1.3.5.30 Proteinase-K solution, containing 20 mg/ml, dissolved in sterile water.

Do not autoclave. Store at –20 °C, but avoid repeated freezing and thawing.

A.1.3.5.31 Ethanol solution, $\phi(C_2H_5OH)$ = 70 %.

Store and use at −20 °C.

A.1.3.5.32 Sodium acetate solution, $c(C_2H_3O_2Na)$ = 3 mol/l.

Adjust the pH to 5,2 with glacial acetic acid.

A.1.3.5.33 TE buffer, c(Tris) = 0,010 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.1.3.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.1.3.6.1 Centrifuge, capable of achieving an acceleration of 12 000 g. In some steps a refrigerated centrifuge is required.

A.1.3.6.2 Water bath or incubator.

A.1.3.6.3 Vacuum dryer (optional).

A.1.3.6.4 Mixer, e.g. Vortex®[2)].

A.1.3.7 Procedure

A.1.3.7.1 General

Once the matrix test portion is prepared, apply the following DNA extraction/purification protocol. Some scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.1.3.7.2 Extraction procedure

Shake the yoghurt well. Transfer 250 µl of yoghurt to a 2-ml reaction vessel. Add 80 µl of sodium citrate solution (A.1.3.5.18). Add 150 µl of NaOH solution (A.1.3.5.19) and mix well. Centrifuge at about 12 000 g for 2 min.

The pellet should not be much bigger than 0,7 cm in diameter and about 100 µl in volume. Otherwise these steps (adding 80 µl of sodium citrate solution and 150 µl of NaOH) should be repeated.

Discard the upper layer of fat and the aqueous supernatant and re-suspend the pellet in 500 µl of 5× SSC solution (A.1.3.5.20). Centrifuge for at least 2 min at about 12 000 g and discard the supernatant. Resuspend the pellet in 500 µl of 5× SSC solution. Centrifuge for 2 min at about 12 000 g and discard the supernatant.

Resuspend the pellet in 500 µl of extraction/lysis buffer (A.1.3.5.28). Add 50 µl of lysozyme solution (A.1.3.5.25). Incubate at 37 °C for 1 h. If results are not satisfying, lysozyme may be combined with 10 U of mutanolysin (A.1.3.5.24), but the matrix-specific effect of this should be tested prior to routine application.

Add 25 µl of SDS solution (A.1.3.5.29) and 25 µl of proteinase-K solution (A.1.3.5.30). Incubate for 10 min at 60 °C. Add 500 µl of phenol/chloroform/isoamyl alcohol (A.1.3.5.23) and mix. Centrifuge for 3 min at about 12 000 g. Transfer the upper aqueous phase to a new reaction vessel. Add 1 volume of chloroform/isoamyl alcohol (A.1.3.5.22) and mix. Centrifuge for 3 min at about 12 000 g.

Transfer the upper phase to a new reaction vessel. Add 0,1 volume of sodium acetate solution (A.1.3.5.32) and 1 part by volume of isopropanol (A.1.3.5.1). Keep for at least 30 min at room temperature. Centrifuge for 15 min at about 12 000 g. Discard the supernatant. Wash the pellet carefully in at least 500 µl of ethanol solution (A.1.3.5.31). Centrifuge for 10 min at about 12 000 g. This step is essential for the removal of the precipitating salts that could interfere with the subsequent analysis (e.g. PCR). Discard the supernatant.

Dry the pellet and re-dissolve it in 100 µl of water or appropriate buffer, e.g. TE buffer (A.1.2.5.33). This is the "DNA master stock".

A.1.3.8 List of examples

See Table A.3.

Table A.3 — List of matrices for which the method has been successfully applied

Matrices successfully analysed	Contents, additives etc.	Micro-organism	Reference
Plain yoghurt	0,3 % fat, 3,5 % fat	*Streptococcus thermophilus*	[9], [11]
Fruit yoghurts	1,5 % fat, modified starch, hazelnuts, gelatine 1,5 % fat, 3,8 % protein, aspartam, acesulfam, pineapple 3,5 % fat, flavour, gelatine, peach, coconut 10 % fat, modified starch, lemon, flavour, almonds, pectin, carotin, riboflavin	*Streptococcus thermophilus*	[9]
Thermally treated plain yoghurt	3,5 % fat	*Streptococcus thermophilus*	[12]

A.1.3.9 Validation

The validation data in Table A.4 have been elaborated in a collaborative study carried out by the working group “Development of methods for identifying foodstuffs produced by genetic engineering techniques” of the Commission of the former German Federal Health Board for the implementation of methods according to article 35 of the German Foodstuffs Act [11].

At this collaborative trial, two laboratories did not carry out the verification by hybridization. *Mutanolysin* was not used for the trial.

Table A.4 — Validation data

Number of laboratories participating	Number of yoghurt samples per laboratory	Number of total samples	Number of samples correctly identified
20	10	200	200 (99 control samples and 101 GMO samples)

A.1.4 Phenol/chloroform method: Protocol for yeasts and/or filamentous fungi harvested from foodstuffs

A.1.4.1 General

This method describes a one-step extraction and purification of PCR-quality DNA from yeast or filamentous fungi[13], or isolated microbial populations. It is well suited for the tracing of the DNA from genetically modified microorganisms in highly complex matrices[14], [15]. It can be used either to extract total DNA from the matrix[14], [15], or from the microbial fraction either directly segregated from a matrix or harvested from starter cultures (liquid or agar colonies).

NOTE Separation of the microbial fraction from the test portion yields the most reliable results if done prior to DNA extraction.

Total DNA from the matrix may also be extracted by the alternative protocols of Annex A. However, these protocols do not guarantee that DNA from all microorganisms (especially from lysis-resistant fungi, or Gram-negative bacteria) would be randomly isolated with a reliable yield. Although the present protocol may be used for total DNA extraction in matrices such as yoghurt, milk or cheese, the extraction efficiency has been shown to be reliable for extensively ground or milled solid matrices only and shall always be checked on a matrix basis.

A.1.4.2 Validation status

This DNA extraction method has been applied and validated[13] for 25 fungal genera representing 325 species (including yeasts used for bakery or wine production, and *Penicillia* spp.[16] used by blue cheese producers) under mycelium or spore forms (among which the most breakage- or lysis-resistant species such as *Aspergillus fumigatus* and *Cryptococcus neoformans*). The method has been designed to avoid inter-portion and laboratory contamination making it suitable for large-scale routine DNA extractions and PCR applications. No variation in template quality in qualitative PCR was observed after long-term storage at −20 °C after up to 5 years, or between different DNA preparations from the same organism. Although the quality of the DNA is suitable for qualitative PCR, it can be poorly digestible with some restriction enzymes. For purposes such as quantitative PCR, the DNA obtained with the present method shall be further purified using another biochemical principle such as the one described in A.4 as an example.

This method applied to microbial pellets or mycelium mats has been submitted for interlaboratory validation exercises [17].

A.1.4.3 Principle

Basically, bacteria, yeasts or mycelia are disrupted and the DNA is simultaneously extracted by high-speed agitation in the presence of glass beads in a mixture of Tris-phenol-chloroform-EDTA-SDS followed by precipitation with ethanol.

A.1.4.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.1.4.5 Reagents

A.1.4.5.1 Ethanol, $\phi(C_2H_5OH) = 96$ %.

Store and use at −20 °C.

A.1.4.5.2 Glacial acetic acid (CH_3COOH).

A.1.4.5.3 Sulfuric acid, $\phi(H_2SO_4) > 90$ %.

A.1.4.5.4 Potassium bicarbonate ($KHCO_3$).

A.1.4.5.5 Potassium acetate ($C_2H_3O_2K$).

A.1.4.5.6 Hydrochloric acid, $\phi(HCl) = 37$ %.

A.1.4.5.7 Isoamyl alcohol [$(CH_3)_2CHCH_2CH_2OH$].

A.1.4.5.8 Phenol (C_6H_5OH).

A.1.4.5.9 Chloroform ($CHCl_3$).

A.1.4.5.10 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.1.4.5.11 Ethylenediaminetetraacetic acid dipotassium salt (K_2EDTA) ($C_{10}H_{14}N_2O_8K_2$).

A.1.4.5.12 Potassium hydroxide (KOH).

A.1.4.5.13 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.1.4.5.14 RNase-A, DNase-free, from bovine pancreas, approximately 50 Kunitz Units/mg lyophilisate.

A.1.4.5.15 Equilibrated phenol, adjusted to pH > 7,8.

Phenol (A.1.4.5.8) prepared according to, for example, Reference [5] and (optional) finally equilibrated against extraction buffer (A.1.4.5.18) without SDS, or according to the manufacturer's recommendations.

A.1.4.5.16 Chloroform-isoamyl alcohol

Mix 24 volume parts of chloroform (A.1.4.5.9.) with 1 volume part of isoamyl alcohol (A.1.4.5.7).

A.1.4.5.17 Phenol-chloroform-isoamyl alcohol

Prepare by mixing 1 volume part of equilibrated phenol (A.1.4.5.15) with 1 volume part of chloroform-isoamyl alcohol (A.1.4.5.16).

A.1.4.5.18 Extraction/lysis buffer, c(Tris) = 0,050 mol/l, c(K_2EDTA) = 0,050 mol/l, ρ(SDS) = 30 g/l.

Adjust the pH to 8,0 with HCl or KOH.

A.1.4.5.19 TE buffer, c(Tris) = 0,010 mol/l, c(K_2EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or KOH.

A.1.4.5.20 RNase-A solution, ρ(RNase-A) = 10 mg/ml lyophylisate.

Store at −20 °C.

A.1.4.5.21 Ethanol solution, ϕ(C_2H_5OH) = 70 %.

Store and use at −20 °C.

A.1.4.5.22 Potassium acetate solution, c($C_2H_3O_2K$) = 3 mol/l.

Adjust to pH 5,2 with glacial acetic acid. Do not autoclave. If necessary, filter through a 0,22 µm filter.

A.1.4.5.23 Conditioned glass beads

Incubate glass beads of 0,2 mm to 0,5 mm diameter overnight in concentrated H_2SO_4 (A.1.4.5.3). Wash them with autoclaved water, boil in $KHCO_3$ solution (A.1.4.5.24), wash again with autoclaved water, and dry at 80 °C under vacuum.[13], [14]

A.1.4.5.24 Potassium bicarbonate solution, ρ($KHCO_3$) = 50 g/l.

Prepare freshly in water.

A.1.4.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.1.4.6.1 Cell disruptor, for 2 ml screw-capped polyethylene microtubes, with a beating frequency of at least 100 beats/min [e.g. Mini-BeadBeater™ 4)].

A.1.4.6.2 Microtubes, O-ring-secured, screw-capped 2 ml polyethylene tubes.

A.1.4.6.3 Filtering device, for 25 mm diameter-glass fibre filters.

A.1.4.6.4 Centrifuge, capable of holding 2 ml microtubes at a minimum acceleration of 10 000 g.

In some steps a refrigerated centrifuge is required.

A.1.4.6.5 Water bath or incubator.

A.1.4.6.6 Vacuum dryer (optional). It is recommended for the preparation of item A.1.4.5.23.

A.1.4.6.7 Mixer, e.g. Vortex® 2).

A.1.4.7 Procedure

A.1.4.7.1 Preparation of the test portion and the microbial fraction

Protocols for the isolation of the microbial fraction, occasionally coupled to an enrichment step on agar or in liquid media for food microbiological quality controls, can be exploited for further DNA extraction from the microbial population, being defined as an ingredient.

Starting from 1 ml to 2 ml of the laboratory sample as standard portion, the microbial population is isolated appropriately (see A.1.3). Alternatively, yeasts or filamentous fungi isolated from the test portion may be grown as starter cultures. In both cases, the microorganisms are harvested and further processed according to A.1.4.7.2 or stored at −20 °C until processing is carried out.

A.1.4.7.2 DNA extraction

A.1.4.7.2.1 For mycelia collected on glass fibre filters, wash the mycelial mat twice with lysis buffer (A.1.4.5.18) not containing SDS. Peel off the fungal mat from the filter and transfer it to a 2 ml screw-capped microtube (A.1.4.6.2) containing conditioned glass beads (A.1.4.5.23) (half a tube), 600 µl of lysis buffer (A.1.4.5.18) and 600 µl of phenol-chloroform-isoamyl alcohol (A.1.4.5.17). Further processing is described in A.1.4.7.2.3.

A.1.4.7.2.2 For pellets of either total microbial population, bacteria, mycelium, yeasts or yeast-like micro-organisms, wash the cells once with 1 ml of lysis buffer (A.1.4.5.18) not containing SDS, centrifuge at 10 000 g to 13 000 g for 10 min, repeat at least once then resuspend in 600 µl of lysis buffer (A.1.4.5.18) and transfer to a microtube containing glass beads and phenol-chloroform as in A.1.4.7.2.1.

A.1.4.7.2.3 After step A.1.4.7.2.1 or A.1.4.7.2.2, agitate the microtube at least at 100 beats/min on a cell-disrupter (A.1.4.6.1) for 1 min to 2 min, then incubate immediately at 65 °C for 30 min to 120 min. Centrifuge at 10 000 g to 13 000 g for 10 min. Transfer the supernatant to a new microtube.

Optionally for further quantitative PCR, after 30 min of incubation, centrifuge at 10 000 g to 13 000 g for 15 min. Transfer the supernatant into a common microtube, add RNase-A (A.1.4.5.20) at a final concentration of 0,001 mg/ml and incubate further for 30 min to 90 min at 65 °C.

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4) Mini-BeadBeater is an example of a suitable product available commercially from Biospec products. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Add potassium acetate solution (A.1.4.5.22) at 0,3 mol/l final concentration. Mix, add 1,2 ml of ethanol (A.1.4.5.1) and incubate at −20 °C overnight or for 1 h at −80 °C. Pellet the DNA at 10 000 g to 13 000 g for 15 min at 4 °C.

Wash carefully the pelleted DNA with ethanol solution (A.1.4.5.21). Drain the microtube upside down on paper and dry the microtube under vacuum. Dissolve the DNA in 50 µl to 100 µl of water. Long-term (up to 5 years) storage at −20 °C is permitted. The use of water instead of TE buffer (A.1.4.5.19) has been validated [13]. This is the DNA master stock.

A.1.4.8 List of examples

The number of species/strains investigated is indicated within brackets:

Absidia corymbifera (1), *Acremonium* spp. (2), *Aspergillus* spp. (119), *Candida* spp. (7), *Cladosporium* spp. (2), *Cryptococcus* spp. (6), *Epidermophyton floccosum* (1), *Fusarium solani* (1), *Malbranchea pulchella* (1), *Geotrichum* spp. (2), *Microsporum canis* (1), *Paecilomyces* spp. (2), *Penicillium* spp. (20), *Pityrosporum ovale* (1), *Rhizopus* spp. (2), *Saccharomyces cerevisiae* (1), *Schizosaccharomyces pombe* (1), *Scopulariopsis brevicaulis* (1), *Trichoderma* spp. (124), *Trichophyton* spp. (2), *Trichosporon* spp. (2), *Ulocladium botrytis* (1), *Verticillium tenerum* (1).

A.1.4.9 Validation

The effectiveness of the method has been validated in the case of fungi[13] Quantitative analysis of the extraction efficiency has shown that the use of glass milling was most effective[18].

A.2 Preparation of PCR-quality DNA using polyvinyl-pyrrolidone (PVP)-based DNA extraction methods

A.2.1 Basic PVP method

A.2.1.1 General

This simple, fast and cheap method[19] is suitable for a wide range of matrices, in particular the ones containing high amounts of polyphenolic compounds.

A.2.1.2 Validation status

The method has been in-house validated and is used for routine DNA preparations in many laboratories, although it has not yet been evaluated in official interlaboratory studies.

A.2.1.3 Principle

The method consists of a lysis step (thermal lysis in presence of sodium dodecyl sulfate and a high EDTA content), followed by the removal of contaminants such as polyphenolic molecules, polysaccharides, metabolites and soluble proteins, from the DNA-containing liquid phase in combination with PVP and ammonium acetate. A final alcohol-precipitation step allows concentration of the DNA and the elimination of salts, see also References [19], [20], [21], [22] and [23].

A.2.1.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.2.1.5 Reagents

A.2.1.5.1 Ethanol, $\phi(C_2H_5OH)$ = 96 %.

Store and use at −20 °C.

A.2.1.5.2 Isopropanol ($CH_3CHOHCH_3$).

A.2.1.5.3 Polyvinylpyrrolidone (PVP), molecular mass M = 360 000 D; intrinsic viscosity (K value) = 80 to 100 [5)].

A.2.1.5.4 Glacial acetic acid (CH_3COOH).

A.2.1.5.5 Hydrochloric acid, ϕ(HCl) = 37 %.

A.2.1.5.6 Sodium chloride (NaCl).

A.2.1.5.7 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.2.1.5.8 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA).

A.2.1.5.9 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.2.1.5.10 Ammonium acetate ($C_2H_3O_2\ NH_4$).

A.2.1.5.11 Ethanol solution, $\phi(C_2H_5OH)$ = 70 %.

Store and use at −20 °C.

A.2.1.5.12 Extraction buffer, pH 8,0, c(Tris) = 0,2 mol/l, c(NaCl) = 0,250 mol/l, $c(Na_2EDTA)$ = 0,025 mol/l, ρ(SDS) = 50g/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.2.1.5.13 Ammonium acetate solution, $c(NH_4C_2H_3O_2)$ = 7,5 mol/l.

Dissolve in sterile water and possibly sterilize by filtration through a 0,22 µm filter.

A.2.1.5.14 TE buffer, c(Tris) = 0,010 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.2.1.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.2.1.6.1 Centrifuge, capable of achieving an acceleration of 10 000 g.

In some steps a refrigerated centrifuge is required.

A.2.1.6.2 Water bath or incubator.

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5) SIGMA P-5288 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

A.2.1.6.3 Vacuum dryer (optional).

A.2.1.6.4 Freeze dryer (optional).

A.2.1.6.5 Mixer, e.g. Vortex®[2)].

A.2.1.7 Procedure

A.2.1.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.2.1.7.2 Extraction procedure

Weigh 0,25 g of milled, crushed or liquid material into a vial. Add 1 ml of extraction buffer (A.2.1.5.12). Agitate the suspension at 65 °C for 1 h, cool to room temperature. Mix sequentially the suspension with 60 mg of PVP powder (A.2.1.5.3) and with 0,5 volume of ammonium acetate solution (A.2.1.5.13). Incubate on ice for 30 min.

Centrifuge at 10 000 g for 10 min and transfer the supernatant to a fresh tube. Mix the lysate with 1 volume of isopropanol (A.2.1.5.2) and incubate at −20 °C for 30 min. Centrifuge at 10 000 g at 4 °C for 10 min and carefully discard the supernatant.

Wash the DNA pellet with 2 volumes of ethanol solution (A.2.1.5.11). This step is essential for the removal of any salts that could interfere with the subsequent analysis (e.g. PCR). Carefully discard the supernatant (in the case of a loose pellet, centrifuge at 10 000 g at 4 °C for 10 min). Dry the pellet and redissolve it in 100 µl of water or appropriate buffer, e.g. TE buffer (A.2.1.5.14). This is the DNA master stock.

A.2.1.8 List of examples

The method has been successfully applied to extract DNA[6)] from the following matrices:

baby biscuits[6)], baby milk[6)], Belgian paté, breadcrumbs (wheat or corn) from fish sticks, brownies[6)], canned maize, cereal bars[6)], cheese croquettes, chicken nuggets, chicken, chocolate cookies[6)], chocolate paste[6)], corn flakes[6)], crisps, dessert creams[6)], infant formula, maize biscuits[6)], maize flour, fresh and cooked meat (beef, pork, chicken and turkey), minced meat, muesli[6)], pop corn, powder milk, sausages (slicing[6)] and cocktail[6)]), schnitzel, soya sprouts[6)], soup balls, soya protein in meat preparations[6)], soya lecithin[6)], soya drinks[6)], soya bean cream, spaghetti sauces[6)], speculoos, tofu, vegetarian hamburgers, waffles with chocolate[6)], waffles[6)], yoghurts[6)].

A.3 Preparation of PCR-quality DNA using the CTAB-based DNA extraction methods

A.3.1 Basic CTAB method

A.3.1.1 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 A.3.1.8).

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6) Repeatability may depend on the batch of the matrix and/or to its production technology. In some cases, DNA could not be found or was degraded in such a way that PCR results were below the limit of detection of the method, irrespective of the PCR primers/protocols used. This may be a source of low reproducibility between laboratories.

A.3.1.2 Validation status

This method has been ring tested (see A.3.1.9).

The method is commonly used for routine DNA preparations in many laboratories.

A.3.1.3 Principle

The method consists of a lysis step (thermal lysis in the presence of CTAB), followed by several extraction steps in order to remove contaminants, such as polysaccharides and proteins[24].

For some matrices, it is helpful to perform different enzymatic steps as outlined in A.3.1.7. Alpha-amylase is added to the lysis buffer to digest the starches in case of amylaceous matrices. Treatment of samples with proteinase-K is necessary in a variety of matrices to eliminate proteins. Also treatment with RNase is usually recommended for those matrices where RNA co-precipitation may disturb the subsequent analytical test.

The salt concentration during the extraction steps is very important for the removal of the contaminants, since a CTAB-nucleic acid precipitate will occur if the salt concentration drops below approximately 0,5 mol/l at room temperature and/or if the temperature drops below 16 °C. By increasing the salt concentration (e.g. addition of sodium chloride), the removal of denaturated proteins and polysaccharides complexed to CTAB is achieved, while the nucleic acids are solubilized. Chloroform is used to further separate the nucleic acids from CTAB and polysaccharide/protein complexes.

Finally, the nucleic acids are purified by isopropanol precipitation and washing with ethanol.

A.3.1.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.3.1.5 Reagents

A.3.1.5.1 **α-Amylase** (optional), type IIa from *Bacillus* species, 1 500 to 3 000 units/mg of protein.

A.3.1.5.2 **Chloroform** ($CHCl_3$).

A.3.1.5.3 **Ethanol**, $\phi(C_2H_5OH)$ = 96 %.

A.3.1.5.4 **Ethylenediaminetetraacetic acid disodium salt** (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.3.1.5.5 **Hexadecyl-trimethyl-ammonium-bromide** (CTAB) ($C_{19}H_{42}BrN$).

A.3.1.5.6 **Hydrochloric acid**, ϕ(HCl) = 37 %.

A.3.1.5.7 **Isopropanol** [$CH_3CH(OH)CH_3$].

A.3.1.5.8 **Proteinase-K** (optional), approximately 20 Units/mg of lyophilisate.

A.3.1.5.9 **RNase A, DNase-free**, (optional) from bovine pancreas, approximately 50 Units/mg of lyophilisate.

A.3.1.5.10 **Sodium chloride** (NaCl).

A.3.1.5.11 **Sodium hydroxide** (NaOH).

A.3.1.5.12 **Tris(hydroxymethyl)-aminomethane** (Tris) ($C_4H_{11}NO_3$).

A.3.1.5.13 α-Amylase solution (optional), c(α-amylase) = 10 mg/ml.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.3.1.5.14 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.

A.3.1.5.15 CTAB-precipitation buffer, ρ(CTAB) = 5g/l, c(NaCl) = 0,04 mol/l.

A.3.1.5.16 Sodium chloride solution, c(NaCl) = 1,2 mol/l.

A.3.1.5.17 Ethanol solution, ϕ(C_2H_5OH) = 70 %.

A.3.1.5.18 Proteinase-K solution (optional), ρ= 20 mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.3.1.5.19 RNase-A solution (optional), ρ(RNase A) = 10 mg/ml.

Store in aliquots at −20 °C.

A.3.1.5.20 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.

A.3.1.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.3.1.6.1 Oven or incubator, preferably with a shaker.

A.3.1.6.2 Centrifuge, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000 g.

In some steps a refrigerated centrifuge is required.

A.3.1.6.3 Mixer, e.g. Vortex®) [2].

A.3.1.6.4 Vacuum dryer (optional).

A.3.1.7 Procedure

A.3.1.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.3.1.7.2 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 (A.3.1.5.14) and mix. (In some cases a higher amount of buffer may be required to suspend the matrix.) Add 10 µl of α-amylase solution (A.3.1.5.13, optional), 10 µl of RNase A solution (A.3.1.5.19, optional) and mix gently. Incubate for 30 min at 65 °C, under

agitation. Add 10 µl of proteinase-K solution (A.3.1.5.18, 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 (A.3.1.5.2) and mix thoroughly.

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

A.3.1.7.3 CTAB-precipitation

Add 2 volumes of the CTAB precipitation buffer (A.3.1.5.15). 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 (A.3.1.5.16). Add 350 µl of chloroform (A.3.1.5.2) and mix thoroughly. Centrifuge for 10 min at 12 000 *g*. Transfer the aqueous phase into a new tube.

NOTE CTAB-precipitation is not necessary for all matrices, only for protein- and polysaccharide-rich matrices. Alternatively, a solid-phase purification of the DNA (e.g. by the use of spin columns) is possible assuming the results are equivalent.

A.3.1.7.4 DNA precipitation

Add 0,6 volume of isopropanol (A.3.1.5.7), 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 (A.3.1.5.17) 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 (A.3.1.5.20). This is the DNA master stock.

A.3.1.8 List of examples

The method has been successfully applied to extract DNA[7] from the following matrices:

baby food (powder), baby food, baking mix, biscuits, bouillon cubes[7], sweet and sour candies, canned corn, caramel cream[7], cattle cakes, cereal grains (rice, wheat, oat, rye, buckwheat, millet), chocolate bars[7], chocolate cream[7], chocolates[7], chocolate cookies[7], cookies, corn beer[7], cornflakes[7], dessert cream, dextrose[7], fillings of pralines, fine pastries, fish[7], fish fingers[7], flakes of whole soya bean, French fries, gravy[7], boiled ham, honey[7], instant meals, maize ear, maize flour, maize germs[7], maize gluten feed, maize grids, maize leaves, maize native starch[7], maize oil (native)[7], maize proteins[7], maize seeds/grains, maize semolina, margarine[7], fresh meat, milk powder, milk, mixed pet food, muesli[7], mungbean seeds, mustard leaves, popcorn (raw), potato chips, potato starch (native), potato tubers, rape leaves, rape press cake, rape seed oil (crude/native)[7], rape seed seeds, raw soya lecithin[7], ready-meals, salami (high fat content), salty snacks (maize), sausages, seasoning agents[7], modified starches (some types of)[7], sour cream with onion[7], soya flour, soya germs (preserved, frozen), soya protein[7], soya drinks[7], soya bean seeds/grains, soya bean tofu, soya beans (acidified)[7], sugar-beet leaves, sugar-beet seeds, sunflower seeds, surimi with soya[7], sweet corn, taco shells, taramas (paste of fish roes), tobacco, tomato ketchup[7], tomato-concentrate[7], tomatoes (fruits), tortilla chips[7], vegetarian hamburgers, waffles[7], wheat starch (native), yoghurts[7].

A.3.1.9 Validation

The validation data in Table A.5 have been elaborated in collaborative studies carried out by the working group “Development of methods for identifying foodstuffs produced by genetic engineering techniques” of the Commission of the former German Federal Health Board for the implementation of methods according to article 35 of the German Foodstuffs Act (see References [25], [26], [27]). The matrices tested were potatoes, soya beans and tomatoes. In these interlaboratory studies, the procedure was carried out with 100 mg of sample material. The CTAB precipitation step was necessary for the analysis of soya bean and soya flour. The enzymatic steps were not carried out in these interlaboratory studies.

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7) Repeatability may depend on the batch of the matrix and/or to its production technology. In some cases, DNA could not be found or was degraded in such a way that PCR results were below the limit of detection of the method, irrespective of the PCR primers/protocols used. This may be a source of low reproducibility between laboratories.

For the collaborative study on soya beans, two participants used strongly modified protocols and in one laboratory the testing of the five samples was discontinued. So 22 out of 25 participants identified all 110 samples correctly.

For the collaborative study on potatoes, three samples were identified false negative and one sample false positive. Three samples were not assessed due to ambiguous results between both replicates.

Table A.5 — Validation data

Matrix	Number of laboratories participating	Number of samples per laboratory	Number of total samples	Number of samples correctly identified
Soya beans[25]	25	5	125	110
Potatoes[26]	18	10	180	173
Tomatoes[27]	18	5	90	90

A.4 Preparation of PCR quality DNA using the silica-based DNA extraction methods

A.4.1 Basic silica method

A.4.1.1 General

The method is suitable for DNA extraction from a wide range of matrices (see examples in A.4.1.8). This method can also be employed as a further purification step of DNA solutions obtained after DNA extraction with other methods.

The method is adapted from a published protocol[28]. It has some advantages for the matrices for which it is suitable, in particular for the avoidance of heavily toxic reagents. Furthermore, the procedure can be easily adapted for manual high-throughput analyses and for automation, in particular because of the lack of labile interfaces (such as water-chloroform) and because low-speed centrifugation is required.

The method is usually not recommended to extract DNA from fat-rich matrices.

A.4.1.2 Validation status

The method has been in-house validated and is used for routine DNA preparations in many laboratories, although it has not yet been evaluated by official interlaboratory studies. The principle of this method has also been implemented in various kit-based methods that were successfully ring tested (see References [29], [30] and [31]).

A.4.1.3 Principle

The method consists of a lysis step (thermal lysis in the presence of sodium dodecyl sulfate in a buffered solution), followed by a purification step realised by means of a silica resin, in the presence of the chaotropic reagent guanidine-hydrochloride. The principle of the method is the binding of nucleic acids to silica under low water activity due to an entropic effect[32]. Contaminants are washed from the resin by isopropanol, while the DNA stays attached. A final elution step with a low-salt buffer solution permits DNA recovery.

Users of this International Standard are made aware that silica-based methods may be covered by patent rights[33].

A.4.1.4 Safety precautions

A fume hood is necessary for handling organic chemicals.

A.4.1.5 Reagents

A.4.1.5.1 Sodium chloride (NaCl).

A.4.1.5.2 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

A.4.1.5.3 Ethylenediaminetetraacetic acid-disodium salt (Na_2EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.4.1.5.4 Hydrochloric acid, ϕ(HCl) = 37 %.

A.4.1.5.5 Sodium hydroxide (NaOH).

A.4.1.5.6 Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$).

A.4.1.5.7 Proteinase-K, approximately 20 U/mg of lyophilisate.

A.4.1.5.8 Guanidine hydrochloride (CH_5N_3-HCl).

A.4.1.5.9 Potassium chloride (KCl).

A.4.1.5.10 Disodium hydrogen phosphate (Na_2HPO_4).

A.4.1.5.11 Potassium dihydrogen phosphate (KH_2PO_4).

A.4.1.5.12 Isopropanol [$CH_3CH(OH)CH_3$].

A.4.1.5.13 Silica (SiO_2), silicon dioxide with particle size distribution between 0,5 and 10 µm (80 % between 1 µm to 5 µm)[8].

A.4.1.5.14 RNase-A, DNase-free, approximately 100 Kunitz Units/mg of lyophilisate.

A.4.1.5.15 Proteinase-K solution, ρ = 20 mg/ml.

Dissolve the enzyme in autoclaved water or buffer as described in Reference [34]. Do not autoclave this solution. Store in aliquots at −20 °C, but avoid repeated freezing and thawing.

A.4.1.5.16 Guanidine hydrochloride solution I, c(CH_5N_3-HCl) = 5 mol/l.

Autoclave for a maximum of 15 min at 121°C.

A.4.1.5.17 Guanidine-HCl solution II, c(CH_5N_3-HCl) = 6 mol/l.

Autoclave for a maximum of 15 min at 121 °C.

A.4.1.5.18 PBS-buffer solution, c(NaCl) = 0,157 mol/l, c(KCl) = 0,0027 mol/l, c(Na_2HPO_4) = 0,010 mol/l, c(KH_2PO_4) = 0,0018 mol/l.

Adjust the pH to 7,5 with HCl.

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8) SIGMA S-5631 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

A.4.1.5.19 Silica suspension

Weigh 5 g of silica (A.4.1.5.13) into a 50 ml tube and add 50 ml of PBS-buffer (A.4.1.5.18). Mix well and allow to settle for 2 h. Remove the supernatant by aspiration with a pipette. Add another 50 ml of the PBS-buffer, mix well and allow to settle again for 2 h. Remove the supernatant by aspiration. Centrifuge for 2 min at 2 000 g. Discard the remaining supernatant. Resuspend the pellet up to 50 ml in guanidine-HCl solution II (A.4.1.5.17). Use within 2 to 5 months. Mix well before use.

A.4.1.5.20 TNE-SDS extraction buffer, c(NaCl) = 0,150 mol/l, c(Tris) = 0,002 mol/l, c(Na_2EDTA) = 0,002 mol/l, ρ(SDS) = 10g/l.

Adjust the pH to 8,0 with HCl or NaOH and autoclave before adding the SDS.

A.4.1.5.21 Isopropanol solution, $\phi[CH_3CH(OH)CH_3]$ = 80 %.

A.4.1.5.22 TE-buffer solution, c(Tris) = 0,010 mol/l and c(Na_2EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.4.1.5.23 RNase-A solution, ρ(RNase) = 10 mg/ml.

Store in aliquots at −20 °C, but avoid repeated freezing and thawing.

A.4.1.6 Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

A.4.1.6.1 Centrifuge, capable of achieving an acceleration of at least 2 000 g.

In some steps a refrigerated centrifuge is required.

A.4.1.6.2 Oven or incubator, with a working temperature of 60 °C.

A.4.1.6.3 Shaker, to be put inside the oven/incubator.

A.4.1.6.4 Mixer, e.g. Vortex®[2)].

A.4.1.6.5 Centrifugation vials, 50 ml, for preparation of the silica suspension.

A.4.1.7 Procedure

A.4.1.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.4.1.7.2 Extraction procedure

Weigh 200 mg to 300 mg of milled or crushed material into a vial. Add 2 ml of extraction buffer (A.4.1.5.20) and 20 µl of Proteinase-K solution (A.4.1.5.15). Incubate for 1 h to 5 h in the oven set at 60 °C. During the incubation time, shake the samples vigorously (approximately 250 min^{-1}). Centrifuge for 15 min at 2 000 g. Transfer 550 µl of the supernatant to a new tube.

Treat the transferred supernatant with 2 µl of RNase solution (A.4.1.5.23) for 5 min at 37 °C (this hydrolysing RNA step is recommended before the silica binding, otherwise the hydrolysed RNA and the resulting nucleotides may interfere with subsequent UV-spectrometry measurements). Add 55 µl of the guanidine-HCl solution I (A.4.1.5.16) plus 100 µl of the silica suspension (A.4.1.5.19) to the supernatant. Mix carefully several times. Leave the tubes on the bench for approximately 1 min.

Centrifuge for 2 min at approximately 800 *g*. Discard the supernatant and add 500 µl of the isopropanol solution (A.4.1.5.21). Close the tubes and mix, possibly with the aid of the mixer (A.4.1.6.4), to completely resuspend the pellet.

Centrifuge for 2 min at approximately 1 500 *g*. Discard the supernatant and dry the pellet. Add 100 µl of TE-buffer solution (A.4.1.5.22). Mix carefully in order to resuspend the pellet. Incubate the samples at 60 °C for 5 min. Centrifuge for 5 min at 2 000 *g*. Transfer 80 % of the supernatant to a new tube. Be careful not to transfer any silica particles because of their inhibiting activity on enzymes (e.g. DNA polymerase, restriction endonucleases).

Treat the transferred supernatant with 2 µl of RNase solution (A.4.1.5.23) for 1 h at 37 °C, or overnight at room temperature. This is the DNA master stock.

A.4.1.8 List of examples

The method has been successfully applied to extract DNA[9)] from the following matrices:

germs from maize[9)], maize flour, maize gluten feed[9)], maize leaves, maize-modified starches[9)], maize native starch[9)], maize seeds, maize semolina, protein from soya beans[9)], soya beans, soya bean leaves, sugar beet (fresh root), sugar beet (frozen pulp), sugar beet leaves.

A.5 Preparation of PCR quality DNA using guanidinium–chloroform-based DNA extraction methods

A.5.1 Basic guanidine-chloroform method

A.5.1.1 General

This method is suitable for DNA extraction from a wide range of food and feed matrices (see A.5.1.8). A further purification step may be necessary depending on the sample.

A.5.1.2 Validation status

The method has been validated in-house.

A.5.1.3 Principle

The method consists of a thermal and enzymatic lysis in presence of sodium dodecyl sulfate in a denaturating guanidinium buffer, occasionally followed by a purification step depending on the matrix.

Contaminants such as lipids and proteins are eliminated by a chloroform-extraction step after the lysis, then DNA is precipitated by isopropanol before purification.

A.5.1.4 Safety precautions

A fume hood is necessary for manipulation of organic chemicals.

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9) Repeatability may depend on the batch of the matrix and/or to its production technology. In some cases, DNA could not be found or was degraded in such a way that PCR results were below the limit of detection of the method, irrespective of the PCR primers/protocols used. This may be a source of low reproducibility between laboratories.

A.5.1.5 Reagents

A.5.1.5.1 **α-Amylase**, type IIa from *Bacillus* spp., 1 500 to 5 000 Units/mg of lyophilisate.

A.5.1.5.2 **Glacial acetic acid** (CH_3COOH).

A.5.1.5.3 **Chloroform** ($CHCl_3$).

A.5.1.5.4 **Ethanol**, $\phi(C_2H_5OH)$ = 96 %.

A.5.1.5.5 **Ethylenediaminetetraacetic acid disodium salt** (Na_2EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

A.5.1.5.6 **Guanidine hydrochloride** (CH_5N_3-HCl).

A.5.1.5.7 **Hydrochloric acid**, ϕ(HCl) = 37 %.

A.5.1.5.8 **Isopropanol** [$CH_3CH(OH)CH_3$].

A.5.1.5.9 **Proteinase-K**, approximately 20 Units/mg lyophilisate.

A.5.1.5.10 **RNase-A**, DNase-free, from bovine pancreas, approximately 50 Kunitz Units/mg of lyophilisate.

A.5.1.5.11 **Sodium chloride** (NaCl).

A.5.1.5.12 **Sodium dodecyl sulfate** (SDS) ($C_{12}H_{25}O_4SNa$).

A.5.1.5.13 **Sodium hydroxide** (NaOH).

A.5.1.5.14 **Tris(hydroxymethyl)-aminomethane** (Tris) ($C_4H_{11}NO_3$),

A.5.1.5.15 **α-Amylase solution**, ρ = 10 mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.5.1.5.16 **Ethanol solution**, $\phi(C_2H_5OH)$ = 70 %.

A.5.1.5.17 **Extraction buffer**, c(Tris) = 0,1 mol/l, c(NaCl) = 0,15 mol/l, $c(Na_2EDTA)$ = 0,05 mol/l, ρ(SDS) = 10g/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.5.1.5.18 **Guanidine hydrochloride solution**, $c(CH_5N_3\text{-HCl})$ = 5 mol/l.

Autoclave after preparation (maximum 15 min at 121 °C).

A.5.1.5.19 **Proteinase-K solution**, ρ = 20 mg/ml, dissolved in sterile water.

Do not autoclave. Store at −20 °C, but avoid repeated freezing and thawing.

A.5.1.5.20 **RNase-A solution**, ρ = 10 mg/ml.

Store in aliquots at −20 °C.

A.5.1.5.21 **TE buffer**, c(Tris) = 0,01 mol/l, $c(Na_2EDTA)$ = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH.

A.5.1.6 Apparatus and equipment

A.5.1.6.1 Centrifuge, capable of achieving an acceleration of 8 000 g. In some steps a refrigerated centrifuge is required.

A.5.1.6.2 Oven or incubator, preferably with a shaker.

A.5.1.6.3 Mixer, e.g. Vortex®[2)].

A.5.1.7 Procedure

A.5.1.7.1 General

Once the matrix test portion has been prepared, apply the following DNA extraction/purification protocol. Scale-adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

A.5.1.7.2 Extraction procedure

Weigh 200 mg to 500 mg of milled or crushed material into a microtube. Add 1,6 ml of prewarmed (60 °C) extraction buffer (A.5.1.5.17).

Add 10 µl of the RNase-A solution (A.5.1.5.20) and 10 µl of the α-amylase solution (A.5.1.5.15), and mix gently by inverting manually the tube. Incubate for 50 min at 60 °C under gentle agitation. Add 1/10 volume of guanidine-hydrochloride solution (A.5.1.5.18) solution, then mix with the mixer (A.5.1.6.3).

Add 20 µl of the Proteinase-K solution (A.5.1.5.19), mix smoothly by inverting the tube manually and incubate for at least 2 h at 60 °C under gentle agitation. Leave the tubes on the bench for 15 min then centrifuge for 15 min at a minimum acceleration of 8 000 g.

Transfer the supernatant into a new tube. Add 1 volume of chloroform (A.5.1.5.3) and mix with a mixer (A.5.1.6.3). Centrifuge for 15 min at a minimum acceleration of 8 000 g. Transfer the supernatant to a new tube. Add 0,6 volumes of isopropanol (A.5.1.5.8), mix inverting and put the tubes on ice for 50 min.

Centrifuge for 20 min at a minimum acceleration of 8 000 g. Wash the pellet with at least 2 ml of ethanol solution (A.5.1.5.16) and centrifuge for 10 min at a minimum acceleration of 8 000 g. This step is essential for the removal of any salts that could interfere with the subsequent analysis (e.g. PCR). Discard the supernatant. Dry the pellet and redissolve it in 100 µl of water or appropriate buffer, e.g. TE buffer (A.5.1.5.21). This is the DNA master stock. If a further purification step is required, it shall be performed on the DNA master stock.

A.5.1.8 List of examples

The method has been successfully applied to extract DNA[10)] from the following matrices:

> acidified soya bean, canned corn, cattle cakes, chocolate bars[10)], dessert cream[10)], flakes of whole soya bean, maize ears, maize flour, maize germs[10)], maize gluten feed[10)], maize grids, maize modified starches[10)], maize proteins[10)], maize seeds/grains, maize semolina, maize native starches[10)], rapeseed seeds/grains, sauces[10)], soya flour, soya bean tofu, soya lecithin (raw brown[10)] and refined yellow[10)]), soya protein, soya seeds/grains, soya tonyu, tortilla chips[10)].

The method has not yet been successfully applied even on a 1 g test sample size of oils, maltodextrine, D-glucose, maltitol, mannitol or xylitol.

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10) Repeatability may depend on the batch of the matrix and/or to its production technology. In some cases, DNA could not be found or was degraded in such a way that PCR results were below the limit of detection of the method, irrespective of the PCR primers/protocols used. This may be a source of low reproducibility between laboratories.

Annex B
(informative)

Methods for the quantitation of the extracted DNA

B.1 Basic ultraviolet spectrometric method

B.1.1 General

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

B.1.2 Validation status

The method has been widely ring-tested and results published[35]. As an example, it was successfully applied within an interlaboratory study on GMO detection organized by the Federal Office of Public Health, Bern, and the Cantonal Laboratories of Basel and Zurich, Switzerland.

B.1.3 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. For this reason, RNA must be removed enzymatically during DNA extraction before DNA determination. Also, oligonucleotides and nucleotides derived from RNA hydrolysis should be eliminated (e.g. by silica treatment, as outlined in A.4.1.7.2). Oligonucleotides and nucleotides generated by RNase treatment, if not removed (e.g. by silica treatment) can lead to an overestimation of the DNA content of the sample. Moreover, double-stranded DNA absorbs less UV light compared to 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.

The production of a calibration curve is not necessary, provided that an appropriate molar extinction coefficient is chosen as a function of the type of nucleic acid under study and/or its integrity.

However, the calibration of the spectrometer should be verified periodically by measuring the concentration of reference DNA solutions.

B.1.4 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).

NOTE Occasionally, residual compounds (e.g. CTAB from the DNA extraction procedure) may interfere with the UV spectrometric detection at 260 nm, because they absorb at this wavelength.

B.1.5 Reagents

B.1.5.1 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

B.1.5.2 Sodium hydroxide (NaOH).

B.1.5.3 Hydrochloric acid, ϕ(HCl) = 37 %.

B.1.5.4 Carrier DNA, e.g. Herring Sperm DNA[11], or Calf Thymus DNA[11].

B.1.5.5 DNA reference solution

Prepare a DNA stock solution with 10 mg/ml by dissolving 100 mg carrier DNA (B.1.5.4) in 10 ml of dilution buffer (B.1.5.7). 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).

B.1.5.6 Sodium hydroxide solution, c(NaOH) = 2 mol/l.

B.1.5.7 Dilution buffer, c(Tris) = 0,01 mol/l.

Adjust the pH to 9,0 with HCl.

B.1.6 Apparatus and equipment

B.1.6.1 UV-spectrometer, single-beam, double-beam or photodiode array instruments are suitable.

B.1.6.2 Mixer/shaker, e.g. Vortex® [2].

B.1.6.3 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 (1 000 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.

B.1.7 Procedure

B.1.7.1 Measurement of a reference DNA solution

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

— for blank measurement only dilution buffer (B.1.5.7) is used to fill the measurement vessel;

— the measurement vessel is filled with the reference DNA solution (B.1.5.5).

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

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11) These are available from Sigma as D-7290 and D-1501, respectively. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

B.1.7.2 Measurement of a test DNA solution of unknown concentration

For the blank solution, mix dilution buffer (B.1.5.7) plus sodium hydroxide solution (B.1.1.5.13), so that a final NaOH substance concentration of 0,2 mol/l is reached. This mixture is used to fill the measurement vessel.

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 reference DNA solutions at wavelengths of 260 nm and 320 nm. The reading is stable for at least 1 h.

EXAMPLE 1 For the blank solution, mix 90 µl of dilution buffer and 10 µl of sodium hydroxide solution and transfer to a 100 µl measurement vessel.

EXAMPLE 2 For the test DNA solution, mix 80 µl of dilution buffer or water, 10 µl of sodium hydroxide solution and 10 µl of a DNA solution of unknown concentration and transfer to a 100 µl measurement vessel.

B.1.8 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):

$$\rho_{\mathrm{DNA}} = F \times (\mathrm{OD}_{260} - \mathrm{OD}_{320}) \times 37 \qquad (1)$$

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.

EXAMPLE For a calculation with a dilution factor of 10 and an OD_{260} of 0,658 and an OD_{320} of 0,040:

ρ_{DNA} = 10 × (0,658 – 0,040) × 37 µg/ml = 229 µg/ml.

B.2 Agarose gel electrophoresis and ethidium bromide staining method

B.2.1 General

This annex describes a routine method to determine the concentration 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). The method also applies if insufficient DNA is available for spectrometric detection, or if the DNA is not sufficiently purified and may contain substances that absorb ultraviolet radiation[36]. Gel electrophoresis is usually not recommended to quantify DNA if degraded. If this is the case, other methods should be employed.

B.2.2 Validation status

The method has been known for many years and has been widely applied, although it has never been tested in interlaboratory studies for GMO detection in foodstuffs.

B.2.3 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[37].

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. The molecular mass of such standards must be similar to that of the DNA under quantitation, because EtBr intercalation, and thus fluorescence emission, also depends on the length of the DNA fragments. EtBr also stains single-stranded DNA and RNA. For a more precise estimation of the DNA content, RNA must be removed enzymatically.

B.2.4 Application range

The method is applicable to DNA concentrations in the approximate range from 5 ng to 500 ng when photographic image acquisition systems are used. CCD-based systems for video documentation may be more sensitive.

B.2.5 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, see Reference [36].

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

B.2.6 Reagents

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

Usually, molecular biology grade is not necessary for the reagents described in this method. Solutions as described in this method do not usually need to be autoclaved.

B.2.6.1 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA molecules.

B.2.6.2 Boric acid (H_3BO_3), for the TBE buffer system only.

B.2.6.3 Bromophenol blue ($C_{19}H_9Br_4O_5SNa$) and/or xylene cyanole FF ($C_{25}H_{27}N_2O_6S_2Na$).

B.2.6.4 DNA quantity standard, of suitable molecular mass (e.g linearized Lambda Phage DNA for high molecular mass DNA and restriction-digested Lambda Phage DNA for lower molecular mass DNA).

B.2.6.5 DNA molecular mass standard, for example a commercial preparation containing DNA fragments from very high to very low molecular mass.

B.2.6.6 Glacial acetic acid (CH_3COOH), for the TAE buffer system only.

B.2.6.7 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

B.2.6.8 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$).

B.2.6.9 Glycerol ($C_3H_8O_3$).

B.2.6.10 Sodium acetate ($C_2H_3O_2Na$), for the TAE buffer system only.

B.2.6.11 Hydrochloric acid, ϕ(HCl) = 37 %.

B.2.6.12 Sodium hydroxide (NaOH).

B.2.6.13 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

B.2.6.14 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.

B.2.6.15 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.

B.2.6.16 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 (B.2.6.14 or B.2.6.15).

B.2.6.17 Ethidium bromide solution, c(EtBr) = 0,5 mg/l.

It is advisable to store the ethidium bromide solution as a concentrate (e.g. 10mg/ml) at 5° C in the dark (EtBr is light-sensitive). It is also advisable to avoid weighing EtBr. The stock solution should be prepared by dissolving an appropriate amount of water in the vessel already containing the EtBr powder, or alternatively, by employing pre-weighed EtBr tablets. Solubilization of EtBr should be carried out protected from light, under agitation at room temperature. This usually takes approximately 1 h.

B.2.7 Apparatus and equipment

B.2.7.1 Microwave oven or boiling water bath

B.2.7.2 Equipment for agarose gel electrophoresis, with accessories and power supply.

B.2.7.3 Ultraviolet (UV) trans-illuminator or lamp, preferably with wavelength of 312 nm.

Alternatively, equipment for column chromatography of nucleic acids and the according detection system or other similar suitable systems may be used.

B.2.7.4 Recording instrument, for example a photo documentation system with 3 000 ASA films and UV filter adequate for EtBr-emitted fluorescence.

As an alternative, a video-documentation system with CCD camera, adequate UV filter and (optional) quantitative analysis software may be used.

B.2.8 Procedure

B.2.8.1 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.

B.2.8.2 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)[38].

Weigh an appropriate amount of agarose (B.2.6.1) and add it to the electrophoresis buffer solution (B.2.6.14 or B.2.6.15). Allow the solution to boil in a microwave oven or in a water bath (B.2.7.1), 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).

B.2.8.3 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 (B.2.6.16) (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 (B.2.6.16) in the proportion of 20 % with respect to the sample volume) to a suitable amount of the DNA molecular mass standard (B.2.6.5) and carry out electrophoresis in parallel.

To estimate the concentration of the unknown sample, run standard DNA quantity samples in parallel. Such samples contain known amounts (within the dynamic range of the method, i.e. 5 ng to 500 ng) of the DNA quantity standard (B.2.6.4) diluted in water or in electrophoresis buffer (B.2.6.14 or B.2.6.15). It is recommended to use quantitation standards containing at least 5 calibration points (i.e. different amounts of DNA).

B.2.8.4 Submarine electrophoresis

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 (B.2.6.14 or B.2.6.15). Overlay the gel with approximately 2 mm of the same buffer and load the samples using a micropipette.

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.

B.2.8.5 Staining

After completing the electrophoresis, incubate the gel for 15 min to 50 min in the ethidium bromide solution (B.2.6.17) 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 min.

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 ethidium bromide, load the unknown sample and the DNA quantity standard (B.2.6.4) into separate slots produced with the same comb on the same gel. Otherwise the quantity of ethidium bromide 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.

B.2.8.6 Gel recording

Transfer the gel to the trans-illuminator surface, switch on the UV light and record the DNA fluorescence by photography or video-documentation.

B.2.9 Evaluation/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, better, with the aid of a quantitation software able to calculate an adequate calibration curve.

B.3 Real time PCR method for quantitation of extracted DNA

When DNA-poor matrices are extracted, the quantitation of the obtained DNA is not always possible with the usual physical methods (e.g. the ones described in this annex), because of their insufficient sensitivity. Where such quantitation is necessary, a real time PCR approach can be followed. This method also provides information on the PCR amplificability of isolated DNA molecules that cannot be provided by physical measurements of DNA concentrations. For details, see ISO 21570.

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