SOP

Methods of analysis for the detection of plant species and derived products – Qualitative nucleic acid based methods

Federal Institute for Risk Assessment

Thielallee 88-92 – D-14195 Berlin – www.bfr.bund.de

Risiken erkennen – Gesundheit schützen

Author: Dr Ines Laube

Date: August 2007

PART I: Conventional PCR

Introduction

The search for ingredients of plant species origin 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, the amount of DNA is determined (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR).

1 SCOPE

This document describes the procedure to detect a plant species and derived products thereof by analysing the nucleic acids extracted from the sample under study. The focus will be on the polymerase chain reaction (PCR) based amplification methods.

It relates to the general requirements for the specific detection and identification of target nucleic acid sequences (DNA) and for the confirmation of the identity of the amplified DNA sequence.

NOTE: Guidelines, minimum requirements and performance criteria laid down in this document are intended to ensure that comparable, accurate and reproducible results are obtained in different laboratories.

The methods have been established for food matrixes, but could also be applied to other matrices (e.g. seeds and feed).

Specific examples of methods are provided in Annexes A and B.

2 TERMS AND DEFINITIONS

For the purposes of this document, the terms and definitions given in ISO 24276 apply.

3 PRINCIPLE OF THE METHOD

3.1 GENERAL

Qualitative analysis consists of specific detection of target nucleic acid sequences in the test samples. Each method shall specify the target sequence.

A qualitative result shall clearly demonstrate the presence or absence of the plant species under investigation, relative to appropriate controls and within the detection limits of the analytical method used and test portion analysed.

3.2 PCR AMPLIFICATION

Amplification of the target sequence occurs *in vitro* through a reaction catalysed by a DNA polymerase in the presence of oligonucleotide primers and deoxynucleoside triphosphates in a defined reaction buffer [1], [2], [3]. An important prerequisite for the amplification of the target sequence is that the reaction mixture contains no polymerase inhibitors. Amplification of the DNA is a cyclical process consisting of:

- denaturation of the double stranded DNA into single stranded nucleic acid by means of heating;
- annealing of the primers to the target sequence at a suitable temperature;
- extension of the primers, which are bound to both single strands, by a DNA polymerase suitable for PCR, at an appropriate temperature.

3.3 DETECTION AND CONFIRMATION OF PCR PRODUCTS

PCR products are detected by gel electrophoresis or an appropriate alternative, if necessary, after isolation by means of a suitable separation procedure.

The identity of any detected target sequence can be verified by an appropriate technique (e.g. by restriction enzyme analysis, by hybridisation or by DNA sequence analysis).

In case of real time PCR analysis, amplification and detection occur simultaneously.

4 REAGENTS

It is generally advisable to store the reaction solutions required for the analytical method at approximately -20 °C if not specified otherwise.

It may also be appropriate to aliquot the reaction solutions required for the analytical method in order to avoid subjecting them to repeated freeze-thaw cycles, and/or to reduce chances of cross contamination.

4.1 TARGET DNA/CONTROL

4.2 WATER

4.3 DEOXYRIBONUCLEOSIDE TRIPHOSPHATE (DNTP) SOLUTION,

containing dATP, dCTP, dGTP, and dTTP and/or dUTP

NOTE The use of dUTP can interfere with restriction enzyme analyses of PCR products.

4.4 PCR BUFFER SOLUTION

The PCR buffer solution is usually delivered with the DNA-polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentrations are method specific. Ready-to-use reagents may be commercially available. The manufacturer's instructions for use should be considered.

4.5 $MGCL_2$ SOLUTION

4.6 THERMOSTABLE DNA POLYMERASE

4.7 FORWARD PRIMER /REVERSE PRIMER

5 APPARATUS AND EQUIPMENT

See ISO 24276 and Annexes A and B for details.

6 PROCEDURE

6.1 QUALITY, INTEGRITY AND AMPLIFIABILITY OF NUCLEIC ACID EXTRACTS

The nucleic acid solution shall be pure enough for subsequent analysis. The quality and amount of nucleic acid extracted using a given method on a given matrix shall be both repeatable and reproducible.

NOTE The quality, integrity and amount of the DNA template influences the outcome of the PCR, and hence the analytical results obtained. The limit of detection of a specific method may therefore depend on whether the material to be analysed has been processed or refined, and on the degree of degradation of the DNA therein.

Nucleic acids for use in PCR should be substantially free of PCR inhibitors [4]. PCR inhibition controls are to be included as described in ISO 24276.

6.2 PERFORMANCE CRITERIA

General performance criteria are described in ISO 24276.

The values for the performance characteristics are given for each method as outlined in Annexes A and B and should take into account the genome sizes, see Reference [5]

The reaction conditions, especially the $MgCl_2$ concentration and the thermocycling conditions should be optimised for every primer pair and/or system. When any primer system is used for the first time, it is necessary to demonstrate beforehand that the cycle conditions chosen for the particular matrix to be studied avoid undesirable competitive products which would otherwise reduce the sensitivity of the PCR detection.

In an optimal reaction, less than 40 cycles are required to amplify ≥ 10 target molecules to produce a product that is readily detectable by standard methods. As cycle number increases non-specific products could accumulate. The optimised PCR should be able to amplify in 40 PCR cycles from a pure reference sample of 100 copies of template DNA enough copies of the PCR product to be detectable. The characteristic temperature/time profile for each primer system and the reaction mixture appropriate for the apparatus used and the number of cycles shall be strictly adhered to.

In general, specificity of the reaction should be enhanced as much as possible (e.g. by using hot start PCR). Hot start PCR is recommended as a means of reducing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerisation (so it increases specificity).

6.3 CONTROLS

Because of the risk of obtaining false positive and/or false negative results, appropriate controls shall be included in each diagnostic PCR assay (see ISO 24276).
If available and appropriate, certified reference materials should be used as positive and negative controls.

6.4 PCR SET-UP, DETECTION AND CONFIRMATION OF PCR PRODUCTS

The annexes describe details on the specific PCR procedure steps.

NOTE In the case of detection of the PCR-products by gel electrophoresis, the size of the PCR products can be estimated using a suitable DNA size marker of known length to run in parallel with the PCR products under test.

It may be desirable in some cases to confirm a positive or negative result for a specific detection. This may be achieved by employing primers to an alternative target sequence, this is particularly suitable for confirmation of screening test results.

A positive identification of the specific target DNA sequence may be confirmed by an appropriate method other than size determination of the PCR-product, for example:

- by hybridisation of the PCR product with specific probes, or
- by carrying out restriction analyses of the PCR product. The length of the resulting fragments has to correspond to the expected length of the target DNA sequence after restriction, or
- by sequencing of the PCR product, or
- other equivalent confirmation

The assay for the identification of species specific sequences can be combined with a system for a general proof of the presence of plant specific DNA. This plant system can be used as an external amplification control. False-negative results due to an inhibition of DNA polymerase can be excluded by using such a control. Even in case that the targeted species is not detected, the amplification control should result in a positive signal in PCR if material derived from plants is present. An example is given in Annex B.

7 INTERPRETATION

7.1 GENERAL

The PCR result will be either:

a) positive if a specific PCR-product has been detected, and all the controls give results as specified in Table 1;

b) negative if a specific PCR-product has not been detected, and all the controls give results as specified in Table 1.

If the results are ambiguous, the procedure shall be repeated.

Table 1 Examples of PCR results

Test sample	Positive extraction control	Extraction blank control	Negative DNA target control	Positive DNA target control	Interpreted result
+[a]	+	-	-[b]	+	positive
-	+	-	-	+	negative
+	+	+	-	+	inconclusive[c]
-	-	+	-	-	inconclusive[c]
-	-	-	-	-	inconclusive[d]

a PCR product is detectable.

b No PCR product is detectable.

c The procedure is repeated beginning with the extraction step (possible contamination).

d The procedure is repeated using another extraction method or a further purification step (possible inhibition).

7.2 VERIFICATION

Verification of positive or negative results for target sequences can be achieved as described in section 6.6.

8 EXPRESSION OF THE RESULTS AND QUALITY ASSURANCE

8.1 GENERAL

The results shall be expressed unambiguously, e.g. not as "+/-".

A negative result shall never be expressed as "plant species not present".

Ideally, the LOD should be provided with reference to the test sample. However, this requires particular materials, DNA of exceptionally high quality, and/or use of sophisticated laboratory equipment which is not available to all laboratories. Consequently the analysis may become very labour intensive and/or expensive, and therefore not practically applicable for routine purposes. As a minimum, the LOD is to be provided with reference to a reference material, and a relative value based on a specified matrix (preferably a given amount of genomic DNA solution, e.g. 25 pg of 100 % sweet chestnut DNA).

8.2 EXPRESSION OF A NEGATIVE RESULT

The following text shall appear in the test report:

> 'For sample X, target sequence Y and therefore Z (plant species) was not detected.
>
> The LOD of the method is χ % determined with ABC (identify the reference material).'

If it cannot be demonstrated that the amount of target DNA included in the PCR is sufficient for the LOD to be applicable, then the following sentence shall be added.

> 'However, the amount of the target DNA extracted from species X may be/was insufficient for the LOD to be applicable for this sample.'

8.3 EXPRESSION OF A POSITIVE RESULT

The following text shall appear in the test report:

'For sample X, target sequence Y and therefore Z (plant species) was detected.

8.4 QUALITY ASSURANCE REQUIREMENTS

Results from both test portions have to be consistent. If one test portion gives a positive result and the other gives a negative result, then the analysis shall be repeated, if possible, by increasing the quantity of template nucleic acid in the reaction so as to obtain consistent results for both test portions. Moreover, as a

minimum, the purity of the template nucleic acid should be checked by including a PCR inhibition control. Other controls to check the length and integrity of the template nucleic acid may be useful.

9 TEST REPORT

The test report shall be written in accordance with ISO 24276 and shall contain at least the following additional information:

- the limit of detection, and the matrix used to identify the limit of detection;
- description of the specificity of the analytical method;
- the result expressed according to clause 8.

PART II: Real-time PCR

Introduction:

The second part of this SOP describes methods which are based on real-time PCR systems. In this part only variations to PART I are specified. A specific example is provided in Annex C.

PRINCIPLE OF THE METHOD

In real-time PCR the amplification of target specific DNA is measured by the use of fluorescence-labelled specific probes. In the context of this document exclusively the TaqMan™ technology is used for this purpose.

REAGENTS

In TaqMan™ PCR a different buffer is used compared to conventional PCR: TaqMan™ buffer A (containing passive reference ROX; depending from machine used passive reference should not be used). In addition a fluorescence-labelled specific probe is applied.

PROCEDURE

The reaction conditions, in particular the $MgCl_2$, primer and probe concentration should be optimised for each primer and probe system.

Annex A

Specific methods for the detection of plant species in honey and pollen

A.1 GENERAL

These methods describe a routine procedure for the detection of species specific, single copy genes occurring in:

Sweet chestnut (*Castanea sativa*), lavender (*Lavandula* sp.), sunflower (*Helianthus annus*), acacia (*Acacia* sp.), citrus (*Citrus* sp.), erica (*Erica carnea*), rape (*Brassica napus*), lime (*Tilia platyphyllos*), rosemary (*Rosmarinus officinalis*), eucalyptus (*Eucalyptus erythrocorys*), oak (*Quercus* sp.), clover (*Trifolium* sp.), rockrose (*Cistus* sp.), and olive (*Olea europaea*).

These methods can be used to identify DNA from the above mentioned plant species derived products (honey, pollen and other food products as well as seeds and feed).

A.2 VALIDATION STATUS AND PERFORMANCE CRITERIA

A.2.1 Molecular specificity

A.2.1.1 General

This annex fulfils the requirements as outlined in clause 6.

Candidate genes were identified either by database search (GenBank®) or by DNA sequencing using consensus primers in case no sequence entry was available:

Table A.1 GenBank® accession-No. of target sequences

detection of	**plant species**	**target gene**	**GenBank® accession-No.**	*Abbreviations (detection systems)*
sweet chestnut	*Castanea sativa*	*phenylalanine ammonia-lyase (PAL)*	no sequence entry available	Cas_PAL
lavender	*Lavandula sp.*	*Hydroxymethylglutaryl coenzyme A reductase*	no sequence entry available	Lav_HMG2
sunflower	*Helianthus annuus*	*HAPROFILN* allergen	J15210	Heli-all-nes
acacia	*Acacia sp.*	*alcohol dehydrogenase 1 (adh1)*	no sequence entry available	Aca_adh1
citrus	*Citrus sp.*	*alcohol dehydrogenase 1 (adh1)*	no sequence entry available	Cit_adh1
erica	*Erica carnea*	*alcohol dehydrogenase 1 (adh1)*	no sequence entry available	Eri_adh1
rape	*Brassica napus*	*lipase 1*	AY866419	Bras_lip
lime	*Tilia platyphyllos*	*nitrate reductase (nr1)*	AY138811	Til_nr
eucalyptus	*Eucalyptus grandis*	*LEAFY/FLORICAULA (LFY1)*	AY640313	Euc_LFY
rosemary	*Rosmarinus officinalis*	*alcohol dehydrogenase 1 (adh1)*	no sequence entry available	Ros_CSL
clover	*Trifolium pratense*	*actin*	AY372368	Tri_act
rockrose	*Cistus incanus*	*1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)*	AY297794	Cis_DXR
oak	*Quercus suber*	*phenylalanine ammonia-lyase (PAL)*	AY443341	Querc_PAL2
olive	*Olea europaea*	Allergen *Ole e 10*	AY082335	Olea_ole10

A.2.1.2 Theoretical

No sequence similarity with DNA sequences of other plants have been found (NCBI BlastN® search, March 2005).

The number of target sequence copies was not determined, but was presumed to be single-copy genes.

A.2.1.3 Experimental

The specificity of the primer systems given in table A.2 was tested with DNA from twenty-five plant species and two animal species:

acacia (*Acacia decora*, *Acacia longifolia*), almond (*Prunus dulcis*), blackberry (*Rubus fruticosus*), broom (*Cytisus scoparius*), chestnut (*Aesculus hippocastanum*), citrus (*Citrus grandis*, *Citrus limon*, *Citrus sinensis*), clover (*Trifolium alpestre*, *Trifolium medium*, *Trifolium montanum*, *Trifolium rubens*), erica (*Erica carnea*), eucalyptus (*Eucalyptus erythrocorys*), hazelnut (*Corylus avellana*), lavender (*Lavandula angustifolia*, *Lavandula lanata*, *Lavandula latifolia*, *Lavandula stoechas*), lime (*Tilia platyphyllos*), maize (*Zea mays*), melissa (*Melissa officinalis*), oak (*Quercus palustris*, *Quercus pubescens*, *Quercus robur*, *Quercus rubra*, *Quercus suber*), olive (*Olea europaea*), papaya (*Carica papaya*), rape (*Brassica napus*), rockrose (*Cistus ladanifer*, *Cistus monspeliensis*), rosemary (*Rosmarinus officinalis*), sage (*Salvia fruticosa, Salvia lavandulifolia*), sunflower (*Helianthus annus*), sweet chestnut (*Castanea sativa*), thyme (*Thymus vulgaris*), wheat (*Triticum aestivum*) as well as chicken (*Gallus gallus*) and cattle (*Bos taurus*).

No cross reactions occurred using the species specific primer systems except for sweet chestnut. The sweet chestnut specific system amplifies also oak, although the amount of amplified product is significantly lower. This result is comprehensible considering the fact that both plant species are of the same subfamily *Quercoideae*. The PCR band intensity indicates a non-relevant quantity of a plant species in honey as the total DNA yield from honey is very low. All other species specific PCR assays appear to be highly specific for the DNA of appropriate plant species.

A.2.2 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to detect at least 1 ng of appropriate plant species DNA, determined fluorometrically.

A.3 PRINCIPLE

Using the different primer sets fragments of the appropriate genes are amplified by PCR and separated by agarose gel electrophoresis. The appropriate amplicon sizes are given in Table A.2.

A.4 REAGENTS

A.4.1 General

A.4.2 Water

A.4.3 **PCR buffer**, (without $MgCl_2$), 10x[1]

A.4.4 **$MgCl_2$ solution,** *c*($MgCl_2$) = 25 mmol/l

A.4.5 **dNTP solution**, *c*(dNTP) = 2,5 mmol/l (each)

A.4.6 Oligonucleotides

Table A.2 Primers to be used and expected amplicon length

Primer systems and expected amplicon length	
System sweet chestnut	
Primer forward (Cas_PAL-f)	5´- CCA AAG AAG TAG AGG GTG CAA GA -3´
Primer reverse (Cas_PAL-r)	5´- GAA TTC TCT CAC CAG TTA GTA AAC TTG TT -3´
Amplicon (Cas_PAL)	144 bp
System lavender	
Primer forward (Lav_HMG2-f)	5´- CCA TAT CGC TCG TCT TCA ACA G -3´
Primer reverse (Lav_HMG2-r)	5´- TTG CCG GCG ATG GCA -3´
Amplicon (Lav_HMG2)	74 bp
System sunflower	
Primer forward (Heli-all-nes f2)	5´- CGT CAA TAC TTG TTA ATA TTA TTA AGA ATT A -3´
Primer reverse (Heli-all r1)	5´- ATA GCT TGG CCC GTT TTC TT -3´
Amplicon (Heli-all-nes)	106 bp
System acacia	
Primer forward (Aca_adh1-f)	5´- TGG GGA ATG TCC ACA TTG TAA GT -3´
Primer reverse (Aca_adh1-r)	5´- TGG AGA ACC TGG TCT TGC CA -3´
Amplicon (Aca_adh1)	107 bp
System citrus	

[1] 10x means 10-fold, i.e. a PCR buffer containing 1,5 mol/l Tris-HCl pH 8,3

Primer forward (Cit_adh1-f)	5′- TGA CAG AGG CGT CAT GCT TAA C -3′
Primer reverse (Cit_adh1-r)	5′- TGA GGT TCC AAC GAA ATG ATA AAT A -3′
Amplicon (Cit_adh1)	83 bp
System erica	
Primer forward (Eri_adh1-f)	5′- GGA TAG GGG AGT GAT GAT CCA T -3′
Primer reverse (Eri_adh1-r)	5′- GAT GTT CCG ACG AAA TGG TAT ATG -3′
Amplicon (Eri_adh1)	81 bp
System rape	
Primer forward (Bras_lip-f2)	5′- CAC GAC CAC GTT CTT TGT TTT -3′
Primer reverse (Bras_lip-r)	5′- CCA CTA CAA CAT TCC ATC CCC -3′
Amplicon (Bras_lip)	122 bp
System lime	
Primer forward (Til_nr-f)	5′- GGG CAT CGA ACA TGA GCT TT -3′
Primer reverse (Til_nr-r)	5′- TTC AAC GAG TTT GCA TGG GA -3′
Amplicon (Til_nr)	101 bp
System eucalyptus	
Primer forward (Euc_LFY-f)	5′- AGG AGC AAG TGG TGC AGC A -3′
Primer reverse (Euc_LFY-r)	5′- TCC TCT GTT GTT GGG CGC -3′
Amplicon (Euc_LFY)	96 bp
System rosemary	
Primer forward (Ros_CSL-f)	5′- GCG TAT GCG GCC AAC AG -3′
Primer reverse (Ros_CSL-r)	5′- ACT GCC CTT GAA GAA GAA ATG G -3′
Amplicon (Ros_CSL)	74 bp
System rockrose	
Primer forward (Cis_DXR-f)	5′- AAT GTT CGA TGT CCT CCT GTT CTT -3′
Primer reverse (Cis_DXR-r)	5′- GCT CAC TGT TTC CAC CCA CAA -3′
Amplicon (Cis_DXR)	94 bp
System olive	
Primer forward (Olea_ole10-f)	5′- TAC CAA AGC AAG GGT CGA AAT G -3′
Primer reverse (Olea_ole10-r2)	5′- AGG GAT AAA ATT GTT TTA CTT ACT TGG GT -3′
Amplicon (Olea_ole10)	96 bp
System oak	
Primer forward (Querc_PAL2-f)	5′- AAG AAG TAG AGG GCG CAA GG -3′
Primer reverse (Querc_PAL-r)	5′- GAA TTC TCT CAC CAG TTA GTA AAC TTG TT -3′
Amplicon (Querc_PAL2)	141 bp

System clover	
Primer forward (Tri_act-f)	5´- CTG GCC GTG ATC TAA CTG AAT CTT -3´
Primer reverse (Tri_act-r)	5´- ATC AGG AAG CTC ATA GTT TTT CTC AAT T -3´
Amplicon (Tri_act)	191 bp

Abbreviations: Cas_PAL: phenylalanine ammonia-lyase (PAL) gene of sweet chestnut; Lav_HMG2: Hydroxymethylglutaryl coenzyme A reductase gene of lavender; Heli-all-nes: Profilin gene of sunflower; Aca_adh1: adh1 gene of acacia; Cit_adh1: adh1 gene of citrus; Eri_adh1: adh1 gene of erica; Bras_lip: Lipase 1 gene of rape; Til_nr : Nitrate reductase (nr1) gene of lime; Euc_LFY: Leafy/Floricaula (LFY1) gene of eucalyptus; Ros_CSL: adh1 gene of rosemary; Cis_DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) gene of rockrose; Olea_ole10: Allergen Ole e 10 gene of olive; Querc_PAL2: phenylalanine ammonia-lyase (PAL) gene of oak; Tri_act: actin gene of clover.

A.4.7 **Thermostable DNA-Polymerase** (for hot-start PCR), 5 IU/µl

A.5 APPARATUS

A.5.1 Thermal cycler

A.5.2 **Electrophoresis chamber,** with power supply

A.6 PROCEDURE

A.6.1 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Table A.3. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table A.3 have proven to be suitable.

Table A.3 Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA	10 ng to 50 ng[b]	2,00
Water		15,60
10x PCR buffer (without $MgCl_2$)	1 x	2,50
$MgCl_2$-solution[a], 25 mmol/l	2 mmol/l	2,00
dNTP solution, 2,5 mmol/l	0,2 mmol/l	2,00
Primer forward, 20 µmol/l	0,2 µmol/l	0,25
Primer reverse, 20 µmol/l	0,2 µmol/l	0,25
Taq DNA polymerase, 5 IU/µl	2 IU	0,40

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 1,5 mmol/l.

[b] Only small DNA amounts are available in honey samples. Nevertheless dilute the DNA solution at least 1:2.

A.6.2 PCR controls

The following controls should be analysed in parallel:

- NTC (no template control), with ddH_2O instead of sample DNA

  This control is used to demonstrate the absence of contaminating nucleic acids in the reagents.
- Extraction blank control

  The extraction blank control is necessary to check for contamination during the extraction step.
- Positive Control

  The positive control should represent the target sequence under investigation, it is recommended to use 100 % of appropriate plant species.

  This control is used to demonstrate that the PCR reagents are working as intended.
- Negative Control

  The negative control should not contain the target sequence under study, which means 0 % of the plant species under investigation.

  This control demonstrates that the results of analyses of test samples not containing the target sequence will be negative.

  NOTE The Extraction blank control can be used for this purpose.

A.6.3 Temperature-time-programme

The temperature-time-programme as outlined in Table A.4 has been used for the validation study using thermal cycler GeneAmp® 2400, 9600 and AmpliTaq Gold® DNA polymerase[2]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table A.4 Temperature-time-programme

Process	Time [min:s]	Temperature [°C]	Cycles
Activation/initial denaturation	10:00	95	
Denaturation	0:30	95	35
Annealing	0:30	see Table A.5	
Extension	0:30	72	
Final extension	6:00	72	
	∞	4	

[2] GeneAmp® 2400 and 9600 and AmpliTaq Gold® DNA polymerase are examples of suitable products available commercially by Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this Standard. Equivalent products can be used if they can be shown to give equivalent results.

Table A.5 Optimum annealing temperatures determined empirically by temperature gradient

System	Annealing temperature (°C)
Cas_PAL	60
Lav_adh1	60
Lav_HMG2	60
Heli-all-nes	56
Aca_adh1	60
Cit_adh1	60
Eri_adh1	60
Bras_lip	57
Til_nr	58
Euc_LFY	60
Ros_CSL	60
Cis_DXR	60
Olea_ole10	60
Querc_PAL2	60
Tri_act	58

A.7 IDENTIFICATION

The identification is based on PCR product size. The identity of the amplified product can be determined by real-time PCR using a specific oligonucleotide probe labelled with fluorescent dyes (Annex C) or by sequencing of the PCR product and comparison with the GenBank® entries given in A.2.1.1.

A.8 GENERAL QUALITY ASSURANCE AND INTERPRETATION OF THE RESULTS

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence, determined by comparison with products derived from reference materials (positive controls).

For identification purposes see A.7.

The detection of fragments with the appropriate size listed in Table A.2 indicates that the sample DNA-solution contains DNA of appropriate plant species origin within the assessed limitations of specificity described in A.2.1.

Annex B

Specific methods for the detection of DNA sequences generally present in plants

B.1 GENERAL

This is a routine procedure for the detection of DNA sequences generally present in plants.

This method is suitable to check if the DNA extraction of a food sample was successful and to check if the sample contains amplifiable plant DNA free of any inhibition. In case of processed material, the applicability of the method depends on the degree of degradation of the DNA. It needs to be tested whenever a new matrix will be analysed.

Two PCR systems are presented, i) is based on the *tRNA-Leu* (trnL) gene, a chloroplast sequence and ii) is based on the amplification of a region of the *actin* gene. The former is suited for the detection of plants if only small DNA amounts are available. A plant cell normally contains multiple copies of this DNA sequence. The number of copies per cell may vary between plant species and tissues. Therefore this method may not be used as a control for quantitative purposes. In contrast to that the second system is presumed to be based on a single-copy gene and is suitable for a quantitative determination.

B.2 VALIDATION STATUS AND PERFORMANCE CRITERIA

B.2.1 Molecular specificity

B.2.1.1 General

This annex fulfils the requirements as outlined in clause 6.

Candidate genes were identified by database search (GenBank®) and by DNA sequencing using consensus primers in case no sequence entry was available for several species.

The PCR system which is based on the *tRNA-Leu* gene has been designed to target sequences described for example in GenBank® accession No. AJ854556 (*Brassica napus*). The PCR system which is based on the amplification of a region of the *actin* gene has been designed to target sequences described for example in GenBank® accession No. XM_470336 (*Oryza sativa*).

B.2.1.2 Theoretical

For both fragments no significant sequence similarity with DNA sequences of non plant organisms has been found in data bank search (NCBI BlastN® search, March 2005).

The primers were designed to amplify a sequence unique to chloroplast DNA (the *trnL* gene) and unique to the actin gene, respectively, and show no known similarity with non-target sequences.

B.2.1.3 Experimental

Using these PCR systems no amplification has been observed for DNA derived from animals.

Number of target sequence copies: *trnL:* multiple, depending on plant species and tissue type; *actin*: not determined, but presumed to be a single-copy gene.

B.2.2 Limit of detection (LOD)

The absolute LOD has not been determined, but the method has been demonstrated to detect at least 0,1 ng (*trnL)* and 1 ng (*actin*), respectively of e.g. sweet chestnut DNA, determined fluorometrically.

B.3 PRINCIPLE

An approx. 90 bp DNA fragment, occurring in the tRNA gene of chloroplasts or an approx. 103 bp DNA fragment, occurring in the actin gene of plants are amplified by PCR and separated by agarose gel electrophoresis.

B.4 REAGENTS

B.4.1 Water

B.4.2 **PCR buffer** (without $MgCl_2$), 10x

B.4.3 **$MgCl_2$ solution**, *c*($MgCl_2$) = 25 mmol/l

B.4.4 **dNTP solution**, *c*(dNTP) = 2,5 mmol/l (each)

B.4.5 Oligonucleotides

B.4.5.1 Forward primer

Chloroplast tRNA gene (GenBank® accession-No. AJ854556)
Primer forward (plant nes-2-f): 5´- ATT GAG CCT TGG TAT GGA AAC CT -3´

Actin gene (GenBank® accession-No. XM_470336)
Primer forward (act-f): 5´- CAA GCA GCA TGA AGA TCA AGG T -3´

B.4.5.2 Reverse primer

Chloroplast tRNA gene (GenBank® accession-No. AJ854556)
Primer reverse (plant nes-2-r): 5´- GGA TTT GGC TCA GGA TTG CC -3´

Actin gene (GenBank® accession-No. XM_470336)

Primer reverse (act-r): 5´- CAC ATC TGT TGG AAA GTG CTG AG -3´

B.4.6 **Thermostable DNA-Polymerase,** 5 IU/µl

B.5 APPARATUS AND EQUIPMENT

B.5.1 Thermal cycler

B.5.2 **Electrophoresis chamber**, with power supply

B.6 PROCEDURE

B.6.1 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Table B.1. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table B.1 have proven to be suitable.

Table B.1 Addition of reagents

Reagent	Final concentration	Volume per sample (µl)
Sample-DNA	10 ng to 50 ng[b]	2,00
Water		15,60
10x PCR buffer (without $MgCl_2$)	1 x	2,50
$MgCl_2$-solution[a], 25 mmol/l	2 mmol/l	2,00
dNTP solution, 2,5 mmol/l	0,2 mmol/l	2,00
Primer forward, 20 µmol/l	0,2 µmol/l	0,25
Primer reverse, 20 µmol/l	0,2 µmol/l	0,25
Taq DNA polymerase, 5 IU/µl	2 IU	0,40

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 1,5 mmol/l.

[b] Only small DNA amounts are available in honey samples. Nevertheless dilute the DNA solution at least 1:2.

B.6.2 PCR controls

The following controls should be analysed in parallel:

- NTC (no template control), with ddH_2O instead of sample DNA
  This control is used to demonstrate the absence of contaminating nucleic acids in the reagents.

- Extraction blank control

  The extraction blank control is necessary to check for contamination during the extraction step.
- Positive Control

  The positive control should represent the target sequence under study, e.g. *Brassica napus* DNA.

  This control is used to demonstrate that the PCR reagents are working as intended.
- Negative Control

  The negative control should not contain the target sequence under study, e.g. *bos taurus* DNA.

  This control demonstrates that the results of analyses of test samples not containing the target sequence will be negative.

B.6.3 Temperature-time-programme

The temperature-time-programme as outlined in Table B.2 has been used for the validation study using thermal cycler GeneAmp® 2400, 9600 and AmpliTaq Gold® DNA polymerase[3]. The use of other thermal cyclers might make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used. If using a hot-start polymerase, the recommendation of the manufacturer should be adhered to unless the protocol states otherwise.

Table B.2 Temperature-time-programme

Process	Time [min:s]	Temperature [°C]	Cycles
Activation/initial denaturation	10:00	95	
Denaturation	0:30	95	35
Annealing	0:30	*plant-nes-2 system:* 60 *act system:* 57	
Extension	0:30	72	
Final extension	6:00	72	
	∞	4	

[3] GeneAmp® 2400 and 9600 and AmpliTaq Gold® DNA polymerase are examples of suitable products available commercially by Applied Biosystems, previously Perkin Elmer/Applied Biosystems. This information is given for the convenience of users of this Standard. Equivalent products can be used if they can be shown to give equivalent results.

B.7 IDENTIFICATION

Identification is based on PCR product size of approx. 90 bp using plant-nes-2 PCR system or approx. 103 bp using act PCR system. The identity of the amplified product can be determined by real-time PCR using a specific oligonucleotide probe labelled with fluorescent dyes (Annex C).

B.8 GENERAL QUALITY ASSURANCE AND INTERPRETATION OF THE RESULTS

The target sequence is presumed to have been detected if the size of the PCR product corresponds to the expected length of the target DNA sequence, determined by comparison with products derived from reference materials (positive controls).

For identification purposes see B.7.

The detection of fragments with the appropriate size (approx. 90 bp and 103 bp, respectively) indicates that the sample DNA-solution contains amplifiable DNA of plant origin within the assessed limitations of specificity described in B.2.1.

Annex C

Specific methods for the detection of plant species in honey and pollen using real-time PCR

C.1 INTRODUCTION

This annex describes methods for the specific amplification and detection of specific gene fragments of plant species (see Table A.1) and of DNA originating from a specific gene generally present in plants.

For limitations, see C.6.

C.2 VALIDATION STATUS AND PERFORMANCE CHARACTERISTICS

C.2.1 General

These methods describe a routine procedure using real-time PCR for the detection of species specific, single copy genes occurring in:

Sweet chestnut (*Castanea sativa*), lavender (*Lavandula* sp.), sunflower (*Helianthus annus*), acacia (*Acacia* sp.), citrus (*Citrus* sp.), erica (*Erica carnea*), rape (*Brassica napus*), lime (*Tilia platyphyllos*), rosemary (*Rosmarinus officinalis*), eucalyptus (*Eucalyptus erythrocorys*), oak (*Quercus* sp.), clover (*Trifolium* sp.), rockrose (*Cistus* sp.), and olive (*Olea europaea*).

These methods can be used to identify DNA from mentioned plant species derived products (honey, pollen and other food products as well as seeds and feed).

For the general proof of the presence of plants two further real-time PCR systems are described in this document, i) is based on the *tRNA-Leu* (trnL) gene, a chloroplast sequence and ii) is based on the amplification of a region of the *actin* gene. The former is suited for the detection of plants if only small DNA amounts are available. A plant cell normally contains multiple copies of this DNA sequence. The number of copies per cell may vary between plant species and tissues. Therefore this method may not be used as a control for quantitative purposes. In contrast to that the second system is presumed to be based on a single-copy gene and is suitable for a quantitative determination (see also Annex B).

C.2.2 Molecular specificity

C.2.2.1 General

The methods have been designed to target a sequence described in Table A.1.

C.2.2.2 Theoretical

Considering plant species-specific DNA sequences no sequence similarity with DNA sequences of other plants have been found (NCBI BlastN® search, March 2005). For the universal plant sequence no significant sequence similarity with DNA sequences of non plant organisms has been found.
The number of target sequence copies was not determined, but was presumed to be single-copy genes with the exception of the target sequence which is based on tRNA-Leu gene. This sequence is a multi-copy one.

C.2.2.3 Experimental

The specificity of the primer-probe systems in PCR was studied with DNA from twenty-six plant species and two animal species (see A.2.1.3) and additional 5 animals: duck (*Anas platyrhynchos*), goat (*capra hircus*), lamb (*Ovis aries*), pig (*Sus scrofa*) and turkey (*Meleagris gallopavo*). No cross reactions occurred using the species-specific primer-probe systems, except for sweet chestnut and oak. Amplified gene fragments showed high sequence similarity between both plant species. This result is comprehensible considering the fact that *Castanea sativa* and *Quercus sp.* are of the same subfamily *Quercoideae*. A delta C_T-value of about fifteen was detected between oak and sweet chestnut using the oak specific primer-probe system as well as a delta C_T-value of about fifteen for sweet chestnut amplified with this system and the act system. A delta C_T-value of about six was detected between oak and sweet chestnut using the sweet chestnut specific primer-probe system and a delta C_T-value of about five for oak amplified with this system and the act system. These delta C_T-values indicate a non-relevant quantity of a plant species in honey as the total DNA yield from honey is very low. All other species specific PCR assays appear to be highly specific for the DNA of appropriate plant species. Using the PCR systems for the detection of plants in general no amplification has been observed for DNA from animals.

C.2.3 Optimization

Optimization was carried out on the ABI PRISM® 7900 sequence detection system[1] (SDS) using TaqMan® chemistry[4]. Primer and probe design was carried out applying the Primer Express software (Applied Biosystems)[1].

C.2.4 Limit of detection (LOD), Limit of quantitation (LOQ)

This work was done with dilutions series of DNA from the fourteen plant species. Ten-fold dilution series were tested in ten replicates. The results are shown in Table C.1. A LOD of at least 25 pg was

[4] These are examples of suitable products available commercially. This information is only given for the convenience of users of this Standard. Equivalent products may be used if they can be shown to lead to the same results.

determined for each developed system with a probability of 95 % to obtain data with C_T values lower than 45. The limit of quantification (LOQ) is the lowest level of analyte that can be reliably quantified, given a known number of target genome copies (Commission Recommendation 2004/787/EC). In the context of this study the LOQ corresponds to the lowest level of analyte for which relative standard deviation within the laboratory (RSD_r) is 30 % or less. Taking this into consideration the real-time PCR systems do have absolute LOQs of at least 250 pg. The efficiency of the developed systems is between 90 % and 117 % according to:

$E = 10^{-1/s} - 1$ (Vaerman *et al.*, 2004)

E = efficiency; s = slope (log DNA concentration against C_T).

The linearity of at least 3 $\log_{10}$ decades were confirmed by the strong linear relationship between C_T values and the $\log_{10}$ of the starting quantity up to $2{,}5*10^5$ pg ($r^2 > 0.99$) (data not shown). The repeatability was determined by measuring duplicates of three dilution steps on three different days. The results showed a relative standard deviation of up to 33 % (data not shown).

Table C.1 LOD, LOQ, efficiency and linearity determined for each system (genome copies: C)

System	**Specific for**	**LOD (pg)**	**LOQ (pg)**	**Efficiency**
Cas_PAL	sweet chestnut	10	100	90
Lav_HMG2	lavender	0,5	5	117*
Heli-all-nes	sunflower	25	250	97
Aca_adh1	acacia	10	10	98
Cit_adh1	citrus	0,5	5	97
Eri_adh1	erica	10	100	108*
Bras_lip	rape	1	10	102*
Til_nr	lime	25	250	98
Euc_LFY	eucalyptus	5	50	93
Ros_CSL	rosemary	25	250	96
Cis_DXR	rockrose	2,5	25	97
Olea_ole10	olive	25	250	99
Querc_PAL2	oak	10	100	91
Tri_act	clover	10	100	98
act	plant	1	10	107*
Plant nes-2 (multicopy sequence!)	plant	0,1	1	98

* Efficiency can't be above 100%, but uncertainty will always result in a range of efficiency which could be above 100%.

C.3 PRINCIPLE

Species-specific fragments are amplified by PCR using species-specific primer pairs (see Table A.2). The appropriate amplicon sizes are also given in Table A.2.

PCR products are measured over each PCR cycle (real-time) by means of a species-specific oligonucleotide probe. This oligonucleotide probe is labelled with two fluorescent dyes: FAM (6-carboxyfluorescein) as a reporter dye and TAMRA (6-carboxytetramethylrhodamine) as a quencher. For that purpose TaqMan® chemistry[5] is employed.

For the detection of plants in general an approx. 103 bp fragment of the *actin* gene sequence is amplified by PCR in a separate real-time PCR reaction using two *actin* gene specific primers, and the PCR products are measured during each PCR cycle by means of an *actin* gene specific TaqMan® probe[2]. The second plant system used for samples if only small amounts of DNA are available is called plant nes-2 system and amplifies an approx. 90 bp fragment of the trnL gene.

It is recommended to perform real-time PCR run with at least two dilutions of the DNA extracted from test samples (e.g. for honey samples a 1:2 and 1:8 dilution of the DNA solution) in order to monitor amplification capability. The data generated can be used to determine the presence of any PCR inhibitors. The C_T values obtained from two linear dilutions should be proportional to a certain C_T value difference (ΔC_T), e.g. a one-to-four dilution results in a ΔC_T of approximately 2. A smaller ΔC_T value indicates non-linear amplification which might be caused by PCR inhibitors.

[5] These are examples of suitable products available commercially. This information is given for the convenience of users of this Standard. Equivalent products may be used if they can be shown to lead to the same results.

Table C.2 Primers and probes to be used and expected amplicon length

Primer-probe systems and expected amplicon length	
System sweet chestnut	
Primer forward (Cas_PAL-f)	5′- CCA AAG AAG TAG AGG GTG CAA GA -3′
Primer reverse (Cas_PAL-r)	5′- GAA TTC TCT CAC CAG TTA GTA AAC TTG TT -3′
Probe (Cas_PAL-probe)	5′-(FAM)- AAA GCA GCA ATT CCT AAC CCA ATT AAG GAG T -(TAMRA)-3′
Amplicon (Cas_PAL)	144 bp
System lavender	
Primer forward (Lav_HMG2-f)	5′- CCA TAT CGC TCG TCT TCA ACA G -3′
Primer reverse (Lav_HMG2-r)	5′- TTG CCG GCG ATG GCA -3′
Probe (Lav_HMG2-f-probe)	5′-(FAM)- CAC TTG ATG CTC TGA AGC TTG GCG A -(TAMRA)-3′
Amplicon (Lav_HMG2)	74 bp
System sunflower	
Primer forward (Heli-all-nes f2)	5′- CGT CAA TAC TTG TTA ATA TTA TTA AGA ATT A -3′
Primer reverse (Heli-all r1)	5′- ATA GCT TGG CCC GTT TTC TT -3′
Probe (Heli-all-nes probe)	5′-(FAM)- ATG CAT ATT CCT CCA GCT CCC TG -(TAMRA)-3′
Amplicon (Heli-all-nes)	106 bp
System acacia	
Primer forward (Aca_adh1-f)	5′- TGG GGA ATG TCC ACA TTG TAA GT -3′
Primer reverse (Aca_adh1-r)	5′- TGG AGA ACC TGG TCT TGC CA -3′
Probe (Aca_adh1-probe)	5′-(FAM)- AGC ATA ACA CCC CTG TCA GTG TTA ATC CTA AGA A -(TAMRA)-3′
Amplicon (Aca_adh1)	107 bp
System citrus	
Primer forward (Cit_adh1-f)	5′- TGA CAG AGG CGT CAT GCT TAA C -3′
Primer reverse (Cit_adh1-r)	5′- TGA GGT TCC AAC GAA ATG ATA AAT A -3′
Probe (Cit_adh1-probe)	5′-(FAM)- CTT GCC ATT GAT GGA AAA TCT CGA TTT CC -(TAMRA)-3′
Amplicon (Cit_adh1)	83 bp
System erica	
Primer forward (Eri_adh1-f)	5′- GGA TAG GGG AGT GAT GAT CCA T -3′
Primer reverse (Eri_adh1-r)	5′- GAT GTT CCG ACG AAA TGG TAT ATG -3′
Probe (Eri_adh1-probe)	5′-(FAM)- TGC CGT TTT TAG AAA ACC TTG ATT TCC CA -(TAMRA)-3′
Amplicon (Eri_adh1)	81 bp
System rape	

Primer forward (Bras_lip-f2)	5′- CAC GAC CAC GTT CTT TGT TTT -3′
Primer reverse (Bras_lip-r)	5′- CCA CTA CAA CAT TCC ATC CCC -3′
Probe (Bras_lip-probe)	5′-(FAM)- TCA CCA AAA CCG CAG CAA GCA -(TAMRA)-3′
Amplicon (Bras_lip)	122 bp
System lime	
Primer forward (Til_nr-f)	5′- GGG CAT CGA ACA TGA GCT TT -3′
Primer reverse (Til_nr-r)	5′- TTC AAC GAG TTT GCA TGG GA -3′
Probe (Til_nr-probe)	5′-(FAM)- AAA GAA GAT GCG CCT ACA AGA CCT GTT GC -(TAMRA)-3′
Amplicon (Til_nr)	101 bp
System eucalyptus	
Primer forward (Euc_LFY-f)	5′- AGG AGC AAG TGG TGC AGC A -3′
Primer reverse (Euc_LFY-r)	5′- TCC TCT GTT GTT GGG CGC -3′
Probe (Euc_LFY-probe)	5′-(FAM)- TCA GAA AAG GAT CAG CTG GGC AGG G -(TAMRA)-3′
Amplicon (Euc_LFY)	96 bp
System rosemary	
Primer forward (Ros_CSL-f)	5′- GCG TAT GCG GCC AAC AG -3′
Primer reverse (Ros_CSL-r)	5′- ACT GCC CTT GAA GAA GAA ATG G -3′
Probe (Ros_CSL-probe)	5′-(FAM)- TGG CAG ATT CAT CAT TCT CCT TCA TTA GGA CC -(TAMRA)-3′
Amplicon (Ros_CSL)	74 bp
System rockrose	
Primer forward (Cis_DXR-f)	5′- AAT GTT CGA TGT CCT CCT GTT CTT -3′
Primer reverse (Cis_DXR-r)	5′- GCT CAC TGT TTC CAC CCA CAA -3′
Probe (Cis_DXR-probe)	5′-(FAM)- CCA ATG GAT CTC TCA ATG GCG CTG TC -(TAMRA)-3′
Amplicon (Cis_DXR)	94 bp
System olive	
Primer forward (Olea_ole10-f)	5′- TAC CAA AGC AAG GGT CGA AAT G -3′
Primer reverse (Olea_ole10-r2)	5′- AGG GAT AAA ATT GTT TTA CTT ACT TGG GT -3′
Probe (Olea_ole10-probe)	5′-(FAM)- TCG ATT GCG ACT TTT CGG GCA CC -(TAMRA)-3′
Amplicon (Olea_ole10)	96 bp
System oak	
Primer forward (Querc_PAL2-f)	5′- AAG AAG TAG AGG GCG CAA GG -3′
Primer reverse (Querc_PAL-r)	5′- GAA TTC TCT CAC CAG TTA GTA AAC TTG TT -3′

Probe (Querc_PAL-probe)	5′-(FAM)- AAA GCA GCA ATT CCT AAC CCA ATT AAG GAG T -(TAMRA)-3′
Amplicon (Querc_PAL2)	141 bp
System clover	
Primer forward (Tri_act-f)	5′- CTG GCC GTG ATC TAA CTG AAT CTT -3′
Primer reverse (Tri_act-r)	5′- ATC AGG AAG CTC ATA GTT TTT CTC AAT T -3′
Probe (Tri_act-probe)	5′-(FAM)- AAG TTC TTG TTC ATA ATC CAC AGC AAC ATA GGC AA -(TAMRA)-3′
Amplicon (Tri_act)	191 bp
System plant	
Primer forward (plant nes-2-f)	5′- ATT GAG CCT TGG TAT GGA AAC CT -3′
Primer reverse (plant nes-2-r)	5′- GGA TTT GGC TCA GGA TTG CC -3′
Probe (plant nes-2-probe)	5′-(FAM)- TTA ATT CCA GGG TTT CTC TGA ATT TGA AAG TT -(TAMRA)-3′
Amplicon (plant nes-2)	approx. 90 bp
Primer forward (act-f)	5′- CAA GCA GCA TGA AGA TCA AGG T -3′
Primer reverse (act-r)	5′- CAC ATC TGT TGG AAA GTG CTG AG -3′
Probe (act-probe)	5′-(FAM)- CCT CCA ATC CAG ACA CTG TAC TTY CTC TC -(TAMRA)-3′
Amplicon (act)	approx. 103 bp

Abbreviations: Cas_PAL: phenylalanine ammonia-lyase (PAL) gene of sweet chestnut; Lav_HMG2: Hydroxymethylglutaryl coenzyme A reductase gene of lavender; Heli-all-nes: Profilin gene of sunflower; Aca_adh1: adh1 gene of acacia; Cit_adh1: adh1 gene of citrus; Eri_adh1: adh1 gene of erica; Bras_lip: Lipase 1 gene of rape; Til_nr : Nitrate reductase (nr1) gene of lime; Euc_LFY: Leafy/Floricaula (LFY1) gene of eucalyptus; Ros_CSL: adh1 gene of rosemary; Cis_DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) gene of rockrose; Olea_ole10: Allergen Ole e 10 gene of olive; Querc_PAL2: phenylalanine ammonia-lyase (PAL) gene of oak; Tri_act: actin gene of clover; plant nes-2: tRNA-Leu (trnL) gene of plant species; act: actin gene of plant species.
Y = C/T (IUB Code)

C.4 REAGENTS

C.4.1 General

C.4.2 Water

C.4.3 **PCR buffer** (without $MgCl_2$), 10-fold

C.4.4 **$MgCl_2$ solution,** $c(MgCl_2)$ = 25 mmol/l

C.4.5 **dNTP solution,** c(dNTP) = 2,5 mmol/l (each)

C.4.6 **Oligonucleotides** (details see Table A.2, A.4)

C.4.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase[6]

C.4.8 **Uracil *N*-glycosylase** (optional)

C.5 APPARATUS

C.5.1 General

Standard laboratory apparatus should be used throughout unless specified otherwise.

C.5.2 Thermal cycler

The indicated temperature-time profile was originally tested with ABI PRISM® 7900 SDS (Applied Biosystems)[3]. Other real-time PCR detection systems may be used after adaption of the reaction conditions.

C.5.3 Reaction vials

The reaction vials shall be suitable for PCR amplification on a thermal cycler, e.g. MicroAmp® optical tubess/96-well optical Reaction Plate (Applied Biosystems) [3].

[6] These are examples of suitable products available commercially. This information is given for the convenience of users of this Standard. Equivalent products may be used if they can be shown to lead to the same results.

C.5.4 Procedure: PCR set-up

The PCR set-up for the plant gene target sequence and for the plant species-specific target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is described for a total PCR volume of 10 µl per reaction mixture with the reagents as listed in Table C.3.

Table C.3 Amplification reaction mixture in the final volume/concentration per reaction vial

Total volume		10 µl
Template DNA (maximal amount 200 ng)		2 µl
DNA polymerase	AmpliTaq Gold®	1,5 IU
Decontamination system	dUTP	400 µmol/l
	AmpErase uracil *N*-glycosylase	0,5 IU
Reaction buffer	TaqMan® buffer A (containing passive reference ROX)[a]	1 X
	$MgCl_2$	see Table C.4
Primers	see Table C.4	see Table C.4
dNTP	dATP, dCTP, dGTP	200 µmol/l each
Probe	see Table C.4	200 nmol/l

[a] ROX = carboxy-X-rhodamine

Table C.4 Optimum concentration of primers, probes, and magnesium chloride ($MgCl_2$) for TaqMan™ PCR determined by titration

System	Forward primer (nmol/l)	Reverse primer (nmol/l)	Probe (nmol/l)	$MgCl_2$ (mmol/l)
Cas-PAL	300	900	200	7
Lav_HMG2	300	300	200	6
Heli-all-nes	900	300	200	5
Aca_adh1	300	300	200	8
Cit_adh1	300	300	200	5
Eri_adh1	300	300	200	8
Bras_lip	900	50	200	6
Til_nr	300	900	200	7
Euc_LFY	300	900	200	5
Ros_CSL	900	900	200	5,5
Cis_DXR	300	900	200	8
Olea_ole10	300	900	200	4
Querc_PAL2	900	300	200	6
Tri_act	900	300	200	4
act	900	900	200	8

C.5.5 PCR controls

As a positive control 100 % of the appropriate plant may be used.

Any other appropriate controls should be included as described in A6.2.

C.5.6 Temperature-time programme

The temperature-time programme as outlined in Table A.5 was optimized for the ABI PRISM® 7900 sequence detection system (SDS)[7] (Applied Biosystems). In the validation study, it was used in combination with the AmpliTaq Gold® DNA polymerase[4]. The use of other thermal cyclers may require specific adaptation. The time for activation/initial denaturation depends on the polymerase used.

Table C.5 describes the reaction conditions.

[7] These are examples of suitable products available commercially. This information is only given for the convenience of users of this Standard. Equivalent products may be used if they can be shown to lead to the same results.

Table C.5 Procedure: Reaction conditions

Process		**Time [min:s]**	**Temperature [°C]**
Pre-PCR: decontamination		02:00	50
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		10:00	95
PCR (45 cycles)			
Step 1	Denaturation	00:15	95
Step 2	Annealing and elongation	01:00	60

C.6 LIMITATIONS OF THE RESULTS

Polyploidy is widespread in the plant kingdom. Polyploidy is the condition where cells have more than one set of chromosomes, as supposed to haploidy. In most organisms which reproduce by sexual reproduction the usual state of cells is diploidy. Some plants (especially flowering plants) exhibit triploidy and tetraploidy, or even octoploidy, e.g. in strawberry plants and sugar cane. Polyploidy is a route by which plants could increase their chromosome numbers, and the size of their genome. Polyploid plants are often larger and show other accentuated characteristics, making them useful in agriculture and horticulture. (http://wiki.cotch.net/index.php/Main_Page)

Due to different degrees of ploidy of plant species it is mostly impossible to determine the amount of a species in relation to the total plant content in the investigated foodstuff using real-time PCR based on the calculation of haploid genomes.

Analysing honey there is an additional aspect that e.g. lavender pollen is strongly under-represented and even absent in various honeys e.g. in comparison to chestnut. Another approach to characterise honeys is the use of a combination of markers including the detection of bee DNA to characterise the origin of honey.

NOTE If food processing has lead to degradation or removal of DNA, the described method does not yield reliable results.

C.7 INTERPRETATION OF RESULTS

The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline and within the exponential increase phase (which looks linear in the log transformation). The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

The C_T value is related directly to the amount of PCR product and, therefore, related to the original amount of target present in the PCR. A low C_T value means high level, and a high C_T value means low level of initial amount of target DNA.

If quantitative determinations would like to be tested the standard curve method should be used. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. Following calculation is used:

A plot of the logarithm of initial target copy number for a set of standards versus C_T is a straight line. Quantification of the amount of target in unknown samples is accomplished by measuring C_T and using the standard curve to determine starting copy number. The entire process of calculating C_Ts, preparing a standard curve, and determining starting copy number for unknowns is performed by the software of the ABI. Afterwards the amount of the plant species is calculated in relation to the total amount of plant in the corresponding product.

REFERENCES

[1] Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N.: Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 1985, **230**, pp 1350-1354.

[2] Mullis, K.B. and Faloona, F.A.: Specific synthesis of DNA in vitro via a polymerase-catalyzed reaction. *Methods Enzymol.*, 1987, **155**, pp 335-350.

[3] Heaton, P.A.: Quantification of total DNA by spectroscopy in Analytical Molecular Biology: Quality and Validation, ed. Saunders, G.S and Parkes, H.C. *RSC publications*, 1999, U.K.

[4] Bickley and Hopkins: Inhibitors and enhancers of PCR in Analytical Molecular Biology: Quality and Validation, ed. Saunders, G.S and Parkes, H.C. *RSC publications*, 1999, U.K.

[5] Arumuganathan K. and Earle E.D.: Nuclear content of some important plant species, *Plant Mol. Biol. Rep*., **9(3)**, 1991, pp 208-218.