Bundesamt für
Verbraucherschutz und
Lebensmittelsicherheit

Sampling and analysis guidelines for the detection of genetic modifications in rice

This guidance document was established in close co-operation with the § 64 LFGB working group "Development of methods to identify foodstuffs produced by means of genetic engineering techniques" of the Federal Office of Consumer Protection and Food Safety (BVL) and with the assistance of the working group of food chemistry experts of the German federal states and of the BVL (ALS). It is a recommendation and shall contribute to the harmonisation of sampling and analysis of rice products in the framework of the food monitoring conducted by the official authorities of the German federal states (Länder) and as well by the in-house control of the food industry.

Due to recent findings of so far unidentified genetically modified rice lines in rice products (basmati rice) originating from other countries than China, it is appears necessary to recommend minimum requirements for the analysis of rice samples for genetic modifications and to make recently developed detection methods generally available.

The control of rice products originating from China at EU entry points should be performed according to the sampling and analysis procedures provided in the Commission Implementation Decision of 22 December 2011 (2011/884/EU [1]).

Sampling

Sampling of rice used for food purposes is done on the basis of the 'Implementation Decision of the European Commission' [1].

Accordingly, the size of the laboratory sample is 2.5 kg, in case of processed food this sample could be reduced to 500 g.

In the case of grain samples, four sub-samples with 10,000 grains of rice (equivalent to 240 g of [1]) are taken from the thoroughly mixed laboratory sample. If necessary, the 1000-grain weight is determined before sub-sampling. For processed products such as flour, pasta or starch, the number of sub-samples can be reduced.

With this procedure a relative detection limit of 0.03% at a confidence level of 99% is achievable for rice grains.

Sample preparation and DNA extraction

The four sub-samples are each milled finely and analyzed separately in the next steps.

From each analytical sample usually two DNA extracts are made. It is recommended to use at least 1 g of the milled material for the DNA extraction.

Successful DNA extractions have been carried out using the methods given in the appendices of ISO 21571 [2], particularly the DNA extraction using the CTAB method (ISO 21571, Annex A.3.1). A further method, particularly validated for the extraction of DNA from packaged (homogenized) rice products, is described in [3].

Optionally other extraction and purification procedures (kit systems) can be used, if they perform comparable.

Note: If rice grains are analysed and the size of test portion is reduced it is necessary to ensure for the analysis sample that sufficient particles of each milled grain are present. This is achievable only by fine grinding.

PCR analysis

Each DNA extract is at first subjected to the PCR screening methods described in step 1 (see page 2) analysing for the presence of the different genetically modified genetic elements. The amount of DNA in the PCR test should be at least 100 ng (photometrically determined) per reaction.

Assuming a haploid genome weight of approximately 0.47 pg for rice [4], this corresponds to at least 200,000 copies of a single-copy target sequence which should be present in each PCR reaction. For PCR methods targeting genetically modified genetic elements or constructs with a sensitivity of 20 copies or less, a relative detection limit of 0.01% or lower should be achievable.

For the detection of genetically modified rice the following analysis steps applying the given real-time PCR methods are recommended:

Screening (step 1)

Detection of P-35S, T-nos and cry1Ab/Ac (element-specific screening)

- Real-time PCR method for the amplification of a DNA sequence of the 35S promoter (P-35S) from the cauliflower mosaic virus (CaMV) [5,6]
- Real-time PCR method for the amplification of a DNA sequence of the terminator region of the nopaline synthase gene (T-*nos*) from *Agrobacterium tumefaciens* [6,7]
- Real-time PCR method for the amplification of a DNA sequence of cryIAb/cryIAc gene [8, 9, 10]. The procedure is described in detail in **annex 1**.
- Real-time PCR method for the amplification of a fragment of the rice DNA for determining the practical detection limit. For this test several methods based on the detection of *gos9*-, *pld*- or the *sps*-gene are available [3, 11, 12].

Construct-specific detection (step 2)

Detection of Pubi-cry, P35S-hpt (175bp) and cryIAc-T-nos

- Real-time PCR method for the amplification of a DNA sequence of the junction region of a construct consisting of the ubiquitin promoter (*Pubi*) from maize (*Zea mays*) and the Bt-toxin cryIAb/cryIAc gene from *Bacillus thuringiensis* [13]
- Real-time PCR method for the amplification of a DNA sequence of the junction region of the 35S promoter (*P-35S*) from cauliflower mosaic virus (CaMV) into a sequence of the hygromycin-resistance gene (*hpt*) from *Escherichia coli* [14]. The procedure is described in detail in **annex 2**.

  Note: To detect a wide range of P35S-hpt constructs, this method amplifies a larger segment than the 35S-hpt (90bp) method described in [13].

- Real-time PCR method for the amplification of a DNA sequence of the junction region of a construct consisting of the Bt toxin *cryIAc* gene from *Bacillus thuringiensis* and the terminator region of the nopaline synthase gene (T-*nos*) from *Agrobacterium tumefaciens* according to Akiyama et al. [15]. The procedure is described in detail in **annex 3**.
- Real-time PCR method for the amplification of a Bt63 (TT51-1) specific DNA sequence from the transition region of a construct consisting of the Bt toxin *cryIA(c)* gene from *Bacillus thuringiensis* and the terminator region of the nopaline synthase gene (T-*nos*) from *Agrobacterium tumefaciens* [3].

Event-specific detection (step 3, optional)

- Real-time PCR-based event-specific detection of Bt63 (TT51-1) [16]
- Real-time PCR-based event-specific detection of Kefeng6 rice [17]
- Real-time PCR-based event-specific detection of Kefeng8 rice [9]
- Real-time PCR-based event-specific detection of KMD1 rice [18]

Detection of DNA from other species

For the detection of maize, soybean and cotton DNA in sample DNA extracts that showed positive PCR results in step 1 and step 2 , the detection methods for the *hmg*, the *lectin* or the *sah7* reference gene can be used [19, 20, 21].

Interpretation of PCR results

All negative and positive controls shall have the expected result (see ISO 24276 [22]).

In the case of a positive screening result (step 1) the PCR-positive sample DNA extracts are further analyzed in the next step with construct-specific PCR methods (step 2). A positive result from step 2 can be specified in step 3 (optional).

If in at least one of the sub-samples a particular target sequence is detected, this result holds true for the entire laboratory sample.

For positive results it should be shown that the tests for amplifiable DNA from the other relevant plant species such as maize, soybean and cotton or possibly cauliflower mosaic virus (CaMV) are negative [23].

Note: *If all controls for other target species DNAs than rice are negative, it can be considered that genetically modified rice material is present in the sample in the case of positive results solely in step 1.*

To evaluate the PCR results, the screening table for genetically modified rice lines shown in **annex 4** can be used, describing all experimental and theoretical verification data so far determined for the specified detection methods.

Annex 1

Real-time PCR for element-specific detection of cry1Ab/cry1Ac DNA-sequences

Sensitivity: <20 copies (in-house validation)

Specifity:

Detection	experimentally verified	theoretically verified (GenBank Acc.No.)
positive	rice: Bt63, KeFeng6, KMD1 maize: Bt11 soy: MON87701 cotton: MON531, MON15985	rice: Bt-ZJ22 (HQ154128) cotton: GK-12 (GU583854), Xinmian-33B (GU583853), Lumianyan-15(GU583855) eggplant: Event EE-1 (DM460255)
negative	maize: MON810, MIR604, CBH-351, DAS59122, MON863, TC1507, MON88017, MON 89034 cotton: 281-24-236 x 3006-210-23	

Amplicon length: 74 bp

Amplicon sequence:

5′-GAGGAAATGCGTATTCAATTCAACnACATGAACAGCGCCTTGACCACAGCnnnnCCATTGTTCGCAGTCCAGAA-3′

Table 1 — Oligonucleotides

Name	DNA Sequence of the Oligonucleotides	Final concentration in PCR
cry1Ab / cry1Ac as target sequence [8,9,10]		
Bt-F1(mod)	5'-gAg gAA ATg CgT ATT CAA TTC AAC -3'	400 nmol/l
Bt-R	5'- TTC Tgg ACT gCg AAC AAT gg -3'	400 nmol/l
Bt-P	5'-FAM- ACA TgA ACA gCg CCT TgA CCA CAg C-TAMRA-3'[a]	100 nmol/l
[a]Equivalent reporter dyes and/or quencher can be used.		

Table 2 — Addition of Reagents

Total reaction volume	25 µl
Sample DNA (up to 200 ng) or control DNA	5 µl
PCR buffer solution[1] (including $MgCl_2$, dNTPs, and DNA polymerase)	12,5 µl
primer Bt-F1(mod) and Bt-R	see Tabelle 1
probe Bt-P	see Tabelle 1
Water	add to 25 µl

[1] if Taqman Universal Mastermix is used; other mastermix reagents can be used if they yield similar or better results; the temperature-time programme needs then appropriate adaption.

Table 3 — Temperature-time programme[1]

Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial Denaturation		95 ℃	10 min	no	1
2	Amplification	Denaturation	95 ℃	15 s	no	45
		Annealing and Elongation	60 ℃	60 s	yes	

Note:

To obtain better consensus of primer Bt-F1(mod) with known cry1Ab/cry1Ac DNA sequences, this primer sequence is optimised. The first two bases of primer Bt-F1(mod) deviate from the sequence describe in [9] and [10].

Annex 2

Real-time PCR for construct-specific detection of P35S–hpt (175bp) DNA sequences

Sensitivity: <20 copies (in-house validation)

Specifity:

Detection	experimentally verified	theoretically verified (GenBank Acc.No.)
positive	rice: KeFeng6, KMD1, unknown events in basmati rice (RASFF 2011.1646, 2012.0252)	99% homology with binary plant transformation vectors containing a 35S-hpt construct
negative	maize: Bt11, Mon810, Nk603, TC1507 rice: Bt63, LL601 soya: 40-3-2, A2704-12, A5547-127 cotton: Mon15985 papaya: Sunup papaya	rice: KeFeng8 , LL62, LL601, Bt-ZJ22 (HQ154128)

Amplicon length: 175 bp (in KeFeng6); 139bp in KMD1; 135bp in GM basmati

Amplicon sequence in Kefeng6:

5′-GACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGA GGACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCGAGCTTTCGCAGATCCGGGGGGCAATGAGATATGAA AAAGCCTGAACTCA-3′

Table 1 — Oligonucleotides

Name	DNA Sequence of the Oligonucleotides	Final concentration in PCR
P-35S – hpt as target sequence [14]		
35S-F [24]	5‘- gAC gTA Agg gAT gAC gCA CAA -3‘	400 nmol/l
652-R (hpt166-R)	5’– TgA gTT CAg gCT TTT TCA TAT CTC AT -3’	400 nmol/l
35S-T [24]	5‘-FAM-CCC ACT ATC CTT CgC AAg ACC CTT CC-TAMRA-3‘[a]	100 nmol/l
[a]Equivalent reporter dyes and/or quencher can be used.		

Table 2 — Addition of Reagents

Total reaction volume	25 µl
Sample DNA (up to 200 ng) or control DNA	5 µl
PCR buffer solution[1] (including $MgCl_2$, dNTPs, and DNA polymerase)	12,5 µl
primers 35S-F and 652-R (hpt166-R)	see Tabelle 1
probe 35S-TP	see Tabelle 1
Water	add to 25 µl

[1] if Taqman Universal Mastermix is used; other mastermix reagents can be used if they yield similar or better results; the temperature-time programme needs then appropriate adaption.

Table 3 — Temperature-time programme[1]

Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial Denaturation		95 ℃	10 min	no	1
2	Amplification	Denaturation	95 ℃	15 s	no	45
		Annealing and Elongation	60 ℃	60 s	yes	

Note:

To detect a broader spectrum of P-35S-hpt constructs, this method amplifies a larger fragment as compared to the 35S-hpt (90bp) method described in [13].

Annex 3

Real-time PCR for construct-specific detection of cry1Ab/Ac- T-nos DNA sequences (KeFeng-like rice lines)

Sensitivity: <20 copies (in-house validation)
Specifity:

Detection	Experimentally verified	Theoretically verified (GenBank Acc.No.)
positive	rice: KeFeng6, unknown Events in basmati rice (RASFF 2011.1646, 2012.0252)	
negative	rice: Bt63, KMD1, LL62 maize: Bt11	rice: Bt-ZJ22 (HQ154128) maize : Bt176, MON810

Amplicon length: 142 bp
Amplicon sequence in Kefeng6:
5′-GCAGGAGTGATTATCGACAGATTCGAGTTCATTCCAGTTACTGCAACACTCGAGGCTGAATGAGAATTCGGTACCCCGACCTGCAGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTT-3′

Table 1 — Oligonucleotides

Name	DNA Sequence of the Oligonucleotides	Final concentration in PCR
cry1Ab/Ac – T-nos as target sequence [15]		
T51-SF-2	5‘- gCA ggA gTg ATT ATC gAC AgA TTC -3‘	750 nmol/l
OsNOS-R2	5‘- AAg ACC ggC AAC Agg ATT CA -3‘	750 nmol/l
NGMr-Taq	5‘-FAM- AAT gAg AAT TCg gTA CCC CgA CCT gCA -BBQ-3‘[a]	150 nmol/l
[a]Equivalent reporter dyes and/or quencher can be used.		

Table 2 — Addition of Reagents

Total reaction volume	25 µl
Sample DNA (up to 200 ng) or control DNA	5 µl
PCR buffer solution[1] (including $MgCl_2$, dNTPs, and DNA polymerase)	12,5 µl
primer T51-SF-2 and OsNOS-R2	see Tabelle 1
probe NGMr-TAQ	see Tabelle 1
Water	add to 25 µl

[1] if Taqman Universal Mastermix is used; other mastermix reagents can be used if they yield similar or better results; the temperature-time programme needs then appropriate adaption.

Table 3 — Temperature-time programme[1]

Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial Denaturation		95 ℃	10 min	no	1
2	Amplification	Denaturation	95 ℃	20 s	no	45
		Annealing and Elongation	60 ℃	60 s	yes	

Note: To obtain better consensus of primer T51-SF-2 with known DNA sequences (see GenBank Nr. HQ161057 = Kefeng6) this primer sequence is optimised. The last four bases of primer T51-SF-2 deviate from the sequence described in [15].

Annex 4

Screening table of genetically modified (gm) rice events (of 06.03.2012)

Event	Origin	P-35S [5, 6]	T-nos [6, 7]	cry1Ab/ cry1Ac [8]	35S-bar [25]	P-ubi-cry1A(b) [13]	35S-hpt (90bp) [13]	P35S-hpt (175bp) [14]	cpti-Tnos [13]	cryIA(c)-Tnos [3]	cry1Ab/Ac-Tnos [15]
Bt63	**China**	**N**	**P**	**P**	**-**	**N**	**N**	**N**	**N**	**P**	**N**
Bt-ZJ22	**China**	**-**	**+**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
KeFeng 6	**China**	**P**	**P**	**P**	**-**	**P**	**P**	**P**	**P**	**N**	**P**
KeFeng 8	**China**	**+**	**+**	**+**	**-**	**+**	**-**	**-**	**+**	**-**	**+**
KMD1	**China**	**P**	**P**	**P**	**-**	**P**	**N**	**P**	**N**	**N**	**N**
LL62	**USA**	**P**	**N**	**N**	**P**	**-**	**-**	**-**	**-**	**-**	**-**
LL601	**USA**	**P**	**N**	**N**	**P**	**-**	**-**	**-**	**-**	**-**	**-**
LL604	**USA**	**+**	**+**	**-**	**+**	**-**	**-**	**-**	**-**	**-**	**-**
gm basmati	**unknown**	**P**	**P**	**P**	**-**	**P**	**N**	**P**	**N**	**N**	**P**

Legend

P = positive, experimentally verified
N = negative, experimentally verified
- = theoretically negative
+ = theoretically positive

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