Concept for seed analysis for genetically modified plant content

Sub-Committee for Method Development of the German National and Federal Länder Joint Committee on Genetic Engineering (LAG)

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Contents

1. Background

1.1. Objective

The Sub-committee for Method Development of the German National and Federal Länder Joint Committee on Genetic Engineering (LAG) proposes a uniform nation-wide procedure for control of genetically modified plant (GMP) content in conventional seeds. Here, it presents a concept for analyzing seeds using PCR methods as well as for evaluating the data and documenting the results.
The concept also essentially applies to the analysis of seeds from approved GMP for the presence of any GMP that have not been authorized for placing on the market in the EU, or where authorization does not cover cultivation purposes.

1.2. Scope

The concept presented here contains recommendations for specific procedures to analyze seeds for genetically modified plant content. It describes sample preparation, analysis methods as well as evaluation and documentation of the analysis results.

Actual sampling is not dealt with in this concept; it should adhere to the recognized regulations for seed trade control. The time point of sample collection should be chosen as early as possible so that the seed analysis is completed before the seeds are sown.

Information about genetically modified plants already authorized or under application for authorization worldwide and their relevant transformation events are available at the following web addresses: http://www.agbios.com, www.gmo-compass.org, http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm,, www.gmo-watch.org, http://gmoinfo.jrc.ec.europa.eu/, www.transgen.de, www.bvl.bund.de.

1.3. Methods

PCR methods are principally recommended for detection of genetic modifications.

The conventional and real-time PCR methods summarized in Appendices 1 and 4 of this concept, unless stated otherwise, have been validated in national or international ring trials.

Due to the expected introduction of threshold values for GMP in seeds, quantitative determination of the GMP content will be required in the future. Therefore, the current concept already addresses such requirements.

Real-time PCR represents a quantitative PCR procedure (see 3.4). However, qualitative PCR detection with appropriate statistical evaluation (sub-sampling, see 3.3) can also be used to determine the probability that a measured content is below or above a threshold value.

2. Selection and size range of seed samples

2.1 Sampling

Sampling should follow seed recognition and seed trading control practices [1] according to the appropriate regulations of the International Seed Testing Association (ISTA). Generally, this is carried out by official samplers of the seed certification and seed trade control offices. Appropriate criteria for creating suitable sampling strategies to ensure obtaining a representative seed sample are published [1-7]. This should minimize potential effects due to non-homogeniety in the seed lot.
In addition to a laboratory sample[1], a file sample should be prepared. This is required for follow-up analysis, if necessary.

2.2 Size of laboratory sample

Great care is usually taken during seed production to ensure that seed lots are as homogeneous as possible. In addition to controlled production, seed processing by the suppliers (pickling, coating) leads to further homogenization. As yet no data is available about the distribution of GMP components in seed lots.
Until such data is available, the concept here assumes that a sample is representative for the lot to be analyzed and that the GMP components are homogenously distributed in the sample.

The laboratory sample should provide sufficient seeds for analysis in the laboratory (see 2.3). Any further analysis should use the file sample of the same size.

The **test sample**[1] is prepared from the laboratory sample in the testing laboratory. The laboratory sample should be homogenized (mixed) before removing the test sample.

2.3 Size of test samples

According to experience the technically feasible detection limit[1] of a PCR reaction to detect genetically modified sequences in seeds is about 0.1%.
However, the exact detection limit for the qualitative PCR reactions (Appendix 1) and real-time PCR procedures (see Appendix 4) suggested here must be determined in each testing laboratory.

Example: Based on the technically feasible detection limit mentioned above, the size of the test sample should be calculated in such a way that a GMP content of at least 0.1% can be measured in the sample with a confidence level of 95%. The size range of the sample is calculated based on [8, 9] where n = number of seeds in all the analyzed test samples, V = maximum assumed GMP content in the seed batch in %, and P = confidence level in %:

$$n = \log [1-(P/100)]/\log[1-(V/100)]$$

Result: A test sample should contain at least 2995 seeds to detect a GMP content of 0.1% with a confidence level of 95%.

In contrast, if qualitative PCR combined with statistical predictions are used to estimate the probability that a test value[1] is below or above a given value, one can apply so-called **sub-**

[1] See Glossary.

sampling procedures according to Remund et al. [9]. Here, qualitative PCR detection is carried out with several test samples taken from a laboratory sample. The number and size of these test samples are based on the given test value (see 3.3).

3. GMP seed analysis

3.1 Test plan

The goal of analyzing seeds for GMP content is to determine the presence of both authorized as well as non-authorized GMP. To ensure that non-authorized GMP content can also be detected, the analysis procedure must be designed so that a single genetically modified seed can be detected in the test sample.

To obtain an analysis result of ≤ 0.1% with a confidence level of 95%, at least 2995 seeds must be analyzed. The procedure used must be able to detect one genetically modified seed in the test sample. Sub-sampling procedures (see 3.3) can mostly exclude the presence of genetically modified components not corresponding to a GMO in the sense of the Genetic Engineering Act (GenTG)[2] in seeds. Analysis can use qualitative PCR (see 3.2), or if available, quantitative PCR procedures (see 3.4).

The following two test plans are recommended:

1. Three test samples taken from the laboratory sample (sub-sampling)

Before grinding, the laboratory sample is divided into three test samples of 1000 seeds each. This procedure can be applied if the detection limit of each PCR used is at least 0.1%.
If no transgenic DNA sequence can be detected in any test sample, then the content of transgenic seeds in the seed sample is below 0.1% with a confidence level of 95%.

If only a few of the test samples give a positive result, it can mostly be excluded that the seeds contain genetically modified components not corresponding to a GMO in the sense of the GenTG.

2. One test sample taken from the laboratory sample

One test sample containing at least 2995 seeds is taken from the laboratory sample.
This procedure can be used if the detection limit of the applied PCR detection system is at least 0.03%.
If no transgenic DNA sequence can be detected in the test sample, then the content of transgenic seeds in the seed sample is below 0.1% with a confidence level of 95%.
In the case of a positive result with the test sample it should be clarified whether this result is caused by genetically modified components that do not correspond to a GMO in the sense of the GenTG. This involves subsequently using the sub-sampling procedure on the file sample (analyzing 3 test samples each with 1000 seeds).

[2] Genetically modified components that do not correspond to a GMO in the sense of the GenTG mean impurities that are not capable of germinating, e.g. pollen from GMOs or treatments containing GMO impurities.

3.2 Qualitative PCR

3.2.1 PCR methods

The seed homogenate is produced from the test sample by carefully grinding the material. A portion of this material is used for DNA extraction, if in-house laboratory method validation ensures that the detection limit (e.g. 0.1%) is reached (at least two DNA extractions = duplicate measurements).
Finally, from the extracted DNA the necessary amount of DNA is used for the PCR if in-house laboratory method validation ensures that the detection limit is reached. Multiple measurements are recommended.

Information about specific qualitative PCR detection (detectable transgenes, gene fragments, primer sequences, PCR parameters) can be found in Appendix 1 (conventional PCR) and Appendix 4 (real-time PCR). These also apply for the use of sub-sampling procedures (see 3.3).

3.2.2 Evaluation and documentation of qualitative PCR results

If the relevant DNA fragment is detected *in all DNA extractions* from a test sample using qualitative PCR, it is suggested that the results for the laboratory sample are presented according to the following format:

Sample description (e.g. variety, identification number)
Genetic modification detected (event(s))

Number of test samples analyzed:
Number of positive test samples:
Number of seeds in the test sample:
Type of detection (qualitative/quantitative):
Test method used (detected gene fragment):
Detection limit of the PCR (e.g. 0.1%):

If the DNA fragment in question *is not detected in any DNA extraction*, it is suggested that the results for the laboratory sample are presented according to the following format:

Sample description (e.g. variety, identification number)
Genetic modification not detected

Number of test samples analyzed:
Number of positive test samples:
Number of seeds in the test sample:
Type of detection (qualitative/quantitative):
Test method used (detected gene fragment):
Detection limit of the PCR (e.g. 0.1%):

Note on evaluating the measured results:
If the relevant DNA fragment *is only detected in one or a few DNA extractions* from a test sample using qualitative PCR, the analyses should be repeated, and if necessary, the results should be verified for reproducibility by using another method.This can include quantification using real-time PCR. If required, the file sample should be analyzed.

3.3 Testing GMP content using sub-sampling

It is possible to estimate the GMP content in the laboratory sample by dividing the laboratory sample into several test samples, followed by qualitative PCR analysis and evaluation of the results using statistical methods. This will reveal the fraction of transgenic seeds in the laboratory sample that contain at least one copy of the gene fragment to be detected.

If qualitative PCR detection (for methods see Appendix 1 and 4) are carried out in order to check a particular threshold, positive results require further sequential analysis of smaller sub-samples from the laboratory sample (sub-sampling). Evaluation of the positive/negative results from a range of test samples as described by Remund et al. [9] allows statistically significant conclusions to be made as to whether these are below or above a given GMP threshold.

The development and selection of an efficient test plan depends on how high the threshold has been set as well as the relative fraction of transgenic seeds, among other factors. This means the number of seeds in the test sample should correlate with the threshold to be controlled (test plan see flowchart in Appendix 2). The required parameters (number of seeds, number of test samples, evaluation of positive results) can be determined using the Excel-based software "seedcalc" [1][7] available from ISTA.

3.3.1 Testing the laboratory sample for GMP content

The first analysis step shall determine whether the laboratory sample contains GMP components. Assuming a detection limit of **0.1%** for the PCR detection system used, and taking this as the **threshold**, one shall analyze test samples containing at least 1000 seeds. This approach shall ensure that one GMP seed in 1000 will be detected. Statistically significant results can be obtained by analyzing at least **3 test samples** each containing **1000 seeds** (compare with 2.3).

If no GMP can be detected in any of the test samples, then this result is below the threshold of 0.1% with a confidence level of 95%. With one or a few GMP positive samples then further analyses are required to measure the probable content in the sample (see Appendix 2).

3.3.2 Testing further threshold values using sub-sampling procedures

Whether a detected GMP content has further consequences depends on the valid threshold value for this particular GMP. It is therefore necessary to identify the GMP before taking further measures.

Currently, there are no thresholds for seeds in the EU. Values between 0.1% and 1% are being discussed. The following examples are therefore only given to demonstrate the principle of the process. To establish the precise threshold to be checked, the number of seeds needed per test sample must be re-evaluated.

[1] Various additional statistic programs are available (as of February 2006: seedcalc3, seedcalc5 and seedcalc7) on the ISTA (International Seed Testing Association) website [7].

The following values were calculated using the Excel spreadsheet "seedcalc3.xls" [7]. The size of the test samples in the following tests is chosen so that the measured value is below the given threshold with a 95% confidence level if no GMP are detected in any of the 3 test samples. In the case of one or more GMP positive test samples, further analyses are required to determine the probable GMP content in the sample.

Example of a calculation using the Excel spreadsheet "seedcalc3.xls" (spreadsheet *Impurity Estimation*):

Threshold 0.3%

A threshold of 0.3% with a confidence level of 95% requires testing of 3 test samples. The number of seeds has to be adapted such that at a confidence level of 95% the upper limit of the GMP content does not exceed 0.3%.

Entries in the spreadsheet "seedcalc3.xls":
# of seed pools = 3;
of seeds per pool = 330;
deviant pools = 0;
desired confidence level = 95%

Result = computed % in sample = 0.00%;
Upper boundary of true % impurity = 0.30
Lower boundary of true % purity = 99.7

Threshold 0.5%

To control a threshold of 0.5%, **3 test samples** each with **200 seeds** shall be analyzed.

Threshold 0.9%

To control a test value of 0.9%, one must analyze **3 test samples** each with **110 seeds**.

If more precise quantification is required, further delimitation of the GMP content shall involve the sub-sampling procedure or, if available, real-time PCR.

Note: The threshold to be controlled should be taken into account in the amount of seeds requested for the laboratory sample (e.g. a threshold of 0.5% = at least 3600 seeds).

3.4 Real-time PCR

3.4.1 Real-time PCR methods

Real-time PCR can be used for qualitative GMP detection as well as for quantifying the genetically modified DNA content.

Information about validated real-time PCR methods currently available can be found in Appendix 4.

3.4.2 Evaluation and documentation of quantitative measurements

The test plan for analyzing seed samples using real-time PCR is shown as a flowchart in Appendix 3.

It has to be considered that real-time PCR involves only *relative* quantitative measurements of the GMP content in the samples. Generally, a species-specific gene (reference gene) is measured in parallel, which serves for "normalization" of the amount of DNA analyzed (determining the ratio between copy numbers of the transgene and the reference gene in the sample).

Quantitative measurements must take into account the measurement uncertainty. This is derived from the measurement uncertainty of the method and the measurement uncertainty due to the number of seeds analyzed.

The measurement uncertainty U_1 of the measurement method is according to [10]:

$$U_1 = \frac{t \times SD \times 100\%}{mean \times \sqrt{n}}$$

(SD = standard deviation, n = number of measurements, t = t-value for n-1 from the t-table)

The measurement uncertainty U_2 arising from the number of analyzed seeds is expressed according to [11] (modified) as:

$$U_2 = \frac{1 + \sqrt{1 + n \times V}}{n \times V} \times 100\%$$

(n = number of seeds analyzed, V = GMP content in the seed lot as a decimal number).

The total measurement uncertainty U_{total} for a measurement result can be derived from these correlations as:

$$U_{total} = \sqrt{U_1^{\,2} + U_2^{\,2}}$$

(U_1 = measurement uncertainty of the method, U_2 = measurement uncertainty due to the number of analyzed seeds).

Example: A test sample of 3000 seeds with a measured GMP content of 1% and measurement uncertainty of the method of 20%, results in a measurement uncertainty due to the number of seeds of 22% and thus a total measurement uncertainty of 30%.

It is suggested that the documentation of quantitative results should give the measurement uncertainty including the corresponding confidence probability P, taking into account the total measurement uncertainty as described above.
It is suggested that quantitative results should be presented in the following format:

Sample description (e.g. variety, identification number)
Genetic modification detected (event{s})

Number of test samples analyzed:
Number of positive test samples:
Number of seeds in the test sample:
Test method used (detected gene sequence):
Measured value:
Measurement uncertainty (with P = 95%):
Limit of quantification of the PCR:

Note: the given transgene content is based on the assumption that the ratio of the reference gene to the detected transgene is 1:X.

If the measurement result is below the limit of quantification[1], the following format is suggested; "Genetic modification detected but cannot be quantified".

The results of quantitative real-time PCR analysis of heterozygous maize grains, based on calculating the ratio of transgenic DNA copies compared to the number of DNA copies in the target species (in haploid genomes), can be considerably influenced by the degree of ploidy as well as the proportional mass of different tissues (endosperm, embryo and pericarp).

If this effect is know in conjunction with a sample to be analyzed, quantitative results should be confirmed with a sub-sampling analysis.

4. Concluding remarks

In justified individual cases the GMP content in seeds may be detected using PCR methods other than those presented here, as long as they can be shown to be equivalent.

In the case of a positive result (GMP detection) it needs to be clarified with those commissioning the work whether the result should be confirmed in a second laboratory. For statistical reasons a second analysis need not show the same result in a second laboratory if the result is within the range of the detection limit.

The Sub-committee for Method Development suggests that the German National and Federal Länder Joint Committee on Genetic Engineering (LAG) recommends the "Concept for seed analysis for genetically modified plant content" presented here to the Federal *Länder* as a working concept.

The Sub-committee for Method Development of LAG will continually develop the concept presented here based on state-of-the-art and best science.

[1] See Glossary

5. Glossary

Limit of quantification: concentration value where the relative error is less than a given threshold (usually 33%) (definition according to [10]).

Limit of detection: the lowest amount of an analyte in a sample that can be detected with a high given probability (mostly P = 95%) (definition according to [10]).

Laboratory sample: a sample prepared for delivery to a laboratory and intended for inspection and analysis (definition according to [3, 12]).

Threshold: stipulated value for the GMP content: samples must be controlled for exceeding this value. Once the threshold value for GMP content in seeds has been defined, this value corresponds to the test value.

file sample: a second laboratory sample intended for subsequent analysis if required.

test sample: a sample prepared for testing or analysis (definition according to [3, 12]); part of the laboratory sample, analyzed as one unit in the laboratory tests.

6. References

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[2] Kruse, M.: Probenahmepläne zur Nachprüfung der Verunreinigung von konventionellem Saatgut mit GVO Saatgut.VDLUFA-Schriftenreihe 57, Teil 2: 562 - 567 (2001)

[3] CEN/TS 15568 Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products – Sampling strategies.

[4] Whitaker, T., Freese, L., Giesbrecht, F. and Slate, A.: Sampling Grain Shipments To Detect Genetically Modified Seed. Journal of AOAC International 84:1941-1946 (2001)

[5] Lischer, P.: Sampling Procedures to Determine the Proportion of Genetically Modified Organisms in Raw Materials. Part I: Correct Sampling, Good Sampling Practice. Mitt. Lebensm. Hyg. 92: 290-304. (2001)

[6] Lischer, P.: Sampling Procedures to Determine the Proportion of Genetically Modified Organisms in Raw Materials. Part II: Sampling from Batches of Grain Mitt. Lebensm. Hyg. 92:305-311. (2001)

[7] ISTA (International Seed Testing Association): http://www.seedtest.org/upload/cms/user/Seedcalc8.zip

[8] http://archive.gipsa.usda.gov/biotech/samplingplan1.xls (Qualitative Test Sample Size Calculator im Tabellenblatt single)

[9] Remund, K., Dixon, D., Wright, D. and Holden L.: Statistical considerations in seed purity testing for transgenic traits Seed Science Research 11, 101-119 (2001)

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[11] Hübner, P., Waiblinger, H.-U., Pietsch, K. and P. Brodmann: Validation of PCR Methods for Quantitation of Genetically Modified Plants in Food. Journal of AOAC International 84: 1855-1864 (2001)

[12] ISO 24276:2006 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions.

13] ISO 21569:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Qualitative nucleic acid based methods.

[14] Official Collection of Methods under article 64 of the German Food and Feed Act (LFGB), Volume I (L), L 00.00-31: Screening method for detection of genetically modified DNA sequences in foodstuffs by detection of DNA sequences frequently present in genetically modified organisms 07-2001. Loose leaf edition as of Juli 2001, Berlin, Köln, Beuth Verlag GmbH

15] Official Collection of Methods under article 64 of the German Food and Feed Act (LFGB), Volume I (L), L 24.01-1: Detection of a genetic modification in potatoes by amplification of the genetically modified DNA sequence by means of the PCR (polymerase chain reaction) and hybridisation of the PCR product with a DNA probe 01-1997. Loose leaf edition as of January 1997, Berlin, Köln, Beuth Verlag GmbH

[16] Allmann et al. (1993), PCR - a possible alternative to immunochemical methods assuring safety and quality of food: Detection of wheat contamination in non-wheat food products. Z. Lebensm. Unters. Forsch. 196: 248 – 251

[17] Official Collection of Methods under article 64 of the German Food and Feed Act (LFGB), Volume I (L), L 15.05-1: Detection of genetic modifications in maize (Zea mays L.) by means of the PCR (polymerase chain reaction) and restriction analysis or hybridisation of the PCR product 02-2002. Loose leaf edition as of May 2002, Berlin, Köln, Beuth Verlag GmbH

[18] Tätigkeitsberichte (2002): Drei Nachweismethoden für transgene Pflanzen; Bericht des Unterausschusses Methodenentwicklung des Länderausschusses Gentechnik Bundesgesundheitsblatt 45: 925-934

[19] Hess N., Ulrich A., Hoffmann T. (2002): Insertionsspezifische Nachweisverfahren für transgene Pflanzenlinien unter Anwendung der inversen PCR. Bundesgesundheitsblatt 45: 626-633

20] Official Collection of Methods under article 64 of the German Food and Feed Act (LFGB), Volume I (L), L 23.01.22–1: Detection of a genetic modification of soybeans by amplification of the modified DNA sequence by means of the polymerase chain reaction (PCR) and hybridisation of the PCR product with a DNA probe. 03-1998. Loose leaf edition as of March 1998, Berlin, Köln, Beuth Verlag GmbH

[

21] ISO 21570:2005 Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods.

See also:
Method collection of the Sub-committee for Method Development of the German National and Federal Länder Joint Committee on Genetic Engineering (LAG): http://www.lag-gentechnik.de/

Appendix 1: Qualitative PCR detection using conventional PCR

The following PCR methods to detect GMP, unless stated otherwise, have been validated in ring trials. The following transgenic plants can be detected with the methods presented here:

- **Maize:** Bt176; Bt11, Bt10; T14; T25; MON810
- **Rapeseed:** Topas 19/2, Falcon GS40/90; Liberator pHoe6/Ac; GT73; GT200; MS1/RF1(RF2); MS8/RF3; fatty acid modified rapeseed with the pCaMV-*nptII* gene cassette
- **Sugar beet:** T120-7; H7-1; #77; T252; #203; GTSB77
- **Soybeans:** Roundup Ready Soybean (GTS 40-3-2)
- **Potatoes:** all events with the *nptII* gene: tBK50-13/tBK50-66; IEH 92-527-1; Bt6/Bt10/Bt12/Bt16/Bt17/Bt23; SPBT02; ATBT04; RBMT21/RBMT22; RBMT15; SEMT15; HLMT15-46

1. Screening methods

1.1 Maize and soybeans

Screening for the CaMV-35S promoter currently detects 16 transgenic maize events and 5 transgenic events of soybean. It should be carried out using the following methods:

- EN ISO 21569, Appendix B.1 [13] and § 64 LFGB, L 00.00-31 [14]
  35S-1 5'-GCT CCT ACA AAT GCC ATC A-3'
  35S-2 5'-GAT AGT GGG ATT GTG CGT CA-3'
  Annealing: 54°C product: 195 bp
  Restriction analysis: *Xmn* I: 115 bp + 80 bp

- EN ISO 21569, Appendix B.2 [13]
  35s-cf3 5'-CCA CGT CTT CAA AGC AAG TGG-3'
  35s-cr4 5'-TCC TCT CCA AAT GAA ATG AAC TTC C-3'
  Annealing: 62°C product: 123 bp

Two further maize events can be detected with additional screening for the NOS-terminator. One of the following methods can be used:

- EN ISO 21569, Appendix B.3 [13]
  HA-nos118f 5'-GCA TGA CGT TAT TTA TGA GAT GGG-3'
  HA-nos118r 5'-GAC ACC GCG CGC GAT AAT TTA TCC-3'
  Annealing: 62°C product: 118 bp

- § 64 LFGB, L 00.00-31 [14]
  NOS-1 5'-GAA TCC TGT TGC CGG TCT TG-3'
  NOS-3 5'-TTA TCC TAG TTT GCG CGC TA-3'
  Annealing: 54°C product: 180 bp
  Restriction analysis: *Nsi* I: 96 bp + 84 bp

1.2 Rapeseed

Screening for the CaMV-35S promoter in rapeseed is not useful, since many GM rapeseed events remain undetected, and the cauliflower mosaic virus (CaMV) can infect rapeseed, thus increasing the chances of false positives.
Currently, 13 known transgenic rapeseed events can be detected as long as four construct-specific tests are performed per sample (see 2.2).

1.3 Sugar beet

Here, screening is not useful due to the work involved. To detect 6 transgenic sugar beet stains, two construction-specific tests must be carried out for each sample (see 2.2).

1.4 Potatoes

The transgenic potatoes listed above can be detected using the following screening methods to detect the *nptII* gene [13, 14]:

- EN ISO 21569, Appendix B.4 [13] and § 64 LFGB, L 00.00-31 [14]
  APH2 short 5'-CTC ACC TTG CTC CTC CCG AGA-3'
  APH2 reverse 5'-CGC CTT GAG CCT GGC GAA CAG-3'
  Annealing: 60°C product: 215 bp
  Restriction analysis: *Rsa* I: 122 bp + 93 bp

- § 64 LFGB, L 00.00-31 [14]
  TN5-1 5'GGA TCT CCT GTC ATC T-3'
  TN5-2 5'-GAT CAT CCT GAT CGA C-3'
  Annealing: 50°C product: 173 bp
  Restriction analysis: *Rsa* I: 136 bp + 37 bp

A further recommended *nptII* detection method uses the following primers (Feldmann, unpublished):

npt-2F 5'-CTG ATG CCG CCG TGT TCC-3'
npt-2R 5'-ATG TTT CGC TTG GTG GTC-3'
Annealing: 53°C product: 234 bp + 89 bp
Restriction analysis: *Pst* I: 234 bp + 89 bp
(This method is not yet validated)

1.5 Control for DNA amplification (i.e. in the case of negative screening results)

As a PCR control, it is suggested to use the ***primers A1/A2*** (conserved chloroplast Leu-tRNA), which produce an amplification product of 531 bp for maize, 384 bp for rapeseed, 550 bp for potatoes and 644 bp for sugar beet.

- EN ISO 21569, Appendix A.2 [13]; § 64 LFGB L 24.01-1 [15] and Subcommittee for Method Development [18]
  A1 5'-CGA AAT CGC TAG ACG CTA CG-3'
  A2 5'-GGG GAT AGA GGG ACT TGA AC-3'
  Annealing: 55°C or 60°C

Alternatively, the ***EU primers*** (highly conserved 18S rRNA) can be used for a control PCR [16]. These produce a 136 bp amplification product in all plants [and other eukaryotes (animals, yeast)]. However, due to the high copy number of this sequence, the sample must be strongly diluted (suggestion 1:25).

EU-- 5'-TCT GCC CTA TCA ACT TTC GAT GGT A-3'
EU + 5'-AAT TTG CGC GCC TGC TGC CTT CCT T-3'
Annealing: 60°C
(This method is not yet validated)

2. Specific detection

2.1 Maize

For maize samples positive in screening tests it is necessary to differentiate between GMP that are authorized and non-authorized in the EU. Specific detection is described in the standard specification EN ISO 21569 [13] and in § 64 LFGB 15.05-1 [17]:

- **Bt 176** (construct-specific detection)

  EN ISO 21569, Appendix C.4 [13] and § 64 LFGB 15.05-1 [17]
  CRY03 5'-CTC TCG CCG TTC ATG TCC GT-3' (CDPK promoter)
  CRY04 5'-GGT CAG GCT CAG GCT GAT GT-3' (*crylA* (b) gene)
  Annealing: 63°C product: 211 bp
  Hybridization probe: DIG-ATGGACAACAACCCCAACATC
  Restriction analysis: *Taq* I: 168 bp + 22 bp + 21 bp

- **Bt 11 / Bt 10** (construct-specific detection)

  EN ISO 21569, Appendix C.3 [13] and § 64 LFGB 15.05-1 [17]
  IVS2-2 5'-CTG GGA GGC CAA GGT ATC TAA T-3' (*IVS2* intron)
  PAT-B 5'-GCT GCT GTA GCT GGC CTA ATC T-3' (*pat* gene)
  Annealing: 64°C product: 189 bp
  Hybridization probe: DIG-TATCTGTCTCAGGGGCAGACTC
  Restriction analysis: *Hinf* I: 73 bp + 116 bp

For further specification in the case of positive samples, a Bt10 event-specific PCR should be carried out: http://gmo-crl.jrc.ec.europa.eu/summaries/Bt10 Detection Protocol version2.pdf

JSF3 5'-CAC ACA GGA GAT TAT TAT AGG G-3'
JSR3 5'-GGG AAT AAG GGC GAC ACG G-3'
Annealing: 62°C product: 130 bp

- **T14 / T25** (construction-specific detection)

  EN ISO 21569, Appendix C.5 [13] and § 64 LFGB 15.05-1 [17]
  T25-F7 5'-ATG GTG GAT GGC ATG ATG TTG-3'
  T25-R3 5'-TGA GCG AAA CCC TAT AAG AAC CC-3'
  Annealing: 64°C product: 209 bp
  Restriction analysis: *Hinf* I: 121 bp + 88 bp
  Mwo I: 141 bp + 68 bp

  or:
  CaMV-F 5'-ATC CTT CGC AAG ACC CTT CCT C-3' (CaMV 35S promoter)

pac3-R 5'-CCC AAC CTT TGA TGC CTA TGT G-3' (*pat* gene)
Annealing: 60°C product: 370 bp
Restriction analysis: *EcoR*V: 140 bp + 230 bp
Sal I: 60 bp + 310 bp [18]

In the case of positive samples, T14 and T25 can be distinguished using an event-specific PCR [19] that is specific for T25:

P 369 5'-TGC TC GCT TGA CCT TGG TTG C-3'
P 370 5'-CTG ATG CGG TAT TTT CTC CTT ACG-3'
Annealing: 60°C product. 549 bp
Restriction analysis: *Ssp* I: 132 bp + 417 bp
(This method is not yet validated. It is not sure to reach the detection limit of 0.1%)

Alternatively, identification of T25 can be achieved by the event-specific real-time PCR of CRL/JRC described in Appendix 4.

- **Mon 810** (event-specific detection)

EN ISO 21569, Appendix D.1 [13] and § 64 LFGB 15.05-1 [17]
VW01 5'-TCG AAG GAC GAA GGA CTC TAA CG-3'
VW03 5'-TCC ATC TTT GGG ACC ACT GTC G-3'
Annealing: 64°C product: 170 bp
Restriction analysis: *Mwo* I: 109 bp + 61 bp
Hae III: 126 bp + 44 bp

2.2 Rapeseed and sugar beet

Qualitative detection of various gene constructs in transgenic rapeseed can use methods of the Sub-committee for Method Development of the LAG [18]. Similarly, 6 GM sugar beet events can be detected with two of these detection methods:

- **Topas 19/2, Falcon GS40/90, Liberator pHoe6/Ac (rapeseed, Aventis), T252 and T120-7 (sugar beet, Aventis)** [18]

CaMV-F 5'-ATC CTT CGC AAG ACC CTT CCT C-3'
pac3-R 5'-CCC AAC CTT TGA TGC CTA TGT G-3'
Annealing: 60°C product: 370 bp
Restriction analysis: *EcoR*V: 140 bp + 230 bp
Sal I: 60 bp + 310 bp

After a positive result in a p35S-pat PCR, detection of *nptII* (see 1.4) allows one to distinguish between **Topas 19/2 rapeseed (Aventis)**, which is authorized in the EU, and other non-authorized Aventis rapeseed varieties.

- **GT73, GT200 (rapeseed, Monsanto) as well as H7-1, #77, #203 and GTSB77 (sugar beet, Monsanto or Syngenta)** [18]

The primer sequences for these tests are confidential!
Enquiries should be directed to the Subcommittee for Method Development.

- **MS1/RF1+2 and MS8/RF3 (rapeseed, PGS)** [18]

  PGS-bar-A2 5'-GAA GTT GAC CGT GCT TGT CT-3' (pSSUAra promoter)
  PGS-bar-B2 5'-CAA GTC CAC CAG GCA AGT AA-3' (*bar* gene)
  Annealing; 54°C product: 624 bp (MS1/RF1) and 454 bp (MS8/RF3)
  Restriction analysis: Hind III: 357 bp + 267 bp (MS1/RF1) or
  187 bp + 267 bp (MS8/RF3)

- **Various rapeseed lines with altered fatty acid patterns [including Laurat rapeseed (pCGN3828) and LPAAT/Trierucin rapeseed (pRESS)]**
  http://www.lag-gentechnik.de/dokumente/ok_laurical_raps.pdf

Construct-specific detection:

35S-X 5'-ACG TTC CAA CCA CGT CTT CA-3' (CaMV 35S promoter)
NPTa 5'-GTC CCT TCC CGCTTC AGT GAC AAC GTC-3' (nptII gene)

For Laurat rapeseed: annealing: 57°C product 450 bp
Restriction analysis: *Eco*R I: 264 bp + 290 bp

For Trierucin rapeseed: annealing: 57°C product 450 bp
Restriction analysis: *Pst* I: 100 bp + 350 bp

- **Laurat rapeseed (pCGN3828)** (construct-specific detection)

  napin5'-F 5'-TGT CAC GCC AGG ACA TGA GCT ACA-3' (napin gene promoter)
  BayTe-R 5'-GGC CAT CAC GAG CCA ACA TTA CAG-3' (thioesterase gene)
  Annealing: 58-65°C product: 314 bp
  Restriction analysis: *Nde* I: 104 bp + 210 bp

- **LPAAT/Trierucin rapeseed (pRESS)** (gene-specific detection)

  LPAAT-F 5'-CCG CAA CAG GAG ACA ACT AAA-3' (acylglycerol-3-phosphate
  LPAAT-R 5'-TAT TGG GAG ATG TGA CTG AAG-3' acyltransferase gene)
  Annealing: 55-60°C product: 603 bp
  Restriction analysis: *Bam* HI: 346 bp + 257 bp

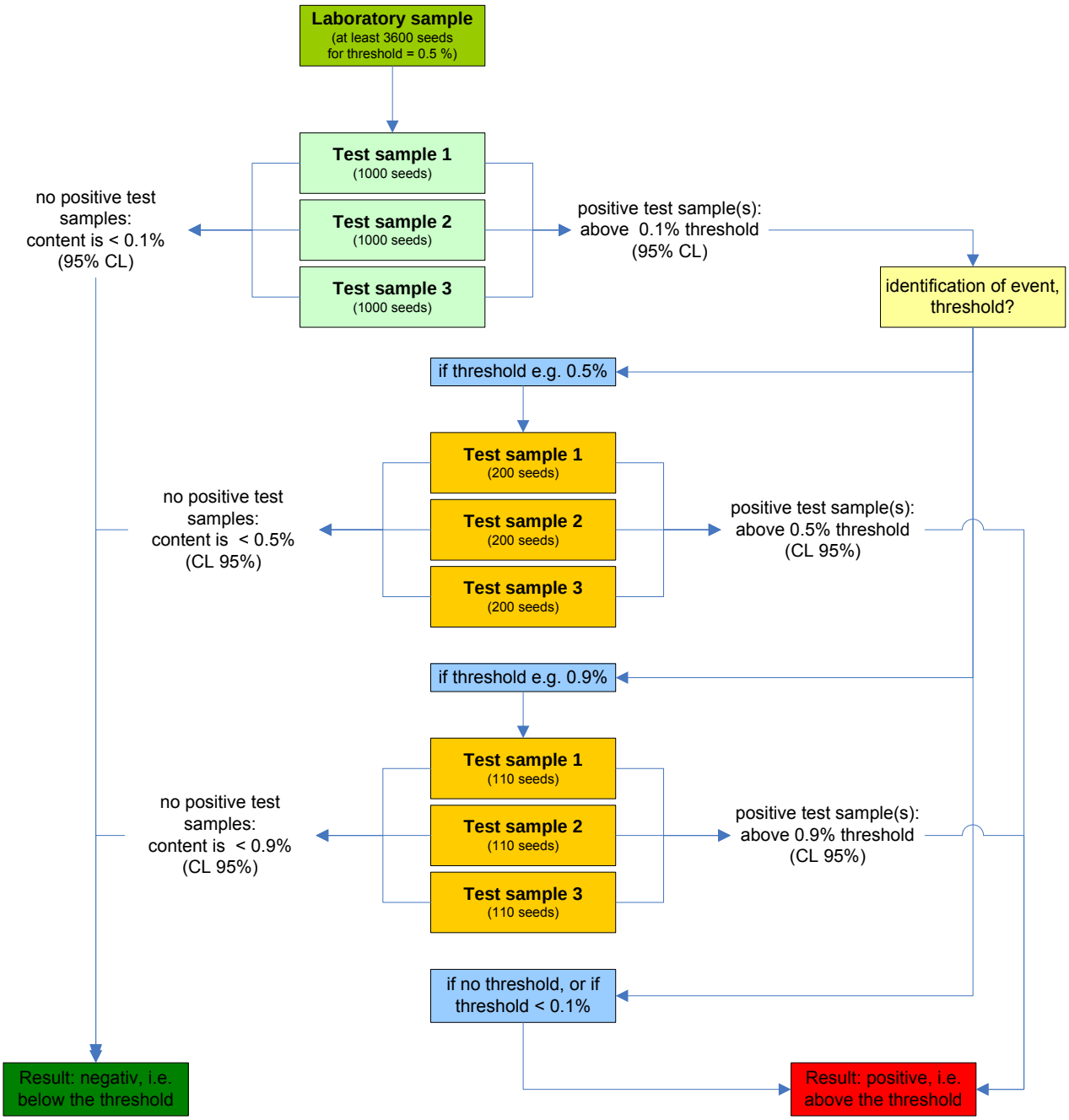
2.3 Soybeans

The following construct-specific method detects the transgenic soybean event GTS 40-3-2 (Monsanto) already authorized in the EU:

prEN ISO 21569, Appendix C.1 [13] and § 64 LFGB 23.01.22-1 [20]
p35s-f2 5'-TGA TGT GAT ATC TCC ACT GAC G-3' (35S CaMV promoter)
petu-r1 5'-TGT ATC CCT TGA GCC ATG TTG T-3' (petunia CTP1)
Annealing: 62°C product: 172 bp
Hybridization probe: FI-5'-GGG TCT TGC GAA GGA TAG TG-3'
Restriction analysis: *Bgl* II: 43 bp + 129 bp

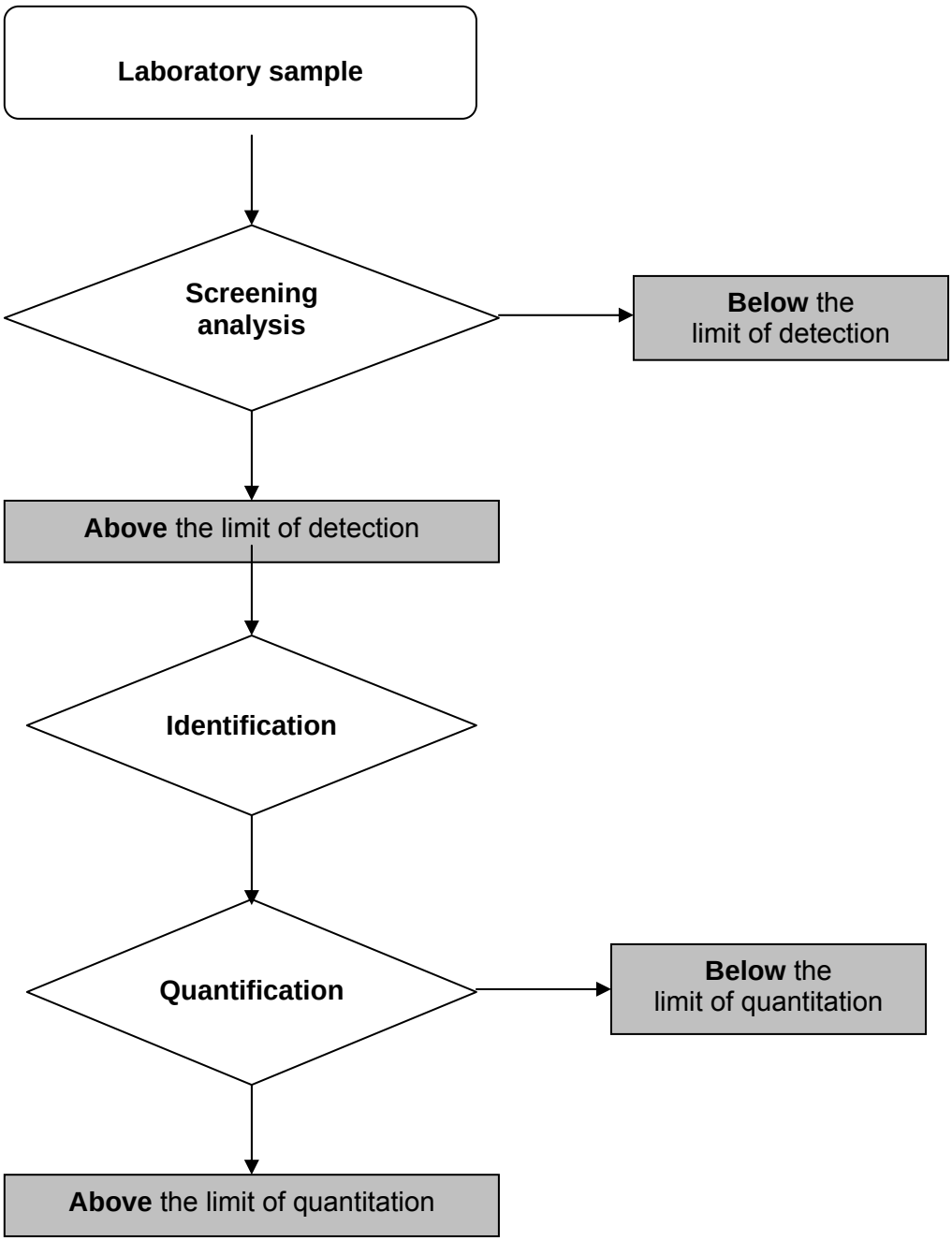
Appendix 2: Test plan for sub-sampling

Example of steps involved in analyzing seed samples for the presence of genetically modified events (sub-sampling)



CL 95% = confidence level of 95%

Appendix 3: Test plan for quantitative PCR detection



Appendix 4: Real-time PCR methods for qualitative and quantitative detection

1. Validated real-time PCR methods associated with authorization according to (EC) No. 1829/2003

Following regulations (EC) No. 1829/2003 and (EC) No. 641/2004 applicants for authorisation of GMO are required to provide a detection method for event-specific identification and quantitation of the GMO. Such a method is then validated by the Community Reference Laboratory (CRL) in cooperation with ENGL in international ring trials. If the method proves suitable it is published. Up to now all submitted methods are real-time PCR methods. The quantitative real-time PCR detection methods as well as the corresponding validation reports for the following GMOs are now published at: http://gmo-crl.jrc.it/statusofdoss.htm (as of March 2006):

Genetically modified maize events:	Bt11, NK603, GA21, MON863, MON810, TC1507, T25, 1507 x NK603, 59122, NK603 x MON810, MON863 x MON810, MON863 x NK603, MON863 x MON810 x NK603
Genetically modified sugar beet event:	H7-1

2. Methods according to DIN EN ISO 21570

The Appendices of standard DIN EN ISO 21570 describe real-time PCR methods for detecting the 35S promoter of the soybean event GTS 40-3-2 as well as various genetically modified maize events [21]. Quantitation should use the validated real-time PCR systems (combination of reference and specific system) of DIN EN ISO 21570.

Reference systems for maize

Alcohol dehydrogenase 1 gene (*adh1*):

Appendix A.1 For the taxonomic target group, specific methods for absolute quantitation of DNA of the *adh1* gene from maize

ADH-FF3	5'-CGT CGT TTC CCA TCT CTT CCT CC-3' (300 nmol/l)
ADH-RR4	5'-CCA CTC CGA GAC CCT CAG TC-3' (300 nmol/l)
ADH1-MDO	5'-*FAM*-AAT CAG GGC TCA TTT TCT CGC TCC TCA-*TAMRA*-3'[a] (200 nmol/l)
Product:	134 bp

High-mobility-group protein gene (*hmg*):

Appendix D.2 Event-specific method for quantitation of DNA from the maize event MON810

ZM1-F	5'-TTG GAC TAG AAA TCT CGT GCT GA-3' (300 nmol/l)
ZM2-R	5'-GCT ACA TAG GGA GCC TTG TCC T-3' (300 nmol/l)
Probe ZM1	5'-*FAM*-CAA TCC ACA CAA ACG CAC GCG TA-*TAMRA*-3'[a] (160 nmol/l)
Product:	79 bp

Maize starch synthase IIb gene (*zSSIIb*):

Appendix C.9	Construct-specific method for quantitation of DNA from the maize event T25	
	zSSIIb 1-5'	5'-CTC CCA ATC CTT TGA CAT CTG C-3' (500 nmol/l)
	zSSIIb 1-3'	5'-TCG ATT TCT CTC TTG GTG ACA GG-3' (500 nmol/l)
	zSSIIb-Taq	5'-*FAM*-AGC AAA GTC AGA GCG CTGCAA TGC A-*TAMRA*-3'[a] (200 nmol/l)
	Product:	151 bp

Reference systems for soybeans

Lectin gene (*le1*):

Appendix C.1	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	Lectin-F	5'-TCC ACC CCC ATC CAC ATT T-3' (900 nmol/l)
	Lectin-R	5'-GGC ATA GAA GGT GAA GTT GAA GGA-3' (900 nmol/l)
	Lectin-TMP	5'-*FAM*-AAC CGG TAG CGT TGC CAG CTT CG-*TAMRA*-3'[a] (100 nmol/l)
	Product:	81 bp
Appendix C.2	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	Lectin gene (le1):	
	GM1-F	5'-CCA GCT TCG CCG CTT CCT TC-3' (600 nmol/l)
	GM1-R	5'-GAA GGC AAG CCC ATC TGC AAG CC-3' (600 nmol/l)
	Probe GM1	5'-*FAM*-CTT CAC CTT CTA TGC CCC TGA CAC-*TAMRA*-3'[a] (120 nmol/l)
	Product:	74 bp
Appendix C.4	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	Lectin gene (le1):	
	Le1n02-5'	5'-GCC CTC TAC TCC ACC CCC A-3' (500 nmol/l)
	Le102-3'	5'-GCC CAT CTG CAA GCC TTT TT-3' (500 nmol/l)
	Le1-Taq	5'-*FAM*-AGC TTC GCC GCT TCC TTC AAC TTC AC-*TAMRA*-3'[a] (200 nmol/l)
	Product:	118 bp

Specific systems

Appendix B: Screening methods

B.1	Screening method for relative quantitation of 35S promoter DNA from the soybean event GTS 40-3-2	
	35S-F	5'-GCC TCT GCC GAC AGT GGT-3' (300 nmol/l)
	35S-R	5'-AAG ACG TGG TTG GAA CGT CTT C-3' (300 nmol/l)
	35S-TMP	5'-*FAM*-CAA AGA TGG ACC CCC ACC CAC G-*TAMRA*-3'[a] (100 nmol/l)
	Product:	82 bp

Appendix B: Construct-specific method

C.1	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	RRS-F	5'-GCC ATG TTG TTA ATT TGT GCC AT-3' (900 nmol/l)
	RRS-R	5'-GAA GTT CAT TTC ATT TGG AGA GGA C-3' (900 nmol/l)

	RRS-TMP	5'-*FAM*-CTT GAA AGA TCT GCT AGA GTC AGC TTG TCA GCG-*TAMRA*-3'[a] (100 nmol/l)
	Product:	83 bp
C.2	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	RR1-F	5'-CAT TTG GAG AGG ACA CGC TGA-3' (600 nmol/l)
	RR1-R	5'-GAG CCA TGT TGT TAA TTT GTG CC-3' (600 nmol/l)
	Probe RR1	5'-*FAM*-CAA GCT GAC TCT AGC AGA TCT TTC-*TAMRA*-3'[a] (125 nmol/l)
	Product:	74 bp
C.3	Construct-specific method for quantitation of DNA from Event176 maize	
	CRY2-F	5'-CCC ATC GAC ATC AGC CTG AGC-3' (300 nmol/l)
	CRY2-R	5'-CAG GAA GGC GTC CCA CTG GC-3' (300 nmol/l)
	Probe BTSYN	5'-*FAM*-ATG TCC ACC AGG CCC AGC ACG-*TAMRA*-3'[a] (160 nmol/l)
	Product:	129 bp
C.4	Construct-specific method for quantitation of DNA from the soybean event GTS 40-3-2	
	RRS 01-5'	5'-CCT TTA GGA TTT CAG CAT CAG TGG-3' (500 nmol/l)
	RRS 01-3'	5'-GAC TTG TCG CCG GGA ATG-3' (500 nmol/l)
	RRS-Taq	5'-*FAM*-CGC AAC CGC CCG CAA ATC C-*TAMRA*-3'[a] (200 nmol/l)
	Product:	121 bp
C.5	Construct-specific method for quantitation of DNA from the maize event MON 810	
	MON 810 2-5'	5'-GAT GCC TTC TCC CTA GTG TTG A-3' (500 nmol/l)
	MON 810 2-3'	5'-GGA TGC ACT CGT TGA TGT TTG-3' (500 nmol/l)
	MON 810-Taq	5'-*FAM*-AGA TAC CAA GCG GCC ATG GAC AAC AA-*TAMRA*-3'[a] (200 nmol/l)
	Product:	113 bp
C.6	Construct-specific method for quantitation of DNA from the maize Event176	
	E176 2-5'	5'-TGT TCA CCA GCA GCA ACC AG-3' (500 nmol/l)
	E176 2-3'	5'-ACT CCA CTT TGT GCA GAA CAG ATC T-3' (500 nmol/l)
	E176-Taq	5'-*FAM*-CCG ACG TGA CCG ACT ACC ACA TCG A-*TAMRA*-3'[a] (200 nmol/l)
	Product:	100 bp
C.7	Construct-specific method for quantitation of DNA from the maize event Bt11	
	Bt11 3-5'	5'-AAA AGA CCA CAA CAA GCC GC-3' (500 nmol/l)
	Bt11 3-3'	5'-CAA TGC GTT CTC CAC CAA GTA CT-3' (500 nmol/l)
	Bt11-2-Taq	5'-*FAM*-CGA CCA TGG ACA ACA ACC CAA ACA TCA-*TAMRA*-3'[a] (200 nmol/l)
	Product:	127 bp
C.8	Construct-specific method for quantitation of DNA from the maize event GA21	
	GA21 3-5'	5'-GAA GCC TCG GCA ACG TCA-3' (500 nmol/l)
	GA21 3-3'	5'-ATC CGG TTG GAA AGC GAC TT-3' (500 nmol/l)
	GA21-2-Taq	5'-*FAM*-AAG GAT CCG GTG CAT GGC CG-*TAMRA*-3'[a] (200 nmol/l)
	Product:	133 bp
C.9	Construct-specific method for quantitation of DNA from the maize event T25	

	T25 1-5'	5'-GCC AGT TAG GCC AGT TAC CCA-3' (500 nmol/l)
	T25 1-3'	5'-TGA GCG AAA CCC TAT AAG AAC CCT-3' (500 nmol/l)
	T25-2-Taq	5'-*FAM*-TGC AGG CAT GCC CGC TGA AAT C-*TAMRA*-3'[a] (200 nmol/l)
	Product:	149 bp

Appendix D Event-specific methods

D.1	Event-specific method for quantitation of DNA from the maize event Bt11	
	Bt113JFor	5'-GCG GAA CCC CTA TTT GTT TA-3' (750 nmol/l)
	Bt113Jrev	5'-TCC AAG AAT CCC TCC ATG AG-3' (750 nmol/l)
	Bt113JFT	5'-*FAM*-AAA TAC ATT CAA ATA TGT ATC CGC TCA-*TAMRA*-3'[a] (250 nmol/l)
	Product:	70 bp
C.8	Event-specific method for quantitation of DNA from the maize event MON 810	
	Mail-F1	5'-TCG AAG GAG GAA GGA CTC TAA CGT-3' (300 nmol/l)
	Mail-R1	5'-GCC ACC TTC CTT TTC CAC TAT CTT-3' (300 nmol/l)
	Probe Mail-S2	5'-*FAM*-AAC ATC CTT TGC CAT TGC CCA GC-*TAMRA*-3'[a] (180 nmol/l)
	Product:	92 bp

3. Methods validated by the Subcommittee for "Method Development"

Real-time PCR for quantitation of genetically modified rapeseed events in conventional seeds using the 35S/pat gene construct

Construction-specific method:

Reference system for rapeseed (pep gene)

	pep-F2	5'-GAG AAC TGA ATG AGA GGT GCA TTG T-3' (200 nmol/l)
	pep-R2	**5'-AGT TCC TAA ATT CTT GAG ACG IGT T-3'** (200 nmol/l)
	pep-S	5'-*FAM*-ACA CGC TCG TTG ATT CCA ATG TTC TTC A-*TAMRA*-3'[a] (100 nmol/l)
	Product:	

Specific system (p35S-CaMV/pat):

	35SP0.f	5'-AAG TTC ATT TCA TTT GGA GAG GAC A-3' (200 nmol/l)
	pat-7.r	5'-CGG CCA TAT CAG CTG CTG TAG-3' (200 nmol/l)
	GSS01.s	5'-*FAM*-CCG GAG AGG AGA CCA GTT GAG ATT AGG C-*TAMRA*-3'[a] (100 nmol/l)
	Product:	111 bp

[a] FAM: 6-carboxyfluorescin, TAMRA: 6-carboxy-tetramethyl-rhodamine