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Report on the Verification of the Performance of a Simplex Endpoint Event-specific Method for the Detection of Event MON71800 in Wheat Using Real-time PCR

European Union Reference Laboratory for Genetically Modified Food and Feed

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EURL European Union Reference Laboratory for GM Food & Feed

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Report on the Verification of the Performance of a Simplex Endpoint Event-specific Method for the Detection of Event MON71800 in Wheat Using Real-time PCR

6 October 2016

European Union Reference Laboratory for GM Food and Feed

Executive Summary

Following the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) announcement that test results confirmed the finding of unauthorised GM glyphosate-resistant wheat volunteers harbouring the event MON71800 on a farm in Oregon, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) was requested to provide the GMO National Reference Laboratories (NRLs) of the EU Member States with a method for detecting this genetically modified organism (GMO) in wheat consignments.

In response, the EURL GMFF designed a testing strategy, based on readily available screening tests, that was published in June 2013 on the respective web page (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). In July 2013 the EURL GMFF also published a 'Report on the Verification of the Performance of a Testing Strategy' at the same URL address.

In May 2013 Monsanto had provided to the EURL GMFF a duplex endpoint TaqMan® PCR procedure for detection of "Roundup Ready® Wheat MON71800 and the acetyl-coenzyme A carboxylase *Acc-1* reference gene that had previously been made available to and used by USDA. The method provided is an event-specific assay spanning the 5' insert-to-plant junction. The EURL GMFF verified the method on positive control samples consisting of MON71800 crude lysate, also provided by Monsanto, and published a report in December 2013. The assay had a sensitivity no lower than 0.5% (expressed as ratio between GM- and target taxon-specific DNA copy numbers) and was therefore not in line with the method acceptance criteria established by the ENGL (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm).

A second event-specific simplex endpoint TaqMan® PCR method spanning the 3' insert-to-plant junction of wheat MON71800 and a new taxon-specific simplex method for the *Acc-1* control were made available by Monsanto. The EURL GMFF tested this method in September 2013 with the following results:

1. The method is event-specific, i.e. can reliably differentiate between the MON71800 and a wide selection of GM events.

2. The method detects MON71800 event at concentrations of 0.03-0.06% expressed as copy number ratio of GM-wheat/non-GM wheat.

The sensitivity of this event-specific method is therefore within the same range of the testing strategy proposed by the EURL GMFF in June 2013 and verified in July 2013 (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). That strategy, based on readily available screening methods, has a relative LOD between 0.03% and 0.06%, expressed as ratio between GM and target taxon-specific DNA copy numbers.

For detection of GM glyphosate-resistant wheat (MON71800) in wheat grain or in food/feed products containing wheat flour originating or consigned from the US, the EURL GMFF suggests following the testing strategy proposed in June 2013 or the present event-specific method, provided DNA of acceptable quality can be obtained.

Note

This report is published for information purposes only. It contains intellectual property and information owned or controlled by Monsanto Company and covered by one or more patents. Consent is not granted or implied by publication of the MON71800 event-specific detection method for any other use or application by any party or entity other than the European institutions and Member States official control laboratories, nor is any right or license granted or implied to the information, material, or intellectual property contained or referenced therein.

Quality assurance

The EURL GMFF is accredited ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://gmo-crl.jrc.ec.europa.eu/accredited_methods.html.

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EURL GMFF quality system.

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1. Introduction

Following the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) announcement that test results confirmed the finding of unauthorised GM glyphosate-resistant wheat volunteers harbouring the event MON71800 in a farm in Oregon, the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) was requested to provide National Reference Laboratories (NRLs) of the EU Member States with a method for detecting this genetically modified organism (GMO) in wheat consignments.

In response the EURL GMFF developed and published in June 2013 a testing strategy, based on readily available screening methods (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). The experimental verification of this strategy, published in July 2013 at the same URL address, showed an approximate limit of detection (LOD) of 0.03%, expressed in terms of copy numbers.

In December 2013, the EURL GMFF published the verification of the performance of an event-specific method, submitted by Monsanto, spanning the 5' insert-to-plant junction of MON71800. The company also provided control samples consisting of crude lysate from MON71800 wheat and sequence information on the event. The method was confirmed to be event-specific but displayed an approximate LOD of 0.5%, expressed as ratio between GM- and target taxon-specific DNA copy numbers (Report on the Verification of the Performance of a Method for the Detection of Event MON71800 in Wheat Using Real-Time PCR, http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm) which is not in line with the method acceptance criteria established by the ENGL.

A second event-specific method spanning the 3' insert-to-plant junction of event MON71800 was made available by Monsanto. This document reports on the *in-house* verification of this method carried out by the EURL GMFF.

2. Experimental design, materials and methods

2.1. Control samples, DNA extraction and DNA concentration

Control samples

The EURL GMFF tested the event specific method spanning the 3' insert-to-plant junction of event MON71800 on positive control sample provided by Monsanto and consisting of crude lysate from MON71800 seeds. The lysates were re-suspended by the EURL GMFF in 0.1x TE buffer to a final volume of 500 μL.

According to information provided by Monsanto, the MON71800 control sample should be considered homozygous. The EURL GMFF did not perform zygosity tests by digital PCR to verify this information.

DNA extraction

In addition to the control samples provided by Monsanto, the EURL GMFF prepared suitable DNA from other sources:

- Genomic DNA extracted from certified seeds of *T. aestivum*, variety Apache, using the NucleoSpin food kit (MACHEREY-NAGEL catalogue number: FC140945).

- Genomic DNA used in the specificity tests (section 2.3.3) was extracted either with Nucleospin food kit or with a CTAB procedure modified from ISO2157 A.3 from IRMM[1] or AOCS[2] certified reference materials, with the exception of LLCotton25xGHB614 genomic DNA.

- Conventional DNA of cotton, rice, maize, soybean and oilseed rape was extracted from control samples stored at EURL GMFF or purchased from local retailers.

DNA extracts were checked for integrity using DNA agarose gel electrophoresis. DNA was quantified and assessed for absence of inhibitory compounds.

Inhibition runs were carried out as described in the ENGL guidance document "Verification of analytical methods for GMO testing when implementing interlaboratory validated methods" [a]
using the respective taxon-specific validated reference systems to rule out possible inhibitory effects. The inhibition runs on conventional wheat genomic DNA were performed using a published wheat reference system (primers Wx012F/Wx012R and probe Wx-Taq 1)[3].

DNA concentration
The concentration of the extracted DNA was determined by fluorescence detection, using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). The respective DNA concentrations were determined as the arithmetic mean of 10 readings on the basis of a five-point standard curve using a VersaFluor Fluorometer (Bio-Rad) as fluorescence detector.

2.2. Description of the event-specific method for MON71800 wheat as provided by MONSANTO (ESM71800) and adapted for verification by the EURL GMFF

The method "Roundup Ready Wheat MON71800 3'-Junction Event Specific endpoint TaqMan® PCR with *acc* Control for Seed Pools of 200" developed by Monsanto makes use of a simplex endpoint PCR system coupled with the use of labelled oligonucleotides (probes) for event-specific detection of MON71800 and of the acetyl-coenzyme A carboxylase, *Acc-1,* reference target (Genebank: AF029897.1). The procedure is designed for detection of the MON71800 event in genomic DNA extracted from wheat seed pools of 200 seeds. The method is optimised for 96-well or 384-well format using an Applied Biosystems GeneAmp PCR System 9700 or MJ Research DNA Engine PTC-225. After PCR amplification the fluorescence is detected via a specific plate reader or via an "Allelic discrimination" assay (e.g. ABI real-time series instruments)[4].

Since plate readers or allelic discrimination analysis are not standard procedures in EU control laboratories, the EURL GMFF ran the method on real-time PCR equipment ABI 7900HT, a widely used type of instrument in GMO testing laboratories, under the conditions described by the method developer and analysed the data in the modality 'Absolute Quantification' (AQ) assay.

Limitations of the "Allelic Discrimination" assay have been already described in the EURL GMFF report on the verification of an event-specific method for detection of MON71800 based on the 5' insert-to-

[a] http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

plant junction, previously proposed by Monsanto (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). In the present study two parameters were investigated: specificity and limit of detection (LOD).

For the event-specific detection of MON71800, a 97-bp fragment of the 3′ region spanning the insert-to-plant junction in wheat event MON71800 is amplified using specific primers. PCR products are detected during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as reporter dye at its 5′ end, and TAMRA (carboxytetramethylrhodamine) as quencher at its 3′ end. A second potential amplification site covering the same portion of the 3′ insert-to-plant junction and a longer segment of the genomic flanking region identified by bioinformatics analysis (see section 3.2.1) was shown not to be amplified (see section 3.2.2).

For the taxon-specific detection of wheat, a reference-specific system amplifies a 94-bp fragment of the *Triticum aestivum Acc-1* gene, by means of specific primers and a probe labelled with FAM as reporter dye at its 5′ end, and TAMRA as quencher at its 3′ end.

2.2.1. Primers and probes

Primers and probes for the two simplex PCR, as defined by Monsanto, used in the EURL GMFF in-house verification, are reported in Table 1.

Table 1. Oligonucleotide sequences for detection of event MON71800 and *Acc-1* reference gene

Oligonucleotides	Name	DNA Sequence (5′ to 3′)	Length (nt)
MON71800 3′ - insert-to-plant junction			
Forward primer	SQ50540	5′-CGC CTT CAG TTT AAA CTA TCA G-3′	22
Reverse primer	SQ50541	5′-CGA CGT GAT GCC TAT GTA TTG-3′	21
Probe	PB50160	5′-6FAM™-CAG TGC CTG GAC ATC GTC ATA ATT ATT TGA GGT TC -TAMRA™ -3′	35
Acc-1			
Forward primer	SQ50542	5′- GCC TAC CCC CTT CAA CAA GA -3′	20
Reverse primer	SQ50543	5′- ATG TAC GCG CTT GAA CCC TT-3′	20
Probe	PB50161	5′- 6FAM™-CCA CCG ACG AGT TAA AAC CAA AGA TAC ACG -TAMRA™ -3′	30

2.2.2. Reaction set up

Final concentrations for reagents of event-specific and wheat specific amplification systems used in the *in-house* verification are shown in Table 2 and 3.

Table 2. Amplification reaction mixture in final volume/concentration per reaction well for the MON71800 method

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)*	1x	5
SQ50540/SQ50541 (20 µM each primer)	0.5 µM	0.25
PB50160 (10 µM)	0.125 µM	0.125
DNase free water	#	2.15
DNA	#	2.475
Total reaction volume:		10 µL

* Applied Biosystems Cat. # 4304437

Table 3. Amplification reaction mixture in final volume/concentration per reaction well for the *Acc-1* method

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)*	1x	5
SQ50542/SQ50543 (20 µM each primer)	0.5 µM	0.25
PB50161 (10 µM)	0.125 µM	0.125
DNase free water	#	2.15
DNA	#	2.475
Total reaction volume:		10 µL

* Applied Biosystems Cat. # 4304437

2.2.3. Cycling parameters

The reaction was run by the EURL GMFF in a touchdown mode, with starting annealing and extension temperature at 64 °C and final annealing and extension temperature at 54 °C (Table 4).

Table 4. Cycling program for the MON87701/*Acc-1* simplex system used by the EURL GMFF.

Step	Stage		T°C	Time (sec)	Acquisition	Cycles
1	UNG*		50°C	120	No	1x
2	Initial denaturation		95°C	600	No	1x
3	Amplification/Touch down	Denaturation	95°C	15	No	10x
		Annealing & Extension	64°C -1 °C/cycle	60	Yes	
4	Amplification	Denaturation	95°C	15	No	40x
		Annealing & Extension	54°C	60	Yes	

*UNG: Uracil-N-glycosylase

2.3. Specificity

2.3.1. Bioinformatics analysis

Bioinformatics analyses were conducted on sequence data provided by Monsanto for the event MON71800 wheat. Similarity searches with BLASTN 2.2.15[5] were performed against the GMO sequence database maintained at the JRC (CCSIS), the NCBI nt nucleotide sequence database, the NCBI patent nucleotide sequence database, as well as the whole genomes of *Brassica rapa, Glycine max, Oryza indica, Oryza sativa, Solanum lycopersicum* and *Zea mays* using the e-PCR prediction tool. Results of the analyses are reported in chapter 3.

2.3.2. Experimental

Specificity tests were conducted to evaluate the cross-reactivity of the method against genomic DNA extracted from the following GMOs: soybean CV127 (AOCS 0911-D), MON87701 (AOCS 0809-A), MON87705 (AOCS 0210-A), MON89788 (AOCS 0906-B), A2704-12 (AOCS 0707-B4), A5547-127 (AOCS 0707-C3), FG72 (AOCS 0610-A2); oilseed rape Ms1 (AOCS 0711-A), Ms8 (AOCS 0306-F3), Rf1 (AOCS 0711-B), Rf2 (AOCS 0711-C), Rf3 (AOCS 0306-G3), T45 (AOCS 0208-A4), Topas 19/2 (AOCS 0711-D), GT73 (AOCS 0304-B); maize MON87460 (AOCS 0709-A), MIR162 (AOCS 1208-A), MON88017 (AOCS 0406-D), MON89034 (AOCS 0906-E), MON810 (ERM-BF413-5), GA21 (ERM-BF414-5), Bt176 (ERM-BF411R-5), NK603 (ERM-BF415-5), MIR604 (ERM-BF423d), Bt11 (ERM-BF412R-5); cotton GHB119 (ERM-BF428c), GHB614xLLCotton25, conventional wheat, maize, soybean, rice, oilseed rape and cotton (ERM-BF428a).

Reactions were conducted in triplicate with 2,500 genome copies of GM DNA per reaction.

2.4. Estimation of the sample size in the determination of the limit of detection

The optimal sample size to determine the absolute or relative LOD was determined by estimating the number *n* of replicates per GM level that would generate a 95% confidence interval around the proportion of GM-negative samples with an upper boundary not exceeding 5%.

For an accurate estimate of the 95% confidence interval (depending on the degrees of freedom used to compute p), the F-distribution was used based on the relationship between such distribution and the binomial distribution[6]. This method, derived from Bliss[7] and recently re-proposed by Zar[8], leads to an estimate of $n = 100$. Additionally, the standard approach based on the normal approximation was also considered, as suggested by Cochran[9]. This alternative method returns an estimate of $n = 60$. Further details are provided in Annex 1.

Given the experimental design for a LOD study where it is required to test a large number of replicates in each sample characterised by defined analyte content (DNA copy number content) over a linearly decreasing series of concentrations, the Cochran approach was accepted as the most feasible. Hence, every sample (GM concentration level) was tested in 60 replicates.

In accordance with the Plant DNA C-values Database of the Royal Botanic Garden[10] the weight of the 1-C value of wheat genome was considered to be equivalent to 17.33 pg.

2.5. Limit of Detection

In official controls in the EU it is necessary to test for presence of MON71800 in grains or food and feed samples, quantifying the amount of the GM event in relation to the total wheat DNA in the sample. According to standard ISO (21569:2005) the LOD shall be provided with reference to a relative value based on a specified matrix, 'preferably a given amount of genomic DNA solution'. Accordingly the EURL GMFF carried out tests to estimate the limit of detection of the method relatively to a defined amount of wheat genomic DNA, extracted at EURL GMFF (LOD_{rel}). For the estimation of the LOD_{rel}, respectively 10, 5, 1 and 1/10 of a solution containing 1 haploid genome copy of MON71800 (corresponding to a theoretical value of 0.1 MON71800 haploid genome per reaction) were added to 17,311 wheat haploid genome copies (corresponding to 300 nanograms of non-GM wheat DNA) and analysed as described above (2.2.2 and 2.2.3) and detailed in Table 5. Each GM level was tested in 60 replicates following the statistical model outlined above. No template controls and positive controls were included.

Table 5. Experimental set-up for the estimation of the Limit of Detection

MON71800 haploid genome copies per reaction	**Added background Wheat haploid genome copies**	**GM% in haploid genome copies**
10	17,311	0.06
5	17,311	0.03
1	17,311	0.01
0.1	17,311	-

3. Results

3.1 Quality of the extracted DNA

Extracted genomic DNA showed acceptable DNA concentrations and passed inhibition tests. The concentration of the MON71800 crude lysate was estimated at 1.4 ng/µL further to re-suspension as previously described; given the low concentration this DNA was not subjected to inhibition tests.

3.2. Specificity of the method

3.2.1. Bioinformatics analysis

Molecular structure of MON71800

The characterised insertion site for event MON71800 contains two copies of the chloroplast transit peptide linked to the 5-enolpyruvyl shikimate-3-phosphate synthase, one copy for the promoter P-35S and two copies of the T-nos terminator. The molecular structure of the MON71800 insert in the wheat genome is shown in Figure 1. The P-35S promoter contains an internal duplication of the enhancer region. Therefore, event MON71800 can be detected with specific methods targeting these respective elements (see 'Report on the Verification of the Performance of a Testing Strategy for the Detection of Wheat MON71800 Event Using Real-Time PCR', http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm).

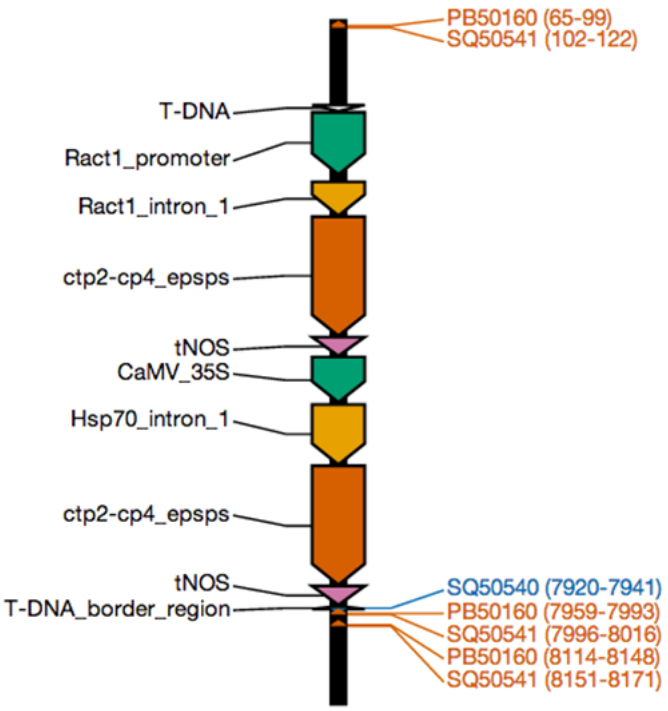
Annealing sites for primers and probe of the MON71800 event-specific system targeting the 3' insert-to-plant junction

According to bioinformatics analysis performed on the sequence of the MON71800 event provided by Monsanto, the method spans the junction between the transgenic insert and the 3' genomic region. The first method for event-specific detection of MON71800 in wheat provided by the company was designed instead on the 5' insert-to-plant junction as previously verified by the EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm).

The SQ50540 primer binds in the transgenic insert, in a region corresponding to the T-DNA border region while the SQ50541 primer and the PB50160 probe bind in the genomic flanking region.

However, the SQ50541 primer and the PB50160 probe have additional perfect binding sites on neighbouring genomic DNA sequence. Therefore, this set of primers could theoretically produce two amplicons of 97 bp and 252 bp. A third binding site for the primer and probe was identified, but on the 5' genomic border, and in an orientation that should not produce additional PCR amplicons (See Figure 1).

Figure 1. Position and orientation of the binding sites found for the primers SQ50540 and SQ50541 and for the probe PB50160 in MON71800. Blue: binding in the sense orientation, Orange: binding in the antisense orientation



Verification of specificity

The sequences of the largest (252 bp) and shortest (97 bp) amplicons were analysed by similarity searches using the BLAST algorithm against the "patents" and NCBI "nt" databases. Significant similarities were only found with sequences from Monsanto patents. In the 'nt' database, fragments of wheat family genomes show similarity with the genomic part of the amplicon, but none of the fragments include both primers binding sites.

In addition, the primers were assessed against other GM events present, as well as against local copies of the whole genomes of *Brassica rapa, Glycine max, Oryza indica, Oryza sativa, Solanum lycopersicum* and *Zea mays*, downloaded from Ensembl[11] No potential amplicon was identified using the e-PCR prediction tool[12].

Wheat-specific reference system

The described taxon-specific method targets the *Triticum aestivum* (common wheat) acetyl-coenzyme A carboxylase (*Acc-1*) gene (GenBank N. AF029897.1). The primers and probe sequences used in this method are different from the ones previously submitted by Monsanto for the *Acc-1* reference system and already evaluated by the EURL GMFF for verification of the duplex endpoint PCR method targeting the 5' insert-to-plant junction. The current simplex *Acc-1* assay targets an intron region of the gene with an expected amplicon size of 94 bp, instead of an exon as with the previous system (according to the annotations of AF029897.1).

The genome of the wheat family is complex, with four types of genomes, symbolised by the letters A, B, D and G. *Triticum aestivum* has an AABBDD genome configuration, produced by hybridization of tetraploid emmer wheat (AABB, *T. dicoccoides*) with *Aegilops tauschii* (DD).

For the analyses, the genome assemblies for the three individual sub-genomes (from the International Wheat Genome Sequencing Consortium, IWGSC) were downloaded individually from the Institute of Bioinformatics and Systems Biology ftp site[13].

Alignments of the amplicon sequence with these sub-genomes identify three regions with significant similarity, one per sub-genome (Figure 2).

Figure 2. Alignment of the amplicon sequence of the acetyl-coenzyme A carboxylase detection method proposed by Monsanto with the regions of significant sequence similarity found in the wheat genome. The sequences of the primers and probes are shown in green; differences at binding sites are highlighted.

Genomic region 1:

```
Amplicon            GCCTACCCCCTTCAACAAGATGACCGAACACCACCGACGAGTTAAAACCAAAGATACACG
Genomic region 1    GCCTACCCCCTTCAACAAGATGACCGAACACCACCGACGAGTTAAAACCAAAGATACACG
                    ************************************************************

Amplicon            GGACCTGCC-AAAAAAAGGGTTCAAGCGCGTACAT
Genomic region 1    GGACCTGCCAAAAAAAAGGGTTCAAGCGCATACAT
                    ********* ****************** *****
```

Genomic region 2:

```
Amplicon            GCCTACCCCCTTCAACAAGATGACCGAACACCACCGACGAGTTAAAACCAAAGATACACG
Genomic region 2    GCCTACCCCCGTCAACAAGATGACCGAACACCACCGGCGACTTAAAACCAAAGATGCACA
                    ********** ************************ ***  ************** ***

Amplicon            GGACCTGCCAAAAAAAGGGTTCAAGCGCGTACAT
Genomic region 2    GAACGCGCAAAACAAAGGGCTCAAGCGTGTACAG
                    * **  ** *** ****** *******  *****
```

Genomic region 3:

```
Amplicon            GCCTACCCCCTTC-AACAAGATGACCGAACACCACCGACGAGTTAAAA-CCAAAGATACA
Genomic region 3    GCCTACCCCTTTTTAACAAGATGATCGAACACCATCGACGAGTTAAAAACCAAAGATGCA
                    ********* **   ********** ********* ************* ******** **

Amplicon            CGGGACCTGCC---AAAAAAAGGGTTCAAGCGCGTACAT
BAC                 CCTGACCTGCCAAAAAAAAAAGGGTTCAAGCGCGTACAC
                    *  ********   ************************
```

Consistent with these analyses, the genomic region from subgenomes A and B were also found, identical, in bacterial artificial chromosomes (BACs) from *Triticum durum* (also AABB) while the genomic region from subgenome D was found, identical, in the Ensembl sequence for *Aegilops tauschii*

As shown on Figure 2, according to the current version of the wheat genome, there is a discrepancy between the genomic sequence of that region and one base of the reverse primer. In addition, the amplicon size from region A would be one nucleotide base shorter (93 bp).

The size difference is caused by a stretch of seven or eight adenines (A), and most probably represents a sequencing error in one of the two sequences (genomic or AF029897). For this, as for the difference in the primer sequence, it is therefore not possible to know which sequence is correct, i.e. whether the amplicon size is 93 bp or 94 bp, and whether there is a mismatch in the suggested reverse primer binding region.

BLAST analysis of the amplicon sequence against the Ensembl genomes of other cereals, such as *B. dystachion*, *H. vulgare* (barley), *S. italica* (millet) and *S. bicolor* (sorghum) did not recognise any additional region of significant similarity.

The primers were also tested against local copies of the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*). Using the e-PCR prediction tool (as referenced in 'Bioinformatics verification of the GM-assay specificity'), no potential amplicon was identified.

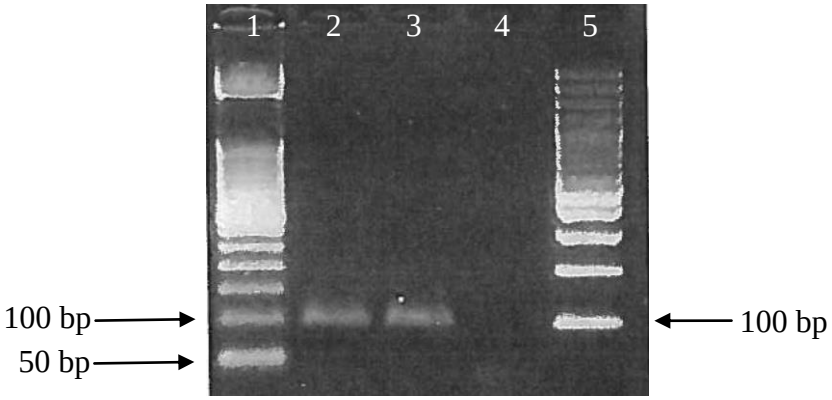
In conclusion, bioinformatics analyses suggest that the three highly similar regions found in the Ensembl genomic sequence of *T. aestivum* may represent the target *Acc-1* genes from the A, B and D genomic backgrounds of *T. aestivum*; the first region, almost identical to AF029897.1 and to the primers and probe sequences of the current *Acc-1* system, seems to be present in both *T. aestivum* and *T. durum genomes*. The method therefore should not be able to differentiate between these two species. A mismatch may be present between the proposed reverse primer (SQ50543) and the corresponding binding site in the genome.

No other potential amplification sites were identified in other plant species.

3.2.2. Verification of the amplicon size in the MON71800-specific system

Bioinformatics analyses showed (3.2.1) that the SQ50541 primer and the PB50160 probe have an additional binding site in the neighbouring flanking genome sequences. It is expected that this set of primers can produce two amplicons, of sizes 97 bp and 252 bp. To verify whether in the experimental conditions described in the protocol (sections 2.2.2 and 2.2.3) amplicons are generated, the following experiment was conducted: 180 copies of MON71800 in two replicates were tested with MON71800-specific system and loaded on a 2% agarose gel electrophoresis. Figure 3 shows that only one amplicon is produced, whose band is aligned to the bands at about 100 bp from both molecular weight markers run in parallel. Therefore, it can be concluded that the amplicon at 97 bp is the only or largely predominant MON71800-specific amplicon in reaction.

Figure 3. Agarose gel electrophoresis of MON71800 samples amplified with the MON71800-specific system



Legend: 1 = 50 bp ladder; 2 and 3 = 180 copies of MON71800; 4 = No template control; 5 = 100 bp ladder

3.2.3. Experimental testing of specificity

In addition to the bioinformatics analysis, a number of GM events were tested with the MON71800 method to verify its specificity. The reference system *Acc-1* was also tested on conventional DNA extracted from crops commonly used as food or feed: maize, soybean, oilseed rape, cotton, rice and wheat.

Each specificity test was run in triplicate with 2,500 GM- or conventional-DNA genome copies per reaction. Detection was recorded when the amplification curve crossed the threshold within the number of cycles described in the method. Results indicating the outcome of tests and the relative quantification cycle (Cq) are shown in Tables 6 and 7.

Table 6 shows that the MON71800 method does not cross-react with any of the GMOs tested and confirms the bioinformatics analysis previously described (section 3.2.1).

Table 6. Specificity of the MON71800 detection method. The Cq number is the mean of three replicates.

Event Name	Cq values event-specific MON71800 system
CV127	n.d.
MON87701	n.d.
MON87705	n.d.
MON89788	n.d.
A2704-12	n.d.
A5542-127	n.d.
FG72	n.d.
GHB119	n.d.
GHB614xLLCotton25	n.d.
MON87460	n.d.
MIR162	n.d.
MON88017	n.d.
MON89034	n.d.
MON810	n.d.
GA21	n.d.
Bt176	n.d.
NK603	n.d.
MIR604	n.d.
Bt11	n.d.
MS1	n.d.
MS8	n.d.
RF1	n.d.
RF2	n.d.
RF3	n.d.
T45	n.d.
Topas 19/2	n.d.
GT73	n.d.
Conventional cotton	n.d.
NTC	n.d.
Positive Control*	28.1

n.d.: not detected, i.e. no amplification observed when the method is applied to the specified analyte
NTC: no template control
* Positive Control: 100 genome copies of MON71800 DNA

Similarly, the *Acc-1* method did not show cross-reactivity with genomic DNA from any of the species tested at 2,500 haploid genome copies (Table 7). No template controls (NTCs) were added for each test. All NTCs tested negative.

Table 7. Specificity of the *Acc-1* wheat reference method. The Cq number is the mean of three replicates.

Crop Name	Cq values* ***Acc-1*** reference system	Cq values/ taxon specific reference system
Cotton	n.d.	26.4 / AdhC
Maize	n.d.	26.1 / HMG
Oilseed rape	n.d.	27.2 / FatA
Rice	n.d.	26.2 / PLD
Soybean	n.d.	25.8 / Le1
Wheat	18.9	Acc-1
NTC	n.d.	Acc-1, CruA, HMG, FatA, PLD, Le1

n.d.: not detected, i.e. no amplification observed when the method is applied to the specified analyte

3.2 Limit of detection

For the estimation of the LOD_{rel}, known quantities of MON71800 were added to 300 nanograms of non-GM wheat genomic DNA and tested in 60 replicates. The levels investigated are shown in Table 8.

Table 8. Results of the determination of the LOD_{rel} of the MON71800 method

MON71800 haploid genome copies	Mean Cq for MON71800/ Standard Deviation	Positive / total amplifications
10	32.6 / 1.34	60/60
5	34.1 / 1.42	58/60
1	31.2 / 1.42	17/60
0.1	29.1 / 1.52	4/60

As shown in Table 8, samples containing 10 and 5 MON71800 copies resulted positive respectively in 60 and 58 replicates out of 60. According to the statistical approach described in section 2.4 the LOD is defined as the GM level where the target is detected in 59 out of 60 replicates. Therefore, for samples containing 300 nanograms of wheat genomic DNA the LOD_{rel} is estimated to be between 5 and 10 copies of MON71800 event. This corresponds to a LOD_{rel} between 0.03% and 0.06% expressed as ratio between GM and target taxon-specific DNA copy numbers.

4. Conclusions

Based on the above described results, the EURL GMFF concludes the following:

3. The wheat MON71800 endpoint TaqMan® PCR method spanning the 3' insert-to-plant junction provided by Monsanto is event-specific, i.e. can differentiate between the MON71800 and a wide selection of GM events.

4. The method is reliably able to detect the MON71800 event at concentrations of 0.03-0.06% expressed as copy number ratio of GM-wheat/non-GM wheat.

The sensitivity of this event-specific method is therefore in the same range of the testing strategy proposed by the EURL GMFF in June 2013 (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). That strategy, which is based on readily available screening methods, has a relative LOD between 0.03% and 0.06%, expressed as ratio between GM- and target taxon-specific DNA copy numbers.

ANNEX 1. Estimation of the sample size in the determination of the Limit of Detection

According to the method from Bliss[7] and Zar[8], in a sample of (n) data, (X) of which showing the character of interest, confidence limits (L_1: lower limit, L_2: upper limit) of a proportion (p) are computed as follows:

$$L_1 = \frac{X}{X + (n - X + 1) \cdot F_{\alpha/2,\nu1,\nu2}}$$

$$L_2 = \frac{(X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}{n - X + (X + 1) \cdot F_{\alpha/2,'\nu1,'\nu2}}$$

where the degrees of freedom v1 and v2 are:

$$\nu1 = 2 \cdot (n - X + 1)$$

$$\nu2 = 2 \cdot X$$

and the degrees of freedom ‘v1 and ‘v2 are:

$$'\nu1 = \nu2 + 2$$

$$'\nu2 = \nu1 - 2$$

Based on this method, with X = 1, α= 0.05, and L_2 = 0.05, (n) is equal to 100.

According to Cochran[9] the simplest approach to estimate the confidence interval of a sample proportion (p), is the use of the normal distribution (z) and its standard deviation p (1 - p):

$$L_1 = p - z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1 - p)}{n - 1}}$$

$$L_2 = p + z_{\alpha/2} \cdot \sqrt{\frac{p \cdot (1 - p)}{n - 1}}$$

Based on this simplified approach, with X = 1 and α= 0.05, L2 = 0.05 (n) would be equal to 60, thus resulting for determining the absolute LOD in an experimental set at 59 positive tests (n - X) over 60 replicates.

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