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JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Report on the Verification of the Performance of a Method for the Detection of Event MON71800 in Wheat Using Real-Time PCR

4 December 2013

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 "volunteer" plants harbouring the event MON71800 on a farm in Oregon, the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) was requested to provide as soon as possible a method to test wheat consignments for the presence of this genetically modified organism (GMO) to the National Reference Laboratories (NRLs) for GMOs of the EU Member States.

In response, the EU-RL GMFF put together a testing strategy, based on readily available screening tests which was published here (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm).

Upon request, Monsanto provided in May 2013 the EU-RL GMFF with the procedure “Roundup Ready® Wheat MON71800 Event Specific Endpoint TaqMan® PCR with *acc* Internal Control for Seed Pools of 1:15” that had previously been made available to, and was used by USDA. The EU-RL GMFF tested this protocol on positive control samples consisting of MON71800 crude lysate, also provided by Monsanto.

Our results can be summarised as follow:
The method is event-specific. Our specificity-tests did not show cross-reactivity on genomic DNA from a wide selection of similar GMO.

The sensitivity of the method was found to be in agreement with previous findings of USDA, i.e. the relative limit of detection lies at 0.5% (expressed as ratio between GM- and target taxon-specific DNA copy numbers), in a background of 301 ng of wheat genomic DNA. The absolute limit of detection (LOD_{abs}) was determined between 5 and 10 copies of MON71800 target.

For seed/grains the application of a sub-sampling strategy could allow detection below 0.5% expressed in terms of copy number ratio but it would require significant additional efforts, including the analysis of numerous sub-samples.

Additional tests showed that detection of 100 copies of MON71800 DNA in a background of increasing amounts of non-GM wheat genomic DNA occurred with proportionally decreasing reaction efficiency.

Based on the scientific evidence described in the present report, the EU-RL GMFF suggests that its testing strategy (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm), making use of validated element and construct-specific methods, is found more sensitive and should be used to test for presence of MON71800 GM-wheat.

The verified event specific method of Monsanto could be used to confirm positive findings at GM-target concentration equal or above 0.5% or it could be used for detection of GM-event MON71800 below 0.5% but it would require a costly sub-sampling strategy, which, in addition, is only possible in seeds/grains.

Important Note

This verification report is published for information purposes only. The publication entitled "Report on the Verification of the Performance of a Method for the Detection of Event MON71800 in Wheat Using Real-Time PCR" contains intellectual property and information owned or controlled by Monsanto Company and covered by one or more patents and 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.

The EU-RL GMFF recommends that the testing strategy developed and published (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm) by the EU-RL GMFF, which conforms to EU testing requirements and available testing equipment used by control laboratories, is used to test for presence of MON 71800 GM-wheat.

Quality assurance

The EU-RL 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 EU-RL GMFF quality system.

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Content

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 "volunteer" plants harbouring the event MON71800 in a farm in Oregon, the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) was requested to provide National Reference Laboratories (NRLs) of the EU Member States as soon as possible with a method to test wheat consignments for the presence of this Genetically Modified Organism (GMO).

In response the EU-RL developed and published a testing strategy, based on readily available screening methods (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). This testing strategy has an LOD around 0.03%, expressed in terms of copy numbers (see report).

Monsanto provided the EU-RL GMFF with the protocol “Roundup Ready® Wheat MON71800 Event Specific EndPoint TaqMan® PCR with *acc* Internal Control for Seed Pools of 1:15” (ESM71800) and control samples. Monsanto also provided sequence information on the MON71800 GMO.

This document reports on the in-house verification of this method and information that was carried out by the EU-RL GMFF.

2. Experimental design, materials and methods

2.1. Control samples, DNA extraction and DNA concentration

Control samples

The EU-RL GMFF tested the event specific method (ESM71800) against control samples, provided by Monsanto, consisting of crude lysate prepared from MON71800 seeds and of crude lysate prepared from conventional wheat seeds. These DNA samples were re-suspended at the EU-RL GMFF in a final volume of 500 µL in 0.1x TE buffer.

According to information provided by Monsanto on the zygosity of the MON71800 control sample, it was considered homozygous. The EU-RL GMFF did not run any zygosity tests in digital PCR to verify this information.

DNA extraction

In addition to the samples provided by Monsanto, the EU-RL GMFF prepared suitable DNA from other sources:

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

- Other genomic DNA used in the specificity tests (section 2.3.3) were extracted from IRMM[1] or AOCS[2] certified reference materials with the exception of LL25xGHB614 genomic DNA and of conventional cotton DNA that were extracted from control samples.

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 inter-laboratory validated methods"[a] using the respective taxon-specific validated reference systems to rule out possible inhibitory effects. The inhibition run on conventional wheat genomic DNA was run with 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, after extensive homogenisation, using the PicoGreen dsDNA Quantitation Kit (Molecular Probes) by 10 readings. The respective DNA concentrations were determined 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 EU-RL GMFF

The method "Roundup Ready® Wheat MON71800 Event Specific EndPoint TaqMan® PCR with *acc* Internal Control for Seed Pools of 1:15" developed by Monsanto makes use of a duplex endpoint PCR system coupled with the use of labelled oligonucleotides (probes) for event-specific detection of MON71800 and of the *acc* reference target. The method was optimised for use in 96-well or 384-well format using an Applied Biosystems GeneAmp PCR System 9700 or MJ Research DNA Engine PTC-225. At the end of the PCR the fluorescence is detected via a specific plate reader or via an "Allelic discrimination" assay (e.g. ABI real-time series instruments)[4]. *It should be noted that plate readers or allelic discrimination analysis are not standard in EU control laboratories.*

For practicability reasons, the EU-RL GMFF therefore ran the method on real-time PCR equipment ABI 7900HT – a widely used type of instrument in GMO testing laboratories, allowing to run the method in the conditions described by the method developer and to analyse data in two modalities: 'Absolute Quantification' (AQ) assay and the 'Allelic Discrimination' (AD) assay. The 'AQ' assay allows storing real-time data for subsequent 'AD' assay application. The AD assay can be used to verify presence/absence of the MON71800 target in reaction, by measuring the fluorescence levels (FAM for the MON71800 target and VIC for the *acc*- target) of the reporter dyes. However, also the AQ assay can be used for the same purpose by assessing the amplification curve profile as crossing the threshold within the amplification cycles described in the protocol. Indeed, in certain cases the AD assay did not provide clearly interpretable results (see chapter 3) and then the AQ assay was preferred.

For the event- specific detection of MON71800, a 95-bp fragment of the 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 MGB-NFQ (minor groove binding non-fluorescent quencher) as quencher at its 3' end.

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

For the taxon-specific detection of wheat, a reference-specific system amplifies a 54-bp fragment of *Triticum aestivum* acetyl-coenzyme A carboxylase (AF029897.1), an '*acc*' specific assay, by means of specific primers and a probe labelled with VIC as reporter dye at its 5' end, and MGB-NFQ as quencher at its 3' end.

2.2.1. Primers and probes

Primers and probes for the duplex PCR, as defined by Monsanto and used in the EU-RL GMFF in-house verification, are reported in Table 1.

Table 1. Oligonucleotide sequences for detection of MON71800 and *acc*

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
MON71800			
MON71800	SQ0718	5'- TTC TTC TCT CTC TTT GAA TCT CAA TAC AA -3'	29
	SQ0719	5'- CCC CCA TTT GGA CGT GAA -3'	18
	PB0101	6-FAM 5'- TCC CCC TCT CTA ATT C- MGB 3'	16
acc			
Wheat reference system *acc*	SQ0716	5'- GGG AGG CAT GCT TCG CT -3'	17
	SQ0717	5'- GCC GCC CAA TGC CAT A -3'	16
	PB0100	VIC 5'- TCT AAG GTT GTT GAA TTT T- MGB 3'	19

2.2.2. Reaction set up

Final concentrations for reagents of event-specific and wheat specific assays that were used by the EU-RL GMFF in its in-house verification are shown in Table 2.

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

Component	Final concentration	µL / reaction
TaqMan® Universal PCR Master Mix (2x)*	1x	5
SQ0718/SQ0719 (20 µM each primer)	0.5 µM	0.25
PB0101 (10 µM)	0.1 µM	0.1
SQ0716/SQ0717 (20 µM each primer)	0.5 µM	0.25
PB0100 (10 µM)	0.1 µM	0.1
DNase free water	#	1.3
DNA	#	3
Total reaction volume:		10 µL

* Applied Biosystems Cat. # 4304437

2.2.3. Cycling parameters

The reaction was run by the EU-RL GMFF in a touchdown mode, with starting annealing and extension temperature at 64 °C and final annealing and extension temperature at 54 °C (Table 3).

Table 3. Cycling program for MON87701/*acc* duplex system used by the EU-RL 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-verification

2.3.1. Bioinformatics analysis

Bioinformatics analyses were conducted by the EU-RL GMFF on the basis of the sequence data for the event MON71800 wheat that were provided by Monsanto. These analyses where carried out by similarity searches, BLASTN 2.2.15[5], with the sequences of the primers SQ0718- SQ0719 (separated by a generic nucleotide 'N') and with the corresponding amplicon against *i*) the GMO sequence database maintained at the JRC (CCSIS), *ii*) the NCBI nt nucleotide sequence database, *iii*) the vector sequences from synthetic (syn) division of GenBank, *iv*) the NCBI patent nucleotide sequence database, *v*) the GMO Detection Method Database (GMDD) of the Shanghai GMO platform *vi*) the plant sequence data section of the EMBL database, and *vii*) the plant genomes available on Ensembl (plants.ensembl.org), including the published *Triticum_aestivum* genome. For the results see chapter three.

2.3.2. Experimental specificity tests

Specificity tests were conducted to evaluate the cross-reactivity of the MON71800 duplex endpoint method against genomic DNA extracted from the following GMO: soybean CV127 (AOCS 0911-D), MON87701 (AOCS 0809-A), MON87705 (AOCS 0210-A), MON89788 (AOCS 0906-B), A2704-12 (AOCS 0707-B4), A5542-127 (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), MON863 (ERM-BF416-3), GA21 (ERM-BF414-5), Bt176 (ERM-BF411R-5), NK603 (ERM-BF415-5), MIR604 (ERM-BF423d), Bt11 (ERM-BF412R-5), 3272 (ERM-BF420c); cotton GHB119 (ERM-BF428c), GHB614xLL25, non-GM cotton (ERM-BF428a).

Reactions were conducted in triplicate with 2,500 copies of GMO 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 (number of replicates (n) per GM level) was estimated. The size is defined by the number (n) of samples at which the GM level (p) is correctly detected at least 95% of the times, thus allowing for ≤5% false negative results. With other words, the number of experiments/replications (n) was estimated that would generate a 0.95 confidence interval with an upper boundary that does not exceed 5%.

For an accurate estimate of the 0.95 (1-α) 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]. The method is derived from Bliss[7] and recently re-proposed by Zar[8]. According to this method, 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 (see: "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"[10]).

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[11] the weight of the 1-C value of wheat genome was considered to be 17.33 pg.

2.5. Absolute Limit of Detection

The EU-RL GMFF carried out tests to estimate the absolute Limit of Detection (LOD_{abs}) of the method. The LOD_{abs} was established by amplifying the positive control sample MON71800 at 10 and 5 copies per reaction and one tenth of a dilution at 1 copy per reaction.

2.6. Relative 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 it relative to the total wheat DNA in the sample. ISO (21569:2005) [12] requires that the LOD is provided with reference to a relative value based on a specified matrix, 'preferably a given amount of genomic DNA solution'. Accordingly, the EU-RL GMFF also carried out tests to estimate the Limit of Detection of the method (LOD_{rel}) relatively to a defined amount of wheat genomic DNA, extracted at EU-RL GMFF as previously described. For the estimation of the LOD_{rel}, respectively 84 and 10 haploid genome copies of MON71800 where added to 17311 wheat haploid genome copies (corresponding to 300 nanograms of non-GM wheat DNA (Table 4). Each level was tested in 60 replicates following the statistical model outlined above.

Table 4. Experimental set-up for the estimation of the relative Limit of Detection

MON71800 haploid genome copies	**Added background Wheat haploid genome copies**	**GM% in haploid genome copies**
84	17311	0.5
10	17311	0.06
NTC	-	-
Positive Control	84	100

3. Results

3.1 Quality of the extracted DNA

No measurable inhibition was detected.

3.2. Specificity of the method

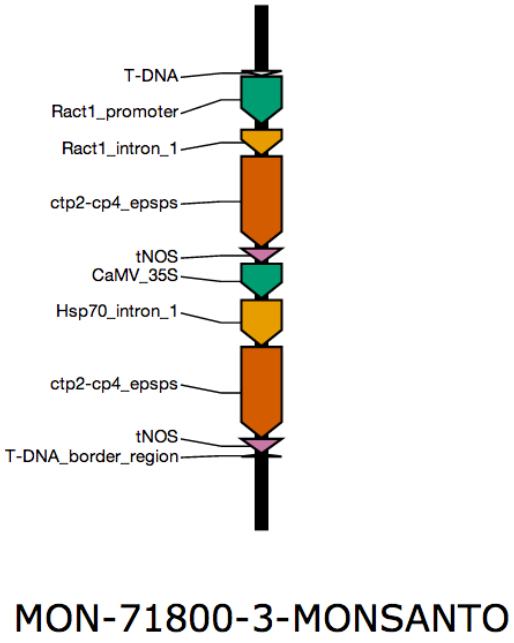
3.2.1. Bioinformatics analysis

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 was drawn at the MBG Unit of the JRC (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.

Analysis on the molecular structure of event MON-00603-6 (NK603) maize indicated that it shares all the elements of MON71800, in the same order.

Figure 1. Schematic representation of the molecular structure of MON71800



Bioinformatics verification of specificity

Similarity searches against the JRC-GMO databases did not show significant annealing for the two primers provided by Monsanto, which otherwise would have indicated a risk of cross-reactivity of the method against other, similar GMOs events that are approved in the EU or under EU regulatory approval: One primer binds to the genomic region, the other to the T-DNA of the event, and the probe is overlapping the two regions.

Wheat-specific reference system

For the "Internal Control", the method targets the *Triticum aestivum* acetyl-coenzyme A carboxylase (AF029897.1), for an amplicon size of 54 bp. No information on the performance of this set of primers and probes was made available by Monsanto. Although sets of primers targeting the same gene were found in the literature, the sequences of the primers are different and target a different section of the gene.

It seems that there are differences in the variants found in each of the three sets of chromosomes[13] (the *Triticum aestivum* genome is hexaploid, AABBDD), and that there is a one base difference compared to the probe sequence in two of the three variants (Figure 2). The exact origin of the three variants is unclear, but the variant showing perfect similarity (AF029897.1) seems to be of the A genome, according to currently published A genome sequence[14]. Regions with high similarity with the *acc* amplicon were found in some other plants genomes, such as barley and rice, but not, for example, in soybean.

Figure 2 - Alignment of the amplicon sequence of the acetyl-coenzyme A carboxylase detection method proposed by Monsanto with the records found in GenBank. The sequences of the primers and probes are shown in bold

```
Amplicon        ------------------------------------------------------------
AF029897.1      CTTTTTTCTCTAGTGGGTCCGAAGAACCCAGGGGCTCCTACCAAATGAATGGGATACTGA
AF029895.1      ------TTTCACATGGGTCCGAAGAACCCAGGGGCTCCTACCAAATGAATGGGATACTGA
AF029896.1      CTTTTTTCTCTAGTGGGTCCGAAGAACCCAGGGGCTCCTACCAAATGAATGGGATACTGA


Amplicon        --------------GGGAGGCATGCTTCGCTGTCTAAGGTTGTTGAATTTTGTATGGCAT
AF029897.1      ATGAAGCACATAATGGGAGGCATGCTTCGCTGTCTAAGGTTGTTGAATTTTGTATGGCAT
AF029895.1      ATGAAGCACATAATGGGAGGCATGCTTCGCTGTCTAAGGTTGTCGAATTTTGTATGGCAT
AF029896.1      ATGAAGCACATAATGGGAGGCATGCTTCGCTGTCTAAGGTTGTCGAATTTTGTATGGCAT
                              **************************** ****************

Amplicon        TGGGCGGC------------------------------------------------------
AF029897.1      TGGGCGGCAAAACACCAATTCACAGTGTATTAGTTGCGAACAATGGAATGGCAGCAGCTA
AF029895.1      TGGGCGGCAAAACACCAATTCACAGTGTATTAGTTG-----------------------
AF029896.1      TGGGCGGCAAAACACCAATTCACAGTGTATTAGTTGCGAACAATGGAATGGCAGCAGCTA
                ********
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The bioinformatics analysis therefore concluded that there is good reason to assume, based on the available sequence data, that the method proposed by Monsanto is indeed event specific.

3.2.2. Experimental testing of specificity

In addition to the bioinformatics analysis of specificity, a selection of GMO events was tested against the duplex MON71800/*acc* method in order to verify the specificity of that method also experimentally. Data were assessed in AQ assay and detection was declared when the amplification curve crossed the threshold within the number of cycles described in the method. For each reaction 2,500 GM copies were tested. Results indicating the outcome of tests and the quantification cycle (Cq) are shown in Table 5.

From Table 5 it is concluded that the experimental verification confirmed the finding of the bioinformatics analysis that the MON71800 method is indeed event-specific. The MON71800 amplification system did not show cross-reactivity with any of the GMO tested. Also the reference system *acc* did not show cross-reactivity with the materials tested except when assayed with DNA from soybean MON87701: an amplification curve at high Cq was generated in one out of three replicates. However, this finding was probably due to a slight contamination during experimental set-up because no other soy event reacted with the *acc* system, and similarity searches did not identify any similarity of *acc* amplicon in the soybean genome.

Table 5. Specificity of the MON71800 detection method. The Cq number is the average of three wells.

Event Name	Cq values event-specific reference system (MON71800)	Cq values taxon-specific reference system (*acc*)
CV127	n.d.	n.d.
MON87701	n.d.	36.1 (1/3 replicates)
MON87705	n.d.	n.d.
MON89788	n.d.	n.d.
A2704-12	n.d.	n.d.
A5542-127	n.d.	n.d.
FG72	n.d.	n.d.
Ms1	n.d.	n.d.
Ms8	n.d.	n.d.
Rf1	n.d.	n.d.
Rf2	n.d.	n.d.
Rf3	n.d.	n.d.
T45	n.d.	n.d.
Topas 19/2	n.d.	n.d.
GT73	n.d.	n.d.
MON87460	n.d.	n.d.
MIR162	n.d.	n.d.
MON88017	n.d.	n.d.
MON89034	n.d.	n.d.
MON810	n.d.	n.d.
MON863	n.d.	n.d.
GA21	n.d.	n.d.
Bt176	n.d.	n.d.
NK603	n.d.	n.d.
MIR604	n.d.	n.d.
Bt11	n.d.	n.d.
Event 3272	n.d.	n.d.
GHB119	n.d.	n.d.
GHB614xLL25	n.d.	n.d.
Conventional cotton	n.d.	n.d.
NTC	n.d.	n.d.
Positive Control	25.2	20.4

n.d.: not detected, i.e. the verified method was applied to the event in question but did not detect any presence
NTC: no template control
Positive Control: 1.6 ng of MON71800 DNA, about 90 haploid wheat genome copies

3.2 Absolute Limit of detection (LOD_{abs})

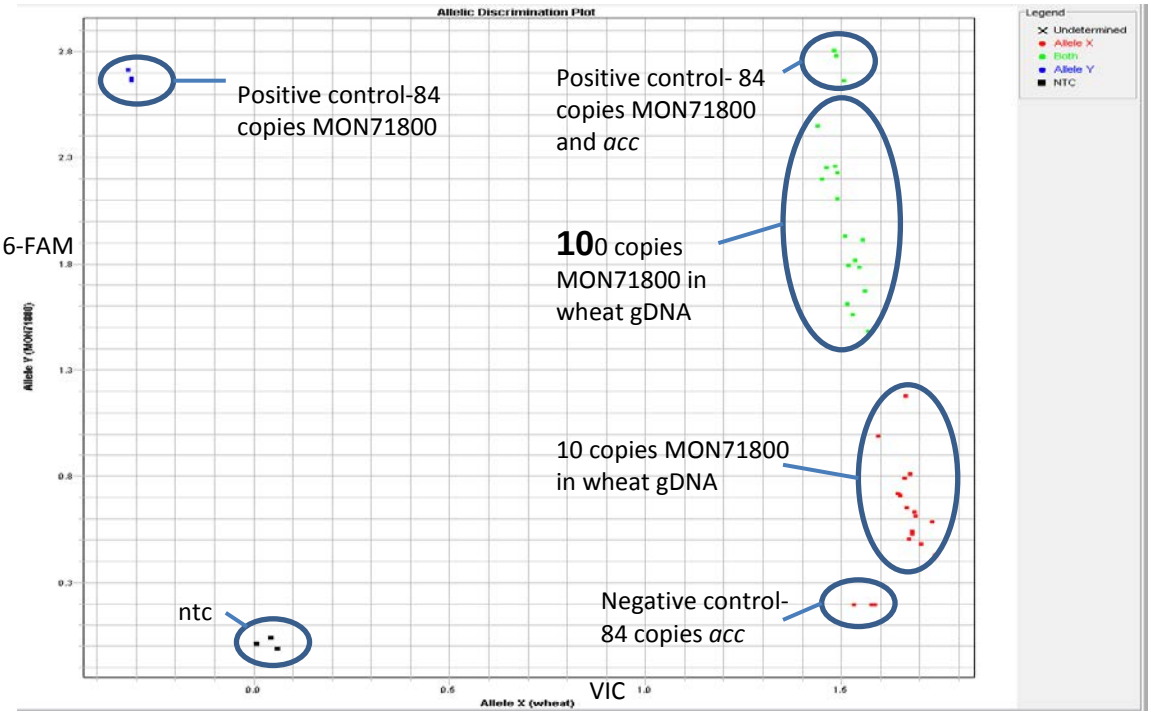
The ability of the method to detect MON71800 with the Allelic Discrimination (AD) assay was assessed by testing, in 10 replicates, two sets of positive samples, containing respectively 100 and 10 copies of MON71800 per reaction.

The FAM and VIC fluorescence values, measured at the end of the PCR cycling for the MON71800 PCR and the *acc* control PCR, are plotted by the software against each other on a scatter graph and are visually scored based on their clustering and the position of these cluster compared to known positive and negative control samples (Figure 3).

Figure 3 shows that samples containing 100 copies of MON71800 displayed high VIC signals and high FAM signals, consistent with known positive control samples, reacting with both MON71800 and *acc* systems, and were classified ('called') positive for the MON71800 event (green dots).

The figure also shows that for samples that contained only 10 copies of the MON71800 target, low FAM signals and high VIC signals were obtained (red dots). This is consistent with known negative control samples, and hence samples with 10 copies in reaction were misclassified as "negative".

Figure 3 shows the results of the detection of the target in samples at 100 and 10 copies evaluated according to the AD assay.



In light of this result the absolute LOD_{abs} was not established by means of the AD-assay but by means of the absolute quantification assay (AQ), i.e. on the basis of the amplification curves. The resulting values for the LOD_{abs} are shown in Table 6.

Table 6. LOD_{abs} of the duplex MON71800 method determined with the AQ-assay

MON71800 haploid genome copies	Average Cq for MON71800 /Standard Deviation	Positive/total amplifications
10	26.7/0.82	60/60
5	27.7/0.88	58/60
1/10 dil. of 1 copy	30.7/1.14	3/60
NTC	-	0/9*
Positive Control	24.5	6/6*

* Total of three runs

Positive Control: 50 copies of haploid MON71800

The absolute LOD (LOD_{abs}) of the event specific method provided by Monsanto was therefore found to be between 5 and 10 copies of MON71800 per reaction.

3.3 Relative Limit of Detection (LOD_{rel})

For the estimation of the LOD_{rel}, known quantities of MON71800 were added to 300 nanograms of non-GM wheat genomic DNA. The resulting samples were then tested with the absolute quantification assay (AQ). Each of the levels shown in Table 7 was tested in 60 replicates.

Table 7. Results of the determination of the LOD_{rel} of the duplex MON71800 method

MON71800 haploid genome copies in 300 nanograms of wheat genomic DNA	Average Cq for MON71800 /Standard Deviation	Positive/total amplifications
84	32.1/1.9	59/60
10	n.d.	0/60
NTC	n.d.	0/6*
Positive Control	29.7	3/3

* Total of two runs

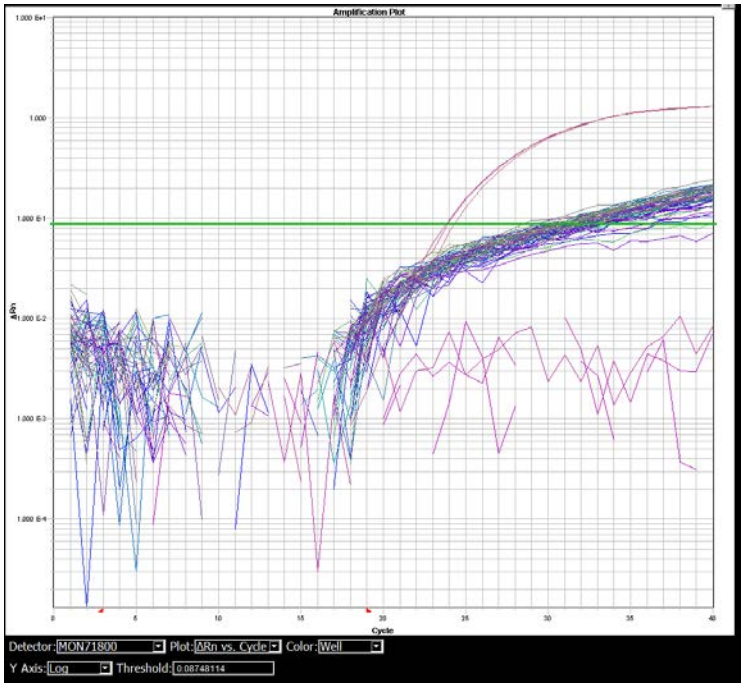
NTC = no template control

As shown in Table 7, fifty-nine out of sixty replicates were positive for samples that contained 84 MON71800 copies. On the other hand, all replicates of samples containing 10 MON71800 copies were negative.

Based on the statistical approach described above, according to which the experimental absolute LOD is the GM-level where the target is detected in 59 out of 60 replicates (Paragraph 2.4), the LOD_{rel} is therefore 0.5% expressed as ratio between GM- and target taxon-specific DNA copy numbers, equal to 84 MON71800 copies in 17,395 wheat genome copies.

Figure 4 shows the trend over cycles of the amplification curves of the samples tested in the LOD_{rel} experiments. It can be noted that, despite the curves cross the threshold line, they are flat, indicating poor efficiency, despite the wheat genomic DNA used as background DNA was tested free from inhibition.

Figure 4. Amplification curves of the LOD_{rel} experiments, MON71800 positive samples



It is noteworthy mentioning that the same samples analysed in the Allelic Discrimination did not reach the LOD_{rel} of 0.5%. In fact, the population of 60 replicates containing 84 copies of MON71800 in 300 ng wheat genomic DNA was misclassified and put into the group of samples only containing the *acc* target; indeed it appeared as a population of samples with high VIC fluorescence due to the *acc* wheat detection system while the fluorescence emission for the FAM reporter was not high enough to allow classification of the sample population as also containing MON71800 target. Therefore the results are identified as false negatives.

3.4. Additional testing

To elucidate better the dependence of the efficiency of the event-specific method (EMS71800) of Monsanto in function of the total DNA amount in reaction, additional tests were run with the AQ and AD assays.

Therefore, 100 copies and 10 copies of MON71800 were added to increasing amounts of wheat genomic DNA, i.e. to 100, 150, 200, 250 and 300 nanograms of non-GM wheat genomic DNA. Each sample was run in three replicates. The results of the experiment are presented in Table 8.

Table 8. Results of the tests conducted on samples containing two levels of MON71800 spiked in increasing amount of wheat background DNA.

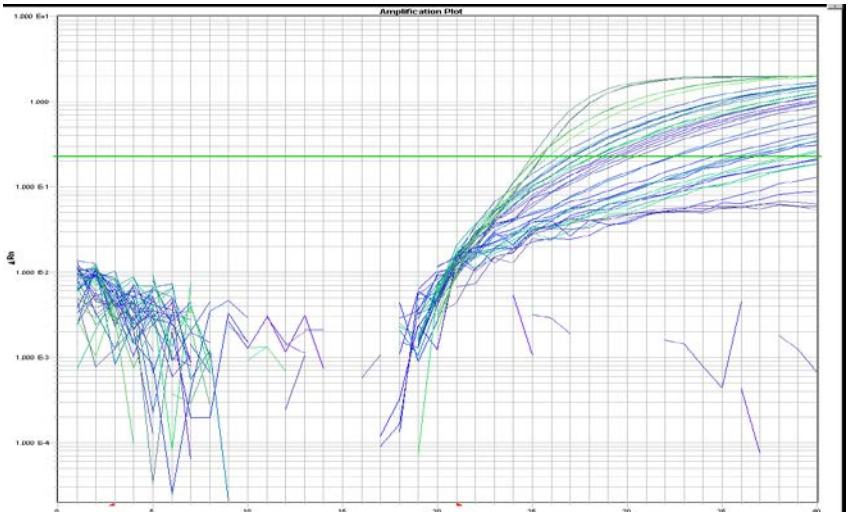
MON71800 haploid genome copies	Amount of background wheat genomic DNA in nanograms	AD assay Number of correct call/Number replicates	AQ assay Number of positive replicates/number replicates (average Cq values)
100	100	3/3	3/3 (26.8)
100	150	3/3	3/3 (27.4)
100	200	3/3	3/3 (28.5)
100	250	3/3	3/3 (29.2)
100	300	3/3	3/3 (29.8)
10	100	0/3	3/3 (35.7)
10	150	0/3	2/3 (34.3)
10	200	0/3	2/3 (36.2)
10	250	0/3	2/3 (38.9)
10	300	0/3	0/3

Table 8 shows that at increasing amounts of wheat genomic DNA the method loses efficiency in detecting the MON71800 target. When 100 or 10 copies of the MON71800 event are present, the Cq values increase with the increasing quantity of background DNA (AQ assay).

Table 8 also shows that with 100 MON71800 copies being present both the AD and AQ assays produce true positives while, when only 10 MON71800 copies are present, no target is detected in the AD assay at any of the tested background DNA amounts.

Figure 5 illustrates the appearance of the 30 amplification curves for the MON71800 sample population on which Table 8 is based. Flatter and more delayed curves correspond to samples containing increasing amounts of background DNA.

Figure 5. 30 amplification curves of the MON 71800 probe as summarised in Table 8.



4. Conclusions

Based on the above described bioinformatics and experimental verification, the EU-RL GMFF concludes as follows:

1. The wheat MON71800 Event Specific EndPoint TaqMan PCR method (EMS71800) provided by Monsanto, is event specific, i.e. can reliably differentiate between the MON71800 and a wide selection of GMO events as shown elsewhere in the present report.

2. The method is reliably able to detect presence of MON71800 at concentrations of 0.5% expressed as copy number ratio of GM-wheat/non-GM wheat and higher.

3. The absolute LOD is between 5 and 10 copies per reaction. Based on this evidence, it may be assumed that a sub sampling strategy could be designed to reach substantially lower detection levels. However, this would require significant additional efforts.

4. Additional tests showed that detection of 100 copies of MON71800 DNA in a background of increasing amounts of non-GM wheat genomic DNA (i.e. 100, 150, 200, 250 and 300 nanograms) occurred with proportionally decreasing reaction efficiency (paragraph 3.4), thus limiting the method's practical usability for GMO control purposes.

Given this performance of the method, and taking account of the fact that it is not generally established in EU GMO control laboratories, thus requiring significant additional effort, the EU-RL GMFF cannot recommend it for routine testing for GM wheat.
The EU-RL GMFF suggests using the testing strategy recently published (http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm). That strategy is based on readily available, established screening methods, and has a relative LOD between 0.03% and 0.06%.

In case a sample would test positive against the screening strategy, the herewith verified event specific method can be employed for counterchecking whenever the expected concentration is 0.5% or higher. For seeds/grains, sub-sampling could allow detecting lower concentrations but that would be rather demanding.

5. References

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