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JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

In-house verification of a qualitative, event-specific, qPCR-based method for detection and identification of GM wheat MON71200

European Union Reference Laboratory for
Genetically Modified Food and Feed

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EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
Food & Feed Compliance

In-house verification of a qualitative, event-specific, qPCR-based method for detection and identification of GM wheat MON71200

28 August 2018

European Union Reference Laboratory for GM Food and Feed

Executive summary

On 15 June 2018 the JRC – EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) was informed by DG SANTE of the findings of unauthorised plants of GM wheat MON71200 on an access road in Southern Alberta, Canada, and has been requested to assess and verify a method for detection and identification of MON71200.

Between 18 and 25 June 2018 the EURL GMFF received from the Canadian Food Inspection Agency (CFIA) a protocol of a qPCR-based method for qualitative, event-specific identification of GM wheat MON71200[1] and a limited amount of genomic DNA harbouring the GM locus MON71200, reagents and sequence information. The EURL GMFF performed an *in silico* specificity assessment of the MON71200 method and an experimental study to assess method specificity and the limit of detection (LOD). According to the *in silico* specificity analyses the method provided by CFIA is event-specific for event MON71200. Moreover, the wheat GM event should be detected by methods targeting the elements or constructs P-35S, CP2-EPSPS and T-nos.

Experimental testing showed that the MON71200 method can reliably detect the MON71200 wheat event. It does not respond to other unauthorised GM wheat lines (e.g. MON71800), to other GMOs hosting the *epsps* gene or to some common crops used in food and feed stuff. The LOD of the method is estimated to correspond to 5 haploid genome copies. In view of its specificity and sensitivity, the method provided by CFIA for the detection of wheat MON71200 is fit for purpose, i.e. it could detect very low concentrations of the corresponding GMO contamination.

Quality assurance

The EURL GMFF is applying procedures which are ISO 17025:2005 accredited [certificate number: Belac 268 TEST (Flexible Scope for DNA extraction, DNA identification and real Time PCR)] and ISO 17043:2010 accredited (certificate number: Belac 268 PT, proficiency test provider).

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

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Introduction

On 15 June 2018 the JRC – EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) was informed by DG SANTE of the findings of unauthorised plants of GM wheat MON71200 on an access road in Southern Alberta, Canada and was requested to assess and verify a method for detection and identification of MON71200.

The initial incident was reported to the Canadian Food Inspection Agency (CFIA) as wheat plants survived spraying treatment for weeds[1]. After screening a database of all GM crops that had been previously planted in confined research field trials in Canada, CFIA contacted companies who had field-tested GM wheat lines in past trials, to obtain methods and materials for detecting their respective GM wheat lines.

CFIA confirmed that the Alberta wheat sample was a match for a Monsanto GM wheat line (MON71200) used in multiple confined research field trials. CFIA sequenced the GM wheat's DNA and developed a PCR-based method to selectively amplify the DNA sequence at the junction between the wheat genome and the inserted DNA[2]. On 22 June 2018, CFIA confirmed that, based on the physical characteristics, as well as on genotyping data, the isolated finding of a few GM wheat plants, announced on June 14th, 2018, was not corresponding to the durum wheat (*Triticum durum*) species. Between the 18th and 25th of June 2018 the EURL GMFF received from CFIA a protocol for qualitative event-specific qPCR-based identification of GM wheat MON71200[1] and a limited amount of genomic DNA harbouring the GM locus MON71200, reagents and sequence information.

The EURL GMFF performed an *in silico* specificity assessment of the MON71200 method and an experimental study regarding the method specificity and its limit of detection (LOD). In addition the laboratory tested the ability of another two methods (an event-specific real-time PCR assay for GM wheat events MON71800 and a construct-specific method detecting both GM wheat events MON71700 and MON71800) to discriminate between those events and the MON71200 wheat event.

Materials, methods, experimental design

MON71200 wheat sample

The EURL GMFF received from CFIA genomic DNA harbouring the GM locus MON71200 (homozygous), wheat flour at 0.5% MON71200 and a linear synthetic positive control sample DNA. The total DNA concentration of the MON71200 DNA sample was measured in two readings (2 µL each) with the PicoGreen dsDNA Quantitation Kit (Molecular Probes) on a Versafluor fluorometer (BioRad), on the basis of a four-point standard curve ranging from 1 to 500 ng/µL

Control samples

Genomic DNA used in the specificity tests were from conventional maize, soybean, cotton, oilseed rape, sugar beet and rice or from GM events containing the *epsps* gene. They were previously obtained as control samples for validation studies or verification of methods for

detection or identification of the unauthorised wheat events MON71800 and MON71700. The material for event MON71700 was only available as 0.5% flour sample. Genomic DNA for maize MON87411, NK603, MON87427, soybean GTS 40-3-2, MON89788, MON87705, oilseed rape GT73 and MON88302, cotton MON88913, sugar beet H7-1 were available as 100% GM DNA solutions while genomic DNA for maize event MON88017 and cotton MON1445 were obtained as 10% GM material. The NK603 sample was available in a stack combination MON87427xMON89034xNK603.

Flour samples of 0.5% MON71800, 0.5% MON71700, conventional wheat and a crude genomic extract of 100% MON71800 were previously obtained by the EURL GMFF for verification of methods for detection of unauthorised GM wheat events. The wheat flour samples were extracted with NucleoSpin food kit (MACHEREY-NAGEL catalogue number: FC740945) while the crude genomic DNA from MON71800 was further purified with the following procedure. It was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuged for 30 minutes at 16,000xg at 4°C and subject to a second extraction with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation for 30 minutes at 16,000xg at 4°C the aqueous layer was precipitated with 1/10 volume of 3M sodium acetate (pH 5.2) and 3 times volume of cold absolute ethanol, incubated at -20°C for 1 hour. After centrifugation at maximum speed, the pellets were washed twice with 70% ethanol, dried and dissolved in 100 µl TE 0.1X buffer. Further purification was achieved using Nucleospin columns as above.

In silico assessment of the method specificity

The EURL GMFF carried out an *in silico* assessment on the specificity of the method for detection and identification of event MON71200 according to the sequence data provided by CFIA. The sequence of the amplicon was analysed by BLASTN, ver 2.3.0+, (NCBI)[3-4] against local copies of the "nt" and "patents" databases. The primers were tested against the sequences of the other GMO events present in the Central Core Sequence Information System (CCSIS) of the JRC, as well as 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 (NCBI)[5].

Experimental testing of specificity

The EURL GMFF performed the PCR reactions for testing the specificity of the MON71200 method according to the conditions specified in the protocol (see Annex 1) with the following adaptations: all tests were carried out on an ABI 7500 platform; ROX was selected as a passive reference dye; the channel VIC® was employed for the HEX™ reporter fluorophore while 'none' was selected for the double quencher ZEN-IowaBlack® FQ of the probe.

The amplification systems for the other GM events and conventional crop genomes used in the specificity tests were implemented according to their validated protocols available on the EURL GMFF website. As an exception the glutamine synthase (*GS*) sugar beet-specific system and the H7-1 system were amplified with the ABI TaqMan® Universal PCR Master Mix with UNG (with decontamination at 50 °C, 2 minutes) instead of PCR buffer I, while the PCR reactions for the maize event NK603 were run in a final volume of 25 µL instead of 50 µL. Taxon-specific methods employed respectively *acc-1* for wheat[6], *hmg* for maize[7], *Le1* for soybean[8], *adhC* for cotton[9], *CruA* for oilseed rape[10], *PLD* for rice[11] and *GS* for sugar beet[12].

According to the ENGL requirement *'specificity tests should be conducted with approximately 2500 copies of non-target DNA and with at least 100 copies of target DNA*' (ENGL, 2015)[14]. The

DNA amount was optimised to meet the limited availability of some positive control samples. Specificity tests for the MON71200 method were generally conducted with 100 ng of transgenic or plant gDNA (soy, maize, cotton, oilseed rape, rice, sugar beet) per reaction. Conversely were employed 200 ng of genomic DNA for the specificity test with event MON88017, 120 ng for the assays with 0.5% MON71700, 0.5% MON71800 and conventional wheat samples and 40 ng with the 100% MON71800 wheat sample. Twenty (20) ng of MON71200 DNA per reaction (corresponding to about 1154 MON71200 copies) were used for testing the MON71200 event-specific method and the *acc-1* taxon-specific system, while 40 ng per reaction (2308 MON71200 copies) were used for assessing the ability of the MON71800 event-specific method to discriminate between the GM MON71800 and MON71200 targets. A linear synthetic control sample DNA prepared by CFIA was also tested with the MON71200 method at a concentration of 0.1-1.0 fg/µl (total of 4 µl per reaction). Tests were conducted in triplicate reactions if not otherwise stated.

Limit of Detection

To determine the absolute limit of detection (LOD_{abs}), defined as the amount of analyte that is detected at least 95% of the times, thus ensuring ≤5% false negative results, the EURL GMFF tested the wheat MON71200 genomic DNA sample in 60 replicates per level at 1, 5, 10 and 50 copies per reaction. The number of copies was calculated under the assumption that the genomic DNA was extracted from plants harbouring the GM locus in a homozygous status and that one haploid genome of wheat[17] corresponded to a mass of 17.33 pg. The LOD is the lowest concentration yielding at least 59 positive results[15-16].

Results

In silico specificity assessment by the EURL GMFF

Bioinformatics analysis performed by the EURL GMFF indicated that the MON71200 detection method spans the 3' insert-to-plant junction of the GM wheat event MON71200. In particular the MON71200 forward and reverse primers were found to anneal respectively to the insert and the wheat genomic border region adjacent to the insertion site, while the probe resulted to bind to the junction between the insert and the 3' genomic region. The ensuing amplicon size is expected to be 139 bp. BLAST (NCBI)[3-4] analyses of the entire amplicon against local copies of the "nt" and "patents" databases revealed no significant similarity with any other published sequence. Similarity values of 98%-100% were found between nucleotide 1 to 57 of the amplicon sequence and GM constructs hosting the cp4epsps gene, GM events MON71800, MON87411, NK603, MON88017, GTS 40-3-2 and a putative sequence of GM Event ASR-368 (SMG-36800-2) in the regions hosting the *CP4-epsps* transgenic sequence. Similarity values of 93%-99% were also found between nucleotide 63 to 139 of the MON71200 amplicon and the *Triticum aestivum*, *urartu* and *Aegilops* genomes. Analysis performed with the e-PCR prediction tool (NCBI)[5] where the primers were tested against the sequences of the other GMO events present in the JRC CCSIS database as well as the whole genomes of more than 80 plants (including *Brassica rapa*, *Glycine max*, *Oryza sativa*, *Solanum lycopersicum* and *Zea mays*) did not identify any potential amplicon.

A list of methods of the EU Database of Reference Methods[18] predicted to react with the MON71200 event includes those targeting the element P-35S (QT-ELE-00-004 and QT-ELE-00-001, QL-ELE-00-001, QL-ELE-00-004, QL-ELE-00-005, QL-ELE-00-012, QL-ELE-00-017), the construct CTP2-CP4EPSPS (QL-CON-00-008), and most methods targeting the element T-nos (QL-ELE-006, QL-ELE-00-007, QL-ELE-00-009, QL-ELE-00-018), while method Ql-ELE-00-011 shows an imperfect probe alignment. Overall, this is similar to the findings obtained for the unauthorised wheat event MON71800 previously tested by the EURL GMFF[19-20].

Experimental verification of the method specificity

The event-specificity of the MON71200 method was experimentally verified against the unauthorised wheat events MON71800 and MON71700 and the genomic DNA of wild type wheat, soybean, maize, oilseed rape, rice, cotton, sugar beet and of a selection of GM events (Tables 1, 2 and 3).

Table 1. Specificity of MON71200 method and MON71800/71700 methods against conventional and GM wheat events

Method	Sample	Cq Mean / St Dev
MON71200	MON71200	25.2 / 0.08
Acc-1	MON71200	17.9 / 0.02
MON71200	MON71200*^	29.6 / 0.56
MON71800	MON71200	n.d.
MON71700/71800[#]	MON71200^	n.d.
MON71700/71800[#]	MON71800, 0.5%^	26.1 / 0.02
MON71200	Wheat	n.d.
Acc-1	Wheat	15.8 / 0.06
MON71200	MON71800	n.d.
MON71800	MON71800	21.2 / 0.21
MON71200	MON71700, 0.5%	n.d.
MON71700/71800[#]	MON71700, 0.5%	26.1 / 0.26
Acc-1	MON71700, 0.5%	15.8 / 0.08

n.d.: not detected, i.e. no amplification observed when the method is applied to the specified sample
*Linear Synthetic DNA positive control
[#]Construct-specific method targeting MON71800 and MON71700, under publication by the EURL GMFF
^ duplicate reactions

Table 2. Specificity of MON71200 method against genomic DNA of conventional crops

Method	Sample	Cq Mean / St Dev
MON71200 ^	Maize	n.d.
Hmg	Maize	24.0 / 0.02
MON71200 ^	Cotton	n.d.
adhC	Cotton	24.5 / 0.02
MON71200 ^	Oilseed rape	n.d.
CruA	Oilseed rape	24.1 / 0.04
MON71200 ^	Rice	n.d.
PLD	Rice	23 / 0.03
MON71200 ^	Sugar beet	n.d.
GS	Sugar beet	23.1 / 0.04
MON71200 ^	Soybean	n.d.
Le1	Soybean	23.0 / 0.02

n.d.: not detected, i.e. no amplification observed when the method is applied to the specified sample
^ duplicate reactions

Table 3. Specificity of MON71200 method against gDNA of selected GM events

Method	Sample	Cq Mean / St Dev
MON71200 ^	MON87411 maize	n.d.
MON87411	MON87411 maize	26.2 / 0.05
MON71200 ^	NK603 maize	n.d.
NK603	NK603 maize	26.0 / 0.07
MON71200 ^	MON88017 maize	n.d.
MON88017	MON88017 maize	29.0 / 0.04
MON71200 ^	GTS 40-3-2 soybean	n.d.
GTS 40-3-2	GTS 40-3-2 soybean	25.3 / 0.01
MON71200 ^	GT73 oilseed rape	n.d.
GT73	GT73 oilseed rape	24.1 / 0.02
MON71200 ^	H7-1 sugar beet	n.d.
H7-1	H7-1 sugar beet	24.8 / 0.27
MON71200 ^	MON1445 Cotton	n.d.
MON1445	MON1445 Cotton	29.1 / 0.01
MON71200 ^	MON89788 Soybean	n.d.
MON89788	MON89788 Soybean	23.0 / 0.01
MON71200 ^	MON88913 Cotton	n.d.
MON88913	MON88913 Cotton	26.3 / 0.07
MON71200 ^	MON87705 Soybean	n.d.
MON87705	MON87705 Soybean	25.1 / 0.05
MON71200 ^	MON88302 oilseed rape	n.d.
MON88302	MON88302 oilseed rape	25.2 / 0.02
MON71200 ^	MON 87427 Maize	n.d.
MON87427	MON 87427 Maize	26.1 / 0.05

n.d.: not detected, i.e. no amplification observed when the method is applied to the specified sample
^ duplicate reactions

The MON71200-specific method responds to the sample of MON71200 DNA and not to DNA from MON71800 and MON71700 or plant species DNA. The functioning of the amplification systems and the amplifiability of the DNA samples is shown in control reactions. The 'no template control' reactions were all negative.

Limit of Detection of the MON71200 method

The sensitivity of the MON71200 event-specific method was evaluated through the determination of the LOD_{abs}, assessed on the MON71200 positive control DNA sample received from CFIA.

Table 3 reports the results of the LOD_{abs} determination.

Table 3. Results for LOD_{abs} of the MON71200 method

MON71200 haploid genome copies per reaction (homozygous)	Positive/Total reactions
50	60/60
10	60/60
5	59/60
1	31/60

The LOD is the lowest concentration level yielding at least 59 positive reactions[15-16]**.** According to the results presented in Table 3, the LOD_{abs} of the MON71200 event-specific method corresponds to five haploid genome copies of event MON71200. As the probability distribution suggests, in case of 60 replicated tests, 1 copy should give approximately from 24% to 49% negative results at 95% CI[14]. Under the assumption that one haploid genome of wheat (*T. aestivum*) has a mass of 17.33 pg[12] and that the MON71200 sample is homozygous for the GM insertion, the level estimated at 1 copy of MON71200 per reaction yielding 48% (29/60) negative results is in line with the expected theoretical range for negative results.

Conclusions

Bioinformatics analysis performed by the EURL GMFF indicated that the detection method provided by CFIA is event-specific for wheat event MON71200. Experimental testing confirmed that the method for detection of MON71200 wheat (simplex) can reliably and specifically detect the MON71200 wheat event. It does not react with other unauthorised GM wheat lines (MON71700 and MON71800), other crops (maize, soy, cotton, rice, oilseed rape, sugar beet and wheat) and a selection of GM events under the reaction conditions described in the protocol.

According to *in silico* specificity analyses the MON71200 event should provide positive amplification also to methods targeting the elements or constructs P-35S, CTP2-CP4EPSPS and T-nos.

The LOD_{abs} of the method is estimated at 5 haploid genome copies under the assumptions of a GM locus in a homozygous status and a mass of 17.33 pg for one haploid wheat genome[17]. In view of its specificity and sensitivity the method provided by CFIA for the detection of wheat MON71200 is therefore considered fit for purpose, i.e. it could detect very low concentrations of the corresponding GMO contamination.

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Annex 1. Event-Specific Diagnostic Method for the Detection of MON71200 Wheat

Method Developer
Canadian Food Inspection Agency

Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Event-Specific Diagnostic Method for the Detection of MON71200 Wheat (Simplex)

0. General Information

Protocol Title	Event-Specific Method for the Detection of MON71200 Wheat Using Real-time PCR
Source:	Genotyping – Botany Laboratory, Ottawa Plant Laboratory, CFIA
Event-Specific:	MON71200 Wheat Event
Transgene Target Assay:	MON71200 - 3'end junction, 139 bp
Limit of detection (LOD):	0.1% in 100 ng of total wheat DNA
Wheat Endogene Target :	Wheat *acc-1*, 94 bp

1. DNA Extraction

SOP Reference:	OPL-PR126
Method:	Modified QIAGEN DNeasy Plant Mini Kit
Amount:	200 mg
Elution Volume:	100 uL

2. RT-PCR Conditions

2.1 Event-Specific

Primers Identification:

	Primer ID	Name	Oligonucleotide DNA Sequence (5' to 3')
Trans Assay	1049	MON71200-3 ' junction-1F	CAC GAC GGT CAT CGA GC
	1050	MON71200-3 ' junction-1R	CCG TTC GTC ATT GAC TGT T
	1051	MON71200-3 ' junction-P	5HEX/CAT ACG GAA/ZEN/AAG ATG CTG CAG GGA ATA TAT TGA AC/3IABkFQ

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RT-PCR Master Mix:

Transgene Assay		
Reagents	1 X*	Final [ ]
2X NEB Luna Universal Probe qPCR Master Mix	10	1X
10 uM MON71200-3 ' junction-1F	0.8	400 nM
10 uM MON71200-3 ' junction-1R	1.6	800 nM
10 uM MON71200-3 ' junction-P	0.4	200 nM
25mM $MgCl_2$	0.8	1 mM
sdH_2O	1.4	-
Vol mix 1X	15	
DNA (20 ng/uL)	5	-
Vol final PCR	20	

*Volumes are expressed in uL.

RT-PCR Profile:

Machine: ViiA 7 Real-Time PCR System (Life Technologies)
Protocol: wheat_TD60-55

Initial Denaturation	1 cycle	95 °C	60 sec
Denaturation	5 cycles	95 °C	15 sec
Annealing & Extension		60 °C – 1 °C/cycle	30 sec
Denaturation	40 cycles	95 °C	15 sec
Annealing & Extension		55 °C	30 sec + plate read

2.2 Wheat endogen target

Primers Identification and Real-time PCR conditions:

Wheat *acc-1*

http://gmo-crl.jrc.ec.europa.eu/doc/EURL-EM-02-13-VR-3.pdf

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3. Results

Samples Tested for Event-Specific Cross-Reactivity:

Canola events: GT73, HCN92 (topas 19/2), Ms1-Rf2, Ms8-Rf3, OXY-235, HCN28/T45

- No cross-reactivity observed

Corn events: MON810, NK603, MON863, MON863/810, MON88017

- No cross-reactivity observed

Soybean events: GTS 40-3-2, MON89788, DP356043, DP305423, A2704-12, A5547-127

- No cross-reactivity observed

Wheat events: MON71700, MON71800, 2mepsps

- No cross-reactivity observed

4. Testing scheme and Data Interpretation

Step1:

1) Detection of the MON71200 Wheat Event with one real-time PCR assay.
2) Confirmation of wheat DNA extraction quality with a wheat-specific real-time PCR assay.

Decision Chart:

	Event Assay	Wheat Assay	Result	Notes
Sample 1	–	+	–	Results released as **Not Detected** for MON71200 wheat
Sample 2	+	+	+	Results released as **Detected** for MON71200 wheat

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5. Low-level Presence Sampling Strategy

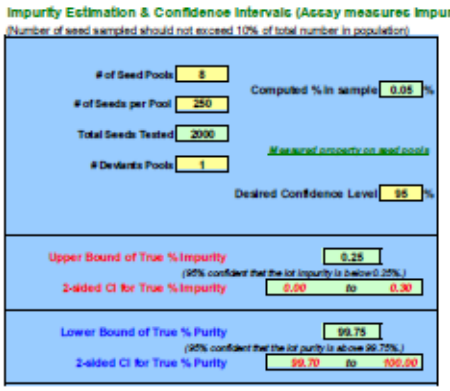
The overall CFIA low-level presence testing strategy is based on ISTA (International Seed Testing Association) Rules for GMO detection. General test procedure involves the division of a seed sample into a certain number of pools that are treated as independent samples for processing and testing. The use of sub-samples allows for a better estimate of the % of GM presence in a sample, and also a higher confidence in the level of impurity found in a sample.

CFIA testing strategy is described below. This strategy is based on calculations made using the statistical tool for seed testing Seedcalc8 (ISTA - https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--1143--279.html).

8 pools of 250 seeds: 2000 seeds tested
If 0/8 pools have GM detected: there is 0% detected in the *sample*
Seed Lot purity estimation: 95% confident that there is <0.15% in the seed lot, 95% CI: 0-0.18% - meaning there could be between 0 and 0.18% in the Seed Lot.

Overall:

- % of GM presence detected in the sample varies between 0.05% (1/8+) and 0.83% (7/8+)
- With 1 detected pool: 95% confident that the seed lot impurity is below 0.25% (confidence interval= 0.00% to 0.30% - i.e. could be up to 0.30%)
-



JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

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