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

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

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European Union Reference Laboratory for GM Food and Feed

Executive Summary

In response to a request of DG SANCO to provide National Reference Laboratories (NRLs) as soon as possible with a method to test soft white wheat consignments for the presence of unauthorised GM glyphosate-resistant wheat harbouring the event MON71800, the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) developed, in collaboration with the European Network of GMO Laboratories (ENGL), a testing strategy[a] intended to be immediately implementable by EU NRLs. The testing strategy is based on a combination of three validated screening methods that allow excluding (detectable) presence of Monsanto's GM glyphosate-resistant wheat (MON71800) in wheat grain or food/feed products and confirming its presence whenever other GMOs can be excluded.

The present report describes the results of the tests carried out by the EU-RL GMFF to verify the testing strategy proposed; the tests were conducted using the positive control sample represented by a crude DNA lysate of MON71800 provided by Monsanto and genomic DNA samples of genetically modified organisms harbouring the CTP2-CP4epsps element for which a validated event-specific method is available.

The sensitivity of the three methods was assessed by verifying the relative limit of detection (LOD_{rel}) on MON71800 wheat DNA. The LOD_{rel} is approximately 0.03% for the P-35S and for T-nos methods and 0.06% for the CTP2-CP4epsps method in 300 nanograms of wheat genomic DNA.

Further experimental evidence confirmed that the three methods react against genomic DNA extracted from GM events containing the CTP2-CP4epsps element for which a validated event-specific method is available.

The experimental verification hereby reported confirmed the validity of the EU-RL GMFF guidance on testing for GM glyphosate-resistant wheat (MON71800) in wheat grain or in food/feed products containing wheat flour originating or consigned from the US, provided that DNA of acceptable quality can be obtained.

[a] http://gmo-crl.jrc.ec.europa.eu/doc/Wheat_EURL_testingstrategy_V2.pdf

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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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 in a farm in Oregon harbouring the event MON71800, DG SANCO asked the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) to provide National Reference Laboratories (NRLs) with a method to test soft white wheat consignments for the presence of this GMO.

As an event-specific method is still under assessment by the EU-RL GMFF, an interim strategy[1] was developed that should be replaced or amended by an event-specific method when this will be considered suitable for the purpose.

Further to bioinformatics analyses conducted on sequence information provided by Monsanto, event MON71800 was shown to contain the cassette of the construct CTP2-CP4epsps, the CaMV 35S promoter (P-35S) and the nopaline synthase terminator (T-nos). Based on such knowledge, the EU-RL GMFF developed, in collaboration with the European Network of GMO Laboratories (ENGL), a testing strategy intended to be immediately implementable by EU official control laboratories: it is based on three validated screening methods targeting the above mentioned genetic elements and constructs, allowing excluding (detectable) presence of Monsanto's GM glyphosate-resistant wheat (MON71800) in wheat grain or food/feed products and confirming its presence whenever other GMOs can be excluded.

2. Materials, methods and experimental design

2.1 Bioinformatics analysis

Bioinformatics analyses were conducted by the EURL-GMFF on the basis of the sequence of event MON71800 wheat provided by Monsanto. The determination of the molecular structure was based on annotations provided by Monsanto, complemented with information from patents US6689880[2] and US7268274[3] and confirmed by comparisons with the sequences of other previously characterised events.

Determination of element and construct-specific methods that could detect MON71800 was performed using specific bioinformatics software that perform simulations of polymerase chain reactions (ePCR[4] and primersearch[5]) using primers and probes sequences from available methods[6].

2.2 Control samples and DNA extraction

Genomic DNA from MON88017, NK603, MON89788, H7-1, MON1445 and MON88913 was already available at the EU-RL GMFF.

DNA extracted from MON71800 wheat material was provided by Monsanto as crude lysate and was resuspended according to instructions.

Genomic DNA was extracted from GT73 and from conventional soybean, maize, cotton, sugar beet, oilseed rape and wheat.

DNAs were extracted according to a modified CTAB DNA extraction method[7] followed by additional purification on a Qiagen Genomic Tip 20[8], where necessary.
Conventional wheat genomic DNA (*T. aestivum)* was extracted using the NucleoSpin® kit (Macherey-Nagel).

The concentration of the DNA samples was determined by fluorescence detection, after extensive homogenisation, using PicoGreen dsDNA Quantitation Kit (Molecular Probes). The DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/mL to 500 ng/mL using a VersaFluor fluorometer (Bio-Rad) as fluorescence detector.

Inhibition runs were performed as described in the ENGL guidance document "Verification of analytical methods for GMO testing when implementing interlaboratory validated methods"[2] using the respective taxon-specific validated reference systems. DNA integrity was verified by agarose-gel electrophoresis.

2.3 Specificity of the methods applied in the 'MON71800 testing strategy'

The three validated methods summarised in Table 1 were applied to DNA extracted from GM-events containing the CTP2-CP4epsps element for which a validated event-specific method is available (Table 2).

Table 1. Methods used to verify the testing strategy and reference to the GMO method database

Target genetic element	Oligonucleotide sequences	Reference in the GMO Method Database	Ref.
CTP2-CP4epsps	PF: 5'-GGGATGACGTTAATTGGCTCTG-3' PR: 5'-GGCTGCTTGCACCGTGAAG-3' PROBE: FAM-CACGCCGTGGAAACAGAAGACATGACC-TAMRA	QL-CON-00-008	9
T-nos	PF: 5'-CATGTAATGCATGACGTTATTTATG-3' PR: 5'-TTGTTTTCTATCGCGTATTAAATGT-3' Probe: 5'-FAM-ATGGGTTTTTATGATTAGAGTCCCGCAA-TAMRA-3'	QL-ELE-00-011	10
P-35S	PF: 5'-GCCTCTGCCGACAGTGGT-3' PR: 5'-AAGACGTGGTTGGAACGTCTTC-3' Probe: 5'-FAM-CAAAGATGGACCCCCACCCACG-TAMRA-3'	QT-ELE-00-004	11

http://gmo-crl.jrc.ec.europa.eu/gmomethods/entry?db=gmometh&id=ql-con-00-008&rq=QL-CON-00-008

http://gmo-crl.jrc.ec.europa.eu/gmomethods/entry?db=gmometh&id=ql-ele-00-011&rq=QL-ELE-00-011

http://gmo-crl.jrc.ec.europa.eu/gmomethods/entry?db=gmometh&id=qt-ele-00-004&q=QT-ELE-00-004

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

Table 2. GM events containing the CTP2-CP4epsps element for which a validated event-specific method is available.

Crop	Event	CTP2-CP4epsps		
			P-35S	T-nos
Maize	MON88017	+	+	+
Maize	NK603	+	+	+
Soybean	MON89788	+	-	-
Rapeseed	GT73	+	-	-
Sugar Beet	H7-1	+	-	-
Cotton	MON1445	+	+	+
Cotton	MON88913	+	+	-

The experimental design implied the use in reaction of respectively 100 GM copies and 10 GM copies of each GMO in a background of 300 ng and 100 ng of the respective species genomic DNA as detailed in Table 3.

Table 3. Experimental design to test the strategy on GMOs listed in Table 2.

Crop	GM-DNA	Method	Number of GM copies in reaction	Total ng DNA in reaction	Replicates
Maize	MON88017, NK603	*Hmg* 35S T-nos CTP2-CP4epsps	100 and 10	300 and 100	3
Soybean	MON89788	*Lec1* 35S T-nos CTP2-CP4epsps	100 and 10	300 and 100	3
Oilseed rape	GT73	*CruA* 35S T-nos CTP2-CP4epsps	100 and 10	300 and 100	3
Sugar Beet	H7-1	*GS* 35S T-nos CTP2-CP4epsps	100 and 10	300 and 100	3
Cotton	MON1445, MON88913	*AdhC* 35S T-nos CTP2-CP4epsps	100 and 10	300 and 100	3

Methods for crop specific reference systems [respectively, *hmg* (high mobility group) for maize, *lec1* (lectin 1) for soybean, *CruA* (cruciferin A) for oilseed rape, *GS* (glutamine synthetase) for sugarbeet and *AdhC* (alcohol dehydrogenase C) for cotton], were applied as described in the respective validated methods[12].

2.4 Determination of the relative Limit of Detection (LOD_{rel}) of P-35, T-nos and CTP2-CP4epsps methods on MON71800 sample

The relative limit of detection (LOD_{rel}) of the methods targeting P-35S, T-nos and CTP2-CP4epsps was determined on DNA extracted from MON71800 provided by Monsanto in a background of respectively 300 ng and 100 ng of conventional wheat (*T. aestivum*) DNA extracted by the EU-RL GMFF. To carry out the assessment of the LOD_{rel} the weight of the 1-C value of wheat genome was considered to be 17.33 pg in accordance with the Plant DNA C-values Database of the Royal Botanic Garden[13].

The zygosity of the MON71800 control sample was not determined by Monsanto nor zygosity tests in digital PCR were run by the EU-RL GMFF. However, the control sample was considered homozygous for MON71800 based on information received from Monsanto.

Three levels containing respectively 10 copies, 5 copies and 1/10 dilution of 1 copy of MON71800 in background wheat DNA were tested in ten replicates, according to the recommendations of the ENGL guidance on method verification[14]. The estimated LOD is the lowest concentration where all replicates are positive. Positive control reactions with a wheat-specific reference system[15] were run to ensure that the amplification of the wheat DNA worked correctly.

3. Results

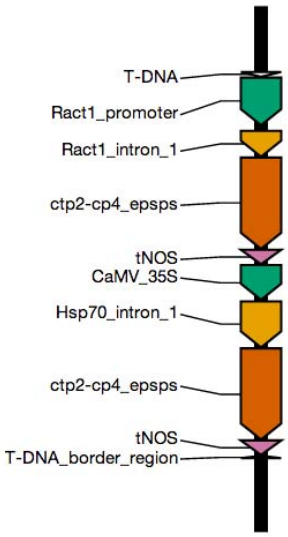
3.1 Bioinformatics analyses

3.1.1 MON71800

Bioinformatics analyses indicated that 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 P-35S and two copies of the T-nos (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



3.1.2 Wheat-specific reference system

The event MON71800 was inserted in white soft wheat (*T. aestivum*). The major cultivated species of wheat *Triticum aestivum* (common wheat) and *T. spelta* are hexaploid, represented as AABBDD genome type, and *T. durum* and *T. dicoccum,* which are tetraploid, represented as AABB. International literature reports about optimisation of several wheat reference systems. However, the selection of a target sequence for specific detection of *T. aestivum* requires in-depth analysis.

For the purpose of the present testing strategy, the EU-RL GMFF made use of the reference system targeting the *T. aestivum* gene Wx-D1 (accession number AF113844) coding for the granule-bound starch synthase and developed by Iida *et al*[15]. The system is species-specific (it does not react with *T. durum* and other cereals), and it is stable across varieties of the same species.

Bioinformatics analysis revealed differences between the published primers and probe sequences (Table 1 of Iida *et al*[15]) and the corresponding sequences of *T. aestivum* (Figure 2 of Iida *et al*[15] and the currently published version of the wheat genome[16]).

Therefore, the EU-RL GMFF made use of the primers (Wx012F/Wx012R) and probe (Wx-Taq 1) sequences reported by the same authors in a subsequent publication[17] and patent US 2009/0011411 A1[18] that are different in one nucleotide from those on Table 1 of Iida *et al*.

3.2 Control samples and DNA extraction

Genomic DNA extracted from MON88017, MON89788, H7-1, MON1445, MON88913 were already available, whilst genomic DNA of GT73 and of conventional soybean, rapeseed, maize, sugar beet, cotton and wheat were extracted as shown in Table 4 and previously described.

Table 4 provides the details on the DNA extraction method use and on the DNA concentration of the stock solutions obtained.

Table 4. DNA extraction methods and DNA concentration of genomic DNA samples

	Extraction method	Stock (ng/uL)
MON71800	-	0.8
MON88017	-	37.3
NK603	-	13.2
MON89788	-	20.5
GT73	CTAB + Tip20	58.3
H7-1	-	26.9
MON1445	-	93.5
MON88913	-	126.2
Soy Wild Type	CTAB	190.9
Rapeseed Wild Type	CTAB + Tip20	57.8
Maize Wild Type	CTAB	332.3
Sugar beet Wild Type	CTAB + Tip20	186.4
Cotton Wild Type	CTAB + Tip20	103.0
Wheat Wild Type	Nucleospin	191.0

Inhibition runs were carried out with the respective taxon-specific validated methods to rule out possible inhibitory effects (data not shown). No inhibition test was run on MON71800 control sample given the low amounts of MON71800 in reaction (e.g. 100 copies or 10 copies), as envisaged in the experimental determination of the LOD.

3.3 Specificity of the methods applied in the 'MON71800 testing strategy'

The specificity of the methods composing the testing strategy was experimentally verified on GM events containing the CTP2-CP4epsps element for which a validated event-specific method is available using 100 and 10 GM copies of GM DNA in 300 ng and 100 ng conventional genomic DNA, as detailed in Table 3 above.

Results are expressed as mean Cq (quantification cycle) value of three replicates +/- standard deviation for the different targets.

Table 6. Mean Cq value of three replicates +/- standard deviation for P-35S, T-nos and CTP2-CP4epsps methods and for the taxon-specific method.

100 GM copies in 300 ng background wheat DNA

	CTP2-CP4epsps	P-35S	T-nos	Taxon-specific
MON88017	33.36+/-0.38	32.24+/-0.11	36.57+/-0.10	21.46+/-0.09
NK603	31.53+/-0.13	32.00+/-0.16	34.67+/-0.22	21.51+/-0.04
MON89788	31.46+/-0.20	*37.80**	n.d.	20.26+/-0.09
GT73	30.57+/-0.09	*39.04+/-1.10*	n.d.	20.50+/-0.16
H7-1	33.36+/-0.35	n.d.	n.d.	21.07+/-0.08
MON1445	32.45+/-0.24	32.35+/-0.26	37.41+/-0.56	22.94+/-0.05
MON88913	31.68+/-0.22	31.67+/-0.12	*43.44*	22.93+/-0.04

n.d., not detected within the validated number of cycles e.g. 45 cycles

* one positive out of three replicates

Table 7. Mean Ct value of three replicates +/- standard deviation for P-35S, T-nos and CTP2-CP4epsps methods and for the taxon-specific method.

10 GM copies in 300 ng background wheat DNA

	CTP2-CP4epsps	P-35S	T-nos	Taxon-specific
MON88017	36.20+/-0.21	35.84+/-0.91	40.43+/-1.45	21.42+/-0.06
NK603	34.88+/-0.42	34.27+/-0.47	38.52+/-0.94	21.03+/-0.05
MON89788	34.93+/-0.29	*36.33**	*43.6**	20.35+/-0.10
GT73	33.88+/-0.17	*38.16+/-0.45*	n.d.	20.42+/-0.16
H7-1	35.98+/-0.46	n.d.	n.d.	20.97+/-0.03
MON1445	35.87+/-0.77	35.26+/-0.23	41.21+/-0.86	22.79+/-0.10
MON88913	35.03+/-0.18	34.76+/-0.40	*43.92+/-2.53*	22.76+/-0.15

n.d., not detected within the validated number of cycles e.g. 45 cycles

* one positive out of three replicates

Results shown in Tables 6 and 7 demonstrate that the method targeting the CTP2-CP4epsps construct reacted with the GM events even when the GM content in reaction was as low as 10 copies. The increase in Cq between samples containing the same GMO are consistent with the decreased number of targets in reactions, respectively 100 and 10 copies.

Similarly, maize MON88017, NK603 and cotton MON1445, MON88913 reacted with the P-35S method and maize MON88017, NK603 and cotton MON1445 reacted with the T-nos method.

Unexpected cross-reactivity at very high Cq values were detected for the P-35S method with the DNA from MON89788 and GT73 and for the T-nos method with MON88913 and MON89788. However, only one out of three replicates was positive for P-35S with 100 copies of MON98788 and for T-nos with 10 copies of MON89788. In addition, it is noted that the Cq for these positive reactions did not show increase in values at decreasing the target GM-content in reaction (Tables 6 and 7), thus suggesting low contamination of samples.

3.3.1 Analysis of unexptected cross-reactivities

In order to confirm (cross-) contamination or aspecific reactions, a number of additional plates was run in which (I) the suspect reactions from Tables 6 and 7 were re-run using the same DNA mix, (II) GMO and wild type DNA were tested separately using both Taqman and SYBRgreen chemistries, followed by melting analysis.

These tests quickly indicted that the unexpected T-nos positive results were likely caused by cross contamination as none of the additional tests showed amplification before cycle 50 (see Table 4). SYBRgreen analysis indicated that the P-35S primers may generate aspecific products with a different melting temperature than the P-35S target. It is hypothesised that these products may cause runaway probe degradation in oilseed rape background.

Table 8. Analysis of cross-reactivities

	MON88913	**MON89788**	**GT73**		
	T-nos (Taqman)	T-nos (Taqman)	P-35S (Taqman)	P-35S (SYBR)	
	Cq	Cq	Cq	Cq	Tm
Mixture (10 GM-copies in 300ng species-DNA)	Not detected	Not detected	38.5	n.a.	n.a.
	Not detected	Not detected	36.3	n.a.	n.a.
	Not detected	Not detected	39.51	n.a.	n.a.
Pure GMO DNA (50ng)	Not detected	Not detected	37.18	35.82	78.2
	Not detected	Not detected	Not detected	35.85	77.9
	Not detected	Not detected	Not detected	36.77	77.6
Pure WT DNA (300ng)	Not detected	Not detected	41.69	41.55	77.6
	Not detected	Not detected	38.3	43.9	77
	Not detected	Not detected	40.23	Not detected	-
NTC	Not detected	Not detected	Not detected	43.95	71.8
	Not detected	Not detected	Not detected	42.43	71.8
	Not detected	Not detected	Not detected	Not detected	-
Pos Ctrl (NK603 50ng)	24.83	-	23.91	22.49	80.7
	24.98	-	23.9	23.19	80.4
	24.99	-	23.92	25.18	80.4

n.a., not available, the test was not run

3.4 Relative Limit of Detection of P-35, T-nos and CTP2-CP4epsps methods in MON71800 DNA

The sensitivity of the three methods used in the MON71800 testing strategy was evaluated through the determination of the relative limit of detection (LOD_{rel}) tested on different amounts of MON71800 in a background of 300 ng of wheat genomic DNA, corresponding to 17,331 haploid genome copies (with 1-C value of wheat genome equal to 17.33 pg). Table 5 reports the results of the experiments carried out.

As positive control for the PCR reactions 50 nanograms of genomic DNA of NK603 were amplified in parallel with all the targets.

Table 5. LOD_{rel} for the three methods of the testing strategy in MON71800 DNA

GM-levels	CaMV35S promoter (P-35S)	Nopaline synthase terminator (T-nos)	CTP2-CP4epsps	Average Cq value +/- Standard Deviation
	Positive Reactions/Total reactions			
10 copies MON71800 in 300 ng wheat DNA	10/10	10/10	10/10	P-35S: 37.27+/1.02 T-nos: 38.87+/-1.30 CTP2-CP4epsps: 36.42+/-0.38
5 copies MON71800 in 300 ng wheat DNA	10/10	10/10	9/10	P35S:38.70+/-1.25 T-nos: 39.23+/-0.65 CTP2-CP4epsps: 37.90+/-0.47
1/10 of 1 copy MON71800 in 300 ng wheat DNA	1/10	0/10	1/10	P35S: 38.34 T-nos: nd CTP2-CP4epsps: 38.66
NK603 (PC)	6/6	3/3	3/3	P35S: 25+/-0.06 T-nos: 28.21+/-0.65 CTP2-CP4epsps: 23.42+/-0.42
NTC	0/3	0/3	0/3	-

Considering the results presented in Table 5, ten out of ten replicates of 5 MON71800 copies reacted with P-35S and T-nos methods and ten out of ten replicates of 10 MON71800 copies reacted with the CTP2-CP4epsps method in a background of 300 ng wheat genomic DNA.

The LOD_{rel} value is approximately 0.03% for P-35S and T-nos methods and 0.06% for the CTP2-CP4epsps, both expressed in terms of copy numbers and mass ratios, given the positive control sample homozygous for the MON71800 locus.

4. Conclusions

Further to the finding of unauthorised GM glyphosate-resistant wheat "volunteer" plants in a farm in Oregon harbouring event MON71800, the European Union Reference laboratory for Genetically Modified Food and Feed (EU-RL GMFF) developed an *interim* testing strategy to test wheat consignments for the presence of this GMO. The strategy is based on three validated screening methods targeting the CTP2-CP4epsps construct, the P-35S, and the T-nos.

The EU-RL GMFF performed bioinformatics analysis and experimental testing of the three validated methods to determine their specificity and sensitivity.

Information on sensitivity was obtained by determining the relative limit of detection (LOD_{rel}) for the three methods on MON71800 wheat DNA. The LOD_{rel} value was approximately 0.03% for P-35S and T-nos methods and 0.06% for the CTP2-CP4epsps, both expressed in terms of copy numbers and mass ratios, given the positive control sample homozygous for the MON71800 locus.

The specificity of the methods composing the testing strategy was verified on GM events containing the CTP2-CP4epsps element for which a validated event-specific method is available.

The experimental verification hereby reported confirmed the validity of the EU-RL GMFF guidance on testing for GM glyphosate-resistant wheat (MON71800) in wheat grain or in food/feed products containing wheat flour originating or consigned from the US, provided DNA of acceptable quality can be obtained.

5. References

1) EU-RL GMFF guidance on testing for GM glyphosate-resistant wheat (MON71800) in wheat grain or in food/feed products containing wheat flour originating or consigned from the US. Http://gmo-crl.jrc.ec.europa.eu/GM_wheat.htm.

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Title: EU-RL GMFF guidance on testing for GM glyphosate-resistant wheat (MON71800) in wheat grain or in food/feed products containing wheat flour originating or consigned from the US

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Abstract
In response to a request of DG SANCO to provide National Reference Laboratories (NRLs) as soon as possible with a method to test soft white wheat consignments for the presence of unauthorised GM glyphosate-resistant wheat harbouring the event MON71800, the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) developed, in collaboration with the European Network of GMO Laboratories (ENGL), a testing strategya intended to be immediately implementable by EU NRLs. The testing strategy is based on a combination of three validated screening methods that allow excluding (detectable) presence of Monsanto's GM glyphosate-resistant wheat (MON71800) in wheat grain or food/feed products and confirming its presence whenever other GMOs can be excluded.
The present report describes the results of the tests carried out by the EU-RL GMFF to verify the testing strategy proposed; the tests were conducted using the positive control sample represented by a crude DNA lysate of MON71800 provided by Monsanto and genomic DNA samples of genetically modified organisms harbouring the CTP2-CP4epsps element for which a validated event-specific method is available.

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