

# Report on the Verification of the Performance of a Method for the Detection of DAS-59132-8 (Event 32) in Maize Using Real-Time PCR

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Joint Research Centre  
Institute for Health and Consumer Protection  
Biotechnology & GMOs Unit

## Executive Summary

In February 2008 Dow AgroSciences LLC announced the voluntary retrieval from the US channels of distribution of hybrid corn seeds potentially contaminated by GM-maize line DAS-59132-8 (Event 32 or E-32). E-32 is declared to be a 'sister' event to GM-maize HERCULEX™ RW *Insect Protection* (59122, unique identifier, DAS-59122-7). According to Dow Agrosciences LLC, identical genetic material has been inserted into the two maize lines, which integrated into different regions of the genome.

The European Commission requested to Dow AgroSciences LLC control samples and a detection method.

On 29<sup>th</sup> February 2008, the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) received from Dow AgroSciences LLC a DNA extraction protocol and a real-time PCR protocol for event-specific detection of E-32.

On 6<sup>th</sup> March 2008, the CRL-GMFF received the E-32 positive control sample and a negative control sample (conventional maize).

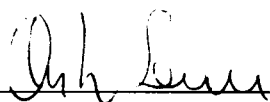
The CRL-GMFF defined the experimental design, extracted the DNA from the control samples following the method provided and conducted specificity assessment by bioinformatic analysis and experimental testing. The CRL-GMFF determined the sensitivity of the E-32 method as Limit of Detection (LOD) by analysing decreasing amounts of E-32 DNA spiked in a constant amount of conventional maize genomic DNA.

The CRL-GMFF observed that the method provided does not react with other GM events tested under the conditions reported; moreover, the CRL-GMFF verified that the method for the detection of event 59122, previously validated (for validation report and description of the method see <http://gmo-crl.jrc.it/statusofdoss.htm>) does not react with DNA of maize line E-32.

The limit of detection (LOD) established is at least 5 haploid genome copies of E-32 in a total of fifty nanograms of maize DNA, corresponding to a relative DNA content of at least 0.03%.

*Drafted by:*

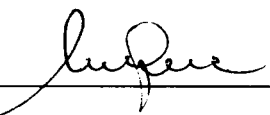
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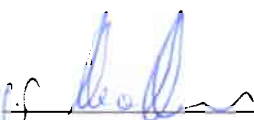
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## 1. Introduction

In February 2008 Dow AgroSciences LLC announced the voluntary retrieval from the US channels of distribution of hybrid corn seeds potentially contaminated by GM-maize line DAS-59132-8 (Event 32 or E-32). E-32 is declared to be a 'sister' event to GM-maize HERCULEX™ RW *Insect Protection* (59122, unique identifier, DAS-59122-7). According to Dow AgroSciences LLC, identical genetic material has been inserted into the two maize lines, which integrated into different regions of the genome.

On 29<sup>th</sup> February 2008, the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) received from Dow AgroSciences LLC a DNA extraction protocol and a Real-time PCR protocol for event-specific detection of E-32.

On 6<sup>th</sup> March 2008, the CRL-GMFF received the E-32 positive control sample as semi-ground seeds of an E-32 inbred line and a negative control sample (conventional maize) as semi-ground hybrid seeds. Dow Agrosciences LLC declared that the negative control sample had been tested to be absent of GM traits at the 0.1% level, 95% confidence.

The CRL-GMFF defined the experimental design, extracted the DNA from the samples following the DNA extraction protocol provided by Dow Agrosciences LLC and conducted specificity assessment by both bioinformatic analysis and experimental testing. The CRL-GMFF determined the sensitivity of the E-32 method as limit of detection (LOD) by detecting decreasing amounts of E-32 DNA spiked in a constant amount of conventional maize genomic DNA.

The present report describes the outcome of the tests carried out.

## 2. Experimental design, materials and methods

### 2.1. DNA extraction

The CRL-GMFF received from Dow Agrosciences LLC two samples of semi-ground maize seeds. One sample of conventional maize declared to be free from GM traits at the 0.1% level, 95% confidence; the second sample consisted of E-32 maize homozygous for the insertion locus.

E-32 and conventional maize seeds were further ground to a fine powder and mixed thoroughly. Ten grams of each flour sample were processed according to the protocol based on the DNeasy plant kit, Qiagen cat. no. 69106

The DNA extraction protocol is attached in Annex 1 to this report.

## 2.2. DNA concentration and integrity

The concentration of the DNA extracted was determined by fluorescence detection, after extensive homogenization, using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Suitable dilutions of each DNA extract were prepared in 10 replicates and mixed with the PicoGreen reagent.

DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/mL to 500 ng/mL using a Modulus (Turner Biosystems) as fluorescence detector.

DNA integrity was verified by agarose-gel electrophoresis.

## 2.3. Linearity and inhibition tests

Linearity and inhibition tests were performed as follows: maize DNA was diluted to a level corresponding to the DNA concentrations intended to be used in the subsequent real-time PCR method (10 ng/ $\mu$ L). From this sample, named "undiluted", a dilution series was prepared. To assess the presence of inhibitors, the Ct values of the diluted samples were plotted against the logarithm of the dilution factor, and the Ct value for the undiluted sample was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the sample was compared with the measured Ct.

Linearity tests were conducted on the E-32 maize line DNA with the GM-specific system (see Annex 1 for description of the method) and on the non-GM maize DNA with an *hmg* specific reference system previously validated by the CRL-GMFF<sup>(1)</sup>. Criteria for DNA quality acceptance were based on ENGL minimum acceptance criteria: slope between -3.1 and -3.6 and linearity above 0.98<sup>(2)</sup>. In addition, the  $\Delta$ Ct between extrapolated and measured Ct number on the least diluted sample should be less than 0.5.

## 2.4. Specificity

### 2.4.1. Bioinformatic analysis

Bioinformatic analyses were conducted by homology searches, BLASTN 2.2.16<sup>(3)</sup>, with the sequences of the primers 32f-32r and with the corresponding amplicon against *i)* the GMO database maintained at the JRC (CCSIS), *ii)* the non-redundant Genebank database and *iii)* the Univec database.

### 2.4.2. Experimental testing

The detection method was tested against 50 ng genomic DNA from a selection of GMOs for which the prediction of total or partial homologies with one or both E-32 primers arose from bioinformatic analysis: maize events 59122, 3272, MIR604xGA21 and soybean 305423. The same GMOs were also tested with the respective target-taxon reference systems, i.e. *hmg* for maize and lectin, according to the method submitted to the CRL-GMFF under Reg. (EC) No 1829/2003, for soybean 305423. DNA of E-32 was tested with the event-specific detection method for event DAS 59122 to verify possible cross-reactivity.

## 2.5. Limit of Detection

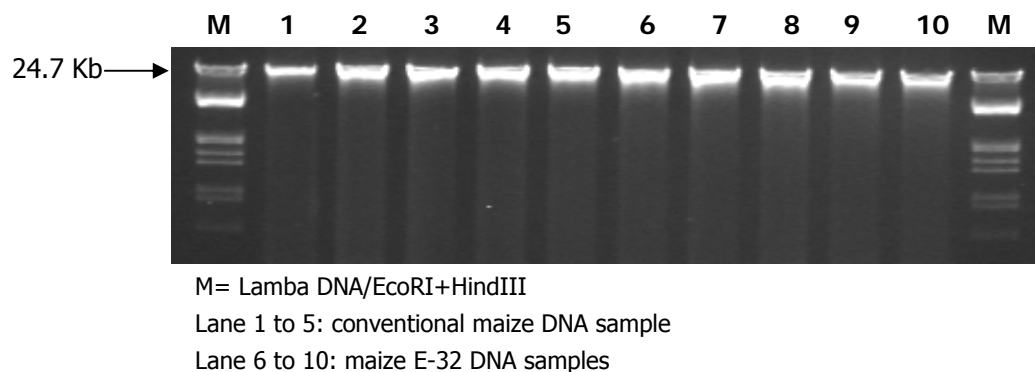
The sensitivity of the method was assessed through the determination of the limit of detection (LOD). An E-32 DNA sample was diluted to 10 haploid genome copies in one microliter. Subsequently a serial dilution was prepared from the first diluted sample to obtain concentrations of respectively 5, 2, 1, 0.2 and 0.02 copies/ $\mu$ L. Five  $\mu$ L of each sample of the dilution series were spiked in 50 ng of non-GM maize DNA and loaded for reaction to test the LOD at 50, 25, 10, 5, 1 and 0.1 haploid genome copies. Twenty-one replicates were tested for each dilution. Each replicate contained a total of approximately 18,348 copies of maize haploid genome based on the 1C value for maize genome as 2.725 pg<sup>(4)</sup>.

## 3. Results

### 3.1. DNA integrity

After DNA extraction, five DNA solutions were obtained respectively from 10 grams each of maize E-32 and 10 grams of conventional maize. Samples were analysed on a 1% agarose gel electrophoresis run at 70 V for 1 h. The results of this assessment for DNA integrity are shown in Figure 1.

Fig. 1. Gel electrophoresis of maize DNA samples



As shown in Fig. 1, the maize E-32 DNA and conventional maize DNA were mostly intact and appeared as high molecular weight samples.

### 3.2. Linearity and inhibition tests

The results of linearity and inhibition tests are shown in Table 1.

Table 1. Linearity and inhibition test results per species-specific reference system

Sample*	PCR system	Total gDNA (ng)					Linearity (R <sup>2</sup> )	Slope	ΔCt
		50	12.5	3.1	0.8	0.2			
		Ct number (mean of 3 wells)							
E-32 maize	E-32	22.5	24.7	26.6	28.5	30.4	0.99	-3.17	0.23
Conventional maize	<i>hmg</i>	25.4	27.4	30.1	31.9	33.7	0.98	-3.42	0.27

\* samples were run on different plates

For both DNA samples the correlation coefficient (R<sup>2</sup>) was above 0.98. Also the slopes were within the acceptance criterion. The test for inhibition provided a ΔCt value of 0.23 and 0.27 for the E-32 maize and the conventional maize DNA extracts, respectively.

Altogether these data suggest a suitable quality of the DNA extracted for subsequent applications of real-time PCR.

### 3.3. Specificity

#### 3.3.1. Bioinformatics analysis

The E-32 detection method was declared 'event-specific' by Dow Agrosience LLC.

The forward primer 32f showed complete matches to entries corresponding to vector sequences as assessed against the 'nt' database of genebank and Univetor databases and partial homologies to maize genome hits.

The reverse primer (32r) showed complete match with one entry from the maize genome database (zmg\_5) and partial matches to entries of the Univec database but not to the Vector database.

Therefore, it appears that the forward primer of the E-32 method is directed to common vector sequences and the reverse primer targets a maize genome sequence.

The blasting of the two primers against the GMO database (CCSIS) maintained at JRC revealed relevant homologies of the forward or reverse primer against entries from the 59122 maize event, the 3272 maize, maize MIR604 and soybean 305423 (Table 3), which were therefore deemed to deserve further investigation through experimental testing.

Table 2. Top five relevant matches for 32f and 32r primers against the GMO-JRC database (CCSIS)

GM-line	Primer 32f (27-bp long)		Primer 32r (19-bp long)	
	Primer match	Destination match	Primer match	Destination match
59122 maize	1-27	Left T-DNA border region from Ti plasmid of <i>Agrobacterium tumefaciens</i>	-	-
3272 maize	1-27	Likely sequence from pNOV7013	-	-
MIR604 maize	1-18	3' non-translated region of the nopaline synthase gene	-	-
GA21 maize	-	-	2-19	3' flanking sequence of the transformation event GA21
305423 soybean	7-17	PHP19340A fragment	-	-

Based on the considerations summarized above, the CRL-GMFF decided to test the E-32 method specificity on the following GM lines: maize 59122, 3272, MIR604xGA21 and soybean 305423.

### 3.3.2. Experimental testing of specificity

Further to the bioinformatics analysis, a selection of GMOs was tested against the E-32 method to verify any possible cross-reaction of such GMOs with the method developed to detect maize line E-32 (Table 3).

Table 3. Specificity results for GMO individually tested with the E-32 detection method. Ct number is the average of three wells.

Event Name	E-32 method (Ct number)	Taxon-specific reference system (Ct number/Ref system)
59122	n.d.	24.9 / hmg
3272	n.d.	25.8 / hmg
MIR604xGA21	n.d.	24.7 / hmg
E-32	23.7	24.9 / hmg
305423	n.d.	23.6 / lectin
NTC	n.d.	n.d.*

n.d. = not determined

NTC = no template control

\* tested with both *hmg* and lectin assays

In a second experiment the maize line E-32 DNA was tested with the event-specific method validated by the CRL-GMFF for detection of maize event 59122, and available at <http://gmo-crl.jrc.it/statusofdoos.htm>. Results are summarised in Table 4.

Table 4. Specificity results for maize line E-32 tested with 59122 event-specific method. Ct number is the average of three wells.

Event Name	DAS 59122 event-specific method (Ct number)	<i>hmg</i> system (Ct number)
E-32	n.d.	31.4
DAS 59122	23.9	29.6
NTC	n.d.	n.d.

n.d. = not determined

NTC = no template control

Altogether these data indicate that, under the conditions described, the E-32 detection method does not react with GMO for which the bioinformatics analysis suggested homologies with the E-32 primer pairs, namely events 59122 maize, 3272 maize, MIR604xGA21 maize and DP-305423 soybean.

In addition, the tests conducted showed that the 59122 event-specific detection method does not react with the DNA of maize line E-32.

### 3.4. Limit of detection (LOD)

The sensitivity of the E-32 detection method was evaluated through determination of the Limit of Detection tested on E-32 DNA spiked in a total of 50 ng non-GM maize DNA and based on haploid genome copy numbers as described in the section Experimental Design. Table 5 reports the results of this experiment.

According to the definition of the European Network of GMO Laboratories (ENGL), the LOD is the lowest amount or concentration of an analyte in a sample which can be reliably detected but not necessarily quantified, as demonstrated by single laboratory validation. Methods should detect the presence of the analyte at least 95% of times at the LOD, ensuring  $\leq 5\%$  false negative results <sup>(3)</sup>.

Table 5. Result of LOD of the E-32 method\*

Samples	E-32 Copy number	Average Ct/Standard Deviation	Positive/total amplifications
Maize E-32 gDNA spiked in non-GM maize DNA	50	32.1 / 0.2	21/21
	25	33.4 / 0.3	21/21
	10	34.8 / 0.4	21/21
	<b>5</b>	<b>35.6 / 0.5</b>	<b>21/21</b>
	1	37.5 / 0.7	17/21
	0.1	38.2 / 0.6	3/21
E-32 positive control (plate #1)	null	21.9/ 0.07**	3/3
E-32 positive control (plate #2)	null	21.0/ 0.05**	3/3
Non-GM maize	null	n.d.	0/3
NTC	null	n.d.	0/6

n.d. = not determined

NTC = no template control

\* Tests conducted in two runs, threshold set at 0.11 in both cases

\*\* based on measurable data

Therefore, having regard to the above definition and to the results presented in Table 5, the LOD of the E-32 detection method is at least 5 copies of E-32 in 50 ng of maize genomic DNA, corresponding to a relative DNA content of at least 0.03%, based on the published 1C value for maize genome of 2.725 pg<sup>(1)</sup>.

## 4. Conclusions

Further to Dow Agrosiences LLC's announcement that the un-authorized GM maize line DAS-59132-8 (Event 32 or E-32) had inadvertently contaminated US channels of distribution of maize hybrid seeds, the European Commission requested to Dow Agrosiences LLC control samples and a method for detection of event E-32.

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF) received a sample of ground seeds of E-32, conventional maize seeds and the protocol of the detection method.

The CRL-GMFF performed bioinformatics analysis and the experimental testing of method specificity and determined the method sensitivity as Limit of Detection (LOD).

The CRL-GMFF concludes that the detection method provided does not react with other GM events tested under the conditions reported. In addition, the CRL-GMFF concludes that the method for the detection of event 59122, previously validated (for validation report and description of the method see <http://gmo-crl.jrc.it/statusofdoss.htm>) does not react with DNA of maize line E-32.

The Limit of Detection (LOD) established is at least 5 haploid genome copies of E-32 in a total of fifty nanograms of maize DNA, corresponding to a relative DNA content of at least 0.03%.

## 5. References

1. "Event-specific method for the quantification of maize line TC1507 using real-time PCR-Protocol". [Http://gmo-crl.jrc.it/statusofdoss.htm](http://gmo-crl.jrc.it/statusofdoss.htm)
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## 6. Annex 1: Recommended PCR assay for detection of Event 32 maize

### ***Recommended PCR Assay for Detection of Event 32 Maize***

#### **Introduction**

The PCR assay has been optimized for use on the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). PCR product formation is measured real-time during each cycle by means of target specific oligonucleotide probes labeled with two fluorescent dyes: FAM as a reporter dye at the 5' end and TAMRA as a quencher dye at the 3' end. The 5' nuclease activity of *Taq* DNA polymerase cleaves the probes and liberates the fluorescent moiety during the amplification process. The resulting increase in fluorescence during amplification is monitored and recorded. The method is designed for qualitative detection of an event 32 specific target sequence, which straddles an integration site of transgenic DNA in the host genome.

#### **Sample homogenization**

Grind approximately 1 kg of corn until no coarse particles remain. Use equipment that can be rigorously cleaned after each use, so as to avoid carryover between samples. Depending on equipment used, it may be required to grind in several portions, which are then combined and blended thoroughly to obtain 1 kg of ground sample. Alternatively, a representative sub-sample from an initial coarse grind of 1 kg corn can be pulverized using a ball mill (e.g. Retsch Mixer Mill MM 200).

#### **DNA extraction**

A recommended DNA extraction method follows. Other extraction methods suitable for samples of ground corn as demonstrated by validation may be used.

#### **Reagents, solutions:**

DNeasy plant kit, Qiagen cat. no. 69106 *Note: depending on volumes used for initial lysis, it may be required to purchase additional buffer AP1 and buffer AP2*

HPLC H<sub>2</sub>O

#### **Procedure:**

- Extract all unknown samples in duplicate.
- Place 10 g of uniformly ground kernel sample in a polypropylene centrifuge tube (50 ml), add 15 ml of AP1 buffer previously warmed at 65 °C and 20 µl of RNase A; mix vigorously to pulverize lumps of the sample. *Note: for extremely fine grinds, e.g. from a ball mill, use 2 g of the pulverized sample, add 10 ml of AP1 buffer previously warmed at 65 °C and 20 µl of RNase A.* Leave the mixture at 65°C for 15 minutes and shake the sample by overturning the centrifuge tube 2-3 times, or subject them to continuous rotating agitation.
- Add 5000 µl of AP2 buffer (or 3250 µL of AP2 buffer for 2 g pulverized sample in 10 ml AP1), leave the mixture on ice or at 4 °C for 5 minutes, and centrifuge it at 3,000 x g or more for 5 minutes.
- Then, apply 500 µl of the supernatant to a QIAshredder spin column, and perform centrifugation at 10,000 x g or more for 4 minutes and place the eluate in a micro centrifuge tube (15 ml).
- After repeating this procedure, add 1.5 times the eluate volume of AP3 buffer/ethanol mixture\*. Apply 500 µl of this solution to a Mini spin column, and perform centrifugation at 10,000 x g or more for 1 minute.

- Apply 500 µl of the remaining solution to the same Mini spin column, perform centrifugation under the same conditions, and dispose of the eluate. Repeat the same procedures until all of the solution is used.
- Apply 500 µl of AW buffer to the column, perform centrifugation at 10,000 x g or more for 1 minute, and dispose of the eluate.
- Apply the AW buffer again and repeat the same procedures.
- After disposing of the eluate, subject the Mini spin column to centrifugation at 10,000 x g or more for 15 minutes to dry it.
- Transfer the Mini spin column to a centrifuge tube of the kit, add 70 µl of previously warmed water, leave the mixture for 5 minutes, and centrifuge it at 10,000 x g or more for 1 minute to elute DNA.
- Add water again and repeat the same procedures, put the obtained eluates together, and use this as the DNA sample stock solution.
- Measure the DNA concentration of the obtained solution by appropriate method, such as comparison to known DNA amount standards using a fluorometer or ethidium bromide stained agarose gel, or UV spectrophotometer absorption at 260/280 nm.

\* AP3 buffer/ethanol mixture Mix AP3 buffer and ethanol (96%-100%) at 1:2 and use this as the AP3 buffer/ethanol mixture.

#### Controls:

Negative control: blank extraction without sample, processed in duplicate and in parallel with unknown samples throughout the entire protocol starting with buffer AP1

or

DNA extraction from a known event free sample of ground corn in duplicate, extracted in parallel with unknown samples

Positive control: DNA extraction from a known sample of 0.1 % ground event 32 in event free ground corn in duplicate, extracted in parallel with unknown samples

## PCR

#### Reagents, solutions:

TaqMan Universal PCR Master Mix, Applied Biosystems cat. no. 4326708

32f: 5'- CCG CAA TGT GTT ATT AAG TTG TCT AAG -3'

32r: 5'- GGT GAA TGT CGC CGT GTG T -3'

32probe: 5'-FAM- CAA TTT GTT TAC ACC AGA GGC CGA CAC G -TAMRA-3'

HPLC H<sub>2</sub>O

Event free DNA solution (10 ng / µl)

0.1 % event 32 DNA in event free corn DNA solution (10 ng / µl)

and /or

event 32 DNA solution (0.25 ng / µl)

**Procedure:**

Adjust the concentration of sample DNA solutions to approx 10 ng /  $\mu$ l.

*Note: When using certain DNA extraction protocols, e.g. protocols that involve a phenol / chloroform extraction step, concentration of the obtained DNA solutions may be considerably overestimated when using UV spectrophotometer absorption. It is advisable to perform plausibility checks by comparison with other DNA quantification methods.*

Combine the following components:

Component	concentration	Final concentration	volume per reaction* ( $\mu$ l)	example 100 reactions
HPLC H <sub>2</sub> O			5*	500
Universal MM	2 x	1 x	12.5	1250
32f	10 $\mu$ M	0.4 $\mu$ M	1	100
32r	10 $\mu$ M	0.4 $\mu$ M	1	100
32probe	10 $\mu$ M	0.2 $\mu$ M	0.5	50
Subtotal			20	2000
DNA	Approx 10 ng / $\mu$ l	50 ng / reaction	5	
Total			25 $\mu$ l	

\*for inhibition control reactions, replace 0.2  $\mu$ l H<sub>2</sub>O / reaction with 0.2  $\mu$ l event 32 DNA solution of 0.25 ng /  $\mu$ l.

Dispense 20  $\mu$ l of the reaction mixture into reaction tubes / wells.

Samples:

- Add 5  $\mu$ l of a 10 ng /  $\mu$ l DNA solution. Prepare one PCR reaction for each of the duplicate DNA solutions.

**PCR profile**

data collection	Time	temperature	Cycles
No	2 min	50	1
No	10 min	95	1
No	15 sec	95	40
Yes	1 min	60	