EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific method for the quantitation of sugar beet line H7-1 using real-time PCR

Validation Report

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

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Executive Summary

The JRC as Community Reference Laboratory (CRL) for the GM Food and Feed (see Regulation EC 1829/2003), in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the H7-1 transformation event in sugarbeet DNA (unique identifier KM-ØØØH71-4). The collaborative trial was conducted according to internationally accepted guidelines.

Monsanto provided the method-specific samples (seeds of sugarbeet line H7-1, 100% event H7-1 and seeds of non-GM conventional sugarbeet line, 0% event H7-1) whereas the JRC, upon extraction of the respective DNA, prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved thirteen laboratories from ten European countries.

The results of the collaborative trial fully met ENGL's performance requirements and the scientific understanding about satisfactory method performance. Therefore, the JRC as Community Reference Laboratory considers the method validated as fit for the purpose of regulatory compliance.
The results of the collaborative study are publicly available under http://gmo-crl.jrc.it/. The method will also be submitted to CEN, the European Standardisation body, to be considered as international standard.

Contents

Document Approval		
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1. Introduction

The Joint Research Centre (JRC, Biotechnology and GMOs Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for the GM Food and Feed (see Regulation EC 1829/2003) organised the collaborative trial of the event-specific method for the detection and quantification of H7-1 sugarbeet. The study involved thirteen laboratories, members of the European Network of GMO Laboratories (ENGL).

Upon reception of methods, samples and related data, the JRC carried out the scientific evaluation of documentation and the in-house testing of the methods, according to the requirements of Regulation (EC) 641/2004 and following its operational procedures.
The internal tests were carried out in March-May 2005.
Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in June 2005.

A method for DNA extraction from sugarbeet seeds, submitted by the applicant, was evaluated by the JRC; laboratory testing of the method was carried out in order to confirm its performance. The protocol was employed for the extraction of DNA samples used in this validation study. The protocol for DNA extraction and a report on method testing is available under http://gmo-crl.jrc.it/.

The operational procedure of the collaborative study comprised the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event H7-1 DNA to total sugarbeet DNA. The procedure is a simplex system, in which a sugarbeet GS (*glutamine synthetase*) endogenous assay (reference gene) and the target assay (H7-1) are performed in separate wells. The PCR assay has been optimised for use in real-time PCR instruments for plastic reaction vessels.

The ring-trial was carried out in accordance with the following internationally accepted guidelines:

- ✓ ISO 5725 (1994).
- ✓ The IUPAC “Protocol for the design, conduct and interpretation of method-performance studies” (Horwitz, 1995).

2. List of Participants

The method was tested in thirteen ENGL laboratories to determine its performance. Each laboratory was requested to carefully follow the protocol provided. The participating laboratories are listed in Table 1 in alphabetical order.

Table 1. ENGL laboratories participating in the validation study of sugar beet H7-1.

Laboratory	Country
Bundesinstitut fuer Risikobewertung (BfR)	Germany
CRA-W, Dépt Qualité des productions agricoles	Belgium
Danish Institute for Food and Veterinary Research	Denmark
Danish Plant Directorate	Denmark
Ente Nazionale Sementi Elette	Italy
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy
Laboratoire MDO – Unité PMDV	France
Laboratoire National de Santé	Luxembourg
LAV Sachsen-Anhalt	Germany
National Food Administration	Sweden
National Institute of Food Hygiene and Nutrition GMO lab	Hungary
Swiss Federal Research Station for Animal Production and Dairy Products	Switzerland
Umweltbundesamt GmbH	Austria

3. Materials

For the validation of the quantitative event-specific method, seeds from sugarbeet line RM-HybH7-A constituted the positive control for event H7-1 and conventional sugarbeet seeds from line RM-HybConv-B were the negative control. Following DNA extraction, samples containing mixtures of 0% and 100% H7-1 sugarbeet genomic DNA at different GMO concentrations were prepared by the JRC.

The participants received the following materials:

- ✓ Four calibration samples (180 µl of DNA solution each) labelled from S1 to S4.
- ✓ Twenty unknown DNA samples (90 µl of DNA solution each), labelled from U1 to U20.
- ✓ Amplification reagent control was used on each PCR plate.
- ✓ Reaction reagents as follows:

  - ❑ PCR buffer I 10X, one tube : 1.5 ml
  - ❑ Rox Reference Dye (25µM), one tube: 500 µl
  - ❑ $MgCl_2$ (25mM), two tubes: 1.7 ml each
  - ❑ dATP (10mM), one tube: 320 µl
  - ❑ dCTP (10mM), one tube: 320 µl
  - ❑ dGTP (10mM), one tube: 320 µl
  - ❑ dUTP (20mM), one tube: 320 µl
  - ❑ Ampli Taq Gold (5U/µl), one tube: 112 µl
  - ❑ Distilled sterile water, one tube: 5 ml

- ✓ Primers and probes (1 tube each) as follows:

  GS system

  - ❑ GluA3-F primer (100 µM): 11 µl
  - ❑ GluA3-R (100 µM): 11 µl
  - ❑ GluD1TaqMan® probe (100 µM): 7 µl

  H7-1 sugar beet system

  - ❑ H7PLT1 primer Forward. (100 µM): 28 µl
  - ❑ ZRH7-R2 primer Reverse (100 µM): 28 µl
  - ❑ ZRH7-prob1 TaqMan® probe (100 µM): 7 µl

Table 2 shows the GM contents of the unknown samples over the dynamic range.

Table 2. H7-1 GM contents

H7-1 GM % **[GM copy number/sugar beet genome copy number (*100)]**
0.10
0.50
0.90
2.00
5.00

4. Experimental design

Twenty unknown samples (ten for each of two plates), representing five GM levels, were used in the validation study. On each PCR plate, samples were analyzed in parallel with both the H7-1 and GS gene specific system. Two plates in total were run. Each GM level was quantified in quadruplicate and each sample was analyzed in three replicates. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided (Excel spreadsheet).

5. Method

Description of the operational steps

For specific detection of event H7-1 genomic DNA, a 108-bp fragment of the recombination region of parts of the construct inserted into the plant genome is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as a reporter dye at its 5′ end and TAMRA as a quencher dye at its 3′ end.

For relative quantification of event H7-1 DNA, a sugarbeet-specific reference system amplifies a 121-bp fragment of GS (glutamine synthetase) a sugar beet endogenous gene, using a pair of GS gene-specific primers and a GS gene-specific probe labelled with FAM and TAMRA.
The standard curves are generated both for the GS and H7-1 specific system by plotting the Ct-values measured for the calibration points against the logarithm of the DNA copy numbers, and by fitting a linear regression line into these data.
Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves.

For the determination of the amount of H7-1 DNA in the unknown sample, the H7-1 copy number is divided by the copy number of the sugarbeet reference gene (GS) and multiplied by 100 to obtain the percentage value (GM% = H7-1/GS * 100).

The calibration sample S1 is was derived from a 10% H7-1 sample, prepared by mixing the appropriate amount of H7-1 DNA from the stock solution in control non-GM sugar beet DNA. This corresponds to 10,000 GM copies in 125 ng of DNA. The absolute copy numbers in the calibration curve samples are determined by dividing the sample DNA weight (nanograms) by the published average 1C value for sugar beet genome (1.25 pg) (Arumuganathan & Earle, 1991). The copy number values, which were used in the quantification, are provided in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount (ng) of DNA in reaction	125	25	5	1
Sugarbeet genome copies	100,000	20,000	4,000	800
H7-1 GM copies	10,000	2,000	400	80

6. Deviations reported

Seven laboratories reported no deviations from the protocol.
The volume for the master mix was calculated for the effective number of wells to be loaded, without any extra-volume to account for random error pipetting. As a consequence, two laboratories prepared extra-tubes of master mix to replenish the control samples. One laboratory reduced by 7% the volume of master mix and DNA to be added to each well. One laboratory performed the runs a second time after modifying the master mix volume but lost a sample corresponding to one GM-level.

One laboratory performed twice the same run due to an erroneous manipulation of one plate during a first loading.

One laboratory had problems with the optical cover and lost a few repetitions of the S-samples (4 in total) without affecting the quality of the standard-curves.

7. Summary of results

PCR efficiency and linearity

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1)*100] of the standard curves and of the R^2 (expressing the linearity of the regression) reported by participating laboratories for both PCR systems and runs (reference gene and GM specific, plate A and B), are summarised in Table 4.

Table 4. Values of standard curve slope, PCR efficiency and linearity (R^2) for the reference gene (GS) and the GM specific (H7-1) systems

		H7-1			GS		
LAB	**PLATE**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**	**Slope**	**PCR Efficiency (%)**	**Linearity (R^2)**
1	**A**	-3.58	90.40	1.00	-3.61	89.10	1.00
	B	-3.48	93.70	0.99	-3.52	92.39	1.00
2	**A**	-3.46	94.39	1.00	-3.38	97.74	1.00
	B	-3.30	99.24	1.00	-3.43	95.83	1.00
3	**A**	-3.26	97.56	1.00	-3.39	97.32	1.00
	B	-3.38	97.80	0.98	-3.41	96.31	1.00
4	**A**	-5.39	53.33	0.98	-3.74	84.98	0.99
	B	-4.97	58.91	0.99	-3.81	83.12	0.99
5	**A**	-2.68	63.82	0.91	-3.40	96.68	0.99
	B	-2.97	82.69	0.86	-3.24	96.25	0.98
6	**A**	-3.32	99.81	0.99	-3.43	95.56	0.99
	B	-3.42	95.98	0.99	-3.56	91.07	1.00
7	**A**	-3.58	90.40	0.98	-3.29	98.65	1.00
	B	-3.35	98.83	0.98	-3.36	98.31	1.00
8	**A**	-3.54	91.59	1.00	-3.49	93.30	1.00
	B	-3.17	106.65	0.99	-3.12	90.81	1.00
9	**A**	-3.90	80.41	0.99	-3.68	87.11	1.00
	B	-3.76	84.46	1.00	-3.39	97.28	1.00
10	**A**	-3.32	99.94	0.99	-3.59	89.75	1.00
	B	-3.37	97.95	0.98	-3.49	93.29	1.00
11	**A**	-3.54	91.71	1.00	-3.58	90.16	1.00
	B	-3.59	90.05	1.00	-3.55	91.33	1.00
12	**A**	-3.33	99.58	1.00	-3.42	96.04	1.00
	B	-3.30	99.14	0.99	-3.36	98.33	1.00
13	**A**	-3.49	93.46	1.00	-3.46	94.57	1.00
	B	-3.61	89.23	1.00	-3.54	91.64	1.00
Mean		**-3.54**	**90.04**	**0.98**	**-3.47**	**93.34**	**1.00**

Data reported in Table 4 confirm the performance characteristics of the method tested.
The mean PCR efficiency for the event-specific system was 90%, while that of the endogenous reference-system was 93.34%.

Two laboratories reported important deviations from the mean of the remaining laboratories. When those data were not taken into account, the mean of PCR efficiency in the H7-1 system rose to 94.65% and that of the GS system remained basically unchanged (93.9%).

The linearity of the method was very good being 0.98 in the H7-1 system and almost 1.00 in the GS system. Again, after eliminating the deviating values reported by two laboratories, the mean of the linearity in the H7-1 system increased to 0.99.

GMO quantitation

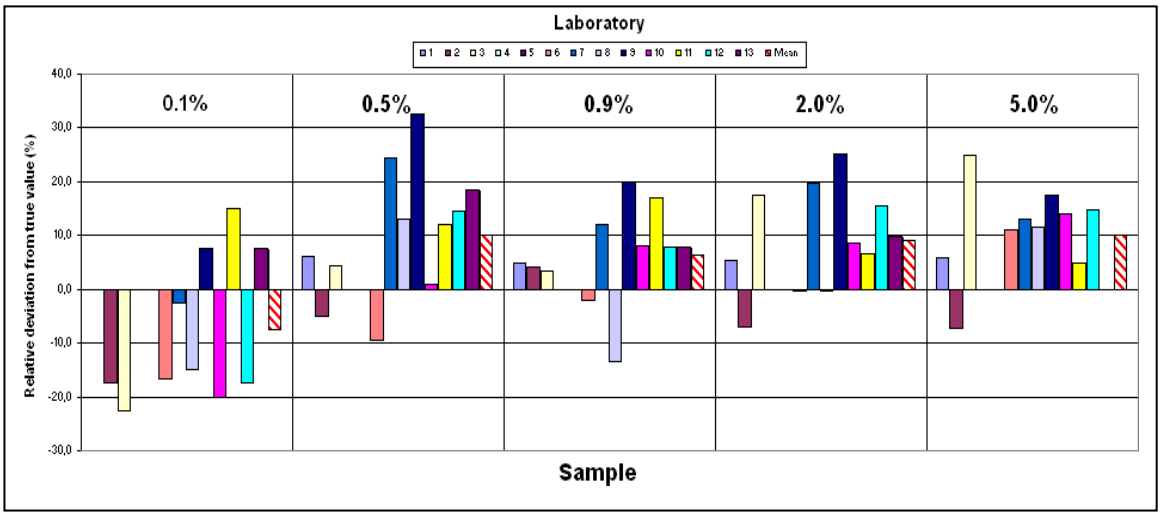
Table 5 shows the mean values of the four repetitions for each GM level as provided by all laboratories. Each mean value is the average of three PCR replicates.

Table 5. Replicates' mean value by laboratories and by all unknown samples.

	GMO content of samples (GM% = GM copy number/sugar beet genome copy number *100)																			
LAB	0.1%				0.5%				0.9%				2.0%				5.0%			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.11	0.11	0.10	0.08	0.59	0.54	0.49	0.50	1.10	0.96	0.84	0.88	2.35	2.35	1.98	1.74	5.08	5.04	5.19	5.84
2	0.09	0.09	0.08	0.07	0.50	0.49	0.44	0.47	0.83	1.02	1.08	0.82	1.68	1.87	2.02	1.87	4.34	4.41	4.54	5.27
3	0.09	0.07	0.07	0.08	0.49	0.50	0.57	0.53	0.94	1.01	0.85	0.92	2.38	2.42	2.35	2.25	5.90	6.13	6.06	6.89
4	0.63	0.76	0.29	0.51	2.40	2.90	1.60	1.88	2.57	2.40	1.93	2.18	5.57	5.49	3.85	5.29	10.5	7.76	7.97	10.94
5	0.07	0.14	0.18	0.13	0.22	1.94	2.16	1.41	1.20	1.11	2.54	3.21	3.84	3.87	5.09	15.85	10.78	16.70	19.09	23.00
6	0.08	0.08	0.09	-	0.53	0.36	0.45	0.47	1.00	1.21	0.87	0.45	2.49	2.36	1.65	1.47	7.01	5.81	4.35	5.05
7	0.10	0.10	0.09	0.10	0.41	0.75	0.64	0.69	0.69	1.03	1.12	1.19	1.94	2.38	2.38	2.88	5.05	6.05	6.24	5.29
8	0.11	0.10	0.06	0.07	0.45	0.61	0.49	0.71	0.93	1.01	0.64	0.54	2.56	2.47	1.41	1.54	5.48	5.85	3.77	7.20
9	0.13	0.09	0.09	0.12	0.73	0.61	0.57	0.74	0.99	1.08	1.16	1.09	2.30	2.68	2.30	2.73	5.74	5.47	6.01	-
10	0.08	0.05	0.09	0.10	0.59	0.45	0.51	0.47	0.96	0.96	1.01	0.96	1.90	2.19	2.65	1.94	5.88	5.71	5.19	6.05
11	0.11	0.10	0.12	0.13	0.51	0.55	0.56	0.62	1.05	0.95	1.09	1.12	1.98	2.20	2.28	2.06	5.34	5.46	5.09	5.10
12	0.10	0.08	0.07	0.08	0.63	0.56	0.61	0.49	0.87	1.03	0.78	1.20	2.03	2.34	2.42	2.46	5.63	5.17	5.86	6.27
13	0.11	0.11	0.11	0.10	0.54	0.59	0.61	0.63	0.80	0.77	1.07	1.24	2.04	2.11	2.30	2.34	4.51	4.86	4.99	5.62

In Figure 1 the relative deviation from the true value for each GM level and per laboratory is shown. The coloured bars represent the relative GM quantification obtained in the participating laboratories; the red bar represents the overall mean. In this computation, the values of two laboratories for each GM-level were excluded since they were identified as outliers following Cochrane and Grubbs analysis. The mean bias of the GM quantitation was modest at each GM-level, indicating that the good correlation between estimated and true value. Only one laboratory showed a deviation from the true value greater than 25% at the 0.90% level. No overall overestimation/underestimation trend can be observed.

Figure 1. Relative deviation (%) from the true value of H7-1 for all laboratories



8. Method performance requirements

The results of the collaborative trial are reported in table 6. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by ENGL and adopted by CRL. In table 6 estimates of both repeatability and reproducibility for each GM level are reported, after identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2.

Table 6. Sugar beet H7-1 validation data.

	Expected value (GMO %)				
Unknown sample GM%	**0.10**	**0.50**	**0.90**	**2.00**	**5.00**
Laboratories having returned results	13	13	13	13	13
Samples per laboratory	4	4	4	4	4
Number of outliers	2	2	2	2	2
Reason for exclusion	2C	2C	1C, 1G	1C, 1G	1C 1G
Mean value	**0.093**	**0.551**	**0.957**	**2.183**	**5.501**
Repeatability relative standard deviation (%)	15.65	13.81	18.19	14.72	12.22
Repeatability standard deviation	0.01	0.08	0.17	0.32	0.67
Reproducibility relative standard deviation (%)	19.74	16.46	18.19	15.54	13.23
Reproducibility standard deviation	0.02	0.09	0.17	0.34	0.73
Bias (absolute value)	-0.007	0.051	0.057	0.183	0.501
Bias (%)	-7.4	10.2	6.3	9.1	10.0

C. test = Cochran's test; G. test = Grubbs' test

The *relative reproducibility standard deviation (RSD_R),* that describes the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range. As it can be observed in table 6, the method fully satisfies this requirement at all GM level tested. In fact, the highest value of RSD_R (%) is 19.74 at the 0.10% level, well within the acceptance criterion.

In the same table the *relative repeatability standard deviation (RSD_r)* values are also reported, as estimated from ring trial results for each GM level. In order to accept methods for collaborative trial evaluation, the CRL requires that RSD_r is below 25%, as indicated by ENGL. As it can be observed from the values reported in table 6, the method satisfies this requirement throughout the whole dynamic range tested.

In table 6 measures of method *bias*, which allow estimating *trueness*, are also shown for each GM level. Bias is estimated according to ISO 5725 data analysis protocol. According to ENGL method performance requirements, trueness should be ±25% throughout the whole dynamic range. In this case the method excellently satisfies such requirement throughout the whole dynamic range tested.

9. Specificity of the GS (glutamine synthetase) reference gene

Experimental tests conducted by the applicant have shown that the GS reference system cannot discriminate between sugar beet (*Beta vulgaris*) and autumn beet (*Brassica rapa*).
Out of the six autumn beet varieties, chosen to represent the genetic variability of *Brassica rapa*, two reacted with the sugar beet reference system. However, in these cases, Ct figures of autumn beet, in subsequent tests carried out by the applicant upon request of the CRL, differ significantly from those obtained from samples of sugar beet when the same amount of DNA was analyzed, corresponding to a *Brassica rapa* reactivity from two thousand to at least thirty-two thousand times less than that of *Beta vulgaris*.

10. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (available under http://gmo-crl.jrc.it). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the collaborative study.

The results obtained during the collaborative trial indicate that the method can be considered as fit for enforcement purposes with respect to its intra- and inter-laboratory variability, trueness and with the observations on the reference system reported above.

10. References

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