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JOINT RESEARCH CENTRE
INSTITUTE FOR HEALTH AND CONSUMER PROTECTION
COMMUNITY REFERENCE LABORATORY FOR GM FOOD AND FEED

Event-specific Method for the Quantification of Event EH92-527-1 Potato 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 EH92-527-1 potato transformation event in potato DNA (unique identifier BPS-25271-9). The collaborative trial was conducted according to internationally accepted guidelines.

BASF Plant Science GmbH provided the method-specific samples (genomic DNA from 100% EH92-527-1 freeze-dried potato tubers), and genomic DNA extracted from fresh potato tubers of the non-GM potato line Prevalent), GeneScan Analytics GmbH provided the report of method optimisation, whereas the JRC prepared the validation samples (calibration samples and blind samples at unknown GM percentage). The trial involved twelve laboratories from ten countries of the European Union.

The results of the collaborative trial 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. Considerations are reported regarding the UGPase reference system.

The results of the collaborative study are publicly available under http://gmo-crl.jrc.it/. The method may also be submitted to ISO for consideration as international standard.

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Document Approval		
Name / *Function*	**Date**	**Signature**
Marco Mazzara *Sector Head*	**14/09/2006**	**Signed**
Stephane Cordeil *Quality Manager*	**14/09/2006**	**Signed**
Guy Van den Eede *B&GMOs Unit Head*	**14/09/2006**	**Signed**

Address of contact laboratory:

European Commission, Joint Research Centre

Institute for Health and Consumer Protection (IHCP)

Biotechnology and GMOs Unit – Community Reference Laboratory

Via Fermi 1, 21020 Ispra (VA) - Italy

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 EH92-527-1 potato. The study involved twelve 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 January-August 2006.
Following the evaluation of the data and the results of the laboratory tests, the ring trial was organized and took place in August-September 2006.

A method for DNA extraction from potato tubers, 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 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 EH92-527-1 DNA to total potato DNA. The procedure is a simplex system, in which a potato UGPase (*UDP-glucose pyrophosphorylase*) endogenous assay (reference gene) and the target assay (EH92-527-1) 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 twelve 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 EH92-527-1.

Laboratory	Country
AGES - Austrian Agency for Health and Food Safety - Competence Centre Biochemistry	Austria
Behoerde fuer Umwelt und Gesundheit	Germany
Centro Nacional de Alimentación - Agencia Española de Seguridad Alimentaria.	Spain
Chemisches und Veterinäruntersuchungsamt Freiburg	Germany
Finnish Customs Laboratory	Finland
Institute of Public Health	Belgium
Istituto Superiore di Sanità, ISS	Italy
Istituto Zooprofilattico Sperimentale Lazio e Toscana	Italy
LGC	UK
National Food Administration	Sweden
National Institute of Biology	Slovenia
Research Institute of Crop Production, Reference Laboratory of the Ministry of Agriculture	Czech Republic

3. Materials

For the validation of the quantitative event-specific method, non-GM potato genomic DNA (Prevalent) and EH92-527-1 potato GM-DNA was provided by BASF Plant Science GmbH. Samples containing mixtures of 0% and 100% EH92-527-1 potato genomic DNA at different GMO concentrations were prepared by the JRC.
Participants received the following materials:

- ✓ Four calibration samples (200 µl of DNA solution each) labelled from S1 to S4.
- ✓ Twenty unknown DNA samples (100 µl of DNA solution each), labelled from U1 to U20.
- ✓ Reaction reagents as follows:
    - ❑ PCR buffer II 10X, one tube 1.5 ml
    - ❑ Rox Reference Dye (25µM), one tube 300 µl
    - ❑ Tween-20, one tube 150 µl
    - ❑ Glycerol (20%), one tube 600 µl
    - ❑ $MgCl_2$ (100mM), one tube 700 µl
    - ❑ 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 120 µl
    - ❑ Distilled sterile water, one tube 6 ml
- ✓ Primers and probes (1 tube each) as follows:

    UGPase system
    - ❑ UGP-af7 primer (10 µM) 300 µl
    - ❑ UGP-ar8 (10 µM) 300 µl
    - ❑ UGP-sf1 probe (10 µM) 150 µl

    EH92-527-1 system
    - ❑ event527-bf1 (10 µM) 225 µl
    - ❑ St527-R1 (10 µM) 225 µl
    - ❑ St527-S2 probe (10 µM) 120 µl
    - ❑
- ✓ Amplification reagent control was used on each PCR plate.

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

Table 2. EH92-527-1 GM contents

EH92-527-1 GM % **(GM copy number/potato genome copy number *100)**
0.10
0.40
0.90
2.20
5.50

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 EH92-527-1 and the UGPase system. Two plates in total were run, with two replicates for each GM level analysed on each plate. The PCR analysis was triplicated for all samples. 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 EH92-527-1 genomic DNA, a 134-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 EH92-527-1 DNA, a potato-specific reference system amplifies an 88-bp fragment of UGPase (UDP-glucose pyrophosphorylase), a potato endogenous gene, using a pair of UGPase gene-specific primers and an UGPase gene-specific probe labelled with FAM and TAMRA.

The standard curves are generated both for the EH92-527-1 and the UGPase 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 EH92-527-1 DNA in the unknown sample, the EH92-527-1 copy number is divided by the copy number of the potato reference gene (UGPase) and multiplied by 100 to obtain the percentage value (GM% = EH92-527-1/UGPase * 100).

Calibration sample S1 is prepared by mixing the appropriate amount of EH92-527-1 DNA from the stock solution in control non-GM potato DNA to obtain 10% EH92-527-1 in a total of 200 ng potato DNA. Samples S2-S4 are prepared by 1:5 serial dilutions of the S1 sample.

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 potato genome (1.8 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	200	40	8	1.6
potato genome copies	111111	22222	4444	889
EH92-527-1 GM copies	11111	2222	444	89

6. Deviations reported

One participating laboratory left one well of the reference system triplicate empty by mistake.

One laboratory run the samples in a total volume of 20 µl, to adapt the protocol to a 384-well template on 9700HT instrument. Only the amount of water added to the master mix was reduced.

One laboratory, loaded the UGPase system in the upper half of the plate and the event-specific system in the lower half.

One laboratory loaded twice the same well for the reference system and left another one empty.

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). Data are reported as two-figure decimals.

		EH92-527-1			UGPase		
Lab	Plate	Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.53	91.94	1.00	-3.54	91.78	1.00
	B	-3.46	94.60	1.00	-3.48	93.82	1.00
2	A	-3.49	93.30	1.00	-3.28	98.33	1.00
	B	-3.41	96.59	1.00	-3.29	98.80	1.00
3	A	-3.49	93.52	1.00	-3.35	98.89	0.99
	B	-3.45	94.96	1.00	-3.54	91.71	1.00
4	A	-3.48	93.89	1.00	-3.37	97.95	1.00
	B	-3.31	99.68	1.00	-3.48	93.89	1.00
5	A	-3.33	99.50	1.00	-3.29	98.80	1.00
	B	-3.30	98.90	1.00	-3.22	95.60	1.00
6	A	-3.43	95.51	1.00	-2.95	81.96	0.99
	B	-3.33	99.46	1.00	-3.33	99.80	1.00
7	A	-3.41	96.52	1.00	-3.34	99.34	1.00
	B	-3.39	97.34	1.00	-3.33	99.60	1.00
8	A	-3.52	92.51	1.00	-3.13	91.52	1.00
	B	-3.50	93.21	1.00	-3.19	94.05	1.00
9	A	-3.36	98.60	1.00	-3.42	96.07	1.00
	B	-3.52	92.44	1.00	-3.48	93.96	1.00
10	A	-3.30	99.17	1.00	-3.31	99.54	1.00
	B	-3.50	92.93	1.00	-3.13	91.29	1.00
11	A	-3.40	96.76	0.99	-3.54	91.49	1.00
	B	-3.40	96.98	0.98	-3.39	97.26	1.00
12	A	-3.38	97.53	1.00	-3.23	95.99	1.00
	B	-3.33	99.58	1.00	-3.18	93.71	1.00
	Mean	**-3.42**	**96.06**	**1.00**	**-3.32**	**95.21**	**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 as high as 96%, while that of the endogenous reference-system was above 95%.

The linearity of the method was very good being close to 1.00 for both the EH92-527-1 and the UGPase system.

GMO quantitation

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

Table 5. GM% mean value determined by laboratories for all unknown samples.

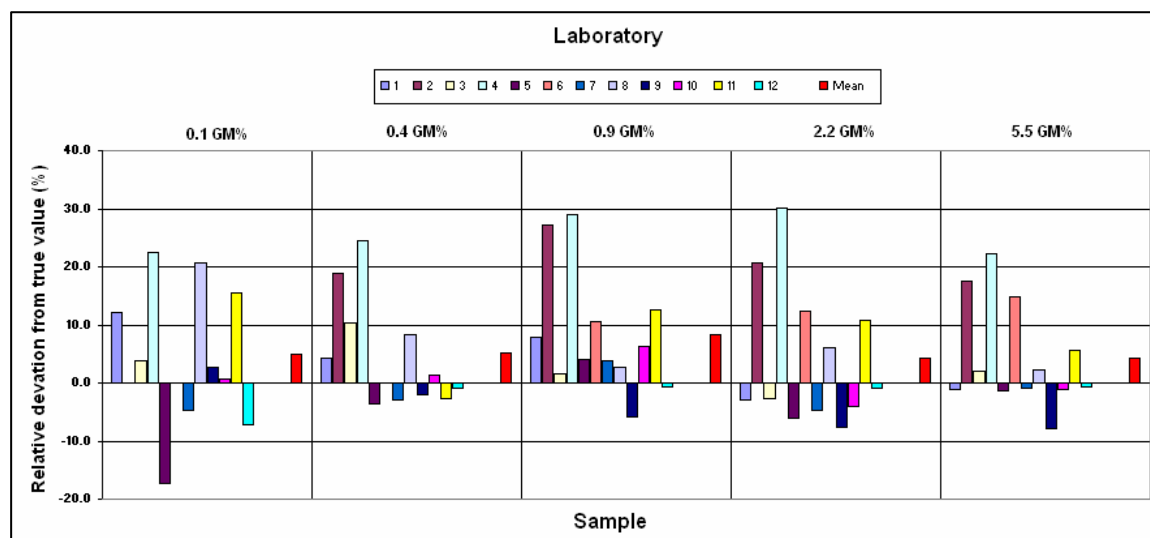
	Sample GMO content (GM% = GM copy number/potato genome copy number *100)																			
LAB	**0.1%**				**0.4%**				**0.9%**				**2.2%**				**5.5%**			
	REP 1	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**	**REP 1**	**REP 2**	**REP 3**	**REP 4**
1	0.10	0.12	0.12	0.10	0.39	0.43	0.47	0.39	0.93	0.90	0.99	1.06	2.02	2.33	2.19	2.01	5.74	5.48	4.83	5.70
2	0.12	0.13	0.13	0.42	0.50	0.48	0.46	0.47	1.15	1.11	1.20	1.12	2.69	2.56	2.69	2.69	6.21	6.45	6.61	6.59
3	0.12	0.12	0.08	0.09	0.42	0.43	0.49	0.42	0.94	0.96	0.82	0.94	1.80	2.30	2.11	2.36	4.58	5.84	6.45	5.58
4	0.12	0.10	0.15	0.12	0.55	0.55	0.39	0.51	1.08	1.04	1.43	1.09	2.87	2.51	3.08	3.00	6.54	5.89	7.61	6.86
5	0.08	0.09	0.08	0.08	0.39	0.35	0.33	0.47	0.76	0.94	1.11	0.93	1.77	2.14	1.84	2.52	4.41	5.53	5.01	6.77
6	0.12	0.19	0.13	0.11	0.51	1.11	0.42	0.43	0.92	0.80	1.17	1.09	2.67	2.41	2.19	2.61	6.46	5.78	7.05	5.98
7	0.10	0.10	0.10	0.08	0.33	0.35	0.42	0.45	0.92	0.94	0.90	0.98	1.63	2.10	2.10	2.56	4.91	5.99	5.36	5.51
8	0.13	0.11	0.13	0.12	0.42	0.48	0.44	0.39	1.04	0.96	0.84	0.85	2.16	2.34	2.38	2.45	6.00	5.52	5.51	5.48
9	0.09	0.11	0.10	0.11	0.35	0.42	0.41	0.39	0.79	0.84	0.80	0.97	1.99	1.88	2.21	2.05	4.96	5.06	5.23	5.01
10	0.10	0.10	0.09	0.11	0.41	0.43	0.38	0.39	1.04	0.93	0.92	0.94	2.36	2.33	1.85	1.90	6.21	5.56	4.70	5.28
11	0.13	0.13	0.09	0.11	0.52	0.41	0.32	0.31	0.97	1.10	0.99	1.00	2.88	2.47	2.11	2.29	6.30	6.65	4.72	5.55
12	0.10	0.09	0.09	0.09	0.38	0.38	0.42	0.40	0.95	0.93	0.85	0.85	2.15	2.18	2.23	2.16	5.28	5.21	5.71	5.64

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 by the participating laboratories; the red bar represents the overall mean for each GM level (%).

As observed in Figure 1, only a few laboratories overestimated importantly (bias higher than 25%) the true value of the 0.90% and 2.00% GM-levels.

The mean value of relative deviation from the true GM% (represented by the red bar) is well within the limit of trueness acceptance level (25%) at each GM-level, indicating an excellent correlation between estimated and true value, thus a good accuracy of the method (maximum bias 8.23%).

Figure 1. Relative deviation (%) from the true value of EH92-527-1 for all laboratories (colored bars) and the overall mean (red bar)



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. EH92-527-1: summary of validation results.

Unknown sample GM%	Expected value (GMO %)				
	0.10	**0.40**	**0.90**	**2.20**	**5.50**
Laboratories having returned results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	2	1	0	0	0
Reason for exclusion	2C	1C	-	-	-
Mean value	**0.105**	**0.420**	**0.974**	**2.293**	**5.735**
Repeatability relative standard deviation (%)	12.44	11.91	10.14	10.36	10.20
Repeatability standard deviation	0.01	0.05	0.10	0.24	0.59
Reproducibility relative standard deviation (%)	16.27	13.73	13.04	14.56	12.42
Reproducibility standard deviation	0.02	0.06	0.13	0.33	0.71
Bias (absolute value)	0.005	0.020	0.074	0.093	0.235
Bias (%)	4.88	5.07	8.23	4.24	4.28

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 16.27% 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 allows 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. The method fully satisfies such requirement for all GM values tested, since the maximum bias is 8.23% at the 0.9% GM-level.

9. Specificity of the UGPase (UDP-glucose pyrophosphorylase) reference gene

Experimental tests conducted by the method developer have shown that the UGPase reference system reacted at very low level with other plant species belonging to the *Solanaceae* family, namely tomato, sweet pepper, egg-plant and tobacco.

The extent of the cross-reactivity in the conditions described in the method (http://gmo-crl.jrc.it/statusofdoss.htm) was such that when 200 ng of genomic DNA of the mentioned species were amplified, Ct figures of 36.4, 33.7, 39.6, 37.2 were respectively observed, compared to a Ct of 17.8 for potato lines as an average. Thus the reactivity of the UGPase primer/probe combination in these *Solanaceae* species varied from around a hundred of thousands to almost two millions times less than that found in potato.

Attempts to characterize alternative potato reference systems had been performed by the method developer through review of the scientific literature and tests carried out on a selection of target sequences: TBP gene (TATA-binding protein, Holdsworth *et al*. 1992) and PCI (metallo-carboxypeptidase inhibitor gene, Hernandez *et al*. 2003): the use of PCR

systems designed on these target sequences was excluded due to suboptimal performance or poor stability among potato lines. As such, the primer/probe combination developed for the target UGPase was chosen as the current best available reference system for potato. The CRL agrees that at this moment in time and with the current knowledge this is the best choice for a potato species specific reference gene. For further details see Annex 1 (report on the selection of a potato-specific reference PCR system).

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 throughout the dynamic range and with the considerations reported above regarding the UGPase reference system.

In conclusion, the method is considered complying with the current labeling requirements in Europe.

10. References

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11. Annex 1: report on the selection of a potato-specific reference PCR system

Report:

Selection of a potato-specific reference PCR system

1. Introduction

The establishment of the UGPase reference system for potato (see Annex II) was the last step in a long and difficult development process towards identifying a potato-specific reference gene. Several attempts to identify a suitable target reference gene and develop a reference system, that would meet the acceptance criteria by DG JRC-CRL for the validation of a quantitative detection method were unsuccessful.

Because the different commercially important starch and table potato varieties display significant variability, it is difficult to identify a target reference gene, which is sufficiently conserved across all varieties. On the other hand potato is extremely similar at the DNA level to other *Solanaceae* species. For example, nearly all tomato restriction fragment length polymorphism probes that have been mapped in tomato were found to cross-hybridize with potato DNA and were successfully used for potato mapping (Bonierbale et al., 1988). Potato and tomato genes generally share a high degree of identity, especially in the protein-coding regions, where levels of identity can be 90%. Sequence differences between the two species in protein-coding regions often correspond to point mutations that tend to be silent or result in conservative amino acid substitutions. Therefore the establishment of a potato-specific PCR system which does not show any cross-reactivity with other *Solanaceae* species and at the same time detects all commercially important starch and table potato varieties is very challenging.

In the following sections efforts undertaken to identify a 'perfect' potato endogenous reference gene for a quantitative PCR-based detection system are summarized.

2. Development of a potato-specific detection system

Certain candidate genes were excluded from system development because of reports in the literature on cross-reactivity e.g. patatin (Jaccaud et al., 2003). Other candidate genes were excluded since they might constitute trait genes in existing genetically modified plants or those that are being developed e.g. sucrose synthase, branching enzymes.

2.1 TATA-binding protein gene (TBP)

The first target reference gene chosen for system development was the gene coding for the TATA-binding protein (TBP). TBP seemed to be an ideal candidate for the development of a potato-specific reference system for the following reasons:

1. TBP is known to be a low copy number gene (Holdsworth et al., 1992). For maize, wheat and Arabidopsis the existence of two TBP genes is described. However, discrimination between these two different genes within each species should be possible, as the nucleotide

sequences of the two types of TBP genes are rather different. Remarkably in the case of TBP the inter-species similarity between TBP genes of the same type is much higher than the intra-species similarity between the different types of TBP genes.

2. TBP is highly conserved. Therefore TBP seemed to provide the possibility of easily generating sequence information from different species (e.g. closely related species from the *Solanaceae* family) using suitable highly conserved exon primers.
3. TBP contains introns and intron sequences tend to differ significantly between different species. Therefore TBP seemed to offer the promise of enabling the establishment of a very specific PCR system targeting (at least partially) intron sequences.

The developmental work started with the amplification of TBP gene fragments from several potato varieties and closely related species from the *Solanaceae* family like tomato, sweet pepper, egg-plant and tobacco. The primers used were directed against highly conserved sequences of two adjacent exons and oriented in such a way, that the intervening intron sequences could be amplified. In some cases several primers had to be tested and reaction conditions had to be optimized before amplification was obtained. Sequencing of the resulting DNA fragments for tomato, sweet pepper and egg-plant resulted in clear and unambiguous sequences. In contrast, in the case of potato and tobacco reproducibly ambiguous results with 'double peaks' were observed at several sequence positions, pointing strongly towards the presence of (at least) two differing TBP genes which were co-amplified during PCR (or alternatively different alleles of the same gene differing at several nucleotide positions). To get deeper insight into the genomic situation in potato and in order to obtain unambiguous sequences for each separate gene present, TBP-primers discriminating between the different sequence variants observed were designed and used for amplification. Whereas in one case sequencing of the resulting amplicons gave clear and unambiguous results, in the other case ambiguous nucleotides were still observed. Comparison of the intron sequences obtained for potato and closely related species from the *Solanaceae* family revealed very high similarity and therefore suboptimal premises for the establishment of a potato-specific PCR system. Because of this the developmental work on the TBP target gene was discontinued.

2.2 Metallo-carboxypeptidase inhibitor gene (PCI)

The second target reference gene chosen was the metallo-carboxypeptidase inhibitor gene (PCI) because a thoroughly validated, highly potato-specific real-time PCR system detecting this gene had been described (Hernández et al., 2003). In addition to the published primers and probe, alternative (only slightly modified) primer-probe combinations were tested and the performance of the different primer-probe combinations compared. Finally the optimal primer-probe combination was identified and the reaction conditions (magnesium concentration, primer concentration, probe

concentration) were thoroughly optimized. However, during the subsequent pre-validation of the PCI-based real-time PCR system the observed variability between potato varieties failed to meet the acceptance criteria since for several potato varieties the PCI amplicon was poorly or not amplified at all. The key experiments are summarized in the following section.

2.2.1 Variability (stability) of the PCI-based potato reference systems

DNA was extracted from 17 potato varieties (9 starch potatoes and 8 table potatoes). DNA concentrations were determined by fluorescence detection using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). The quality of the extracted DNA was confirmed by gel electrophoresis. Confirmation of absence of PCR inhibitors was achieved using a real-time PCR-based assay. Subsequently this quality-checked DNA was analyzed at 10000 copies per reaction with the optimized PCI system described above.
While 12 potato varieties (Bintje, Superb, Saturna, Eloge, Appell, Seresta, Kuras, Dinamo, Oktan, Karnico, Kantara und EH92-527-1) showed uniform CT values (mean CT 24,44), strong deviations from the mean CT were observed and poor, if any, amplification of the PCI target occurred in the case of table potato Sibu (CT 40,30) and starch potatoes Katinka (CT 39,57), Bonanza (CT 42,37), SW93-1214 (CT 43,28) and SW94-1307 (CT 44,00) (Table 1).

Table 1. Analysis of different potato varieties at 10000 copies/reaction with the optimized PCI system

Potato variety	CT (mean of triplicate reactions)	Delta CT from mean of marked lines(*)
Sibu	40,30	15,86
Bintje*	24,96	0,52
Superb*	23,73	0,71
Saturna*	23,37	1,06
Eloge*	24,98	0,54
Appell*	24,57	0,13
SW93-1214	43,28	18,84
SW94-1307	44,00	19,56
Seresta*	24,72	0,28
Kuras*	23,24	1,19
Dinamo*	25,09	0,65
Bonanza	42,37	17,93
Oktan*	24,55	0,11
Karnico*	24,86	0,42

Katinka	39,57	15,13
Kantara*	24,78	0,34
EH92-527-1*	24,41	0,03

In order to make sure that this failure was not due to our modifications of the primer-probe system described by Hernández et al. (2003), the testing for variability between potato varieties was repeated with the original real-time PCR-system described in the literature. However the results (Table 2) confirmed our previous results: the same potato varieties were not at all (SW93-1214 and SW94-1307) or only poorly amplified (Sibu (CT 39,59), Katinka (CT 38,39) and Bonanza (CT 42,32)).

Table 2. Analysis of different potato varieties at 10000 copies/reaction with the PCI system described by Hernández et al.

Potato variety	CT (mean of triplicate reactions)	Delta CT from mean of marked lines(*)
Sibu	39,59	15,12
SW93-1214	45,00	20,53
SW94-1307	45,00	20,53
Kuras*	24,10	0,38
Bonanza	42,32	17,85
Oktan*	24,48	0,01
Katinka	38,39	13,92
EH92-527-1*	24,83	0,35

These data clearly indicate that the PCI-based PCR system described by Hernández et al. as well as the newly developed slightly modified variant of this PCI-based PCR system are not suitable as potato-specific reference systems due to problems concerning variability within potato varieties of the respective region of the PCI target.

2.3 UDP-glucose pyrophosphorylase gene (UGPase)

The third target reference gene which was finally chosen for PCR system development is the UDP-glucose pyrophosphorylase gene (UGPase). This gene was reported to be single copy (Borokov et al., 1997) and a UGPase-based qualitative PCR system for detection of potato DNA had successfully been established (Watanabe et al., 2004).

For system development the published nucleotide sequence of the UGPase gene was used (GenBank accession no. U20345). Intron sequence (intron 2) was chosen as a target region for

establishment of a potato-specific PCR system due to the higher variability of intron sequences relative to exon sequences and the resultant better chance of discriminating potato from the other *Solanaceae* species.

Different real-time PCR systems were optimized and subsequently analyzed for specificity and allelic variation. All PCR systems tested showed minor cross-reactivity with other members of the *Solanaceae* family (with CT differences of at least 10 compared to potato) indicating that even the selected intron sequence shows a high degree of conservation resulting in slight cross-reactivity with closely related *Solanaceae* family members. However species of other plant families (e.g. rapeseed, soy, cotton, maize and wheat) were not detected. The real-time PCR system with primers UGP-af7/UGP-ar7 and probe UGP-sf1 showed the highest specificity and most uniform CT values in the testing for variability amongst potato varieties (at an amount of 10000 copies per reaction mean CT 29,13) and was selected for further validation. However analysis of the primer/probe target region of the 17 potato varieties by amplification of the target region with external primers and subsequent sequencing of the PCR products revealed a presumptive mistake in the Genbank sequence used for system development. . The sequences of all 17 potato varieties had a one nucleotide deletion in comparison to the sequence deposited in Genbank (pos. 2250 insertion of A). Unfortunately this mismatch was located in the reverse primer (UGP-ar7). Therefore the reverse primer had to be adapted (UGP-ar8) and the validation had to be repeated. The newly optimized real-time PCR system with primers UGP-af7/UGP-ar8 and probe UGP-sf1 showed only minor cross-reactivity with closely related *Solanaceae* family members like sweet pepper, tomato, egg-plant and tobacco (with CT differences of at least 15 compared to potato). In the testing for intra-species variability all potato varieties were detected uniformly indicating stability of the PCR target across potato varieties. Surprisingly, a strong shift to lower CT values (at an amount of 10000 copies per reaction mean CT 17,86) was observed after correction of the primer mismatch. This is in conflict with the initial assumption of a low copy number for the UGPase target gene.

The UGPase PCR system was selected as potato-specific endogenous reference gene as part of the development of the quantitative detection method of potato event EH92-527-1 and submitted for validation by DG JRC-CRL as it displays very good stability in different genetic potato backgrounds and only minor cross-reactivity with closely related *Solanaceae* family members like sweet pepper, tomato, egg-plant and tobacco and thus is fit for the intended purpose.

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