

## Response by BASF Plant Science to questions raised by the EPA on 13<sup>th</sup> March 2006

1. In relation to the Agency query (question 6 of 13<sup>th</sup> February 2006) on the data supporting the number of inserts, you have confirmed the difficulties pertaining to using the delta Ct values and explained the approach you used to determine copy number insert, i.e. the  $f\tilde{f}CT$  approach (delta delta CT). However, you have not supplied the supporting data to confirm the copy number.

**You are requested to supply this data to confirm copy number.**

In the following table supporting data are provided to confirm the copy number for a representative selection of potato lines transformed with construct VCPMA16. For each GM potato line two samples were analysed.

	Line no	Sample data			Calibrator data			ddCt	Copy no
		Ct ahas	Ct end ctrl	dCt	Ct ahas	Ct end ctrl	dCt		
5	TS-PH05-002-0055	28,34	27,28	1,06	28,01	26,89	1,12	-0,06	1,04
5	TS-PH05-002-0055	28,27	26,92	1,34	28,01	26,89	1,12	0,22	0,86
13	TS-PH05-008-0004	27,37	26,29	1,08	28,01	26,89	1,12	-0,04	1,03
13	TS-PH05-008-0004	27,89	26,89	1,01	28,01	26,89	1,12	-0,11	1,08
14	TS-PH05-009-0001	26,62	25,54	1,00	25,28	24,09	1,19	-0,19	1,14
14	TS-PH05-009-0001	24,38	23,15	1,37	25,28	24,09	1,19	0,18	0,88
16	TS-PH05-009-0051	25,94	24,88	1,22	25,28	24,09	1,19	0,03	0,98
16	TS-PH05-009-0051	26,62	25,54	0,87	25,28	24,09	1,19	-0,32	1,25
38	TS-PH05-010-0041	24,38	23,15	1,05	25,28	24,09	1,19	-0,14	1,10
38	TS-PH05-010-0041	25,94	24,88	1,04	25,28	24,09	1,19	-0,15	1,11
42	TS-PH05-010-0070	26,62	25,54	1,34	25,28	24,09	1,19	0,15	0,90
42	TS-PH05-010-0070	24,38	23,15	1,12	25,28	24,09	1,19	-0,07	1,05
57	TS-PH05-010-0156	25,94	24,88	0,73	25,28	24,09	1,19	-0,45	1,37
57	TS-PH05-010-0156	27,75	26,75	0,80	25,28	24,09	1,19	-0,38	1,31
60	TS-PH05-010-0178	28,35	26,98	1,36	25,28	24,09	1,19	0,17	0,89
60	TS-PH05-010-0178	29,29	27,83	0,86	25,28	24,09	1,19	-0,33	1,26
61	TS-PH05-010-0186	27,75	26,75	1,17	25,28	24,09	1,19	-0,02	1,02
61	TS-PH05-010-0186	28,35	26,98	0,92	25,28	24,09	1,19	-0,27	1,20
71	TS-PH05-010-0303	29,29	27,83	1,32	25,28	24,09	1,19	0,13	0,91
71	TS-PH05-010-0303	27,75	26,75	1,28	25,28	24,09	1,19	0,09	0,94
80	TS-PH05-010-0466	28,35	26,98	1,07	25,28	24,09	1,19	-0,12	1,08
80	TS-PH05-010-0466	29,29	27,83	1,46	25,28	24,09	1,19	0,27	0,83
81	TS-PH05-010-0471	27,35	26,40	1,25	25,28	24,09	1,19	0,07	0,96
81	TS-PH05-010-0471	29,27	28,27	1,15	25,28	24,09	1,19	-0,03	1,02
82	TS-PH05-010-0472	28,49	27,31	0,98	25,28	24,09	1,19	-0,21	1,15
82	TS-PH05-010-0472	27,35	26,40	1,05	25,28	24,09	1,19	-0,14	1,10
93	TS-PH05-011-0026	29,27	28,27	1,68	25,28	24,09	1,19	0,49	0,71
93	TS-PH05-011-0026	28,49	27,31	1,15	25,28	24,09	1,19	-0,04	1,03
94	TS-PH05-011-0027	27,35	26,40	1,12	25,28	24,09	1,19	-0,07	1,05
94	TS-PH05-011-0027	29,27	28,27	1,33	25,28	24,09	1,19	0,14	0,91

95	TS-PH05-011-0029	28,49	27,31	1,13	25,28	24,09	1,19	-0,06	1,04
95	TS-PH05-011-0029	27,04	25,82	1,36	25,28	24,09	1,19	0,17	0,89
96	TS-PH05-011-0048	26,42	25,56	1,11	25,28	24,09	1,19	-0,08	1,06
96	TS-PH05-011-0048	25,71	24,72	0,97	25,28	24,09	1,19	-0,22	1,16
100	TS-PH05-011-0088	27,04	25,82	1,19	25,28	24,09	1,19	0,00	1,00
100	TS-PH05-011-0088	26,42	25,56	1,42	25,28	24,09	1,19	0,23	0,85
107	TS-PH05-011-0124	25,71	24,72	1,19	25,28	24,09	1,19	0,00	1,00
107	TS-PH05-011-0124	27,04	25,82	1,22	25,28	24,09	1,19	0,03	0,98
112	TS-PH05-012-0008	25,93	25,17	0,75	28,01	26,89	1,12	-0,37	1,29
112	TS-PH05-012-0008	28,69	27,63	1,06	28,01	26,89	1,12	-0,06	1,04
125	TS-PH05-015-0001	26,42	25,56	1,45	25,28	24,09	1,19	0,26	0,84
125	TS-PH05-015-0001	25,71	24,72	1,00	25,28	24,09	1,19	-0,19	1,14
129	TS-PH05-015-0018	25,28	24,09	1,06	25,28	24,09	1,19	-0,13	1,09
129	TS-PH05-015-0018	26,79	25,94	1,03	25,28	24,09	1,19	-0,16	1,11
148	TS-PH05-016-0044	25,11	24,00	1,11	28,01	26,89	1,12	-0,01	1,01
148	TS-PH05-016-0044	25,26	24,45	0,82	28,01	26,89	1,12	-0,30	1,23
166	TS-PH05-017-0004	27,10	25,86	0,95	25,28	24,09	1,19	-0,24	1,18
166	TS-PH05-017-0004	25,28	24,09	0,85	25,28	24,09	1,19	-0,34	1,27
177	TS-PH05-018-0011	26,79	25,94	1,20	25,28	24,09	1,19	0,01	0,99
177	TS-PH05-018-0011	27,10	25,86	1,46	25,28	24,09	1,19	0,27	0,83
187	TS-PH05-018-0093	25,28	24,05	1,23	28,01	26,89	1,12	0,11	0,93
187	TS-PH05-018-0093	25,04	23,75	1,29	28,01	26,89	1,12	0,17	0,89
207	TS-PH05-019-0023	27,71	26,76	0,95	28,01	26,89	1,12	-0,17	1,12
207	TS-PH05-019-0023	24,74	23,97	0,77	28,01	26,89	1,12	-0,35	1,27
218	TS-PH05-020-0032	25,28	24,09	1,11	25,28	24,09	1,19	-0,08	1,06
218	TS-PH05-020-0032	26,79	25,94	1,09	25,28	24,09	1,19	-0,10	1,07
226	TS-PH05-022-0013	25,81	24,56	1,25	28,01	26,89	1,12	0,13	0,91
226	TS-PH05-022-0013	26,43	25,33	1,10	28,01	26,89	1,12	-0,02	1,01
230	TS-PH05-023-0003	24,98	23,71	1,26	28,01	26,89	1,12	0,14	0,91
230	TS-PH05-023-0003	27,39	26,07	1,32	28,01	26,89	1,12	0,20	0,87
236	TS-PH05-025-0038	27,10	25,86	1,08	25,28	24,09	1,19	-0,11	1,08
236	TS-PH05-025-0038	24,95	23,77	1,04	25,28	24,09	1,19	-0,15	1,11
240	TS-PH05-025-0067	28,95	27,62	1,22	25,28	24,09	1,19	0,03	0,98
240	TS-PH05-025-0067	27,78	26,85	1,00	25,28	24,09	1,19	-0,18	1,14
266	TS-PH05-030-0034	28,54	27,42	1,12	28,01	26,89	1,12	0,00	1,00
266	TS-PH05-030-0034	28,89	27,74	1,14	28,01	26,89	1,12	0,02	0,98
272	TS-PH05-034-0004	24,95	23,77	1,60	25,28	24,09	1,19	0,41	0,75
272	TS-PH05-034-0004	28,95	27,62	1,27	25,28	24,09	1,19	0,08	0,94
274	TS-PH05-035-0006	25,95	24,99	0,96	28,01	26,89	1,12	-0,16	1,12
274	TS-PH05-035-0006	25,57	24,29	1,28	28,01	26,89	1,12	0,16	0,89
279	TS-PH05-036-0007	27,78	26,85	1,47	25,28	24,09	1,19	0,28	0,82
279	TS-PH05-036-0007	24,95	23,77	1,32	25,28	24,09	1,19	0,13	0,91

**Detection methodology:**

- In relation to the Agency request to include a method for the endogenous gene, you stated that the qualitative detection method for AHAS supplied in the annex does not rely on an endogenous gene as reference. It is true that a reference gene for quantitation is not required in a qualitative method. However, I wish to point out that an endogenous control PCR is normally used as a quality control

procedure to confirm the amplifiability of the extracted DNA and it is normal practise to do so in many laboratories.

I wish to point out that the alternative method given in your response to answer question 15 of 13<sup>th</sup> February 2006, is not **properly** documented.

**Consequently, you are requested to submit a method for detection to include all stages of the procedure including initial sample preparation and DNA extraction. It should be fully documented in standard operating procedure format to ensure consistent application so that a laboratory can easily replicate the exact procedure used by your company. Please refer to ISO 17025, on the type of information that should be included in such a detection method.**

A step-by-step protocol describing the real-time PCR method for qualitative detection of the GM potato lines using primers and probe against the *Rpi-blb2* as well as the *ahas* gene is attached in the Annex to this document. The protocol also includes an assay for an endogenous control gene to enable confirmation of the quality of the DNA extraction and PCR reaction.

3. Please provide a numerical statement of the frequency/rate of out breeding among the respective parental varieties under comparable field growing conditions in order to evaluate the upper level of out breeding risk in the proposed trial.

The parental potato varieties P698, P835 and P880 are classified regarding flowering from 'middle' to 'abundant' and regarding berry formation from rarely to frequently. Flowering and berry formation are determined by the genotype. In addition to the flowering rate, the extent of pollen dispersal and successful out-crossing relate to the type of pollination and the availability of receptive ovules. Potato is mostly (>80 %) self-pollinating. The remaining 0 to 20 % potato pollen can be transported by insects, mostly by bumblebees (due to the absence of nectar not by honey bees) though only over short distances. Further, dissemination by wind is considered of no importance (OECD, 1997). An examination of cross-pollination levels from herbicide tolerant GM plants by Tynan (Eastham and Sweet, 2002) showed that the frequency of transgenic seedlings among the progeny of non-GM potato plants growing within 4.5 m of a GM trial was 0.05 %. In a similar experiment McPartlan and Dale (Eastham and Sweet, 2002) analysed the frequency of herbicide tolerant seedlings obtained from non-GM potato plants grown at a distance of 10 and 20 m to GM potato plants. At 10 m the frequency of cross-pollination was 0.017 % and dropped to zero at 20 m. Considering the frequency rating of flowering for the three parental varieties, we assume that viable pollen is formed from 'middle' to 'abundant', however the risk management measure of installing an isolation distance of at least 20 m to any commercial potato field will reduce the frequency of outcrossing under field conditions between GM potato lines and non-GM commercial plants to zero. Therefore the risk of out-crossing to commercial potato plants is considered negligible. Further in the unlikely event of out-crossing there will be no manifestation of the conferred trait in the pollinated plant, since the commercial product is the tuber, in addition potatoes are propagated vegetatively. So the consequences of a potential out-crossing will also be negligible.

4. Please confirm if you have applied to the Irish Department of Agriculture and Food for a phytosanitary status report/certificate regarding the proposed planting materials in advance of importation to Ireland, pending consent from the Agency.

We received confirmation from the Irish Department of Agriculture and Food and that according to applicable EU legislation the requirements for importation into Ireland of potato material for a field release is that the material to be accompanied by a valid plant passport, which also takes into account Ireland's status as a high grade seed potato region. Furthermore, the receiver of the material according to national Irish legislation is required to inform the Irish Department of Agriculture and Food about the introduction of the material.

5. Provide an explanation as to why data confirming the absence of the LB primer genes in the insert for VCPMA16 and VCPMA19 lines has not been given?

**Provide this information to verify its exclusion from the GM lines.**

The primary intention to show absence of the RB primer genes in the insert for the VCPMA16 and VCPMA19 potato lines was to confirm the absence of integration of the *aadA* gene. In the following Table complementary data for a random selection of potato lines transformed with construct VCPMA16 also confirming the absence of the LB primer genes is given.

	Line no	Ct LB	Ct end ctrl	dCt	Result
5	TS-PH05-002-0055	40,00*	26,53	13,47	negative
13	TS-PH05-008-0004	40,00*	25,67	14,33	negative
14	TS-PH05-009-0001	40,00*	27,13	12,87	negative
16	TS-PH05-009-0051	40,00*	24,40	15,60	negative
38	TS-PH05-010-0041	40,00*	23,64	16,36	negative
42	TS-PH05-010-0070	40,00*	27,83	12,17	negative
57	TS-PH05-010-0156	40,00*	25,78	14,22	negative
60	TS-PH05-010-0178	40,00*	24,54	15,46	negative
61	TS-PH05-010-0186	40,00*	26,58	13,42	negative
71	TS-PH05-010-0303	40,00*	24,89	15,11	negative
80	TS-PH05-010-0466	40,00*	26,89	13,11	negative
81	TS-PH05-010-0471	40,00*	27,34	12,66	negative
82	TS-PH05-010-0472	40,00*	27,21	12,79	negative
93	TS-PH05-011-0026	40,00*	26,30	13,70	negative
94	TS-PH05-011-0027	40,00*	24,52	15,48	negative
95	TS-PH05-011-0029	40,00*	22,77	17,23	negative
96	TS-PH05-011-0048	40,00*	26,88	13,12	negative
100	TS-PH05-011-0088	40,00*	27,38	12,62	negative
107	TS-PH05-011-0124	40,00*	26,25	13,75	negative
112	TS-PH05-012-0008	40,00*	24,48	15,52	negative
125	TS-PH05-015-0001	40,00*	27,04	12,96	negative
129	TS-PH05-015-0018	40,00*	25,42	14,58	negative
148	TS-PH05-016-0044	40,00*	23,84	16,16	negative
166	TS-PH05-017-0004	40,00*	27,37	12,63	negative
177	TS-PH05-018-0011	40,00*	26,66	13,34	negative

187	TS-PH05-018-0093	40,00*	23,65	16,35	negative
207	TS-PH05-019-0023	40,00*	26,02	13,98	negative
218	TS-PH05-020-0032	40,00*	25,14	14,86	negative
226	TS-PH05-022-0013	40,00*	24,33	15,67	negative
230	TS-PH05-023-0003	40,00*	23,32	16,68	negative
236	TS-PH05-025-0038	40,00*	24,94	15,06	negative
240	TS-PH05-025-0067	40,00*	23,48	16,52	negative
266	TS-PH05-030-0034	40,00*	26,48	13,52	negative
272	TS-PH05-034-0004	40,00*	27,47	12,53	negative
274	TS-PH05-035-0006	40,00*	24,08	15,92	negative
279	TS-PH05-036-0007	40,00*	26,00	14,00	negative
	neg ctrl, P698	40,00*	23,89	16,11	negative
	neg ctrl, P835	40,00*	25,07	14,93	negative
	positive ctrl	29,29	28,87	0,42	positive
	positive ctrl	29,58	28,98	0,60	positive

\* no signal detected after 40 cycles

6. The literature suggests that true potato seed (TPS) will survive up to 8 years post-harvest and the described process of harvesting in the notification will facilitate the entry of the true potato seed into the soil.

**Please provide both a post-release monitoring strategy to ensure eradication for both TPS and groundkeepers which includes a case-specific and general surveillance component.**

The parental potato varieties P698, P835 and P880 are classified regarding berry formation from 'rarely' to 'frequently'. It is therefore assumed that derived GM potato lines will, to a certain extent, produce berries that in turn will produce true seeds.

True potato seeds (TPS), embedded in the soil of the deliberate release plot, can survive and germinate in the soil and the resultant potato plants might produce tubers. However, due to genetic segregation, the true seed derived plants differ from their parental plants. Their agronomic performance is poor and they show lower competitiveness. Further seed-derived plants show a lower early vigour as the nutritional resources in the seed are much lower than those in the tubers. Therefore plants potentially arising out of those seeds are usually weak, with poor agronomic performance and low competitiveness.

Further so-called ground-keepers can remain in soil after harvest. As the tubers are generally frost sensitive, their survivability and reproduction is dependent on temperature. Under European conditions the tubers persist poorly in cold wet soils and plants rapidly become infected with a range of fungal and viral diseases (Eastham and Sweet, 2002). The survivability is also limited by cultivation practices such as ploughing, harrowing and application of herbicides and by competition from other crops in the crop rotation.

Both the survival of tubers grown from true seeds and ground-keepers depends on cultivation practices and crop rotation. The risk management measure proposed in the e.r.a. in order to control potential persistence in the field are conventional agricultural practice and volunteer management (monitoring for volunteers and removal/destruction of volunteers in the field, crop rotation). This is also outlined in the monitoring plan below.

The first year following the release the volunteer monitoring programme starts and the field plot will either remain fallow or will be cultivated with a species that facilitates weed management of the area that year. For the duration of the volunteer monitoring programme no potatoes will be planted on the field plot, however other crops (except for the first year) can be planted as long as they allow volunteer monitoring. Emerging volunteers will be recorded and destroyed by herbicide treatment (systemic herbicide e.g. glyphosate) prior to flower setting. The monitoring for volunteers will continue till no volunteers emerge. Thus, if volunteers (seed-derived or tuber-derived) emerge, the monitoring period will be prolonged by another year. In that way the monitoring will continue until no volunteers appear in the respective year. The cultivation of the release site in the years after the monitoring programme has concluded will be according to local crop rotation practice for potatoes.

According to conventional agricultural practice also non-GM potato plants are cultivated under a crop rotation and volunteer monitoring programme in order to control fungal infections like *Phytophthora infestans*. Therefore in the unlikely event that volunteers derived from true seeds emerged only after the conclusion of the volunteer monitoring programme, conventional agricultural practice would also lead to their destruction.

### Monitoring plan

The following monitoring plan is based on the conclusions of the environmental risk assessment and aims at early observation and identification of intended and unintended effects related to the release of the GM potato plants.

<b>Assumptions of risk assessment</b>	<b>Observations performed by notifier</b>
<b>Case specific monitoring</b>	
No selective advantage due to improved resistance to <i>P. infestans</i>	Monitoring for volunteers
No selective advantage or disadvantage conferred to sexually compatible plant species	Monitoring for volunteers
Intended effects on target organism <i>P. infestans</i>	Observations on changes in tolerance to <i>P. infestans</i>
No impact on the environment due to interactions with non-target organisms	Observations on changes in susceptibility to insects and pests
<b>General Surveillance</b>	
No differences in general characteristics of the plant: size, shape, flowering, development	Observations on general plant characteristics and agronomic performance, on effects of soil and climate
No differences in disease and pest susceptibility	Observations on changes in susceptibility to insects and pests

No difference in competitive behaviour	Monitoring for volunteers
Limitations of the potato to the release site	Control (notebook, restricted access) over implementation of risk management measures

### **Baselines**

The performance of the genetically modified potato lines will be compared to the performance of the recipient varieties grown in parallel at the same release site.

### **Time period**

During the course of the entire vegetation period (from about April to October) of the potato lines the area of release will be visited by the compliance liaison and trained personal to observe the release at defined intervals (at least once a month). The compliance liaison or trained personnel will observe the area post-release at defined intervals for the duration of the volunteer monitoring program.

### **Responsibilities**

The notifier is responsible for the monitoring plan. Case-specific and general surveillance will be carried out by BASF Plant Science and contracted individuals including compliance liaison and trained personal.

### **Area**

It is the site of release and the individual release plots that will be monitored.

### **Inspections**

The area of release will be visited by the compliance liaison and trained personal. Inspections may also be performed by the responsible authority.

### **Data collection and evaluation**

BASF Plant Science will be responsible for all records of observations and analyses performed in accordance with the monitoring plan. Data will be collected and analysed according to specifications by BASF Plant Science in accordance with international guidelines (e.g. UPOV for general plant characteristics). Field notebooks are kept during the period of release.

### **Reporting**

Information regarding any unexpected occurrences of relevance regarding potential adverse effects on the environment and human health directly related to the genetically modified potato lines will be communicated to the appropriate Authority and required measures will be implemented accordingly. A report summarising the observations during the field trial will be submitted annually.

7. It is noted that you did not provide an answer to question 20 of those included in Agency letter of 13<sup>th</sup> February 2006:

- how do you propose to control any fruit that might occur at the proposed trial site?

**You are requested to provide an answer to this question.**

No specific measures are being proposed to control fruits. Should berries be formed on the GM potato lines as the plants mature they will undergo the same treatment as the entire plant. According to conventional agricultural practice all green parts of the potato plants will be burned down by herbicide treatment (Reglone) prior to harvest and left at the ground for decomposition. Please refer to question 6 above for the proposed volunteer management programme to control true-seed derived tubers.

8. In answer to Q 26 of 13<sup>th</sup> February 2006, you stated:

It is not planned to apply special bird protection measures during the course of the growing season as there have been no reports and no observation from other release trials that potatoes have been disseminated by birds or small animals or constitute a preferred food thereof.

**The Agency is of the view that dispersal of groundkeepers by birds post-harvest is an issue that can facilitate tuber loss from potato fields.**

**You are required to forward logistical measures to minimise tuber loss at the proposed site from bird intrusion.**

The following procedures apply during harvest and post-harvest at the location of the release and are documented in a compliance notebook that is kept at the site of release by the field manager.

- All personnel entering the field and performing activities will be trained with regard to the procedures to follow during and after the release.
- Presence of personnel at harvest and post-harvest and running harvesting machinery will deter birds from entering the field at those times.
- All machinery or equipment used for harvest will be inspected and cleaned prior to and after harvest in order to remove any remaining tubers or pieces of tubers.
- Tubers are harvested either manual or by small machinery as applicable to the size of the trial plots and are removed carefully and as completely as possible from the ground in order to avoid volunteer development.
- After harvest the soil is loosened in such a way that tubers close to the surface emerge and can be collected by hand.
- All harvested tubers (GM potatoes and non-GM comparator lines) are immediately packed and labelled (closed, double containment) at the release site in order to limit exposure.
- All packed tubers are transported in a closed transport vehicle from the release site directly to the site of analysis and destruction outside of Ireland.

The above procedures ensure that the number of tubers that are exposed during harvest and after harvest is minimised and therefore the risk of tuber loss due to bird intrusion becomes negligible.

9. Provide an **Identity Preservation System** to verify the absence of admixture with non-GM potato that may be harvested from adjacent fields, pending consent from the Agency.

The BASF Plant Science (BPS) Compliance System ensures that the identity of the GM and non-GM potato lines to be release is preserved and that there is no admixing of commercial potato varieties from neighbouring fields into the experimental release plot nor admixing of GM potato lines with commercial conventional potatoes in the food or feed chain. Compliance instructions, forms and regulatory guidance documents together with the consent will form the basis for the compliance notebook, which is kept at the site of the release by the field manager. Further all steps taken during the release will be documented in the notebook and are complemented by a system of visits and audits at the release site. Compliance instructions guide all steps to be taken during receipt and transport of the potato material, at planting and during harvest and post-harvest. A BPS internal traceability and detection system verifies the identity of the potato material to be released prior to planting and prior to analysis after harvest. All material transported to and from the release site will be clearly labelled (identity, GM) and packaged in double containment. A separate transport vehicle is used for transport of material intended for the experimental release to and off the release site. Receipt and condition of the material are documented prior to planting. All personnel involved in the release will be trained according to the compliance instructions and the conditions of the consent. The release site will be clearly labelled and access will be restricted to authorized personnel only. All machinery and equipment used when handling the potato material will be inspected and cleaned prior to and after planting and prior to and after harvesting. All relevant observations (see monitoring plan) will be recorded. A response plan outlines the process in case of any unintentional release caused by e.g. acts of nature or vandalism. Any unintentional admixture with non-GM commercial potatoes from neighbouring fields, should it occur via e.g. vandalism, will be treated as being of GM material and will be destructed after transportation off site and outside of Ireland.

## ANNEX

### Detection Protocol

**Method:** *Sample preparation and real-time PCR analysis for qualitative detection of constructs VCPMA16 and VCPMA19 in potato leaves or tubers.*

**Scope:** *Sample collection, tissue homogenisation, DNA extraction and real-time PCR analysis of potato material for construct verification*

### Protocol:

*Always work with gloves during all steps of handling plates and samples.*

1) With forceps and/or puncher and/or scissors, sample 25-50 mg of potato material (lower amount for leaves, higher amount for tubers) in 96-well blocks (Corning Inc. – Costar 3957, 0.5ml). Between samples sterilise instruments using Ethanol and flame or bead steriliser.

Never touch the tissue with your hands. Leaf samples should be taken from young leaves.

2) Seal plate with 96-plate mat (ABGene AB-0566). If storing plate before extraction, store at  $-80^{\circ}\text{C}$ .

3) Homogenisation:

- a) Add sterile stainless steel beads<sup>1</sup> to each well in the 96-well plate.
- b) Homogenise samples at 25Hz using Retsch MM300 Mixer Mill, 2 x 30 sec. If material is not properly homogenised, repeat homogenisation.

4) DNA Extraction:

Extract the DNA using the Wizard Magnetic 96 DNA Plant System (Promega) following the protocol provided by the manufacturer.

For extraction of DNA from tubers, the first steps should be held cool and 1.5 volumes of lysis buffer should be used. Should starch still be present in the eluted extract, please re-extracted using the same method.

5) Real-time PCR analysis:

Depending on assay to be run either the setup for Mastermix from Applied Biosystems or Mastermix from Sigma should be used (see specification for each assay below).

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<sup>1</sup> Stainless steel beads might not be available commercially, however will be provided by BASF Plant Science GmbH upon request.

a-1) PCR-setup for Mastermix from Applied Biosystems:

I) Mix the reagents according to the tables below.

Set up for Gene of Interest, GOI

	Final conc.
TaqMan 2xPCR Mastermix (4318157, Applied Biosystems)	1x
GOI primer for	900 nM
GOI primer rev	900 nM
GOI probe	200 nM
Millipore water	

Set up for Endogenous Control, EC

	Final conc.
TaqMan 2xPCR Mastermix (4318157, Applied Biosystems)	1x
Human 18S rRNA, 20x (4310893, Applied Biosystems)	1x
Millipore water	

a-2) PCR-setup for Mastermix from Sigma

I) Prepare the JumpStart Taq Ready Mix (P-2893, Sigma) by adding 1M Magnesium chloride (A4998,0500, AppliChem) to a final concentration of 14 mM.

II) Make a stock of 15µM Sulforhodamine, ROX (S-7635, Sigma).

III) Mix the reagents according to the tables below.

Set up for Gene of Interest, GOI

	Final conc.
JumpStart Taq Ready Mix (P-2893, Sigma)	1x
GOI primer for	900 nM
GOI primer rev	900 nM
GOI probe	200 nM
ROX, 15µM	300 nM
Millipore water	

Set up for Endogenous Control, EC

	Final conc.
JumpStart Taq Ready Mix (P-2893, Sigma)	1x
Human 18S rRNA, 20x (4310893, Applied Biosystems)	1x
ROX, 15µM	300 nM

Millipore water	
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- b) Mix, spin down and pipette 8 µl into 384-Well Optical Reaction Plate (4326270, Applied Biosystems).
- c) Dilute the DNA 2 times and add 2 µl to each well. Add 2 µl water to at least one well as a negative mastermix control.
- d) Seal the plates with Adhesive Optical Covers (4311971, Applied Biosystems) and centrifuge the 384-plates at 3500 rpm for 5 minutes.
- e) Real-time PCR, using the instrument ABI 7900HT. Set up the instrument with the following conditions:

	Stage 1	Stage 2	
Temperature	95°C	95°C	60°C
Time	5 min	15 s	1 min
		40 cycles	

- f) Analyse data according to instructions in “User Guide, Basic Operation and Maintenance” for “ABI PRISM 7900 HT Sequence Detection System and SDS Enterprise Database” (Applied Biosystems).

### Specification for each assay:

#### *Gene of Interest, GOI assays:*

##### A) AHAS-gene:

Forward primer: GATCCTCAGGTAAACCAGGTATCTGT

Reverse primer: ATCGGCTAATCCGCTAACGA

Probe: CCACTTCAGGTCCCGGA

Analyse using Mastermix from Applied Biosystems.

##### B) blb2 - gene

Forward primer: TTCAAACCCCAAATAAGTTTCAAC

Reverse primer: CCATGCTTGCTGTACTTTGCA

Probe: CGTTACCCAGTCCTTCGGCG

Analyse using Mastermix from Sigma.

#### *Endogenous Control, EC, assay:*

##### C) Endogenous control (reference):

Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems, Part Number 4310893E)

Analyse using Mastermix from Applied Biosystems or Sigma.