

**Tom McLoughlin**

# Appendix 1

**From:** christine.wandelt@basf.com  
**Sent:** 24 February 2006 07:16  
**To:** Tom McLoughlin  
**Subject:** Antwort: Request for further information

**Attachments:** RESPONSE\_Request for further information4th\_CW.doc



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Dear Dr. McLoughlin,

in response to your request from 13th February regarding our notification B/IE/06/01 we hereby submit the following information.

With best regards,  
Christine Wandelt

(See attached file: RESPONSE\_Request for further information4th\_CW.doc)

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Manager Regulatory Affairs

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"Tom McLoughlin"  
<T.McLoughlin@epa  
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02/13/2006 05:08  
PM

<christine.wandelt@basf.com> An  
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Thema  
Request for further information

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1. Please confirm that the GM potato lines that BASF propose to release are tetraploid?

All commercially cultivated potato varieties are tetraploid ( $4n = 48$ ) (OECD, 1997). As the GM potato lines intended for the small-scale release are derived from commercial tetraploid potato varieties, these lines are tetraploid as well.

2. Page 7 of the notification, Point No 3 (b) it is stated that '*Under field conditions the berries do not mature as well as the seeds*'.  
**Is this not a contradictory statement considering that seed comes from berries? Please clarify.**

Revised wording: 'Under field conditions the berries do not mature as well as the tubers. The berry-derived seeds, however can continue to mature when left in the soil.'

3. Under the same paragraph it is stated: '*Plants potentially arising out of those seeds are usually weak, with poor agronomic performance and low competitiveness*'.  
**Please supply information that supports this statement as in Ireland seed that originates from berries can survive and germinate and produce tubers under field conditions.**

True potato seeds (TPS) 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.

4. Page 11, it is stated '*There are no components of the vectors known to code for harmful substances*'.  
**Provide evidence to verify this statement.**

The coding regions present on the T-DNA are those listed in the Table of genetic elements (see page 11), i.e. the *Rpi-blb2*, *Rpi-blb1* and the *ahas* genes. Based on database comparisons none of these genes are known to code for any harmful substances and none show similarity to known toxins or allergens.

5. Page 14, 2 (a) it is stated: *According to analysis with real-time PCR all genes of the T-DNA; the Rpi-blb1, Rpi-blb2 as well as ahas gene are confirmed to be present in all of the transgenic lines intended for the field trial.*  
**Provide relevant molecular data to verify this statement.**

For all GM potato lines data confirming the presence of the *ahas* gene integrated into the potato genome are presented in *Tables 1 and 2 of Annex 1*. As, during the integration process, the *ahas* gene is the last genetic element

of the T-DNA to be inserted into the potato genome, these data serve as prove for the complete integration of the T-DNA. Additionally, all GM potato lines listed in *Tables 1* and *2* were analysed for presence of the *Rpi-blb2* and *Rpi-blb1* genetic elements and their presence was confirmed positive. The Table below illustrates those data based on a random selection of GM potato lines transformed with construct VCPMA16 and analysed for the presence of *Rpi-blb2* and *Rpi-blb1* genes.

	Line no	Insert analysis - Rpi-blb2				Insert analysis - Rpi-blb1			
		Ct blb2	Ct end ctrl	dCt	Result	Ct blb1	Ct end ctrl	dCt	Result
14	TS-PH05-009-0001	26,92	26,02	0,90	positive	26,95	26,02	0,92	positive
16	TS-PH05-009-0051	24,69	23,98	0,71	positive	24,49	23,92	0,56	positive
38	TS-PH05-010-0041	23,86	22,94	0,92	positive	23,96	23,27	0,70	positive
42	TS-PH05-010-0070	27,21	26,70	0,51	positive	27,41	26,99	0,42	positive
57	TS-PH05-010-0156	25,40	24,52	0,88	positive	25,82	25,28	0,54	positive
60	TS-PH05-010-0178	24,62	23,81	0,81	positive	24,70	24,18	0,52	positive
61	TS-PH05-010-0186	25,26	24,48	0,78	positive	26,06	25,56	0,51	positive
71	TS-PH05-010-0303	23,46	22,55	0,91	positive	25,23	24,44	0,78	positive
80	TS-PH05-010-0466	26,78	26,00	0,78	positive	27,19	26,68	0,51	positive
81	TS-PH05-010-0471	27,63	26,58	1,05	positive	27,85	26,80	1,05	positive
82	TS-PH05-010-0472	27,25	26,24	1,01	positive	26,95	26,42	0,52	positive
93	TS-PH05-011-0026	26,46	25,64	0,82	positive	26,67	26,03	0,64	positive
94	TS-PH05-011-0027	23,73	22,50	1,23	positive	24,38	23,58	0,80	positive
95	TS-PH05-011-0029	23,99	23,28	0,71	positive	23,11	22,44	0,67	positive
96	TS-PH05-011-0048	25,66	24,66	1,00	positive	26,76	26,11	0,65	positive
100	TS-PH05-011-0088	26,77	25,78	0,99	positive	27,46	26,99	0,46	positive
107	TS-PH05-011-0124	26,04	24,74	1,30	positive	26,75	25,79	0,96	positive
125	TS-PH05-015-0001	25,75	24,82	0,94	positive	27,28	26,65	0,63	positive
129	TS-PH05-015-0018	25,30	24,33	0,97	positive	25,04	24,58	0,46	positive
166	TS-PH05-017-0004	26,08	25,23	0,84	positive	26,96	26,41	0,55	positive
177	TS-PH05-018-0011	26,53	25,62	0,91	positive	26,59	25,86	0,74	positive
218	TS-PH05-020-0032	24,16	23,22	0,95	positive	24,87	24,44	0,43	positive
236	TS-PH05-025-0038	25,43	24,51	0,93	positive	25,14	24,63	0,51	positive
240	TS-PH05-025-0067	25,58	24,73	0,85	positive	23,92	23,29	0,63	positive
272	TS-PH05-034-0004	27,39	26,42	0,98	positive	27,50	26,84	0,66	positive
279	TS-PH05-036-0007	26,39	25,14	1,26	positive	26,31	25,52	0,80	positive
	neg ctrl, P835	40,00*	22,84	17,15	positive	40,00*	22,10	17,89	positive
	positive ctrl	26,47	25,85	0,62	positive	28,86	27,99	0,87	positive

\* no signal detected after 40 cycles

6. On page 14, it is stated that the lines intended for release contain 1 or 2 copies of the AHAS gene. This statement would appear to be inconsistent with the RTPCR data given in pages 37 to 45, where there are several delta Ct values approaching and greater than 2. Information on the PCR efficiencies is not provided and the copy number of the endogenous gene has not been given to allow for interpretation. Assuming the PCR reactions were both highly efficient, a delta Ct of 2 would indicate 4 copies of an insert if the endogenous gene was present in single copy.

**You are requested to explain this apparent inconsistency and provide support for your statement on the number of inserts.**

Please note, that the insert copy numbers are not determined from the delta Ct values as this requires a known copy number for the endogenous control and identical Ct values for both reactions at a given copy number and identical efficiencies. This is not always the case. Instead, the copy numbers are calculated as compared to a calibrator sample with known copy number analysed in parallel with the sample to be determined, as this is a more robust method. In the following the method used to determine the copy number is explained in detail:

#### Transgene copy number determination, potato

Transgene copy numbers were determined by real-time PCR on the instrument ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The application of the fluorogenic 5' nuclease (TaqMan) assay for quantitative determination of transgene copy number was published by Ingham et al, 2001. For the transgene copy number determination in the potato plants primers and probes for the endogenous gene, used for normalisation, as well as for the *ahas* gene were designed using the Primer Express software (Applied Biosystems). PCR reactions were multiplexed, using the endogenous genomic gene for normalisation. Calibrator samples with known copy numbers are analysed in parallel. The software SDS 2.0 (Applied Biosystems) was used for the calculations of C<sub>T</sub> values and calculations of transgene copy number were performed using the so-called comparative C<sub>T</sub> method ("User Bulletin #2:ABI PRISM 7700 sequence Detection System", Applied Biosystems.). The method uses arithmetic formulas to achieve the result for relative quantification eliminating the need for a standard curve and the adverse effect of any dilution errors made in creating the standard curve samples.

With the comparative C<sub>T</sub> method the amount of target, normalized to an endogenous control and relative to a calibrator is given by:

$$2^{-\Delta\Delta CT}$$

where

C<sub>T</sub> = the fractional cycle number at which the amount of amplified target reaches a fixed threshold

$\Delta CT = C_{T,X} - C_{T,R}$ , the difference in threshold cycles for target and reference

$\Delta\Delta CT = \Delta CT_{q} - \Delta CT_{cb}$ , where q is any sample and cb is the calibrator

7. Page 15, 3 (a), it is stated: '*In the genetically modified lines a low level of expression of both Rpi-blb1 and Rpi-blb2 has been demonstrated by real-time PCR analysis in leaves, stems, tubers and roots.*

**Do low levels of gene expression give tolerance to the blight fungus?  
Please clarify.**

Yes, only low levels of gene expression of the inserted *Rpi-blb1* and *Rpi-blb2* genes are needed to confer resistance against *Phytophthora infestans*. The R-gene products exert no direct activity towards *Phytophthora infestans*. Instead, they are acting as "guards" that recognize pathogen-derived

molecules and upon recognition initiate the resistance response including a localized cell death trapping the pathogen. For this recognition to take place only small amounts of R-gene products are required. NBS-LRR genes are usually expressed at low levels (Michelmore, 2000). Massively parallel signature sequencing (MPSS; Meyers et al., 2004) data of NBS-LRR proteins in *Arabidopsis* and rice obtained from Plant MPSS Databases (<http://mpss.udel.edu/>) further confirms low expression levels for many more such genes.

Further, the GM potato plants that express the inserted *Rpi-blb1* and *Rpi-blb2* genes at low levels are able to resist infection by *Phytophthora infestans* when challenged in the greenhouse.

8. Page 15, under Survivability it is stated: '*potatoes expressing the AHAS protein have been evaluated for frost tolerance in field, but no alterations compared to the parental varieties were observed*'.

**Please provide information in support of this statement.**

GM potato lines expressing the AHAS protein, though not harbouring the *Rpi-blb1* and *Rpi-blb2* genes, and non-GM comparator potato lines were exposed to conditions of frost in the field. During those so-called overwintering trials in the season 2004-2005 potato tubers were left in the ground from autumn to spring the following year at field trial locations in Sweden, the Netherlands and Germany. Whereas at locations in Sweden and Germany no sprouting potatoes (GM and non-GM comparator) could be detected in the spring, the estimated survival rate for the GM potatoes in the Netherlands (under conditions of a milder winter) ranged between 25 and 99 % and did not significantly differ from the survival rates of the non-GM comparator varieties.

9. Page 15, *Potato already contains many other R-genes of the NBS-LRR class. Some NBS-LRR genes are introgressed from Solanum demissum and confer resistance to some races of P. infestans.*

- **Do the 3 potato breeding lines that you transformed have any of these R- genes from *S. demissum* using traditional breeding methods?**

Crosses of *Solanum tuberosum* with *Solanum demissum* were first introduced in the early 20th century as a source for resistance against *P. infestans* and have been widely used in potato breeding programmes in Europe (Scotland, Germany) and the US. Thus, many potato cultivars contain *S. demissum* R-genes. Disappointingly, the resistance introgressed from *S. demissum* turned out to be rather short-lived and the broad breeding programmes based on *S. demissum* genes were discontinued (Wastie, 1991). However, many older potato varieties contain *S. demissum* genetic material and therefore also more recent potato varieties derived from crosses with these may still contain *S. demissum* genes. The three breeding varieties that were used for the transformations may very well contain genetic material from *S. demissum*, though due to the low efficiency against late blight those R-genes are not always detectable in resistance screenings.

- **Please indicate whether R-genes from *S. demissum* or from *S. bulbocastanum* have been sequenced?**

The two R-genes, *Rpi-blb1* and *Rpi-blb2*, from *S. bulbocastanum* that are described in the notification have undergone DNA sequence analysis. From *S. demissum*, two genes conferring resistance against *P. infestans*, *R1* and *R3a*, have been cloned and sequenced (Ballvora et al., 2002; Huang et al., 2005).

- **Where sequencing has been carried out indicate the level of homology (DNA) between the different R-genes from the different potato species?**

The upper part of the *Rpi-blb1* gene has 91% sequence identity (at DNA level) to an EST from potato and 81-90% sequence identity to partial NBS fragments of tomato (van der Vossen et al., 2003). The *Rpi-blb2* gene is 90% identical to the *Mi-1* R-gene from tomato (van der Vossen et al., 2005).

DNA sequence homology (introns excluded) comparing four R-genes from *S. demissum* and *S. bulbocastanum* using AlignX function in Vector NTI 9.0 (InforMax, MD, USA) are indicated in the table below:

	R1 demissum	R3a demissum	Rpi-blb1 bulbocastanum	Rpi-blb2 bulbocastanum
R1 demissum	100%	31%	29%	48%
R3a demissum		100%	43%	40%
Rpi-blb1 bulbocastanum			100%	36%
Rpi-blb2 bulbocastanum				100%

10. Page 16, No 7, *Information on any toxic, allergenic or harmful effects on human health and the environment arising from the genetic modification.* **Please indicate if the following studies have been addressed/ examined/taken into consideration. Where the studies have not been undertaken please explain why.**

- **comparison of homology (bioinformatic database search) between amino acid sequence of all the inserted genes (blight and herbicide tolerance genes) and amino acid sequence of known toxic/allergenic proteins?**

The AHAS, *Rpi-blb1* and *Rpi-blb2* protein sequences were compared *in silico* to all proteins in a downloaded Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (version 6.00) via a BlastP (version 2.2.6) analysis. None of the submitted proteins showed 35% or greater identity over 80 amino acids to a known allergen. Additionally, no submitted protein shared a sequence of eight consecutive identical amino acids with a known allergen.

The AHAS, Rpi-blb1 and Rpi-blb2 proteins were also analyzed for sequence homology to known toxins via a BlastP search of the downloaded June 2005 GenBank non-redundant peptide sequence database. This BlastP analysis did not identify any known toxins with significant homology to the submitted proteins.

- **possible epigenetic or pleiotropic effects pertaining to the introduced DNA?**

Potential epigenetic or pleiotropic effects in plant development generally relate to heritable changes in gene expression that are not associated with alterations in DNA sequence. These effects can be exerted at transcription level or posttranscriptionally. Many epigenetic changes depend on the recognition of sequence homology at the DNA or RNA level. This recognition can lead to transcriptional or posttranscriptional gene silencing. None of the genetic elements present on the T-DNA to be integrated into the potato genome show sufficient DNA sequence homology to respective endogenous potato genes in order to be able to trigger any epigenetic or pleiotropic effects. Therefore the likelihood of the occurrence of possible epigenetic or pleiotropic effects in the GM potato lines pertaining to the introduced DNA is remote.

- **have any of the proposed 340 GM potato lines been measured for the main toxic or anti-nutritional substances (glycoalkaloids and nitrates) found in potatoes?**

None of the GM potato lines intended for the release have been analysed for tuber composition, i.e. glycoalkaloids or nitrates. The genetically modified potatoes differ from conventional potato varieties in their resistance to *Phytophthora infestans* conferred by the introduced R-genes. Potato already contains a large number of resistance genes conferring resistance against other plant diseases where the majority of those genes belong to the NBS-LRR class. Included are also genes introgressed from wild potato species. None of the genes are known to exert any toxic or allergenic effects to human health. The mode of action is a hypersensitive response upon infection by the fungus leading to plant cell necrosis. The introduced genes are expressed by their endogenous promoters at very low levels that are comparable to those from other endogenous resistance genes.

The introduced selection marker gene is expressed as the enzyme AHAS, which is an enzyme found in all plant species and not known to confer any toxic or allergenic properties. The safety of different crop species with AHAS-mediated tolerance to imidazolinones has been assessed by the Canadian Food Inspection Agency.

The potato plants are intended to be released within the scope of a small-scale field trial, are not for human consumption and measures taken with regard to planting, harvest, storage and transportation will minimize any contact to humans. Therefore the overall impact on human health is negligible.

Moreover we would like to point out that the release, if consent is granted, will be a small-scale experimental trial at one location to be conducted in two

phases (A) screening of events (proof of concept; duration 1 to 2 years) and (B) development (stability of trait, safety studies; duration 2 to 3 years). During the course of the trial the following will be observed and recorded: Phase (A): agronomic performance (e.g. plant vigour, yield, susceptibility to climatic factors), altered agronomic properties (e.g. disease susceptibility), event screening (susceptibility to *Phytophthora infestans*), plant characteristics (e.g. emergence, leaf shape and colour, flowering, ground coverage, maturation), management of volunteers and Phase (B): stability of expression (e.g. sampling of plant tissue at various developmental stages in order to conduct gene expression studies), potential effects on non-target organisms (e.g. soil flora, potato-related insects), altered qualitative properties (e.g. tuber composition, nutrients, anti-nutrients, feeding studies). Therefore the field trial will enable the generation of GM potato material grown under Irish field conditions for compositional analyses.

- **Have animal feeding trials been carried out on any of these GM potato lines?**

No animal feeding trials have been carried out on any of the GM potato lines intended for the release. However, the field trial will enable the generation of sufficient GM potato material grown under field conditions in Ireland that may be used for animal feeding studies within the frame of safety studies (see point above). During the course of the trial measures taken under current release practice will protect the trial against damage by wild animals (e.g. fences) and also ensure that seed stock and plant material are harvested, stored, transported or disposed of (e.g. cleaning of machinery, packaging) in such a way to minimise contact to animals. Measures will also be taken in order to prevent that GM potatoes enter the food or feed chain.

11. Page 17 No 7, it is stated: *The expression of the introduced R-genes (Rpi-blb1 and Rpi-blb2) in the genetically modified potatoes, under the control of the respective endogenous promoters is at very low levels and comparable to those from other resistance genes.*

**What other resistance genes are you referring to? Please clarify this statement.**

We are referring to R-genes in general and especially those of the NBS-LRR group, which is the most common group of R-genes. NBS-LRR genes are generally found to be constitutively expressed at low levels, based on the data available (Michelmore, 2000). As an example, Grant et al. (1995) have in detail studied one NBS-LRR gene, the *Rpmi* gene, and noticed a very low constitutive expression.

12. *Detection methodology:*  
**Please provide a protocol that fully describes the detection method (including DNA extraction) in tuber and leaf tissue matrices. It is essential that the method for the endogenous gene be included and that positive controls are also supplied.**

As indicated in the notification (Section D.12) a qualitative detection method is provided in Annex I, section 3: *Detection of genetically modified potato lines (ahas-gene)*. The described method allows for detection and identification of the GM potato lines intended for the release and distinction from non-GM potato lines. Since the method is a qualitative detection method (standard PCR) it does not rely on an endogenous gene as reference. In case of inspections of the experimental release by the authority at time of planting or at any later stage, leaf or tuber tissue may be collected by the authority in order to verify that potato lines released carry the T-DNA described in the notification. If required, the notifier will provide positive control samples at that point in time. Please also find an alternative detection method outlined in the response to question 15.

13. The method that you have supplied targets the AHAS gene. This method could give a positive response for other GM events and conceivably for non-potato plant tissue, given that the AHAS enzyme 'is an enzyme found in all plant species'? **Please clarify.**

The use of the *ahas*-gene as selection marker in potato is developed in-house (patent application no WO 2004/005516 A1) and to our knowledge not used by any other company.

The method given has been tested carefully on non-GM potato lines showing no cross-reaction with endogenous *ahas* genes. To our knowledge no potato *ahas* gene has so far been cloned and sequenced, but at the protein level the identity between the *A. thaliana* AHAS protein and potato AHAS is around 80%. Generally, dicot AHAS proteins share a high degree of similarity (>70%) at the amino acid level. The method provided has not been tested on other plant species, since as described in the notification, the method serves the purpose to detect and identify GM-potato lines to be released in the environment for experimental purposes only and according to the specific handling conditions described in the notification.

14. Page 17, No 7, it is stated that: '*Due to the specificity of the response reaction no effects on other organisms than P. infestans are expected other than those that also apply to the interaction of non-genetically modified potatoes with non-target organisms under conventional agricultural practice*'. Also, on page 25, H (v) you conclude that: '*The overall impact on non-target organisms is considered negligible*'.

- **Please supply information on what is known about the potential effects of GM potatoes on beneficial soil microbes e.g., Mycorrhizal fungi?**

No changes in *Mycorrhizal* interactions are anticipated as the R-genes function only in living plant cells, and can only affect potato-penetrating organisms. NBS-LRR gene products are localized in the cytoplasm of the cells. Further the triggering of the plant's defence response requires an organism to introduce elicitor proteins into the cytoplasm. This injection of elicitor proteins into the cytoplasm of potato cells can only be

accomplished by potato pathogens, not by loosely associated microorganisms. In addition there is no evidence suggesting soil-dwelling or mycorrhizal fungi to transfer proteins to the cytoplasm of plants they are interacting with.

- **Outline the studies, if any, that have been carried out with these GM potato lines on soil biodiversity?**

**If such studies have not been carried out heretofore and taking account of the precautionary principle, you are advised to strongly consider such studies be carried out under Part B notifications submitted in different Member States, in particular, to include one for this notification as it would be pertinent for Irish soil conditions, pending consent from the Agency.**

**As you are probably aware the information gleaned from such a study will be a requirement for a Part C product application under Directive 2001/18/EC that your company might consider placing on the market in the EU in the future.**

As described in Section F.1. of the notification the purpose of the experimental release is to evaluate and to screen the genetically modified potato lines for improved resistance to *P. infestans*. Additionally, the release is aiming at, in the context of safety studies, collecting necessary data in comparison to unmodified recipient varieties and to conventional potato varieties. Data on agronomic properties and also on environmental interactions will be generated. Further within the frame of the monitoring plan any changes in susceptibility to insects and pests, effects of soil and climate will be observed according to conventional agricultural practice and recorded. The intended release therefore will provide the unique opportunity to conduct studies relating to soil biodiversity under Irish soil conditions and the notifier intends to conduct those studies as part of the safety studies that will be carried out during the course of the planned release period from 2006 to 2010.

15. Page 18, No 12, *Description of detection and identification techniques for the genetically modified plant.*

Mention is made of assays based on real-time PCR that have been developed for the *Rpi-blb1*, the *Rpi-blb2* as well as the *ahas* gene. However, only a qualitative method based on the *ahas* gene is given.

- **Please explain this omission?**

The conventional PCR method described can be used universally in any laboratory for detection of our transgenic material. The real-time PCR assays that we have used in our laboratory are developed specifically for and have only been tested on our ABI Prism 7900HT Sequence Detection System. A description of the real-time PCR assays can only be handed over under confidentiality.

- **It is noted that the qualitative method given for the *ahas* gene as outlined in Annex 1 contains only an element within the construct**

**as opposed to a construct specific method. The point at issue is, can the GM potatoes BASF propose to use be identified using this method, in particular, if other companies use a similiar *ahas* gene insert? Please clarify.**

A reason for targeting the *ahas* gene is that both *Rpi-blb1* and *Rpi-blb2* have to some extent been used in breeding programmes and may also be used by other parties in developing late blight resistant potatoes via applying plant biotechnology approaches. The *ahas*-gene as selection marker in potato is a development of the notifier (patent application no WO 2004/005516 A1) and to our knowledge not used by any other company. At the described field trial location in Arodstown only BASF Plant Science intends to release genetically modified potato lines. We are not aware of any other entity conducting field releases with similar constructs or traits in genetically modified potatoes close to the intended site of release or in Ireland at all. The conditions of the release as described in Section G 1. to 6. of the notification and measures taken with regard to control, monitoring and treatment of the release are outlined in order to assure that at all times GM potatoes are handled separately from non-GM potatoes. The qualitative detection method (as described below and in Annex 1 of the notification) will be able to serve as tool of control for the respective authorities to detect and identify the GM potato lines in the framework of the release experiment intended to be performed by BASF Plant Science between 2006 and 2010.

***DNA Extraction:***

DNA from leaf tissue was extracted using the Wizard Magnetic 96 DNA Plant System (Promega) according to protocol from manufacturer.  
DNA extraction from tubers is generally difficult due to the presence of starch. For extraction of DNA from tubers, the protocol above can be used if the first steps are held cool and a larger volume of lysis buffer is used.

***Real-time PCR :***

**1) Conditions:**

*Real-time PCR ABI 7900 HT*

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

**2) Assays:**

**A) AHAS gene:**

Forward primer: GATCCTCAGGTAAACCAGGTATCTGT  
Reverse primer: ATCGGCTAATCCGCTAACGA  
Probe: CCACTTCAGGTCCCGGA

**B) *Rpi-blb2* gene**

Forward primer: TTCAAACCCCAAATAAGTTTCAAC  
Reverse primer: CCATGCTTGCTGTACTTTGCA

Probe: CGTTACCCAGTCCTTCGGCG

C) Endogenous control (reference):

Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems, Part Number 4310893E)  
(Suitable for qualitative analysis, but not for copy number determinations.)

16. Page 18, No 13, *Genetically modified potato lines carrying the Rpi-blb2 gene were released in the field in Sweden in 2005 (consent B/SE/05/450). During these trials no unforeseen effects as compared to conventional potato varieties have been observed.*

**Please provide the results and any additional information relating to this trial. What if any monitoring studies were carried out?**

Three genetically modified potato lines carrying the Rpi-blb2 gene were released in the field in Sweden in 2005. Throughout the trial the performance of the GM lines in comparison to the non-GM parental potato variety and the variety Bintje with regard to resistance against *Phytophthora infestans* was followed. Growth and development of the GM lines was recorded as normal and comparable to the non-GM comparator lines and no significant differences were observed till the early flowering stage. Upon early flowering the plants became infected with *Phytophthora infestans*. While the non-GM comparator plants suffered substantial defoliation by the disease, the three GM potato lines tested showed the intended phenotype of late blight resistance. Apart from the late blight infection no other pests or diseases were recorded for the duration of the field trial. Monitoring for volunteers as part of the monitoring plan is ongoing.

17. Page 19. under E, No 3, it is stated: *No potatoes will be cultivated in close vicinity (< 20 m) to trial.*

**It should be noted that at least 20 m (if not greater) would be required to limit gene dispersal, primarily, mechanical admixture.**

Revised wording: 'No potatoes will be cultivated in close vicinity (at least or > 20 m) to the trial.'

18. Page 20, No 4, *In addition Phytophthora infestans inoculation might be carried out. Seed potatoes will be planted directly into the field.*

**Provide written confirmation that regulatory approval is not required to release an indigenous fungal pathogen into an Irish soil from the relevant authority in Ireland (Department of Agriculture and Food).**

Revised wording: 'Seed potatoes will be planted directly into the field.'

For the purpose of evaluating the GM potato lines for late blight resistance non-GM potato lines sensitive to *Phytophthora infestans* infection (variety Bintje) will be planted next to the GM potato lines in order to attract and spread the Irish indigenous strains present in the field to the GM lines. No artificial inoculation is planned to be carried out, due to the presence of sufficient *Phytophthora infestans* spores in the Irish agriculture environment and the favourable climatic conditions. The notifier does not intend to import *Phytophthora infestans* strains from outside of Ireland.

19. Page 20, F 1, *additionally, the release is aiming at, in the context of safety studies, collecting necessary data in comparison to unmodified recipient varieties and to conventional potato varieties. Data on agronomic properties and also on environmental interactions will be generated.*

**Specify the safety studies and studies on environmental interactions you propose to carry out, if consent is granted?**

If consent is granted the following studies are proposed to be carried out within the timeframe of 5 years (2006 to 2010) for selected GM potato lines. We would like to point out that the release, if consent is granted, will be a small-scale experimental trial at one location to be conducted in two phases (A) screening of events (proof of concept; duration 1 to 2 years) and (B) development (stability of trait, safety studies; duration 2 to 3 years). During the course of the trial the following will be observed and recorded: Phase (A): agronomic performance (e.g. plant vigour, yield, susceptibility to climatic factors), altered agronomic properties (e.g. disease susceptibility), event screening (susceptibility to *Phytophthora infestans*), plant characteristics (e.g. emergence, leaf shape and colour, flowering, ground coverage, maturation), management of volunteers and Phase (B): stability of expression (e.g. sampling of plant tissue at various developmental stages in order to conduct gene expression studies), potential effects on non-target organisms (e.g. soil flora, potato-related insects), altered qualitative properties (e.g. tuber composition, nutrients, anti-nutrients, feeding studies).

20. Page 21, G. 1 (a), *the year directly following the release the field plot will either remain fallow or will be cultivated with a species that facilitates weed management of the area.*

- **Is one year sufficient for post release monitoring considering tubers and seed can survive in the soil for a number of years?**
- **how do you propose to control any fruit that might occur at the proposed trial site?**

**It should be noted that there would be little or no control of groundkeepers with frost under Irish soil conditions having regard to the fact that Irish winters have been very mild over the last number of years. In view of the above outline in detail an amended proposal for post release monitoring.**

Section G 2. outlines: *The release site will be managed according to conventional agricultural practice. 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 destroyed by herbicide treatment (systemic herbicide e.g. glyphosate) prior to flower setting. The monitoring for volunteers will continue till no volunteers emerge. 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.*

From the above it should be understood that in the case that volunteer plants appear the year after the trial has been carried out, those plants will be destroyed and the monitoring period will be prolonged by another year. In that way the monitoring will continue until no volunteers appear in the respective year.

21. Page 21, G. 2. (b), *any excess potato material (tubers after planting, after harvest) will be inactivated (e.g. via heat or via chopping).*

- **What type of heat will be used to inactivate the tubers?**
- **At what location will heat inactivation of the tubers be carried out?**
- **How will heat inactivated tubers be disposed of?**
- **Are you referring to the inactivation of both GM lines and non-GM controls that you propose to use in the experimental site as well?**

**It should be noted that the chopping of potato material is not considered an appropriate method of inactivation as this could lead to the propagation of tubers. Please clarify.**

Section G. 3. outlines: *Harvested tubers will be transported from the release site to certified contained facilities in and outside Ireland. Any leftover tubers identified on the release area after harvest, will be collected and inactivated e.g. via heat, via chopping. Above ground green parts will be inactivated prior to harvest either chemically or mechanically. Any remaining dead leaves and stems will be left at the release site for decomposition.*

Revised wording: 'Harvested tubers will be transported from the release site to certified contained facilities outside of Ireland.'

All potatoes grown within the release trial will be handled as GM potato lines irrespective of being GM potato lines or comparator lines. Therefore no distinction with regard to handling and destruction is made between GM and non-GM potato lines. Any excess tubers (remainders from planting, after harvest) will be transported from the release site outside of Ireland and steamed or autoclaved at respective facilities approved for the purpose of handling GM material. The inactivated tubers will be placed for decomposition at approved contained facilities.

22. Page 21, G. 3, *Harvested tubers will be transported from the release site to certified contained facilities in and outside Ireland.*

- **Please indicate whether this refers to both GM and non-GM potatoes within the experimental site?**
- **What method(s) will be used to differentiate between the GM and the non-GM lines before planting during planting and at/post harvest?**

**Please note that the storage of the GM lines in a contained facility in Ireland requires a consent from the EPA in accordance with Part III of the GMO (Contained Use) Regulations, S.I. No 73 of 2001.**

Revised wording: 'Harvested tubers will be transported from the release site to certified contained facilities outside of Ireland.' All potatoes grown within the release trial will be handled as GM potato lines irrespective of being GM potato lines or comparator lines. Therefore no distinction with regard to handling and destruction is made between GM and non-GM potato lines.

Any excess tubers (remainders from planting) and also the complete harvest will be transported from the release site, either processed for analytical purposes or steamed/autoclaved at respective contained facilities approved for the purpose of handling GM material outside of Ireland. The inactivated tubers will be placed for decomposition at approved contained facilities.

23. Page 21, *Description of post-release treatment methods for the genetically modified plant material including wastes.*

**What Standard Operating Procedures do you propose to use for the transportation of the GM material on/off site. Provide details.**

A compliance field notebook in use at the trial location will contain instructions, forms and guidance documents for each step of the process from packaging and labelling, to transport, receiving and handling, to planting, harvesting and shipping (including cleaning of equipment) as well as monitoring. All personnel handling GM material will receive compliance training prior to entering the release site. During transport on and off site the genetically modified potatoes are packaged in secure closed containment in order to prevent any spillage or unintentional release. The containment will be clearly labelled as containing GMO. For planting the potatoes are unpacked at the release site. Transport vehicles are inspected and cleaned on site. GM material intended for the release is always to be transported clearly separated from any conventional non-GM potatoes. The transportation is accompanied by shipping documentation including the consent for the release. Harvested potatoes are packaged on site in secure closed containment, labelled and shipped in transport vehicles outside of Ireland again accompanied by the appropriate shipping documentation.

24. Page 22, *under Emergencies Plan.*

**Please note that the ‘ploughing in’ would not be an acceptable method of inactivation as this could lead to propagation of the tubers.**

“Ploughing in” in this context applies to the green parts of the plants. As far as tubers are already build at this moment in time, they would be removed and shipped off for disposal or alternatively remain in the soil and be treated as volunteers the years after.

25. Page 22, No 6, *Methods and procedures to protect the site.*

**Please provide a proposal to provide a more secure fence rather than that proposed in the notification. Electric fences will not adequately protect the proposed field trial from intrusion by animals especially small animals.**

Revised wording: The release site may be fenced (non-electric fence) – if required – to protect against damage by animals.

26. Page 22, *Methods and procedures to protect the site.*

**Please provide a method to protect the tubers (planting, during the growing season, at harvest) from possible bird intrusion (e.g., crows) to**

**reduce the possibility of dissemination of the tubers to neighbouring fields by birds and small mammals.**

At planting as well as at harvesting a number of employees will be present/working at the release site, which will make it unlikely that birds (e.g. crows) will enter the place at this time. After planting seed tubers will be covered with soil and after harvest tubers will be removed from the site in secure closed containment. It is not planned to apply special bird protection measures during the course of the growing season. Up to our knowledge there is no report and no observation from other release trials that potatoes have been disseminated by birds or small animals or constitute a preferred food thereof.

27. Page 26, H (vi), *None of the genes are known to exert any toxic or allergenic effects to human health.*

Also page 30, ERA, it is stated that *the potential effect on human or animal health due to introduced R-genes is negligible. NBS-LRR genes not known to confer toxic or allergenic properties and that the overall impact is negligible. Provide data to support these statements and explain how you concluded in your determination of the risk of the GMOs (in the ERA) that the overall impacts are negligible, if you cannot support these statements with relevant data/studies? See also question 10 above.*

The Rpi-blb1 and Rpi-blb2 protein sequences were compared *in silico* to all proteins in a downloaded Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (version 6.00) via a BlastP (version 2.2.6) analysis. None of the submitted proteins showed 35% or greater identity over 80 amino acids to a known allergen. Additionally, no submitted protein shared a sequence of eight consecutive identical amino acids with a known allergen.

The Rpi-blb1 and Rpi-blb2 proteins were also analyzed for sequence homology to known toxins via a BlastP search of the downloaded June 2005 GenBank non-redundant peptide sequence database. This BlastP analysis did not identify any known toxins with significant homology to the submitted proteins.

As NBS-LRR proteins in general are not known to be toxic or allergenic and as none of our introduced R-proteins shows any homology to known allergens or toxins we concluded in the e.r.a. that the overall impact to human or animal health is negligible.

28. Page 26, H (vii), *Measures taken under current release practice will protect the trial against damage by wild animals (e.g. fences) and also ensure that seed stock and plant material are harvested, stored, transported or disposed of (e.g. cleaning of machinery, packaging) in such a way to minimise contact to animals. Therefore the overall impact on animal health is negligible.*

**Describe precisely these measures.**

The following measures will be taken: Transport to and from the site of release will be in secure close containment in order to prevent any unintentional release and thereby exposure to animals. Any equipment used

for transport, planting and harvesting will be inspected and cleaned on site after use. Any remaining tuber material will be destructed via heat. The release site may be fenced if required. Transport of GM material will always be clearly separated from non-GM conventional potatoes in order to prevent any mixing. The material will be packaged and labelled appropriately in order to prevent it from entering the food or feed chain.

29. On page 27, *The overall impact on biogeochemical processes is negligible. Provide data to support this statement?*

Effects on biogeochemical processes have not been measured previously with the genetically modified late blight resistant potato lines intended for the release. Therefore the small-scale field trial, if consent is granted, will provide the unique opportunity to verify the trait of increased late blight resistance conferred to the GM potatoes under Irish agricultural conditions and to perform studies that can reveal any potential effects on biogeochemical processes via measuring parameters such as e.g. soil fertility.

30. Page 27, *Therefore the overall impact on the environment is negligible and comparable to the effect of the cultivation of non-genetically modified potatoes with a potentially positive impact on soil microflora.*

**Please indicate if these parameters have been measured previously and the results thereof.**

Environmental impacts due to specific cultivation and management practices have not been measured previously, since it will be for the first time that the genetically modified potato lines can be tested under field conditions in Ireland. Therefore the small-scale experimental field trial, if consent is granted, will provide the unique opportunity to verify the trait of increased resistance to late blight under Irish agricultural conditions and to perform studies that explore potential environmental effects. Only under those conditions will it be possible to determine if specific cultivation and management practices need to be applied and if so, what the potential effects might be on the soil and its flora.

31. In the ERA part of the notification you are requested to comment on the following potential effect:

**Please comment on whether the introduction of your GM blight resistant potatoes might alter the pathogenicity of the potato blight fungus under Irish soil conditions thus facilitating the dissemination of infectious diseases and/or creating new reservoirs or vectors.**

The mechanism of response conferred to the genetically modified potatoes by the R-genes is one of hypersensitivity and very specific to the interaction of the host and the pathogen. R-genes encode receptors that will recognize specific elicitors injected by the pathogen into the plant cell. This recognition will through a signalling network trigger both local and systemic defense responses. The local response aims at trapping the pathogen in the cells by localized cell death thus stopping further penetration and spreading.

Further the durability of the conferred resistance and therefore the lack of the pathogen to alter its pathogenicity is also supported by a large body of evidence. Schilde-Rentschler et al. (2002) produced somatic hybrids between *S. tuberosum* and *S. bulbocastanum*. The hybrid material containing the *blb1* gene has been tested in several countries (Germany, Spain, Bolivia, Columbia, Costa Rica and Ecuador). Furthermore, Helgeson et al. (1998) reported the production of somatic hybrids containing *blb1*. This material was tested at Hancock, Wisconsin and in Mexico for several years, whereas the progeny showed remarkable high levels of late blight resistance. Breeding clones containing *blb2* have shown high levels of resistance in experimental organically grown fields in the Netherlands from 1999 to 2002 (not published). The basic material from which those breeding clones were derived has been tested already in the eighties for resistance in Mexico (Hermsen, 1983) and proven highly resistant.

In addition to the evidence for the durability of the resistance, the genetically modified late-blight resistant potatoes are intended to be released into the environment within the frame of a small experimental field trial. Due to the scale (size, timing) of the trial as well as the presence of conventional potato varieties with a susceptibility to *P. infestans* the selection pressure exerted onto the oomycete will be negligible and the likelihood of a change in its pathogenicity is considered negligible.

32. **Please confirm if the potato donor of the R-genes (*S. bulbocastanum*) is a table potato consumed in some countries or is it a wild potato species not consumed by any humans or animals?**

*S. bulbocastanum* (Ornamental nightshade) is a wild potato species and based on our current knowledge generally not consumed by humans or animals.

## References

- Ballvora A., Ercolano M.R., Weiss J., Meksem K., Bormann C.A., Oberhagemann P., Salamini F. and Bebbhardt, C. (2002).** The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. - *Plant J.* 30:361-371.
- Grant M.R., Godiard L., Straube F., Ashfield T., Lewald J., Sattler A., Innes R.W. and Dangl J.L. (1995).** Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. - *Science* 269:843-846
- Helgeson, J.P., J.D. Pohlman, S. Austin, G.T. Haberlach, S.M. Wielgus, D. Ronis, L. Zambolim, P.Tooley, J.M. McGrath, R.V. James, and W.R. Stevenson (1998).** Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. *Theor. Appl. Genet.* 96, 738-742.
- Hermesen, J.G. (1983).** Report of the XXVI Planning conference. Dec. 12-14, 1983. International Potato Center, Lima (CIP), Peru, pp 115-132.)
- Huang S., van der Vossen E.A.G., Kuang H., Vleeshouwers V.G.A.A., Zhang N., Borm T.J.A., van Eck H.J., Baker B., Jacobsen E. and Visser R.G.F. (2005).** Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. *Plant J.* 42:251-261.
- Ingham D.J., Beer S., Money S. and Hansen G. (2001).** Quantitative real-time PCR assay for determining transgene copy number in transformed plants. - *BioTechniques* 31:132-140
- Meyers B.C., Vu T.H., Tej S.S., Ghazal H., Matvienko M., Agrawal V., Ning J. and Haudenschild C.D. (2004).** Analysis of the transcriptional complexity of *Arabidopsis thaliana* by massively parallel signature sequencing. *Nature Biotechnology* 8:1006-1011.
- Michelmore R. (2000).** Genomics approaches to plant disease resistance. - *Curr. Opin. Plant Biol.* 3:125-131.
- Schilde-Rentschler et al. (2002).** EAPR Conference on Potato Research, Hamburg, Germany 2002, paper 102.
- Van der Vossen, E., Sikkema, A., te Lintel Hekkert, B., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W. and Allefs, S. (2003).** An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. - *Plant J.* 36:867-882
- van der Vossen E.A., Gros J., Sikkema A., Muskens M., Wouters D., Wolters P., Pereira A. and Allefs S. (2005).** The *Rpi-blb2* gene from *Solanum bulbocastanum* is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato.- *Plant J.* 44:208-222

**Wastie, R.L. (1991).** Breeding for resistance. - *Advances in Plant Pathology* 7: 193-224

## Tom McLoughlin

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**From:** christine.wandelt@basf.com  
**Sent:** 22 March 2006 07:31  
**To:** Tom McLoughlin  
**Subject:** Response to request B/IE/06/01

**Attachments:** BPS RESPONSE to EPA request 22-03-06.doc



BPS RESPONSE  
EPA request 22

Dear Dr. McLoughlin,

please find attached our response to the request by EPA raised on 13th March 2006.

Please do not hesitate to contact me should there be additional questions.

With best regards,  
Christine Wandelt

(See attached file: BPS RESPONSE to EPA request 22-03-06.doc)

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**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  $f'f'$ 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 Irelands 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.

Assumptions of risk assessment	Observations performed by notifier
<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 °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  $\mu$ l into 384-Well Optical Reaction Plate (4326270, Applied Biosystems).
- c) Dilute the DNA 2 times and add 2  $\mu$ l to each well. Add 2  $\mu$ l 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: CCACTTCAGGTCCCAGGA

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.