Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3136865 A1 2020/10/22

(21) 3 136 865

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2020/04/06
(87) Date publication PCT/PCT Publication Date: 2020/10/22
(85) Entrée phase nationale/National Entry: 2021/10/14
(86) N° demande PCT/PCT Application No.: EP 2020/059807
(87) N° publication PCT/PCT Publication No.: 2020/212190
(30) Priorité/Priority: 2019/04/16 (EP19169555.0)

(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01), *C12Q 1/6895* (2018.01)

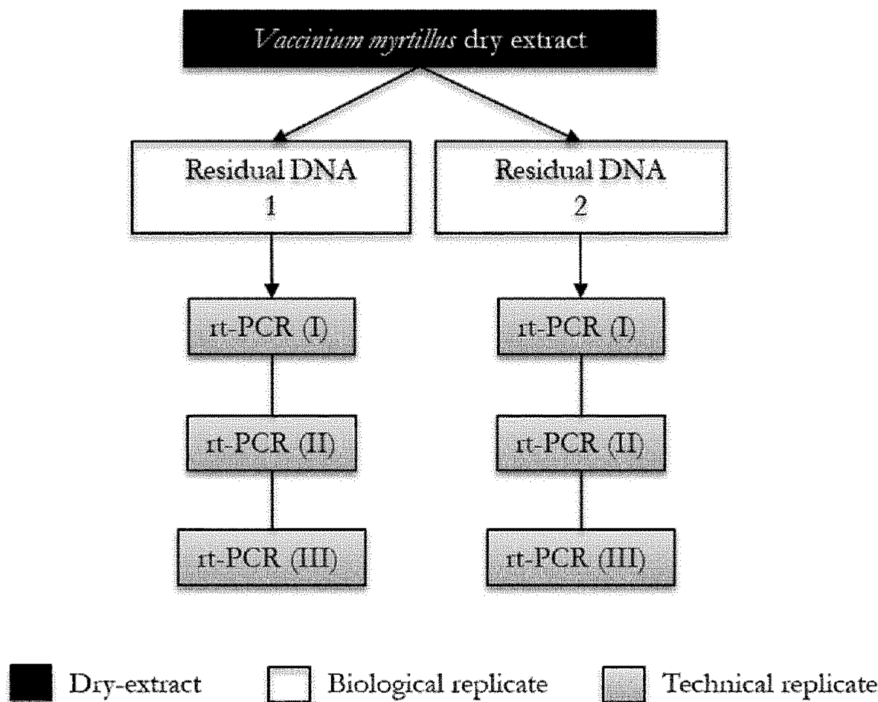
(71) Demandeur/Applicant:
INDENA S.P.A., IT

(72) Inventeurs/Inventors:
LONGO, VALERIA, IT;
BERLANDA, DAVIDE, IT

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : PROCEDE ET KIT D'IDENTIFICATION DE VACCINIUM MYRTILLUS
(54) Title: METHOD AND KIT FOR THE IDENTIFICATION OF VACCINIUM MYRTILLUS

Figure 7



(57) Abrégé/Abstract:
The invention provides a method for the identification of Vaccinium myrtillus in a botanical composition and a kit specifically designed for its implementation. The method of invention is based on the detection, using PCR amplification, of nucleic acid fragments within a genomic region of Vaccinium myrtillus, namely the genomic region within the internal transcribed spacer 1, 5.8S ribosomal RNA genomic region and the internal transcribed spacer 2.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 October 2020 (22.10.2020)

(10) International Publication Number
WO 2020/212190 A1

(51) International Patent Classification:
C12N 15/82 (2006.01) *C12Q 1/6895* (2018.01)

(21) International Application Number:
PCT/EP2020/059807

(22) International Filing Date:
06 April 2020 (06.04.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
19169555.0 16 April 2019 (16.04.2019) EP

(71) Applicant: INDENA S.P.A. [IT/IT]; Viale Ortles, 12, 20139 MILANO (IT).

(72) Inventors: LONGO, Valeria; c/o Indena S.p.A., Viale Ortles, 12, 20139 MILANO (IT). BERLANDA, Davide; c/o Indena S.p.A., Viale Ortles, 12, 20139 MILANO (IT).

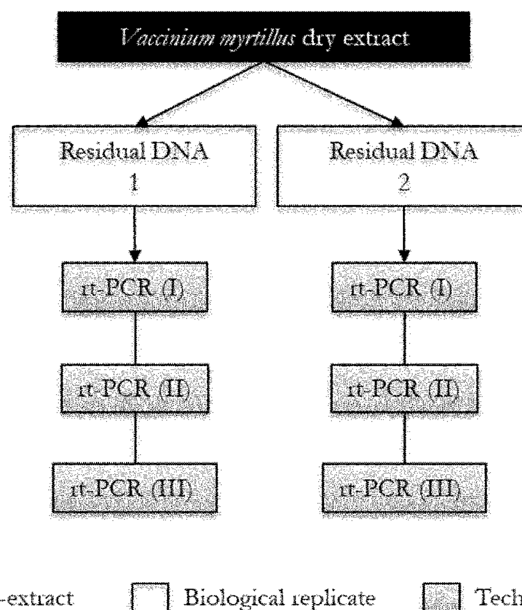
(74) Agent: BANFI, Paolo; c/o Bianchetti Bracco MInoja S.r.l., Via Plinio, 63, 20129 MILANO (IT).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: METHOD AND KIT FOR THE IDENTIFICATION OF VACCINIUM MYRTILLUS

Figure 7



(57) Abstract: The invention provides a method for the identification of *Vaccinium myrtillus* in a botanical composition and a kit specifically designed for its implementation. The method of invention is based on the detection, using PCR amplification, of nucleic acid fragments within a genomic region of *Vaccinium myrtillus*, namely the genomic region within the internal transcribed spacer 1, 5.8S ribosomal RNA genomic region and the internal transcribed spacer 2.

WO 2020/212190 A1

[Continued on next page]

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

METHOD AND KIT FOR THE IDENTIFICATION OF *VACCINIUM MYRTILLUS*

The present invention provides a method for the identification of *Vaccinium myrtillus* in a botanical composition, which is based on the detection of specific genomic fragments using PCR amplification. The invention further provides a kit specifically designed for implementing the method of invention.

Background of the invention

Plant extracts are widespreadly used in the medical, nutraceutical, cosmetic and food industry. One of the main issues encountered when dealing with plant extracts is that of determining not only their chemical composition, but also their botanical origin, in order to exclude the risk of counterfeit.

Genetic-based methods for determining the botanical origin of plant materials are known in the art (Parker, J., et al., *Field-based species identification of closely-related plants using real-time nanopore sequencing.* Sci Rep, 2017. **7**(1): p. 8345; Group, C.P.W., *A DNA barcode for land plants.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12794-7; Fazekas, A.J., et al., *DNA barcoding methods for land plants.* Methods Mol Biol, 2012. **858**: p. 223). Such methods are based on the comparison of the DNA present in the plant material with known DNA sequences present in publicly available databases. For example, WO 2006/020147 (The Regents of the University of California) discloses a method for identifying individual biological genetic components present in a botanical mixture, said method being based on a combination of genomic-locus specific PCR, single strand conformation polymorphyspm (SSCP), and sequence analysis. The method is said to be able to provide information on the biologic components of the composition without requiring prior knowledge as to which botanicals may be present and to detect and identify unknown biologic components that may be present in the mixture.

Methods for the genetic identification of plants from botanical samples are also disclosed in CN102146477, CN106119394, CN1372005, CN107142329, CN107653330, CN105624291, CN105603107, ES2176066, CN104673930, CN102222969, CN102732513, CN105063203,

JP2007282626. In some cases, the methods for the identification of botanical species are based on the detection, by PCR amplification, of specific sequences located within the nuclear ribosomal RNA-encoding locus containing the internal transcribed spacer (ITS) regions ITS-1 and/or ITS-2. In certain cases (CN1052429, CN105603107, ES2176066), the methods are aimed at identifying adulterations in commercialised products containing plant materials.

Jaakola L et al., Food Chemistry vol. 123, no. 2 (2010) pp. 494-500, discloses the identification of commercially important berry species by means of a combined approach of DNA barcoding and HRM (High Resolution Melting) analysis, using designed primer pairs which enable the species-specific identification of wild berries. *Vaccinium myrtillus* is identified through HRM analysis of an amplicon located in the ITS (Internal Transcribed Spacer) region obtained with primers ITSVm2f and ITSVm2r.

CN108642207 discloses the construction of an allelic map of the bilberry plant, and a method for the identification of blueberry varieties and related species using primer-specific PCR-amplification.

Marieschi M. et al, Food Chemistry vol. 202 (2016) pp. 438-444 discloses a method based on Sequence Characterized Amplified Regions (SCARs) to detect the presence of V. myrtillus and adulterating species useful for multiple batches analysis.

Koskima Ki JJ et al., European Journal of Plant Pathology, Kluwer Academic Publishers - vol. 125 no. 4 (2009) pp. 629-640 discloses the relative expression of bilberry genes quantified by Real-Time PCR with SYBR-green as the fluorescent reporter.

When plant materials are processed and, in particular, when they are subjected to extraction procedures, the DNA degrades giving rise to fragments of variable size and amount according to the extraction method and which cannot be directly compared with known DNA sequences, thereby making it difficult, if not practically impossible, to apply to extracts the genomic identification methods that can be applied on the starting materials.

Vaccinium myrtillus extracts are largely used in pharmaceutical, cosmetic, nutraceutical and dietary products due to their known health-beneficial properties. The clinical benefits of *V.*

myrtillus as both a dietary supplement and a therapeutic have been attributed to the presence of abundant amounts of flavonoids and anthocyanins. For extract manufacturers, it is important to guarantee that *V. myrtillus* extracts have the required specifications in terms of chemical components and the declared pure botanical origin. It would therefore be desirable to provide a method that allows to identify *V. myrtillus* in a botanical composition, e.g. in a plant extract, securing high levels of accuracy and species-specificity particularly when *V. myrtillus* is in admixture with closely related contaminant species.

Description of invention

These objectives are achieved by the present invention, which provides a method for the specific and accurate identification of *Vaccinium myrtillus* in a botanical composition through detection of a nucleic acid fragment which is contained in the residual DNA of *V. myrtillus* extracts.

Specifically, the method of invention comprises detecting, in a sample of botanical composition, a *V. myrtillus*-specific nucleic acid fragment located within the internal transcribed spacer 1, 5.8S ribosomal RNA gene and the internal transcribed spacer 2, wherein said nucleic acid fragment consists of either SEQ ID NO:1 or a sequence comprising SEQ ID NO:1 which is selected from the group of SEQ ID NOs:2, 3 and 4.

In a preferred embodiment, the primers used for PCR-amplification are selected from the following pairs:

(i) SEQ ID NO:5 and SEQ ID NO:6;

(ii) SEQ ID NO:7 and SEQ ID NO:8;

(iii) SEQ ID NO:9 and SEQ ID NO:10;

(iv) SEQ ID NO:11 and SEQ ID NO:12;

In a particularly preferred embodiment, the PCR is a real-time PCR (rtPCR) and the method of invention comprises the following steps:

(a) isolating nucleic acids from a sample of botanical composition;

(b) conducting a rt-PCR on the isolated nucleic acids, using:

- a pair of primers selected from the group consisting of:

(i) SEQ ID NO:5 and SEQ ID NO:6;

(ii) SEQ ID NO:7 and SEQ ID NO:8;

(iii) SEQ ID NO:9 and SEQ ID NO:10;

(iv) SEQ ID NO:11 and SEQ ID NO:12;

and

- a probe annealing within the nucleic acid region amplified by the primers, said probe having sequence SEQ ID NO:13;

(c) determining the presence of the amplification product,

whereby detection of the amplification product is indicative of the presence of *Vaccinium myrtillus* in the botanical composition.

According to the invention, the botanical composition is a mixture of plants or parts thereof, e.g. leaves, fruits, bark, roots, including plant extracts and particularly fruit extracts, which are intended for consumption or therapeutic use. In a preferred embodiment, the botanical composition is a product containing an extract of fruits of *Vaccinium myrtillus*, alone or in combination with related species such as *Empetrum nigrum*, *Sambucus nigra*, *Vaccinium oxycoccos*, *Vaccinium corymbosum* and *Vaccinium macrocarpon*.

The isolation of nucleic acids involves their separation and purification from other components of the plant mixture or extract and it can be conducted with conventional techniques using commercially available kits. In particular, the genomic DNA may be isolated using extraction-precipitation protocols, silica-membrane- or anion-exchange-based procedures.

Real-time PCR technology is known in the art and it combines the polymerase chain reaction chemistry with the use of fluorescent reporter molecules in order to monitor the production of amplification products during each cycle of the PCR reaction. The amplification of the target DNA is obtained by repeated cycles of denaturation followed by primer- and probe-annealing and by DNA polymerase-catalyzed primer extension. DNA amplification is

monitored at each cycle of PCR by measuring a fluorescent signal which is produced for instance by non-specific fluorescent dyes that intercalate with double-stranded DNA or by sequence specific DNA probes consisting of oligonucleotides labelled with a fluorescent reporter which allows for detection after probe hybridization with its complementary DNA target. Suitable intercalating dyes include SYBR® (Green I, Green II, Gold), LCGreen®, SYTO-(9, 13, 16, 60, 62, 64, 82), BOBO-3, LCGreen®, POPO-3, BEBO, TO-PRO3, PicoGreen®, SYTOX Orange and similar commercially available fluorescent dyes (fluorophores).

The oligonucleotide probe is labeled with a fluorescent reporter (fluorophore) at one end and a quencher of fluorescence at the opposite end of the probe. The 5' exonuclease activity of the polymerase cleaves the probe releasing the reporter molecule resulting in an increase of the fluorescence intensity. Examples of fluorophores include 5- or 6-carboxyfluorescein (5- or 6-FAM), tetrachlorofluorescein (TET), hexachloro-6-carboxyfluorescein (HEX), 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein succinimidyl ester (JOE), tetramethylrhodamine (TAMRA), 5-carboxytetramethylrhodamine (TAMRASE), carboxy-X-rhodamine (ROX), 4-(dimethylaminoazo)benzene-4-carboxylic acid (DABCYL). Examples of quenchers include those of the BHQ (Black Hole Quencher®) family, NFQ-MGB (non-fluorescent quencher and minor groove binder), QSY 7 or 21 carboxylic acid succinimidyl ester.

The parameters and conditions of the rtPCR, such as the temperature and the length of each cycle of denaturation and annealing, can be adjusted depending on the nucleic acid fragment to be amplified, on the set of primers used in the amplification and on other variables, as known to anyone skilled in the art. In a preferred embodiment of the invention, the nucleic acid fragments herein disclosed are amplified with primers (i) through (iv) applying the following conditions:

- initial denaturation step at 95°C for 180 sec
- 2-step cycles of 15 sec at 95°C (1st step) and 15 sec at 62-68.5°C (2nd step) repeated

forty (40) to fifty (50) times.

The specific combinations of primers and probe according to the invention allows for the specific identification of *Vaccinium myrtillus* in botanical compositions containing closely related species such as *Empetrum nigrum*, *Sambucus nigra*, *Vaccinium oxycoccos*, *Vaccinium corymbosum* and *Vaccinium macrocarpon*. As reported in the experimental section, the use of a probe different from the *Vaccinium myrtillus*-specific probe SEQ ID NO:13 and likewise annealing with fragments SEQ ID NOs:1-4, abolishes the system ability to identify *Vaccinium myrtillus* in admixture with *Empetrum* using the same primers and rt-PCR conditions disclosed above. This denotes the specificity of the selected combination of primers, probe and the effectiveness of the rtPCR conditions according to the invention.

Another aspect of the invention regards a kit for the identification of *Vaccinium myrtillus* in a botanical composition. The kit of invention comprises at least one pair of primers selected from (i) through (iv) and the probe as above defined. In addition the kit may comprise, in separate containers, reagents needed for running the (rt)PCR, particularly the deoxynucleotides and the DNA polymerase, and reagents for isolating, purifying and optionally quantifying DNA. The kit may also contain DNA of *Vaccinium myrtillus* as positive control and nuclease-free water or buffer as negative control, as well as a leaflet with the instructions for performing the PCR assay.

In a preferred embodiment of invention the kit contains:

- one tube or vial containing all reagents necessary to perform the analysis (DNA Polymerase, dNTPs, Buffer, Probe chemistry, Primers and Probe)
- one tube or vial containing the positive control (DNA of *Vaccinium myrtillus*)
- one tube or vial containing the negative control DNA (Nuclease-free water)

The kit can be used with all commercially available Real-time PCR System.

Description of the figures

Figure 1: rt-PCR amplification protocol.

Figure 2: rt-PCR amplification results (a) and melt curve analysis (b) of genomic DNA

isolated from *Vaccinium myrtillus* frozen fruit.

Figure 3: Standard curve analysis for *Vaccinium myrtillus.*

Figure 4: Probe-based rt-PCR amplification with M-FAM probe specific for *V. myrtillus* NTC: negative control.

Figure 5: (a) Probe-based rt-PCR amplification with M-FAM probe specific for *V. myrtillus* in a mixed samples with a ratio reported in the legend. NTC: negative control; (b) Probe-based rt-PCR amplification with E-HEX probe specific for *E. nigrum* in a mixed samples with a ratio reported in the legend. NTC: negative control.

Figure 6: Correlation between Cq Mean and percentage of target species for *V. myrtillus*.

Figure 7: Experimental scheme used for rt-PCR analysis of dry extract samples.

Figure 8: rt-PCR amplification of residual DNAs isolated from *Vaccinium myrtillus* dry-extract samples. The positive control is the gDNA extracted from *Vaccinium myrtillus* frozen fruit. a) Primer set L; b) Primer set S.

Figure 9: Agarose gel analysis of rt-PCR amplicons.

Figure 10: Alignment analysis of sequenced amplicons (top) and relative sequence identity matrix (bottom).

Figure 11: rt-PCR of *Vaccinium myrtillus* E. ET. 36%. Amplification and melt curves.

Figure 12: rt-PCR of *Vaccinium myrtillus* E. ET. 36% with probe-based method. PTC: positive control (gDNA extracted from frozen fruit of *V. myrtillus*); NTC: negative control.

EXPERIMENTAL SECTION - general procedures

Extraction of genomic DNA (gDNA)

The DNA extraction was performed by using the NucleoSpin® plant II protocol as described by the supplier (Macherey nagel. Cat. 740770.250 - July 2014/Rev.09).

Purification of residual DNA from the dry extract.

The first purification was done by using the kit NucleoSpin® plant II Maxi protocol as described by the supplier (Macherey nagel. Cat. 740770.250 - July 2014/Rev.09), with some

modification reported below.

- Weigh 3-5 g of dry extract in a 50 ml conical tube
- Add 3 ml of distilled water
- Add 9 ml of lysis buffer
- Vortex for 30 sec
- Transfer the sample to a NucleoSpin® Filter Maxi
- Centrifuge 5 min at 4500 x g, collect the clear flow-through and discard the NucleoSpin Filter Maxi
- Add 20 ml binding buffer
- Vortex for 30 sec.
- Load sample on a NucleoSpin® Plant II Maxi Column
- Centrifuge for 3 min at 4500 x g and discard the flow-through.
- Repeat the loading for all the resting sample
- Add 4 ml wash buffer (PW1) to the NucleoSpin® Plant II Maxi Column
- Centrifuge for 3 min at 4500 x g and discard the flow-through.
- Add 10 ml wash buffer (PW2) to the NucleoSpin® Plant II Maxi Column
- Centrifuge for 3 min at 4500 x g and discard the flow-through.
- Add 2 ml wash buffer (PW2) to the NucleoSpin® Plant II Maxi Column
- Centrifuge for 12 min at 4500 x g and discard the flow-through.
- Place the NucleoSpin® Plant II Maxi Column into a new collection tube (50 ml)
- Pipette 1000 µl elution buffer (PE) (65°C) onto the membrane.
- Incubate the NucleoSpin® Plant II Maxi Column for 5 min at 65°C
- Centrifuge for 3 min at 4500 x g to elute the DNA

The second purification was done by using the kit ReliaPrep™ DNA Clean-UP and Concentration System protocol as described by the supplier (Promega. Cat. A2893).

Quantification of DNA

The DNA was quantified through the NanoQuant Plate™ instrument. The quantification

was performed by using the UV-method. The 260 nm absorbance was used to quantify the DNA as 1 OD at 260 nm correspond to 50 µg/µl of DNA. The 260 nm/280 nm absorbance ratio was determined for the assessment of DNA purity.

rt-PCR and Melt curve analysis

The rt-PCR amplification was performed by using the SYBR Green or probe based chemistry as described by the supplier (SsoAdvanced™ Universal SYBR® Green Supermix, BioRad Cat. N. 1725272; SsoAdvanced™ Universal Probes Supermix, BioRad Cat. N. 1725281), with 3-step based amplification protocol, as reported in **Figure 1**.

Real-time PCR

Prepare the mix as follos, final volume 20 µl:

Probe Mastermix (BioRad or equivalent) 2X	10 µl
Primer F 10 µM	0.5 µl
Primer R 10 µM	0.5 µl
Probe M-FAM 10 µM	0.5 µl
DNA 0.5 – 30 ng/ µl	2 µl
Nuclease-free water	6.5 µl

Load the sample in a real-time instrument (BioRad or equivalent) and set the following method:

<table>
<tr><td>95°C</td><td colspan="2">180 sec</td></tr>
<tr><td>95°C</td><td>15 sec</td><td rowspan="2">50 X</td></tr>
<tr><td>62 C - 68.5°C</td><td>15 sec</td></tr>
</table>

Acquisition after the second step of cycling.

DNA sequencing

The amplified DNA was purified on agarose gel and the purified fragment was sequenced through the generation of two sequences for each sample: one is generated by using forward primer and the other one by using reverse primer. Each sequencing tube was prepared

by mixing the purified DNA and TRIS-HCl 5 mM pH 8.0 in order to obtain the concentration requested for the sequencing (depending on the length of the sequence, 2-5 ng/µL).

The sequences were analysed by using BioEdit or BLAST software in order to compare and identify the sequences.

EXAMPLES

Example 1 - Method validation

The gDNA was purified and quantified (**Table 1**) for the *Vaccinium myrtillus* frozen fruit and its contaminant/related species hereafter reported:

Table 1. Quantification of gDNA extracted for all species tested in the present report.

	Ref. QdL	DNA (ng/µl)	Ratio (260/280)
Vaccinium myrtillus	1072/10/12	7.9	1.61
Empetrum nigrum	1072/10/6	29	2.07
Sambucus nigra	1072/10/4	12	2
Vaccinium macrocarpon	1072/10/7	3.4	2.83
Vaccinium oxycoccos	1072/10/1	6.9	1.25
Vaccinium corymbosum	1072/10/3	10.2	1.23

The set-up of rt-PCR reaction parameters, in terms of Cq (quantification cycle) and Tm (melt temperature) peak, were initially evaluated by using the gDNA extracted from *Vaccinium myrtillus* frozen fruit (Figure 2). The rt-PCR results showed that the designed primers allow the amplification of a single DNA region for all primer set (Tables 2 and 3).

Table 2

Region	Name	Sequence (5'→3')	Tm (°C)	Amplicon (bp)
Large sequence (L)	Vac-ex_LF	CCATCGAGTCTTTGAACGCA	57.3	275
	Vac-ex_LR	CACTTCAGGGTCAAATGGGC	59.4	
Small sequence (S)	Vac-ex_sF	GCATTGCGTCACCCACTC	58.2	131
	Vac-ex_sR	ACTTGTCGTTGAATGTCCGTCA	57.3	
Large sequence 2 (L2)	Frw_Large 2	TTGCAGAATCCCGTGAACCA	57.3	230
	Rev_Small 2	TTTAGCAACCACCACTTGTCGT	58.4	
Small sequence 2 (S2)	Frw_Small 2	TGAAGGCACGTCTGCCTG	58.2	162
	Rev_Small 2	TTTAGCAACCACCACTTGTCGT	58.4	

Table 3

Primer set	Sample	Cq	Cq Mean	Cq Std. Dev
L2	1072/10/12	25.61	25.37	0.212
		25.28		
		25.21		
S2	1072/10/12	26.17	25.89	0.247
		25.70		
		25.80		
L	1072/10/12	24.88	25.11	0.203
		25.20		
		25.25		

S	1072/10/12	25.97	26.15	0.166
		26.19		
		26.29		

The rt-PCR was also performed with DNA isolated from *V. myrtillus* contaminant/related species and the results showed that it is possible to distinguish the different DNA by using the primer sets and particularly the small 2 primers (Table 4).

Table 4 - melt curve peak results

Sample	Large2	Small2	Large	Small
V. myrtillus	90.50	90.50	90.50	89.50
E. nigrum	88.50	88.00	90.50	89.00/89.50
S. nigra	88.50	87.50	89.50	87.50
V. oxycoccos	91.00	91.00	91.00	89.50/90.00
V. corymbosum	89.00	89.00	89.00	88.00
V. macrocarpon	91.50	91.50	91.00	90.50

The linearity of the amplification curve was also evaluated with the standard curve generation for *Vaccinium myrtillus* by using the small 2 primer set (**Figure 3**). It is possible to see that the linearity has been ensured in the tested range of concentration (almost 0.0625 - 8.00 ng/µl).

In order to improve the method capability to distinguish between *Vaccinium myrtillus* and contaminant/related species, the rtPCR was conducted with the Minor Groove Binding-Probe (M-FAM -SEQ ID NO:13) specifically designed to enable the amplification of V. myrtillus sequences.

In a comparative experiment, the rtPCR was conducted with simultaneous use of the Minor Groove Binding-Probes SEQ ID NO:13 (M-FAM) and SEQ ID NO:14 (E-HEX).

To test the probe-based method different subsets of experiments have been carried out, summarized in the table below.

Table 5

Probe	Sample	Expected result	Result
M-FAM	*V. myrtillus*	Amplification of *V. myrtillus*	Figure 4
	E. nigrum		
	S. nigra		
	V. oxycoccos		
	V. corymbosum		
	V. macrocarpon		
M-FAM	*V. myrtillus* 100%	No Amplification	Figure 5(a)
	E. nigrum 100%	Amplification	
	V. myrtillus 95% *E. nigrum* 5%	Proportional amplification with the percentage of *V. myrtillus*	
	V. myrtillus 98% *E. nigrum* 2%		
	V. myrtillus 99% *E. nigrum* 1%		
E- HEX	*V. myrtillus* 100%	No Amplification	Figure 5(b)
	E. nigrum 100%	Amplification	
	V. myrtillus 95% *E. nigrum* 5%	Proportional amplification with the percentage of *E. nigrum*	
	V. myrtillus 98% *E. nigrum* 2%		
	V. myrtillus 99% *E. nigrum* 1%		

The amplification results were proportional to the content of the target species_(**Figure 6**).

Example 2 – *Vaccinium myrtillus* dry-extract residual DNA identification

For each sample, two independent isolations of residual DNA were performed (biological replicates) and for each extracted DNA three technical replicates were tested, **Figure 7**.

The whole procedure was initially performed on four samples: 32549/H76, 32549/H80, 32549/H83, 32549/H84. After the residual DNA isolation and quantification (**Table 6**), these

samples were analysed for their rt-PCR amplification characteristics (Cq and Tm) compared with that of positive control (**Figure 8 and Table** 7).

Table 6 - Residual DNA quantification

Sample	Biological replicate	DNA (ng / µL)	Ratio (260 / 280)
32549/H76	1	0	3.5
	2	1	1.25
32549/H80	1	0	0
	2	1.1	0.55
32549/H83	1	0.7	1.4
	2	0	2
32549/H84	1	10.3	1.41
	2	10.3	1.38

Table 7 - rt-PCR Summary results

Sample	DNA (ng/µL)	Cq. Mean	Cq. Std. Dev	Melt Temp
32549/H76_2	1.0	32.05	0.180	89.50
32549/H83_1	0.7	35.26	0.181	89.00
32549/H84_1	10.3	28.96	0.119	89.50
32549/H84_2	10.6	28.44	0.092	89.50
Negative ctrl	0.0	None	None	None
Positive ctrl	24.8	23.60	0.040	89.50

The results of rt-PCR amplification with all samples showed that:

- the DNA was amplified for the positive control as well as for all tested samples;

- the negative control (no DNA) showed no amplification signal;
- positive control and samples showed equal values for Tm peaks.

This result indicates that the amplicons have the same characteristics in terms of length and/or nucleotide bases composition.

Moreover the Cq results are correlated with the DNA amount tested, meaning that the amplification is specific for the selected target.

To verify if the generated amplicons have the same sequence of the positive control, all amplified sequences were purified on agarose gel (**Figure 9**) and the purified fragments were sequenced (**Figure 10**).

The agarose gel analysis confirmed the differences of the amplicons length: the fragment generated with primer set S shows a length of about 130 bp, while the fragment generated with primer set L shows a length of about 270 bp. Moreover, from gel agarose analysis it is possible to see also the presence of unspecific rt-PCR products, as in **Figure 9,** lane 4 for the sample 32549/H83_1 where two bands are visible, in good accord with Tm peak results (**Figure 8, b**).

All generated sequences were aligned by considering only the portion with high quality sequencing parameters. The sequencing results (**Figure 10**) showed that all amplicon sequences (small and large) are identical to the sequence of the *Vaccinium myrtillus* standard reference.

Example 3 - *Vaccinium myrtillus* 36% dry ethanolic extract (E. ET.) residual DNA identification

The residual DNA analysis was also performed on samples with Indena code 9042202, MIRTILLO (V. MYRTILLUS) E. ET. 36% after the dry-powder mixing phase, 32788/M1, 32786/M2, 32788/M2. The previous samples 32549/H76, 32549/H80 and 32549/H83 were tested again as control samples.

In order to optimize the purification procedure, after the first step of DNA purification the isolated residual DNA was processed with ReliaPrep™ Kit (Promega). The results in terms of DNA quantity (ng/ µL) and quality (260/280 ratio) on the two purification steps (**Table 8**) revealed that the concentration as well as the purification are better introducing the second step.

Table 8 - Residual DNA quantification - E. ET. 36%

Sample	I purification		II purification	
	DNA (ng/µL)	**Ratio (260/280)**	**DNA (ng/µL)**	**Ratio (260/280)**
32549/H76	0	-	2.3	1.77
32549/H80	0	2	0.2	0.5
32549/H83	0	0.84	2.1	1.75
32788/M1	1.2	0.71	3.4	1.55
32786/M2	0	-	2.5	1.67
32788/M2	0.3	0.75	3.6	1.5

The rt-PCR analysis was performed by using SYBR green (**Figure 11**) and probe-based methods (**Figure 12**), according to the protocols described above.

The results indicate that:

(a) all tested samples showed the same Tm peak of the positive control (**Figure 11** and **Table 9**) when analysed with SYBR green method:

Table 9 - rt-PCR Summary results, SYBR green method – E. ET. 36%

Sample	DNA (ng/µL)	Cq. Mean	Cq. Std. Dev	Melt Temp
32549/H76	2.3	31.59	0.099	89.50
32549/H80	0.2	31.87	0.107	89.50
32549/H83	2.1	33.61	0.276	89.50
32788/M1	3.4	31.85	0.286	89.50
32786/M2	2.5	31.88	0.011	89.50
32788/M2	3.6	31.36	0.144	89.50

Negative ctrl	0	0	0	None
Positive ctrl	24.8	23.75	0.016	89.50

(b) all tested samples were detected with the probe specific for the sequence of *V. myrtillus* (**Figure 12** and **Table 10**) when analysed with probe-based method.

Table 10 - rt-PCR Summary results, Probe-based method - E. ET. 36%

Sample	DNA (ng/μL)	Cq. Mean	Cq. Std. Dev
32549/H76	2.3	31.52	0.306
32549/H80	0.2	31.38	0.073
32549/H83	2.1	33.44	0.326
32788/M1	3.4	31.10	0.098
32786/M2	2.5	31.52	0.156
32788/M2	3.6	30.53	0.197
Negative ctrl	0	0	0
Positive ctrl	7.45	24.84	0.177

Example 4 - Kit for the analysis

The kit is composed by:

- One 1.5 ml tube containing all reagents necessary to perform the analysis (DNA Polymerase, dNTPs, Buffer, Probe chemistry, Primers and Probe)
- One 1.5 ml tube containing the positive control (DNA of *Vaccinium myrtillus*)
- One 1.5 ml tube containing the negative control DNA (Nuclease-free water)

The kit can be used with all commercially available Real-time PCR System (es: BioRad CFX96™, BioRad CFX96 ™, bCube®, Roche LightCycler® 480, etc)

<u>**List of sequences**</u>

Nucleic acid fragments

GCATTGCGTCACCCACTCCCCCCGTGCCCCAAGCGGGCACGTCGGAGCGTGGGCG
GATATTGGCCCCCCGTTCGCATCCGTGCGCGGTCGGCCTAAAAAACGGGTCCCCA
ATGACGGACATCACGACAAGT (**SEQ ID NO:1)**

TGAAGGCACGTCTGCCTGGGCGTCACGCATTGCGTCACCCACTCCCCCCGTGCCCC
AAGCGGGCACGTCGGAGCGTGGGCGGATATTGGCCCCCCGTTCGCATCCGTGCGC
GGTCGGCCTAAAAAACGGGTCCCCAATGACGGACATCACGACAAGTGGTGGTTGC
TAAA (**SEQ ID NO:2)**

TTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCTGAAGCCAT
TAGGTTGAAGGCACGTCTGCCTGGGCGTCACGCATTGCGTCACCCACTCCCCCCGT
GCCCCAAGCGGGCACGTCGGAGCGTGGGCGGATATTGGCCCCCCGTTCGCATCCG
TGCGCGGTCGGCCTAAAAAACGGGTCCCCAATGACGGACATCACGACAAGTGGTG
GTTGCTAAA (**SEQ D NO:3)**

CCATCGAGTCTTTGAACGCAAGTTGCGCCTGAAGCCATTAGGTTGAAGGCACGTC
TGCCTGGGCGTCACGCATTGCGTCACCCACTCCCCCCGTGCCCCAAGCGGGCACG
TCGGAGCGTGGGCGGATATTGGCCCCCCGTTCGCATCCGTGCGCGGTCGGCCTAA
AAAACGGGTCCCCAATGACGGACATCACGACAAGTGGTGGTTGCTAAACCGTCGC
GTCACGTCGTGCATGCCATCGTTTGTTGCGGGTTGGCCCATTTGACCCTGAAGTG
(**SEQ ID NO:4)**

Primers

"S"

F GCATTGCGTCACCCACTC **(SEQ ID NO:5)**

R ACTTGTCGTGATGTCCGTCA **(SEQ ID NO:6)**

“S2”

F TGAAGGCACGTCTGCCTG **(SEQ ID NO:7)**

R TTTAGCAACCACCACTTGTCGT **(SEQ ID NO:8)**

“L2”

F TTGCAGAATCCCGTGAACCA **(SEQ ID NO:9)**

R TTTAGCAACCACCACTTGTCGT **(SEQ ID NO:10)**

“L”

F CCATCGAGTCTTTGAACGCA **(SEQ ID NO:11)**

R CACTTCAGGGTCAAATGGGC **(SEQ ID NO:12)**

Probes

M-FAM

ACGTCGGAGCGTGGGC **(SEQ ID NO:13)**

E-HEX

TAGGGCGGGTAAGTGAGT **(SEQ ID NO:14)**

CLAIMS

1. A method for the identification of *Vaccinium myrtillus* in a botanical composition, which comprises detecting from a sample thereof, by means of PCR-amplification, a *V. myrtillus* nucleic acid fragment located within the internal transcribed spacer 1, 5.8S ribosomal RNA genomic region and the internal transcribed spacer 2, said method comprising the following steps:

(a) isolating nucleic acids from said sample;

(b) conducting a real-time PCR on the isolated nucleic acid, using:

- a set of primers selected from the group consisting of:

(i) SEQ ID NO:5 and SEQ ID NO:6;

(ii) SEQ ID NO:7 and SEQ ID NO:8;

(iii) SEQ ID NO:9 and SEQ ID NO:10;

(iv) SEQ ID NO:11 and SEQ ID NO:12;

and

- a probe annealing within the nucleic acid region amplified by the primers, said probe consisting of the sequence SEQ ID NO:13;

(c) determining the presence of the amplification product,

whereby the detection of the amplification product is indicative of the presence of *Vaccinium myrtillus* in the botanical composition.

2. The method of claim 1, wherein the set of primers (i) is used in step (b).

3. The method of claims 1-2, wherein said rt-PCR is conducted under the following conditions:

- initial denaturation step at 95°C for 180 sec;

- 2-step cycles of 15 sec at 95°C (1st step) and 15 sec at 62-68.5°C (2nd step) repeated 40 to 50 times.

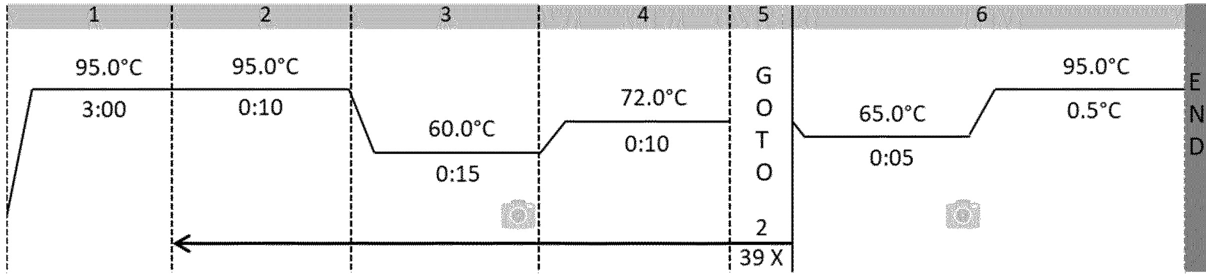
4. The method of claims 1-3, wherein the botanical composition is a plant extract.

5. A kit for the identification of *Vaccinium myrtillus* in a botanical composition, comprising a set of primers and a probe as defined in claim 1.

6. The kit of claim 5, further comprising a DNA polymerase, a mixture of deoxynucleotides (dNTP), buffer solutions.

7. The kit of claims 5-6, further comprising, in separate containers, a sample of *Vaccinium myrtillus* nucleic acid for use as positive control and nuclease-free water or buffer solution as negative control.

Figure 1



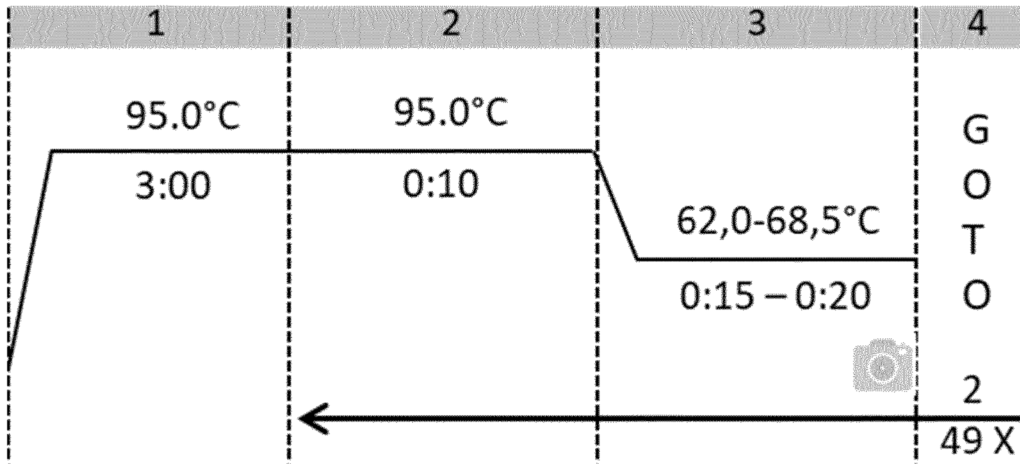


Figure 2

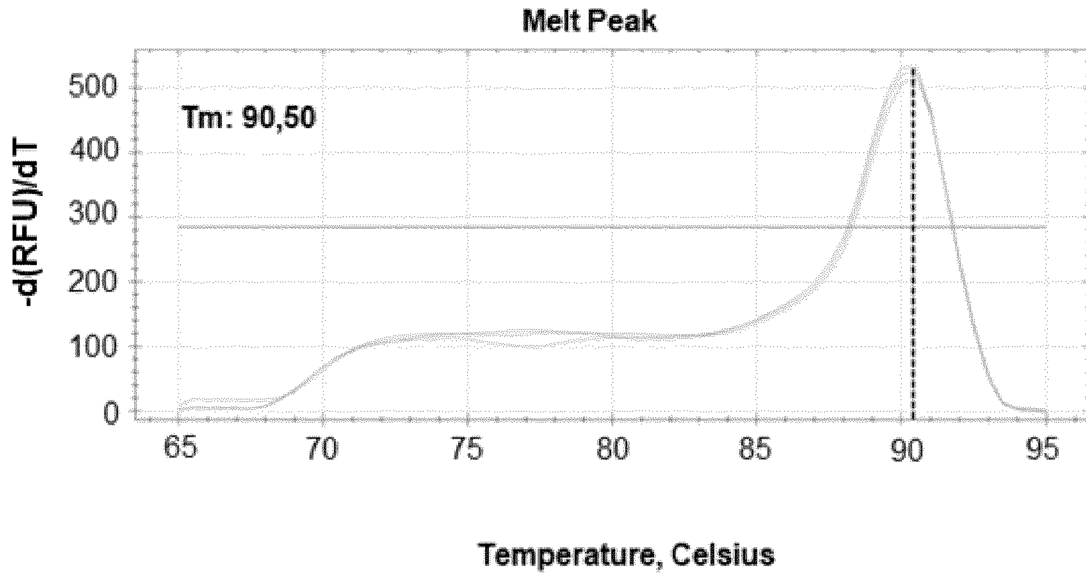
a)

Primer set	Sample	Cq	Cq Mean	Cq Std. Dev
L2	1072/10/12	25.61	25.37	0.212
		25.28		
		25.21		
S2	1072/10/12	26.17	25.89	0.247
		25.70		
		25.80		
L	1072/10/12	24.88	25.11	0.203
		25.20		
		25.25		
S	1072/10/12	25.97	26.15	0.166
		26.19		
		26.29		

Figure 2 (continued)

b)

Primer set **L2**



Primer set **L**

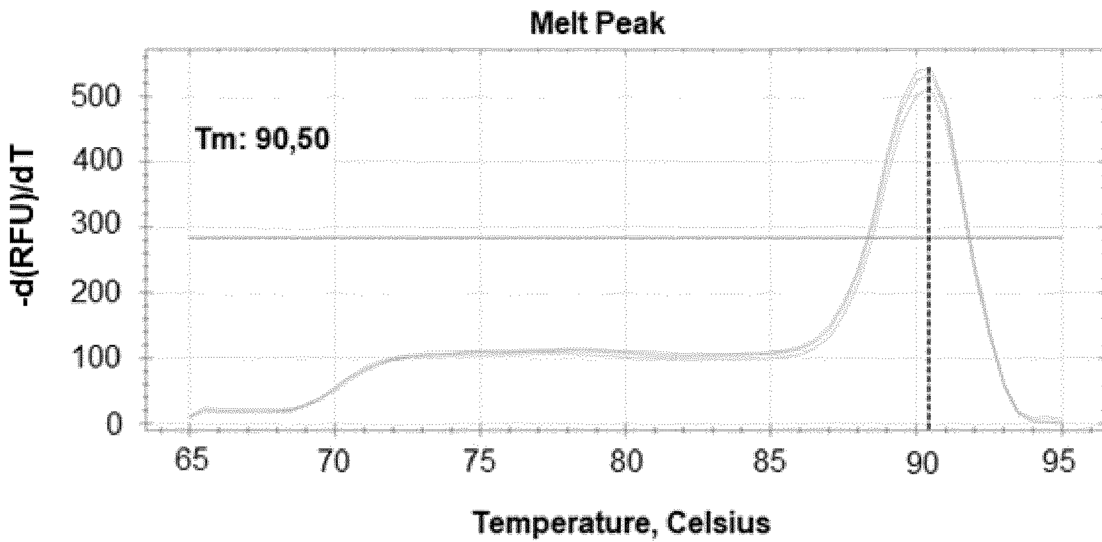
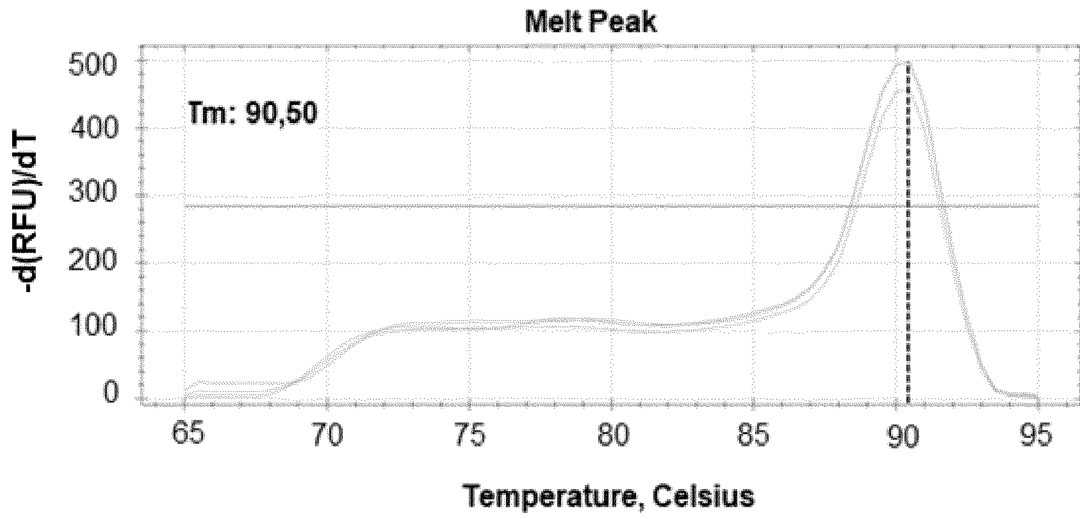


Figure 2 (continued)

Primer set **S2**



Primer set **S**

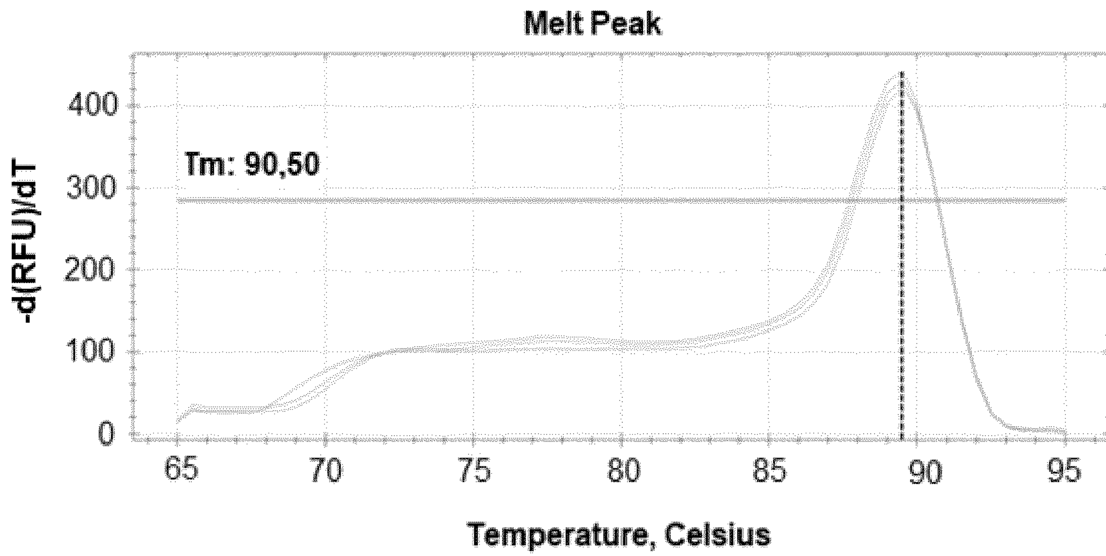
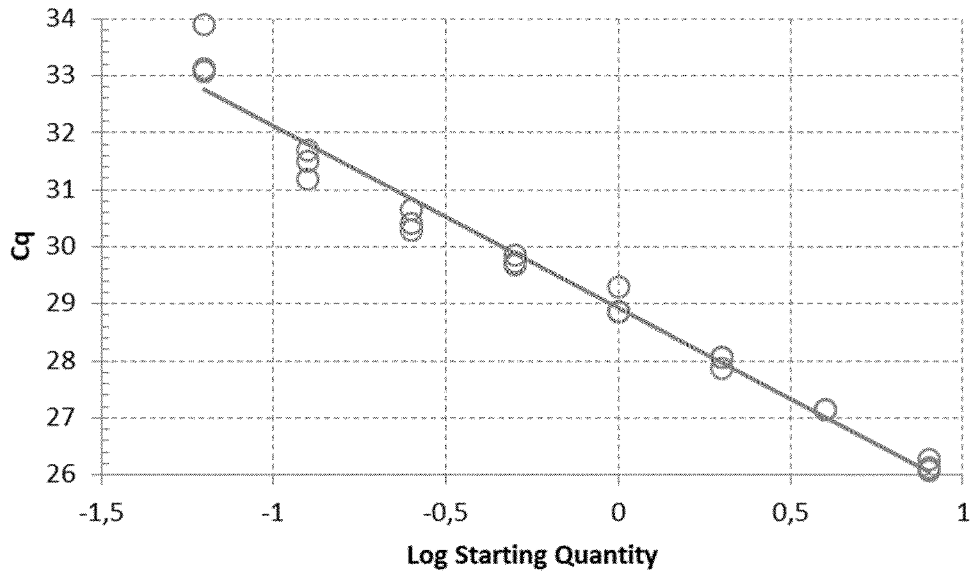


Figure 3



○ Standard

— SYBR

E=106,1% R^2=0,975 Slope=-3,183 y-int=28,931

Figure 4

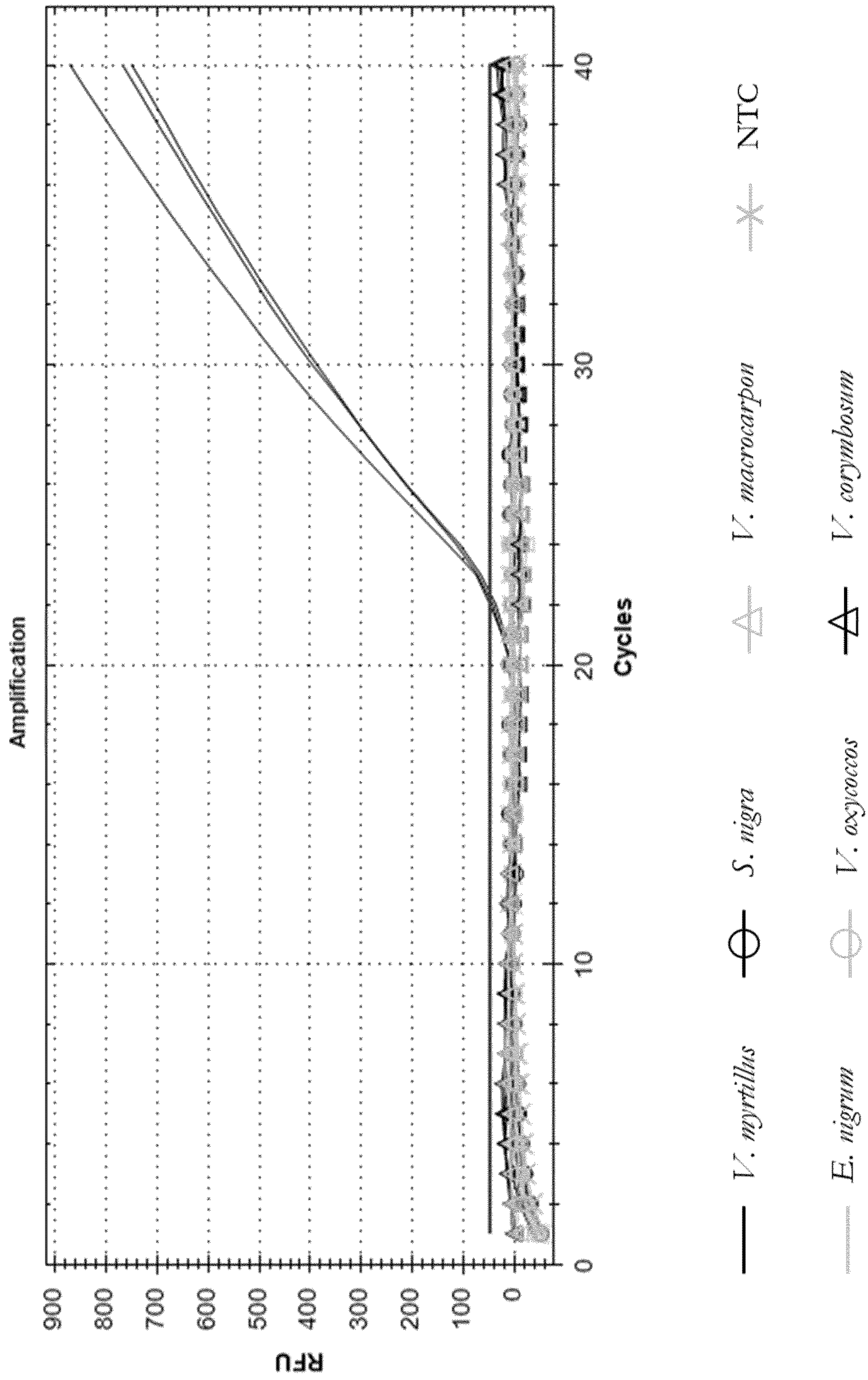
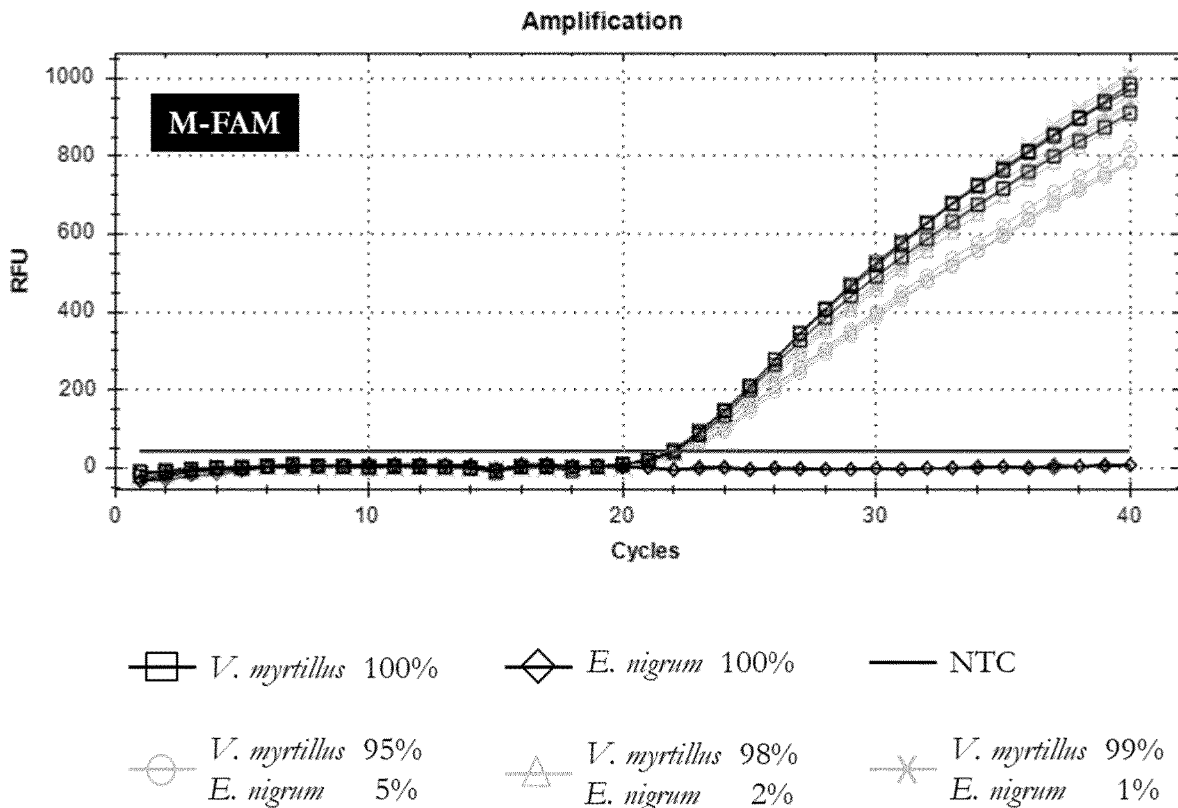


Figure 5

(a)

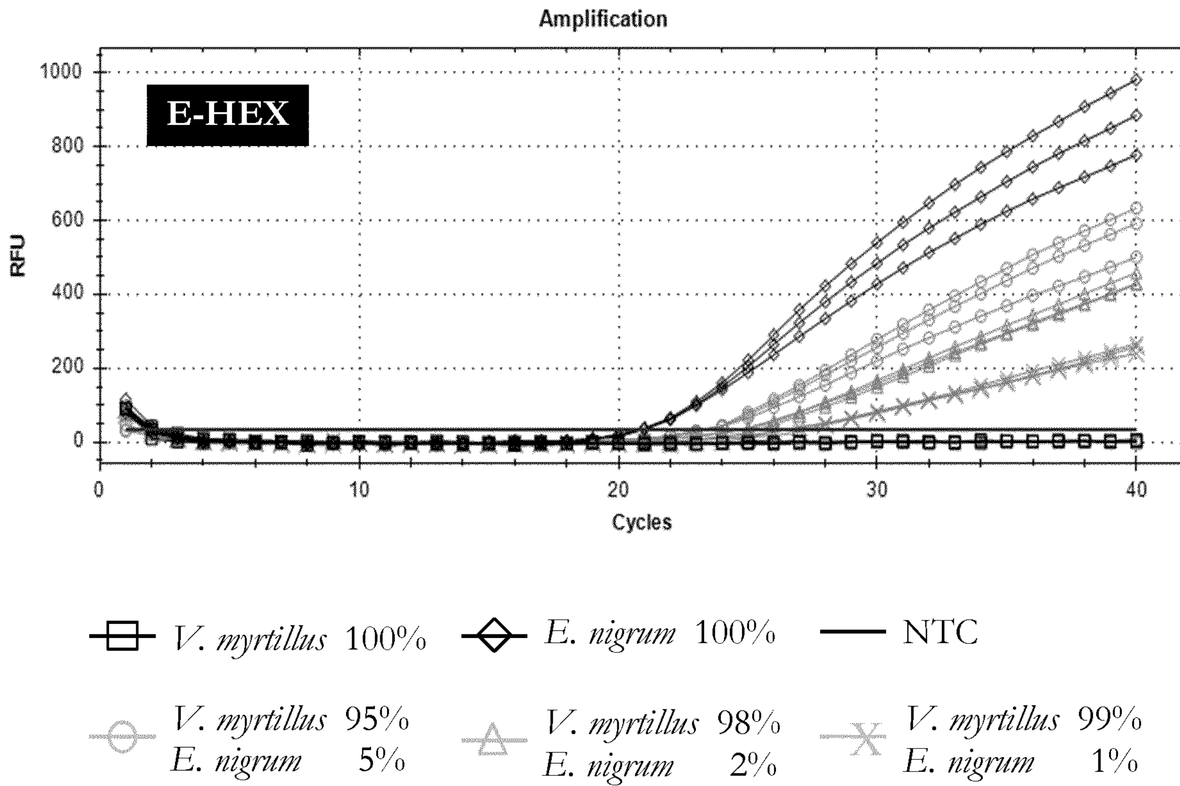


V. myrtillus 100% *E. nigrum* 100% NTC

V. myrtillus 95% *E. nigrum* 5% *V. myrtillus* 98% *E. nigrum* 2% *V. myrtillus* 99% *E. nigrum* 1%

Figure 5 (continued)

(b)



V. myrtillus 100% *E. nigrum* 100% NTC

V. myrtillus 95% *E. nigrum* 5% *V. myrtillus* 98% *E. nigrum* 2% *V. myrtillus* 99% *E. nigrum* 1%

Figure 6

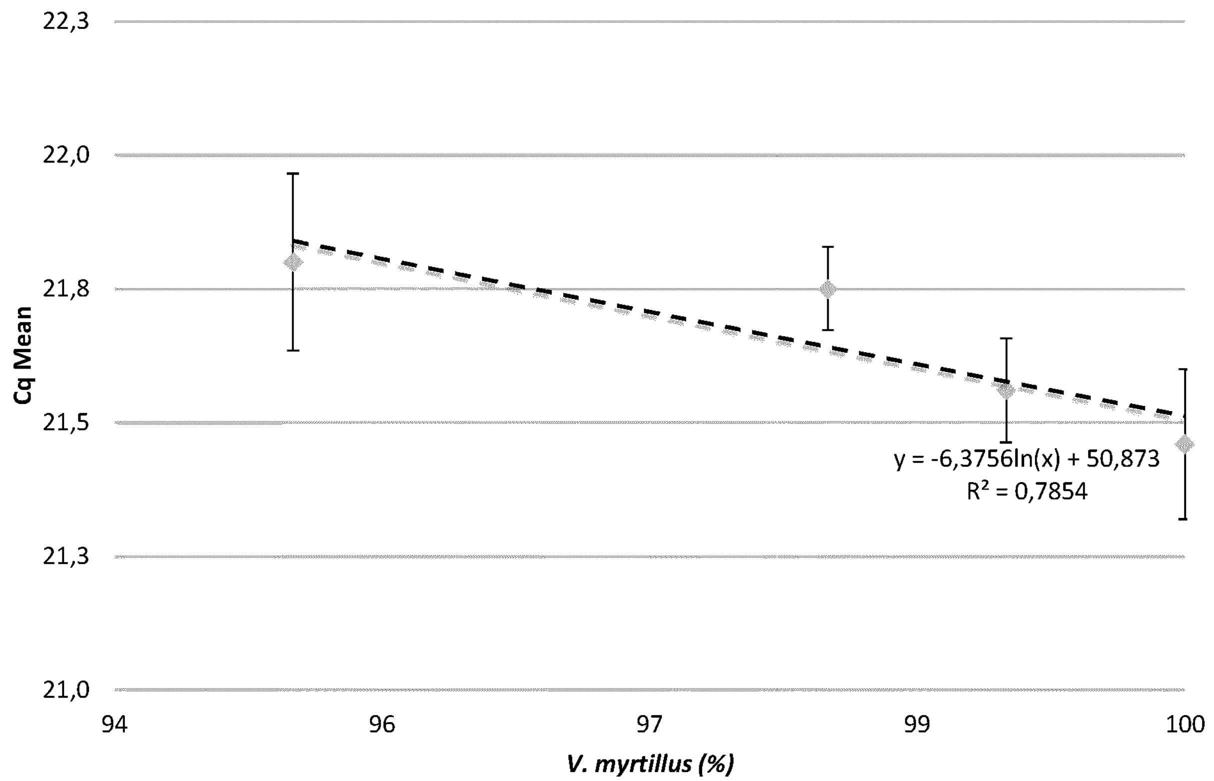


Figure 7

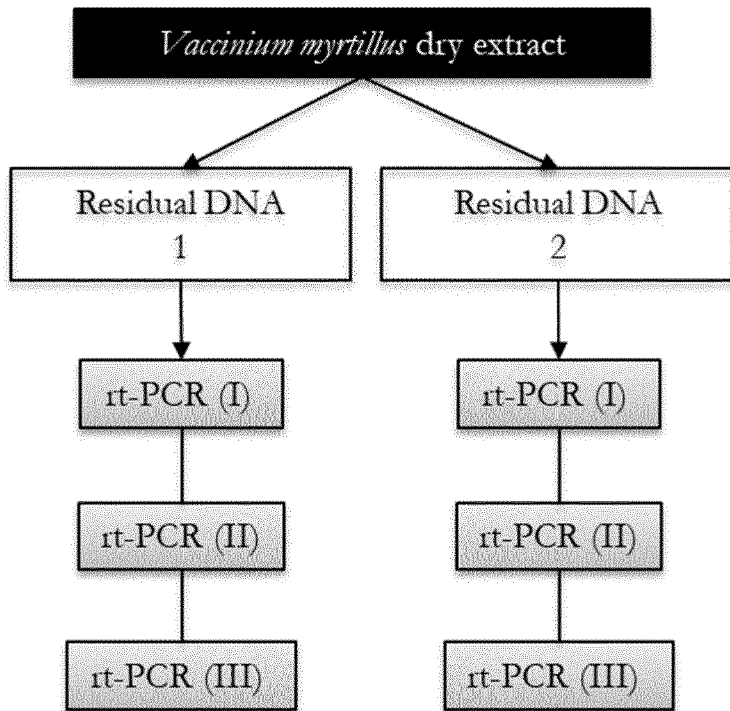
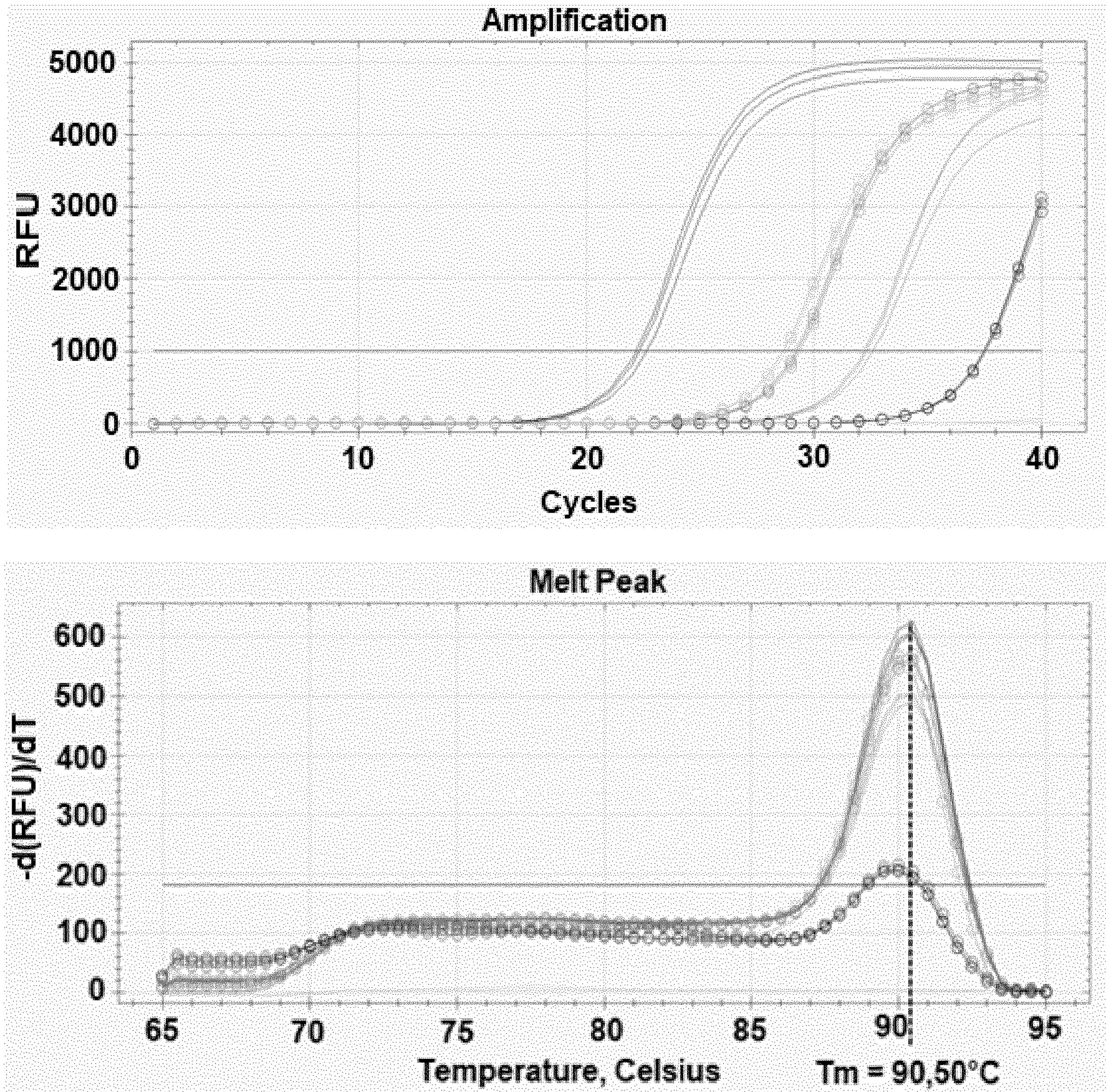


Figure 8

a)



V. myrtillus fruit sample

Negative control

32549/H76_2

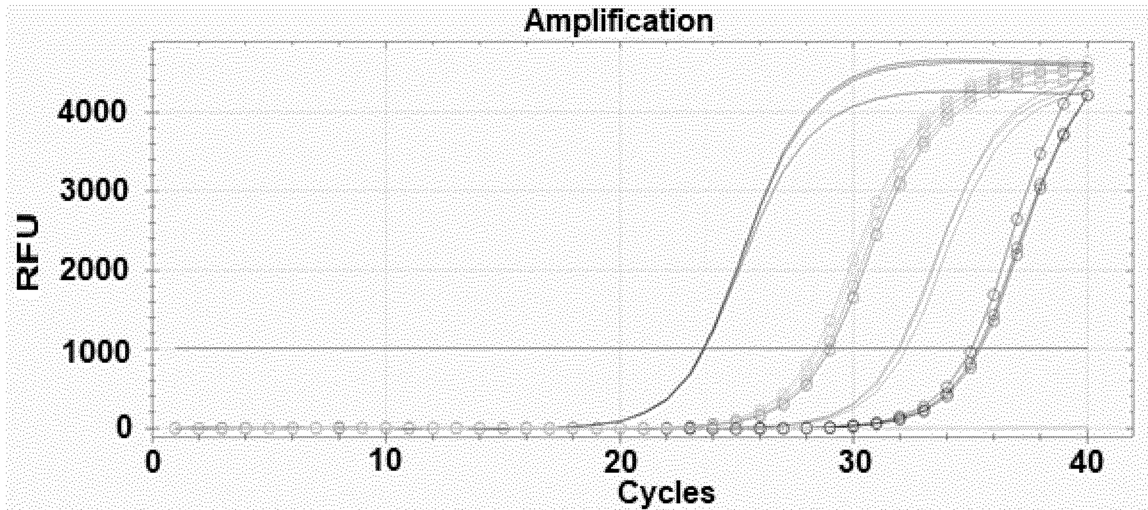
32549/H83_1

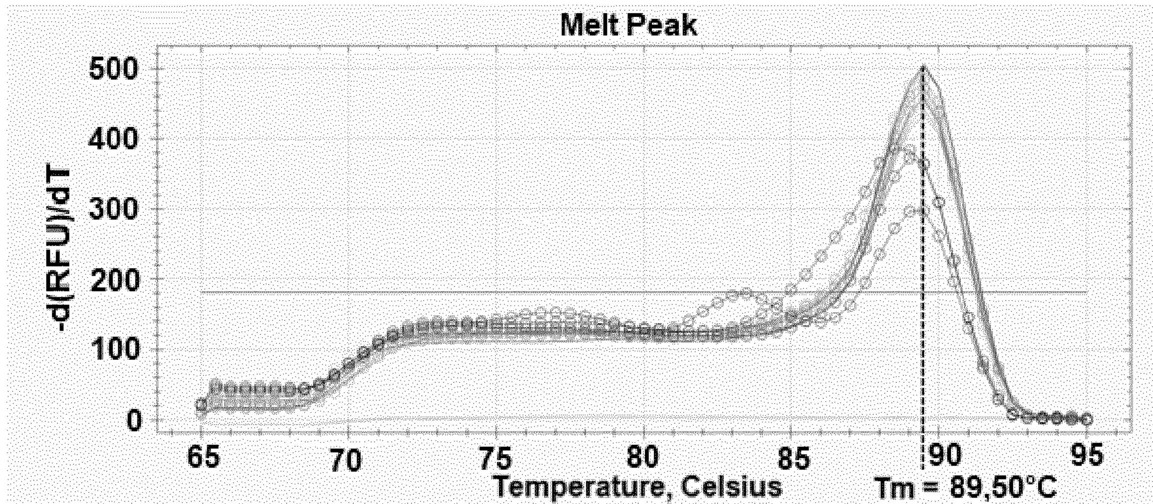
32549/H84_1

32549/H84_2

Figure 8 (continued)

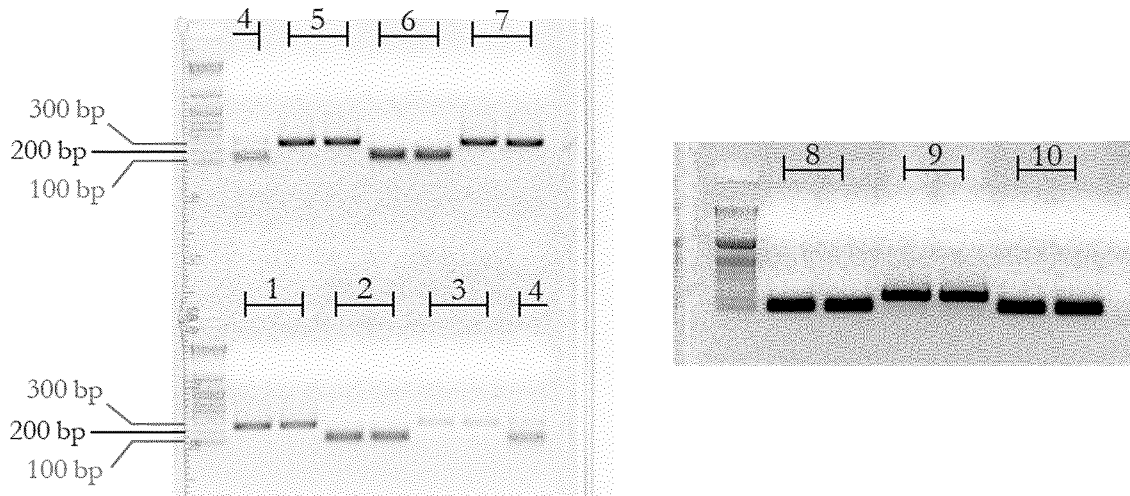
b)





V. myrtillus fruit sample
Negative control
32549/H76_2
32549/H83_1
32549/H84_1
32549/H84_2

Figure 9



1. 32549/H76_2 Large
2. 32549/H76_2 Small
3. 32549/H83_1 Large
4. 32549/H83_1 Small
5. 32549/H84_1 Large
6. 32549/H84_1 Small
7. 32549/H84_2 Large
8. 32549/H84_2 Small
9. Positive control Large
10. Positive control Small

Figure 10

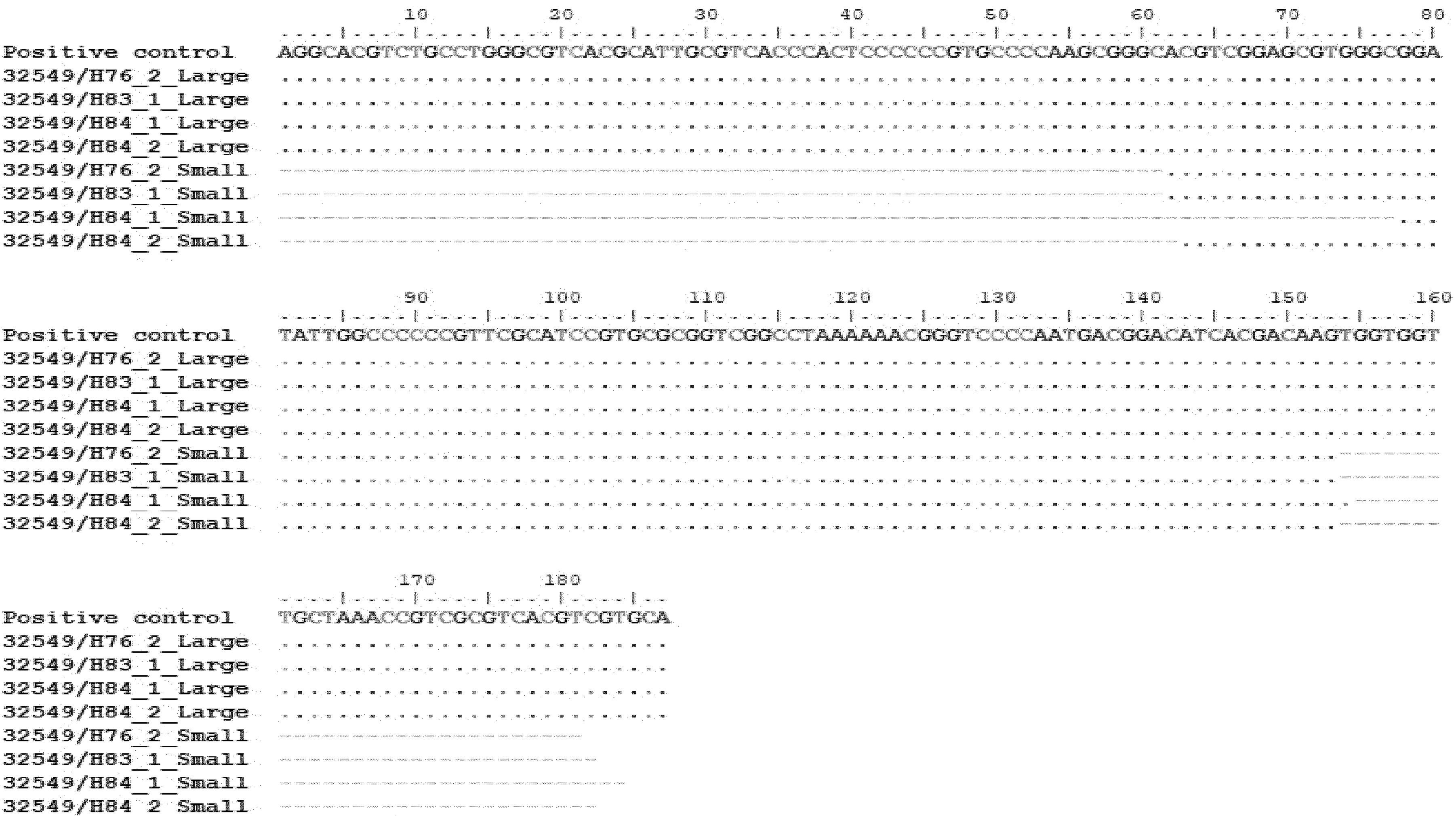
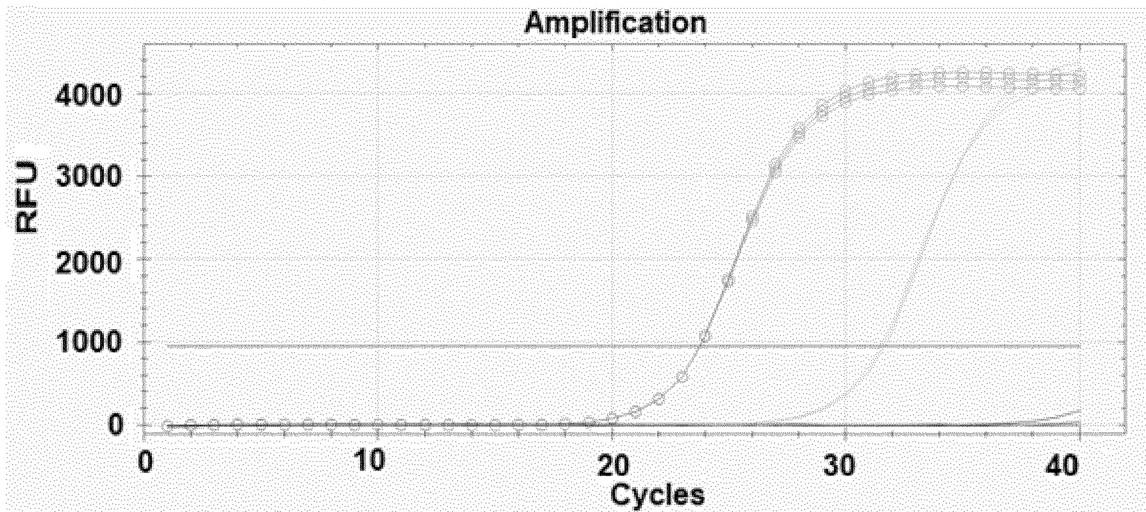


Figure 11

32549/H76



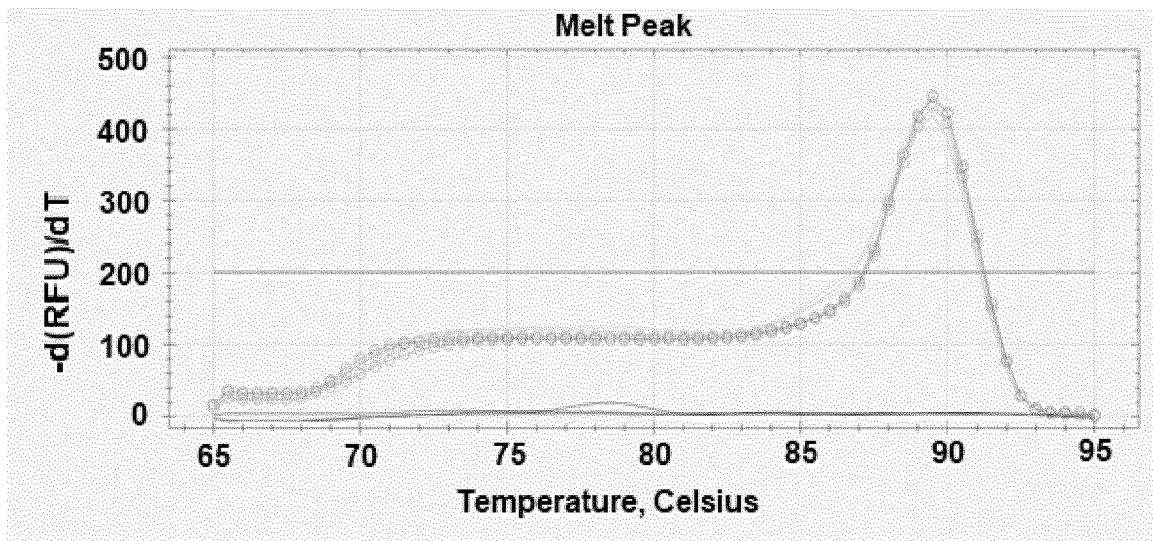
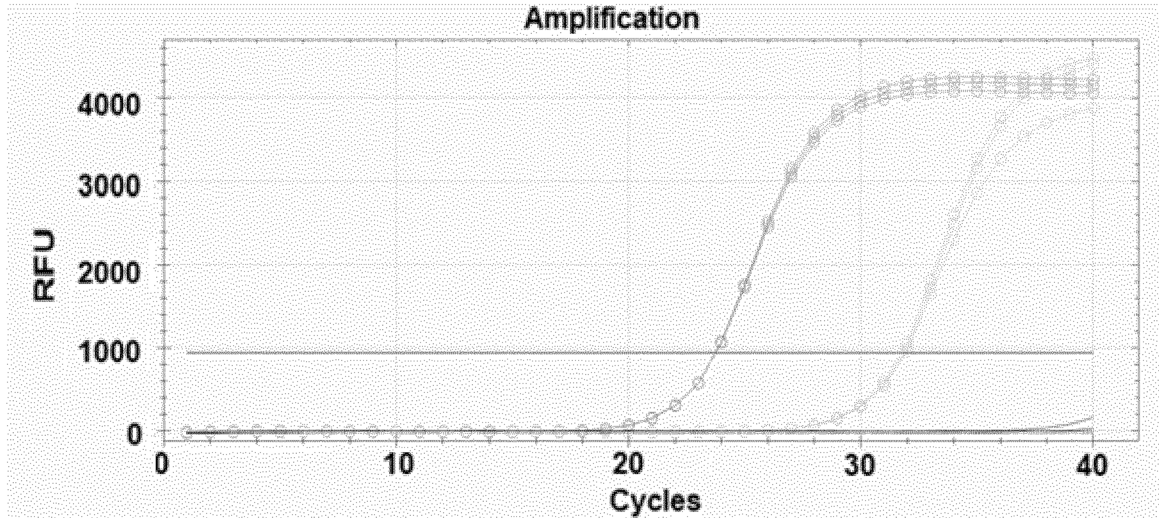


Figure 11 (continued)

32549/H80



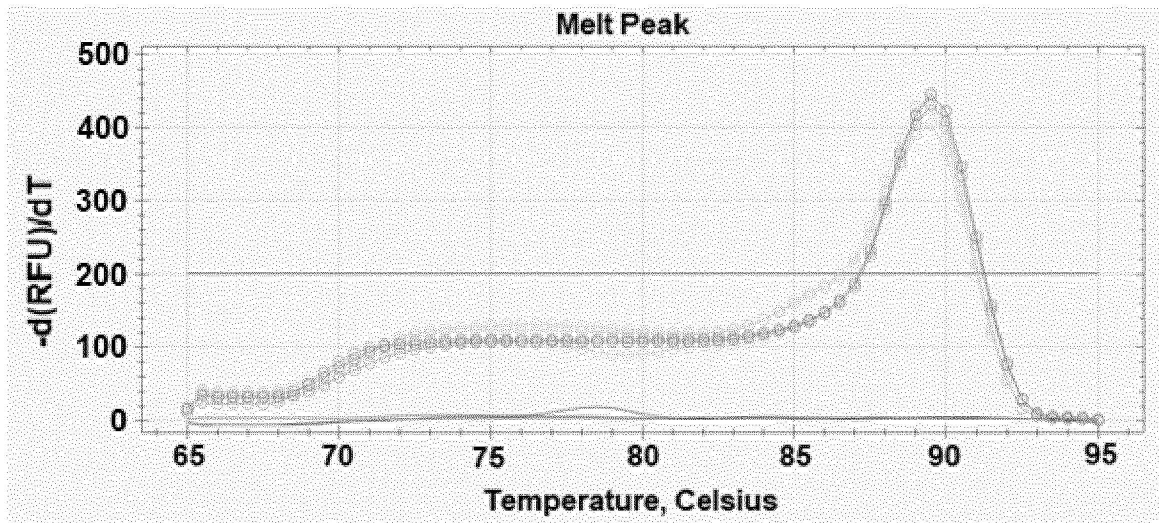


Figure 11 (continued)

32549/H83

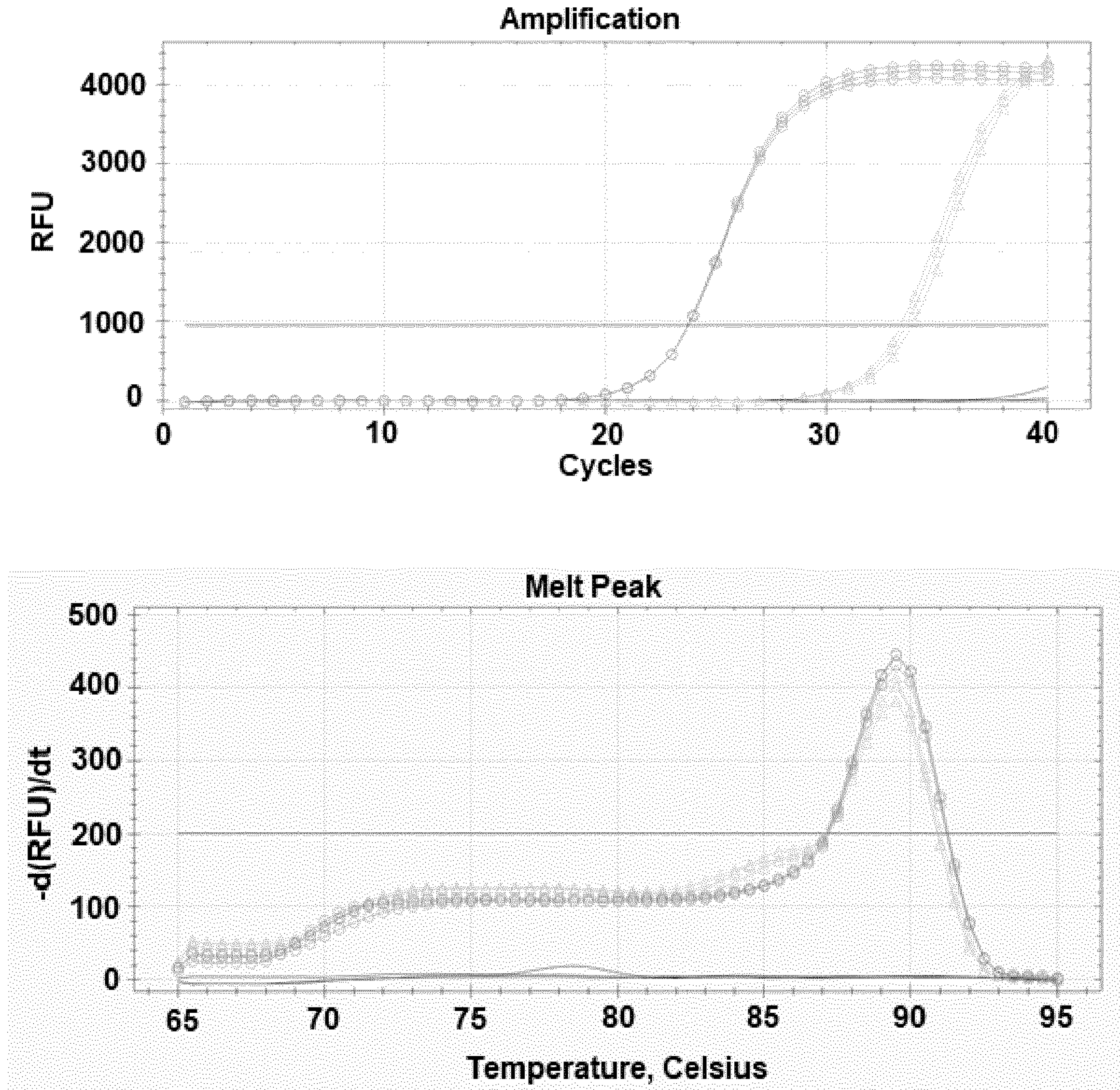
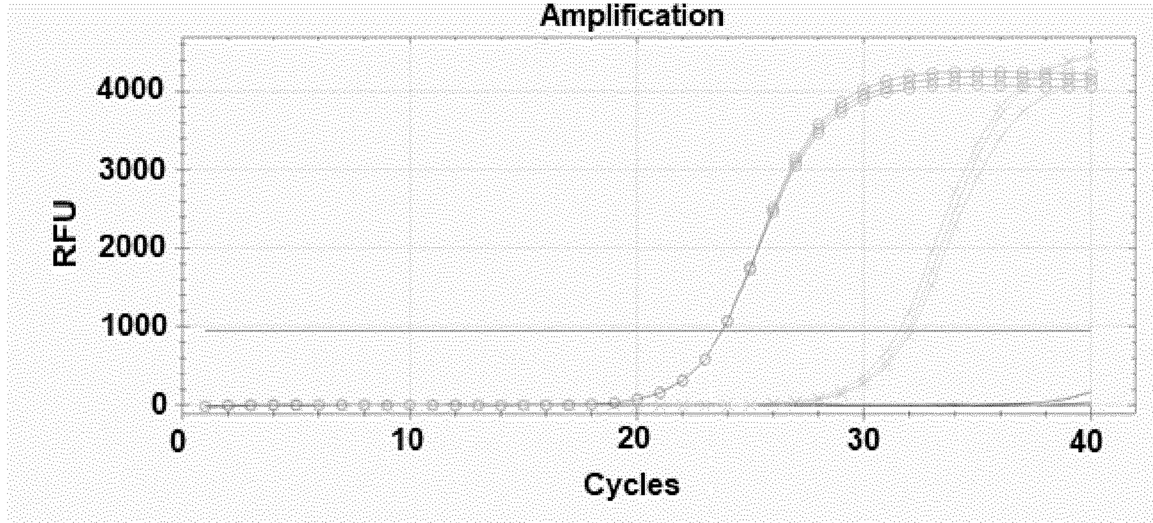


Figure 11 (continued)

32788/M1



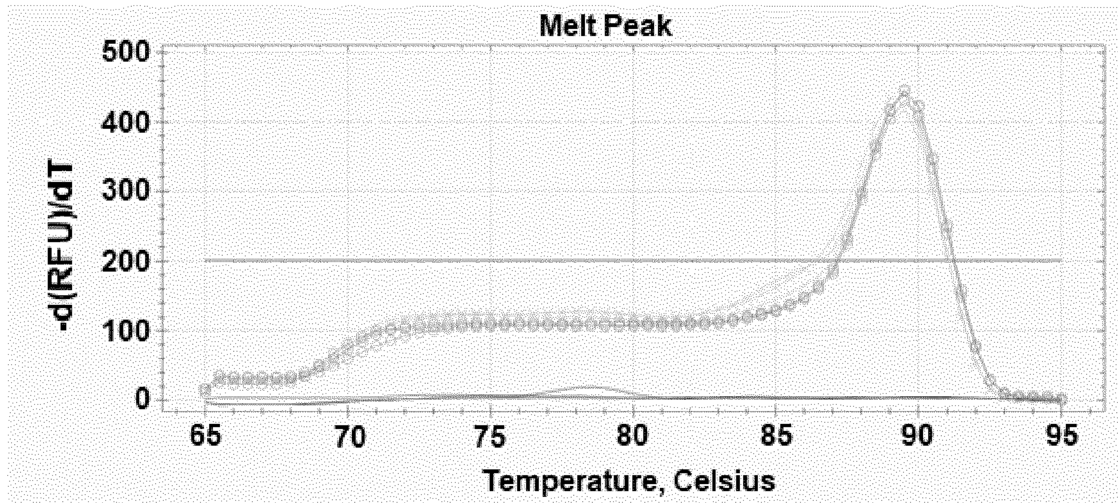
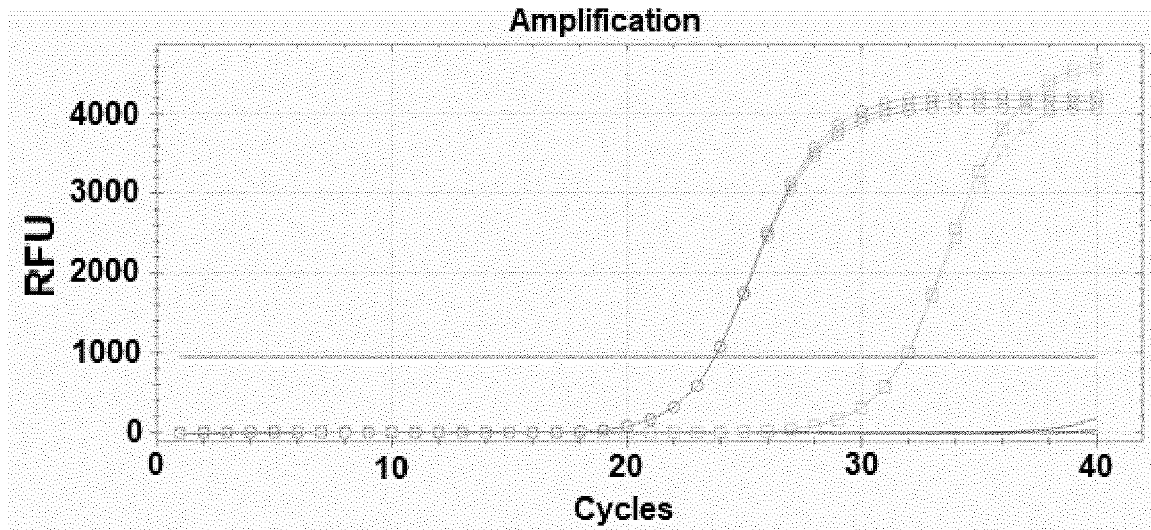


Figure 11 (continued)

32786/M2



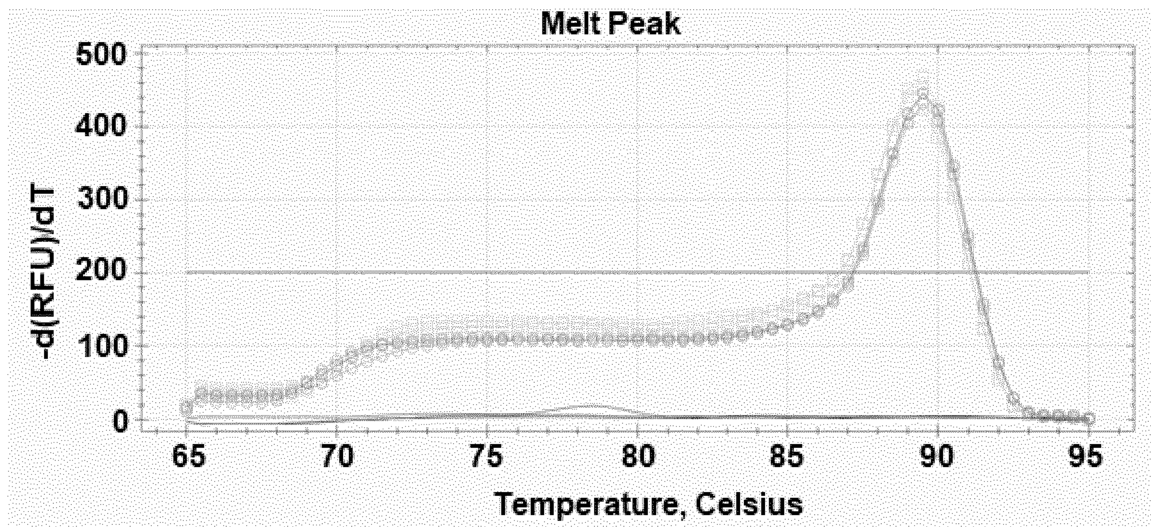
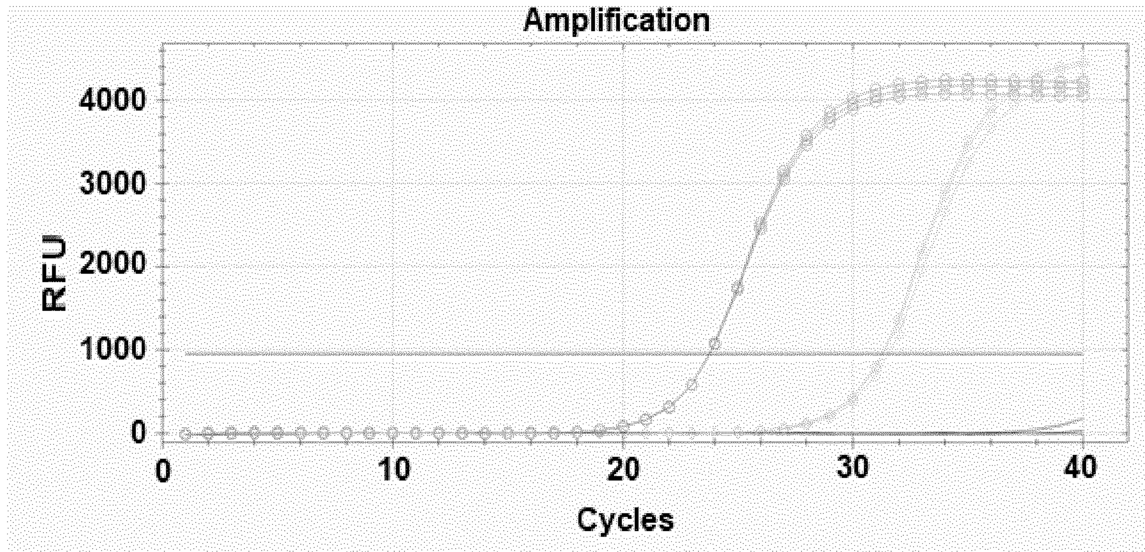


Figure 11 (continued)

32788/M2



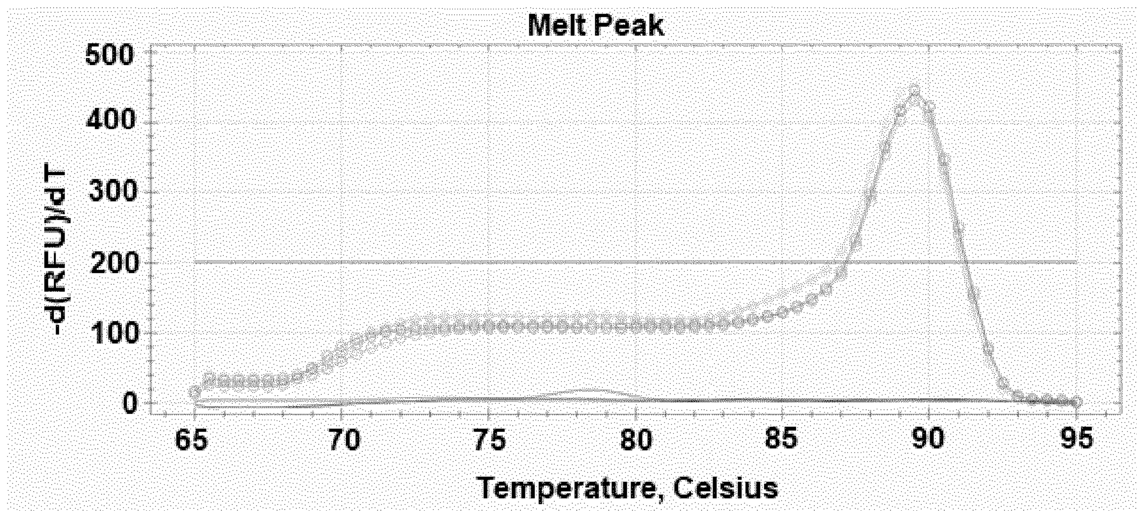
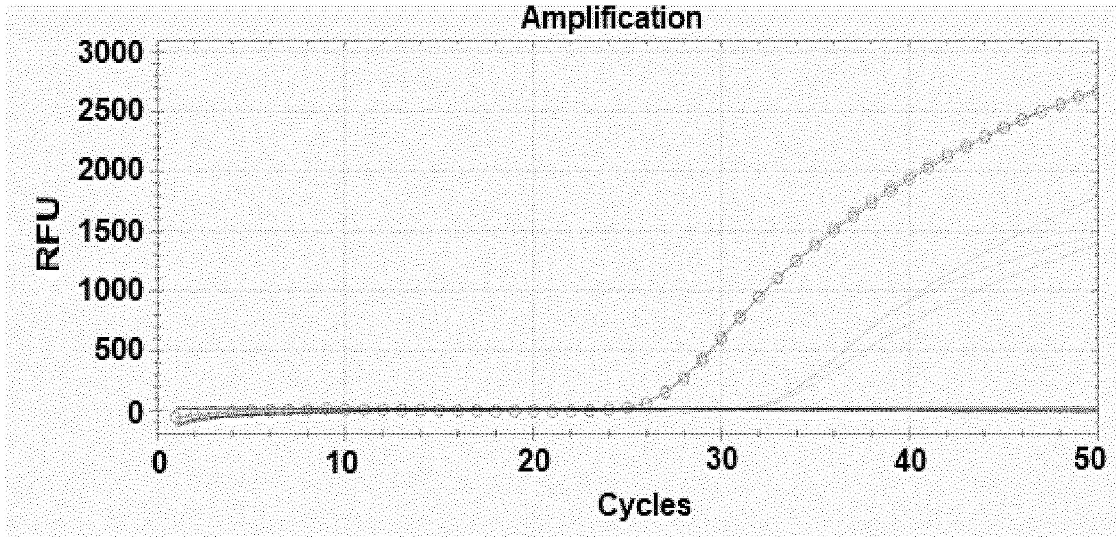
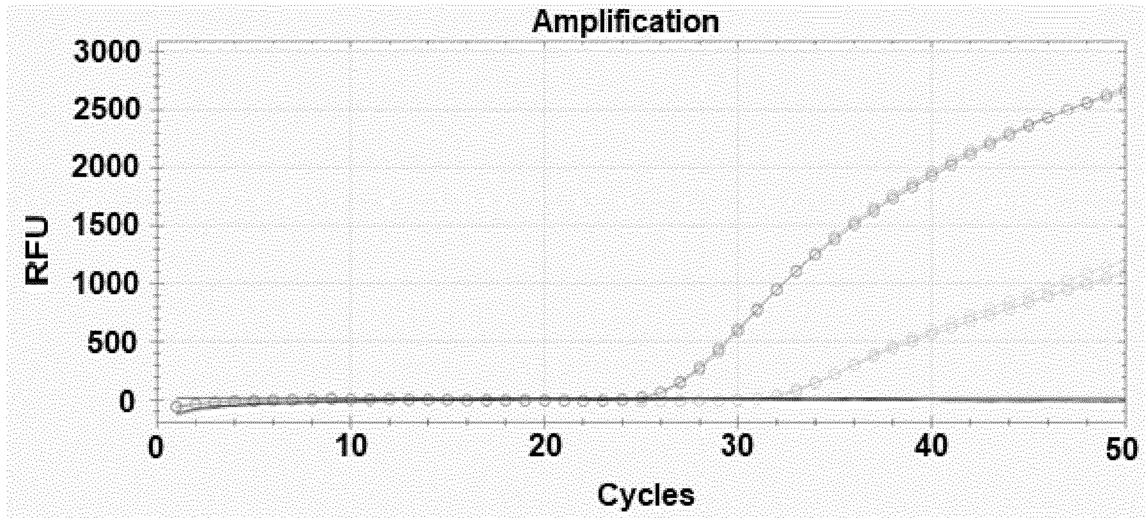


Figure 12

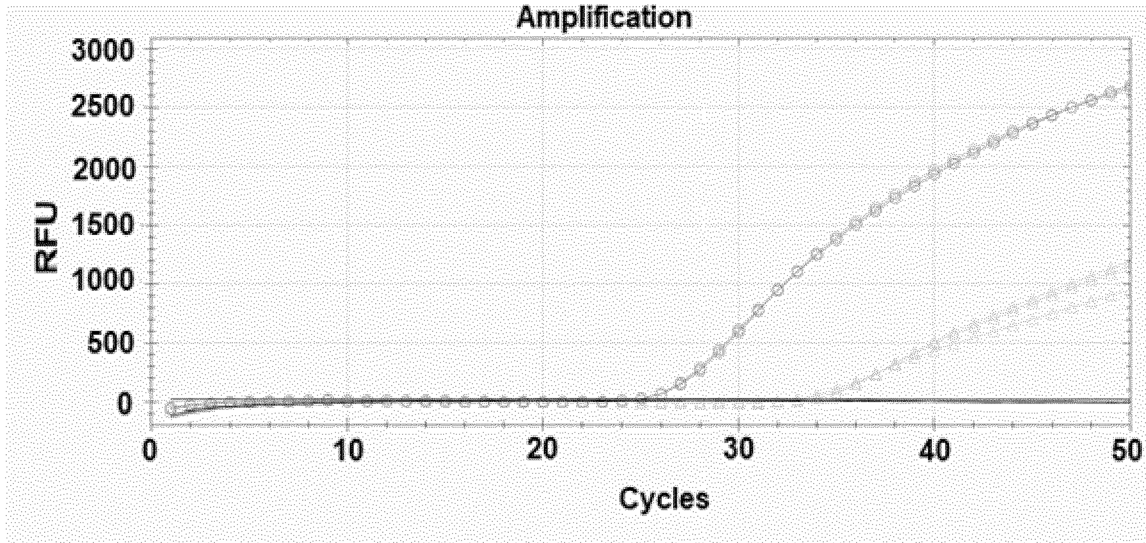


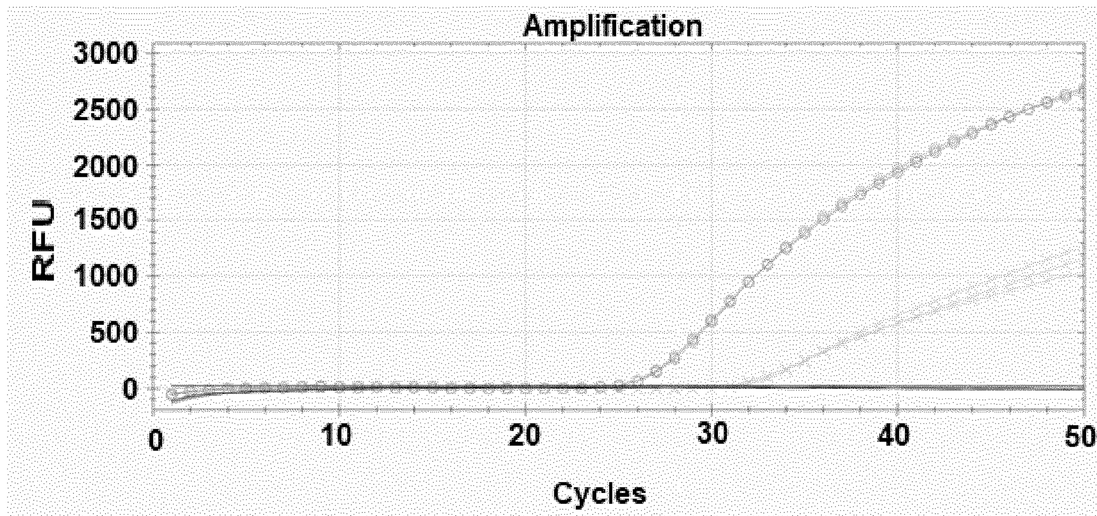


32549/H76 32549/H80 32549/H83 PTC

32788/M1 32786/M2 32788/M2 NTC

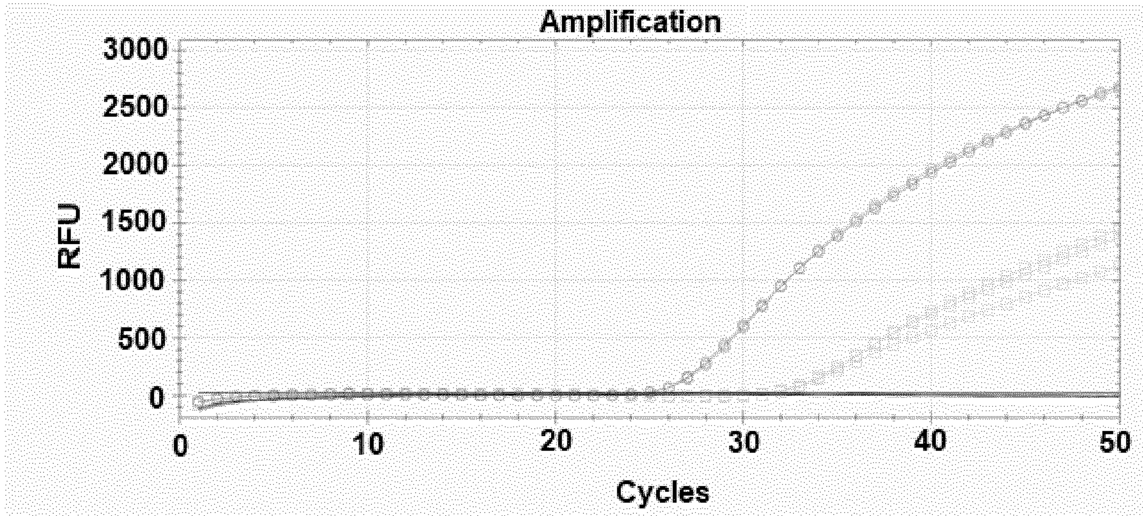
Figure 12 (continued)

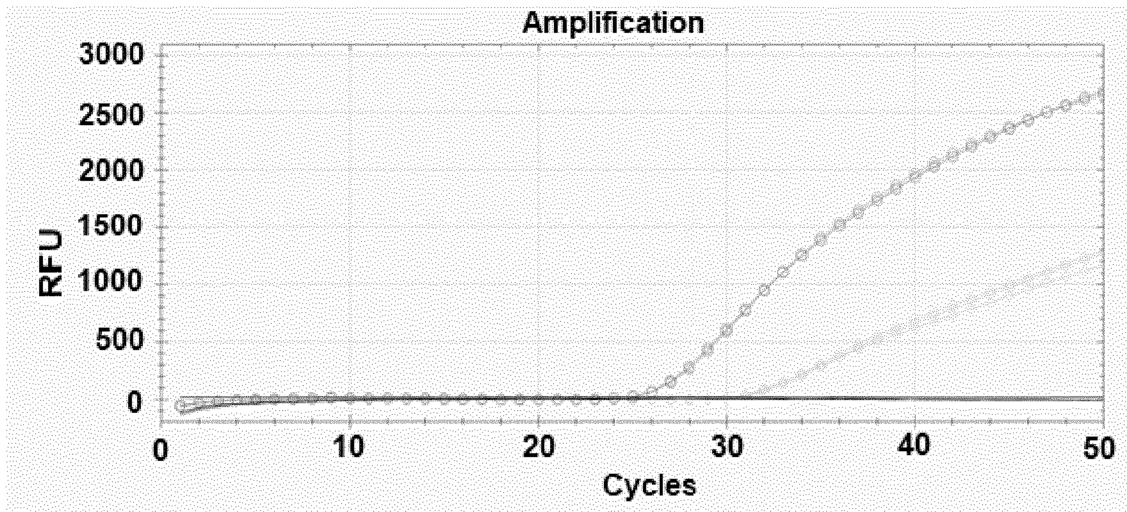




32549/H76 32549/H80 32549/H83 PTC
32788/M1 32786/M2 32788/M2 NTC

Figure 12 (continued)

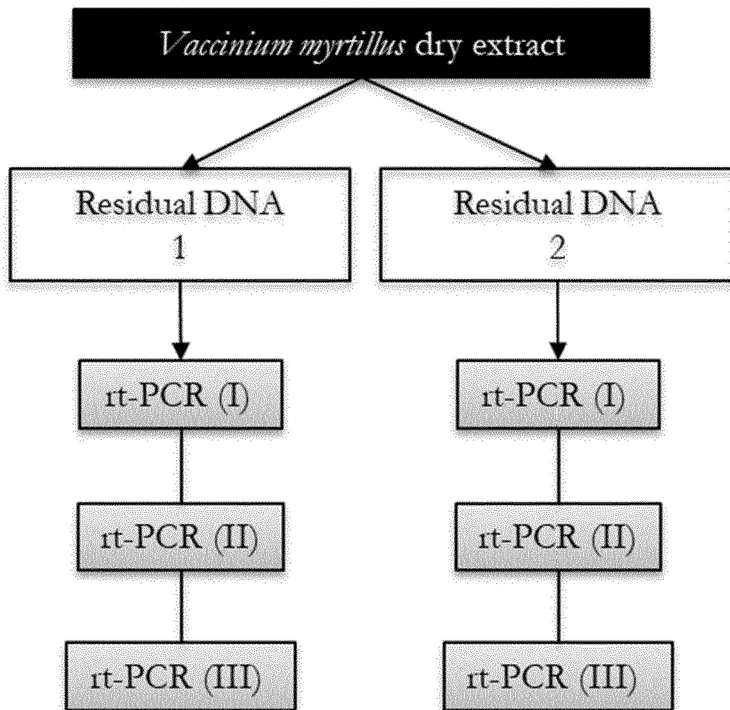




32549/H76 32549/H80 32549/H83 PTC

32788/M1 32786/M2 32788/M2 NTC

Figure 7



Dry-extract Biological replicate Technical replicate