Development and Verification of PCR based Assay to Detect and Quantify Garden Pea *lec* Gene

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Abstract

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Genetically modified organisms (GMOs) entering the food chain have became its part, which is necessary to monitor. GMO analyses are used as a control mechanism according to valid acquis communautaire for traceability and labeling of GMOs. Generally, approved PCR based protocols are used and they require stepwise procedures that use amplification of species specific gene as initial point. This study aims to develop and verify PCR based assay for amplification of garden pea lectin gene (*Pisum sativum* L.) as reference one. Lectin gene was analysed *in silico*, selected region was amplified and sequenced and new set of species specific primers for identification of garden pea was designed. Conditions of conventional PCR as well as real-time PCR were optimised and specificity of new primer set on DNA extracted from garden pea cultivars as well as DNA extracted from other selected species from *Fabaceae* family was tested. Quantification of garden pea lectin gene using real-time PCR based on SYBR Green I was optimised and performance characteristics recorded. The characteristics fit to method acceptance criteria range. Plasmid with garden pea lectin sequence was developed and plasmid is available as a positive control.

Keywords: GMO; lectin; PCR detection; real-time PCR

There are three fundamental types of agriculture in Europe considered. Conventional agriculture, ecological agriculture and GM (genetic modified) plants cultivation. Coexistence measures must be applied for these three types of agricultural.

Basic rules are carried out by technical and agronomical arrangements, e.g. use of buffer zones with specific variety structure, registry of GM plants cultivation, establish of GM plants cultivation methods and accidental admixtures of GM and non-GM crops definition. Regulation are in place for GM derived food and feed (e.g. EU Regulations – EC 1829/2003 and EC 1830/2003). Methods are in place that allows efficient control of in EU approved GMO.

The most used methods for GMO detection in food and feed are based on Polymerase Chain Reaction (PCR). Principles of these methods are described in the international standards (e.g. EN ISO 21571:2005) and validated protocols are avail-

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able on the JRC (Joint Research Centre) website covering mostly GM soybean, maize, cotton, rapeseed or rice (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm – accessed 27. 7. 2011).

DNA extraction from representative samples constitutes the first step of PCR based GMO analysis. The DNA should meet certain criteria for further analysis. Several procedures have been described that allow efficient DNA extraction (EN ISO 21571:2005; ENGL 2008). Among them extraction exploiting selective precipitation in cetyltrimethylammonium bromide (CTAB) (Murray & Thompson 1980) was found to be an appropriate method, which is in line with preceding recommendation for DNA isolation from plants containing higher content of polysaccharides, lipids and polyphenols (Porebski *et al.* 1997; Ovesna *et al.* 2010).

After DNAs are isolated, they are subjected either to conventional or real-time PCR. In both cases, DNA amplifiability is verified by plant/species specific gene(s) amplification. This procedure is used for verifying if the PCR ran through and for the control of false negatives results in further analyses (Hemmer 1997).

In real-time PCR, nucleic acid increase is measured and the standard curve is used as a reference standard for extrapolating quantitative information for starting amount of nucleic acid (Paoletti *et al.* 2006). The resulting percentage of GMO in the sample is then calculated from the proportion of species-specific (endogenous) DNA and the transgenic target.

For correct GMO quantification, the reaction has to be fully optimised and reaction efficiency should reach at least 90% (Querci *et al.* 2005). The PCR efficiency can be affected by several factors as e.g. primer design, composition of reaction mixture, reaction profile and presence of inhibitors/enhancers (Holst-Jensen *et al.* 2006; Hodek *et al.* 2009; Bergerová *et al.* 2011).

As increasing number of various GM plants has appeared in the environment released deliberately for research or commercial purposes, appropriate assays are required for efficient control.

Also garden pea (*Pisum sativum* L.) has been already transformed and the cultivars are undergoing field tests (Rakousky *et al.* 2004; Švábová *et al.* 2005).

Development of PCR based assay for detection and quantification of garden pea species specific gene, verification of method performance and specificity was the main aim of the study.

A gene coding for lectin was selected as multiple sequencing data were available in the databases (Chrispeels & Raikhel 1991; Lioi *et al.* 2005). Moreover, garden pea lectin is a one-copy gene (Gatehouse *et al.* 1987; Kaminski *et al.* 1987; Galasso *et al.* 2003) and amplification of part of pea lectin gene as a control element was already used in several studies (Brežná *et al.* 2006; Hrnčírová *et al.* 2008).

MATERIAL AND METHODS

Plant material. The 13 cultivars of commercially available pea (*Pisum sativum* L.) seeds (Raman, Alderman, Arvika, Bajka, Hero, Dalila, Havel, Oskar, Pegaz, Rondo, Kelvedon Wonder, Ambrosia and Delikata) and selected species of the pea family (*Fabaceae*) vetch (*Vicia villosa* ROTH), soybean (*Glycine max* L.), lentil (*Lens culinaris* Med.), mung bean (*Vigna radiata* L.) and bean (*Phaseolus vulgaris* L.) were used. All used seeds were stored at room temperature until processed.

DNA isolation. Seeds were ground in liquid nitrogen to obtain a homogenous powder. The CTAB (cetyltrimethyl ammonium bromide) method for DNA extraction was performed according ISO standard (EN ISO 21571:2005). DNA was extracted from 200 mg of powdered sample. Two independent extractions were performed from each sample. Extracted DNAs were eluted in 60 μl of ultrapure DNA and RNA free H_2O.

The extraction of plasmid DNA was performed using High Pure Plasmid Isolation Kit (Roche, Basel, Switzerland).

Assessment of DNA quality. The quality of extracted DNA (its integrity and rough estimation of quantity) was evaluated using electrophoretic separation on 0.8% agarose gel with ethidium bromide staining. The spectrophotometer NanoPhotometer (Implen GmbH, Munich, Germany) was used for measurement of DNA quantity. Absorption at 260 and 280 nm was measured and the spectra profile (from 190 nm to 760 nm) was recorded. DNA quantity was calculated from the absorption at wavelength 260 nm and DNA purity calculation was based on A_{260}/A_{280} ratios.

Primers design. Free-software Primer3Plus was used for oligonucleotide primer designing (Untergasser *et al.* 2007). First, primers for amplification of 658 bp long part of pea lectin gene (primers Lec658) were designed according to DNA sequence

Table 1. Primers used in PCR

Primer	Nucleotide sequence (5'→3')	Product size (bp)	Source
Pla F	CGAAATCGGTAGACGCTACG	ca 600	TABERLET *et al.* (1991)
Pla R	GGGGATAGAGGGACTTGAAC		
Lec658 F	CCGAACAACCTCGAAGAAATAC	658	this paper
Lec658 R	ACTCTGCGCTATTGAAAACTCC		
Lec101 F	CCCGACCAACAAAACCTAAT	101	this paper
Lec101 R	TAGAGGGCTCTGCCAACAGT		

of pea lectin (EU825771.1). Amplicons were used for cloning and for subsequent DNA sequencing.

Primers Lec101 were designed according to sequenced DNA of 658 bp long part of pea lectin gene. Primers Lec658 and Lec101 are shown in Table 1.

The specificity of primers Lec101 was analysed *in silico* in ClustalW2 free-software for the multiple sequence alignment and in free accessible database of the Center for Biotechnology Information (NCBI) with using Basic Local Alignment Search Tool (BLAST) (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Conventional Polymerase Chain Reaction (PCR). Amplification reactions (25 μl) were performed using AmpliTaq Gold PCR reagents (Applied Biosystems, Carlsbad, USA). For the verification of DNA amplifiability of all samples used in this study, primers (Pla primers) for amplification of specific region within gene coding plastid t-RNA were used (TABERLET *et al.* 1991) (Table 1).

PCR for preparation of 658 bp long part of pea lectin gene (with Lec658 primers) was performed with DNA extracted from seeds of garden pea cv. Raman.

PCR with Lec101 primers was used for reaction specificity test with all samples used in this study (all garden pea cultivars as well as chosen species belonging to the family *Fabaceae*).

Final concentration of PCR's components were as follow: Gold buffer 1×, $MgCl_2$ 1.5mM, dNTPs (Fermentas, Burlington, Canada) 0.2mM, AmpliTaq Gold polymerase 1U, and primers both of Pla primers 0.5μM, both of Lec658 primers 0.5μM or both of Lec101 primers 0.24μM. The volume was adjusted with water for PCR to 20μl. PCR was performed using 5μl of template DNA. Several controls were included in each reaction set – positive control of PCR run, control of extraction reagents (CTRL EX) and control of amplification reagents (CTRL MM and CTRL MM open).

All reactions were performed in an MJTB-96 PCR thermal cycler (MJ Research, Watertown, USA) and the thermal profile was: 12 min at 95°C; 40 cycles at 95°C for 30 s, at 60°C for 30 s (Pla primers and Lec658 Primers) or at 65°C for 30 s (Lec101 primers), at 72°C for 30 s and final extension was at 72°C for 10 minutes. Used primers were distributed by GENERI BIOTECH, s.r.o. (Hradec Králové, Czech Republic).

Results of PCR were visualised using electrophoretic separation on 2% agarose gel with ethidium bromide staining.

Cloning. Positive plasmid control (Plasmid658) was prepared by cloning of 658 bp long amplicon of pea lectin gene. Cloning was performed using TOPO® TA Cloning Kit (Invitrogen, Grand Island, USA). Plasmids were linearised with the restriction enzyme *Hind*III (Fermentas, Burlington, Canada) before using in PCR – circular form of plasmid is not appropriate for that purpose (HOU *et al.* 2010).

DNA sequencing. Plasmid658 was used for DNA sequencing 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, USA). The sequencing was performed using BigDye® Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA) according to the manufacturer's protocol. The sequencing reaction was performed independently twice with forward primer Lec658 and independently twice with reverse primer Lec658.

Real-time PCR. Real-time PCR was performed with Lec101 primers in the 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA). All reactions were done in triplicates using SYBR® Green (Applied Biosystems, Carlsbad, USA) detection. Final concentration of real-time PCR's components were as follow: SYBR® Green 1×, both of Lec101 primers 0.05μM, volume was adjusted with water for PCR to 45 μl. The thermal profile of real-time PCR consisted of 10 min at 95°C; 35 cycles at 95°C for 15 s, and at 60°C for 45 seconds.

***Real-time PCR characterisation*.** The occurrence of inhibitions or enhancers was controlled using 10-fold dilution of template DNA, when the difference between Ct values in no inhibited reaction should be around 3.3 Ct.

The real-time PCR efficiency was determined for genomic DNA as a template using serial of dilutions. The starting amount of DNA in reaction was 200 ng (corresponding to approx. 41 000 DNA copies; Bennet & Smith 1976), sample was diluted 4-times with ultrapure H_2O for PCR at ratio of 1:3. Limit of Detection (LOD) and Limit of Quantification (LOQ) were defined. Specificity of used primers Lec101 was tested in real-time PCR with all of used samples in this study (all garden pea cultivars and chosen species belonged to the family *Fabaceae*).

Efficiency of reaction was calculated according to the formula:

$$\text{Efficiency} = 100 - \text{ABS}\,(100 - (10^{-1/\text{slope}} - 1) \times 100) \quad (1)$$

Data from 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA) were evaluated in Microsoft Office Excel.

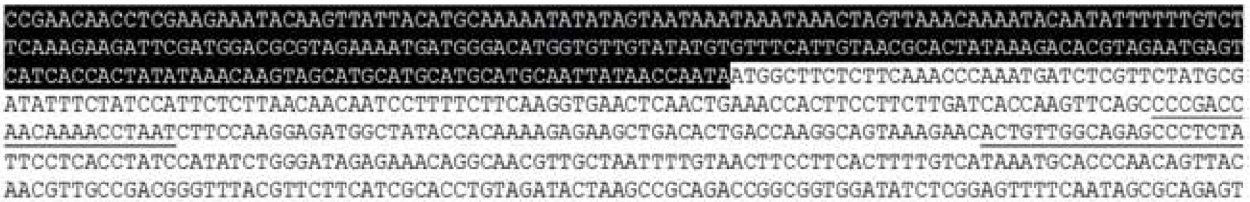
RESULTS AND DISCUSSION

The CTAB method was proved to be suitable for DNA extraction from seeds of the *Fabaceae* family. CTAB based method thus represents an adequeate alternative to GeneSpin protocol exploited by e.g. Brežná *et al.* (2006). DNA amplifiability that serves an independent check of its quality was verified using a tRNA-leu specific PCR assay (Taberlet *et al.* 1991) and confirms required DNA purity.

Using cv. Raman DNA as a template in PCR with Lec658 primer pair we amplified 658 bp long fragments. Fragments were cloned into the plasmid vector to prepare Plasmid658 control DNA. Insert representing part of the lectin gene in the Plasmid658 was sequenced and DNA sequences were *in silico* analysed against NCBI database. Results confirm 100% homology for all of 658 nts with genomic DNA sequence of *Pisum sativum* L. lectin gene (EU825771.1, X66368.1), 98% homology (646/658 nts identity) was observed with genomic DNA sequence of *Pisum sativum* L. lectin gene

Table 2. Accesion numbers of nucleotide sequences used for testing of Lec101 primers specifity

Accession number	Description
EU825771.1	*Pisum sativum* lectin (*psl*) gene, complete cds
X66368.1	*Pisum sativum psl* gene for Psl lectin
Y00440.1	*Pisum sativum lecA* gene for lectin
M18160.1	Pea *PSL1* gene encoding lectin, complete cds
J01254.1	pea lectin (alpha and beta subunits) mRNA
HQ337024.1	*Pisum sativum* lectin gene, 5' upstream region
AY342212.1	*Glycine max* lectin (*Le2*) gene, complete cds
HM348715.1	*Vigna radiata* cv. Wilczek lectin gene, complete cds
AJ277588.1	*Vigna unguiculata* partial mRNA for lectin (*lec* gene)
AJ920065.1	*Phaseolus vulgaris* partial *lec1* gene for lectin precursor
AJ318222.2	*Lens odemensis* lectin gene, clone Ode9-1
AJ318220.2	*Lens ervoides* lectin gene, clone Erv6-22
AJ419573.2	*Lens lamottei* lectin gene
AJ318221.3	*Lens nigricans* lectin gene, clone Nig7-42
AJ421799.2	*Lens culinaris* subsp. *tomentosus* lectin gene
AJ318219.2	*Lens culinaris* subsp. *orientalis* lectin gene, clone Ori4-31
AJ318218.2	*Lens culinaris* subsp. *culinaris* lectin gene, cv. Eston, clone Est1-17
AJ318217.2	*Lens culinaris* subsp. *culinaris* lectin gene, cv. Laird, clone Lai2-71
DQ005103.1	*Lens culinaris* cv. L-4076 lectin mRNA, complete cds
AJ318216.1	*Lens culinaris* subsp. *culinaris* lectin gene, partial
AJ438490.2	*Vicia faba lec2* gene for lectin
AJ438593.2	*Vicia faba lec1* gene for lectin



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TTCCTCACCTATCCATATCTGGGATAGAGAAACAGGCAACGTTGCTAATTTTGTAACTTCCTTCACTTTTGTCATAAATGCACCCAACAGTTAC
AACGTTGCCGACGGGTTTACGTTCTTCATCGCACCTGTAGATACTAAGCCGCAGACCGGCGGTGGATATCTCGGAGTTTTCAATAGCGCAGAGT
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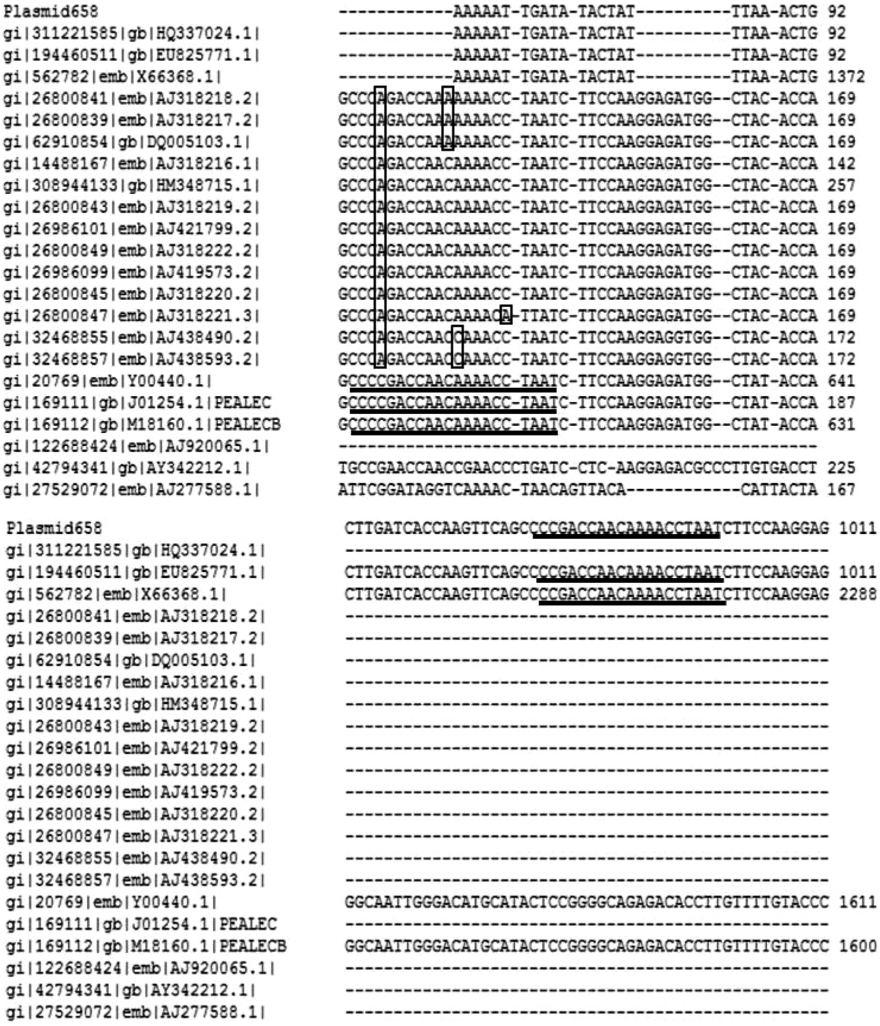
Figure 1. DNA sequence of 658 bp long part of garden pea lectin gene. Part 5'-upstream region DNA is labelled black, the exon part of lectin gen is without labelling. Target regions for Lec101 primers are labelled with a black line

Y00440.1 (recorded DNA sequence contained 12 nts deletion compared to our DNA sequence), 98% homology (651/661 nts identity) was observed with mRNA sequence of *Pisum sativum* L. lectin gene M18160.1 and the 98% homology (414/415 nts identity) was observed with mRNA sequence of *Pisum sativum* L. lectin gene J01254.1.

The results document that this sequence fragment corresponding to the garden pea (*Pisum sativum* L.) lectin gene is highly conservative and thus suited for designing of specific primers. Sequenced DNA includes border part of 5'-upstream region DNA (labeled black) with the exon part of lectin gene (Figure 1).

Primers Lec101 were designed to be specific to the exon part of lectin gene. Specificity of Lec101 primers was tested: (1) *in silico* by comparison with NCBI recorded DNA sequences corresponding to lectin gene in tested species of the family *Fabaceae* (Table 2); (2) experimentally in the conventional PCR after the assay had been optimised.



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gi|194460511|gb|EU825771.1|         ------------AAAAAT-TGATA-TACTAT----------TTAA-ACTG 92
gi|562782|emb|X66368.1|             ------------AAAAAT-TGATA-TACTAT----------TTAA-ACTG 1372
gi|26800841|emb|AJ318218.2|         GCCCAGACCAAAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
gi|26800839|emb|AJ318217.2|         GCCCAGACCAAAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
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gi|14488167|emb|AJ318216.1|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 142
gi|308944133|gb|HM348715.1|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 257
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gi|26986101|emb|AJ421799.2|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
gi|26800849|emb|AJ318222.2|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
gi|26986099|emb|AJ419573.2|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
gi|26800845|emb|AJ318220.2|         GCCCAGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAC-ACCA 169
gi|26800847|emb|AJ318221.3|         GCCCAGACCAACAAAACA-TTATC-TTCCAAGGAGATGG--CTAC-ACCA 169
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gi|20769|emb|Y00440.1|              GCCCCGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAT-ACCA 641
gi|169111|gb|J01254.1|PEALEC        GCCCCGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAT-ACCA 187
gi|169112|gb|M18160.1|PEALECB       GCCCCGACCAACAAAACC-TAATC-TTCCAAGGAGATGG--CTAT-ACCA 631
gi|122688424|emb|AJ920065.1|        --------------------------------------------------
gi|42794341|gb|AY342212.1|          TGCCGAACCAACCGAACCCTGATC-CTC-AAGGAGACGCCCTTGTGACCT 225
gi|27529072|emb|AJ277588.1|         ATTCGGATAGGTCAAAAC-TAACAGTTACA------------CATTACTA 167

Plasmid658                           CTTGATCACCAAGTTCAGCCCCGACCAACAAAACCTAATCTTCCAAGGAG 1011
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gi|26800839|emb|AJ318217.2|          --------------------------------------------------
gi|62910854|gb|DQ005103.1|           --------------------------------------------------
gi|14488167|emb|AJ318216.1|          --------------------------------------------------
gi|308944133|gb|HM348715.1|          --------------------------------------------------
gi|26800843|emb|AJ318219.2|          --------------------------------------------------
gi|26986101|emb|AJ421799.2|          --------------------------------------------------
gi|26800849|emb|AJ318222.2|          --------------------------------------------------
gi|26986099|emb|AJ419573.2|          --------------------------------------------------
gi|26800845|emb|AJ318220.2|          --------------------------------------------------
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gi|32468855|emb|AJ438490.2|          --------------------------------------------------
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gi|27529072|emb|AJ277588.1|          --------------------------------------------------
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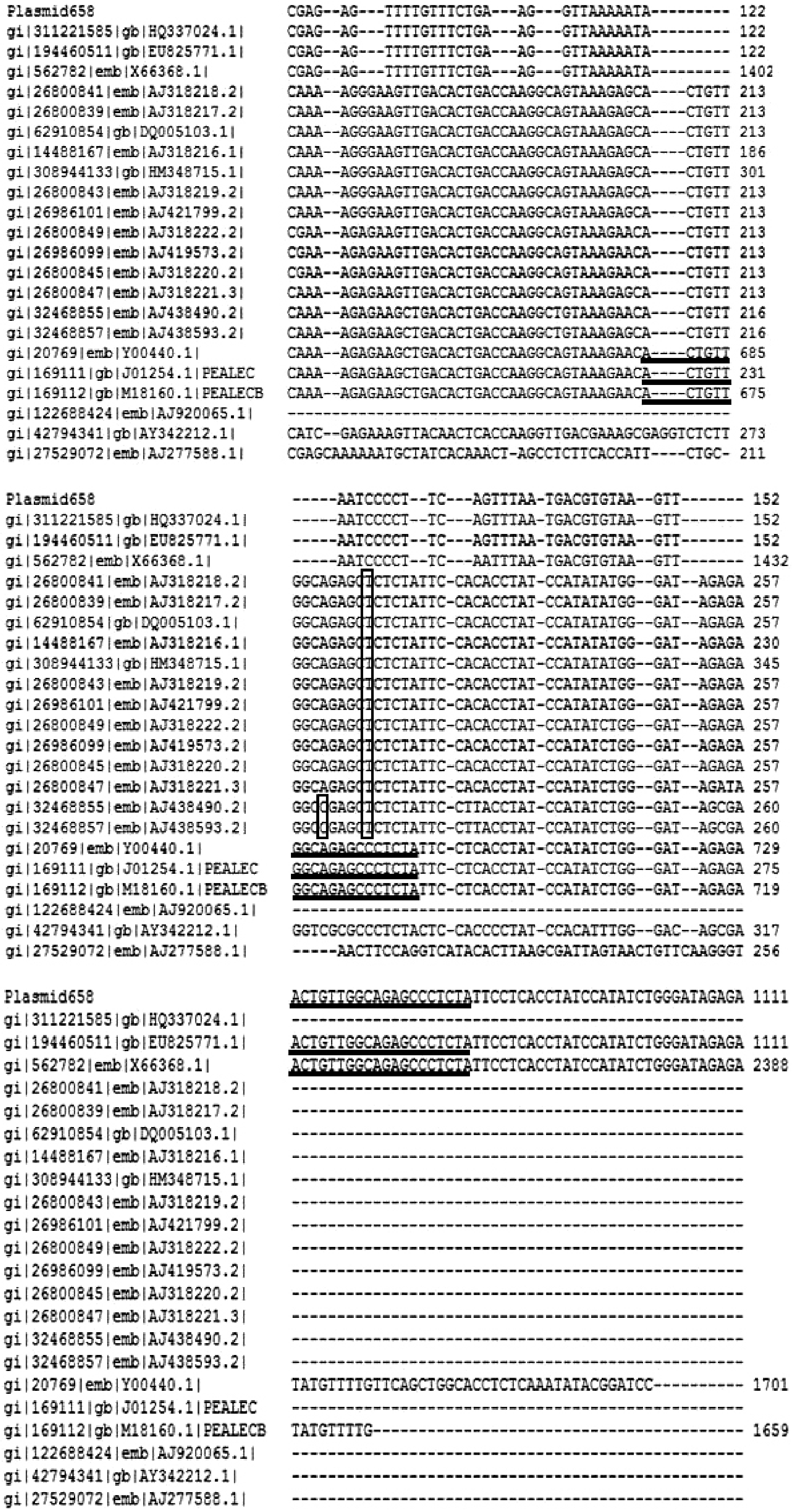
Figure 2. The alignment of forward primer Lec101 with sequences of the lectin gene of selected species of the family *Fabaceae.* Target sequence for the primer is underlied. Mismatches in target sequences are labelled in a black box



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gi|32468855|emb|AJ438490.2|      GGCCGAGCTCTCTATTC-CTTACCTAT-CCATATCTGG--GAT--AGCGA 260
gi|32468857|emb|AJ438593.2|      GGCCGAGCTCTCTATTC-CTTACCTAT-CCATATCTGG--GAT--AGCGA 260
gi|20769|emb|Y00440.1|           GGCAGAGCCCTCTATTC-CTCACCTAT-CCATATCTGG--GAT--AGAGA 729
gi|169111|gb|J01254.1|PEALEC     GGCAGAGCCCTCTATTC-CTCACCTAT-CCATATCTGG--GAT--AGAGA 275
gi|169112|gb|M18160.1|PEALECB    GGCAGAGCCCTCTATTC-CTCACCTAT-CCATATCTGG--GAT--AGAGA 719
gi|122688424|emb|AJ920065.1|     --------------------------------------------------
gi|42794341|gb|AY342212.1|       GGTCGCGCCCTCTACTC-CACCCCTAT-CCACATTTGG--GAC--AGCGA 317
gi|27529072|emb|AJ277588.1|      -----AACTTCCAGGTCATACACTTAAGCGATTAGTAACTGTTCAAGGGT 256

Plasmid658                       ACTGTTGGCAGAGCCCTCTATTCCTCACCTATCCATATCTGGGATAGAGA 1111
gi|311221585|gb|HQ337024.1|      --------------------------------------------------
gi|194460511|gb|EU825771.1|      ACTGTTGGCAGAGCCCTCTATTCCTCACCTATCCATATCTGGGATAGAGA 1111
gi|562782|emb|X66368.1|          ACTGTTGGCAGAGCCCTCTATTCCTCACCTATCCATATCTGGGATAGAGA 2388
gi|26800841|emb|AJ318218.2|      --------------------------------------------------
gi|26800839|emb|AJ318217.2|      --------------------------------------------------
gi|62910854|gb|DQ005103.1|       --------------------------------------------------
gi|14488167|emb|AJ318216.1|      --------------------------------------------------
gi|308944133|gb|HM348715.1|      --------------------------------------------------
gi|26800843|emb|AJ318219.2|      --------------------------------------------------
gi|26986101|emb|AJ421799.2|      --------------------------------------------------
gi|26800849|emb|AJ318222.2|      --------------------------------------------------
gi|26986099|emb|AJ419573.2|      --------------------------------------------------
gi|26800845|emb|AJ318220.2|      --------------------------------------------------
gi|26800847|emb|AJ318221.3|      --------------------------------------------------
gi|32468855|emb|AJ438490.2|      --------------------------------------------------
gi|32468857|emb|AJ438593.2|      --------------------------------------------------
gi|20769|emb|Y00440.1|           TATGTTTTGTTCAGCTGGCACCTCTCAAATATACGGATCC---------- 1701
gi|169111|gb|J01254.1|PEALEC     --------------------------------------------------
gi|169112|gb|M18160.1|PEALECB    TATGTTTTG----------------------------------------- 1659
gi|122688424|emb|AJ920065.1|     --------------------------------------------------
gi|42794341|gb|AY342212.1|       --------------------------------------------------
gi|27529072|emb|AJ277588.1|      --------------------------------------------------
```

Figure 3. The alignment of reverse primer Lec101 with sequences of the lectin gene of selected species of the family *Fabaceae*. Target sequence for primer is underlied. Mismatches in target sequences are labelled in a black box

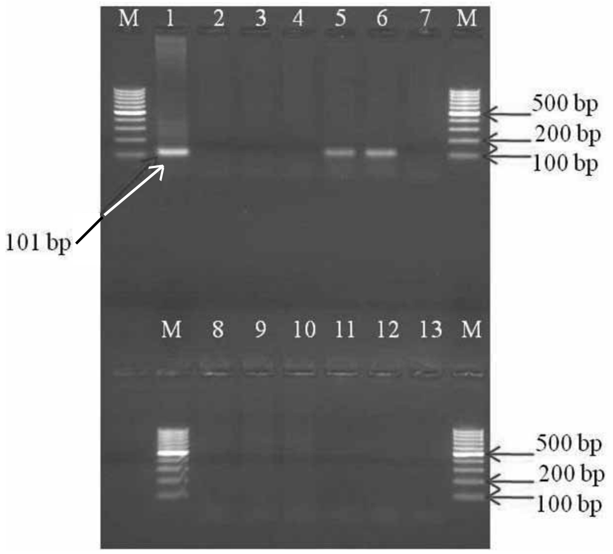


Figure 4. Verification of Lec101 primers specificity via PCR on *Fabaceae* plants: 1 – plasmid control, 2 – vetch, 3 – soybean A, 4 – soybean B , 5 – pea (Raman) A, 6 – pea (Raman) B, 7 – lentil A, 8 – lentil B, 9 – bean A, 10 – bean B, 11 – CTRL EX, 12 – CTRL MM, 13 – CTRL MM – open

Results of *in silico* analysis showed that both Lec101 primers show 100% homology only for the garden pea exon part of lectin gene (Y00440.1, J01254.1, M18160.1, EU825771.1, and X66368.1). However, both primers show certain homology to several clones of lentil, mung bean and vetch (HM348715.1, AJ318222.2, AJ318220.2, AJ419573.2, AJ318221.3, AJ421799.2, J318219.2, AJ318218.2, AJ318217.2, DQ005103.1, AJ318216.1, AJ438490.2, AJ438593.2). Mismatches were identified within available sequences corresponding to primer sites (Figures 2 and 3).

In experimental test, no PCR products were recorded when vetch, soya-bean, lentil and bean DNAs were used as template DNA, while amplification of all pea DNA isolates showed the expected amplification products (Figures 4 and 5).

To allow quantification, optimised protocol was used in real-time PCR with SYBR® Green detection system regardless its disadvantage, i.e. tendency to inhibit PCR at higher concentration (Karsai *et al.* 2002; Giglio *et al.* 2003; Gasparic *et al.* 2010).

SYBR® Green real-time PCR was specific also only for samples of garden pea – no amplification signal was recorded when the DNAs from the family *Fabaceae* were used as a template that confirmed previous results obtained by conventional PCR.

To allow quantification, optimised protocol was used in real-time PCR with SYBR® Green detection

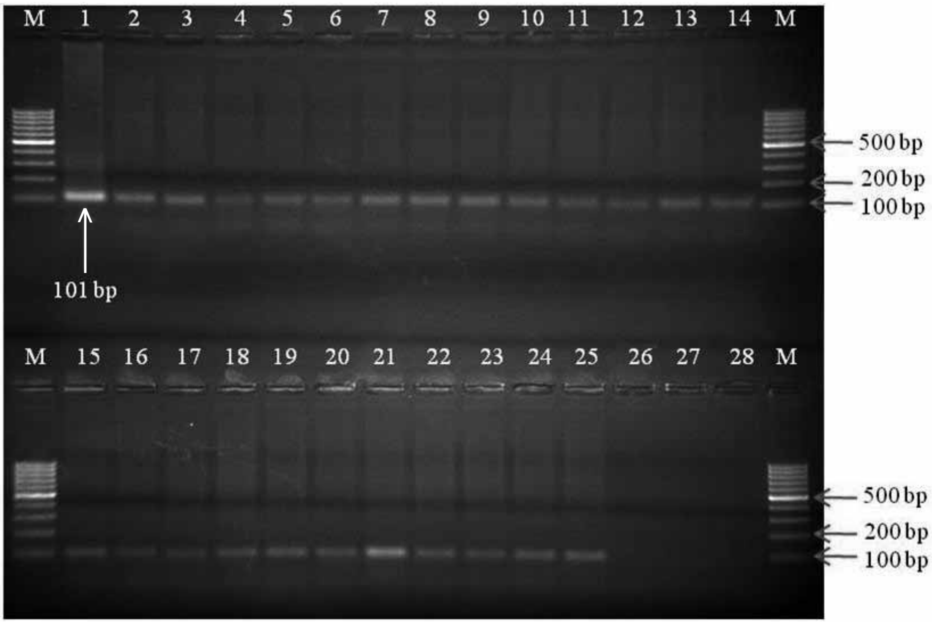


Figure 5. Verification of Lec101 primers specificity via PCR on selected pea species: 1 – plasmid control, 2 – Alderman A, 3 – Alderman B, 4 – Arvika A, 5 – Arvika B, 6 – Bajka A, 7 – Bajka B, 8 – Hero A, 9 – Hero B, 10 – Dalila A, 11 – Dalila B, 12 – Havel A, 13 – Havel B, 14 – Oskar A, 15 – Oskar B, 16 – Pegaz A, 17 – Pegaz B, 18 – Rondo A, 19 – Rondo B, 20 – Kelvedon Wonder A, 21 – Kelvedon Wonder B, 22 – Ambrosia A, 23 – Ambrosia B, 24 – Delikata A, 25 – Delikata B, 26 – CTRL EX. 27 – CTRL MM, 28 – CTRL MM – open

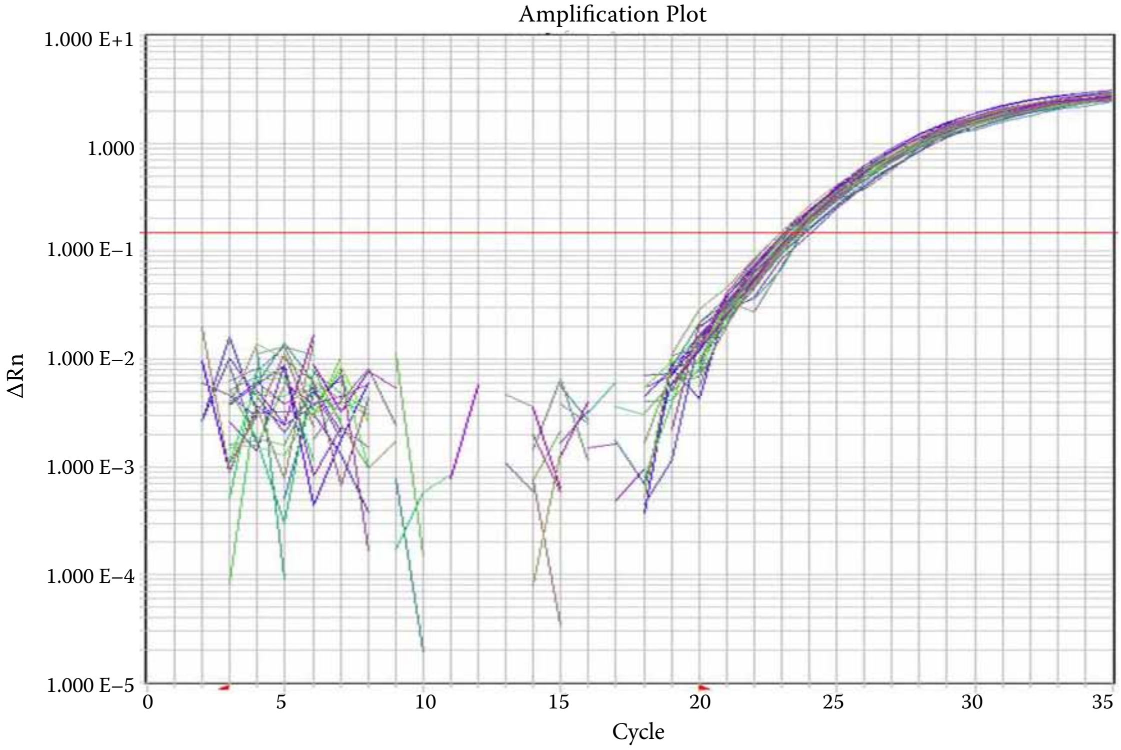


Figure 6. Fluorescent signal of garden pea cultivars with the average value of 23.56 ± 0.36 Ct

system regardless its disadvantage, i.e. tendency to inhibit PCR at higher concentration (Karsai *et al.* 2002; Giglio *et al.* 2003; Gasparic *et al.* 2010).

SYBR® Green real-time PCR was specific also only for samples of garden pea – no amplification signal was recorded when the DNAs from the family *Fabaceae* were used as a template that confirmed previous results obtained by conventional PCR.

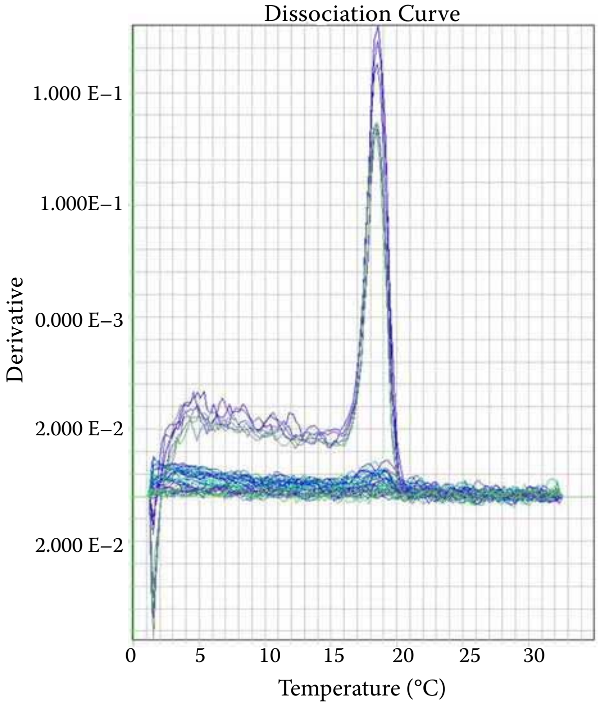


Figure 7. Dissociation curves of real-time PCR, selected plants of the family *Fabaceae* and pea cultivars

Obtained Ct values of reaction based on garden pea DNA (200 ng of DNA in reaction, corresponding to approx. 41 000 genome copies) reached on average 23.56 ± 0.36, which is an acceptable value for analytical assay (Figure 6).

Dissociation melting curves of final PCR products confirmed that reaction lead to a unique specific product amplification (Figure 7).

The specific product size of 101 bp was also checked by gel electrophoresis (data not shown). A difference of 3.23 Ct between the signal of pea samples at a DNA concentration of 40 ng/µl (23.28 Ct) and the same 10-fold diluted samples (26.51 Ct) exclude inhibition by template (Figure 8). The results confirm that DNAs quality is fitting for the purpose.

Real-time PCR efficiency was calculated for genomic DNA as a template. The efficiency was 99% and the average value of R^2 was 0.997 (Figure 9).

On the base of dilution, limit of quantification was defined to be 160 copies of garden pea DNA. That is the lower limit of linear range, in which the reaction fulfils an acceptance criteria for real-time PCR based GMO quantification (ENGL 2008). The

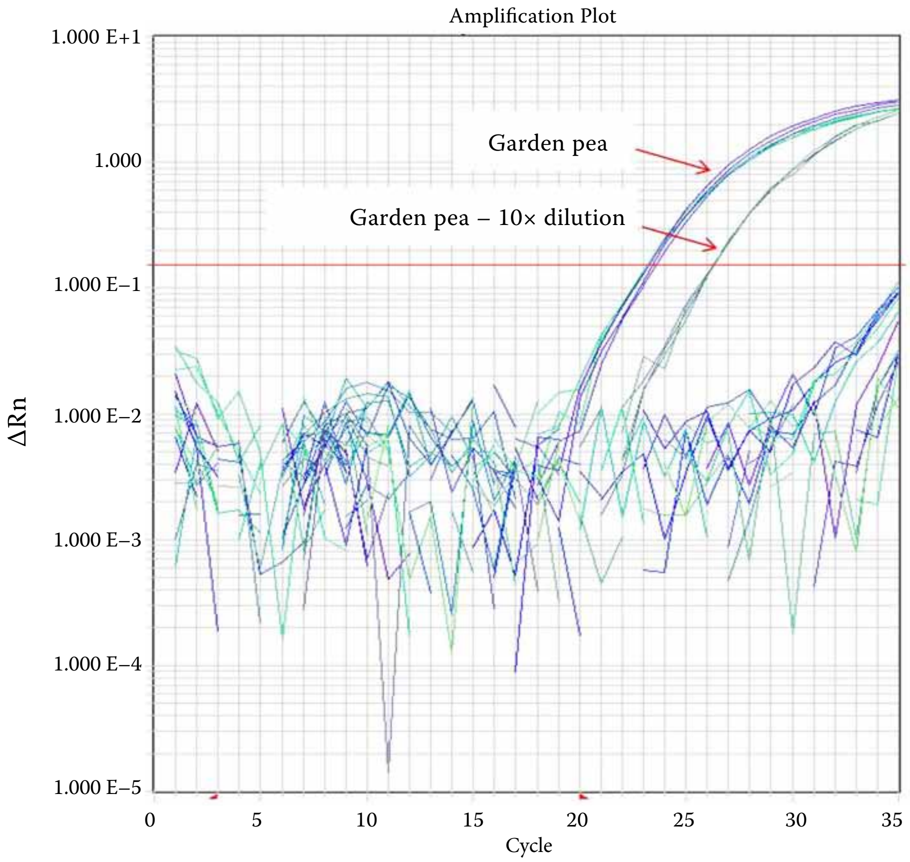
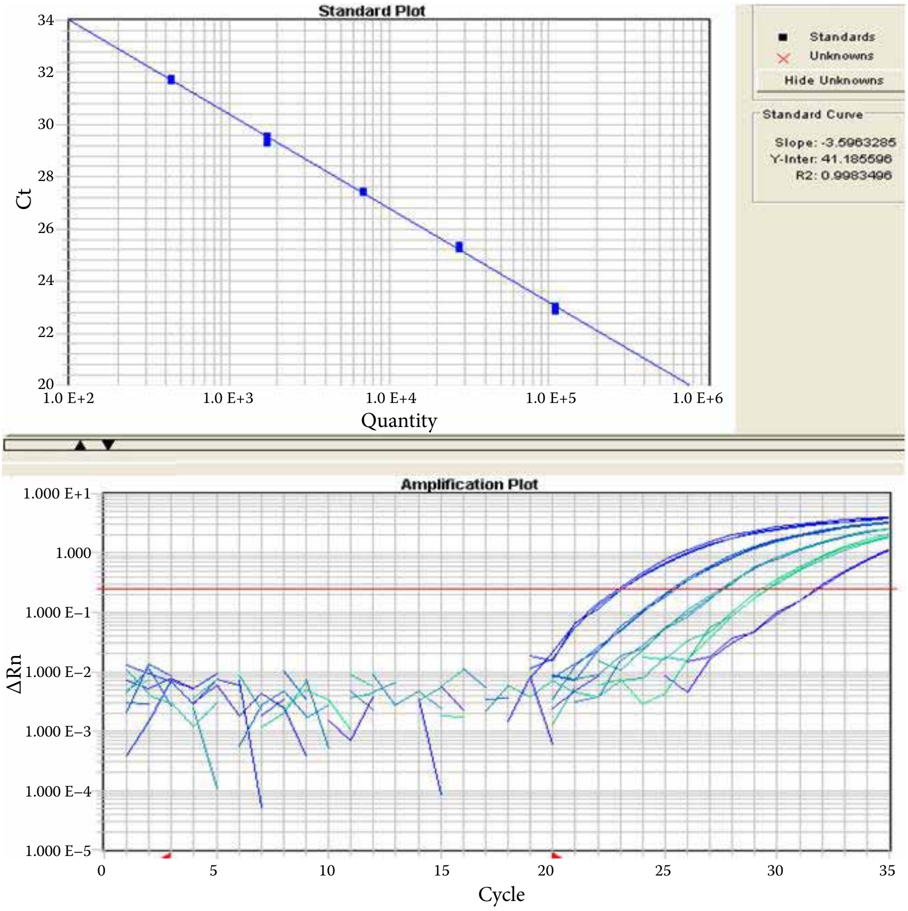


Figure 8. Control of the reaction inhibition signal of pea samples at DNA concentration of 40 ng/µl (23.28 Ct) and the same 10-fold diluted samples (26.51 Ct)



Standard Curve

Slope: -3.5963285
Y-Inter: 41.185596
R2: 0.9983496

Figure 9. Serial of dilution of genomic DNA for the calculation of real-time PCR efficiency

the limit of detection was revealed to be 40 copies of garden pea DNA.

As the reaction fulfill also criteria described by Ahmed (2002) and Anklam *et al.* (2002) we verified that the amplicon suits as a comparator for the quantification in real-time PCR.

In conclusion, we designed and optimised the real-time PCR based assay for specific detection and quantification of garden pea lectin gene (*P. sativum* L.). This reaction was developed to be used as a reference system for the quantification of GM pea varieties on the market. Although the SYBR Green assay is based on non-specific fluorescent detection, the characteristic of reaction meets requirements for GMO detection defined by the Community Reference Laboratory (CRL) for GM food and feed (ENGL 2008). The assay could be used as well for relative quantification of pathogens as it was shown for other species (Leisova *et al.* 2006) or allergens (Wensing *et al.* 2003). Moreover, positive plasmid control containing part of garden pea lectin gene is available and can be used according currently available standards.

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