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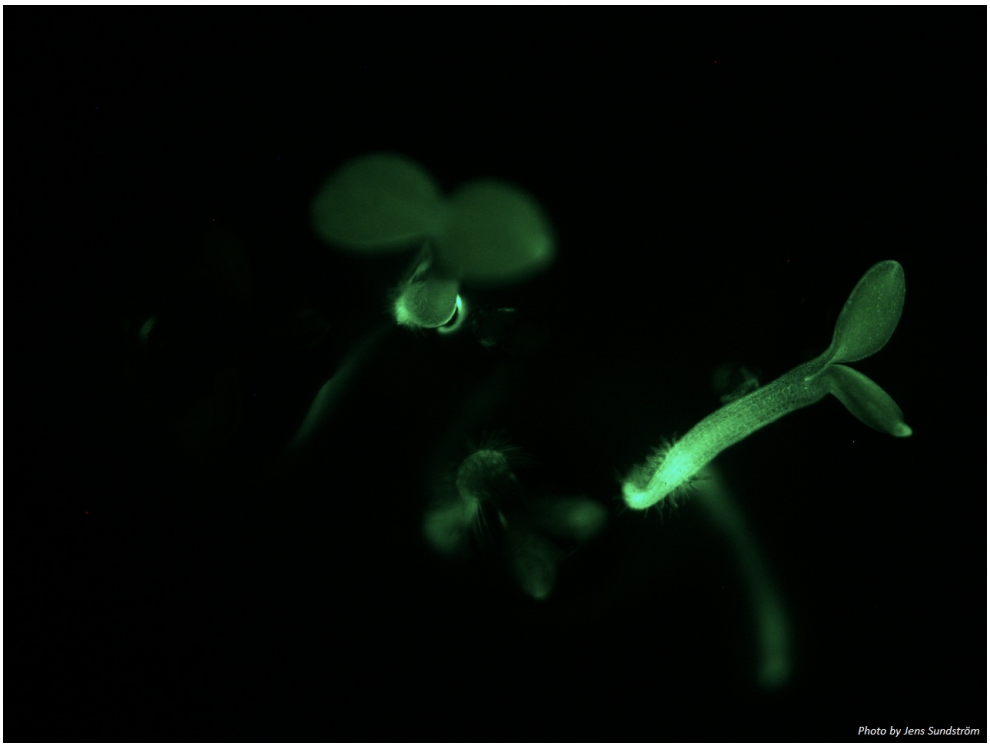
Proof of concept for a transgenic DNA-tag in genetically modified plants for non food or non feed purposes



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Abstract

Within the current regulatory framework in the EU all genetically modified (GM) food has labeling and traceability requirements. To this date all approved GM-crops have undergone thorough risk assessment with the conclusion that they do not have adverse effects on human or animal health or the environment in comparison with its parental line. GM-plants that are constructed for a technical or industrial purpose, designated as non-food/non-feed GM (NFGM) are not primarily intended as food or feed and may in some cases even pose a threat to consumers or livestock if occurring in food and feed. The need for a standardized, inexpensive and reliable method for detection and characterization of NFGM-plants has been recognized by national and international competent authorities. A DNA-tag was designed to fill this need with the name Plant-Made Industrial or Pharmaceutical Products Tag (PMIP-T). PMIP-T is envisioned to act as a silent DNA identifier to facilitate identification and characterization of NFGM-plants in food or feed samples. This projects principal aim was to deliver proof of concept that the PMIP-T-tag could be detected in different kinds of groceries (wheat flour, soy, raw and fried meatballs) at an accredited GMO laboratory. This goal was achieved with the conclusion that the PMIP-T-tag is a stable and reliable way to identify NFGM.

Introduction

Background on the legislation of genetically modified organisms in Europe and the *de facto* moratorium from 1998 to 2004

The introduction of genetically modified organisms (GMO) used for food and feed to the European market in 1996 could not have come in a worse time. Only months before, the great scare of the "Mad Cow disease" had shaken the public's trust in the science community (Jasanoff, 1997). Media outlets covered the introduction of GMOs rigorously and even though the issues were non-related, GM-crops were associated with something fundamentally wrong in the eye of the public. This was a prequel to the *de facto* moratorium on GMOs in Europe during 1998 to 2004, when the first GMO in over 5 years was approved (Scholderer, 2005). The moratorium, though never officially agreed upon, was later recognized and deemed illegal by the world trade organization (WTO, 2010).

During the moratorium new and extensive regulations regarding GMOs was put in place. It has been argued that there was a shift from science-based legislations to policy-based legislation, taken the public opinion into consideration where before the concerns had been primarily scientific (Johansson, 2009). The new regulatory framework consists of primarily two legal instruments: Directive 2001/18/EC and Regulation (EC) No 1829/2003. Directive 2001/18/EC outlines the principles for, and regulates deliberate release of GMOs into the environment in the EU. Regulation (EC) No 1829/2003 outlines the principles for and regulates the placing on the market of food and feed consisting of, containing, or produced from GMOs (Plan, Wan den Eede, 2012).

The European Food Safety Authority (EFSA) is the agency responsible for the scientific risk assessment on food and feed in Europe. Food- and feed-plants are evaluated with respect to adverse effects on human or animal health or the environment, in relation to its parental comparator (EFSA, 2011). Upon a favorably opinion from the EFSA a draft decision is presented to the Regulatory Committee composed of representatives from the member states. If a qualified majority is not obtained, the draft decision is passed on to the Council of ministers for another vote. If neither rejection nor authorization is determined within three months, the commission shall adopt the decision (Plan, Wan den Eede, 2012).

Because of the strict regulations, only three plants are allowed to be cultivated in Europe at the moment, two events of maize: MON810 (MON-ØØ81Ø-6), an insect resistant event and T25 (ACS-ZMØØ3-2), and an herbicide tolerant event. A potato with modified starch property, EH92 (BPS-25271-9), is also approved for feed but intended for industrial purposes (European Commission A, 2012). There is however more than 40 GMOs approved for food and feed that are imported to the EU, a majority of which are maize events.

The strict regulations on GMOs for food and feed purposes in Europe is perhaps best illustrated in the opinion made by the EFSA on non-food/non-feed GM-plants (NFGM), plants that are made for pharmaceutical or technical applications (European Food Safety, 2009). The conclusion is that although special attention is to be applied to NFGM, the

comparative method used in the ordinary risk assessment on GMO's is adequate (EFSA, 2011). The NFGM have completely different applications in comparison with the food- and feed plants. Where food- and feed-plants are meant for human or animal consumption, the NFGM are constructed to have a medical or technical application through, for example, amplification of a transgene's product up to 5000 times (Spok, 2007). Worth noting is that only because a plant is intended for an industrial purpose and not for food and feed, like the BPS-25271-9 potato with a modified starch composition, it does not necessarily pose any threat to the environment or animal health, recognized through the approval of the potato for feed. But some may pose very real dangers to human or animal health.

Project description

Hence, with the introduction of GMOs intended for pharmaceutical or technical applications there is a need for a standardization of the methods for detection and identification of the NFGM entering the market. The current project aims to meet this need by providing proof-of-concept for an identification-tag, envisaged to occur as a transgenic silent DNA identifier in NFGM-plants. Thus making the detection of the NFGM-plants simple and inexpensive, as specified in the review paper that laid the foundation for the project (Alderborn et al., 2010). The DNA-tag, with the proposed name Plant-Made Industrial or Pharmaceutical Products Tag (PMIP-T), is suggested to be incorporated in all future industrial GM-plants that are going to be placed on the European market. In designing it four different specific criteria was targeted:

• The method of detection, i.e. the proposed real-time PCR platform, should conform to standard GMO detection procedures in European accredited GMO laboratories.

• One single pair of PCR primers should be able to detect the presence of any PMIP-T fragment, regardless of specific identification code.

• The combination of PCR primers used should specifically target the PMIP-T fragment, thereby not producing any unspecific PCR product.

• The DNA-tag should contain an identification code, being unique for each NFGM-event.

The suggested method is based upon real-time PCR-detection in line with the methods in place at accredited European GMO laboratories. The design of the PMIP-T construct with two right primers is due to the optimization of detection using a Taqman probe in real-time PCR, with a 70 nucleotide long synthesized amplicon, represented by the distance between the left primer (LP) and the first right primer (RP1). The amplicon produced in a PCR reaction using the left primer and the second right primer (RPII) is 150 nucleotides long and implemented to allow for identification of an event specific DNA-tag using either Sanger- or Pyro-sequencing, but also for detection in end-point PCR (figure 1).

Figure 1. The construct of the PMIP-T fragment proposed to be used as primers for detection of NFGM. The left primer (LP) and the first right primer (RPI) contains the 70 nucleotides long synthesized amplicon Taqman probe optimized for detection in real-time PCR. The inclusion of the second right primer (RPII) is primarily for Pyro- and Sanger- sequencing, and end-point PCR detection.

The entire recombinant DNA molecule inserted into Arabidopsis in the current project consists of two antibiotic markers, hygromycin and kanamycin flanking the PMIP-T-fragment. (figure 2).

Figure 2. The schematic order of the sequence inserted in wild type *A. thaliana*. LB is the left border, KAN represents the Kanamycin-gene marker, PMIP-T represents the insert, and HYG the hygromycin-marker gene, and RB the right border.

To ensure that the primers are only specific to a certain set of sequences, randomly aligned DNA-sequences from an extinct species, *Mammuthus primigenius*, were used. The primers have been exposed to thorough BLAST (Basic Local Alignment Search Tool) searches against all of the currently available public databases and if similar sequences were found, alterations in the nitrogen bases were made. They were also tested for cross reaction with real-time PCR from a selection of 40 species that is common in food and feed, both plants and animals, none of which amplified.

For fast detection and characterization a unique and event specific code was constructed, based on a quaternary number system with the nitrogen bases, as follows: A=0, C=1, G=2 and T=3. With nine nitrogen bases it is possible to construct 262144 unique sequences, by far exceeding the necessity of labeling in the foreseeable future. It could be noted that although the NFGM-tag is only intended for industrial or pharmaceutical crops the number of possible combinations exceeds, by far, the number of GM-crops currently being developed.

The project is a collaboration with researchers at the Swedish National Food Agency (Martin Sandberg, Ulf Hammerling, Christer Andersson) , Uppsala University (Anders Alderborn) and Swedish University of Agricultural Sciences (Jens Sundström). At BioCenter (SLU) the construct was inserted into two separate lines, named #1477 and #1932 (the years Uppsala University and The Swedish University of Agricultural Sciences were founded), of the model organism *A. thaliana* via a cloning vector.

The reason for doing two separate lines were partly that it would allow us to replicate the detection in the real-time PCR, and to make crosses between the two lines to create a stacked event and determine the level of detection on stacked events. Prior to my involvement in the

project, single insert events of the PMIP-T #1477-line have been identified and used in qRT-PCR experiments, this thesis focuses on the PMIP-T line #1932- and serves as an independent repetition of previous experiments.

The development of the transgenic lines was made at BioCenter (SLU), as well as a Non-radioactive DIG-Southern Blot to identify transgenic PMIP-T lines that only harbor one insert.

To simulate real-life conditions the transgenic Arabidopsis-plant with one insert were diluted into a wild type Arabidopsis-plant, and processed according to standard food procedures for industrial food processing. Since the concerned agency for GMO detection procedures in Sweden is the GMO accredited laboratory at SLV the experiments regarding detection were conducted there. The absolute limit of detection was determined with a controlled number of genomic copies of the PMIP-T- insert mixed in different foods: raw and fried meatballs, wheat flour and in soy.

Aim

The short-term goal is to demonstrate a proof-of-concept that the PMIP-T-tag is stable and can be used to detect a NGGM-plant in processed food according to methods used in accredited European GMO laboratories. The long-term goal for the whole project is to make the PMIP-T-tag a mandatory requirement for all NFGM-plants in Europe.

Results

Concentration and sample purity of DNA from PMIP_T#1932-events and wild type *A. thaliana* ecotype L.(er) extracted for Southern Blot

The concentration on the DNA extracted from the from PMIP_T#1932-events and wild type *A. thaliana* ecotype L.(er) to use on the Southern Blot was not sufficient (table 1). The DNA was concentrated through SpeedVac.

Table 1. Concentration and sample purity of extracted DNA from PMIP_T#1932-events and wild type *A. thaliana* ecotype L.(er)

1932-event	DNA conc. ng/µl	260/280
619	10.9	1.7
729	26.6	1.8
2614	16	1.82
WT	15	1.77

Southern Blot on PMIP_T#1932-events and wild type *A. thaliana* ecotype L.(er)

In order to establish how many inserts of the PMIP-T fragment was present three selected events of PMIP-T #1932 were tested on a Southern Blot. DNA from the three PMIP-T #1932 events 619, 729 and 2614 was cleaved using either XhoI or HindIII. Both enzymes cleave once within the inserted DNA and lanes with only one band is indicative of single insert events. The membrane was hybridized with a DIG labeled ntpII probe which bind to the kanamycin marker gene present within the t-DNA (figure 2). The PMIP-T #1932 event 619 gave a signal corresponding to a ~20 kbp cleavage product with the Xho1 endonuclease (figure 3). 729 has one signal at ~20 kbp in the XhoI endonuclease reaction, and one signal at ~3.5 kbp in the HindIII reaction. 2614-event showed multiple signals on both reactions and has multiple inserts. This experiment have been repeated with an independent DNA-extraction from the same #1932-events and a DIG-probe that binds to the Hygromycin marker, the 619 indicated a single insert in a analogues way (Supplemental figure 1).

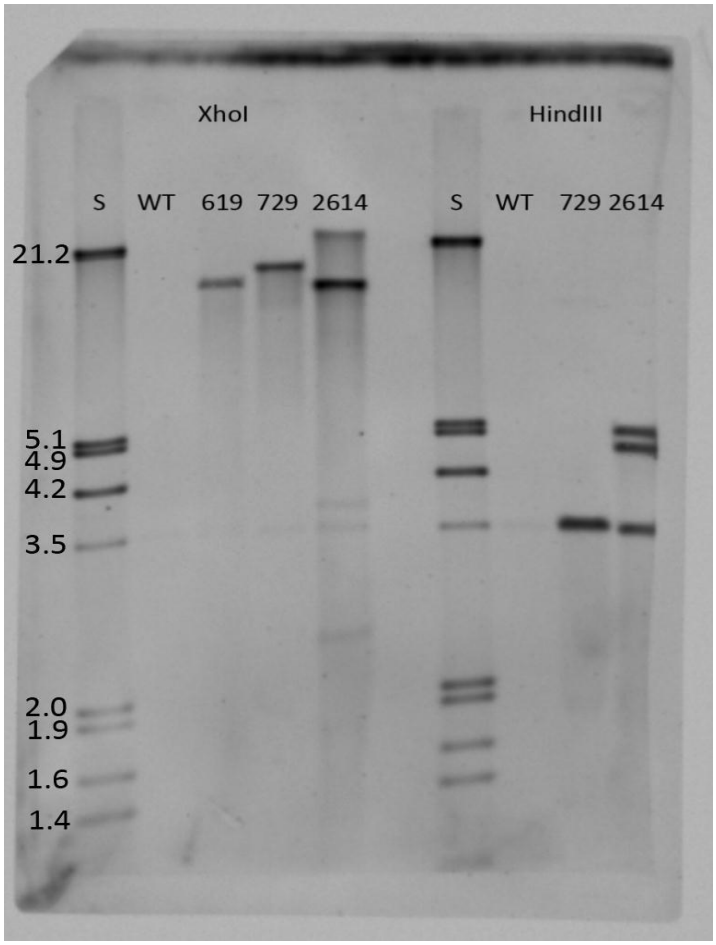

Figure 3. The results from the Southern Blot on the PMIP_T#1932-events (619, 729 and 2614) and wild type *A. thaliana* ecotype L.(er) (WT). The S is the ladder and the number represents kbp. XhoI and HindIII are the endonuclease enzymes that were used for cleavage. The 619- and 729-events gives a single signal at ~ 20 kbp in the XhoI-reaction. The 619-event have previously been cleaved with HindIII and a hygromycin probe showing one band (Supplemental figure 1). This shows that the 629-event have one insert of the PMIP-T-sequence . The 2614-event seems to have three inserts at least.

DNA extraction on PMIP_T#1932 and wild type *A. thaliana* ecotype L.(er)-strands for use on PCR

DNA was extracted from all strands except the WT-strand using the Qiagen DNeasy® Plant Mini Kit method. DNA concentration was then measured according to the DNA concentration measurement method (Table 2).

Table 2. Concentration and sample purity (260/280) on extracted DNA from PMIP-T-events and wild type *A. thaliana*.

1932-event	DNA conc. ng/µl	260/280
619	55.64	1.69
729	50.16	1.82
2614	53.38	1.87
Control	2.58	1.43

Real-time PCR on #1932-events

To determine if the results from the Southern Blot could be repeated a real-time PCR was made on the #1932-events. The 619 and 729 events reached the threshold at approximately the same Ct cycle (~20) whereas event 2614 amplified at Ct cycle 19 indicating that 2614 may harbor additional copies of the PMIP-T fragment in accordance with the southern blot experiments above (Figure 3). According to these data the 619- and 729-events have the PMIP-T-insert (Table 3).

Table 3. Number of cycles to reach the threshold in real-time PCR on PMIP_T#1932-events

1932-event	Cycles (C_t)
619	20.8
619	20.7
729	20.4
729	20.8
2614	18.9
2614	19
control	34.9
control	34.9
H2O	undetermined
H2O	undetermined

The material the results of the real-time PCRs is based upon is interpreted from the raw data from SDS 2.3 software (Supplemental figure 2).

The WT brought from SLU was prepared in the same way as the 1932-events but on a separate occasion. Final DNA concentration was 86.5 ng/µl and the sample purity (260/280) was 1.93.

Absolute LOD on PMIP_T#1932:619-event

To determine the lowest quantity of the substance, the PMIP-T-fragment, that could be detected an absolute LOD was performed. The 1932:619-event was prepared in 10 levels according to the method described in Absolute Limit of Detection, with 3 wells per sample. The lowest positive 3/3 detection was 6.25 copies, although 1/3 copies were detected on the 0.78 level (Table 4)

Table 4. Absolute limit of detection on the #1932:619-event

Genome-copies	PCR-replicates	Positive amount
5000	3	3/3
500	3	3/3
100	3	3/3
50	3	3/3
25	3	3/3
12.5	3	3/3
6.25	3	3/3
3.125	3	2/3
1.563	3	0/3
0.781	3	1/3

The 1932:619-event Absolute Limit of detection test using real-time PCR showed a 3/3 detection rate down to 6,25 genome-copies in the sample. The sequence was detected at 0,781 copies however, insinuating that detection level for the sequence in real-time PCR in extracted DNA from plant tissue is one copy.

The DNA extracted from the WT seems to be contaminated and the sequence was detected at fairly low Ct-level, thus arising suspicion that it was pre-contaminated.

This was confirmed with a real-time PCR consisting of WT from previous batch and the new WT (table 5). Since both have been contaminated but the already existing batch had such a small amount of contamination it was considered insignificant.

Table 5. Real-time PCR confirming wild type contamination of PMIP_T-sequence

Sample	Cycle threshold for detection of PMIP sequence
WT old 1	40.2
WT old 2	41
WT new 1	35.2
WT new 1	34.9

The table shows the results of the Real-time PCR confirming that the WT brought from SLU (New) have been contaminated with PMIP sequence, detection in the 41th and 40.2th cycles of the old WT suggesting insignificant contamination.

DNA-extraction from wheat flour samples

DNA was then extracted from the wheat flour samples according to the DNA extraction using the National Food Agency method SLV K2-m237.9-f. Extracted DNA was then measured according to the method specified in DNA concentration measurement (Table 6).

Table 6. Concentration and sample purity on extracted DNA from wheat flour dilution series

Dilutions of #1932:619 in wheat flour in %	DNA conc. ng/µl	260/280
0.1	242	2.03
0.01	454	2.01
0.001	262	2.01
0.0001	191	1.99
0.00001	189	2.04
WT 1%	389	1.97

LOD in wheat flour

#1932:619-event was diluted in wheat flour ranging from 0.1% to 0.00001%. Relative LODs was made to determine the detectability of the PMIP-T-sequence in wheat flour. It was detected to 0.001% in 6/8 PCR replicates (table 7).

Table 7. Detection of #1932:619-event in wheat flour

% of 1932:619 mixed in wheat flour	PCR-replicates	Positive Amount
0.1	8	8/8
0.01	8	8/8
0.001	8	6/8
0.0001	8	2/8
0.00001	8	0/8

The table shows the results from two separate relative LODs (3 replicates and 5 replicates) of the 1932:619 mixed in wheat flour. The 1932:619-event was detected at 0.001% with 6/8 positive hits.

End-point PCR on wheat flour samples

We then made an end-point PCR according to end-point PCR protocol on the wheat flour samples, and thereafter an electrophoresis according to the Gel electrophoresis-protocol (figure 4). The limit of detection was lower in the real-time PCR than in the end-point PCR, 0.001% respectively 0.01%.

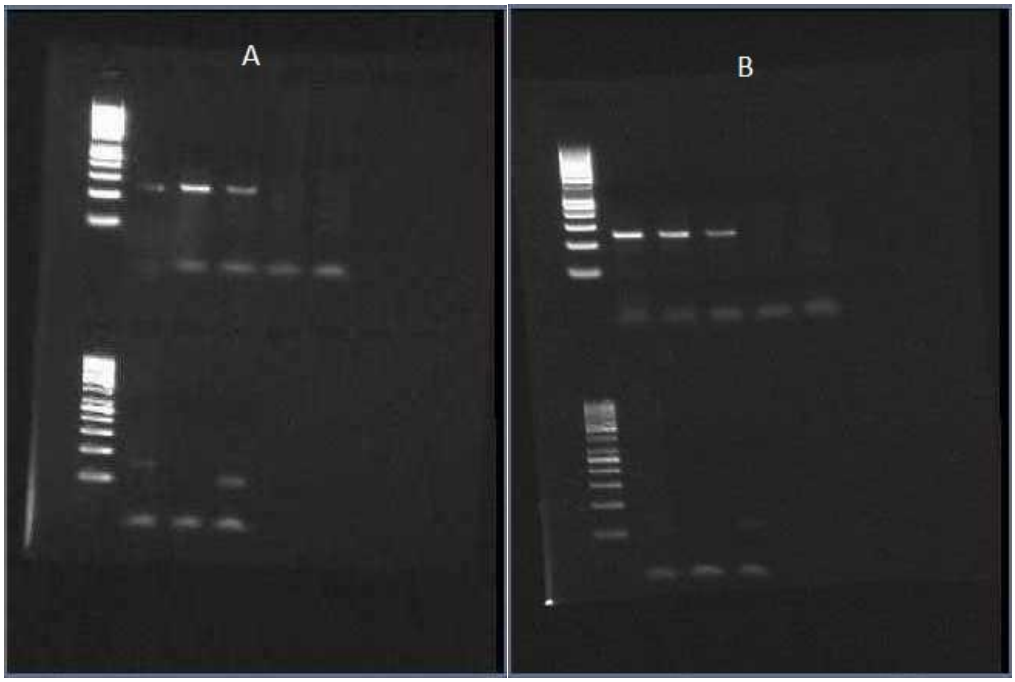

Figure 4.Gel electrophoresis showing the detection of the PMIP-sequence. The first three wells from the upper left in A & B indicates the detection with 1 % #1932:619 in WT *A. thaliana*, and 0.1 and 0.01 % detection of #1932:619 in wheat flour.

LOD in soy DNA

#1932:619-event was diluted in soy-DNA ranging from 0.1% to 0.00001%. Relative LODs was made to determine the detectability of the PMIP-T-sequence in soy-DNA. It was detected to 0.001% in 10/10 PCR replicates and 5/10 replicates at 0.00001% (table 8).

Table 8. Detection of #1932:619-event in soy-DNA

% of 1932:619 mixed in soy-DNA	PCR-replicates	Positive Amount
0.1	10	10/10
0.01	10	10/10
0.001	10	10/10
0.0001	10	4/10
0.00001	10	5/10

LOD of raw and fried meatballs

#1932:619-event was diluted in the prepared ground beef ranging from 0.1% to 0.00001%. Relative LODs was made to determine the detectability of the PMIP-T-sequence in raw and fried grounded beef-steaks. It was detected to 0.001% in 5/8 and 7/8 replicates in raw and fried mixes respectively (table 9). It was detected at 1/8 and 2/8 replicates at 0.00001% in raw and fried mixes respectively.

Table 9. Detection of #1932:619-event in meatballs

% of #1932:619-event mixed in ground beef-steaks	Replicates	Positive amount
0,1 raw	8	8/8
0,1 fried	8	8/8
0,01 raw	8	8/8
0,01 fried	8	8/8
0,001 raw	8	5/8
0,001 fried	8	7/8
0,0001 raw	8	2/8
0,0001 fried	8	1/8
0,00001 raw	8	1/8
0,00001 fried	8	2/8
Grounded beef + WT A. th raw	7	0/7

The table shows the results from two separate relative LODs of the #1932:619-event mixed in raw and fried meatballs. The #1932:619-event was detected at 0.001% with 7/8 positive hits on fried meatballs and with 5/8 positive hits on raw meatballs at 0.01%. Both in the raw and fried meatballs the PMIP-T-sequence were detected at 0.00001%.

Methods & Materials

Methods

Preparation of plant tissue from wild type *A. thaliana* ecotype L.(er) and #1932-lines of *A. thaliana*

Transgenic Arabidopsis strands harboring a PMIP-T#1932 construct and wild type *A. thaliana* ecotype L.(er) (WT) was cultivated under short night conditions (8 hours darkness) in the phytotron at BioCenter (SLU). Samples were prepared through grinding in liquid nitrogen. Plant tissue used at SLV was freeze dried for 24 hours in HETO™ freeze dryer prior use.

Non-radioactive DIG-Southern Blot

DIG-Southern Blot was essentially done according to manufacturer's instructions (Roche, 2009). Below is a brief description of the crucial steps.

Digestion Reaction

The extracted DNA from the PMIP_T#1932-events (619, 729, 2614) and wild type *A. thaliana* ecotype L.(er) (WT) were cleaved using endonucleases, HindIII and XhoI, and incubated at 37 ^{o}C. 1µg and 0.5 µg of genomic DNA was cleaved respectively (table 10).

Table 10. Digestion reaction with restriction endonucleases Xho1 (1 µg) and HindIII (0.5 µg) on the #1932-events and WT.

Substrate	**Volume**
DNA 1 µg or 0.5 µg	x µl
Restriction Enzyme	5 µl
Standard Buffer	5 µl
dH_20	y µl
total	50 µl

The table shows the template for the digestion reaction on the DNA extracted from the #1932-events. Since the DNA was not diluted to the same before mixing the volumes differ of DNA and Water differ accordingly. Note that it was 1 µg in the Xho1-reaction, and 0.5 µg in the HindIII-reaction.

Gel Electrophoresis

The remaining digested DNA was run on a 0.6 % Agarose gel in Tris/Borate/EDTA-buffer at 30 V overnight. Molecular weight marker DIG 0.12-231 kb (Roche) was used to visualize band size. Cleavage products and markers were .heated for 1 minute at 65 ^{o}C and then cooled 1 minute on ice prior loading. To each sample of the digested DNA 5 µl of loading buffer was added. For lanes loaded with the molecular weight marker 1,25 µl of loading buffer, 1,25 µl marker and 10 µl mH20 was loaded.

Transfer of DNA to a nylon membrane

Prior transfer, the gel was depurinated in 0.25 M HCL for 15 minutes, denatured in 2x15 minutes in (0.5M NaOH, 1.5M NaCl) and neutralized in 0.5M Tris, 1.5M NaCl for 2x15 minutes. To transfer the DNA in the gel to a nylon membrane the gel was placed on the pieces of pre-cut 3M papers followed by a nylon membrane and 5 additional pre-cut 3M papers. To facilitate transfer paper towels (500 g) were added to the assembly and left for 5 hours with one exchange.

After the transfer the paper towels were discarded and the 3M papers were removed one at a time. To fixate the transferred DNA the membrane was crosslinked twice using a crosslinker. The membrane was prehybridized (DIG Easy Hybridization) in 42 ^{o}C for 20 minutes. A premade kanamycin probe (NPTII) (Kindly provided by) was boiled and thereafter cooled on ice for 5 minutes before applied to the membrane and hybridized overnight at 42 ^{o}C.

Washing of the membrane and detection of hybridization with chemiluminiscent

A series of washes were done, first low stringency wash (2x SSC + 0.1% SDS) for 2 x 5 minutes at room temperature, then a high stringency wash (0.1x SSC + 0.1% SDS) for 2 x 15 minutes at 65 °C. To prevent non-specific binding of the DIG antibody blocking solution (1x Blocking) was added for 45 minutes. To allow detection of the DIG-labeled DNA probe the membrane were incubated with Anti-Digoxigen

in-AP antibodies (1:20 000) for 1 hour at room temperature. A final wash was made with DIG2 (DIG1 + 0.3% TWEEN 20) before adding detection buffer (100 mM Tris, 100mM NaCl), in order to detect the nucleic acids.

The membrane was placed in a plastic bag and a chemiluminscence substrate (CPD star) was applied evenly while closing the bag, covered with paper due to the light sensitivity of CPD star and incubated for 5 minutes. Any excess fluid was removed before the bag was heat-sealed. The detection of the chemiluminscence signal was made in the LAS-3000 lite camera system, with incrementing exposure for 25 minutes

DNA extraction from plant tissue from the #1932-events and wild type *A. thaliana* ecotype L.(er)-strain using the DNeasy® Plant Mini Kit

DNA extractions for Southern Blot at BioC for Southern Blot were made according to Qiagen DNeasy® Plant mini kit, with 2 x 50 µl of the Buffer AE on the 12th step. Due to insufficient DNA concentration the samples were concentrated using a SpeedVac.

DNA extractions from freeze-dried plant material at SLV were made with a few alterations to the protocol provided by the manufacturer. Instead of using the recommended amount of a maximum of 20 mg of dried plant tissue 50 mg were used to increase the final DNA yield. Because of this all solutions up until step 10 were doubled, but incubation and centrifugation times remained the same. Because the volume in step 7 was more than 650 µl x 2, step 8 had to be repeated one extra time. In step 12, 2 x 50 µl of the Buffer AE was used to increase the

DNA yield. To control for contamination during the preparation, 2 negative extraction controls without plant material were used.

DNA extraction from the wheat flour and meatball samples using the National Food Agency method SLV K2-m237.9-f

The method used is based on the Qiagen DNeasy® Blood & Tissue kit. It is slightly modified by SLV to fit different kinds of foodstuff as well as plant tissue, although plant tissue is preferably extracted with the previous method due to some uncertainties regarding this method and pure plant material. This method is accredited by the Swedish Board for Accreditation and Conformity Assessment.

Circa 100 mg of the dried sample was put in the Lysing Matrix A tubes, with 2 negative extraction controls. 720 µl ATL buffer were added to every sample and then homogenized using BIO 101 FastPrep-120® homogenizer for 40 seconds at speed level 4, and centrifuged before they were opened. 80 µl Proteinas K was added, then vortexed and incubated at 55 ^{o}C overnight.

The tubes were centrifuged a short while before they were opened, 400 µl AL buffer was added, thoroughly mixed by vortexing and then incubated at 70 ^{o}C for 10 minutes. The tubes were centrifuged at 16 000 x g for 5 minutes. The supernatant was added to a 2 ml eppendorf-tube with 1 part ethanol so that the final concentration was 50-53 % ethanol, and then vortexed. The DNeasy mini spin column was placed in a collection tube (2 ml), the sample was added to the column, about 600 µl, and centrifuged for 1 minute at 6000 x g. This was repeated until all the sample had passed through the column (it was repeated 3 times). The flow through and the collection tubes were discarded.

The columns were washed by adding 500 µl AW1 buffer and then centrifuged for 1 minute at 6000 x g. The 500 µl AW2 buffer were added and centrifuged for 3 minutes at 16 000 x g in order to dry the membrane. The columns were placed in new sterile eppendorf-tubes (with the caps removed) and 50 µl AE elution buffer were added to the membrane. It was incubated at room temperature for 1 minute, and then centrifuged for 1 minute at 6000 x g. This was repeated once to increase the final DNA yield.

The eluate was transferred to a new sterile eppendorf-tube.

DNA concentration measurement

DNA concentration was measured in the Nanodrop® ND-1000 spectrophotometer according to regulating document SLV K2-i736.3. Software ND-1000 V3.2.1 was used and since the program uses the formula Nucleic Acid concentration= (Absorbance * analysis constant) / (radiation length in cm) and we added 1 part 0.4 M NaOH (pH 9.2) to receive ssDNA, the concentration was multiplied with 2. The constant 37 is used because all DNA is single stranded. Sample purity is estimated through the ratio of absorptions between 260 nm and 280 nm, a number ~1.8 represents insignificant contamination of other molecules.

Real-time PCR

For the real-time PCRs a PCR mix of 40 µl/well and 10 µl of sample/well were made, with negative controls without DNA (table 11). MicroAmp optical 96-well reaction plate was used on the ABI prism 7900HT Real-time PCR system, set on standard mode using the SDS 2.3 software. The thermal profile was: 50 ^{o}C for 2:00 minutes, 95 ^{o}C for 10:00 minutes, and then 45 cycles on 95 ^{o}C for 0:15 minutes followed by 1:00 minute on 60 ^{o}C. All DNA amplified in the real-time PCR was diluted to 20 ng/µl.

Table 11. Real-time PCRmix-template with volume and concentration per well

Reactant	Volume (µl)/well	Final concentration
Primer PMIP-T_FP	5	0.3 µM
Primer PMIP-T_RPI	5	0.3 µM
Probe PMIP-T_Taq	5	0.2 µM
TaqMan® 2x PCR Master Mix	25	1x
DNA	10	
Total	50	

Table shows the template for the real-time PCR-mix with concentration and volume in every well on the MicroAmp optical 96-well reaction plate for every real-time PCR run on the 7900HT and on 9600 emulation.

End-point PCR

The PCR mix for the end-point PCR is different (table 12). MicroAmp reaction tubes with caps were used on the GeneAmp® PCR system 9700, the thermal profile being: 94 ^{o}C in 12 minutes, then 40 cycles of 94 ^{o}C in 0:10 minutes, 53 ^{o}C in 0:20 minutes, 72 ^{o}C in 0:15 minutes, and then 72 ^{o}C for 7:00 minutes followed by 4 ^{o}C indefinitely.

Table 12. End-point PCR mastermix template with volume and concentration per well

Reactant	Volume (µl)/well	Final concentration
10x PCR Buffer	26.75	5.35 x
dNTPs (20 µM)	5	2 µM
PMIP-FP (10 µM)	1	0.2 µM
PMIP-RP2 (10 µM)	1	0.2 µM
MgCl2 (25 mM)	1	1 mM
AmpliTaq Gold (5U/µl)	0.25	1x
Sample	10	
Total	50	

The end-point PCR mastermix template with concentration and volume in every MicroAmp reaction tube for every end-point PCR-run on the 9700-PCR.

Gel electrophoresis

The gel was made according to the National Food Agency method SLV K2-m267.6 with 2% MetaPhor® agarose in a 0,5x TBE buffer. GelRed™ diluted to 1:10000 was added before the gel had set. Then the loading dye solution, 2.2 µl for every sample, was added to a bit of Parafilm®. 20 µl of the PCR-product from the End-Point PCR was then mixed with the loading dye solution, and then administered to a well on the agarose gel, together with the size marker. The electrophoresis then ran for 45 min on 120 V in an 8 x 10 container.

Absolute Limit of Detection (LOD)

To determine how many genome-copies of the sequence the real-time PCR could detect an LOD was made. One haploid *A. thaliana* genome copy weighs circa 0.15 pg and the highest number of copies used was 5000 and the lowest was 0.78 copies (table 13). A dilution series between 5000 copies to 0.78 copies was made on the only transformation event that was known to have the insert in one copy: #1932:619. Detection of copies below one is of course only true in a statistical sense, it is stochastic and depends on whether the copy was transferred in the last dilution event or not.

Table 13. Number of genome copies and the corresponding weight for the Absolute limit of detection.

Copies	5000	500	100	50	25	12.5	6.25	3.125	1.563	0.781
Weight (pg)	750	75	15	7.5	3.75	1.875	0.937	0.468	0.234	0.117

The table show the number of genomic copies and the corresponding weight of the different levels in the first LOD-run. This was made to determine the number of copies the real-time PCR could detect.

LOD in wheat flour

To determine the relative detection limit of the #1932:619-event, it was diluted in wheat flour. A 1 % material was produced by mixing the freeze dried #1932:619-event with WT Arabidopsis. This was then serially diluted in wheat flour from 0.1 %, and then 10 times every step until 0.00001%. DNA was extracted according to the "DNA extraction from the wheat flour and ground beef samples using the National Food Agency method document SLV K2-m237.9-f"-method. The extracted DNA was prepared in 20 ng/µl solutions and amplified in real-time PCR. To study detection with the RPII primer end-point PCR was made, followed by electrophoresis.

LOD in soy DNA

Extracted DNA from the #1932:619-event was mixed with soy-DNA (IRMM Certified reference material) and serially diluted from 0.1% of #1932:619-event in soy-DNA and then ten times every step until 0.0001%. The extracted DNA was prepared in 20 ng/µl solutions and amplified in real-time PCR

LOD in meatballs

Ground beef was freeze-dried for 24 hours in HETO™ freeze dryer, grounded in a IKA-grinder and then sifted through a 1.7 mm sieve. The ground beef was mixed with the prepared mix of 1% #1932:619-event in wild type *A. thaliana*. Both the raw and fried samples were mixed from 0.1% and then ten times every step until 0.00001%. Water was then added to be able to form the ground beef in to meatballs, which were fried in sun flower oil. DNA was extracted using the "DNA extraction from the wheat flour and ground beef samples using the National Food Agency method document SLV K2-m237.9-f"-method. The extracted DNA was prepared in 20 ng/µl solutions and amplified in real-time PCR

Materials

DNA-extraction using DNeasy® Plant mini kit (version 08/2000 at SLV and version at SLU):

- Qiagen DNeasy® Plant mini kit
- MP Biomedicals Lysing Matrix A tubes
- BIO 101 FastPrep-® FP120

DNA-extraction using National Food Agency method SLV K2-m237.9-f:

- Qiagen DNeasy® Blood & Tissue kit including
    - ATL buffer
    - Proteinas K, 20 mg/ml
    - AL buffer
    - AW1 buffer
    - AW2 buffer
    - AE elution buffer
    - Spin columns
    - Collection tubes

- Ethanol 99.5 %
- MP Biomedicals Lysing Matrix A tubes
- BIO 101 FastPrep-® FP120

DNA concentration measurement:

- Nanodrop® ND-1000 spectrophotometer
- Software ND-1000 V3.2.1
- 0.4 M NaOH

Real-time PCR:

PCR mix:

- PMIP-T_FP 5'- AAG CGA CAA ACT CGC ACA AA-3'
- PMIP-T_RP1 5'- ACG TGC CAC ACA CAA CAA CA-3'
- PMIP-T_Taq 5'-(FAM) ACC GCT GAT GCT GCT GGC TGT TC-(TAMRA)-3'

Applied Biosystems TaqMan® 2x PCR Master Mix consisting of:

- PCR buffer
- $MgCl_2$
- AmpliTaq Gold
- dNTPs (dUTP, dATP, dCTP, dGTP)
- AmpErase®UNG

Equipment and software:

- ABI prism 7900HT Real-time PCR system standard mode
- Applied Biosystems MicroAmp optical 96-well reaction plate with bar code MicroAmp® Optical Adhesive Film
- Software SDS (sequence detection system) 2.3

End-point PCR:

PCR mix:

- PMIP-T_FP 5'- AAG CGA CAA ACT CGC ACA AA-3'
- PMIP-T_RP2 5'- AAC GCT GAA AAG CTG AAA TCC-3'
- dNTPs (dTTP, dATP, dCTP, dGTP) 20 mM each
- Applied Biosystems $MgCl_2$ solution (25 mM)
- Applied Biosystems AmpliTaq Gold (5U/µl)
- Applied Biosystems 10x PCR Gold Buffer

Equipment:

- ABI GeneAmp® PCR system 9700
- PE Biosystems MicroAmp reaction tube with cap

Gel electrophoresis:

- BioWhitaker Molecular Applications MetaPhor® agarose
- 0.5x TBE buffer
- DNA size marker with 50 bp ladder
- Fermentas 6x loading dye solution
- Biotium GelRed™ Nucleic Acid Gel Stain, 10.000X in water

Southern Blot:

- DIG 1: Maleic Acid 8.8g/L + NaCl 11.3g/L + NaOH 5.5g/L
- DIG 2: DIG1 + 0.3% TWEEN 20
- DIG 3: 100mM Tris pH 9.5-9.6 + 100mM NaCl
- 20x SSC
- Depurination: 0.25M HCl
- Denaturation: 0.5M NaOH, 1.5M NaCl
- Neutralization: 0.5M Tris, 1.5M NaCl
- 20% SDS
- 1:20,000 Anti-Digoxigenin-AP (ROCHE)
- 10X Blocking solution (ROCHE)
- DIG Easy Hyb (ROCHE)
- Restriction endonucleases: HindIII, XhoI
- Chemiluminscence substrate (CPD star)
- DNA Molecular Weight Marker III, DIG-labeled (ROCHE)

Discussion

Regarding the conducted experiments

The aim of the project was to provide a proof-of-concept on the detection of the PMIP-T-sequence in different foodstuffs in an accredited GMO laboratory. The limit of detection (LOD) of the sequence mixed in the three different groceries was 0.001%, conforming to the approach of the LOD at approximately 50% positive replicates. The LOD for fried and raw food was the same, indicating that the PMIP-T-tag is stable under normal food processing conditions. There are however considerably more harsh process methods that would be interesting to apply, such as deep-frying or conservation during 12 months or more. Particularly, it is known that there is notably more difficult to extract DNA from processed foods such as cornflakes, due to the fact that DNA deteriorates at high temperatures (Ohmori et al., 2008).

For the LODs dilutions were made through serial dilution by transfer of 1/10 of the solution from one tube to the next containing the foodstuff. In the absolute limit of detection, detection of the genomic copies is one at 0.781 but zero at 1.563 (table 8). Due to the stochastic nature of whether the copy is transported from the previous solution or not, the copy will either show up in 1.5683 or 0.781. The conclusion is that 45 cycles in the real-time PCR is sufficient for detection of all genomic copies of PMIP-T, and that if there is a copy, the real-time PCR will detect it.

Based on the results from the Southern Blots and the real-time PCR the number of inserts in #1932:2614-event is probably three, the insert in the 619-event seems to unambiguously be one. The 729-event do probably not have the full construct inserted, however the PMIP-T-tag seems to be there. With the schematic picture of the construct in mind (figure), the results from the two Southern Blots, with kanamycin- and hygromycin probes, indicates that the part of the T-DNA with the hygromycin-section did not recombine in the 729-event. The 729-event will be Sanger sequenced at a later time to determine this.

On the real-time PCR on the #1932-events there is considerable contamination in the control with wild type *A. thaliana*. There is two main suspicions where the contamination may have occurred. In the cultivation in the phytotron at BioCenter (SLU), the WT and the transgenic *A. thaliana* were grown in the same room. Cross-pollination due to insects is possible, emphasizing the need to keep WT- and transgenic lines separate during the whole process. Although this is a possibility, we think that it is far more likely that contamination occurred in the grinding process in liquid nitrogen of the #1932-line and the wild type *A. thaliana*. Whatever the case, the genomic DNA preparations from WT *A. thaliana* used in the experiments was from the batch that detected the presence of the PMIP-T fragment at 40 threshold cycles (C_t). Since the genomic preparation of the #1932-events had a C_t value of 20, the contamination does not interfere with the LOD experiments performed in the present study.

On a general note

The existence of GMOs is an intensely discussed subject all over the world. In the introduction I try to present a short background on the resistance against the technique. There is a lot to be said on the subject, and although this is not a thesis regarding the existence of GMO per se, but rather a method based on the legislation, some remarks may be of interest to the reader, specifically on the labeling and traceability requirements.

The labeling and traceability requirements on GMOs for food and feed are regulated by Regulation (EC) No 1830/2003. This legislation states that the highest percentage of technical intermixing of a GMO in food or feed allowed without labeling of the product as GMO is 0.9%. Its implementation has been criticized for being a political limit rather than a scientific (Nature, 2002, Davison, 2010). The EU-legislation states that the consumer should have the right to choose whether they want to buy a product consisting of, containing, or produced from GMOs (European Commission B, 2006) The public attitude is very concerned about the risk assessment of GMOs and have a low trust in the regulating agencies, and they want to be able to choose if they buy GM-food (Moon and Balasubramanian, 2004).

Although it would be presumptuous to argue that the public should not have the right to choose the food they eat or the feed they give to their animals, one also has to assess if the scientific qualifications imposed on the costumer in requiring an active choice in this matter is fair. The same public that is concerned with the rapid loss of biodiversity, the overuse of pesticides and many off the other problems facing conventional agriculture and the consequences thereof, seems to be the most critical of GMOs. In strong contrast with the position from the scientific community that the technique represents our best scientific chance to overcome these issues (Bruce, 2012, Van Montagu, 2011). Sadly, there is also a conflict between GMO supporters and the supporters of organic farming, although the very merging of GMOs with organic farming techniques represents the most sustainable way to feed the world (Ronald,C. Adamchak,W. 2009). This paradoxical relationship is maintained due to the organizations that approve organic farming as organic, which does not authorize the use of GMO as organic.

It is worth noting that there has been an overwhelming effort from the scientific community to assess beyond reasonable doubt whether the GMOs approved today pose a threat to consumers, animals or the environment. This is perhaps best summarized in the introduction of a thorough review covering the last decade of EU-funded GMO research:

“The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than e.g. conventional plant breeding technologies.” (European Commission C, 2010).

No one argues that GMOs should not be properly regulated and extensively researched prior to environmental release. What could be argued though, is that when a GMO is approved for cultivation as food and feed it should be treated as any other crop, because that is, by all meaningful definitions, what it is.

Conclusion

Because of what is at stake, the conversion of the public's opinion on GMOs is one of the scientific community's great challenges. A small part of this is to have reliable, cost-efficient and fast ways to detect GMOs that may have a considerable adverse effect on human health or the environment. This is the principal objective of the project as a whole. A part of this project was to demonstrate that a PMIP-T tag is stable and can be detected in processed food samples using the established methods implemented at accredited GMO laboratories. This has been demonstrated, reaching the end of this part of the project.

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I wish you all the best!

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Appendix A, Supplemental data

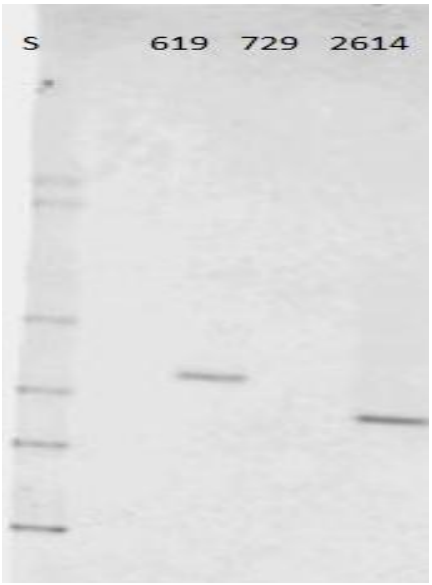

Figure 1. The figure shows a previous Southern Blot conducted with a hygromycin probe and HindIII restriction endonuclease. (unpublished results). S stands for marker, and the numbers represents the different events. The 729-event seems to have the PMIP-T-fragment but not the Hyg-fragment.

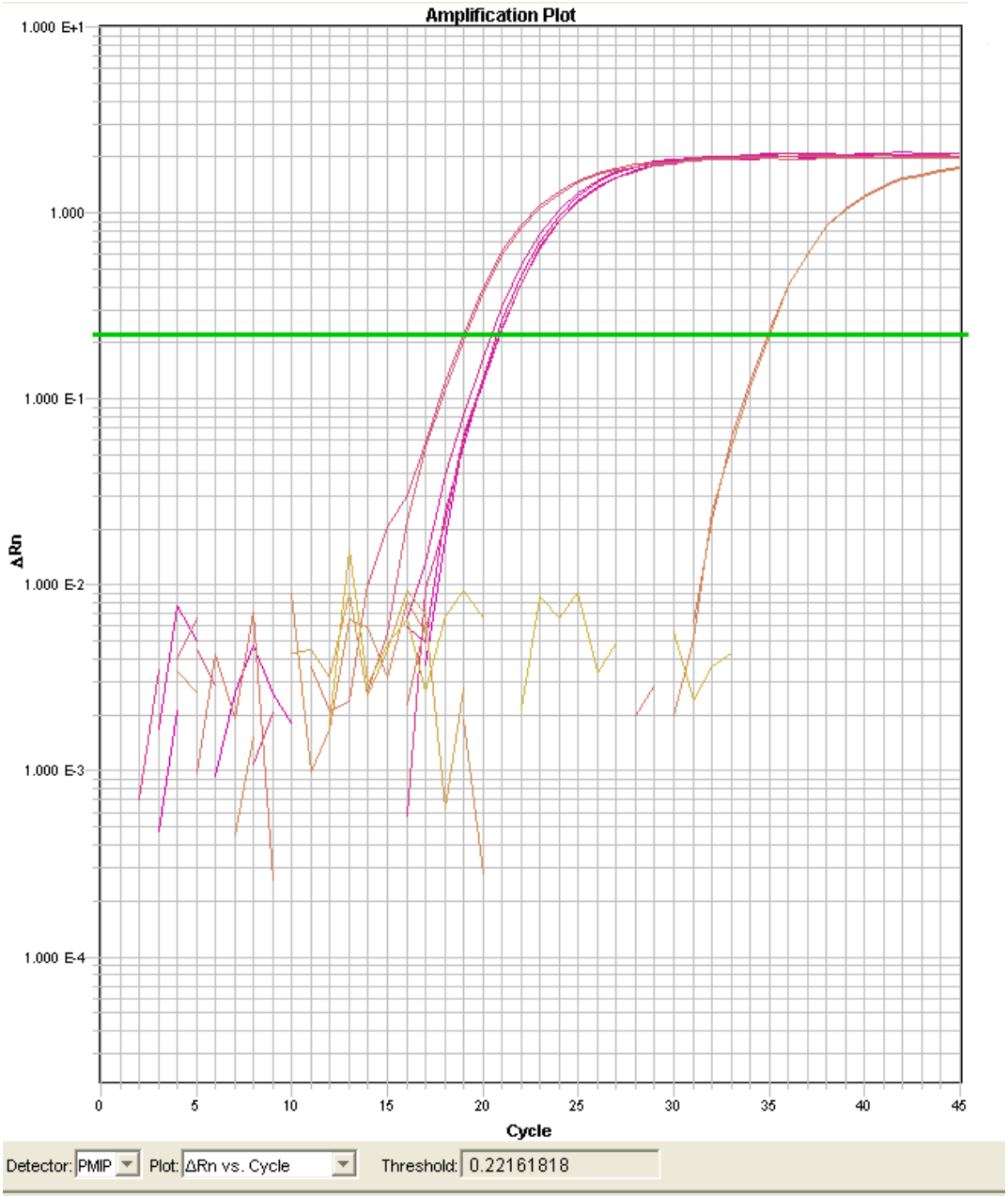

Figure 2. The figure shows the raw data from the real-time PCR on the different 1932-events. The green line represents the cycle threshold . The y-axis shows the Δ Rn, which is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye; i.e. Rn is the reporter signal normalized to the fluorescence signal of ROX™. An amplification plot shows the variation of log (ΔRn) with PCR cycle number. The x-axis represents the number of cycles the PCR-machine has repeated. Detection towards the right indicates a smaller amount of, in this case, copies of the PMIP-T-sequence.