



# Development and validation of a multiplex real-time PCR method for screening genetically modified plants

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## Abstract

The diversity of genetically modified plants used for food and feed is increasing worldwide. For the detection and control of these products, efficient and reliable analytical tools are a prerequisite. This can be done by screening for specific DNA-elements and constructs characteristic of transgenic plants. In the past, numerous methods have already been published. However, several genetically modified plants are not covered by common screening methods. Here, a new qualitative triplex real-time polymerase chain reaction (PCR) method is presented, detecting two transgene flanking sequences and the transition between the Cassava Vein Mosaic Virus Promotor (P-CsVMV) and the phosphinothricin-N-acetyltransferase (pat) gene. These sequences are present in several transgenic plants and therefore, the described triplex method can be used as a screening tool to guide further analysis and increase the efficiency of the analysis strategy for GMO detection. The method is characterized by high specificity, sensitivity and robustness and is provided as a ring-trial validated method in the Official Collection of Methods according to the German Food and Feed Act.

**Keywords** GMO · Qualitative multiplex real-time PCR · Screening · Flanking sequences · Cassava vein mosaic virus promoter · Ring-trial validation

## 1 Introduction

New genetically modified (GM) plants continue to be developed and commercialized. In many countries, especially within the European Union, strict authorization and specific labeling requirements must be met before the marketing of GM plants and their derived products is permitted. Therefore, the need for efficient and reliable analytical methods is

paramount for control and monitoring purposes. For nearly 30 years, screening methods based on polymerase chain reaction (PCR) have been widely employed for detecting the presence of genetic modifications in routine analyses of food, feed, and seeds (European Network of GMO Laboratories 2011; Grohmann et al. 2009; 2015; Holst-Jensen et al. 2003; Waiblinger et al. 2010). These screening methods target DNA sequences that are frequently inserted into GM plants, such as specific promoters, terminators, or genes conferring tolerance to herbicides or protection against certain insects (European Network of GMO Laboratories 2011; Holst-Jensen et al. 2003). Due to the lack of specificity in identifying particular GM events, positive screening results need further confirmation using construct- and/or event-specific methods, if available (ISO 21569:2005).

The most commonly targeted sequences include the 35S promoter (P-35S) derived from the cauliflower mosaic virus (CaMV) or its derivatives, as well as the terminator sequence from the nopaline synthase (T-nos) gene found in *Agrobacterium tumefaciens* (ISO 21569:2005; Waiblinger et al. 2008).

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Until a decade ago, the majority of GM plants listed in databases such as EUGINIUS: European GMO Initiative for a Unified Database System (2023) and ISAAA's GM Approval Database could be detected in an initial screening with no more than 5 screening target sequences (Bahrtdt et al. 2010; Waiblinger et al. 2010). However, over the last 10 years, the increasing diversity of genetic modifications has necessitated the identification of additional screening targets.

To economically and efficiently screen GM plants, multiplex PCR methods for simultaneously detecting various screening targets in a single PCR reaction have become increasingly important (BVL L 00.00-125:2008-12; BVL L 00.00-124:2008-12; BVL L 00.00-148:2014-02; Bahrtdt et al. 2010; Waiblinger et al. 2008). These methods minimize analysis costs and time, providing a convenient way to develop analysis strategies using GMO screening elements (EUGINIUS: European GMO Initiative for a Unified Database System 2023; Federal Office of Consumer Protection and Food Safety 2020b; Waiblinger et al. 2010).

In this study, the development and validation of a novel triplex real-time PCR method that targets 3 distinct DNA sequences found in various GM plants is presented. Ti plasmids are utilized as vectors in *Agrobacterium tumefaciens*-mediated plant transformations. Such transformations result in the integration of the genetic construct, often flanked by short Ti plasmid-derived border sequence motifs located at the left and/or right ends of the transgenic construct. The presence of these border sequence motifs in a plant indicates that the plant has undergone genetic modification.

To identify these border sequence motifs, we conducted a sequence alignment using publicly available information related to the transformation method. We compared sequence information from various GM plants known to have been transformed by *Agrobacterium* using the NCBI BLASTN tool, leading to the identification of two border sequence motifs known as AgroBorder I and AgroBorder II.

The selection of 3 target sequences was based on enhancing the toolbox for routine GMO screening, allowing for the simultaneous detection of a significant number of GM plants in a single PCR run. Furthermore, this additional screening may significantly reduce the need for candidate event-specific tests for GM plant identification when employing common screening matrices (EUGINIUS: European GMO Initiative for a Unified Database System 2023; Waiblinger et al. 2010).

The presented method facilitates the simple, parallel, sensitive, and robust qualitative detection of up to 3 DNA sequences found in transgenic plants. These sequences include the nos promoter sequence (AgroBorder I) from *Agrobacterium tumefaciens* (Ti plasmid type nopaline), a sequence (AgroBorder II) from *Agrobacterium tumefaciens*

(Ti plasmid type octopin, often flanking various transgenic plants), and a genetic construct comprising the cassava vein mosaic virus promoter (P-CsVMV) and the phosphinothricin-N-acetyltransferase gene (pat) sequences from *Streptomyces viridochromogenes* (P-CsVMV-pat). The method has been validated in an international ring trial conducted within the § 64 working group for GMO at the Federal Office of Consumer Protection and Food Safety (BVL). It has also been included in the Official Collection of Test Methods in accordance with the German Food and Feed Act (BVL L 00.00-176:2022-12).

## 2 Materials and methods

### 2.1 Plant material and DNA-samples

Certified plant reference materials were obtained from the Joint Research Centre (JRC) of the European Commission or from the American Oil Chemists' Society (AOCS) as leaf DNA or ground seed material. Materials from 11 different GM cotton events, 4 GM potato events, 24 GM maize events, 10 GM canola events, 1 GM rice event, 18 GM soybean events and 1 GM sugar beet event were used (Table 1). Detailed descriptions of commercial reference materials (catalogue numbers, GM content, status of the materials) are given in a publicly available list (Federal Office of Consumer Protection and Food Safety 2020a).

Further non-GM plant materials for specificity testing were collected from the market.

### 2.2 DNA extraction

DNA was extracted from ground seed materials with either the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) starting with a CTAB extraction (ISO 21571:2005), the NucleoSpin Food Mini Kit (Macherey-Nagel AG, Oensingen, Switzerland) or the Quick-DNA Plant/Seed Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany). The concentration of DNA was estimated fluorimetrically using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. Copy numbers were calculated on the basis of the genome sizes (Arumuganathan and Earle 1991). Zygosity of GM plant material (CRMs) was applied as stated on the respective analytical certificate. When a more precise specification of copy numbers was necessary (e.g. for determination of LOD), the target copy numbers of the DNA extracts of the GM plant materials were quantified by droplet digital PCR on the QX200 system (BioRad Laboratories, Feldkirchen, Germany) using event

**Table 1** Specificity of the triplex PCR method. The theoretical presence of the target sequences in the genome (theor.) and the experimental detectability of the target sequences in reference material (exp.) of GM events are shown

| Species            | Genetically modified event | AgroBorder I  |      | AgroBorder II |      | P-CsVMV-pat |      |   |
|--------------------|----------------------------|---------------|------|---------------|------|-------------|------|---|
|                    |                            | theor.        | exp. | theor.        | exp. | theor.      | exp. |   |
| <b>cotton</b>      | 281-24-236×3006-210-23     | -             | -    | +             | +    | -           | -    |   |
|                    | COT102                     | +             | +    | -             | -    | -           | -    |   |
|                    | GHB119                     | -             | -    | -             | -    | -           | -    |   |
|                    | GHB614                     | -             | -    | -             | -    | -           | -    |   |
|                    | LL25                       | -             | -    | -             | -    | -           | -    |   |
|                    | MON531                     | -             | -    | -             | -    | -           | -    |   |
|                    | MON1445                    | -             | -    | -             | -    | -           | -    |   |
|                    | MON15985                   | -             | -    | -             | -    | -           | -    |   |
|                    | MON88701                   | -             | *    | +             | +    | -           | -    |   |
|                    | MON88913                   | -             | -    | +             | +    | -           | -    |   |
| <b>maize</b>       | T304-40                    | -             | -    | -             | -    | -           | -    |   |
|                    | 3272                       | +             | +    | -             | -    | -           | -    |   |
|                    | 4114-3                     | -             | -    | -             | -    | -           | -    |   |
|                    | 5307                       | +             | +    | -             | -    | -           | -    |   |
|                    | DAS 59122                  | +             | +    | -             | -    | -           | -    |   |
|                    | 98140                      | +             | +    | -             | -    | -           | -    |   |
|                    | Bt11                       | -             | -    | -             | -    | -           | -    |   |
|                    | Bt176                      | -             | -    | -             | -    | -           | -    |   |
|                    | DAS-40278-9                | -             | -    | -             | -    | -           | -    |   |
|                    | DP-202216-6                | +             | +    | -             | -    | -           | -    |   |
|                    | GA21                       | -             | -    | -             | -    | -           | -    |   |
|                    | MIR162                     | +             | +    | -             | -    | -           | -    |   |
|                    | MIR604                     | +             | +    | -             | -    | -           | -    |   |
|                    | MON810                     | -             | -    | -             | -    | -           | -    |   |
|                    | MON863                     | -             | -    | -             | -    | -           | -    |   |
|                    | MON87403                   | -             | -    | +             | +    | -           | -    |   |
|                    | MON87427                   | -             | -    | +             | +    | -           | -    |   |
|                    | MON87460                   | -             | -    | +             | +    | -           | -    |   |
|                    | MON88017                   | -             | -    | +             | +    | -           | -    |   |
|                    | MON89034                   | -             | -    | +             | +    | -           | -    |   |
|                    | MZHG0JG                    | -             | -    | -             | -    | -           | -    |   |
|                    | NK603                      | -             | -    | -             | -    | -           | -    |   |
|                    | T25                        | -             | -    | -             | -    | -           | -    |   |
|                    | TC1507 (DAS 1507)          | -             | -    | -             | -    | -           | -    |   |
|                    | VCO-01981-5                | -             | -    | -             | -    | -           | -    |   |
|                    | <b>potato</b>              | AM04-1020     | [+]  | +             | -    | -           | -    | - |
|                    |                            | AV43-6-G7     | -    | -             | -    | -           | -    | - |
|                    |                            | EH92-527-1    | +    | +             | -    | -           | -    | - |
|                    |                            | PH05-026-0048 | [+]  | +             | -    | -           | -    | - |
|                    | <b>rapeseed</b>            | 73496         | -    | -             | -    | -           | -    | - |
|                    |                            | GT73          | -    | -             | +    | +           | -    | - |
|                    |                            | MON88302      | -    | -             | +    | +           | -    | - |
|                    |                            | MS1           | [+]  | +             | +    | +           | -    | - |
| MS8                |                            | -             | -    | -             | -    | -           | -    |   |
| RF1                |                            | [+]           | +    | +             | +    | -           | -    |   |
| RF2                |                            | [+]           | +    | -             | -    | -           | -    |   |
| RF3                |                            | -             | -    | -             | -    | -           | -    |   |
| T45                |                            | -             | -    | -             | -    | -           | -    |   |
| Topas19/2 (HCN 92) |                            | [+]           | (+)  | -             | -    | -           | -    |   |
| <b>rice</b>        |                            | LL62          | -    | -             | -    | -           | -    | - |

**Table 1** (continued)

| Species           | Genetically modified event | AgroBorder I |      | AgroBorder II |      | P-CsVMV-pat |      |
|-------------------|----------------------------|--------------|------|---------------|------|-------------|------|
|                   |                            | theor.       | exp. | theor.        | exp. | theor.      | exp. |
| <b>soybean</b>    | 305423                     | -            | -    | -             | -    | -           | -    |
|                   | 356043                     | -            | -    | -             | -    | -           | -    |
|                   | A2704-12                   | -            | -    | -             | -    | -           | -    |
|                   | A5547-127                  | -            | -    | -             | -    | -           | -    |
|                   | BPS-CV127-9                | -            | -    | -             | -    | -           | -    |
|                   | DAS-44406-6                | -            | -    | -             | -    | +           | +    |
|                   | DAS-68416-4                | -            | -    | -             | -    | +           | +    |
|                   | DAS-81419-2                | -            | -    | -             | -    | +           | +    |
|                   | FG72                       | -            | -    | -             | -    | -           | -    |
|                   | GMB151                     | -            | -    | -             | -    | -           | -    |
|                   | GTS 40-3-2                 | -            | -    | -             | -    | -           | -    |
|                   | MON87701                   | -            | -    | +             | +    | -           | -    |
|                   | MON87705                   | -            | -    | +             | +    | -           | -    |
|                   | MON87708                   | -            | -    | +             | +    | -           | -    |
|                   | MON87751                   | -            | -    | +             | +    | -           | -    |
|                   | MON87769                   | -            | -    | +             | +    | -           | -    |
|                   | MON89788                   | -            | -    | +             | +    | -           | -    |
|                   | SYHT0H2                    | -            | -    | -             | -    | -           | -    |
| <b>sugar beet</b> | H7-1                       | -            | -    | +             | +    | -           | -    |

“+” = positive result was obtained (approx. 10 copies/PCR of the target sequence were used)

“-“ = negative result was obtained (at least 2,500 copies/PCR of the haploid genome were used)

“\*” = weak amplification (at least 2,500 copies/PCR of the haploid genome were used; C<sub>q</sub> value  $\geq$  35)

“[+]” = one mismatch for theoretical specificity

“(+)” = no unambiguous experimental detection below approx. 25 copies/PCR of the target sequence (5/5 replicates positive at 25 copies/PCR, but 4/5 replicates positive at 10 copies/PCR)

specific methods (Bonfini et al. 2012). For further dilution of extracts, 0.2 x TE buffer was used as diluent.

### 2.3 Oligonucleotides

The primers and probes were developed in this study. *In silico* testing for complementarity or primer dimerization was performed using primer3 software (Untergasser et al. 2012). Each combination of the underlying primer sequences (Table 2) was tested. To theoretically test the specificity of the triplex detection method, a comparison was performed against genome sequences of 1,760 animal and plant species (Benson et al. 2013). A similar alignment was performed against DNA sequences from authorized and non-authorized GMO from the CCSIS (Central Core DNA Sequence Information System) database of the Joint Research Centre (JRC) of the European Commission (retrieved on 10/03/2020) using the e-PCR tool (Schuler 1997).

### 2.4 Real-time PCR analysis

DNA extracts (5  $\mu$ l) were added to 20  $\mu$ l of reaction mix containing SensiFast™ Probe No-ROX Kit (Bioline, Meridian

Bioscience, Cincinnati, USA), primers and probes (for final concentration see Table 2). The DNA concentration used depended on the respective question and is described in more detail in the corresponding sections. Oligonucleotides were purchased from Microsynth AG (Balgach, Switzerland). During the development phase PCR was performed on the Rotor-Gene 6000 (QIAGEN, Hilden, Germany) according to the following cycling protocol: Initial step of 15 min at 95 °C; followed by 45 cycles of 60 s at 94 °C and 90 s at 60 °C. Fluorescence data were collected during the annealing / elongation step at 60 °C. Further reaction master mixes and PCR devices were used as specified during the validation of the method (see 2.5.4).

## 2.5 Validation of the method

### 2.5.1 Specificity testing

For experimental testing of the specificity, i.e., the presence of the individual screening target in the material from the GM events, 10 copies of target DNA and at least 2,500 copies of non-target DNA, respectively, were used according to Table 1. Five replicates were tested for confirmation of the

**Table 2** Sequences, modifications and concentrations of the primers and probes of the triplex qPCR method for the simultaneous detection of DNA sequences from the cassava vein mosaic virus promotor-pat construct, AgroBorder I and AgroBorder II

| Name  | Final concentration | Sequence (5' to 3')                                       | Amplification Length | Reference |
|---|---------------------|---|----------------------|-----------|
| <b>AgroBorder I</b> (GenBank No. MK439386.1)  |                     |   |                      |           |
| AgroBS1 F                                     | 0.20 $\mu$ M        | TGA TCA TGA<br>GCG GAG AAT<br>TAA GGG                     | 76 bp                | this work |
| AgroBS1 R                                     | 0.20 $\mu$ M        | CCA AAC GTA<br>AAA CGG CTT<br>GTC C                       |                      |           |
| AgroBS1                                       | 0.08 $\mu$ M        | HEX-CGT<br>TAT GAC CCC<br>CGC CGA TGA<br>CGC-BHQ1         |                      |           |
| <b>AgroBorder II</b> (GenBank No. CP033030.1) |                     |   |                      |           |
| AgroBS2 F                                     | 0.50 $\mu$ M        | ATT TTG ATA<br>AAA CGA CAA<br>ATT ACG                     | 88 bp                | this work |
| AgroBS2 R                                     | 0.50 $\mu$ M        | TCG AAA TAA<br>AGA TTT CCG<br>AAT TAG                     |                      |           |
| AgroBS2                                       | 0.08 $\mu$ M        | Cy5-CCG TCG<br>TAT TTA TAG<br>GCG AAA GCA<br>ATA AAC-BHQ2 |                      |           |
| <b>P-Cs-VMV-pat</b> (GenBank No. JC006926)    |                     |   |                      |           |
| CsVMV F                                       | 0.20 $\mu$ M        | AGT ACT GAG<br>GAT ACA ACT<br>TCA GAG A                   | 107 bp               | this work |
| patsyn R                                      | 0.20 $\mu$ M        | GCG GCC ATA<br>TCA GCT GCT GT                             |                      |           |
| patsynp                                       | 0.08 $\mu$ M        | 6-FAM-CCG GAG<br>AGG AGA CCA<br>GTT GAG ATT AG<br>-BHQ1   |                      |           |

6-FAM, 6-Carboxyfluorescein; HEX, Hexachlorofluorescein; Cy5, Cyanine 5; BHQ1, Black Hole Quencher 1; BHQ2, Black Hole Quencher 2

presence and duplicates for the confirmation of the absence of target sequences, respectively.

### 2.5.2 Crosstalk

Any potential bleed-through of fluorescence signals between detection channels during multiplex PCR was tested experimentally (ENGL 2021). Mixtures of test samples containing no target DNA of the respective PCR module were tested in the presence of 2,500 copies of target DNA for the other two PCR modules ( $n=3$  per test). Triplex PCR was performed using the QuantiTect Multiplex PCR No ROX Kit (QIAGEN) on a LightCycler®480 Instrument II (LC 480 II, Roche Life Science, Mannheim, Germany). For this purpose, DNA from the GM events maize MIR162

(AgroBorder I), soybean MON87769 (AgroBorder II) and soybean DAS44406-6 (P-CsVMV-pat) was used.

### 2.5.3 Sensitivity

The limit of detection (LOD) was first determined by means of a dilution series of the target DNA according to the guidance document for single laboratory validation (Grohmann et al. 2016). For this purpose, DNA from the GM events maize MIR162 (AgroBorder I), soybean MON87769 (AgroBorder II) and soybean DAS44406-6 (P-CsVMV-pat) was used. Dilution levels of 20, 10, 5, 2, 1 and 0.1 mean copies of each DNA target per PCR were analyzed under symmetric conditions in a background of 100 ng of non-target DNA (UltraPure™ Salmon Sperm DNA, Thermo Fisher Scientific) in 12 replicates each. An additional calibration series was included in the PCR run of 2,500, 500, 100, and 50 copies of each DNA target per PCR in triplicates each. Two different thermocyclers were used: QuantStudio™ 5 (Thermo Fisher Scientific, Wilmington, DE, USA) and Rotor-Gene Q (QIAGEN).

For a first estimation, the lowest dilution level (i.e. the lowest number of copies) for which all 12 replicates are positive can be considered as the LOD<sub>95%</sub>. In addition, the concentration of the target DNA at which an amplification product is detected with a probability of at least 0.95 (LOD<sub>95%</sub>) was determined by means of a probability of detection model (POD curve) using a software tool as described in (Grohmann et al. 2016; Uhlig et al. 2015).

The confirmation of the LOD under asymmetric conditions was performed in accordance with the ENGL guidance document for multiplex PCR (European Network of GMO Laboratories 2021). In a single laboratory, 20 copies of each target were analyzed in the presence of 20,000 copies per reaction of each of the other targets (1:1,000). For each combination, 3 PCR replicates were tested. Additionally, sensitivity under both symmetric and asymmetric conditions were tested in an interlaboratory trial with 7 participating laboratories (see Sect. 2.5.4).

Moreover, the possible influence of background DNA present in routine samples was tested at the level of 30 copies per reaction of each target DNA together with 100 ng background DNA extracted from non-modified maize, canola, and soybean, respectively, corresponding to 0.03 to 0.08% cp/cp, in duplicates (Grohmann et al. 2016).

### 2.5.4 Robustness

Robustness was first tested inhouse under symmetric conditions on the QuantStudio™ 5 system (Thermo Fisher Scientific) using the Bioline SensiFast™ Probe No-ROX Kit. Twenty copies of target DNA in a mixture of DNA from

the GM events maize MIR162 (AgroBorder I), soybean MON87769 (AgroBorder II) and soybean DAS44406-6 (P-CsVMV-pat) were analyzed in a background of 100 ng of non-target DNA (UltraPure™ Salmon Sperm DNA, Thermo Fisher Scientific) per PCR in triplicates using a multifactorial experimental design (European Network of GMO Laboratories 2015; Grohmann et al. 2016). The following factors were changed compared to the protocol: Volume of PCR master mix (19 and 21 µl, 5 µl of sample DNA to be added), annealing temperature (59 and 61 °C), concentration of primers and probes (unchanged and -30%) (Table S1).

Similarly, a robustness test under asymmetric conditions was performed inhouse using the QuantiTect Multiplex PCR No ROX Kit in triplicates with 20 copies of one target DNA in the presence of 20,000 copies per reaction of the other targets (European Network of GMO Laboratories 2021). All reactions were run on a LightCycler®480 Instrument II (Roche Life Science, Mannheim, Germany). The following factors were changed compared to the standard protocol: Volume of PCR master mix (19 and 21 µl, 5 µl of sample DNA to be added), annealing temperature (59 and 61 °C), concentration of primers and probes (-10% and +10%, tested for each module independently) (Table S2).

Prior to the ring trial validation of the method, robustness data were determined by an interlaboratory test with 7 participating laboratories. Dilution levels of 50, 20, 10, 5, 1 and 0.1 mean copies/PCR of each DNA target (MIR162/AgroBorder I, MON87769/AgroBorder II, DAS44406-6/P-CsVMV-pat) under symmetric conditions (n = 12) and additional calibration series of 2,500, 500, 100, and 50 copies/PCR of each DNA target (n = 3) in a background of 100 ng of non-target DNA (UltraPure™ Salmon Sperm DNA, Thermo Fisher Scientific) were analyzed. In addition, a mixture of 10 copies of each target DNA in presence of 2,500 copies of the other 2 target sequences (1:250 asymmetric conditions) were analyzed using the following different combinations of PCR master mixes and real-time PCR cyclers: SensiFast Probe No-ROX Kit on Rotor-Gene 6000 (QIAGEN), Rotor-Gene Q and QuantStudio 5; QuantiTect Multiplex Kit on QuantStudio 5 and LC 480 II; PCRrely Mplex Mix (Gold Standard Diagnostics) on CFX96™ (Bio-Rad), and PerfeCTa® MultiPlex qPCR ToughMix® (Quantabio, MA, USA) on CFX96™ and Mx3005P™ (Agilent, CA, USA).

## 3 Results and discussion

### 3.1 Specificity testing

The specificity of the multiplex PCR method was initially assessed *in silico* to anticipate potential artifacts and false

positive results (European Network of GMO Laboratories 2021). Using Primer3, no increased probability of self-complementarity or primer dimerization was observed. Additionally, querying the Genbank and CCSIS databases did not reveal any unexpected similarities to other target sequences. Combining the primers in the triplex method did not predict the presence of any additional unintended amplicons.

Experimental testing of specificity involved analyzing DNA extracts from available reference materials of GM events. Table 1 provides a list of these events along with their qualitative signals obtained using the triplex method. All results aligned with the expectations based on the available theoretical sequence information. Notably, when DNA extracted from cotton event MON88701 was measured with 2,500 copies per PCR, a weak amplification with a Cq value exceeding approximately 35 was observed in the AgroBorder I - PCR. This result deviates from the theoretical absence of this sequence in this event and may be attributed to traces or contamination of material containing the AgroBorder I sequence in the certified reference material (AOCS 0113-A) used for DNA extraction. In a few events, one mismatch between a primer and the target sequence was theoretically predicted; however, PCR amplification was successful in all cases except for Topas19/2, which did not exhibit unambiguous detection below 25 copies per PCR (Table 1).

DNA extracted from non-GM materials, such as maize, soybean, canola, cotton, potato, sugar beet, and rice, did not yield any positive results.

### 3.2 Crosstalk

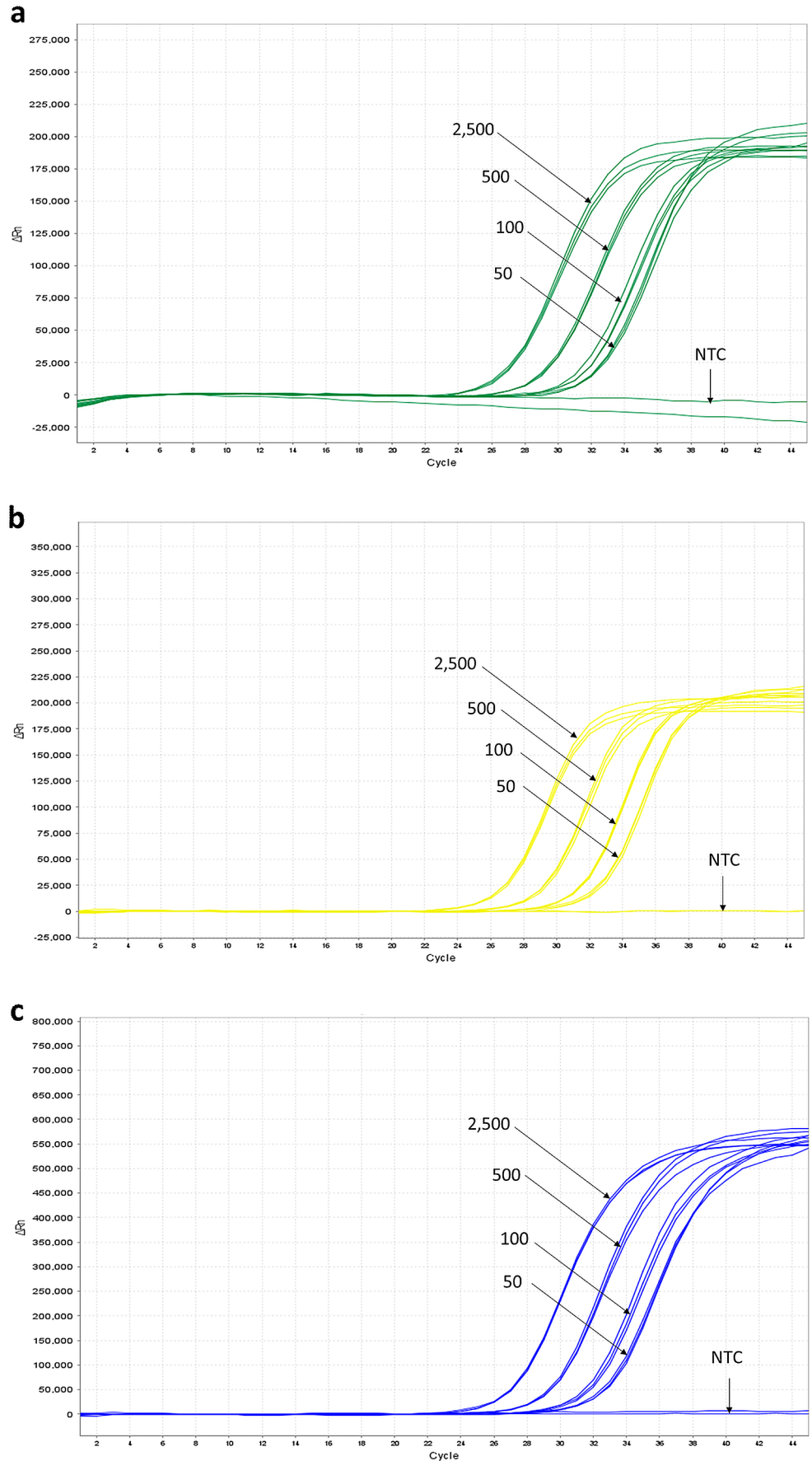
No significant crosstalk was observed in any of the 3 detection channels of the LightCycler®480 Instrument II (Fig. S1). As a result, the acceptance criteria outlined in the ENGL guidelines (European Network of GMO Laboratories 2021) pertaining to crosstalk were met. Moreover, there were no indications of crosstalk on other PCR devices.

### 3.3 Sensitivity

Amplification plots for the standard curves can be found in Fig. 1. The results of the in-house validation of the method's sensitivity are summarized in both Table 3 and Table S3. Performance criteria for standard curves are delineated in several guidelines for GMO analysis (European Network of GMO Laboratories 2015; 2021; Grohmann et al. 2016). According to these guidelines, the Limit of Detection at 95% confidence (LOD<sub>95%</sub>) for a qualitative real-time PCR method should not exceed 20 to 25 copies per PCR.

Furthermore, the slope of the standard curve, which is obligatory only for quantitative methods, should fall within the range of -3.1 to -3.6, with a coefficient of determination

**Fig. 1** PCR amplification of the standard curves. Sample mixtures containing DNA from maize MIR162 (containing AgroBorder I), soybean MON87769 (containing AgroBorder II) and soybean DAS44406-6 (containing P-CsVMV-pat) were measured on a QuantStudio 5 in triplicates under symmetric conditions with copy numbers as indicated. (a) AgroBorder I channel; (b) AgroBorder II channel; (c) P-CsVMV-pat channel. NTC, non-template control



**Table 3** Results of inhouse validation of method sensitivity tested on the QuantStudio 5

|  | AgroBor-<br>der I | AgroBor-<br>der II | P-CsVMV-<br>pat |
|--|-------------------|--------------------|-----------------|
| LOD <sub>95%</sub> (copies/PCR, lowest dilution with 12/12 positives) <sup>1</sup> | 5                 | 5                  | 5               |
| LOD <sub>95%</sub> (copies/PCR, POD modelling)                                     | 7.2               | 8.2                | 9.0             |
| Slope (2,500–50 copies/PCR)  | -3.35             | -3.29              | -3.40           |
| Efficiency (2,500–50 copies/PCR)   | 98.8%             | 101.3%             | 96.8%           |
| R <sup>2</sup> (2,500–50 copies/PCR)   | 0.99              | 1.00               | 0.99            |

<sup>1</sup> Dilution level 10 cp.: 1/12 negative, but same well negative for all 3 targets, most probably due to pipetting error (value omitted from analysis)

(R<sup>2</sup>) equal to or greater than 0.98. The triplex method successfully met all of these requirements. The LOD<sub>95%</sub> was approximately 7 copies/PCR for AgroBorder I, 8 copies/PCR for AgroBorder II, and 9 copies/PCR for P-CsVMV-pat, respectively (Table 3). These results were also confirmed with similar outcomes on another PCR device (Table S3). The coefficients of determination (R<sup>2</sup>) and the slopes of the calibration curves underscore the method's quantitative potential.

Additionally, in accordance with the guidelines for GMO analysis (European Network of GMO Laboratories 2015; 2021; Grohmann et al. 2016), the sensitivity of a multiplex method should be tested individually for each target in the presence of high amounts of the other targets to assess potential competitive effects.

To confirm the method's sensitivity under asymmetric conditions, we conducted an analysis involving 20 copies of each target in the presence of 20,000 copies per reaction of the respective other targets (Fig. S2). Furthermore, sufficient sensitivity under asymmetric conditions was validated in an interlaboratory trial (as discussed in sect. 3.4). Moreover, the presence of background DNA from maize, soybean, or canola (100 ng DNA per PCR), as commonly encountered in routine samples of official control, did not affect the sensitivity of the method (data not shown).

### 3.4 Robustness

According to guidelines (European Network of GMO Laboratories 2015, 2021; Grohmann et al. 2016), robustness should be assessed to simulate the practical implementation of the method, ensuring that sensitivity remains sufficient even in the presence of minor deviations from the experimental conditions.

First, in-house robustness testing was conducted under symmetric conditions by analyzing 20 copies of each target

DNA per reaction using a multifactorial experimental design (Grohmann et al. 2016) with the SensiFast Probe No-ROX Kit on a QuantStudio 5. Various factors, including changes in PCR master mix volume, annealing temperature, and the concentration of primers and probes, were investigated. It was found that these variations did not compromise the detectability of the targets (as shown in Table S4).

Furthermore, we assessed robustness in-house under asymmetric conditions by analyzing 20 copies of each target DNA in the presence of 20,000 copies of the other targets per reaction (at a 1:1,000 ratio) using a multifactorial experimental design (Grohmann et al. 2016) with the QIAGEN QuantiTect Multiplex Kit on a LightCycler 480 Instrument II. In all combinations of experimental factors tested, all PCR replicates consistently produced positive detection signals for the 3 targets (Table S5), demonstrating the method's satisfactory robustness.

Robustness was also verified through an interlaboratory ring trial involving 7 different laboratories. Mixtures containing 10 copies of each target DNA in the presence of 2,500 copies of the other 2 target sequences (asymmetric conditions at a 1:250 ratio) were unambiguously detected by all participating laboratories, despite the use of various combinations of PCR master mixes and real-time PCR devices (Table 4). This trial reaffirmed the high sensitivity of the triplex method, with an average Limit of Detection at 95% confidence (LOD<sub>95%</sub>) of approximately 5 copies/PCR for AgroBorder I, 3 copies/PCR for AgroBorder II, and 5 copies/PCR for P-CsVMV-pat, respectively. Once again, the mean standard curve performance parameters were consistent with the guidelines for GMO analysis (European Network of GMO Laboratories 2015, 2021; Grohmann et al. 2016).

## 4 Conclusions

In this study, we present the development and validation of a novel triplex real-time PCR method designed for the efficient screening of food, feed, and seed products for GM traits encoding transgene flanking sequences known as 'AgroBorder' and the Cassava Vein Mosaic Virus Promoter-pat construct. Our results confirm the theoretically expected presence and absence of the 3 target sequences in 37 GM plants, demonstrating the method's adequate specificity. Additionally, we have demonstrated sufficient sensitivity and robustness, even under challenging asymmetric conditions. Importantly, the multiplex amplification did not exhibit any interference between the individual PCR reactions. This method is already being successfully used for GMO screening in the routine analysis of numerous food and feed samples, as well as in proficiency tests conducted



**Table 4** Results of the interlaboratory trial of the triplex PCR method

| Lab No.                                      | 1                         | 2                       | 3                       | 4                   | 5                   | 6                     | 7a <sup>1</sup>         | 7b <sup>1</sup>       |
|--|---------------------------|-------------------------|-------------------------|---------------------|---------------------|-----------------------|-------------------------|-----------------------|
| PCR device                                   | Rotor-Gene 6000           | Quant-Studio 5          | CFX96                   | Quant-Studio 5      | Rotor-Gene Q        | CFX96                 | LC 480 II               | MX3005P               |
| Master mix                                   | SensiFast Probe<br>No-ROX | QuantiTect<br>Multiplex | PCRrely<br>Mplex<br>Mix | SensiFast<br>No-ROX | SensiFast<br>No-ROX | PerfeCTa<br>MultiPlex | QuantiTect<br>Multiplex | PerfeCTa<br>MultiPlex |
| <b>AgroBorder I</b>                          |                           |                         |                         |                     |                     |                       |                         |                       |
| LOD <sub>95%</sub> <sup>2</sup> (copies/PCR) | 4.4                       | 4.1                     | 4.2                     | 4.4                 | 4.1                 | 6.6                   | 3.4                     | 11.1                  |
| Slope <sup>3</sup>                           | -3.50                     | -3.53                   | -3.08                   | -3.49               | -3.38               | -3.50                 | -3.24                   | -3.28                 |
| Efficiency <sup>3</sup>                      | 93.0%                     | 92.2%                   | 111.0%                  | 93.4%               | 97.5%               | 93.3%                 | 103.5%                  | 102.0%                |
| R <sup>2</sup> ( <sup>3</sup> )              | 0.998                     | 0.989                   | 0.993                   | 0.996               | 0.991               | 0.988                 | 0.998                   | 0.998                 |
| No. of false negatives <sup>4</sup>          | 0                         | 0                       | 0                       | 0                   | 0                   | 0                     | 0                       | 0                     |
| <b>AgroBorder II</b>                         |                           |                         |                         |                     |                     |                       |                         |                       |
| LOD <sub>95%</sub> <sup>2</sup> (copies/PCR) | 2.7                       | 4.7                     | 1.3                     | 3.2                 | 4.1                 | 2.9                   | 2.0                     | 1.5                   |
| Slope <sup>3</sup>                           | -3.60                     | -3.34                   | -3.46                   | -3.49               | -3.22               | -3.56                 | -3.30                   | -3.37                 |
| Efficiency <sup>3</sup>                      | 89.6%                     | 99.3%                   | 94.7%                   | 93.4%               | 104.5%              | 90.8%                 | 101.0%                  | 98.2%                 |
| R <sup>2</sup> ( <sup>3</sup> )              | 0.994                     | 0.985                   | 0.992                   | 0.996               | 0.986               | 0.991                 | 0.997                   | 0.995                 |
| No. of false negatives <sup>4</sup>          | 0                         | 0                       | 0                       | 0                   | 0                   | 0                     | 0                       | 0                     |
| <b>P-CsVMV-pat</b>                           |                           |                         |                         |                     |                     |                       |                         |                       |
| LOD <sub>95%</sub> <sup>2</sup> (copies/PCR) | 4.8                       | 5.4                     | 3.0                     | 3.6                 | 4.4                 | 3.2                   | 5.4                     | 13.6                  |
| Slope <sup>3</sup>                           | -3.35                     | -3.37                   | -3.49                   | -3.49               | -3.37               | -3.76                 | -3.28                   | -3.05                 |
| Efficiency <sup>3</sup>                      | 99.0%                     | 98.1%                   | 93.6%                   | 93.4%               | 98.1%               | 84.6%                 | 101.8%                  | 112.6%                |
| R <sup>2</sup> ( <sup>3</sup> )              | 0.993                     | 0.980                   | 0.992                   | 0.996               | 0.994               | 0.996                 | 0.997                   | 0.990                 |
| No. of false negatives <sup>4</sup>          | 0                         | 0                       | 0                       | 0                   | 0                   | 0                     | 0                       | 0                     |

<sup>1</sup>) One laboratory participated with two different PCR devices and master mixes

<sup>2</sup>) LOD<sub>95%</sub> based on POD modelling,  $n = 12$  for each concentration

<sup>3</sup>) standard curve from 2,500–50 copies/PCR,  $n = 12$  for each concentration

<sup>4</sup>)  $n = 6$  for lowest concentration

by various laboratories. The screening results obtained using this method have been consistently confirmed by subsequent identification of the respective GM plant events (data not shown).

To further evaluate the performance of this method, an international ring trial validation study was conducted within the § 64 working group of the BVL, confirming the method's high sensitivity, specificity, robustness, and practicability (as per BVL L 00.00-176:2022-12).

The triplex real-time PCR method described in this study serves as an efficient screening approach, reducing both analysis time and costs. It is fully suitable for official GMO control of food, feed, and seed products. Furthermore, the method will be incorporated into the BVL GMO screening matrix (Federal Office of Consumer Protection and Food Safety 2020b) and the EUGinius database (EUGINIUS: European GMO Initiative for a Unified Database System (2023)). Information regarding the presence or absence of the 3 target sequences in upcoming new GM plant events will be regularly updated in these databases.

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## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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