REVIEW ARTICLE

DNA-based methods for detection of genetically modified events in food and supply chain

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Efficient detection strategies for genetically modified (GM) crops need to be in compliance with regulatory frameworks and address consumer concerns. The present review describes widely employed DNA-based technologies for GM detection. Polymerase chain reaction (PCR) and real-time PCR (qPCR) are the methods that can be used for qualitative and quantitative analysis of GM crops due to their specificity, sensitivity and robustness. With increase in number and complexity of genetic elements in newly developed GM events, strategies based on matrix approach, real-time PCR-based multi-target system, loop-mediated isothermal amplification, next generation sequencing, have emerged, which could facilitate cost-effective, rapid, on-site or high throughput GM detection.

Keywords: GM detection strategies, GMO matrix, loop-mediated isothermal amplification, next generation sequencing, polymerase chain reaction, real-time PCR.

GENETICALLY modified (GM) crops with desired traits are developed by introducing 'gene or genetic construct of interest' employing recombinant DNA tools. 'Transgene(s)' being introduced in GM crops, either confer a new trait to the plant or enhance an already existing trait. More than 28 countries are commercially growing GM crops with highest cultivation area in the United States (40%), followed by Brazil (23%), Argentina (13%), India (6.4%) and Canada (6.4%). Soybean, maize, cotton and canola are major globally commercialized GM crops, with herbicide tolerance and insect resistance as predominant traits[1]. Stacked traits for both herbicide tolerance and insect resistance have also been deployed in cotton and maize.

GM crops are one of the most promising technologies in ensuring global food security and increased crop productivity; however, before their release in the food and agricultural supply chain, consumers' apprehensions need to be addressed effectively through appropriate risk assessment and management.

The approval of GM crops and GM-derived products is regulated in different countries by respective regulatory bodies. GM crops approved in one country do not necessarily have the same approval status in another country, which may have a considerable impact on international trade and transboundary movements. In some countries as in the United States, labelling of GM products is voluntary; whereas it is mandatory in several countries as in the European Union (EU)[2]. Several countries have implemented labelling thresholds for unintentional presence of approved GM crops defined as 0.9% in the EU and Russia, 3% in Korea, 5% in Japan, Indonesia, Thailand and Taiwan, and 1% in Brazil[3,4]. So far, no labelling threshold has been implemented in India. The Department of Consumer Affairs, Food and Public Distribution, Ministry of Consumer Affairs, Government of India, in an extraordinary gazette notification, made an amendment to enforce GM food labelling for selected food commodities[5]. The regulatory regime for GM crops/products in India has been reviewed extensively[4,6,7]. Regulatory framework has been introduced since 1989 under the provisions of the Environment Protection (EP) Act, 1986, by the Ministry of Environment & Forests and Climate Change (MoEF&CC), Government of India. Under EP Act, 'Rules for Manufacture/Use/Import/Export and Storage of Hazardous Microorganisms, Genetically Engineered Organisms or Cells' were notified. In 2006, Director General of Foreign Trade (DGFT) notification no. 2 (RE-2006)/2004-2009, regarding import policy was published in an Extraordinary Gazette by the Ministry of Commerce and Industry, Government of India.

To implement the regulatory requirements effectively and for labelling purposes, reliable analytical methods for detection and quantification of GM content in seed lots or food commodities are required. DNA-based diagnostics can be employed for monitoring adventitious presence of transgenes and for ensuring post-release monitoring of GM events. To screen for a large number of GM crops/events, it is necessary to develop user-friendly, cost-effective and reliable GM detection strategies.

GM detection methods can target either the transgenic DNA or the novel recombinant protein(s) expressed in GM crops. Protein-based GM detection methods include immunoassays that are based on the specific binding between an antigen and an antibody. Due to specific binding, immunoassays exhibit high level of specificity allowing detection of GM lines expressing the recombinant protein. Commonly employed immunoassays are enzyme-linked immunosorbent assay (ELISA), lateral flow sticks and Western blot. Lateral flow strip tests are easy

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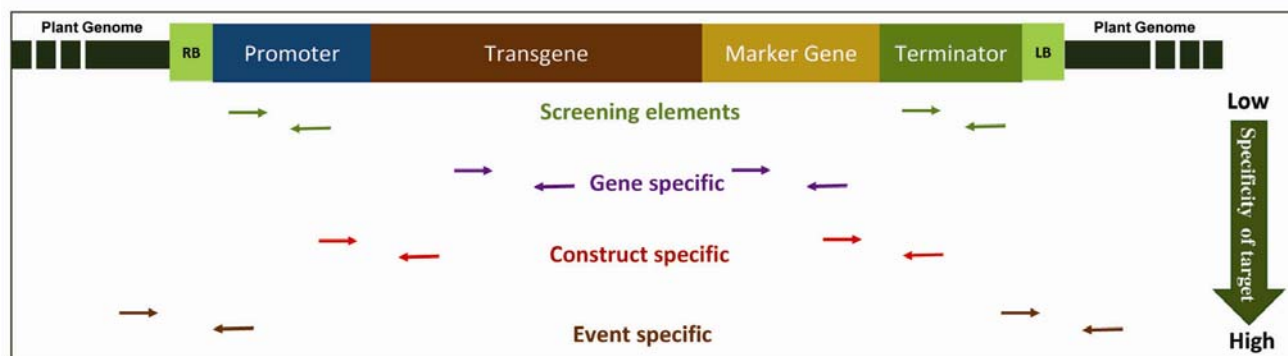

Figure 1. Transgenic construct showing the genetic elements and targets for DNA-based GM detection. Primer positions are depicted by arrows.

and rapid and do not require specialized staff and expensive or sophisticated equipments, so can be employed on-site. Protein-based GM diagnostics have certain advantages and disadvantages, as reviewed thoroughly[8]. These methods are economical in terms of resources and equipment set-up required, relatively fast and simple assays requiring moderate sample preparation and less skill. However, the applicability of protein-based methods is limited to raw or partially processed products as the proteins may be degraded in processed food. DNA-based methods are robust with higher specificity, sensitivity and wider applicability. Based on the target amplification, DNA-based detection methods can be categorized as: Target amplification methods to increase the amount of target DNA, as in polymerase chain reaction (PCR); and signal amplification methods to increase the signal of the target, as in real-time PCR (qPCR)[9].

This study provides an overview of widely employed DNA-based GM detection methodologies, along with recent advances in the area for cost/time efficiency and broad applicability.

Polymerase chain reaction based GM detection methods

With increasing number and complexity of GM events (single as well as stacked), testing for every GM event has become labour-intensive and costly[10–12]. A GM event with single trait can be tested using a simple method, whereas identification and quantification of multiple or stacked traits or GM events require use of combination of high-throughput technologies[10]. PCR, involving amplification of transgenic elements, is a widely employed method for GM detection. A transgenic construct constitutes various genetic elements: a promoter, which enables the expression of inserted gene; the inserted transgene conferring a specific trait to the host plant; a marker gene for selection of transformants; and the terminator, which acts as a stop signal. PCR-based GM detection methods are categorized on the basis of the level of specificity (Figure 1): screening methods; gene-specific methods; construct-specific methods and event-specific methods.

Screening methods

Screening methods, targeting most commonly employed genetic elements, including promoters and terminators, are employed to check the GM status of samples. If a sample is GM, only then further tests are required for identification of a particular GM event. If a sample is non-GM, based on screening results, there is no need for further analysis. Hence, preliminary screening reduces the number of test samples for further confirming the presence of specific GM event/trait. Since majority of plants have been transformed with *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter (*p35S*) and *Agrobacterium tumefaciens* nopaline synthase terminator (*Tnos*), screening methods targeting these elements, can be efficiently employed to check the GM status[13]. In addition to these, other regulatory elements have also been employed such as *A. tumefaciens* nopaline synthase promoter (*pNos*), rice actin promoter (*pAct*), *CaMV 35S* terminator (*t35S*), *Figwort Mosaic Virus* promoter (*pFMV*) and maize ubiquitin promoter (*pUbiZm*). Several PCR/qPCR-based screening strategies have been reported: hexaplex PCR targeting commonly used marker genes, i.e., *aadA*, *bar*, *hpt*, *nptII*, *pat*, *uidA* to check for GM status of a sample irrespective of crop and GM trait; duplex, triplex and pentaplex qPCR targeting *p35S*, *Tnos*, *ctp2-cp4-epsps*, *bar* and *pat*, qPCR assays targeting six promoter and four terminator elements, *viz*., *pFMV*, *pNos*, *pSSuAra*, *pTa29*, *pUbi*, *pRice actin*, *t35S*, *tE9*, *tOCS* and *tg7*; quadruplex qPCR targeting *p35S*, *Tnos*, *nptII* and an endogenous gene[14–17].

Gene-specific methods

Gene-specific methods targeting specific transgenes expressed in a GM crop, are more specific than screening methods. Gene-specific PCR assays are more specific

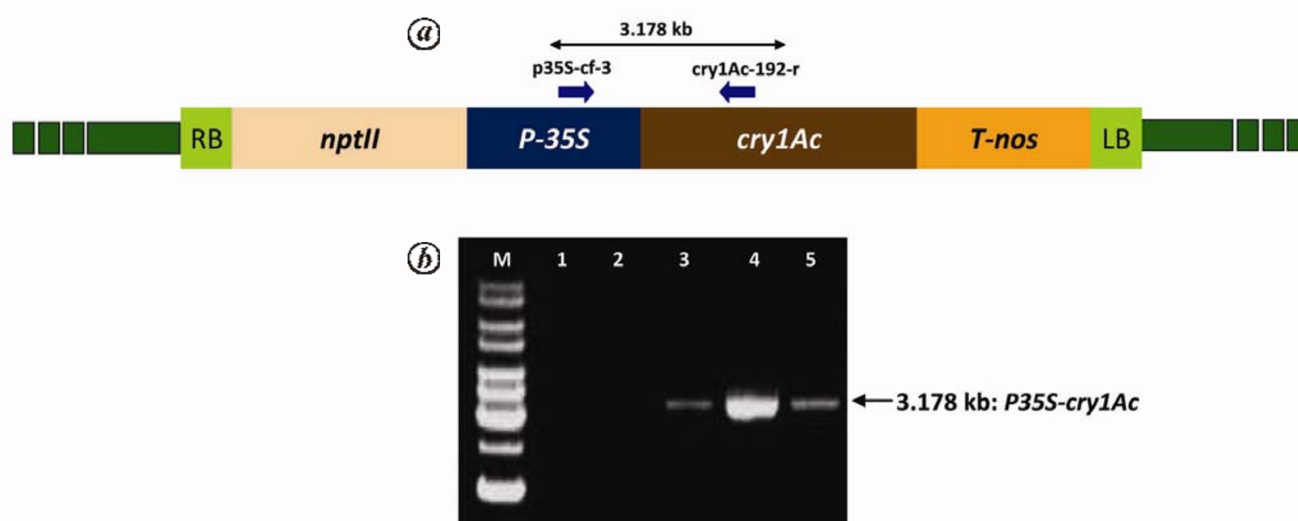

Figure 2. Construct-specific PCR for detection of *p35S-cry1Ac* region in *Bt* rice. ***a***, Transgene construct for *Bt* rice indicating the location of forward and reverse primers. ***b***, Construct-specific PCR using forward primer of promoter and reverse primer of transgene, i.e. *p35S-cf-3/cry1Ac-192-r*: (1) Non-template control; (2) Sample of non-GM rice; (3–5) Replications of samples of *Bt* rice; (M) 1 kb DNA ladder. Source: ref. 33.

than screening assays targeting most commonly employed promoters and terminators present in a large number of GM events, as they target transgene(s) specific to a particular trait being expressed in a GM event. For example, *cry1Ac*, *cry2Ab* genes for insect resistance in Bollgard® II (MON15985) event of cotton, and *cp4-epsps* for herbicide tolerance in Roundup Ready® cotton (MON1445) can be used for testing the presence of these events, after the screening tests targeting *p35S* and *Tnos*, present in both these events (MON15985 and MON1445). Gene-specific PCR/qPCR assays have been developed for *cry1A.105*, *cry1A3*, *cry1Ac*, *cry2Ab2*, *cry9C*, *cry1Ab*, *epsps*, *pat*, *vip3A*, *AmA1*[18–27].

Construct-specific methods

Construct-specific methods target the junction between two DNA elements, for instance, a region of the insert spanning junction between the promoter and transgene. Construct-specific PCR/qPCR assays have been reported, targeting junction regions of *Tnos* and dehydrofolate reductase (*dfr*) gene in GM linseed event FP967, signal peptide and phytase gene in GM maize line BVLA430101, *cry1Ac-cry1Ab* fusion gene and *Tnos* in GM rice[28–30]. Construct-specific assays, targeting *ctp2-cry2Ab2*, *ctp2-cp4epsps*, *p35S-cry1Ac*, *p35S-uidA*, have also been reported[31–34]. Construct-specific PCR targeting the junction between *p35S* promoter and *cry1Ac* gene for detection of *Bt* rice is shown in Figure 2.

Event-specific methods

Event-specific methods, targeting junction region at the integration locus between recipient genome and inserted DNA, exhibit highest specificity for GM identification. Validated qPCR protocols for more than 50 GM events of different crops including maize, soybean, cotton and oilseed rape and one event each of potato, rice and sugar beet are available at GMOMETHODS, an European Union Database of Reference Methods for GMO analysis based on the *Compendium of Reference Methods for GMO Analysis* (http://gmo-crl.jrc.ec.europa.eu/). Event-specific PCR and/or qPCR assays have been developed for event 55-1 of GM papaya, *Bt*63 (TT51-1) and Kefeng events of GM rice; event EE1 of GM eggplant, GM maize events LY038, MON810, *Bt*11, GA21[35–40].

Qualitative and quantitative PCR methods

Qualitative PCR methods including singleplex/multiplex PCR and real-time PCR primarily involve identification of specific GM event(s) in the test samples, while quantitative methods enable absolute quantification of particular GM event(s) employing real-time PCR. For a reliable PCR assay for GM detection, selection and validation of suitable endogenous reference gene to be used as an internal control, for a particular crop, is the pre-requisite, as the per cent GM content can be calculated by ratios of specific GM target sequence to species-specific endogenous reference gene. The endogenous gene should be nuclear, having a stable, low copy number, taxon-specific and highly conserved among the species of a particular genus. Several endogenous reference genes for GM detection purposes have been reported/validated for various crops (Table 1).

Multiplex PCR, a variant of conventional PCR, involves simultaneous amplification of multiple target sequences in a test sample. MPCR-based detection kit,

Table 1. List of endogenous reference genes employed for GM detection and quantification

Crop	Genes	Reference
Maize	*Adh1* (alcohol deydrogenase 1) Zein Invertase	87
Cotton	*SAH7* (IVS of the putative *Sinapis*, *Arabidopsis* Homolog 7)	88
	Sad1 (stearoyl-ACP desaturase)	89
Rice	*SPS* (sucrose phosphate synthase)	90
	PLD (phospholipase D alpha 2)	91
Soybean	*Lectin*	92
Tomato	*LAT52* (late anther tomato)	93
Potato	*UGPase* (UDP-glucose pyrophosphorylase)	19
	ST-LS1	94
Oilseed rape	*BnC1* (cruciferin storage protein)	95
Sugarbeet	*GS2* (glutamine synthetase)	96
Solananceae (Eggplant, potato, pepper, tomato)	β-fructosidase	52
Brassicaceae (Cauliflower and other members)	*SRK* (S-locus receptor kinase)	47

which screens four herbicide-tolerant genes (*cp4-epsps*, *m-epsps*, *pat,* and *bar*) has been developed[41]. A multiplex nested PCR assay targeting four commonly employed transgenic elements, *cp4-epsps*, *cry1Ab*, *bar*, *pat* and an endogenous reference *ribulose bisphosphate carboxylase/oxygenase* large subunit (*RBCL*) gene, has been developed for simultaneous detection of GM soybean, maize and rice in highly processed products[42]. A multiplex PCR assay coupled with capillary gel electrophoresis for amplicon identification by size and colour has been developed for simultaneous detection of five GM cotton events[43]. Multiplex PCR assay has also been employed to detect stacked GM maize events, *Bt*11 × GA21, MON810 × MON863, NK603 × MON863, and NK603 × MON810 × MON863, TC1507 × DAS59122 × NK603, MON810 × MON863 × NK603, MON810 × MON88017, MON810 × GA21, *Bt*11 × MIR604 × GA21[44–46].

Multiplex PCR assays for detection of all commercialized events of *Bt* cotton and GM crops/events under field trials or under different stages of testing in the country, *viz*, GM tomato for salinity and drought tolerance, GM potato with better protein quality, *Bt* crops, including *Bt* cauliflower, *Bt* eggplant. *Bt* okra, *Bt* potato, *Bt* rice have been reported[20,33,34,38,47–51].

Qualitative/quantitative qPCR allows monitoring of products, by measuring fluorescence signal produced during the progress of reaction. Fluorescent signals are detectable using DNA binding fluorescent dyes, for example SYBR Green® or more specific fluorescent probes. qPCR assays for the differentiation of four members of Solanaceae, viz. potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*) have been reported[52]. qPCR assays have been developed and validated for detection of *cry1A.105* and *cry2Ab2* genes in GM maize[18]. For quantification of MON810 and GA21 in maize, qPCR assays were optimized with limits of detection (LOD) and quantification (LOQ) of 3 and 36 copies respectively[53]. Duplex qPCR method has been developed for identification of four GM maize events, *Bt*11, *Bt*176, MON810 and T25[54]. Quadruplex qPCR assay targeting *p35S*, *Tnos* and *nptII* marker gene along with an endogenous gene has been developed for screening of GM tomatoes[17].

qPCR-based multi-target system for GM detection

Taqman® qPCR-based 'ready-to-use multi-target analytical system for detection of GMOs' has been developed, which reduces the number of steps and minimizes handling error and chances of cross-contamination. The system consists of pre-spotted plates containing lyophilized primers and probes for the individual detection of targets, allowing simultaneous amplification of 39 European GM events of six crops, viz. maize, cotton, rice, oilseed rape, soybean, sugar beet and potato along with taxon-specific methods of target crops in a single run[55]. The applicability of this system in processed maize matrices was reported[56].

A multi-target TaqMan® qPCR-based system was developed for checking presence of authorized GM events in India[57]. The developed system consists of a 96-well pre-spotted plate with lyophilized primers and probes for a total of 47 assays in duplicate allowing simultaneous detection of GM events from corn, eggplant, rice, soybean, and cotton; in particular, the system combines 21 event-specific assays, 6 taxon-specific assays, 5 construct regions and 15 element-specific assays.

Technological advancement for rapid and efficient GM detection

Next generation sequencing

Molecular characterization of GM event at the chromosome level includes the copies and localization of inserted transgenic construct, sequences of insert and its flanking genomic regions, which is essential for event-specific detection[58]. For precise information on integrated transgenic construct and their flanking regions, PCR-

based chromosomal or genome walking strategies are being commonly employed. Practical utility of advanced sequencing technologies and reduction in the cost of sequencing contributes to efficient event-specific detection, in case of genome sequences with complex/rearranged modifications. Next generation sequencing has emerged as an efficient tool for testing GM events without any sequence information available. Applicability of next generation sequencing and bioinformatics tools has been demonstrated for molecular characterization of GM soy and rice events[59–61]. A specific and sensitive qPCR assay has been developed for detection of *vip3A* gene in MIR162 event of GM maize and Cot102 event of GM cotton. Site finding PCR in combination with next generation sequencing targeting the flanking DNA sequence of the *vip3Aa20* element in MIR162 has been reported[26].

Microarray technology based approaches

Microarrays or DNA chips are high-throughput systems that allow the analysis of multiple targets in a single assay. Main advantages of microarrays are miniaturization, high sensitivity and screening throughput. Different DNA approaches coupled with multiplex PCR have been reported: multiplex DNA array-based PCR for quantification of GM maize; ligation detection reaction coupled with universal array technology for detection of GM maize event *Bt*11; peptide nucleic acid array approach for detection of five GM events and two plant species; multiplex DNA microarray chip for simultaneous identification of nine GMOs, five plant species and three screening elements, namely, *p35S*, *Tnos* and *nptII*; an event-specific DNA microarray system to detect 19 GM events, two of soybean, thirteen of maize, three of canola and one of cotton, in processed foods[62–66].

Novel multiplex quantitative DNA-based target amplification method, NASBA (nucleic acid sequence based amplification) Implemented Microarray Analysis (NAIMA), was developed for sensitive, specific and quantitative detection on microarray[67,68]. It involves the use of tailed primers allowing the multiplex synthesis of template DNA in a primer extension reaction, followed by transcription-based amplification using universal primers. The cRNA product is ligated to fluorescent dyes labelled dendrimers, allowing signal amplification and then hybridized on an oligonucleotide probe-based microarray for multiplex detection[67].

Matrix-based approach

GM detection laboratories initially undertake PCR-based preliminary screenings followed by more specific identification and quantification, if required. As testing directly for each target is extremely labour-intensive and costly, use of initial screening targeting commonly employed transgenic elements can facilitate time- and cost-efficient discrimination of GM and non-GM samples[10–12]. Matrix-based approach is an efficient and cost-effective strategy to check authorized GM events[10,11,69,70]. GMO matrix is represented in the form of a table, in which each row represents a GM event, whereas columns represent the analytical test methods or vice-versa (http://gmo-crl.jrc.ec.europa.eu/doc/2011-12-12%20ENGL%20UGM%20-WG%20Publication.pdf). While implementing the matrix approach for analysis of samples, the matrix is used as a reference. The results from application of selected screening modules on the sample are compared with the data tabulated in the matrix.

A matrix-based universal screening approach using combination of five target elements was developed for 81 authorized/unauthorized GM events of EU[69]. Combinatory SYBR Green® real-time PCR screening (CoSYPS) is another matrix approach in which SYBR Green® qPCR analysis is based on four parameters, i.e. Ct and Tm values, and the LOD and LOQ of each method[70]. More comprehensive and user-friendly testing strategies based on integrated decision support system such as GMOtrack and GMOseek matrix reduce the cost of GM diagnostics[11,71]. GMO track approach contains a data matrix on GM events on one hand and potential targets for detecting respective GM event on the other hand. While assisting in choosing the most cost-effective GMO testing strategy for a given sample, GMOtrack also supports the interpretation of wet-laboratory results. The core algorithm is freely available on the webpage (http://kt.ijs.si/software/GMOtrack/)[11]. GMOseek matrix has been developed as a comprehensive open-access tabulated database, with information of 328 GM events and 247 genetic elements[71].

GMO screening matrix was developed to check for authorized GM events in India, for detection of 141 GM events of 21 crops based on the information of 106 genetic elements[72]. Out of 106 genetic elements, 10 most frequently present targets were identified to screen these events. The matrix approach facilitates efficient, rapid and cost-effective screening by eliminating the need for development of specific testing methodologies for each individual GM event.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acids amplification technique, in which amplification and detection of target genes are completed in a single step at a constant temperature[73]. LAMP is characterized by the use of four different primers, which recognize six distinct regions on the target. An inner primer pair containing sequences of sense and antisense strands of the target DNA initiates LAMP reaction, which proceeds at a constant temperature, followed by strand displacement DNA synthesis primed by an

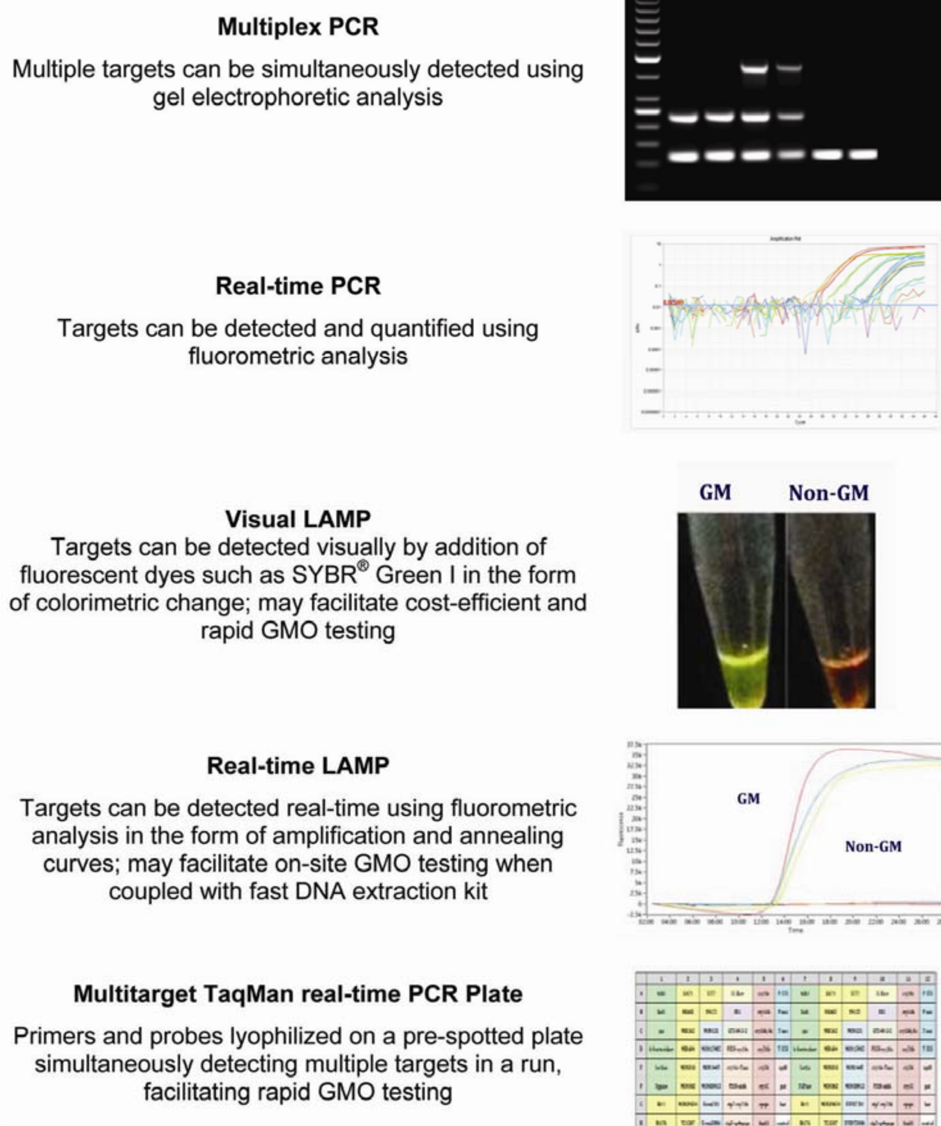

Figure 3. DNA-based GMO detection technologies developed at ICAR-National Bureau of Plant Genetic Resources.

outer primer pair[73–75]. Addition of 'loop' primers increases the specificity and time-efficiency of LAMP assays[76].

LAMP products show ladder-like pattern on agarose gel or can be real-time monitored using turbidimetry or by measuring fluorescence using real-time LAMP[77–79]. The amplicons can alternatively be visualized after completion of the LAMP reactions using nucleic acid staining or fluorescent dyes such as SYBR® Green 1 (refs 80, 81).

LAMP assays have been employed in GM diagnostics in the recent years, due to their time-efficiency, robustness and ease-of-use. A bioluminescent real-time reporter (BART) of LAMP, targeting *p35S*, *Tnos* and *Zea mays* alcohol dehydrogenase (*Adh1*) gene, has been used for screening of GM maize event MON810 (ref. 82). Event-specific LAMP assays were developed for the two soybean events, three GM rice events, and seven GM maize events[80,81,83]. LAMP-based visual and real-time screening assays targeting commonly used promoters, viz. *p35S*, *pFMV* and marker genes, viz. *aadA*, *nptII* and *uidA* were developed employing two chemistries (*Bst* polymerase and isothermal master mix), two detection methods (visual detection using SYBR Green I and real-time monitoring based on fluorescent signals) and four systems (conventional heating block, thermal cycler, real-time PCR system and real-time isothermal system)[79]. LAMP assays employing two chemistries, namely, *Bst* polymerase and ready-to-use isothermal master mix were found specific and sensitive. However, assays performed on real-time isothermal system were faster and most sensitive, detecting up to four copies of target within 35 min. Visual and real-time LAMP assays targeting three

commonly employed transgenes, namely, *cry1Ac*, *cry2Ab2* and *cp4-epesps* were reported[84]. Event-specific visual and real-time LAMP assays for detection of two major commercialized *Bt* cotton events, *viz*., MON531 and MON15985 were developed[85]. Event-specific visual and real-time LAMP assays for detection of six GM maize events were also reported[86]. The flexibility of these LAMP assays facilitates their applicability for reliable GM detection on-site, if combined with a fast DNA extraction method. This approach would be useful for GMO screening by customs authorities to check the consignments at ports of entry or by the field inspectors or farmers in the fields. For on-site testing employing real-time LAMP, portable real-time isothermal system and rapid DNA extraction kits without involving centrifugation steps are required[85].

Cost/time-efficient DNA-based GM detection technologies have been developed by GM detection laboratory at ICAR-National Bureau of Plant Genetic Resources, New Delhi, which are being employed routinely for testing transgenic planting material imported for research purposes (Figure 3).

Conclusions

In India, *Bt* cotton is commercially grown in an area of 11.8 million hectares. More than two hundred consignments of 15 GM crops have been imported for research purposes and several GM events with diversified traits were under field trials. With increase in number of GM events and diversification of traits, cost-effective GM diagnostics could facilitate effective risk assessment and management of GM crops and for their post-release monitoring, to ensure public confidence and solve legal disputes. In developing countries with limited resources, cost-efficient GM diagnostics would be helpful. Cost of an assay is determined on basis of the price of consumables to perform a test and one-time cost of setting up of detection system in the laboratory. Cost-efficiency of different PCR and LAMP based GM detection assays have been compared by our group in a recently published article, where visual LAMP was found to be cost-efficient[85]. PCR and qPCR-based assays are being widely employed for GM detection and quantification because of high specificity, sensitivity and robustness. However, due to involvement of cumbersome and time-consuming electrophoretic analysis in conventional PCR and use of highly sophisticated equipments and TaqMan® probes in qPCR, their application in GMO testing is restricted to specialized laboratories with high availability resources and expertise. In LAMP assays for GMO testing, visual detection of products using SYBR® Green I and real-time analysis using portable equipments could facilitate rapid/cost-efficient GMO testing with on-site applications. Efficient strategies/technologies based on multiplex/real-time PCR, GMO matrix, LAMP and qPCR-based multi-target system need to be utilized by GMO testing laboratories in the country, and further these technologies need to be validated by GMO testing laboratories in an interlinking mode.

1. James, C., *Global Status of Commercialized Biotech/GM Crops*. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca, NY, 46, 2014.
2. Querci, M., Van den Bulcke, M., Žel, J., Van den Eede, G. and Broll, H., New approaches in GMO detection. *Anal. Bioanal. Chem.*, 2010, **6**, 1991–2002.
3. Viljoen, C. D., Detection of living modified organisms (LMOs) and the need for capacity building. *Asian Biotechnol. Dev. Rev.*, 2005, **7**, 55–69.
4. Gruère, G. P. and Rao, S. R., A review of international labeling policies of genetically modified food to evaluate India's proposed rule. *AgBioForum,* 2007, **10**, 51–64.
5. Department of Consumer Affairs, Government of India, The Gazette of India: Extraordinary Part II, Sub-rule (6) of G.S.R. 427(E) notification, Ministry of Consumer Affairs, Food and Public Distribution, 2012.
6. Randhawa, G. J. and Chhabra, R., Import and commercialization of transgenic crops: an Indian perspective. *Asian Biotechnol. Dev. Rev*., 2009, **11**(2), 115–130.
7. Choudhary, B., Gheysen, G., Buysse, J., van der Meer, P. and Burssens, S., Regulatory options for genetically modified crops in India. *Plant J. Biotechnol*., 2014, **12**(2), 135–146.
8. Jasbeer, K., Ghazali, F. M., Cheah, Y. K. and Son, R., Application of DNA and immunoassay analytical methods for GMO testing in agricultural crops and plant-derived products. *ASEAN Food J.*, 2008, **15**(1), 1–25.
9. Csako, G., Present and future of rapid and/or high-throughput methods for nucleic acid testing. *Clin. Chim. Acta*., 2006, **363**(1–2), 6–31.
10. Holst-Jensen, A. *et al.*, Detecting un-authorized genetically modified organisms (GMOs) and derived materials. *Biotechnol. Adv*., 2012, **30**(6), 1318–1335.
11. Novak, P. K., Gruden, K., Morisset, D., Lavra, N., Tebih, D., Rotter, A. and Žel, J., GMOtrack: Generator of cost-effective GMO testing strategies. *J. AOAC Int*., 2009, **92**(6), 1739–1746.
12. Žel, J., Milavec, M., Morisset, D., Plan, D., Van den Eede, G. and Gruden, K., *How to Reliably Test for GMOs*? Springer, New York, 2012, 1st edn.
13. Lipp, M., Broadmann, P., Pietsch, K., Pauwels, J. and Anklam, E., IUPAC collaborative trial study of a method to detect genetically modified soybeans and maize in dried powder. *J. AOAC Int*., 1999, **82**(4), 923–928.
14. Randhawa, G. J., Chhabra, R. and Singh, M., Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. *J. Agric. Food Chem*., 2009, **57**, 5167–5172.
15. Huber, I. *et al.*, Development and validation of duplex, triplex, and pentaplex real-time PCR screening assays for the detection of genetically modified organisms in food and feed. *J. Agric. Food Chem.*, 2013, **61**(43), 10293–10301.
16. Debode, F., Janssen, E. and Berben, G., Development of 10 new screening PCR assays for GMO detection targeting promoters (*pFMV*, *pNOS*, *pSSuAra*, *pTA29*, *pUbi*, *pRice actin*) and terminators (*t35S*, *tE9*, *tOCS*, *tg7*). *Eur. Food Res. Technol.,* 2013, **236**(4), 659–669.
17. Wang, F., Zhang, X., Feng, J., Wang, Z. and Wang, P., Establishment of a quadruplex real-time PCR for screening of genetically modified tomatoes. *Eur. Food Res. Technol*., 2014, **238**(4), 683–690.

18. Dinon, A. Z., Prins, T. W., Van Dijk, J. P., Arisi, A. C., Scholtens, I. M. and Kok, E. J., Development and validation of real-time PCR screening methods for detection of *cry1A.105* and *cry2Ab2* genes in genetically modified organisms. *Anal. Bioanal. Chem.*, 2011, **400**(5), 1433–1442.
19. Rho, J. K., Lee, T., Jung, S. I., Kim, T. S., Park, Y. H. and Kim, Y. M., Qualitative and quantitative PCR methods for detection of three lines of genetically modified potatoes. *J. Agric. Food Chem.*, 2004, **52**(11), 3269–3274.
20. Randhawa, G. J., Chhabra, R. and Singh, M., Decaplex and real-time PCR-based detection of MON531 and MON15985 *Bt* cotton events. *J. Agric. Food Chem.*, 2010, **58**(18), 9875–9881.
21. Randhawa, G. J., Singh, M., Chhabra, R. and Sharma, R., Qualitative and quantitative molecular testing methodologies and traceability systems for *Bt* crops commercialised or under field trials in India. *Food Anal. Meth.*, 2010, **3**(4), 295–303.
22. Windels, P., Bertrand, S., Depicker, A., Moens, W., Van Bockstaele, E. and De Loose, M., Qualitative and event-specific PCR real-time detection methods for StarLink maize. *Eur. Food Res. Technol.*, 2003, **216**(3), 259–263.
23. Yoke-Kqueen, C., Yee-Tyan, C., Siew-Ping, K. and Son, R., Development of multiplex-PCR for genetically modified organism (GMO) detection targeting *epsps* and *cry1Ab* genes in soy and maize samples. *Int. Food Res. J.*, 2011, **18**, 512–519.
24. Noguchi, A. *et al.*, A novel trait-specific real-time PCR method enables quantification of genetically modified (GM) maize content in ground grain samples containing stacked GM maize. *Eur. Food Res. Technol*., 2014, **240**(2), 413–422.
25. Singh, C. K., Ojha, A., Bhatanagar, R. K. and Kachru, D. N., Detection and characterization of recombinant DNA expressing *vip3A*-type insecticidal gene in GMOs – Standard single, multiplex and construct-specific PCR assays. *Anal. Bioanal. Chem.*, 2008, **390**, 377–387.
26. Liang, C. *et al.*, Detecting authorized and unauthorized genetically modified organisms containing *vip3A* by real-time PCR and next-generation sequencing. *Anal. Bioanal. Chem.*, 2014, **406**(11), 2603–2611.
27. Randhawa, G. J., Singh, M. and Sharma, R., Duplex, triplex and quadruplex PCR for molecular characterization of genetically modified potato with better protein quality. *Curr. Sci.*, 2009, **97**(1), 21–23.
28. Grohmann, L., Busch, U., Pecoraro, S., Hess, N., Pietsch, K. and Mankertz, J., Collaborative trial validation of a construct-specific real-time PCR method for detection of genetically modified linseed event 'CDC Triffid' FP967. *Eur. Food Res. Technol*., 2011, **232**, 557–561.
29. Su, C., Sun, Y., Xie, J. and Peng, Y., A construct-specific qualitative and quantitative PCR detection method of transgenic maize BVLA430101. *Eur. Food Res. Technol*., 2011, **233**(1), 117–122.
30. Made, D., Degner, C. and Grohmann, L., Detection of gentically modified rice: A constuct-specific real-time PCR method based on DNA sequences from transgenic *Bt* rice. *Eur. Food Res. Technol*., 2006, **224**, 271–278.
31. Lee, S. H., Kim, J. K. and Yi, B. Y., Detection Methods for Biotech Cotton MON 15985 and MON 88913 by PCR. *J. Agric. Food Chem*., 2007, **55**(9), 3351–3357.
32. Grohmann, L., Brunen Nieweler, C., Nemeth, A. and Waiblinger, H. U., Collaborative trial validation studies of real-time PCR-based GMO screening methods for detection of the *bar* gene and the *ctp2-cp4epsps* construct. *J. Agric. Food Chem*., 2009, **57**, 8913–8920.
33. Randhawa, G. J. and Singh, M., Multiplex, construct-specific and real-time PCR-based analytical methods for *Bt* rice with *cry1Ac* gene. *J. AOAC Int*., 2012, **95**(1), 186–194.
34. Chhabra, R., Randhawa, G. J., Bhoge, R. K. and Singh, M., Qualitative and quantitative PCR-based detection methods for authorized genetically modified cotton events in India. *J. AOAC Int*., 2014, **97**(5), 1299–1309.
35. Kim, S. A. *et al.*, Detection of GM papaya event 55-1 in fresh and processed papaya using duplex PCR. *J. Kor. Soc. Appl. Biol. Chem*., 2010, **53**(2), 237–242.
36. Wu, G., Wu, Y., Nie, S., Zhang, L., Xiao, L., Cao, Y. and Lu, C., Real-time PCR method for detection of the transgenic rice event TT51-1. *Food Chem.*, 2010, **119**, 417–422.
37. Guertler, P., Huber, I., Pecoraro, S. and Busch, U., Development of an event-specific detection method for genetically modified rice Kefeng-6 by quantitative real-time PCR. *J. Verbraucherschutz Lebensmittelsicherheit*, 2013, **7**(1), 63–70.
38. Randhawa, G. J., Sharma, R. and Singh, M., Qualitative and event-specific real-time PCR detection methods for *Bt* brinjal event EE-1. *J. AOAC Int*., 2012, **95**(6), 1933–1739.
39. Mano, J. *et al.*, Development and validation of event-specific quantitative PCR method for genetically modified maize LY038. *Shokuhin Eiseigaku Zasshi*, 2013, **54**(1), 25–30.
40. Takabatake, R. *et al.*, Interlaboratory study of qualitative PCR methods for genetically modified maize events MON810, *Bt*11, GA21, and *CaMV* P35S. *J. AOAC Int*., 2013, **96**(2), 346–352.
41. Kim, J. H., Kim, E. H., Yea, M. C. and Kim, H. Y., Validation of A multiplex PCR detection kit for screening of herbicide-tolerant genes in genetically modified crops. *J. Kor. Soc. Appl. Biol. Chem*., 2013, **56**(2), 251–254.
42. Jinxia, A., Qingzhang, L., Xuejun, G., Yanbo, Y., Lu, L. and Zhang, M., A multiplex nested PCR assay for the simultaneous detection of genetically modified soybean, maize and rice in highly processed products. *Food Cont*., 2011, **22**(10), 1617–1623.
43. Nadal, A., Esteve, T. and Pla, M., Multiplex polymerase chain reaction-capillary gel electrophoresis: a promising tool for GMO screening- assay for simultaneous detection of five genetically modified cotton events and species. *J. AOAC Int*., 2009, **92**(3), 765–772.
44. Xu, W., Yuan, Y., Luo, Y., Bai, W., Zhang, C. and Huang, K., Event-specific detection of stacked genetically modified maize *Bt*11 × GA21 by UP-M-PCR and real-time PCR. *J. Agric. Food Chem.*, 2009, **57**, 395–402.
45. Kim, S. Y., Kim, J. H., Lee, H. and Kim, H. Y., Detection system of stacked genetically modified maize using multiplex PCR. *Food Sci. Biotechnol.*, 2010, **19**(4), 1029–1033.
46. Shin, K. S., Suh, S. C., Lim, M. H., Woo, H. J., Lee, J. H., Kim, H. Y. and Cho, H. S., Event-specific detection system of stacked genetically modified maize by using the multiplex PCR technique. *Food Sci. Biotechnol.*, 2013, **22**(6), 1763–1772.
47. Randhawa, G. J., Chhabra, R. and Singh, M., Molecular characterization of *Bt* cauliflower with multiplex PCR and validation of endogenous reference gene in *Brassicaceae* family. *Curr. Sci.*, 2008, **95**(12), 1729–1731.
48. Kamle, S., Kumar, A. and Bhatnagar, R. K., Development of multiplex and construct specific PCR assay for detection of *cry2Ab* transgene in genetically modified crops and product. *GM Crops*, 74–81; doi:10.4161/gmcr.2.1.16017.
49. Randhawa, G. J., Singh, M., Chhabra, R., Guleria, S. and Sharma, R., Molecular diagnosis of transgenic tomato with *osmotin* gene using multiplex polymerase chain reaction. *Curr. Sci.*, 2009, **96**(5), 689–694.
50. Randhawa, G. J., Chhabra, R. and Singh, M., PCR-based detection of genetically modified tomato with *AVP1D* gene employing seed sampling strategy. *Seed Sci. Technol.*, 2011, **39**, 112–124.
51. Randhawa, G. J., Sharma, R. and Singh, M., Multiplex polymerase chain reaction for detection of genetically modified potato (*Solanum tuberosum* L.) with *cry1Ab* gene. *Indian J. Agric. Sci.*, 2009, **79**(5), 367–371.
52. Chaouachi, M., Malki, R. L., Berard, A., Romaniuk, M., Laval, V., Brunel, D. and Bertheau, Y., Development of a real-time PCR method for the differential detection and quantification of four *Solanaceae* in GMO analysis: Potato (*Solanum tuberosum*),

tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*). *J. Agric. Food Chem.*, 2008, **56**(6), 1818–1828.
53. Marmiroli, N., Multiplex real-time PCR assays for simultaneous detection of maize MON810 and GA21 in food samples. *Food Cont*., 2013, **30**(2), 518–525.
54. Chaouachi, M., Zellama, M. S., Nabi, N., Hafsa, A. B. and Saïd, K., Molecular identification of four genetically modified maize (*Bt*11, *Bt*176, MON810 and T25) by duplex quantitative real-time PCR. *Food Anal. Meth.*, 2014, **7**(1), 224–233.
55. Querci, M., Foti, N., Bogni, A., Kluga, L., Broll, H. and Van den Eede, G., Real-time PCR-based ready-to-use multi-target analytical system for GMO detection. *Food Anal. Meth*., 2009, **2**, 325–336.
56. Kluga, L., Folloni, S., Van den Bulcke, M., Van den Eede, G. and Querci, M., Applicability of the real-time PCR-based ready-to-use multi-target analytical systems for GMO detection in processed maize matrices. *Eur. Food Res. Technol.*, 2012, **234**, 109–118.
57. Randhawa, G. J., Singh, M., Sood, P. and Bhoge, R. K., Multitarget real-time PCR-based system: Monitoring for unauthorized genetically modified events in India. *J. Agric. Food Chem.*, 2014, **62**(29), 7118–7130.
58. Codex Alimentarious Commission Guidelines, Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods. *CAC-GL*, 2010, **74**, 22.
59. Kovalic, D. *et al.*, The use of next generation sequencing and junction sequence analysis bioinformatics to achieve molecular characterization of crops improved through modern biotechnology. *Plant Genome J*., 2012, **5**, 149–163.
60. Wahler, D., Schauser, L., Bendiek, J. and Grohmann, L., Next generation sequencing as a tool for detailed molecular characterization of genomic insertions and flanking regions in genetically modified plants: A pilot study using a rice event unauthorised in the EU. *Food Anal. Meth.*, 2013, **6**(6), 1718–1727.
61. Zhang, D., Characterization of GM events by insert knowledge adapted re-sequencing approaches. *Sci. Rep*., 2013, **3**; doi:10.1038/srep02839.
62. Rudi, K., Rud, I. and Holck, A., A novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. *Nucleic Acids Res.*, 2003, **31**, e62.
63. Bordoni, R. *et al.*, Detection and quantitation of genetically modified maize (*Bt*176 transgenic maize) by applying ligation detection reaction and universal array technology. *J. Agric. Food Chem.*, 2004, **52**, 1049–1054.
64. Germini, A., Rossi, S., Zanetti, A., Corradini, R., Fogher, C. and Marchelli, R., Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. *J. Agric. Food Chem.*, 2005, **53**, 3958–3962.
65. Leimanis, S. *et al.*, Validation of the performance of a GMO multiplex screening assay based on microarray detection. *Eur. Food Res. Technol.*, 2008, **227**, 1621–1632.
66. Kim, J. H., Kim, S. Y., Lee, H., Kim, Y. R. and Kim, H. Y., An event-specific DNA microarray to identify genetically modified organisms in processed foods. *J. Agric. Food Chem.*, 2010, **58**(10), 6018–6026.
67. Morisset, D., Dobnik, D., Hamels, S., Žel, J. and Gruden, K., NAIMA: target amplification strategy allowing quantitative on-chip detection of GMOs. *Nucleic Acids Res.*, 2008, **36**(18), e118.
68. Dobnik, D., Morisset, D. and Gruden, K., NAIMA as a solution for future GMO diagnostics challenges. *Anal. Bioanal. Chem.*, 2010, **396**(6), 2229–2233.
69. Waiblinger, H. U., Grohmann, L., Mankertz, J., Engelbert, D. and Pietsch, K., A practical approach to screen for authorised and unauthorised genetically modified plants. *Anal. Bioanal. Chem.*, 2010, **396**(6), 2065–2072.
70. Van den Bulcke, M., Lievens, A., Barbau Piednoir, E., Mbongolombella, G., Roosens, N., Sneyers, M. and Casi, A. L., A theoretical introduction to combinatory SYBR Green qPCR screening, a matrix-based approach for the detection of materials derived from genetically modified plants. *Anal. Bioanal. Chem.*, 2010, **396**(6), 2113–2123.
71. Block, A. *et al.*, The GMO seek matrix: a decision support tool for optimizing the detection of genetically modified plants. *BMC Bioinform.*, 2013, **14**, 256–269.
72. Randhawa, G. J., Morisset, D., Singh, M. and Žel, J., GMO matrix: A cost-effective approach for screening for unauthorized genetically modified events in India. *Food Cont.*, 2014, **38**, 124–129.
73. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T., Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.*, 2000, **28**(12), e63.
74. Fukuta, S., Mizukami, Y., Ishida, A., Ueda, J., Hasegawa, M., Hayashi, I., Hashimoto, M. and Kanbe, M., Real-time loop-mediated isothermal amplification for the *CaMV*35S promoter as a screening method for genetically modified organisms. *Eur. Food Res. Technol.*, 2004, **218**(5), 496–500.
75. Tomita, N., Mori, Y., Kanda, H. and Notomi, T., Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat. Prot.*, 2008, **3**(5), 877–882.
76. Nagamine, K., Hase, T. and Notomi, T., Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell Probes*, 2002, **16**(3), 223–229.
77. Mori, Y., Nagamine, K., Tomita, N. and Notomi, T., Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem. Biophys. Res. Commun.*, 2001, **289**(1), 150–154.
78. Huang, X., Chen, L., Xu, J., Ji, H. F., Zhu, S. and Chen, H., Rapid visual detection of *phytase* gene in genetically modified maize using loop-mediated isothermal amplification method. *Food Chem*., 2014, **156**, 184–189.
79. Randhawa, G. J., Singh, M., Morisset, D., Sood, P. and Žel, J., Loop-mediated isothermal amplification: Rapid visual and real-time methods for detection of genetically modified crops. *J. Agric. Food Chem.*, 2013, **61**(47), 11338–11346.
80. Guan, X., Guo, J., Shen, P., Yang, L. and Zhang, D., Visual and rapid detection of two genetically modified soybean events using loop-mediated isothermal amplification method. *Food Anal. Meth.,* 2010, **3**(4), 313–320.
81. Chen, X. *et al.*, End-point visual detection of three genetically modified rice events by loop-mediated isothermal amplification. *Int. J. Mol. Sci.*, 2012, **13**(11), 14421–14433.
82. Kiddle, G. *et al.*, GMO detection using a bioluminescent real time reporter (BART) of loop mediated isothermal amplification (LAMP) suitable for field use. *BMC Biotechnol.*, 2012, **12**(1), 15.
83. Chen, L., Guo, J., Wang, Q., Kai, G. and Yang, L., Development of the visual loop-mediated isothermal amplification assays for seven genetically modified maize events and their application in practical samples analysis. *J. Agric. Food Chem.*, 2011, **59**(11), 5914–5918.
84. Singh, M., Randhawa, G. J., Sood, P. and Bhoge, R. K., Loop-mediated isothermal amplification targeting insect resistant and herbicide tolerant transgenes: monitoring for GM contamination in supply chain. *Food Cont*., 2015, **51**, 283–292.
85. Randhawa, G. J., Chhabra, R., Bhoge, R. K. and Singh, M., Visual and real-time event-specific loop-mediated isothermal amplification based detection assays for *Bt* cotton events MON531 and MON15985. *J. AOAC Int.*, 2015, **98**(5), 1207–1214.
86. Bhoge, R. K., Chhabra, R., Randhawa, G. J., Sathiyabama, M. and Singh, M., Event-specific analytical methods for six genetically modified maize events using visual and real-time loop-mediated isothermal amplification. *Food Cont*., 2015, **55**, 18–30.

87. Mazzara, M., Grazioli, E., Savini, C. and Van den Eede, G., Event-specific method for the quantitation of maize line T25 using real-time PCR validation report and protocol. Online publication, 2005.
88. Mazzara, M., Larcher, S., Savini, C., Delobel, C. and Van den Eede, G., Event-specific methods for the quantitation of the hybrid cotton line 281-24-236/3006-210-23 using real-time PCR – validation report and protocol – Sampling and DNA extraction of cotton seeds. Online publication, 2006.
89. Yang, L., Chen, J., Huang, C., Liu, Y., Jia, S., Pan, L. and Zhang, D., Validation of a cotton-specific gene, *Sad1*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic cottons. *Plant Cell Rep*., 2005, **24**(4), 237–245.
90. Jiang, L., Yang, L., Zhang, H., Guo, J., Mazzara, M., Van den Eede, G. and Zhang, D., International collaborative study of the endogenous reference gene, sucrose phosphate synthase (*SPS*), used for qualitative and quantitative analysis of genetically modified rice. *J. Agric. Food Chem*., 2009, **57**(9), 3525–3532.
91. Mazzara, M., Cordeil, S. and Van den Eede, G., Report on the verification of an event-specific detection method for identification of rice GM-event LLRICE601 using a real-time PCR assay. Online publication, 2006.
92. Mazzara, M., Delobel, C., Grazioli, E., Larcher, S., Savini, C. and Van den Eede, G., Event-specific method for the quantification of soybean line A2704-12 using real-time PCR – validation report and protocol – soybean seeds sampling and DNA extraction. Online publication, 2007; doi:10.2788/28149.
93. Yang, L., Pan, A., Jia, J., Ding, J., Chen, J., Cheng, H., Zhang, C. and Zhang, D., Validation of a tomato-specific gene, *LAT52*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic tomatoes. *J. Agric. Food Chem.*, 2005, **53**(2), 183–190.
94. Randhawa, G. J., Singh, M. and Sharma, R., Validation of *ST-LS1* as an endogenous reference gene for detection of *AmA1* and *cry1Ab* genes in genetically modified potatoes using multiplex and real-time PCR. *Am. J. Pot. Res.*, 2009, **86**(5), 398–405.
95. Savini, C., Mazzara, M., Bogni, A., Angers, A. and Petrillo, M., Event-specific method for the quantification of oilseed rape MON88302 using real-time PCR – validation report and validated method. Online Publication, 2013.
96. Mazzara, M., Foti, N., Savini, C. and Van den Eede, G., Event-Specific method for the quantitation of sugarbeet line H7-1 using real-time PCR – validation report and protocol. Online Publication, 2006; doi:10.2788/32035.

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