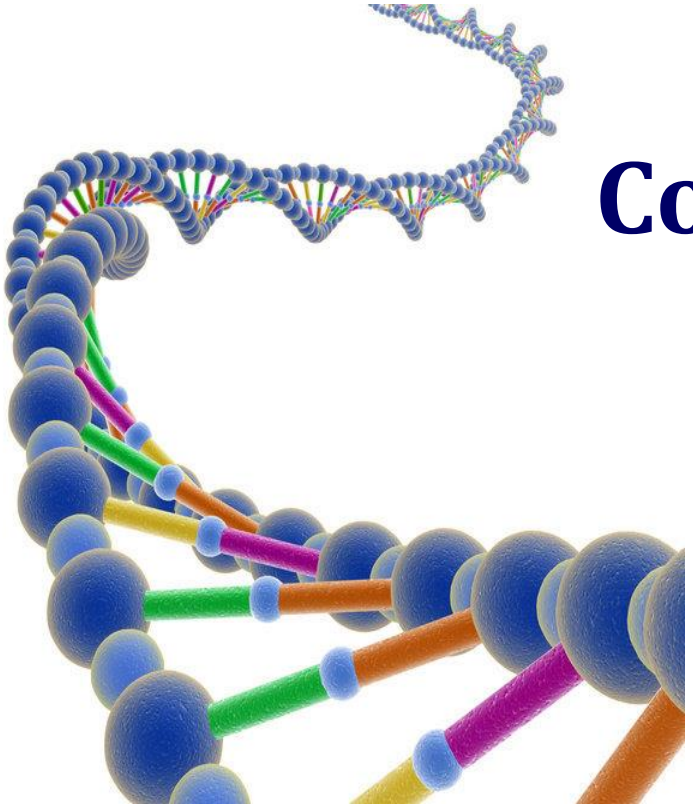

Cost Efficient DNA-based GM Detection

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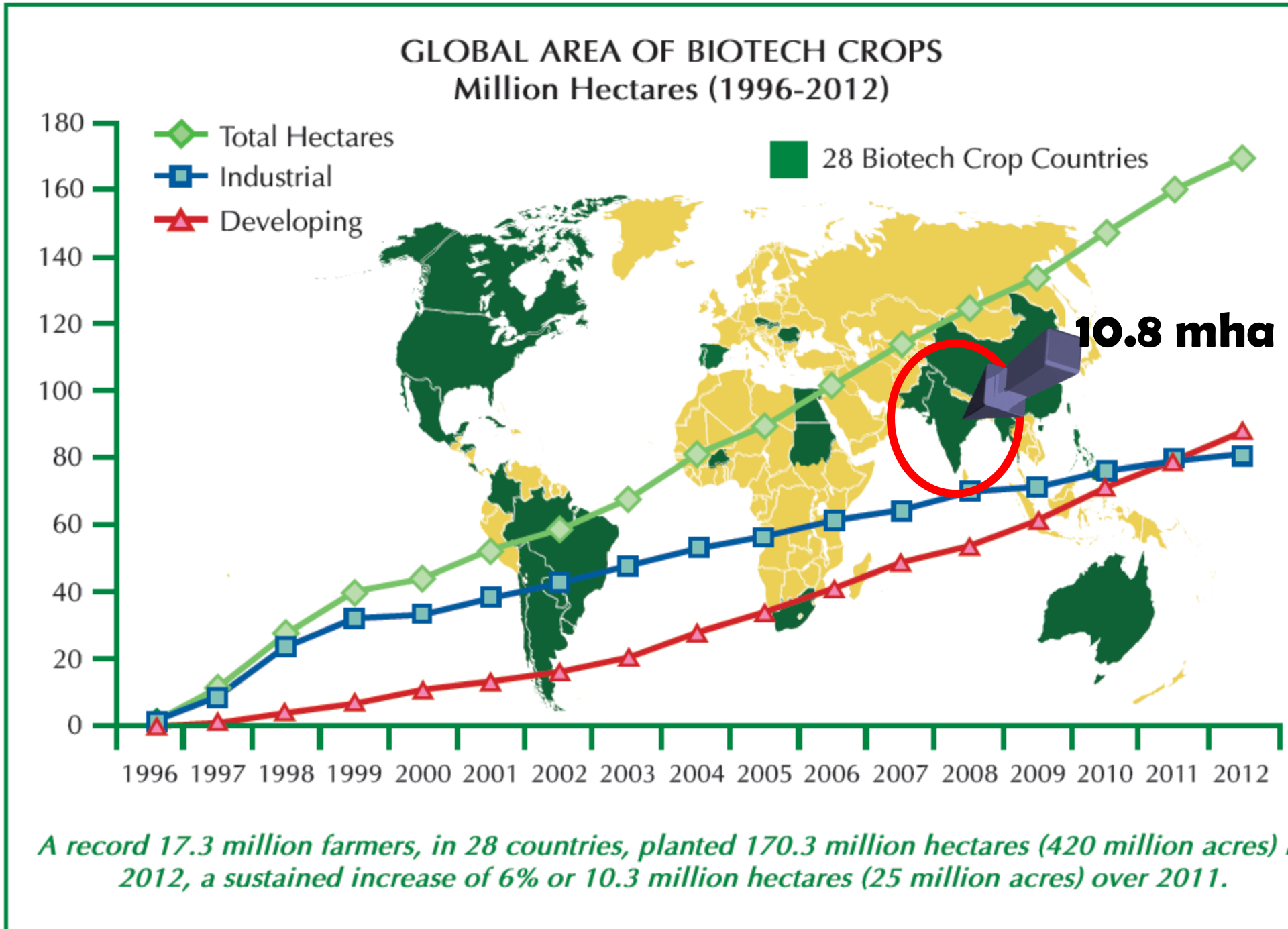
Principal Scientist
National Bureau of Plant Genetic Resources
New Delhi, India

रा पा आ सं ब्यूरो
NBPGR

भाकृअनुप
ICAR

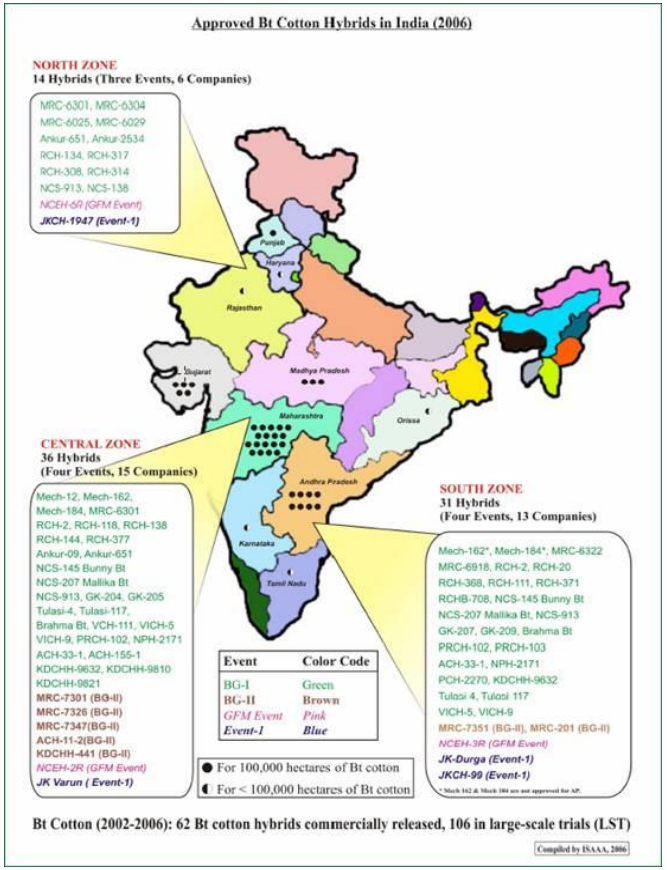


A record 17.3 million farmers, in 28 countries, planted 170.3 million hectares (420 million acres) in 2012, a sustained increase of 6% or 10.3 million hectares (25 million acres) over 2011.

Source: Clive James, 2012.

Status of GM crops in India

- **Commercialized**
  (Area under *Bt* cotton: 10.8 mha)
- **Under development** by public (including ICAR)& private sector
- **Imported** for research purposes
- Under different stages of **field trials**



Event	Developer	Year of Approval
MON-531	Mahyco/Monsanto	2002
MON-15985	Mahyco/Monsanto	2006
Event-1	JK Agri-Genetics	2006
GFM Event	Nath Seeds	2006
BNLA-601	UAS, Dharwad & CICR, Nagpur	2008
MLS-9124	Metahelix Life Sciences	2009

(Source: GEAC, MOEF)

National Bureau of Plant Genetic Resources
New Delhi

Nodal institute for import permit, quarantine processing and issue of phytosanitary certificate for germplasm/transgenic planting material

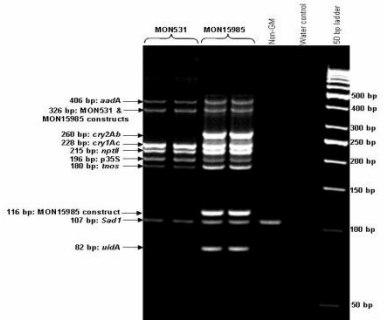
Facilities for GM detection

CL-4 Containment Facility

GM Detection Lab

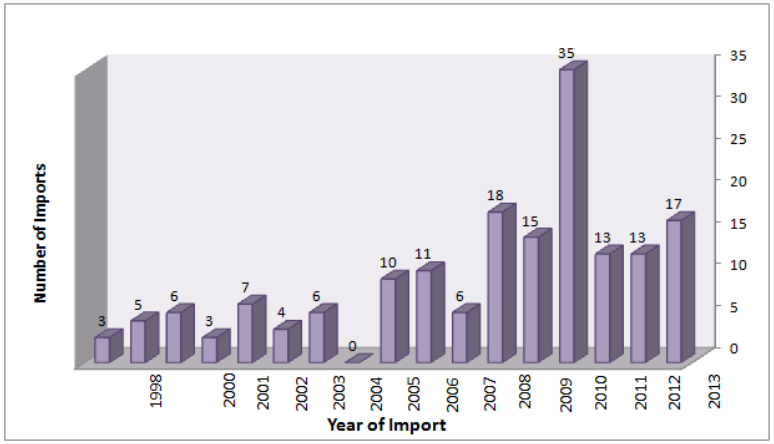
Expertise available for GM detection at NBPGR, New Delhi

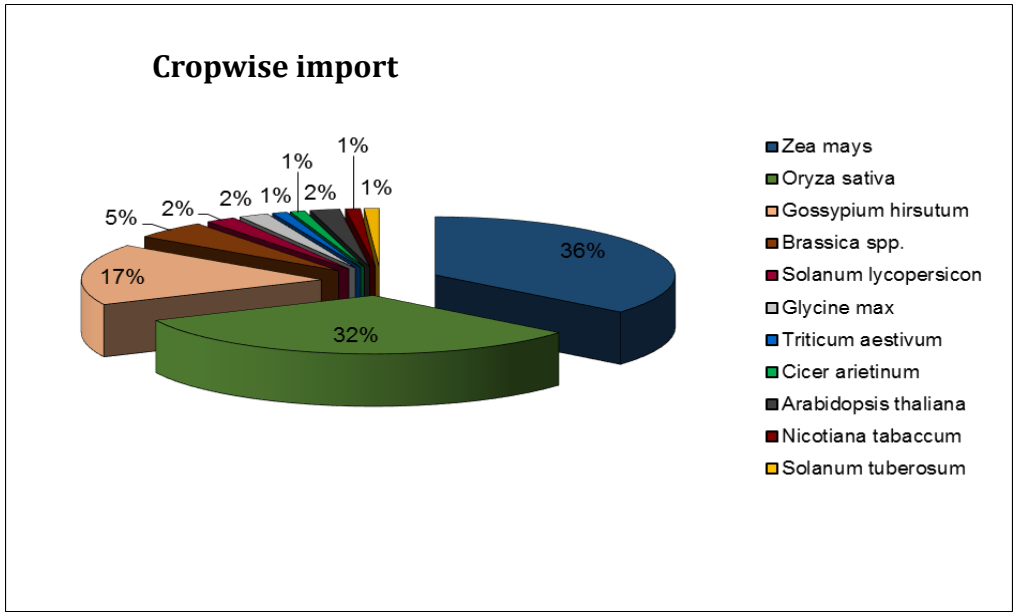
- PCR-based GM diagnostics for more than 10 GM crops
- Real-time PCR based quantitative assays (Transgene/construct/event-specific) for more than 20 GM events
- Visual and real-time LAMP assays for screening of GM crops
- GMO matrix for screening of authorized and unauthorized GM events in India
- Ready-to-use real-time multi-target plate for screening and detection of GM crops/events
- Quality Assurance by regular participation in International Proficiency Testings as per ISO/IEC 17043:2010 Accreditation



Molecular testing of imported transgenic planting material

So far, **172 imports of 14 crops comprising 5,112 accessions** have been tested.





Initial screening through simplex/multiplex PCR targeting marker and reporter genes: To test the GM status of a sample irrespective of crop & GM trait

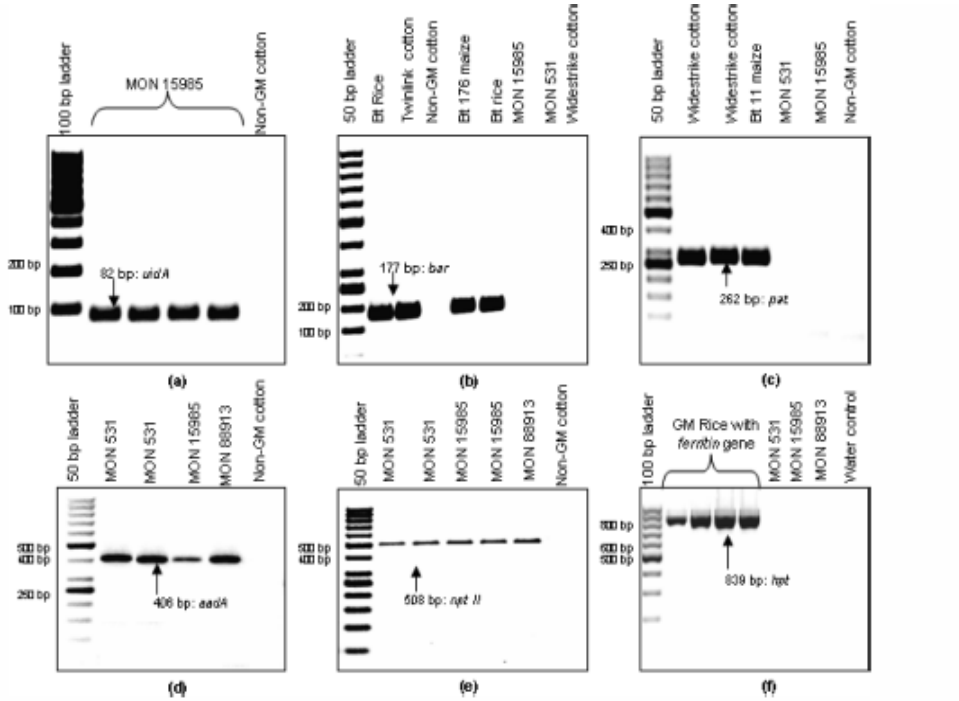


Figure 1. Simplex PCR amplicons in different transgenic seed materials for their respective selectable marker genes using the specific primer pairs for the detection of (a) *uidA*; (b) *bar*; (c) *pat*; (d) *aadA*; (e) *npt II*; and (f) *hpt* genes.

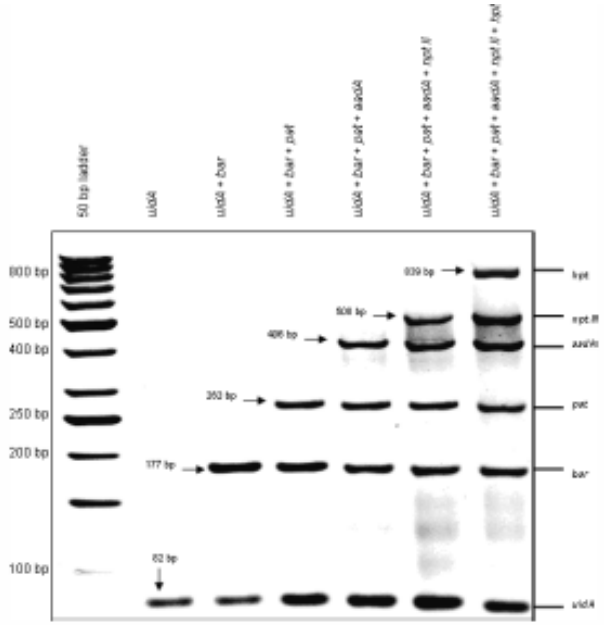
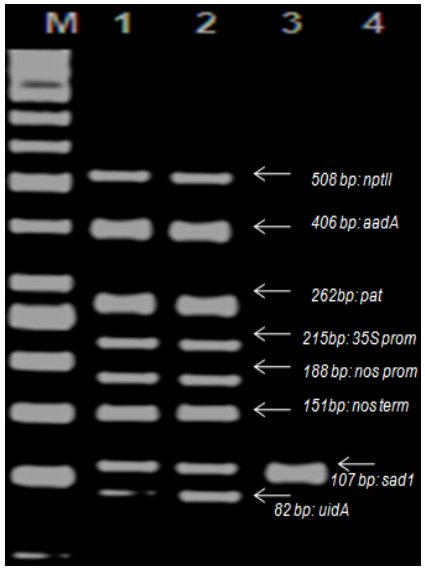


Figure 2. Multiplex PCR assay for testing of primer interference using equivalent DNA mix of six different GM events, i.e., MON 531 of cotton, MON 15985 of cotton, Widestrike cotton, Bt rice, GM rice with the fermitin gene and Bt176 of maize.

Simultaneous amplification of six commonly used marker genes
nptII, aadA, bar, pat, hpt* and *uidA

Randhawa G.J. *et.al* (2009) Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for screening of genetically modified crops. *J. Agri. Food Chem*. 57 (12): 5167-5172.

Combo Octaplex PCR for screening of GM cotton



Lane M: 50 bp ladder; Lane 1 & 2: mixture of MON531, MON15985, GFM cry1A and Widestrike events of GM cotton (covering all the seven regulatory elements used for study), Lane 3: Non-GM cotton, Lane 4: NTC

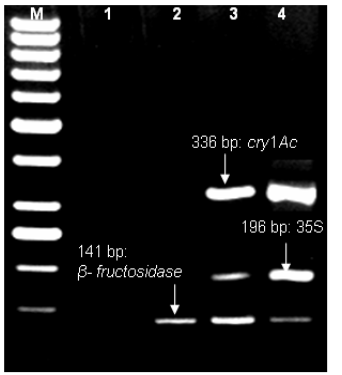
Simultaneous amplification of seven targets

Control elements: *P-35S*, T-*nos*, P-*nos*

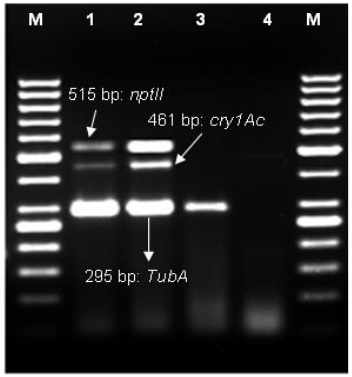
Marker genes: *nptII*, *aadA*, *pat* and *uidA*

Endogenous gene: *Sad1*

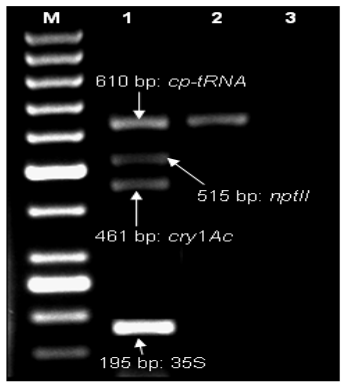
Gene-specific PCR



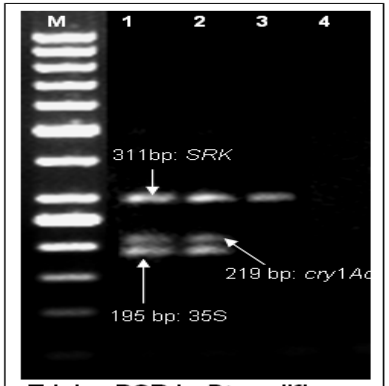
Triplex PCR in *Bt* brinjal with *cry1Ac* gene
1: Water control
2: Non-*Bt* brinjal
3-4: *Bt* brinjal
M: 50bp DNA ladder



Triplex PCR in *Bt* rice with *cry1Ac* gene
1-2: *Bt* rice
3: Non-*Bt* rice
4: Water control
M: 50bp DNA ladder

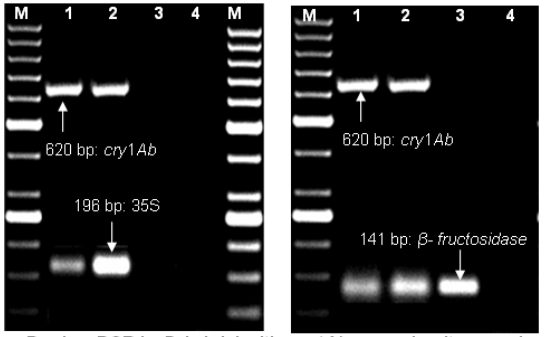


Tetraplex PCR in *Bt* okra with *cry1Ac* gene
1: *Bt* okra
2: Non-*Bt* okra
3: Water control
M: 50bp DNA ladder

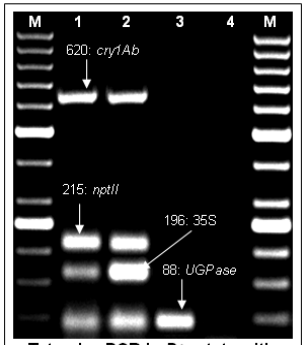


Triplex PCR in *Bt* cauliflower with *cry1Ac* gene
1-2: *Bt* cauliflower
3: Non-*Bt* cauliflower
4: Water control
M: 50bp DNA ladder

Targeting the specific genes (*cry1Ac, cry1Ab*) in the construct



Duplex PCR in *Bt* brinjal with *cry1Ab* gene simultaneously detecting (i) *cry1Ab* and *CaMV* 35S *promoter; and (ii) cry1Ab and endogenous β- fructosidase* gene
1-2: *Bt* brinjal
3: Non-*Bt* brinjal
4: Water control
M: 50bp DNA ladder

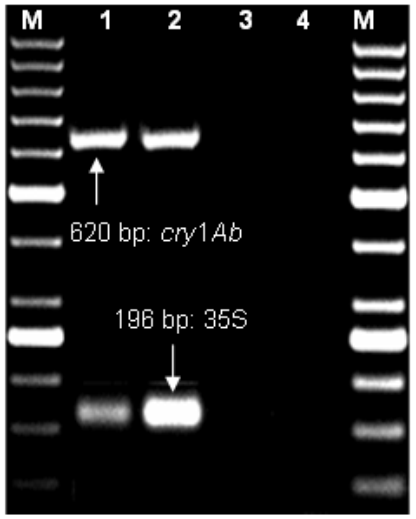


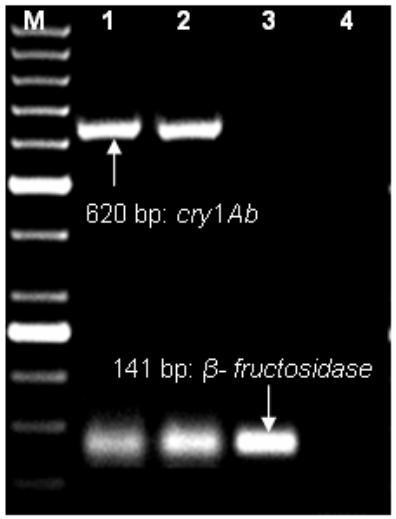
Tetraplex PCR in *Bt* potato with *cry1Ab* gene
1-2: *Bt* potato
3: Non-*Bt* potato
4: Water control
M: 50bp DNA ladder

GJ Randhawa, M Singh, R Chhabra and R Sharma (2010) Qualitative and quantitative molecular testing methodologies and traceability systems for *Bt* crops commercialised or under field trials in India. *Food Analytical Methods* 3 (4), 295-303

Multiplex PCR assays for detection of *Bt* crops with *cry1Ab* gene

Bt Brinjal

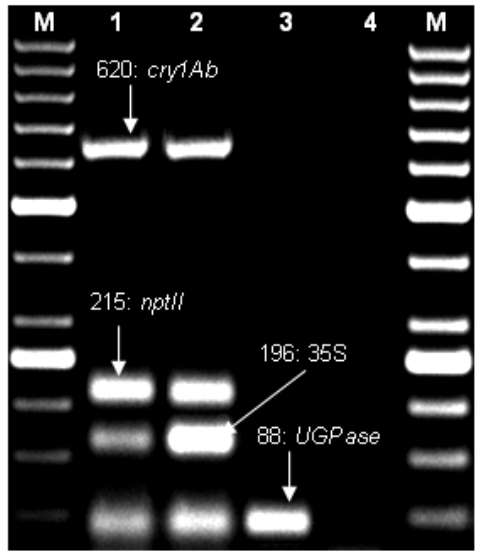




Duplex PCR in *Bt* brinjal with *cry1Ab* gene simultaneously detecting (i) *cry1Ab* and *CaMV* 35S *promoter; and (ii) cry1Ab and endogenous β- fructosidase* gene

1-2: *Bt* brinjal
3: Non-*Bt* brinjal
4: Water control
M: 50bp DNA ladder

Bt Potato



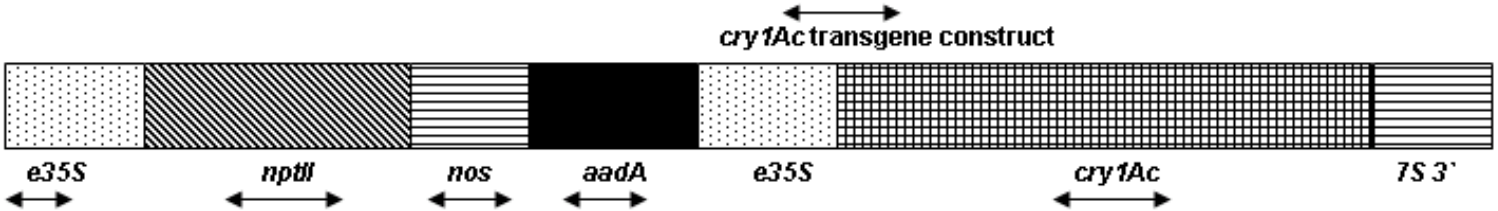
Tetraplex PCR in *Bt* potato with *cry1Ab* gene

1-2: *Bt* potato
3: Non-*Bt* potato
4: Water control
M: 50bp DNA ladder

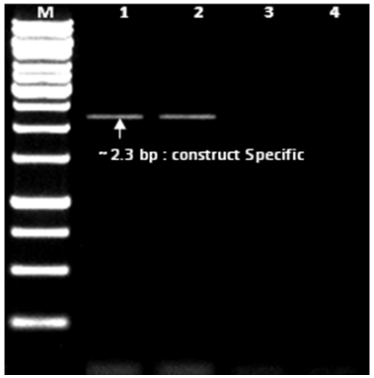
Randhawa G.J., M Singh, R Chhabra and R Sharma (2010) Qualitative and Quantitative Molecular Testing Methodologies and Traceability Systems for Bt Crops Commercialised or Under Field Trials in India. Food Analytical Methods 3 (4), 295-303

Construct-specific PCR

Targeting the junction region of two elements of construct

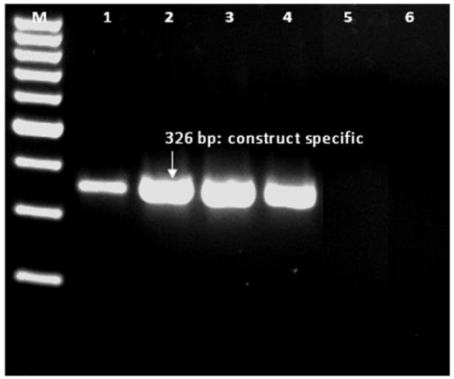


Linear Transgene Construct of MON 531



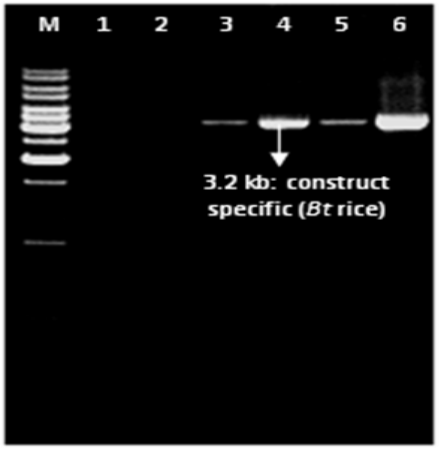
Construct-specific PCR for detection of a part of inserted gene construct in GM Tomato (*avp1* gene)

Lane1-2: GM tomato, Lane 3: Non-GM tomato, Lane 4: Water control, M: 1kb Ladder



Construct-specific PCR for detection of a part of inserted gene construct in GM cotton events *viz.* BGI, BGII, Event1 and GFM- cry1A

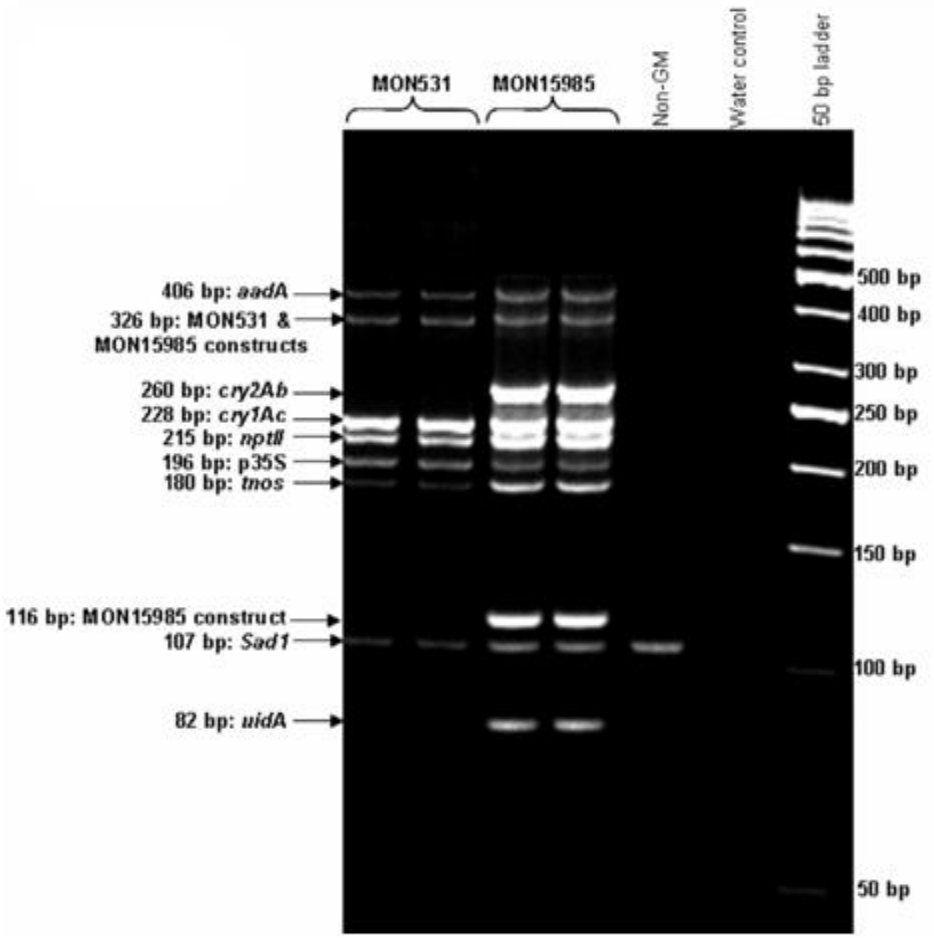
Lane 1: BGI, Lane 2: BGII, Lane 3: Event1, Lane 4: GFM-cry1A
Lane 5: Non-GM cotton, Lane 6: Water control, M: 100 bp ladder



Construct-specific PCR for detection of a part of inserted gene construct in GM rice (*cry1Ac* gene)

Lane 1: Water control, Lane 2: Non-GM rice, Lane 3-6: GM rice, M: 1kb ladder

Decaplex PCR Assay for detection of two major *Bt* cotton events MON531 and MON15985

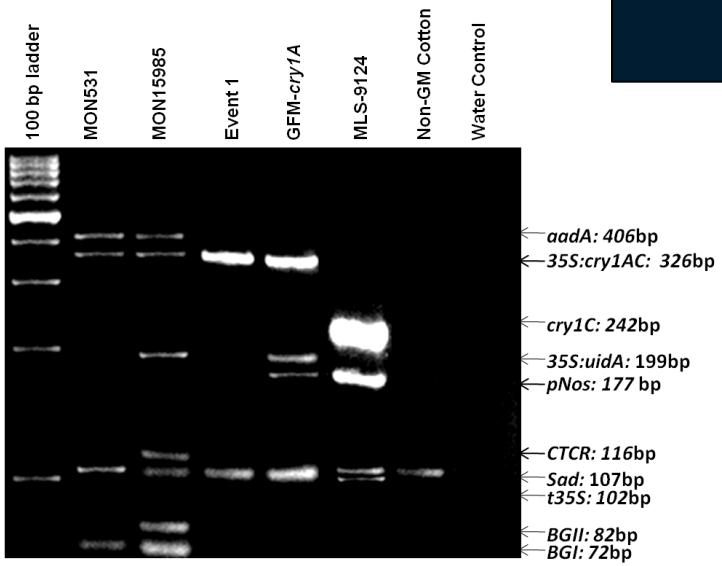


Transgene- and construct-specific multiplex PCR for discrimination of two *Bt* cotton events, using primer pairs for *cry1Ac* and *cry2Ab* transgenes, *nptII, aadA*, and *uidA* marker genes, *CaMV* 35S promoter, *nos* terminator, endogenous *Sad1* gene, and specific gene constructs

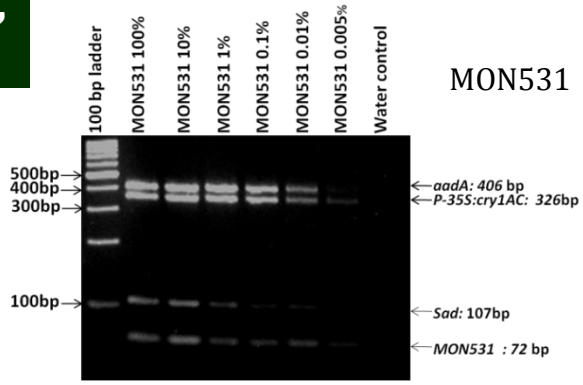
Randhawa G.J., R Chhabra and M Singh (2010) Decaplex and Real-Time PCR Based Detection of MON531 and MON15985 *Bt* cotton events. *J. Agri. Food Chem*. (2010) 58 (18), 9875–9881

Decaplex PCR to screen and discriminate five *Bt* cotton events, viz., MON531, MON15985, Event 1, GFM-cry1A and MLS-9124

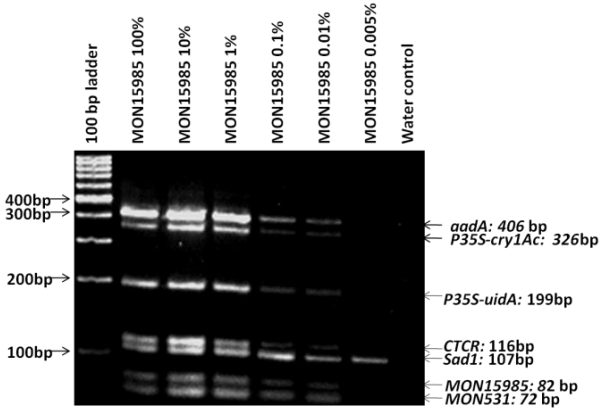
Limit of Detection (LOD): Upto 0.01%



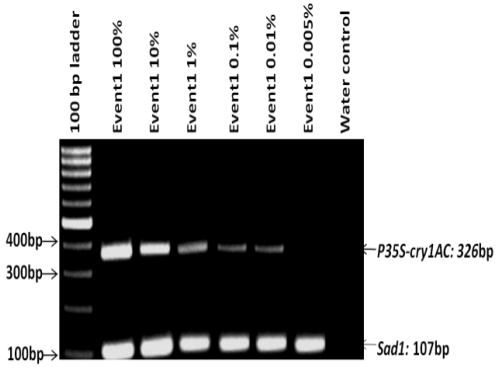
MON531



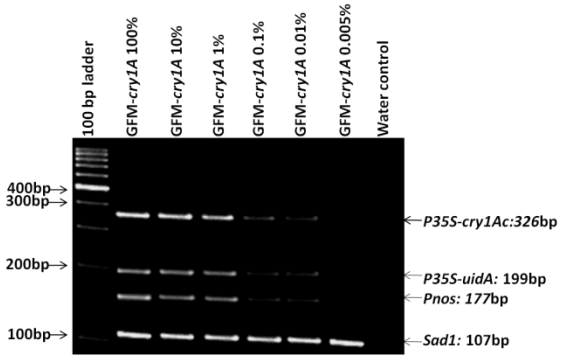
MON15985



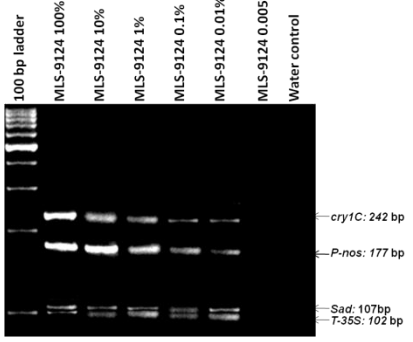
Event 1



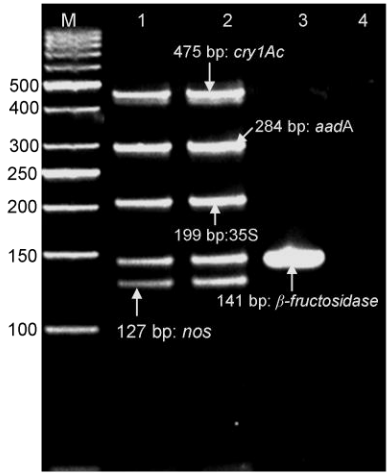
GFM-*cry1A*



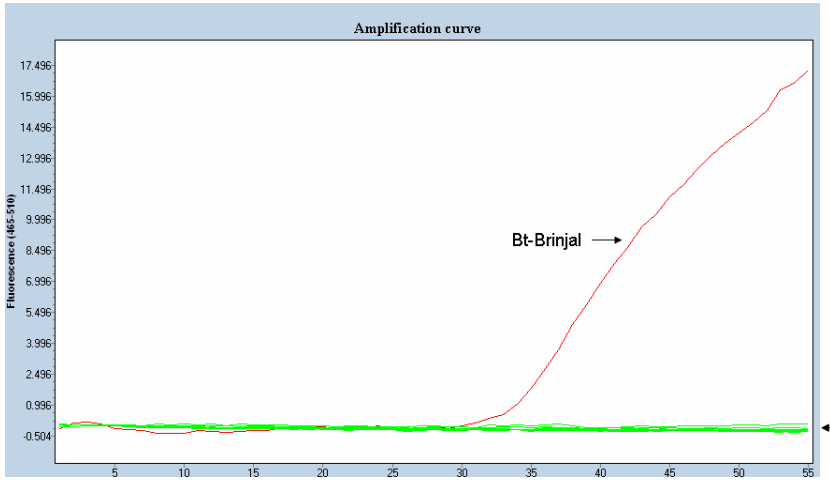
MLS-9124



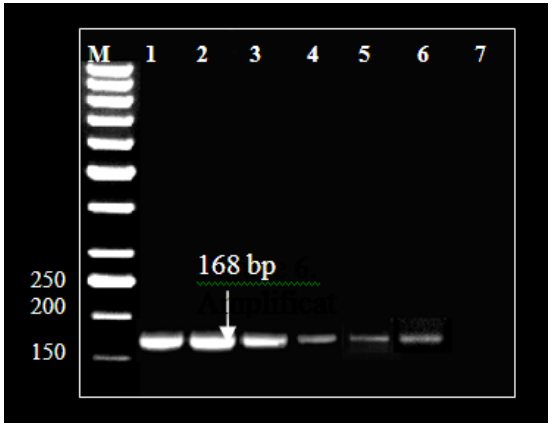
Event-specific detection of *Bt* brinjal event EE-1



Pentaplex PCR detection of *Bt* brinjal event EE-1, M: 50 bp ladder; Lanes 1, 2: Samples of *Bt* brinjal; Lane 3: Sample of non-*Bt* brinjal; Lane 4: Water control



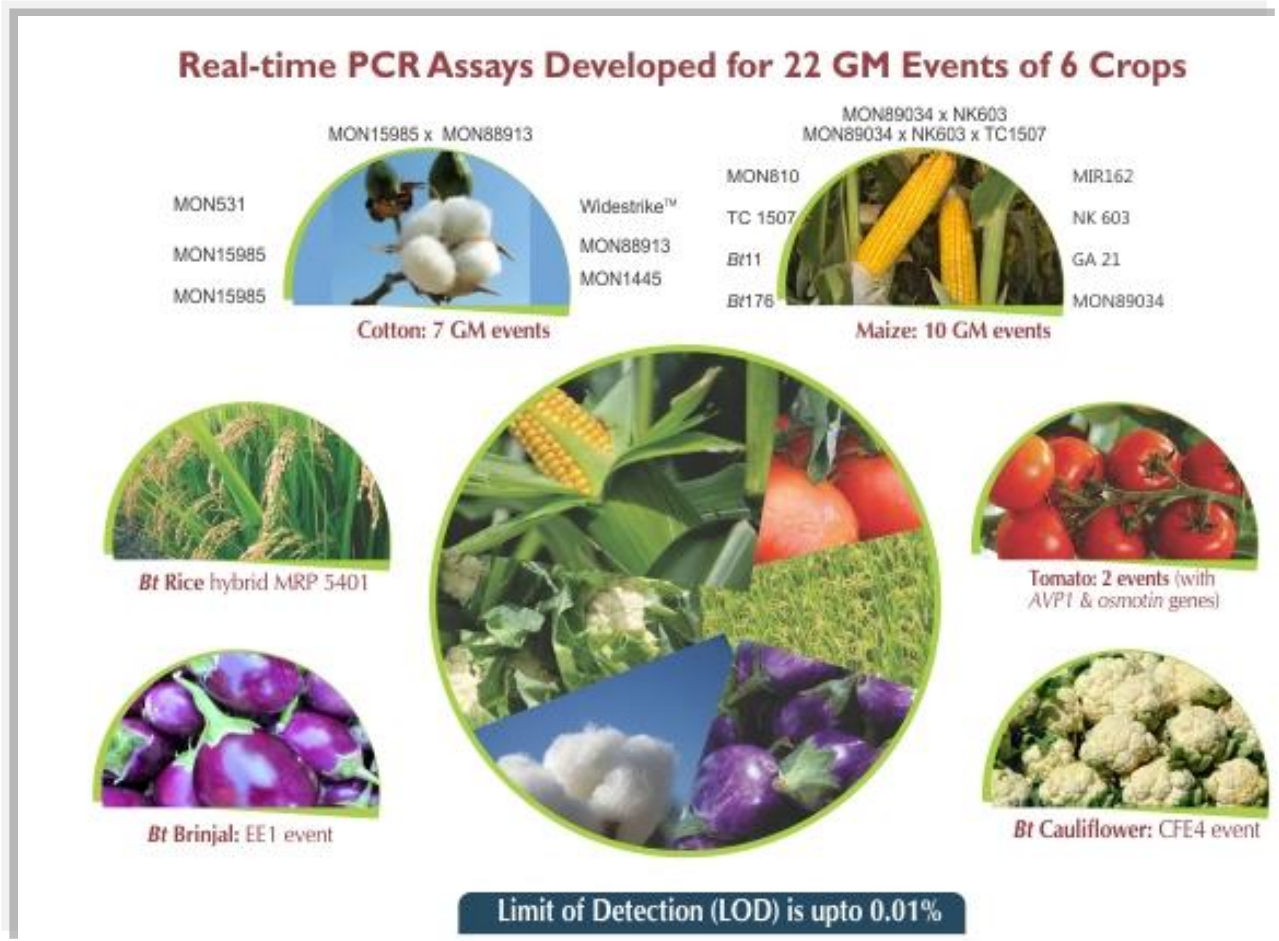
Real-time event-specific PCR for *Bt* Brinjal Event EE-1 using specific probes



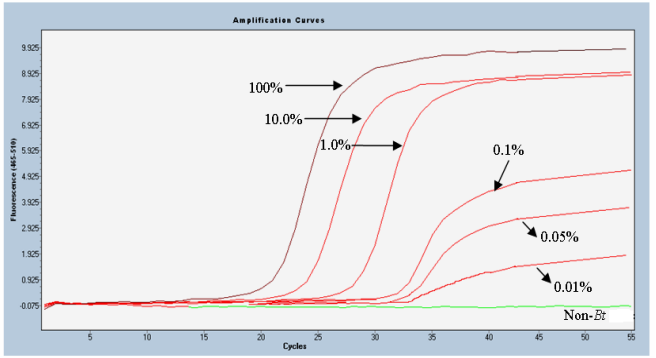
Lane M: 50bp ladder; Lane M: 1kb ladder, Lanes 1-6: Serial dilutions of *Bt* brinjal with 100, 10, 1.0, 0.1, 0.05, 0.01% of GM content, Lane 7: Non-*Bt* brinjal

LOD is upto 0.01%

GJ Randhawa, R Sharma & M Singh (2012) Qualitative and event-specific real-time PCR detection methods for *Bt* brinjal event EE-1. *J. AOAC Int.* 95 (6): 1733-1739



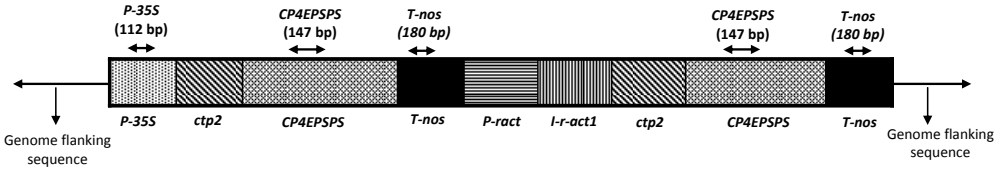
Amplification curves for *EPSPS* gene in Roundup ready cotton (MON 1445)

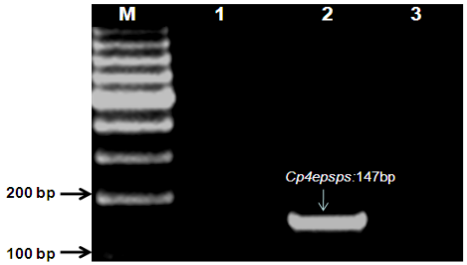


Identification of specific transgene by simplex PCR /multiplex PCR : Development of qualitative and quantitative PCR assays

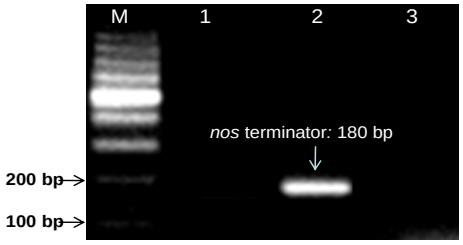
Event	Multiplex PCR	Transgenes + reference gene involved
Bt cotton MON 531	*Heptaplex*	*fs-ACP + cry1Ac + 35S promoter + nos term. + nptII + aadA + cry1Ac construct*
Bt cotton MON 15985	Decaplex	*fs-ACP + cry1Ac + cry2Ab + 35S promoter + nos term. + nptII + aadA + uidA + cry1Ac construct + cry2Ab construct*
Bt Rice	*Pentaplex*	*cry1Ac, nptII, 35S promoter, nos terminator + α-tubulin*
Bt Brinjal	Quadraplex	*cry1Ac, caMV 35S promoter, aadA + β- fructosidase*
Bt Brinjal	Triplex	*cry1Ab, 35S promoter + β- fructosidase*
Bt cauliflower	Triplex	*cry1Ac, 35S promoter + SRK*
Bt Okra	Quadraplex	*cry1Ac, nptII, 35s promoter + chloroplast t-RNA*
GM tomato	Quadraplex	*Avp1, nptII, 35S promoter + LAT52*
GM tomato	Triplex	*Osmotin + 35S promoter + LAT52*
GM potato	Triplex/ Quadraplex	*RB gene, CaMV 35S promoter, npt II marker + UGPase*
GM potato	Triplex/ Quadraplex	*Ama1 gene, CaMV 35S promoter, nos terminator, nptII + UGPase*
GM potato	Triplex/ Quadraplex	*cry1Ab gene, CaMV 35S promoter, nos , nptII + UGPase*

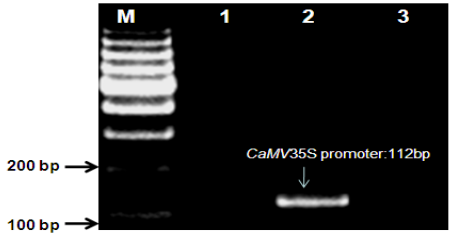
DNA-based diagnostics for GM wheat MON71800

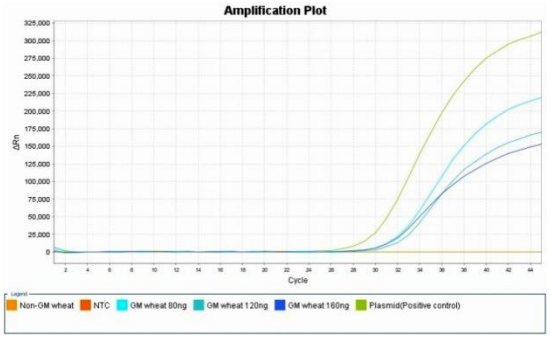




PCR based diagnostics for GM wheat targeting (a) *CP4EPSPS* transgene; (b) *CaMV*35S promoter; (c) *nos* terminator; M: 100 bp ladder; 1: Sample of Non-GM wheat; 2: Sample of GM wheat; 3: Non-template Control





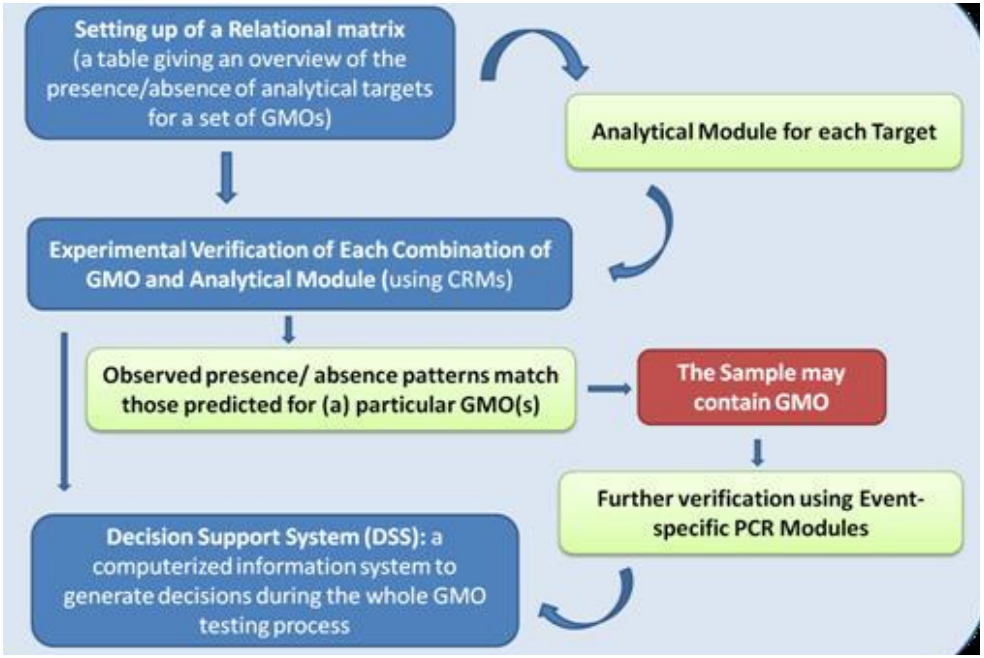


Amplification profile for real-time PCR analysis for *CP4EPSPS* transgene in GM wheat

GMO Matrix: A Cost-effective Screening Approach

Most efficient and cost-effective strategy for detection of authorized and unauthorized GMOs

(Holst-Jensen et al., 2012; Kralj Novak et al., 2009; Van den Bulcke et al., 2010; Waiblinger et al., 2010)



Setting and implementation of matrix is a stepwise process

GMO Screening Matrix using GMO Seek Algorithm

- GMO screening matrix, with the information on **106 genetic element targets for detection of 141 GM events of 21 crops**, has been developed;
- The matrix includes commercially cultivated *Bt* cotton events and other GM events, under field trials during the past six years;
- Ten most frequently present targets, *viz.*, ***P-35S, T-nos, Os-Msca1, cry1Ab, cry1Ac, cry1C, cry2Ab, GA20 oxidase1, nptII, bar*** were identified to screen these events using a GMOseek algorithm.

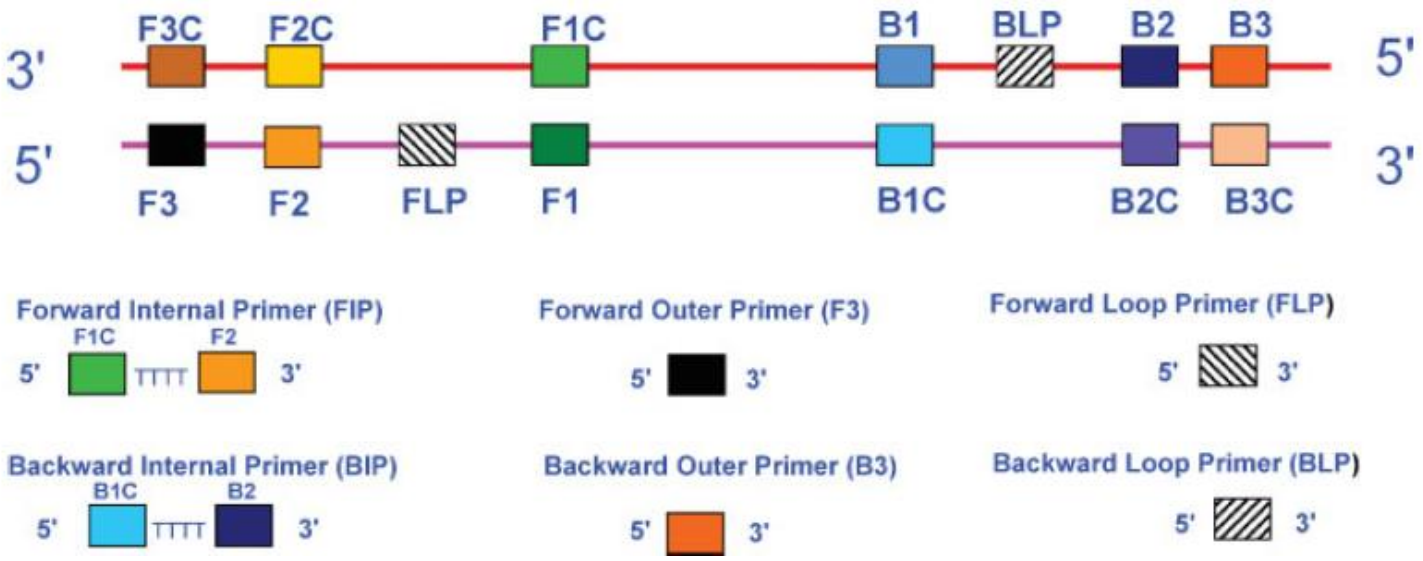
Genetic elements frequently appearing in combinations resulting from the GMO-seek simulations for all plants and the main crop species.

Crops	Status	Genetic elements
All plants	All status	[**P-35S**] [**T-nos**] [**Os-Msca1**] [**cry1Ab**] [**cry1Ac**] [**Cry1C**] [**cry2Ab**] [**GA20 oxidase1**]
	Commercialized	[**P-35S**]
	Field trials	[**P-35S**] [**T-nos**] [**Os-Msca1**] [**cry1Ad**] [**GA20 oxidase1**] [**nptII**]
	Imported	[**P-35S**] [**T-nos**] [*m-epsps*] [**cry1Ab**] [**cry1Ac**] [**cry1C**] [**cry2Ab**] [**bar**] [*gat*]
	Indigenously developed	[**T-nos**] [**Os-Msca1**] [**cry1Ac**] [**cry2Ab**] [*mtlD*] [**GA20 oxidase1**] [**nptII**] [**bar**]
Cotton	All status	[**P-35S**] [*m-EPSPS*] [**cry1Ac**] [*cry1F*]
	Commercialized	[**P-35S**]
	Field trials	[**P-35S**] [*m-EPSPS*] [**cry1Ac**] [*cry1F*]
	Imported	[**P-35S**] [*m-EPSPS*] [**cry1Ac**]
	Indigenously developed	[**T-nos**] [**cry1Ac**]
Rice	All status	[**P-35S**] [**T-nos**] [**cry1Ab**] [**cry1Ac**] [**cry1C**] [**cry2Ab**] [*gat*]
	Commercialized	/
	Field trials	[**T-nos**] [**cry1Ab**] [**cry2Ab**] [cry2Ad]
	Imported	[**P-35S**] [**cry1Ab**] [**cry1Ac**] [**cry1C**] [**cry2Ab**] [*gat*]
	Indigenously developed	[**cry1Ac**] [**cry2Ab**]
Corn	All status	[**P-35S**]
	Commercialized	/
	Field trials	[**P-35S**]
	Imported	[**P-35S**]
	Indigenously developed	/

Randhawa GJ, Morisset D, Singh M,and Žel J (2013) GMO matrix: A cost-effective approach for screening unauthorized GM events in India. Food Control, 38: 124-129

Loop-mediated isothermal amplification (LAMP)

- 'LAMP' is characterised by the use of six different primers specifically designed to recognise eight distinct regions on the target gene.
- The amplification proceeds at a constant temperature using strand displacement reaction. Amplification and detection of a gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature.



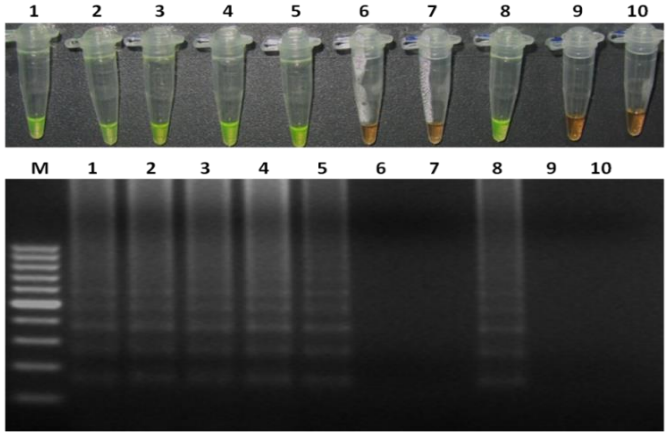
Schematic representation of primer design for LAMP assay showing the position of the six primers spanning the target

Loop-mediated isothermal amplification (LAMP) Assays for GMO Screening

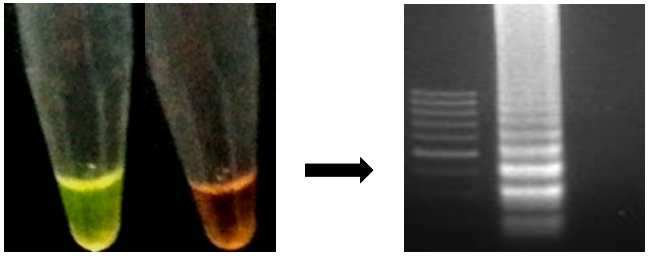
End-point LAMP assays for the screening elements:

Promoters
P-35S
P-FMV
P-nos

Marker genes
aadA
nptII
uidA



Detection of *nptII* marker gene
Green colour: Positive test samples
Orange colour: Negative test samples



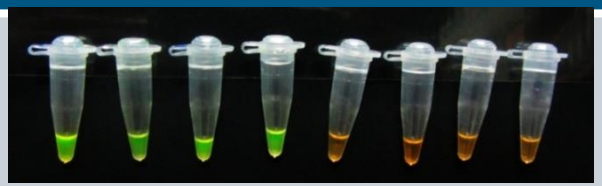
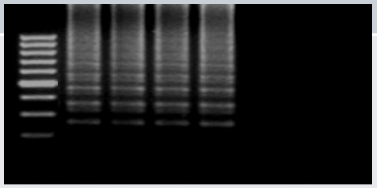
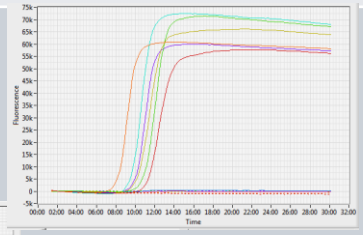
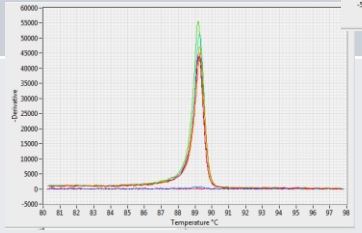
MON531 Non GM cotton

MON531 Non GM cotton

Detection of *CaMV35S* promoter

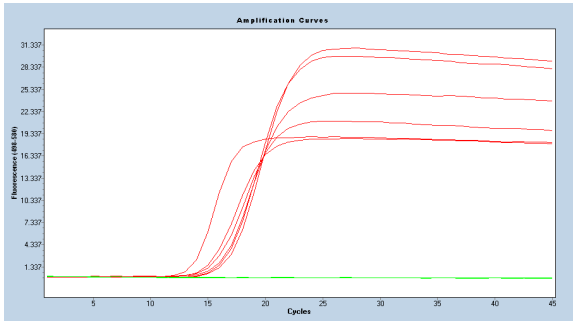
LAMP assays can be used for rapid, cost-effective and on-spot detection of GM crops at port of entry and farmers' fields

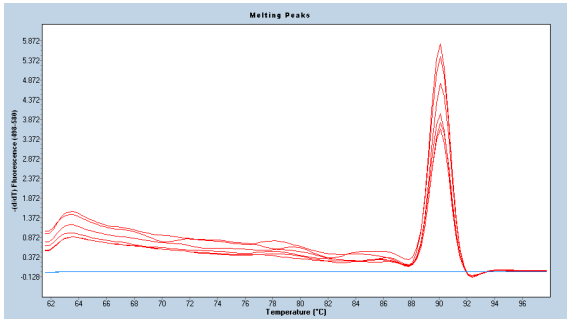
Loop-mediated Isothermal Amplification (LAMP): Screening Tool for GMOs (Targets: *P-35S, P-FMV, aadA, nptII, uidA*)

System	Chemistry	Detection Method	LOD	Completion Time
Heating Block	Bst DNA Pol.		40 copies	75 min
Thermal cycler	Bst DNA Pol.	End Point 	40 copies	75 min
Light Cycler®480 Real-time System	OptiGene Isothermal Master Mix	Real-time 	10 copies	45 min
Isothermal Real-time System (Genie II)	OptiGene Isothermal Master Mix		4 copies	35 min

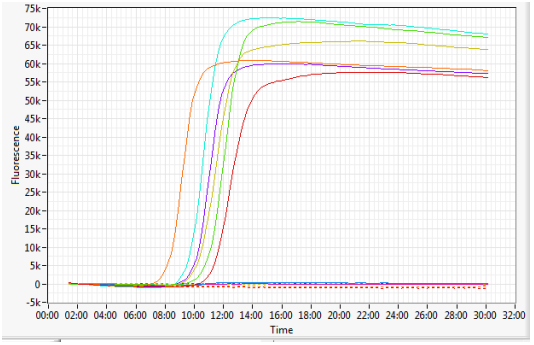
Randhawa GJ, Singh M, Morisset D, Sood P and Žel J (2013) Loop-mediated isothermal amplification: Rapid, visual and real-time methods for detection of GM crops. J Agric. Food Chem. , DOI: dx.doi.org/10.1021/jf4030085

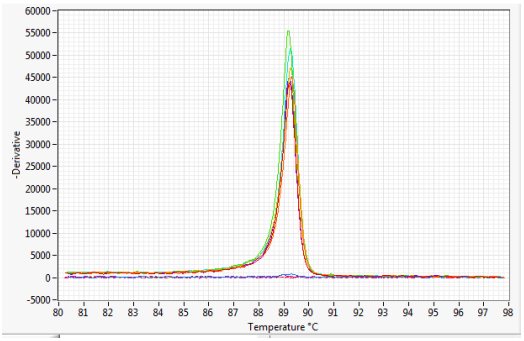
Real-time LAMP for *nptII* marker gene





Amplification profile and melting curves for real-time LAMP products on Light Cycler 480 system





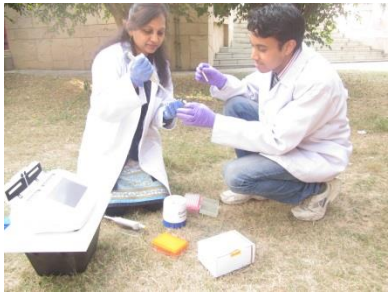
Amplification and annealing curves for LAMP assays on isothermal real-time system (Genie® II)

Randhawa GJ, Singh M, Morisset D, Sood P and Žel J (2013) Loop-mediated isothermal amplification: Rapid, visual and real-time methods for detection of GM crops. J Agric. Food Chem. , DOI: dx.doi.org/10.1021/jf4030085

Advantages of real-time LAMP assays:

- Real Time LAMP - a newer generation of diagnostics
- ideal for end of point diagnostic
- rapidity
- quantitative measurement
- lower volume of sample required
- lower contamination rate
- higher sensitivity
- higher specificity
- easy standardisation
- applicable to crude sampling
- portability

On-site GM detection using RT-LAMP



Ready-to-use real-time multi-target plate for GMO screening

Targets for Multi-target Plate for Screening and Detection of GM Food Crops/Events

Endogenous genes (6): Maize, Cotton, Rice, Brinjal, Soy, Potato

Events (21): Maize (12), Cotton (6), Rice (1), Brinjal (1), Soy (1)

Construct-specific (5)

Transgenes (8)

Control elements (4)

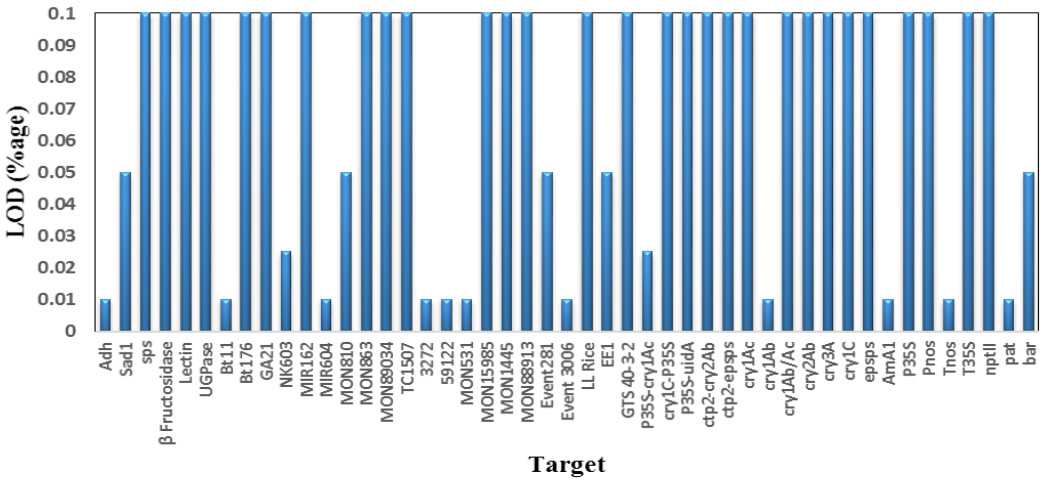
Marker genes (3)

Adh1	GA21	3272	LL Rice	*cry1Ac*	*P-35S*	Adh1	GA21	3272	LL Rice	*cry1Ac*	*P-35S*
Sad1	NK603	59122	EE1	*cry1Ab*	*P-nos*	*Sad1*	NK603	59122	EE1	*cry1Ab*	*P-nos*
sps	MIR162	MON531	GTS 40-3-2	*cry1Ab/Ac*	*T-nos*	*sps*	MIR162	MON531	GTS 40-3-2	*cry1Ab/Ac*	*T-nos*
β-fructosidase	MIR604	MON15985	*P35S - cry1Ac construct*	*cry2Ab*	*T-35S*	*β-fructosidase*	MIR604	MON15985	*P35S - cry1Ac construct*	*cry2Ab*	*T-35S*
Lectin	MON810	MON1445	*cry1C-P35S* construct	*cry3A*	*nptII*	*Lectin*	MON810	MON1445	*cry1C-P35S* construct	*cry3A*	*nptII*
UGPase	MON863	MON88913	*P35S-uidA* construct	*cry1C*	*pat*	*UGPase*	MON863	MON88913	*P35S-uidA* construct	*cry1C*	*pat*
Bt11	MON89034	Event281	*ctp2-cry2Ab construct*	*epsps*	*bar*	Bt11	MON89034	Event281	*ctp2-cry2Ab construct*	*epsps*	*bar*
Bt176	TC1507	Event3006	*ctp2-epsps*	*AmA1*	control	Bt176	TC1507	Event3006	*ctp2-epsps*	*AmA1*	control

Ct value: 24.35±0.87 (*UGPase*) to 38.12±0.40 (*bar*)

47 Targets for ready-to-use real-time multi-target screening plate

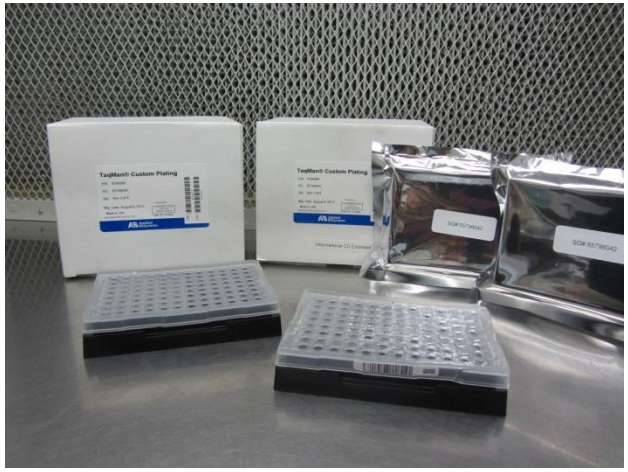
Maize	Cotton	Rice	Brinjal	Soy
Bt11	MON531	LL Rice	EE1	GTS 40-3-2
Bt176	MON15985			
GA21	MON1445			
NK603	MON88931			
MIR162	Event281			
MIR604	Event3006			
MON810				
MON863				
MON89034				
TC1507				
3272				
59122				



Endogenous Gene	Crop
Adh1	Maize
Sad1	Cotton
Sps	Rice
β-fructosidase	Brinjal
Lectin	Soy
UGPase	Potato

Limit of Detection : Upto 0.1-0.01% for different targets

Constructs	Transgenes	Promoters	Terminators	Marker genes
P-35-cry1Ac	*cry1Ac*	*P-35S*	*T-nos*	*nptII*
cry1C-P-35	*cry1Ab*	*P-nos*	*T-35S*	*pat*
P-35-uidA	*cry1Ab/Ac*			*bar*
ctp2-cry2Ab	*cry2Ab*			
ctp2-epsps	*cry1C*			
	epsps			
	AmA1			



Publications on GM Detection work

1. Randhawa GJ, Singh M, Morisset D, Sood P and Žel J (2013) Loop-mediated Isothermal Amplification: Rapid Visual and Real-Time Methods for Detection of Genetically Modified Crops. ***J. Agric. Food Chem***., DOI: 10.1021/jf4030085
2. Randhawa GJ, Morisset D, Singh M and Žel J (2013) GMO matrix: A cost effective approach for screening unauthorized genetically modified events in India. ***Food Control***. DOI: 10.1016/j.foodcont.2013.10.013
3. Randhawa GJ, Sharma R and Singh M (2012) Qualitative and event-specific real-time PCR detection methods for *Bt* brinjal event EE-1. ***J. AOAC Int.*** 95 (6): 1733-173
4. Randhawa GJ and Singh M (2012) Multiplex, construct-specific and real-time PCR based analytical methods for *Bt* rice with *cry1Ac* gene**.** ***J. AOAC Int***. 95 (1): 186-194
5. Randhawa GJ, Chhabra R and Singh M (2011) PCR-based detection of GM tomato with *AVDI* gene employing seed sampling strategy**.** ***Seed Sci. Technol*** . 39, 112-124.
6. Randhawa GJ, Chhabra R and Singh M (2010) Decaplex and real-time PCR based detection of MON531 and MON15985 *Bt* cotton events. ***J. Agric. Food Chem.*** *58* (18), 9875-9881.
7. Randhawa GJ, Singh M, Chhabra R and Sharma R (2010) Qualitative and quantitative molecular testing methodologies and traceability systems for *Bt c*rops commercialised or under field trials in India. ***Food Anal. Meth.*** 3:295-303.
8. Randhawa GJ, Chhabra R and Singh M (2009) Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for screening of GM crops**.** ***J. Agric. Food Chem***. 57: 5167-5172.
9. Randhawa G.J., Singh M. and Sharma R. (2009) Validation of *ST-LS1* as an endogenous reference gene for detection of *AmA1* and *cry1Ab* genes in GM potatoes using multiplex and real time PCR. ***Am. J. Pot. Res.*** 86: 398-405.
10. Randhawa G.J. and Chhabra R. (2009) Import and commercialization of transgenic crops: An Indian perspective. ***Asian Biotechnol. Develop. Rev.*** 11(2): 115-130.

Publications on GM Detection work

11. Randhawa GJ, Sharma R and Singh M (2009) Duplex, triplex and quadruplex PCR for molecular characterization of GM Potato with improved protein quality ***Curr. Sci.*** 97 (1) 21- 23.
12. Randhawa GJ, Sharma R and Singh M (2009) Multiplex PCR for detection of GM potato with *cry1Ab* gene. ***Ind. J. Agric. Sci.*** 79 (5): 368-71.
13. Randhawa GJ, Chhabra R and Singh M (2009) Molecular diagnosis of transgenic tomato with *osmotin* gene using Multiplex PCR. ***Curr. Sci.*** 96 (5): 689-694.
14. Randhawa GJ, Singh M, Chhabra R, Guleria S and Sharma R (2008) Molecular characterization of *Bt* cauliflower with multiplex PCR and validation of endogenous reference gene in *Brassicaceae* family. ***Curr. Sci.*** 95 (12) :1729-31.
15. Randhawa GJ and Firke PK (2006) Detection of transgenes in GM soybean and maize using polymerase chain reaction. ***Ind. J. Biotech.*** 5: 510-513.


Thank You!