

Technical Reference Document on Quantitative PCR-Based Detection and Identification of Living Modified Organisms

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Table of Contents

Technical Reference Document (TRD) on Quantitative PCR-Based Detection and Identification of Living Modified Organisms (LMOs)..... 1

1. Background	3
2. Scope.....	3
3. Principle of Detection	4
4. Laboratory Workflow for GMO Detection	4
4.1 Sample Receipt and Registration	4
4.2 Sample Processing	5
4.3 DNA Extraction	5
4.4 DNA Quality Assessment	5
5. Qualitative GMO Detection Using Conventional PCR	6
6. Quantitative Detection Using Real-Time PCR (qPCR).....	7
6.1 qPCR procedure	7
6.2 Relative Quantification of GMO DNA.....	8
6.3 Interpretation of Results.....	8
7. Quality Assurance and Run Validation	8
8. Reporting of Results	9
9. Contamination Prevention	10
10. Waste Management	10
11. Data Management	10
12. References.....	11

1. Background

Reliable detection and identification of living modified organisms (LMOs), commonly referred to as genetically modified organisms (GMOs), is essential for biosafety monitoring, regulatory compliance, and food safety assurance. Molecular detection methods based on Polymerase Chain Reaction (PCR) are the most widely adopted technologies for GMO identification due to their high sensitivity, specificity, and reproducibility.

The Molecular Biology Laboratory of the Council for Scientific and Industrial Research - Crops Research Institute applies a standardized workflow, combining conventional PCR screening and Quantitative real-time PCR (qPCR) detection for relative quantification of transgene content in food based on the Biotechnology Explorer™ GMO Investigator™ Kit (Catalog no. 166-2500EDU). These activities contribute to National Biosafety Authority (NBA) oversight by providing analytical support to the NBA and other competent bodies in GMO detection.

This technical reference document provides an overview of the laboratory procedures used for the detection and estimation of GMO content in plant-derived samples submitted for testing.

2. Scope

This TRD applies to all Molecular Biology Laboratory personnel performing GMO testing of seeds, grains and processed food products submitted by clients for GMO analysis. This document describes the technical workflow used for:

- Sample Receipt and Processing
- Detection of GMO-associated DNA sequences using PCR
- Confirmation of plant DNA quality using endogenous reference genes
- Estimation of relative GMO content using quantitative real-time PCR

All samples are submitted by clients for GMO analysis.

3. Principle of Detection

The analytical approach relies on amplification of **transgenic regulatory elements** commonly used in GM crops.

Target sequences screened include:

Target Element	Description
CaMV 35S promoter	Viral promoter widely used in GMO constructs
NOS terminator	Gene terminator from <i>Agrobacterium tumefaciens</i>
PS II gene	Endogenous plant gene used as internal reference

Conventional PCR is used for qualitative detection, while SYBR Green-based quantitative real-time PCR (qPCR) is used for estimation of the proportion of GMO DNA relative to plant genomic DNA. Fluorescence generated by SYBR Green binding to double-stranded DNA is monitored during amplification cycles to determine the Cycle threshold (Ct) value, which is proportional to the amount of target DNA present in the sample.

4. Laboratory Workflow for GMO Detection

The laboratory follows a structured workflow to ensure traceability, reproducibility, and reliability of results.

4.1 Sample Receipt and Registration

Client samples are received and recorded in the Sample Receipt Logbook and Client Request Form. Each sample is assigned a unique Laboratory Identification Code (Lab ID) to ensure traceability throughout the analytical process. Samples are inspected for integrity and registered together with accompanying client information. Dry seeds are stored at ambient temperature, while milled/processed samples are stored at -20°C until extraction.

4.2 Sample Processing

Seed and grain samples are ground using a dedicated laboratory grinder to obtain a uniform powder. Processed samples are mixed thoroughly to ensure uniformity. Duplicate sub-samples of approximately 0.5 g are transferred into sterile microcentrifuge tubes and stored at -20°C for DNA extraction.

4.3 DNA Extraction

Genomic DNA is isolated using a cetyltrimethylammonium bromide (CTAB) extraction method based on Doyle and Doyle (1987). This method provides high-quality genomic DNA suitable for PCR-based detection.

The procedure follows the steps below:

1. Add 1 mL pre-warmed CTAB buffer to homogenized sample (~0.5 g).
2. Incubate 60°C , 30 min, gentle mixing.
3. Phenol:chloroform extraction, centrifuge, and transfer supernatant.
4. DNA precipitation with isopropanol, centrifuge, wash with 70% ethanol.
5. Resuspend pellet in 50 μL nuclease-free water; allow 15–30 min dissolution.

4.4 DNA Quality Assessment

DNA concentration and purity are evaluated using a spectrophotometer.

Acceptable quality criteria for GMO testing include:

Parameter	Acceptance Criteria
DNA concentration	$\geq 20 \text{ ng}/\mu\text{L}$
A260/280 ratio	1.7–2.0
A260/230 ratio	≥ 1.8

Samples not meeting these criteria may be re-extracted before analysis. DNA is diluted for PCR as 50 ng/μL, while dilution of 1:10 is prepared for qPCR. Genomic DNA is stored at –20°C, with working dilutions stored at 4°C (≤1 week).

5. Qualitative GMO Detection Using Conventional PCR

Conventional PCR screening is performed to determine whether GMO-associated genetic elements are present.

PCR amplification targets:

- 35S promoter
- NOS terminator

An endogenous plant gene (PS II) serves as an internal control to verify DNA quality.

PCR products are resolved using agarose gel electrophoresis and visualized using nucleic acid staining. Expected fragment sizes confirm successful amplification of target sequences.

The conventional PCR protocol is as follows:

1. Prepare primers: GMO (10 μM), PS II (2.5 μM). Store at –20°C.
2. Prepare master mix on ice; dispense into PCR tubes.
3. Add 2 μL DNA template (~50 ng).
4. Thermocycler conditions:
 - Initial denaturation: 94°C, 2 min
 - 40 cycles: 94°C 30s, 59°C 30s, 72°C 30s
 - Final extension: 72°C 5 min
 - Hold: 4°C
5. Include controls per run: Positive control (GMO DNA), No Template Control (NTC), PS II control.
6. Agarose gel electrophoresis (1.5% agarose, 100 V, 30 min).
7. Visualize bands using UV/blue-light system, document and archive images.

8. Interpret results:

- 203/225 bp band: GMO detected as 35S promoter and NOS terminator sequences
- 455 bp band: PS II gene detected hence DNA quality adequate
- NTC band: implies contamination hence invalidates run

6. Quantitative Detection Using Real-Time PCR (qPCR)

Quantitative detection is performed using SYBR Green–based real-time PCR.

The assay monitors fluorescence emitted during amplification cycles to determine Ct values for both:

- the GMO target gene
- the plant reference gene (PS II)

Each reaction is performed in duplicate to ensure analytical reliability.

A melt curve analysis is conducted following amplification to confirm specificity of the PCR products.

6.1 qPCR procedure

1. Use 1:10 diluted DNA and SYBR Green master mix.
2. Prepare duplicate reactions for each sample, positive control, NTC, PS II control.
3. Thermocycling (Qiagen Rotor Gene Q):
 - Initial Hold: 95°C 4 min
 - 40 cycles: 94°C 60 s, 59°C 60 s (Green), 72°C 60 s (Green)
 - Melt Curve: 55–95°C
4. Analyze amplification curves and melt curves; duplicate Ct variation ≤ 0.5 acceptable.
5. Relative quantification: $\Delta Ct = Ct(\text{GMO}) - Ct(\text{PSII}) \rightarrow \text{Relative GMO} = 2^{(-\Delta Ct)} \times 100\%$.

6. All data is recorded in the qPCR Logbook and raw files are archived.

6.2 Relative Quantification of GMO DNA

Relative quantification is based on comparison of Ct values between the GMO target gene and the plant reference gene.

The difference between Ct values is calculated as:

$$\Delta Ct = Ct_{GMO} - Ct_{PSII}$$

The relative amount of GMO DNA is then estimated using:

$$Relative\ GMO\ Quantity = 2^{-\Delta Ct}$$

To express results as percentage GMO content:

$$\%GMO = Relative\ GMO\ Quantity \times 100$$

This calculation assumes near-optimal PCR amplification efficiency.

6.3 Interpretation of Results

Results are interpreted based on the estimated percentage of GMO DNA relative to plant DNA.

Percentage GMO	Interpretation
< 1 %	Considered Non-GMO
≥ 1 %	Considered GMO

7. Quality Assurance and Run Validation

Quality control measures are implemented to ensure validity of the analytical results.

Each PCR or qPCR run includes:

- Positive control DNA (This is not Certified Reference Materials, but rather in-house non-certified positives).

- No-template control (NTC)
- Internal plant reference gene amplification

Runs are accepted only when:

- Positive controls amplify correctly
- NTC shows no amplification
- PS II reference gene amplifies in all samples
- Duplicate Ct values fall within acceptable limits

Any deviation results in rejection of the run and repetition of the assay.

8. Reporting of Results

GMO test reports are prepared following verification and review of analytical results.

Reports include:

- Client information
- Sample identification
- Target genes analyzed
- Qualitative detection results
- Estimated percentage of gmo dna
- Interpretation of results relative to regulatory thresholds

Reports undergo two levels of authorization:

1. Verification by the Laboratory Supervisor
2. Final approval by the Quality Manager

Only authorized reports are issued to clients.

All analytical records and reports are archived according to institutional record-retention policies.

9. Contamination Prevention

Strict measures are implemented at the Molecular Biology Laboratory to prevent cross-contamination during GMO testing. Sample preparation, DNA extraction, PCR and qPCR setup, and post-PCR analysis are conducted following controlled laboratory practices. Work surfaces and equipment are regularly disinfected using 70% ethanol. Dedicated pipettes and consumables are used for pre-PCR and post-PCR activities. Gloves are changed between samples, and negative controls are included in each analytical run to monitor potential contamination.

10. Waste Management

Laboratory waste generated during DNA extraction, PCR, and qPCR analysis is managed according to the CSIR–Crops Research Institute Laboratory Waste Management procedures. Chemical and hazardous wastes including phenol–chloroform mixtures, β -mercaptoethanol-containing buffers, and Ethidium Bromide waste are collected in designated chemical waste containers and disposed of following institutional hazardous waste guidelines. Biological waste such as used pipette tips, microcentrifuge tubes, agarose gels, and contaminated consumables are discarded in biohazard containers and treated according to approved biosafety protocols. All waste handling procedures comply with institutional laboratory safety and environmental protection regulations.

11. Data Management

All analytical activities performed in the Molecular Biology Laboratory are documented to ensure traceability of results. Records maintained include the Sample Receipt Logbook, Client Request Form, DNA Extraction Logbook, PCR and qPCR Run Logs, Gel documentation archives, and final test reports. Each sample is assigned a unique laboratory identification code that is used throughout the analytical process. Instrument run files, raw data, worksheets, and authorized reports are archived for a minimum period of five (5) years in accordance with institutional record-retention policies.

12. References

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, 19, 11–15.

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