International Seed Federation
Seed is Life

# Best Practices for PCR Assays in Seed Health Tests

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**DEVELOPED BY**
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## BEST PRACTICES AND THEIR PURPOSE

The following Best Practices have been developed to ensure accurate and reliable results when performing PCR Assays and to provide general guidance to laboratories developing and using seed health tests.
These guidelines are not intended to serve as a legal reference. They are not binding on ISHI nor ISF members.

## DISCLAIMER

This document has been developed based on the current technical state of the art. ISF cannot be held liable for any possible claims associated with the application of these guidelines.

## INTRODUCTION

A Polymerase Chain Reaction (PCR) assay assesses the presence of nucleic acid from specific organism(s) by the process of multiplication (amplification) of small nucleic acid templates (PCR product) relative to positive and negative controls. This document describes best practices for the use of Polymerase Chain Reaction (PCR) assays in seed health tests to ensure accurate and reliable results. Best practices include process controls and assay conditions that should be applied to all experiments.

Controls and conditions of the assays are designated either as essential (necessary to perform for proper test execution), recommended (advised to perform for proper test execution) or optional (not necessary to perform for proper test execution).

Specific recommendations that may be essential during PCR assay development and validation can be found in Technical Guidelines for PCR assay design. For detection and identification of RNA viruses and viroids, the PCR is preceded by an enzymatic Reverse Transcriptase (RT) step to transcribe RNA into DNA. Whenever this document refers to PCR, it may apply to both PCR and Real-Time PCR (qPCR).

## 1. PCR TESTS: INDIRECT TESTS FOR THE DETECTION AND IDENTIFICATION OF PATHOGENS

This document encompasses the two following types of PCR tests:

- **Seed Extract (SE)-qPCR assay**: An assay in which a seed extract is used for isolation of the nucleic acid of the pathogen present on or/and in the seed and the subsequent evaluation of the nucleic acid by qPCR assay.
- **Identification PCR**: A PCR assay whereby the nucleic acids used as template originate from single isolate microbial cultures to determine if the isolate is of the target pathogen.

The use of PCR assays for identification of single isolate microbial cultures, is usually applied to a sample with a known and/or controllable concentration and quality of the target nucleic acid. For example, a PCR assay may be performed with a cell suspension of a bacterial isolate from a dilution-plating assay. The density of the cell suspension, and therefore the concentration of the nucleic acid target can be controlled and defined.

In the case of detection using SE-qPCR, the quantity of target nucleic acid in the sample is unknown and quality of the sample is less controllable. For example, a PCR assay may be performed with a DNA or an RNA extract directly from seeds. In the case of direct RNA extraction from seeds, the RNA quality is highly dependent on the seed source and the RNA extraction method.

Due to genetic variability between pathogen isolates, and to reduce the risk of false negative results, the use of two primer sets for each target pathogen, which amplify different regions, is recommended in PCR assays.

A PCR assay detects any trace of DNA and RNA associated with a target pathogen without discriminating whether the source of the nucleic acid is pathogenic, viable, or intact. and is not sufficient to prove pathogenicity. While a negative PCR assay result can be assumed to be conclusive evidence of a healthy seed lot, a positive PCR assay result must be followed by a direct

test to inform about the viability of the pathogen and its pathogenicity (see ISF viewpoint on indirect seed health tests). A PCR assay is considered as a pre-screen performed prior to a direct test, such as a bioassay or grow-out assay (see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods). A PCR assay may also be used to confirm the detection of a target pathogen in suspect plant tissues obtained from a dilution plating, bioassay or grow-out assay.

## 2. CONTROLS AND THEIR PURPOSE

The types of controls for PCR assays are defined in Table 1. Their purpose is to verify both the quality of the material used in the assays and proper test execution, notably for the pathogen extraction, the nucleic acid (DNA or RNA) extraction-and the PCR process. Appropriate negative and positive controls should be included in every assay to ensure reliability of the test results.

**Table 1**. Controls defined for PCR assays.

| | **Non Target (Template) Control (NTC)** |
|---|---|
| **Definition** | Control that contains all PCR reagents used to prepare the sample that is tested and processed using the same assay at the same time as the samples. This control contains no extraction buffer, no target pathogen, and no spike. |
| **Objective** | To verify that the reagents used are free from the target in the absence of extraction buffer and samples. |
| **Expected Result** | No detection of any pathogen, including the target. |
| **Description** | PCR mix free from any pathogen or seed. |
| **SE-PCR for Detection** | Essential |
| **Identification PCR** | Essential |

Note: When using universal bacterial primers, positive reactions may occur due to the presence of residual DNA in Taq enzyme reagents. The Cq values of the IAC from reactions on suspect isolates should at least be 3.3 Cq value lower than the Cq values of the IAC from the NTC reactions.

| | **Negative Buffer Control (NBC)** |
|---|---|
| **Definition** | Control that contains all buffers and reagents used to prepare the sample that is tested and processed using the same assay and at the same time as the samples. This control contains no target pathogen and no spike. |
| **Objective** | To verify the sterility of the buffers in the absence of samples. |
| **Expected Result** | No detection of the target nucleic acid or non-specific products. |
| **Description** | Eluate obtained from the extraction process without seeds or pathogen. |
| **SE-PCR for Detection** | Recommended |
| **Identification PCR** | Recommended |

Note: Users should at least perform either the NBC or NPC.

| | Negative Process Control (NPC) |
|---|---|
| **Definition** | Control that contains a well characterized healthy seed sample (with respect to the target pathogen for SE-PCR or a non-target isolate strain for identification PCR, that is tested and processed using the same assay at the same time as the samples. |
| **Objective** | To verify that no cross-contamination occurred under the assay conditions. |
| **Expected Result** | No detection of the target pathogen DNA or RNA. |
| **Description** | Freshly prepared suspension of a non-target pathogen for identification PCR<br>OR<br>Seed sample that contains no target pathogen |
| **SE-PCR for Detection** | Recommended |
| **Identification PCR** | Recommended |

Note: Users should at least perform either the NBC or NPC.

| | Negative Amplification Control (NAC) |
|---|---|
| **Definition** | Control that contains a well characterized quantity DNA or RNA of one or more non-target pathogen(s) that is added in the DNA or RNA sample and then tested and processed using the same assay at the same time as the samples. |
| **Objective** | To verify that the assay gives a negative result when testing DNA or RNA from a non-target pathogen at the assay stage.<br>For bacterial PCR, it also allows to discriminate that "false" positive results that may be observed in the NTC with primers with broad specificity, for example 16S rRNA gene targeted primers and probe by Wu et al. (2008), are due to bacterial DNA present in the Taq polymerase. |
| **Expected Result** | No detection of target or spike DNA or RNA. |
| **Description** | Known quantity DNA or RNA from one or more non-target pathogen(s). |
| **SE-PCR for Detection** | Not applicable |
| **Identification PCR** | Optional |

| | Internal Amplification Control (IAC) |
|---|---|
| **Definition** | Control that contains non-target DNA or RNA sequence in the same PCR reaction tube as the sample and that is amplified simultaneously with the target sequence. |
| **Objective** | To verify that DNA or RNA extraction conditions are appropriate for amplification by adding a non-target to the sample being tested at the nucleic acid extraction stage. |
| **Expected Result** | The expected product (IAC) should be observed in the expected ratio (Cq) to validate a negative sample PCR result. |
| **Description** | For the SE-PCR, the IC added at the seed extraction stage can act as IAC OR<br>A known quantity DNA or RNA from a non-target pathogen added before nucleic acid extraction ("late spike") OR<br>Amplification of general conserved sequences present in targets and non-targets. |
| **SE-PCR for Detection** | Recommended |
| **Identification PCR** | Essential |

Note: The use of an IAC may reduce the sensitivity of the target PCR. When multiplexing the IAC and target is not possible due to competition, an external amplification control should be run in a separate tube or well.
Note: When using universal bacterial primers, positive reactions may occur due to the presence of residual DNA in Taq enzyme reagents. The Cq values of the IAC from reactions on suspect isolates should at least be 3.3 Cq value lower than the Cq values of the IAC from the NTC reactions.
Note: IAC PCR primers can be adapted according to laboratory's specificities although risks of interactions with the target pathogen primers and probes need to be evaluated before routine use. Internally validated control primers or commercial kits with internal control primers may be used.

| | **Positive Control (PC)** |
|---|---|
| **Definition** | Control that contains a known (isolated) target pathogen in the absence of seed that is tested and processed using the assay at the same time as the samples. |
| **Objective** | To verify that the target pathogen can be detected under the assay conditions in the absence of a seed matrix. |
| **Expected Result** | Detection of the target pathogen. |
| **Description** | Extraction buffer spiked. Eluate obtained from the extraction process with a known reference isolate of the target pathogen. |
| **SE-PCR for Detection** | Recommended |
| **Identification PCR** | Essential |

Note: For SE-PCR, users should at least perform either the PC or PPC.

| | **Positive Process Control (PPC)** |
|---|---|
| **Definition** | Control that contains a well characterized positive seed sample, naturally infected or artificially contaminated with the target pathogen, ~~and~~ that is tested and processed using the same assay and at the same time as the samples. |
| **Objective** | To verify that the target pathogen can be detected under the assay conditions in the presence of a seed matrix. |
| **Expected Result** | Detection of target DNA or RNA. |
| **Description** | A naturally infected or artificially contaminated seed sample OR<br>A seed sample spiked with target pathogen. |
| **SE-PCR for Detection** | Recommended |
| **Identification PCR** | Not applicable |

Note: For SE-PCR, users should at least perform either the PC or PPC.
Note: Spiking with a target pathogen or non-target is possible. When a look-a-like (LAL) is used as a non-target, the LAL resembles the target pathogen on the basis of morphology on (semi-selective) growth media and genetic features.
Note: An example of a LAL is the use of a different subspecies or pathovar of the target pathogen. It is also possible to spike with a non-target that has no relation with the target pathogen e.g., other species. See recommendations for spiking in Section III.

| | Positive Amplification Control (PAC) |
|---|---|
| **Definition** | Control specific for PCR assays that contains a known quantity DNA or RNA of one or more target pathogen that is added in the DNA or RNA sample and then tested and processed using the same assay at the same time as the samples. |
| **Objective** | To verify that PCR assay conditions are appropriate for amplification by adding the target nucleic acid at the PCR stage. |
| **Expected Result** | Detection of target or spike nucleic acid. |
| **Description** | Known quantity DNA or RNA from one or more target pathogen(s) (for each target and including pathogen used as spike). |
| **SE-PCR for Detection** | Essential |
| **Identification PCR** | Recommended |

Note: synthetic RNA or DNA can be used.

| | Inhibition Control (IC) |
|---|---|
| **Definition** | Control that contains an internal spiking of a predetermined quantity of an isolated target pathogen or related species, added directly to the seed samples that is tested and processed using the same assay and at the same time as the samples. |
| **Objective** | To verify that no inhibition due to the sample seed matrix (e.g., chemical residues if treated and saprophytic load if not treated) occurs under the assay conditions. |
| **Expected Result** | Detection of the spiked pathogen. |
| **Description** | Spiking of a predetermined quantity of target pathogen into a split subsample of the seed sample tested ("early spike") OR<br>Spiking of a predetermined quantity of a related species of the target pathogen into the sample tested ("early spike") OR<br>Spiking of a predetermined quantity of target pathogen into the seed sample being tested after the seed extraction step ("late spike"). |
| **SE-PCR for Detection** | Essential |
| **Identification PCR** | Not applicable |

Note: The use of isolates for spike, either target or related species, carries some risk in that the isolate may respond differently than the target pathogen. When a Look-ALike (LAL) is used as a related species, the LAL resembles the target pathogen. Use well-characterised strain(s) of the target that represent the population variations or related pathogen that give similar responses to the target pathogen and genetic features.

## 3. SPIKING RECOMMENDATIONS

To ensure controls provide meaningful data to monitor method performance, spike solutions should be prepared at a concentration following one or more of the guidelines below.

- Spike concentration should result in a pathogen concentration in the sample that is representative of a seed lot that has low levels of natural infection.
- Spike concentration should be 10 - 100 times the concentration of the Limit of Detection.
- If a cut-off value is used, the resulting Ct value from a spiked sample should range 3-7 cycles below the cut-off value.

## 4. PCR SET-UP: RECOMMENDATIONS FOR TECHNICAL REPLICATES OR DUPLICATE REACTIONS

Quantitative values produced with qPCR are susceptible to variation between technical replicates (e.g. multiple identical reactions in separate wells of a reaction plate originating from a single master mix). There are many explanations for variation between replicates, including temperature variation, concentration differences introduced by pipetting errors, and stochastic variation (Bustin *et al.*, 2009). Stochastic variation in particular increases as template copy number decreases because the first few cycles at which the primers bind to the template DNA is more variable at lower copy numbers, since the likelihood of primer binding declines with decreasing template concentration.

As such, because the starting template copy number is unknown in the case of SE-PCR, it is necessary to run at least duplicate (if not triplicate) technical replicates of each qPCR reaction to assure the validity of the results of the detection assay, and to guard against the possibility of false negative results. In the case of colony confirmation PCR, it can generally be assumed that the template copy number is high, and though amplification failure may randomly occur, this would also affect the internal amplification control reaction. In the case of colony confirmation PCR, duplicate reactions are not considered essential (see Table 2).

**Table 2**. Duplicate reactions to be included in methods with a PCR assay.

| | **Duplicate PCR reactions** |
|---|---|
| **Expected Result** | Similar Ct values in both wells. |
| **Description** | Duplicate identical reactions in separate wells of a reaction plate originating from a single master mix and using the same sample. |
| **SE-PCR for Detection** | Essential |
| **Identification PCR** | Optional |

## 5. ESSENTIAL POINTS

Changing certain parameters in a PCR assay can influence the outcome of the test. For example, changing primer and probe sequences can influence detection sensitivity, selectivity, and robustness. Therefore, such parameters must not be modified. Other parameters can be modified to suit specific laboratory conditions without an effect on the outcome of the test. In Table 3 changeable and non- changeable parameters of a PCR assay are specified.

**Table 3**. Changeable and non-changeable parameters in PCR assays.

| Parameters details | Status | Remarks |
|---|---|---|
| Detection target (pathogen) primer sequences | Non-changeable | Probe cannot be added without additional validation due to potential increased selectivity. |
| Detection target (pathogen) probe sequences | Non-changeable | |
| Probe sequence modifications impacting on melt temperatures | Non-changeable | Probe molecular modifications (e.g., MGB, LNA) can significantly change Tm values and specificity of probes. |
| Probe dyes (fluorophore, quencher) not impacting on melt temperatures | Changeable | The impact on the sensitivity and selectivity of the detection method due to protocol changes needs to be validated. |
| Internal amplification control (IAC) PCR primers | Changeable | Internal control PCR primer sequences should be proposed, but flexibility allows laboratories to use internally validated control primers or commercial kits with internal control primers. The laboratory should investigate possible interactions with the specific pathogen primers/probes. |
| PCR conditions (temperature; time) | Changeable | The impact on the sensitivity and selectivity of the detection method due to protocol changes needs to be validated. |
| Reaction mix (Reverse transcriptase, DNA polymerase, buffer, MgCl2, dNTPs) supplier and concentrations | Changeable | |
| PCR product identification (gel-electrophoresis or SYBR green) | Changeable | |
| PCR machine | Changeable | |

## REFERENCES

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