International Seed Federation
Seed is Life

Best Practices for PCR Assays in Seed Health Tests
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Polymerase Chain Reaction (PCR) is currently the most commonly utilized molecular technique in seed health testing. This document provides best practices on the use of PCR assays in seed health testing for pathogen identification and detection by describing essential, recommended and optional controls. For detection and identification of RNA viruses and viroids PCR is preceded by an enzymatic Reverse Transcriptase (RT) step to transcribe RNA into DNA. Therefore, where this document refers to PCR, it may apply to both PCR and real-time PCR.

I PCR for Identification and Detection

The use of PCR assays for identification is usually applied to a sample with a known and/or controllable concentration and quality of the target nucleic acid. For example, a real-time 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, the quantity of target nucleic acid in the sample is unknown and quality of the sample is less controllable. For example, a real-time PCR assay may be performed with a DNA extract from a bio-PCR assay or an RNA extract directly from seeds. In the case of bio-PCR the quantity of target DNA in the sample will vary according to the number of bacteria extracted from the seed sample and bacterial growth during biological amplification. 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 genomic regions is recommended in PCR assays.

A PCR assay will detect any trace of DNA and RNA without discriminating whether the source of the nucleic acid is pathogenic, viable, or even intact. Such “indirect” tests, when used in seed health testing, do not yield any information about pathogen viability and are not sufficient to prove pathogenicity. While a negative PCR result can be assumed to be conclusive evidence of a healthy seed lot, a positive result must be followed by a direct test that confirms viability of the pathogen and its pathogenicity, see http://www.worldseed.org/wp-content/uploads/2015/10/Indirect_Seed_Health_Tests_2013.pdf. In such a situation a real-time PCR assay could be considered as a pre-screen for the subsequent direct test such as a bio-assay or grow-out, see http://www.worldseed.org/wp-content/uploads/2018/03/Real-time_PCR_pre-screens_2018.pdf. A PCR assay may also be used in detection to confirm suspect plant tissue obtained from a bio-assay or grow-out.

II Controls and their Purpose

The number and type of controls necessary for a robust seed health test using PCR assays depend on the application. The current use of PCR assays in seed health testing can be grouped into identification

Note: Version 4 includes a recommendation on the use of two primer sets (section I, paragraph 3).

and detection assays. The best practices described in this document have been established for use in conjunction with routine seed health tests. There are some recommendations that may be essential during PCR assay development and validation, but these have not been defined in this document.

The purpose of controls is to verify proper test execution as well as the quality of the materials and methods used in the pathogen extraction, the nucleic acid (DNA or RNA) extraction process and the PCR process. The proper controls ensure a reliable test result (see Table 1). Examples of controls to be included in protocols for detection and identification are presented in Table 2.

Given that molecular techniques are under constant development and improvement, this document will be updated as needed to reflect the appropriate controls needed to achieve a reliable result.

Table 1. Controls to be included in methods with a PCR assay

Control type	Non Template Control (**NTC**)	
Definition	Control that contains all PCR reagents but no target or spike DNA, RNA or internal amplification control (IAC) nucleic acids	
Expected Result	No detection of target DNA or RNA or non-specific products [a]	
Description	**Bio-PCR or Seed Extract PCR (SE-PCR) for detection**	**Single isolate microbial cultures for identification**
Nucleic acid-free water	**essential**	**essential**

[a] When using universal bacterial primers, positive reactions may occur due to the presence of residual DNA in Taq enzyme reagents. The IAC Ct values from reactions on suspect isolates should indicate at least 10-fold higher concentration of bacterial DNA than the IAC Ct values from the NTC reactions; the difference between Ct values should be more than 3.3.

NOTE: The Ct value may also be termed Crossing point (Cp) value or Quantification cycle (Cq) value.

Control type	Negative Process Control (**NPC**)	
Definition	Control (culture medium, extraction buffer or seed sample) that contains no target organism and is processed in the same way and at the same time as the samples.	
Expected Result	No detection of target DNA or RNA	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Liquid used for preparing microbial DNA or RNA extractions or for preparing suspensions **OR** Freshly prepared suspension of a non-target organism	**not applicable**	**essential**
A seed extraction buffer or seed sample that contains no target organism (known non-infected sample)	**essential**	**not applicable**

Control type	Positive Process Control (**PPC**)	
Definition	Control (Culture medium, suspension or (spiked) seed sample) that contains the target organism and is processed in the same way and at the same time as the samples [a]	
Expected Result	Detection of target DNA or RNA	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Freshly prepared suspension of a reference target organism	**not applicable**	**essential**
A seed sample that contains the target organism (known infected seed sample) **OR** A seed sample spiked with target organism **OR** Seed extraction buffer spiked with target organism	**recommended**	**not applicable**

[a] Spiking with a **target** organism or **non-target** organism is possible. When a look-a-like (LAL) is used as a non-target organism, the LAL resembles the target organism 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 organism. It is also possible to spike with a non-target organism that has no relation with the target organism e.g. other species. See recommendations for spiking in Section III.

Control type	Positive Extraction Control (**PEC**)	
Definition	Control that contains a **parallel spike** with the target organism or an **internal spike** with a non-target organism [a]	
Expected Result	The spike needs to be detected to validate a negative PCR result.	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Internal spiking of a known quantity non-target organism into the sample ("early spike") **OR** *Parallel* spiking of a known quantity target organism into split subsamples of homogenised seed extracts	**essential**	**not applicable**

[a] Spiking with a **target** organism or **non-target** organism is possible. When a LAL is used as a non-target organism, the LAL resembles the target organism on the basis of morphology on (semi-selective) growth media and genetic features.

Control type	Inhibition Control (**IC**) [a]	
Definition	Control that contains a known quantity spike with a non-target organism [b]	
Expected Result	The spiked DNA/RNA needs to be detected. In real-time PCR the Ct values may be used to identify possible inhibition of the PCR on samples.	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Spiking of a known quantity non-target organism into a seed extraction buffer ("early spike") in the absence of seed **OR** in the presence of a known non-infected seed sample	**recommended**	**not applicable**

[a] Inhibition Control is only applicable in the case of an "early spike" PEC

[b] Spiking with a **target** organism or **non-target** organism is possible. When a LAL is used as a non-target organism, the LAL resembles the target organism on the basis of morphology on (semi-selective) growth media and genetic features.

Control type	Internal Amplification Control (**IAC**) [a]	
Definition	Non-target DNA or RNA sequence present in the same PCR reaction tube which is co-amplified simultaneously with the target sequence	
Expected Result	The expected product (IAC) should be observed in expected quantity (Ct) to validate a negative sample PCR result [b]	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
The PEC "early spike" serves as IAC **OR** A known quantity IAC is added *before* nucleic acid extraction ("late spike") **OR** A known quantity IAC is added *after* nucleic acid extraction	**essential**	**optional**
Amplification of general conserved bacterial and fungal sequences present in target and non-target organisms	**optional**	**essential**

[a] 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

[b] When using universal bacterial primers, positive reactions may occur due to the presence of residual DNA in Taq enzyme reagents. The IAC Ct values from reactions on suspect isolates should indicate at least 10-fold higher concentration of bacterial DNA than the IAC Ct values from the NTC reactions; the difference between Ct values should be more than 3.3

Control type	Positive Amplification Control (**PAC**)	
Definition	Control that contains a known quantity DNA or RNA of one or more target organism (for each target and including spike organism)	
Expected Result	Detection of target or spike DNA or RNA	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Known quantity DNA or RNA from one or more target organism (for each target and including spike organism)	**essential**	**recommended**

Control type	Negative Amplification Control (**NAC**)	
Definition	Control that contains a known quantity DNA or RNA of one or more non-target organism	
Expected Result	No detection of target or spike DNA or RNA	
Description	**Bio-PCR or SE-PCR for detection**	**Single isolate microbial cultures for identification**
Known quantity DNA or RNA from one or more non-target organism	**not applicable**	**optional**

Table 2. Examples of controls included in PCR assays for detection and identification

Bio-PCR or SE-PCR for detection		
Example: SE-PCR *Acidovorax citrulli* (Ac) on cucurbit seeds		
NTC	**essential**	nucleic acid-free water
PPC	**recommended**	Ac infected watermelon seed lot **OR** healthy watermelon seed lot spike with Ac
NPC	**essential**	watermelon seed lot free from Ac and *Acidovorax cattleyae* (Acat) **OR** seed extraction buffer free from Ac and Acat
PAC	**essential**	known quantity of Ac and Acat DNA
NAC	**not applicable**	-
PEC	**essential**	Acat cell suspension added to test samples ('early spike')
IC	**recommended**	Acat cell suspension added to seed extraction buffer
IAC	**essential**	PEC serves as IAC

Single isolate microbial cultures for identification		
Example: Colony confirmation *Clavibacter michiganensis* subsp. *michiganensis* (Cmm)		
NTC	**essential**	nucleic acid-free water
PPC	**essential**	freshly prepared suspension of Cmm
NPC	**essential**	freshly prepared suspension of non-suspect colony
PAC	**recommended**	known quantity of Cmm DNA
NAC	**optional**	known quantity of non-target DNA
PEC	**not applicable**	-
IC	**not applicable**	-
IAC	**essential**	universal bacterial primers

III 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

IV PCR Set-up: Recommendations for Technical Replicates or Duplicate Reactions

Quantitative values produced with real time PCR 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[1]). 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 and Bio-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 3).

[1] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, and Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55: 611-622. http://www.clinchem.org/cgi/doi/10.1373/clinchem.2008.112797

Table 3. Duplicate reactions to be included in methods with a PCR assay

PCR set up	Duplicate PCR reactions	
Expected Result	Similar Ct values in both wells	
Description	Bio-PCR or SE-PCR for detection	Single isolate microbial cultures for identification
Duplicate identical reactions in separate wells of a reaction plate originating from a single master mix and using the same sample	essential	optional

V 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 4 changeable and non-changeable parameters of a PCR assay are specified.

Table 4. Changeable and non-changeable parameters in PCR assays

Parameter 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, $MgCl_2$, dNTPs) supplier and concentrations	Changeable	
PCR product identification (gel-electrophoresis or SYBR green)	Changeable	
PCR machine	Changeable	