International Seed Federation
Seed is Life

Real-time PCR, an 'indirect' test used for pre-screening in seed health methods

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Introduction

There is an increasing use of molecular tests such as real-time PCR for pathogen detection on symptomatic plant tissue, as well as for the identification and characterisation of isolated plant pathogens. PCR (conventional and real time) has also been used for many years for different applications in seed health testing, including identification of viable bacteria and fungi isolated from seed, and as an indirect test for the detection of pathogens on seed. Other indirect tests, including ELISA and next generation sequencing (NGS) technologies, are also used or are being investigated for use in pathogen detection. Seed-extract PCR (SE-PCR) detection tests that combine nucleic acid extraction (RNA or DNA) from seed and real-time PCR offer several advantages in seed health testing. Importantly, they can identify non-infected seed lots more rapidly than direct tests. In addition, with the combination of available sequence data and simple low-cost assay design, specific and sensitive SE-PCR tests can be developed rapidly in response to emerging or existing pathogens. However, these indirect tests for pathogen detection do not yield any information about pathogen viability and are not sufficient to prove pathogenicity.

The ISF Viewpoint on Indirect Seed Health Tests adopted in 2013 is that if an indirect test yields a negative result then no confirmatory test is necessary: http://www.worldseed.org/wp-content/uploads/2015/10/Indirect_Seed_Health_Tests_2013.pdf. However, if the indirect test yields a positive result then a second, preferably direct[1], test that confirms viability and pathogenicity of the target organism is crucial. In such a situation SE-PCR should be considered as a pre-screen for a subsequent direct test such as a bio-assay or grow-out. This is particularly important in seed health testing as nucleic acids from dead or inactivated pathogens may remain detectable in seed samples following seed processing and/or seed treatments.

PCR pre-screening in seed health methods

The analytical sensitivity or limit of detection (LOD) of a PCR pre-screening test should be determined during method validation studies. The aim of a pre-screen is to identify seed lots that are not infected with the target pathogen(s). Therefore, the LOD of a fit-for-purpose pre-screen test should be lower than the LOD of the fit-for-purpose confirmatory test. If this requirement is not met, false negatives could occur with the pre-screen test. Consequently, any positive result with a pre-screen test should be classified as a "suspect" result that needs to be investigated further and verified with the confirmatory test.

A positive real-time PCR result is determined when fluorescence of a reaction exceeds the background fluorescence, thus giving a cycle threshold (Ct) value[2]. Real-time PCR methods are routinely described with 40 reaction cycles, which corresponds to an inherent and arbitrary cut-off at Ct40. To ensure the practical relevance of real-time PCR results, a Ct cut-off value other than Ct40 may be identified, beyond which a sample is determined to be negative for the target pathogen(s).

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1 In cases where a direct test is not available, a second indirect test based on different biological principles, e.g. ELISA, should be used.

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

Several methods have been described for identifying Ct cut-off values based on the LOD of the PCR for different applications including pathogen detection (Chandelier et al., 2010; Caraguel et al., 2011; Grosdidier et al., 2017). Due to the inherent sensitivity of the PCR technique, the LOD of PCR tests, which can detect traces of DNA/RNA from live or dead pathogen propagules, can be considerably lower than the LOD of direct tests that detect viable infectious pathogens, or indirect methods with fit for purpose sensitivity, such as ELISA. Therefore, ISHI-Veg recommends applying a Ct cut-off relative to the LOD of a validated and fit-for-purpose confirmatory detection test.

Different approaches may be taken to fix and justify the Ct cut-off values for PCR pre-screening. For example:

- PCR as a pre-screen for an ELISA: Test in parallel the same seed extracts with a PCR test and an ELISA. This is only possible if the same seed extraction method is used for both tests. For example, a dilution series of infected seed extracts can be tested with both methods.
- PCR as a pre-screen for a plating assay and/or bioassay: Conduct comparative tests with the PCR pre-screen test and the reference plating and/or bioassay using the same sample. This approach can be affected by the presence of DNA or RNA from non-viable pathogen propagules that are detected with the PCR pre-screen test.
- PCR as a pre-screen for grow-out: Correlation of results obtained with these two tests cannot be established using the same sample. Therefore, a large data-set from samples divided and tested with both tests is required to obtain the correlation between the real-time PCR Ct values and the direct test (grow-out) result.

Ct values are dependent on reaction efficiency which can be affected by many factors, including the type of PCR equipment, reagents, the nucleic acid extraction method and the nature of the sample being tested (e.g. seed microflora, seed treatments…). The Ct cut-off value applied in routine analysis must be monitored and confirmed for each individual test using appropriate control reactions, as described in the best practices developed by ISHI-Veg for the use of molecular tests in seed health methods: http://www.worldseed.org/wp-content/uploads/2018/01/ISHI-Veg_BestPractices_PCR_2017.pdf.

References

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