| TITLE: | Real-Time PCR for diagnostic purposes – guidance for method validation | |
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| OWNER: | APPROVER: | ISSUE DATE: |

## INTRODUCTION

Real-time PCR technology (also commonly known as TaqMan® PCR) is based on the theory that there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. The kinetics of the reaction are measured via the emission of fluorescence from a labelled probe and this provides distinct advantages over traditional PCR detection: precision, sensitivity, automation and results that are expressed as numbers (rather than gel images).

Real-time PCR is a robust and powerful diagnostic tool that can provide both quantitative and qualitative results. In addition, it allows a generic approach to the way assays are validated, plates are set up and results analysed.

The process of method development and **validation** includes the following: establishment of the assay/method, reference standard preparation, establishing the limitations of the method and application of the method/assay to routine use.

## SCOPE

This document provides an overview of the validation process. The purpose of this document is to highlight the most important stages of the process and documentation of the information. An outline of method validation is also provided in the CSL Quality Manual YQM3.

A flow diagram summarising the whole process is included in **Appendix 1**.

## PROCEDURE

### 1. General method validation

An assay should be reliable and fit for purpose and the validation should be reflected in this. The process of method development and validation includes the following: establishment of the assay/method, reference standard preparation and application of the method/assay to routine use. There are three main areas where data is required (see Appendix 1), performance reliability and specific limitations. The specific limitations of an assay are defined as matrix/analyte (or host/pathogen) combinations where for whatever reason the assay does not perform as well as it should, if at all.

The fundamental parameters for validation include a) sensitivity, b) specificity, c) repeatability/reproducibility and e) applicability (robustness). In addition a limit of detection (LoD) should be established. The LoD is defined as the lowest amount or concentration of analyte in a sample that can be reliably detected. It is important to include this figure when reporting results as it helps to define the limitations of the test.

The techniques used to determine the performance of a method should be one or a combination of the following:

- calibration using reference standards or reference materials;

- comparison of results achieved with other methods;

- interlaboratory comparisons;

- systematic assessment of the factors influencing the result (e.g. host effects);

- assessment of the uncertainty of the results based on scientific understanding of the theoretical principles of the method and practical experience.

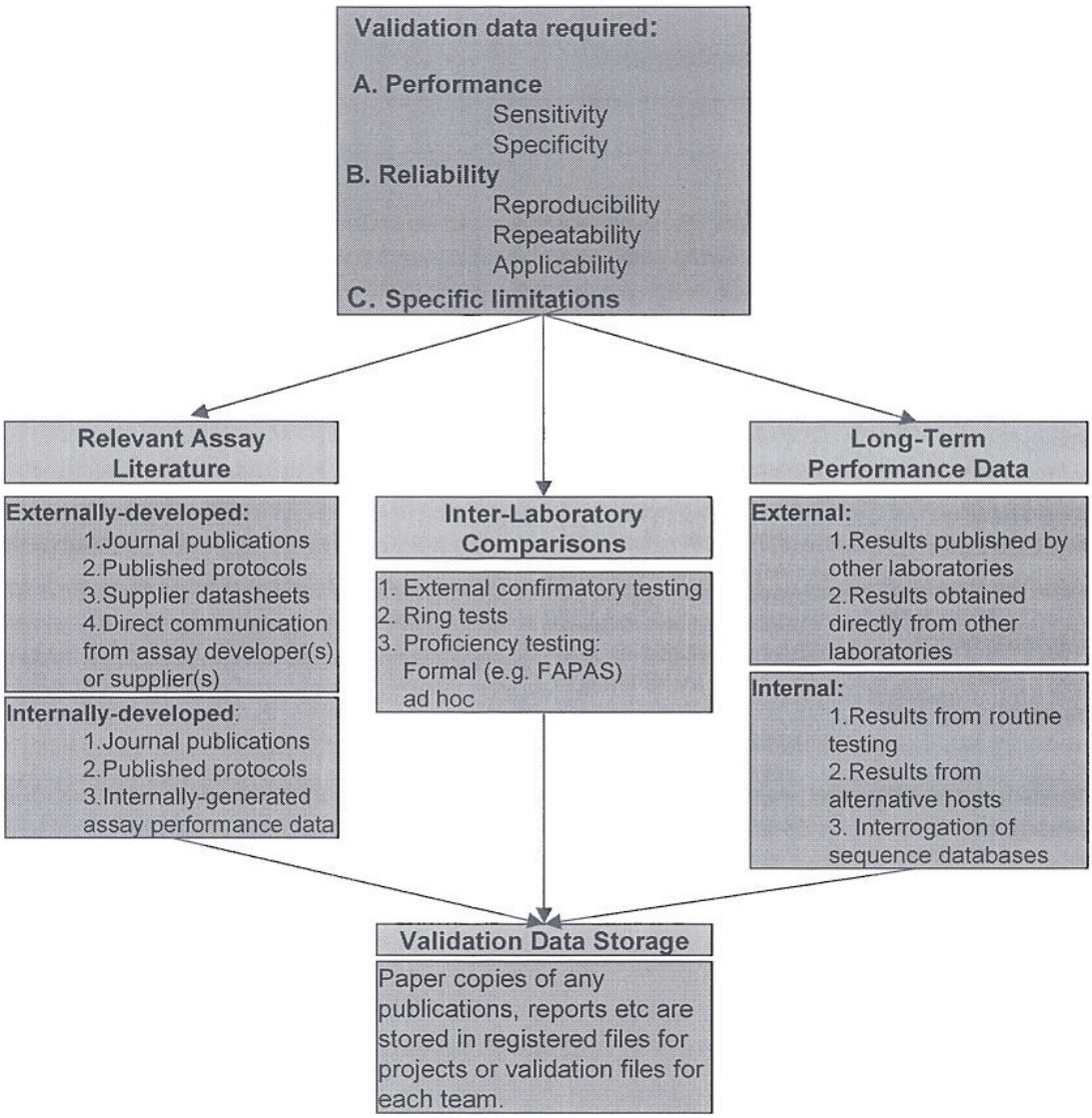
Validated methods can be obtained from external sources as well as being developed 'in-house'. Relevant literature on externally developed assays can be obtained from peer-reviewed publications, supplier datasheets and direct communication from the assay developers or suppliers.

Once a method has been validated and is in routine use it is important to record long-term performance data. This data will provide an indication as to whether the assay is still performing to the same standard. If the results appear to be deteriorating then action can be taken to investigate why e.g. reduced quality of reagents or a change in the characteristic of the pathogen. Plant pathogens will evolve and mutate and an important aspect of long term monitoring of PCR performance is the investigation of published sequence data to ensure that the primer/probe sets will detect the necessary range of isolates. This can be done through a combination of ENTREZ and BLAST searches using the NCBI database. Although there are no specific rules about this, it would be prudent to check for sequence misses every 2-3 years or when new information is published e.g. new pathogen isolates.

## 2. Documenting validation data

A validation sheet has been provided in **Appendix 2**. The purpose of this document is to enable the method developer to gather together the appropriate validation information so that it is easily accessible for end users. In addition to the criteria mentioned above (e.g. sensitivity, specificity), the form also asks for details to help users interpret the data e.g. cut-off points. This information can provide guidance to the end-user in deciding whether an assay has worked well or not.

## Appendix 1



## Appendix 2 - Validation sheet for PCR-based diagnostic methods

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|---|---|
| **Target Organism:** | |
| **Publication details** Enter details of publication | |
| **SOP number(s)** Is the SOP on Workbench? | |
| **ISO accredited?** (Yes/No) | |
| **Intellectual property** Are there any restrictions on use? Are they solely CSL's property? | |
| **Nucleic acid extraction method** Procedure for DNA/RNA extraction (or reference to SOP). | |
| **Sensitivity (units)** What is the limit of detection? | |
| **Specificity** Do these primers cross react with other species? What organisms were the primers tested against? | |
| **Reproducibility** Has someone other than yourself got the same results from the same DNA extractions or used this method? | |
| **Repeatability** Are the reps close? Do you get the same results from the same extraction? | |
| **Matrices tested** What host material was tested (e.g. root, leaves, petioles or soil)? | |
| **Methods for different matrices** Fill in if different matrices need different extraction methods. | |
| **Ring tested** Has this primer set been used in a ring test? Include brief details of the ring test. | |
| **Proficiency tested** Has this primer set been used in a formal proficiency testing scheme? | |
| **Standards/ controls** Detail any Certified reference standards or known reference controls needed for the tests. | |
| **Is it Quantitative?** Yes or no answer: Is there validation data to support this? | |
| **Statistical analysis on batch testing and/or reporting results.** Reference to relevant documentation or spreadsheet. | |
| **Long term monitoring for sequence misses.** Describe the frequency and method of checking. | |