

The challenges and feasibility of validating molecular diagnostic kits on multiple Real-Time PCR platforms

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Background

- Real-Time PCR is an expanding technological area, with multiple manufacturers producing different platforms for use with this technique
- The MycAssay™ family of molecular diagnostic real-time PCR assays are intended to enhance the speed, sensitivity and specificity of fungal diagnosis and are initially validated on the Cepheid SmartCycler®
- For broad application across the fungal community, they need to run on multiple platforms
- The chemistry is designed to work with the majority of Real-Time PCR instruments. MycAssay™ products are CE-marked as in vitro diagnostic devices and as such the performance characteristics of the assay must be faithfully transferred from one platform to another
- To achieve this objective Myconostica has a platform transfer program for each MycAssay™ product

Design Principles for the MycAssay™ Family of Products

- Duplex reaction (pathogen + Internal Amplification Control (IAC))
- Molecular beacon [1] detection of target
- Universal chemistry
- Dyes used located at blue/green end of spectrum
 - FAM: Excitation = 492nm, Emission = 517nm
 - HEX: Excitation = 535nm, Emission = 556nm
- Commonality in procedure for all assays
- 25 µL total volume

Real-Time PCR Data Analysis Methods

- The analysis of Real-Time PCR data is currently based on the "cycle-threshold" method. This is defined as the fractional cycle number in the log-linear region of PCR amplification in which the reaction reaches fixed amounts of amplicon DNA. This is determined in one of two ways (see Table 1);
 - "fit point"; performed by drawing a line parallel to the x-axis of the real-time fluorescence intensity curve (C_T) [2]
 - "second derivative"; calculates the fractional cycle where the second derivative of the real-time fluorescence intensity curve reaches the maximum value (C_P) [3]
- Each manufacturer/platform uses different algorithms to achieve analysis of raw data while still reporting either a C_T or a C_P

		Name	Description
1	C_T	Cycle threshold	The number of cycles require for the fluorescent signal to cross the threshold
2	C_P	Crossing point	The cycle at which the fluorescence of a sample rises above background fluorescence

Table 1: Data analysis methods

Methods for a Platform Transfer

- Each platform is put through a series of steps, gated with decision points, prior to transfer of the CE-marked IVD assay to the new platform (see Figure 1)
- Each platform is assessed for suitability by determining efficiency, as well as the ability to reliably distinguish negative samples using the automated analysis software
- Replicate runs are performed to check that threshold and other analysis settings are robust

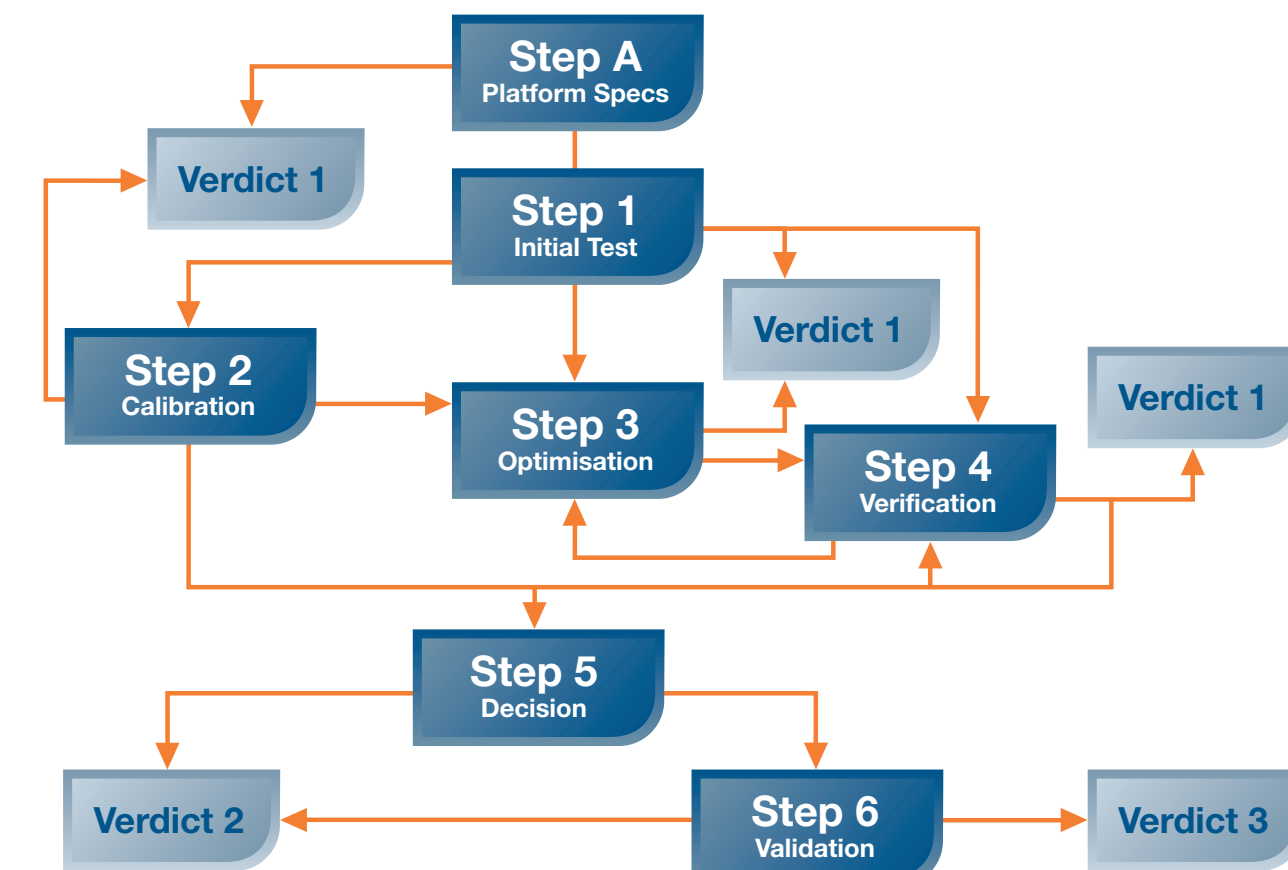


Figure 1: Platform transfer process. Verdict 1 = platform unsuitable; Verdict 2 = platform only suitable for Research Use Only; Verdict 3 = platform suitable for CE-marking

Plasticware choices

- There can be differences in the quality of plasticware available, in terms of;
 - Suitability
 - Contamination with pathogen DNA
- These differences can affect performance (see Figure 2)
- Not all available plasticware is suitable for use with every MycAssay™ product on every platform, and may require local validation prior to use

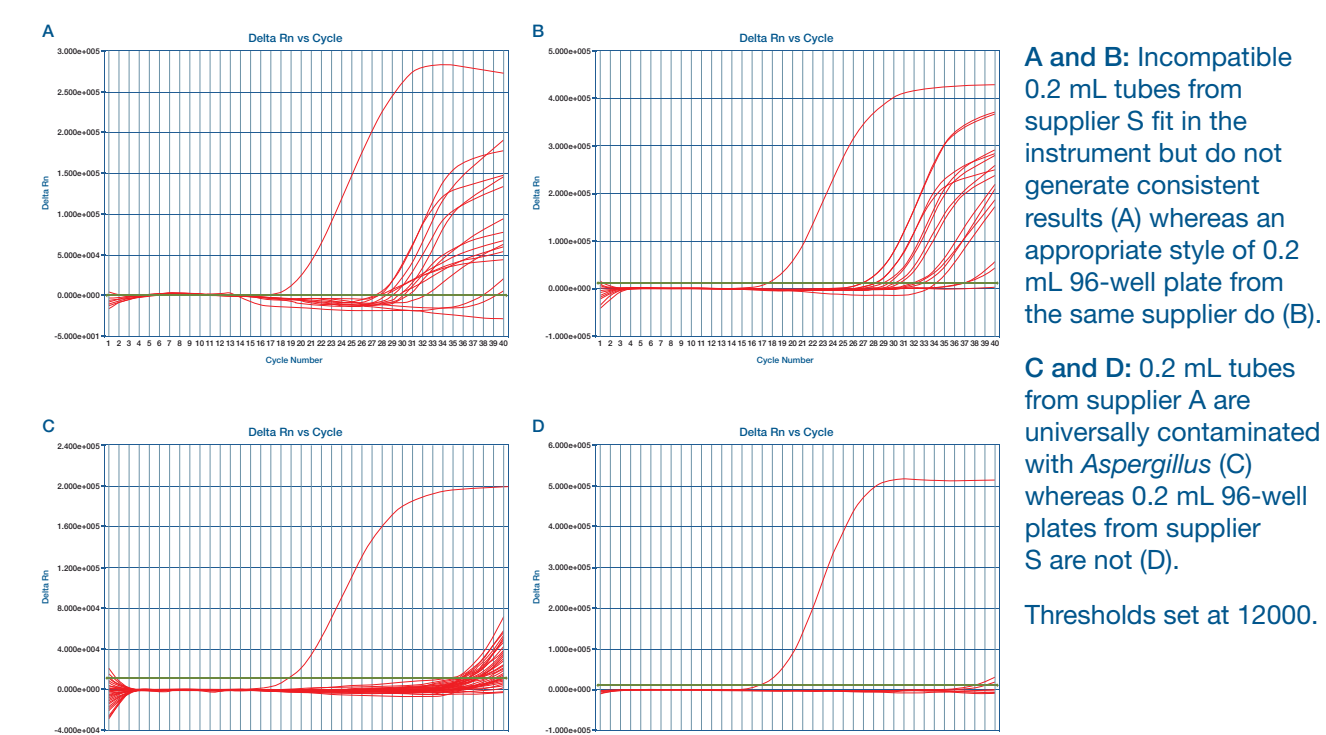


Figure 2: Plasticware choices

RotorGene-Q;

A Case Study

Step A - Platform Specifications

Block format	Volumes	Thermal cycling system	Heating rate (per second)	Cooling rate (per second)	Light source	Detector	Excitation Ranges (nm)	Emission Ranges (nm)
Rotor (in 3 sizes)	5-100 µL	Air	>15 °C	>20 °C	LED / channel	Photomultiplier tube with variable or automatic gain setting	Green: 460-490 Yellow: 525-535 Orange: 580-590 Red: 615-635 Crimson: 675-685 HRM: 445-475	Green: 505-515 Yellow: 550-560 Orange: 605-615 Red: 650-670 Crimson: >712 HRM: 505-515

Table 2: Platform specifications of the Qiagen RotorGene-Q

- Platform specifications indicate that this platform is capable of holding the volume of the assay and can detect both of the probes used in the MycAssay™ products (see Table 2)

CONCLUSION: proceed to STEP 1

Step 1 - Initial Test

- Initial data indicate that this platform does detect both probes and sufficiently discriminates between them
- Positive pathogen reactions give positive C_T results (Figure 3A)
- Negative pathogen reactions do not currently produce consistent results (Figure 3A)
- IAC reactions generate a tight range of C_T values, independent of template concentration is also present (Figure 3B)

CONCLUSION: proceed to STEP 3

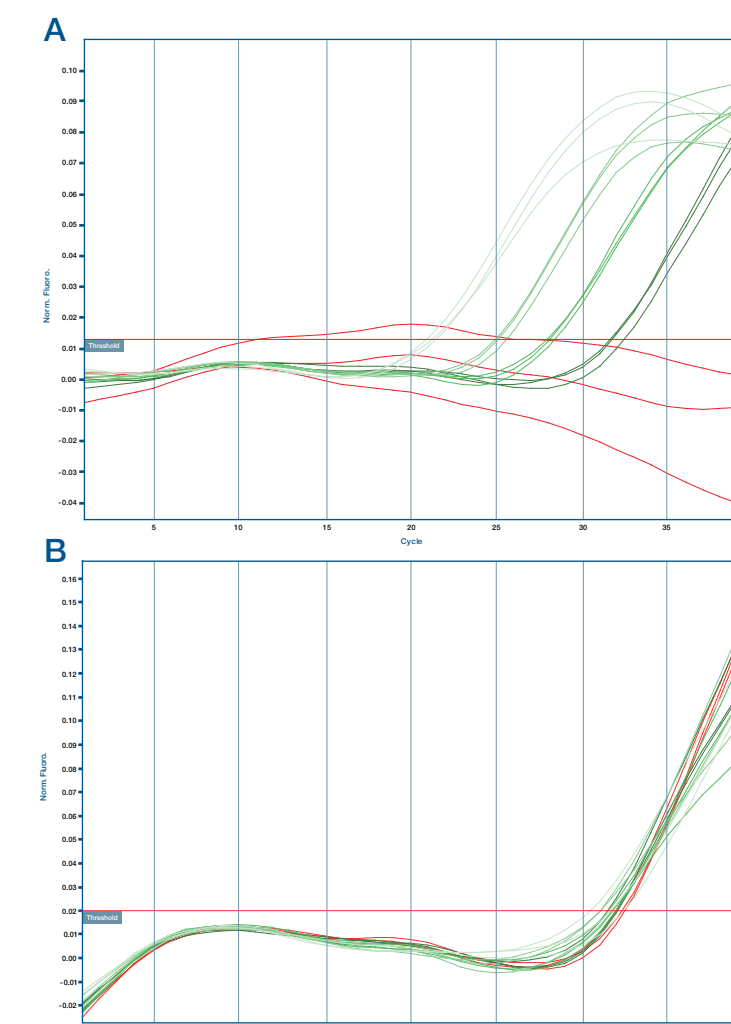


Figure 3: Amplification curves from an initial test using standard curve templates and Negative control reaction, performed in triplicate. Red = Negative. Green = Positive samples (dark-light; low-high concentration). A) Green channel; Pathogen reaction. B) Yellow channel; IAC reaction

Step 3 - Optimisation

- Results from Step 1 suggest that PCR protocol is sufficient for amplification purposes
- Platform specific settings (such as Gain) investigated for optimal signal read (Figure 4)

CONCLUSION: proceed to STEP 4

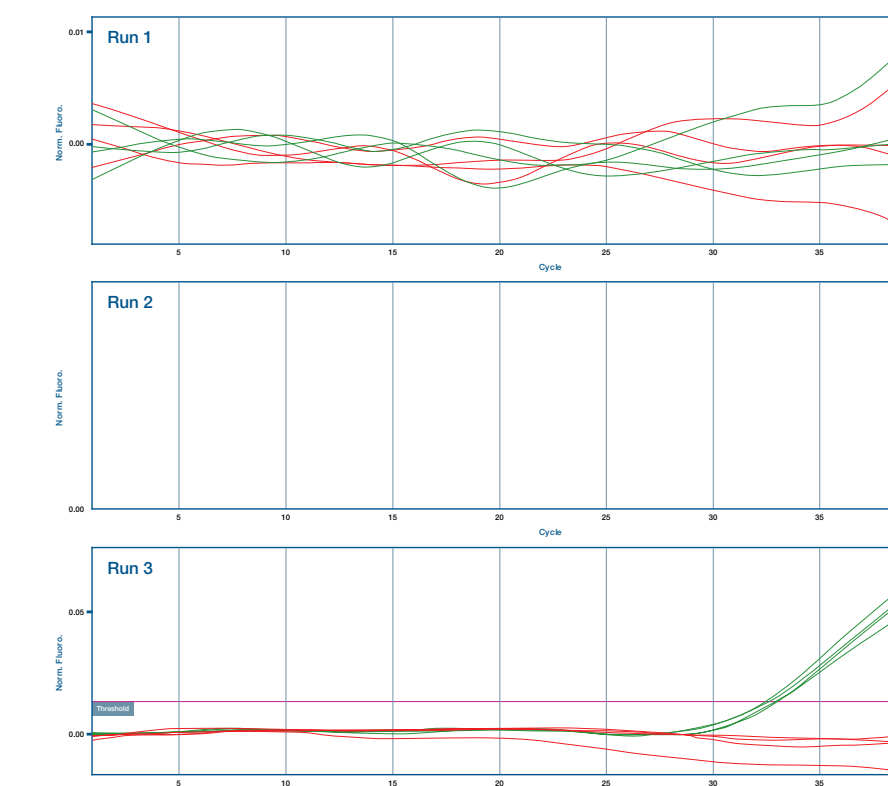


Figure 4: Amplification curves from tests using different Gain settings. Identical run parameters (other than Gain), templates, and analysis settings used for all 3 runs. Green = positive sample, Red = negative samples. Each sample was tested in quadruplicate.

Step 4 - Verification

- Verdict 1 discounted; platform is not unsuitable for the assay chemistry
- Inconsistent within-run and between-run results for all template types (Figures 5 & 6)

CONCLUSION: proceed to STEP 5

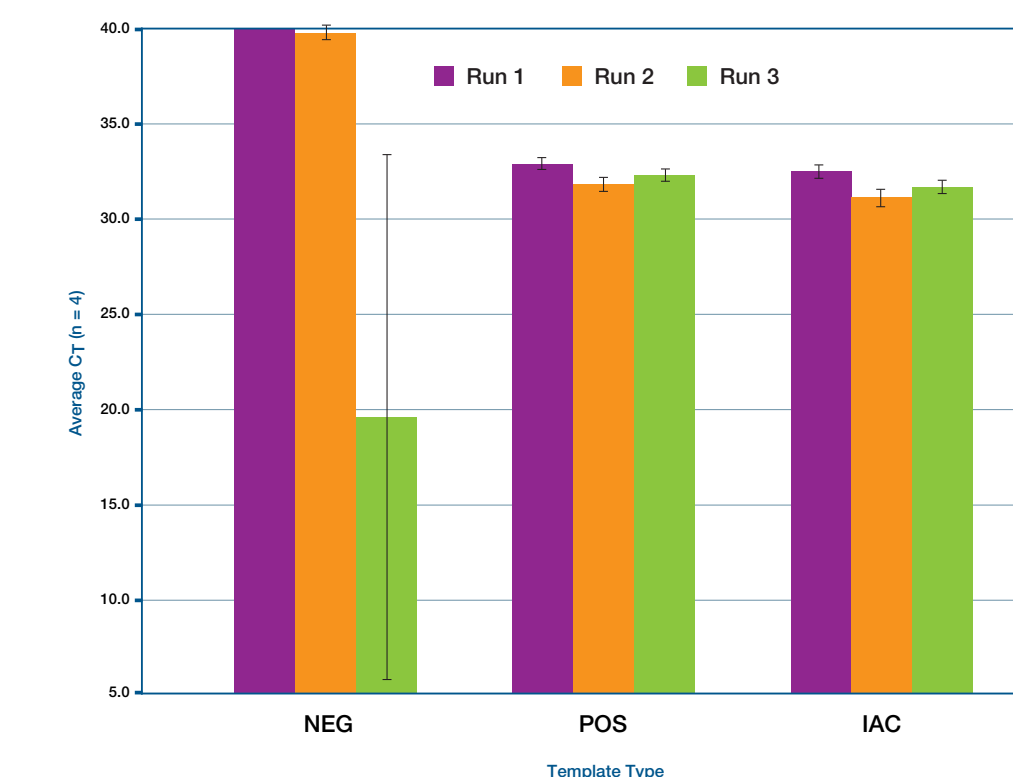


Figure 5: Average C_T values for 3 different template types across 3 replicate runs using standardised analysis settings, as selected in STEP 3. Error bars show standard deviation.

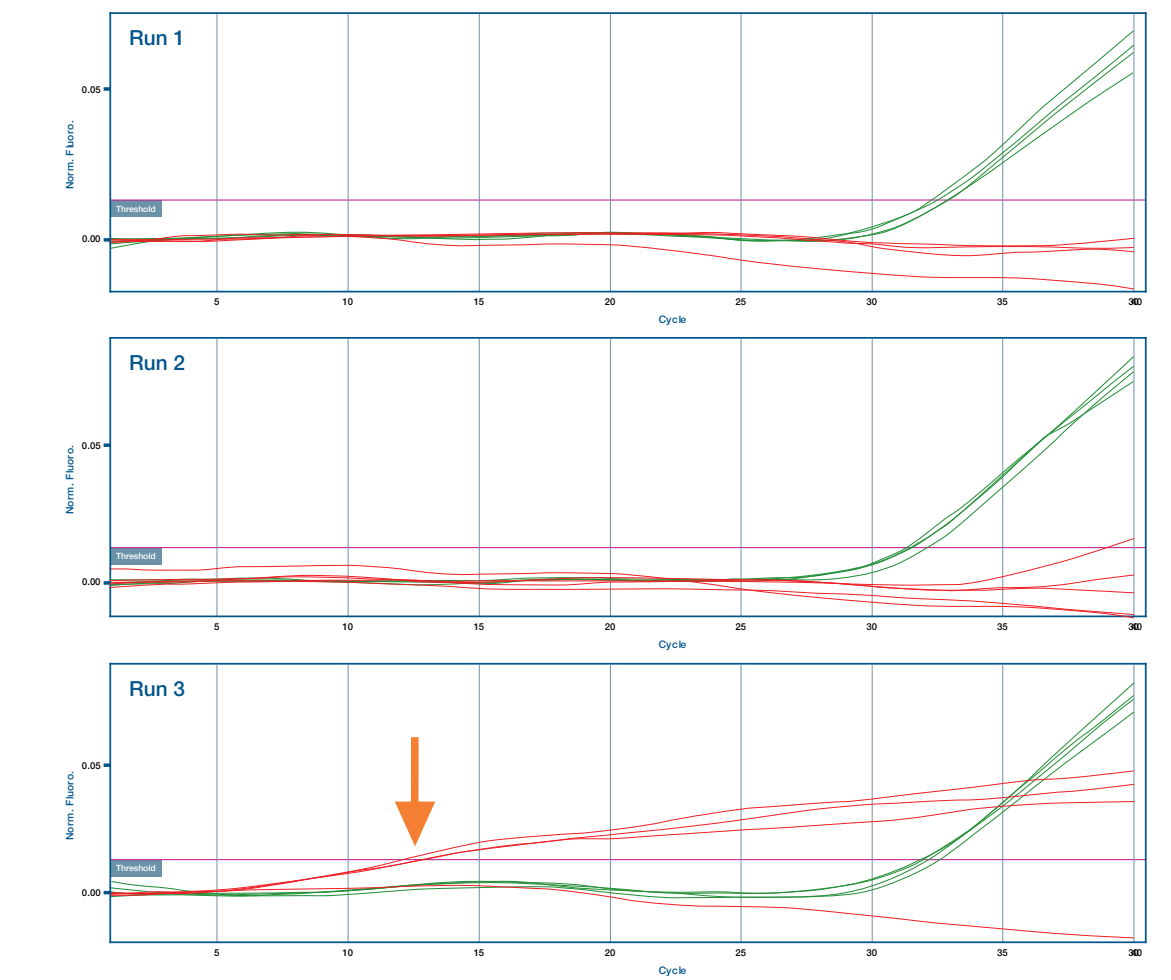


Figure 6: Results in the Green (pathogen) channel. Identical run parameters, templates, and analysis settings used for all 3 runs. Green = positive sample, Red = negative samples. Each sample was tested in quadruplicate. The arrow indicates an inconsistency in the results for the negative reactions between the runs.

Step 5 - Decision

- Data does not currently meet the requirements for CE-marking of an IVD diagnostic device

CONCLUSION: Do not proceed to STEP 6; Consider returning to STEP 3
Platform suitable for Research Use Only

Conclusions

- Transfer of a CE-marked IVD from one platform to another is a complex process.
- Raw data can look normal yet analysis algorithms within each software program can cause the generation of discrepant amplification curves and false positive results, especially in negative control samples.
- As shown in Figure 1, the platform transfer process may pass through several steps successfully, only to be stopped later down the process pathway.

References

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- Pfaffl MW. 2004. Quantification strategies in real time PCR. In: *A-Z of quantitative PCR* Edited by: Bustin SA La Jolla, CA, International University Line.
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