Troubleshooting an ISO 17025 accredited mycoplasma qPCR detection assay.

Authors : Sarvesh Dalvi, Jim Cooper, Edward Burnett, Simone Lilley, Sarah Alexander

Location: Culture Collections, UKHSA, Porton Down.

INTRODUCTION

Mycoplasmas are sub-microscopic, self-replicating organisms and a common contamination of cell cultures. These contaminations can go unnoticed; they cannot be detected with the naked eye or conventional microscopy. Mycoplasma contamination will invalidate *in vitro* research projects, impacting on timelines and leading to substantial financial loss. Ensuring cell cultures used *in vitro* research are mycoplasma-free is essential.

ECACC (An UKHSA Culture Collection) provides authentic cell cultures to a global customer base. Amongst other tests, ECACC uses a highly sensitive 'in-house' ISO 17025 accredited qPCR assay developed in 2018 capable of detecting 56 mycoplasma species using hydrolysis probes. The assay has multiple primers and probes; an internal amplification control (IDT 173 bp 'G Block') (VIC channel) and mycoplasma specific amplification (FAM Channel). The assay was subject to confounding background 'noise' manifested by unexpected non-specific, non-logarithmic amplification in the mycoplasma FAM channel in negative controls at CTs <37, reducing confidence through false positive results. Process improvement and troubleshooting was therefore critical. A systematic series of experiments was executed to determine the root cause of the 'noise' to regain confidence in the assay.

METHODS

To determine if any component(s) of the PCR master mix were not functioning as expected, a 'singular exclusion' approach was employed. PCR reactions were run from multiple master mixes, each excluding a key component, with care being taken to avoid confounding results with any other variables. Once all the runs had been completed the results were compared and analysed 'en masse'.

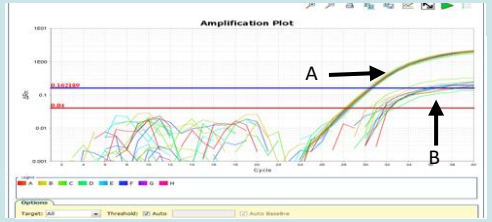

Figure.1. A: Arrow shows logarithmic expression of IC

B: Arrow shows unspecific amplification.

RESULTS

- The principal components of the PCR master mix were: Brilliant III master Mix, Reference dye (ROX), Primers, Probe ('oligos'), Internal Control (IC) and Water.
- The potential of template contamination of reagents was eliminated as a cause by using new batches of water and master- mix, however, non-specific amplification was still observed
- Investigation showed the oligos had been in storage for 5 years leading to a hypothesis that the oligo's might have degraded. All primers and probes were replaced with new sets. This led to some improvement but did not entirely remove the issue.
- New IC (diluted to 5 copies/µL to ensure the assay operates at low template levels) was sourced but despite running this with a complete set of new reagents, non-specific FAM amplification persisted.
- Spurious FAM amplification was not seen when the Internal control was excluded from the PCR master mix, leading to the hypothesis that the IC was the root cause and responsible for the non-specific amplification.
- To further explore the hypothesis that the spurious non-specific amplification (FAM) was caused by the IC, reactions were set up with no mycoplasma template but increasing concentrations of the IC ('old' and 'new').
- Logarithmic ('S' shaped curve) IC-specific amplification (VIC channel) was observed with decreasing Ct accompanying increasing [IC].The spurious FAM "noise" also occurred in an IC 'dose-response' manner (see figure 2)

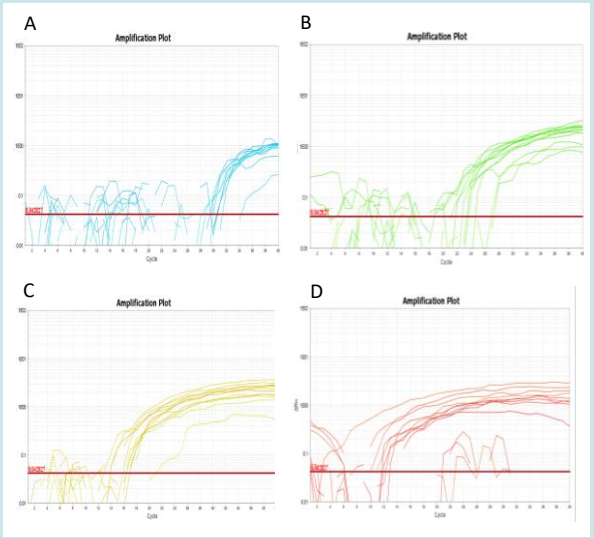

Figure 2. Non specific amplification related to concentration on Internal Control .

A:- 1.38 e3 copies/uL B : 1.38 e5 copies/uL

C: 1.38 e7 copies/uL D: 1.38 e9 copies/uL

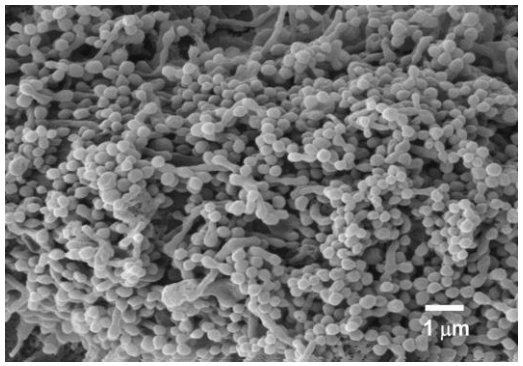

Figure3. Scanning Electron Micrograph. Surface of a Vero cell infected with mycoplasma

DISCUSSION

The experimental approach of focusing on one variable at a time was effective and provided a systemic framework to troubleshoot the assay; both assuring the components were free from template contamination and confirming the IC was the root cause of the problem. A strategy of running samples with and without IC was developed to allow continued assay control but also allowing sample replicates to be run without the confounding effect of the IC.

The internal control is an important component, it acts as an internal QC of the reaction in each well and for each sample, ensuring there are no factors in the sample that might inhibit the PCR reaction.

This strategy has allowed continued assay development whilst maintaining a valid assay for QC release.

CONCLUSIONS

- The G block internal control caused non-logarithmic, non-specific FAM amplification, possibly due to non-specific binding of mycoplasma specific primers and/or probes
- There is a direct correlation with the concentration of IC and the non-specific FAM amplification.
- A 'de-constructed' strategy of running the samples in two separate reactions, with and without the IC has enabled the assay to continue to be employed in ECACC as part of the ISO17025 environment (under deviation), increasing confidence and giving an operational assay whilst further assay development is carried out.

ACKNOWLEDGEMENTS

I would like to thank UKHSA for giving me an opportunity to present my work. Special thanks to Dr. Jim Cooper and Dr. Edward Burnett for their continuous technical support and suggestions. Thanks to the ECACC QC team to co-share responsibilities allowing me to devote time to this assay. Thanks to my manager Dr.Simone Lilley for strength and support. Credits to Jim Cooper for figure 3.

REFERENCES

1. Sung J, Hawkins JR. A highly sensitive internally-controlled real-time PCR assay for mycoplasma detection in cell cultures.
2. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention
Hans G. Drexler and Cord C. Uphoff