The Bio Molecular Systems Magnetic Induction Cycler (Mic) as Applied to Livestock and Soils Research and Diagnostics at the Elizabeth Macarthur Agricultural Institute (EMAI)

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Introduction

The Elizabeth Macarthur Agricultural Institute (EMAI, New South Wales Department of Primary Industries) is the NSW Centre for Animal and Plant Biosecurity that undertakes plant and animal research and diagnostics in a modern laboratory. EMAI utilises nucleic acid based technologies to underpin much of its research and diagnostic work.

DNA-based sciences continue to evolve rapidly. As the technology for these sciences advances so do their applications, not only what they can be used for but also where they can be used. Real-Time Polymerase Chain Reaction continues to develop as a major diagnostic tool and Real-Time PCR thermocyclers that were once cumbersome and costly, are now small, inexpensive and ideally suited to both laboratory and remote diagnostic applications. In 2016, we purchased a Bio Molecular Systems Magnetic Induction Cycler, better known as Mic. I was fortunate enough to have the opportunity to discuss this new technology and its applications to livestock-based veterinary diagnostics on the ABCs NSW Country Hour 70th Anniversary Celebration broadcast live from EMAI (Figure 1). Since then we have been using the Mic to address a broad range of applications.

Figure 1. Ian Marsh holding the Mic while being interviewed on the ABCs NSW Country Hour discussing the future of farming with Michael Condon as part of their 70th Anniversary Celebration (Photo ABC NSW Country Hour).

So why did I choose the Bio Molecular Systems MIC?

As the field of molecular research and diagnostics continues to expand, so do the expectations we have of it. Whilst sensitivity and specificity have been, and continue to be critical, accuracy and precision are now equally important with respect to true quantitative PCR results. That is, how close can we get to the true value (accuracy) and how reproducibly

(precision). When updating our capabilities we needed a thermocycler with the ability to generate accurate and precise data; and software to analyse that data simply and rapidly. We also needed software that facilitated high end research analysis but made day to day diagnostic work easy and understandable. Working in the microbiology field, Real-Time PCR-based diagnostics invariably take the form of low sample numbers but many different assays needing to be run routinely. Other important factors included, price, maintenance and of course speed to meet the ever increasing pressure of turnaround time. The Mic offered 48 samples (with no unnecessary use of consumables for runs of less than 48 samples), no calibration, no maintenance, is extremely affordable and has a very small space requirement. With the Mic we are developing assays that run between 30 and 60 minutes that are highly accurate, precise and increasingly affordable.

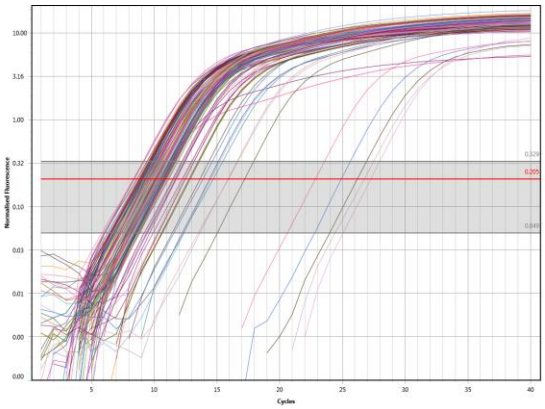
Efficiencies in PCR culture validation

Real-Time PCR is routinely used to confirm bacterial culture from many media types where traditional bacteriological techniques might be ambiguous, slow and/or expensive. We recently introduced a new Real-Time PCR assay to confirm the presence of *Erysipelothrix rhusiopathiae*. When validating the new assay we also evaluated the use of 6 µL Real-Time PCR reactions in the Mic to minimise reagent costs. To determine how effective this was we used the project function of the Mic software to analyse the results. This function allows up to 10 runs to be imported into a single file and be analysed simultaneously. Three Real-Time PCR runs were undertaken on a total of 138 samples (Figure 3). Using the Project function we could immediately see there was uniformity across runs. The majority of samples could be analysed by Linear Regression with an average reaction efficiency of 88.6% and R^2 value of 0.9997. This suggests that the smaller volume is well suited to this purpose.

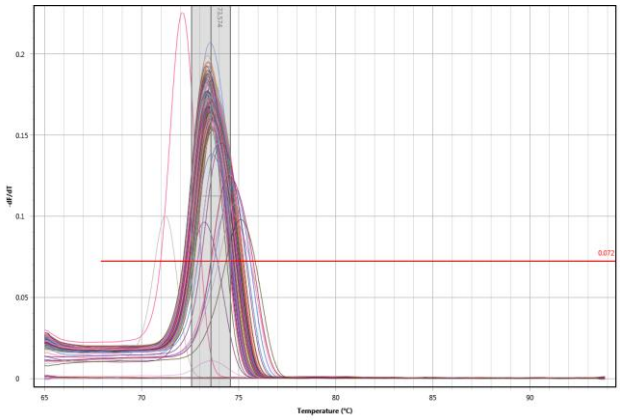
Figure 2. Ian Marsh and Jef Hammond working with the Mic at EMAI (Photo Ian Marsh).

Figure 3: Preliminary validation data from three Real-Time PCR runs used to assess a new assay to confirm culture results (n = 138). All DNA samples originated from culture and DNA was extracted identically for all samples. The Real-Time PCR conditions were identical for all three runs. Data was analysed using the Project function within the MIC software. Panel 1 is the cycling analysis and Panel 2 is the melt analysis.

Panel 1



Panel 2



Pyrosequencing to confirm Real-Time PCR results and/or strain type

The World Organisation for Animal Health (OIE) now states it "would be good practice to" include a subsequent sequencing step to confirm Real-Time PCR positive results, especially when index cases from a previously unaffected source are detected or for samples that produce very high Cq values. At EMAI we are currently incorporating Real-Time PCR on the Mic with pyrosequencing to confirm Real-Time PCR results that exceed the cut off parameters set for assays that target notifiable diseases. Occasionally results are obtained from a sample were the Cq value is beyond the diagnostic cut off. This is most likely the result of inhibition that may have prolonged the initiation of amplification. Using Real-Time PCR assays designed and optimised on the Mic in conjunction with in house pyrosequencing, we can now achieve results in hours that previously took days to weeks when outsourcing the sequencing component. We typically find the combination of a 16 µL Real-Time PCR reaction (to save on cost and reagents) in 30-60 minute runs to be highly efficient whilst not compromising on sensitivity (Figure 4). We have also applied a similar approach using Real-Time PCR and pyrosequencing (allelic discrimination) to differentiate the genomically similar species of Chlamydia (Figure 5). Classified bacteria within the Chlamydiaceae family include: *C. trachomatis, C. muridarum, C. suis, C. psittaci, C. abortus, C. caviae, C. felis, C. pneumoniae* and *C. pecorum*. These cause a variety of diseases in both human and animal hosts and whilst host specificity was thought to be well understood, recent insights in this field have been gained to question this. Given the potential for different species to manifest similarly, the broadening host ranges, and that several of these species are notifiable and or exotic to Australia, diagnosis can be complicated and protracted. Our new Real-Time PCR assay combined with desktop pyrosequencing will detect and identify each species within hours of DNA extraction.

Figure 4: Results demonstrating a combination of Mic-based Real-Time PCR and pyrosequencing to confirm the amplicon sequence at extremely low concentrations of template DNA, ranging from 8.0 to 8.0 x 10^{-4} pg/reaction.

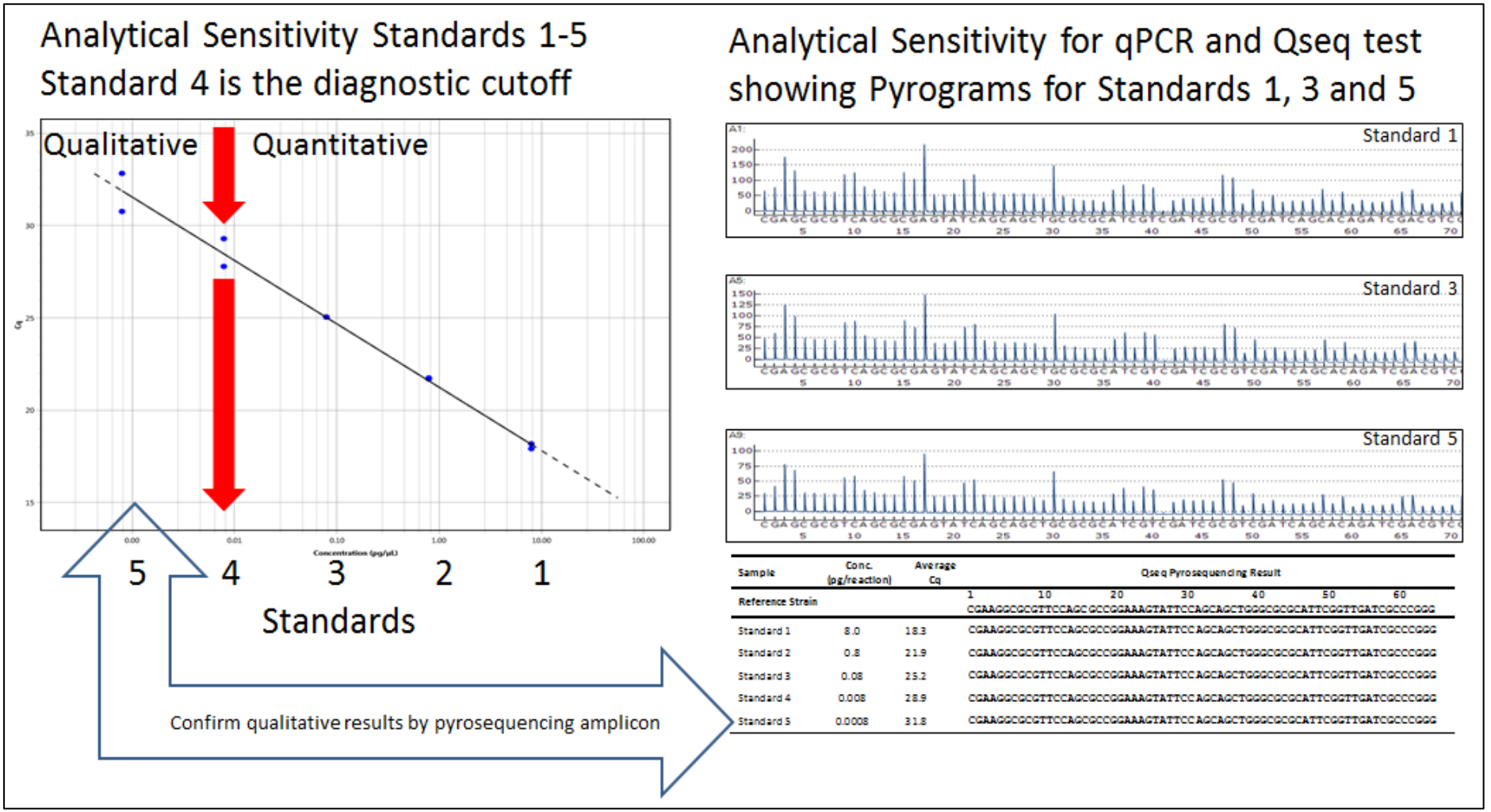
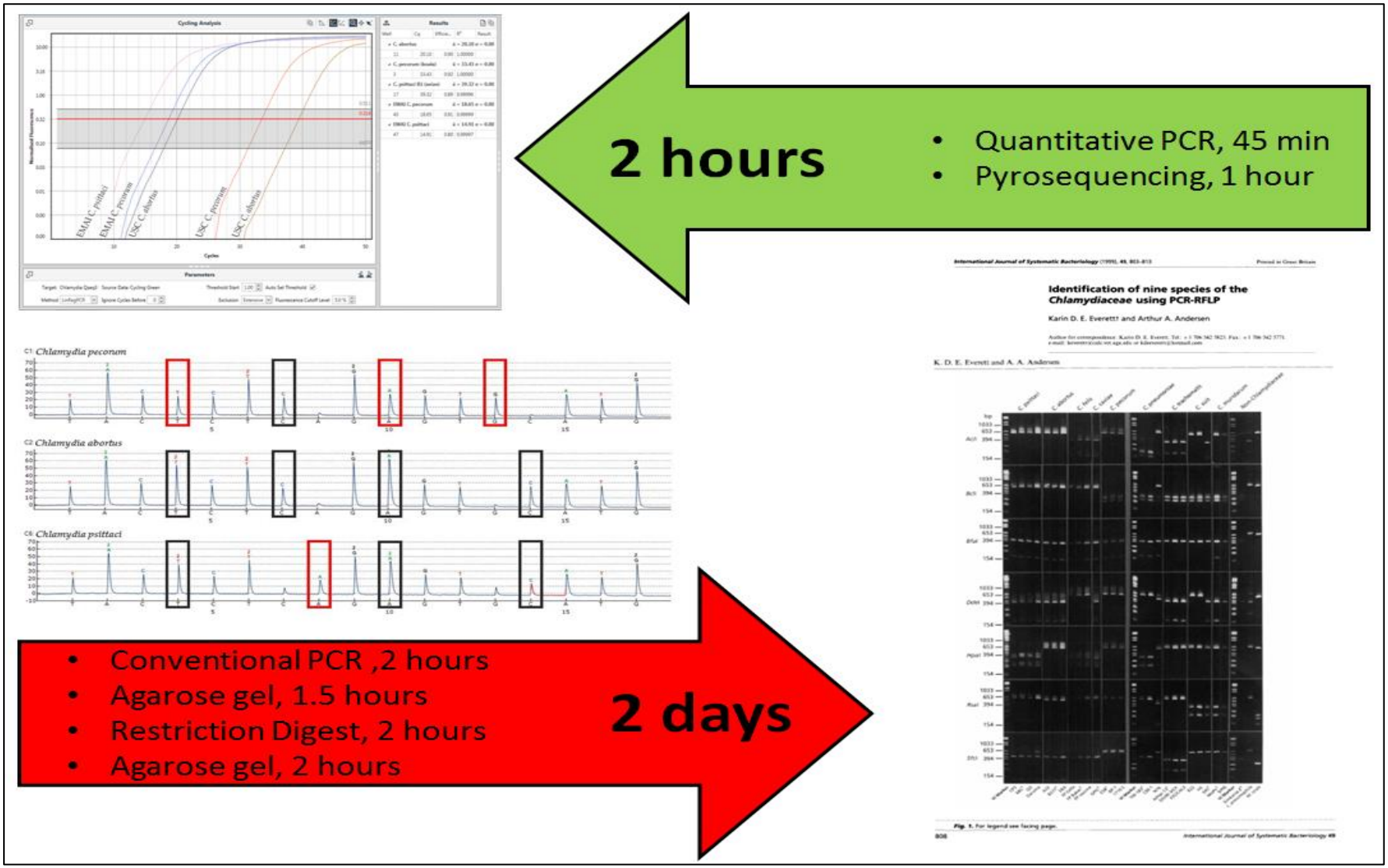


Figure 5: Results demonstrating a combination of Mic-based Real-Time PCR and pyrosequencing to differentiate the genomically similar species of Chlamydia Compared to conventional PCR and restriction enzyme digest analysis which takes up to 2 days to complete.



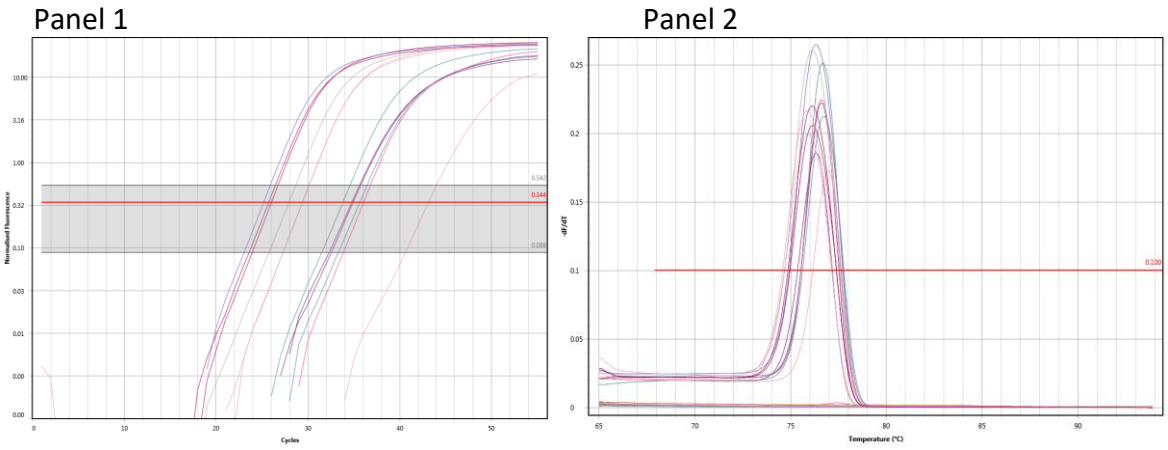
Field-based research using the MIC

Recently, we joined forces with Local Land Services (NSW DPI) and private veterinarians on a cattle property where Real-Time PCR testing using the Mic (Figure 6) was combined with culture to evaluate preputial samples (Figure 7) for bovine Campylobacteriosis. As part of this on farm investigation, the Real-Time PCR results (Figure 8) were integrated with the bacterial culture results to develop a better understanding of the potential of this technology to better diagnose difficult bovine diseases. Relocating the Mic to the farm presented no problems. Bovine Campylobacteriosis is a venereal disease characterised by infertility, early embryonic death, and occasionally abortion in cattle. Bacterial culture is used to examine samples, however, culture may lack sensitivity and specificity. Bovine Campylobacteriosis remains difficult to diagnose and continued problem in cattle production. At this stage the results we achieved with the Mic are very encouraging with regard to the use of this technology in more field-based research.

Figure 6: The Mic being put to use on farm (Photo Ian Marsh)

Figure 7: Local Land Services veterinarian taking a preputial sample (Photo Ian Marsh)

Figure 8: Analysis of cattle (n = 47) sample data from three Real-Time PCR runs used to assess a new assay to detect bacteria in field samples. Data was analysed collectively using the Project function within the Mic software. Panel 1 is the cycling analysis and Panel 2 is the melt analysis.



Improving soils for greater productivity

The Australian Inoculants Research Group (AIRG) within the Soils team of DPI Agriculture curates the National Code of Practice for Legume Inoculants and the 'Green Tick' quality assurance scheme for commercial legume inoculant products. The 'Green Tick' ensures farmers are guaranteed quality legume inoculant product in the paddock.

Rhizobia are specialized bacteria that associate with legumes to 'fix' atmospheric nitrogen into soil. It is estimated that the legume-rhizobia symbiosis fixes 2.7 million tonnes of nitrogen into Australian agricultural systems each year, boosting productivity by $4 billion annually (Drew et al. 2012). The commercial rhizobial collection curated by the AIRG is issued to industry to manufacture commercial quantities of legume inoculant products. Each year the AIRG authenticate and check the performance of up to 40 legume inoculant strains that are used on over 90 host legumes before issue. The commercial product made with these inoculant strains is then assessed by the AIRG to ensure that each batch contains (1) the correct rhizobial strain for the intended host plant (2) sufficient numbers of that strain and (3) that they are still functional (able to associate with the host plant and fix nitrogen).

The AIRG also uses molecular methods to authenticate the strains we issue and isolate from batches assessed by our quality assurance schemes. We use the Mic to generate a PCR product in real time which is then sequenced to confirm the genetic identity of each strain. To achieve this, we generate PCR amplicons greater than 1200 base pairs long and require the entire length to be sequenced to facilitate speciation. Whilst achievable on other platforms, the results were variable and the resulting sequence data was unreliable. The reliability of this process has been vastly improved with the Mic which has resulted in a high degree of success. The Mic software is very simple to use and is highly intuitive allowing us to validate this process and shortening turnaround times significantly compared to sequencing from conventional PCR methods. We anticipate using the Mic to validate new and novel direct strain identification and molecular-based quantitation techniques directly from products in the next phase of our work.

Figure 9: Jessica Rigg and Francesca Galea preparing soil inoculants for industry (Photo Ian Marsh)

Conclusion

Polymerase chain reaction (PCR), both conventional and quantitative, has undoubtedly made a significant contribution to both research and diagnostic applications for microbial pathogens. However, over time its widespread use has led to problems with standardisation

and harmonisation within and between laboratories, especially as the sensitivity of PCR-based tests continually increases. In 2009, an international collaboration was undertaken to establish the Minimum Information for the publication of real-time Quantitative PCR Experiments (MIQE) to assist researchers in the publication of more robust quantitative real-time PCR assays. For those wanting a simple explanation of Real-Time PCR in terms of microbial diagnostics, Kralik and Ricchi published an excellent review in 2017. Fortunately these initiatives underpin the World Organisation for Animal Health (OIE) recommendations for diagnostic test development and validation (Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. NB: Version adopted in May 2013, Figures 9 and 10) and the STAndards for the Reporting of Diagnostic accuracy studies (STARD, www.stard-statement.org/) initiative. These initiatives in conjunction with an increasing focus on measurement of uncertainty should result in diagnostic assays that generate highly reproducible and accurate results. This is becoming increasingly critical for publication or before acceptance at national or international levels. To that end we have found the Mic to be an incredibly valuable tool with which to generate Real-Time PCR data and analyse that data to make sound decisions on progressing our research and diagnostics as describe here with just a few examples here.

Figure 9: Key recommendations taken directly from the World Organisation for Animal Health (OIE) recommendations for diagnostic test development and validation (Chapter 1.1.6)

Assay performance is affected by many factors beginning with optimisation of the assay. After initial optimisation for an intended purpose, characteristics of the performance of the assay will be tested. The assay may need additional optimisation or may be found to be fit for purpose based on the results of the validation work.

Criteria for Assay Development and Validation

i) Definition of the intended purpose(s)
ii) Optimisation
iii) Standardisation
iv) Repeatability
v) Analytical sensitivity
vi) Analytical specificity

vii) Thresholds (cut-offs)
viii) Diagnostic sensitivity
ix) Diagnostic specificity
x) Reproducibility
xi) Fitness for intended purpose(s)

Figure 10: Validation pathway suggested the World Organisation for Animal Health (OIE)(Chapter 1.1.6)

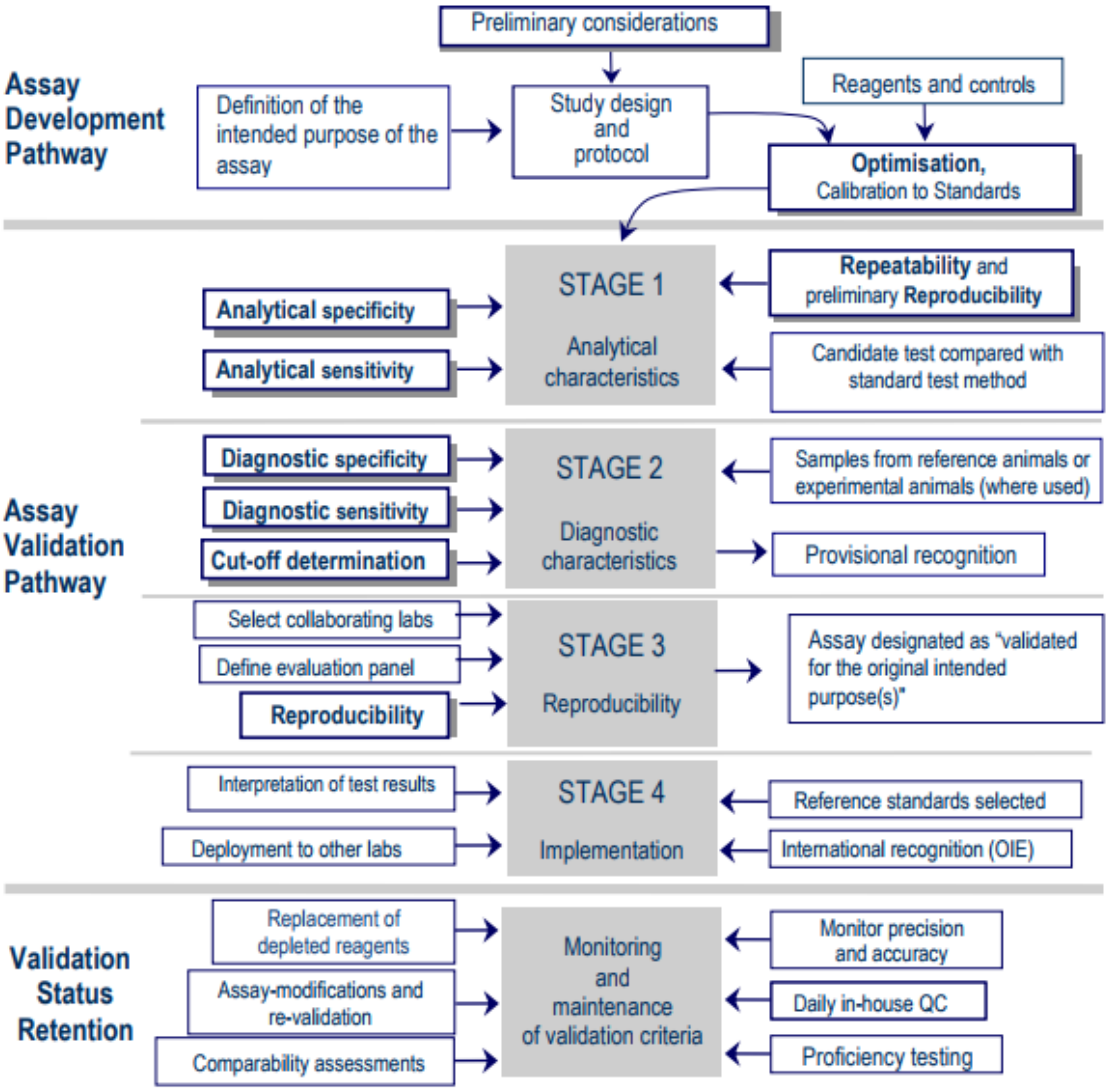


Figure 1. *The assay development and validation pathways with assay validation criteria highlighted in bold typescript within shadowed boxes.*

References

1. OIE Terrestrial Manual, 2013, Chapter 1.1.6 Principles of validation of diagnostic assays for infectious diseases, *www.oie.int/eng/en_index.htm*
2. Kralik, P., & Ricchi, M. (2017). A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. Frontiers in Microbiology, 8, 108.
3. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry. Apr;55(4):611-22
4. Patrick M Bossuyt, Johannes B Reitsma, for the STARD group, The STARD initiative The Lancet, Vol 361, January 4, 2003
5. Patrick M. Bossuyt, Johannes B. Reitsma, David E. Bruns, Constantine A. Gatsonis, Paul P. Glasziou, Les M. Irwig, David Moher, Drummond Rennie, Henrica C.W. de Vet, and Jeroen G. Lijmer, The STARD Statement for Reporting Studies of Diagnostic Accuracy: Explanation and Elaboration, Clinical Chemistry 49:1 7–18 (2003)
6. STARD checklist for the reporting of studies of diagnostic accuracy. First official version, January 2003.