Novel Solution Automates qPCR Diagnostics for Qualitative and Quantitative Data without Loss of Accuracy

Collaborative research by West of Scotland Specialist Virology Centre Gartnavel General Hospital and Azure PCR Limited

Objective: To assess cost-savings delivered by the Azure PCR automated qPCR data-analysis technology in a routine diagnostics setting.

Introduction

Existing methods of real-time PCR data analysis rely on manipulation of output data for visual interpretation purposes[1] as well as time-consuming setting of the reaction parameters such as baseline, threshold, or noise-band range to determine responsive (positive) from non-responsive (negative) curves[2]. The results provided by these methods can lead to a need for sample reprocessing or even to patient misdiagnoses[3,4] as threshold-based methods can incorrectly classify sample curves (delivering false positive or negative results). Setting reaction parameters and visual interpretation of results requires considerable time, expertise and quality control procedures. For all of these reasons, standardisation of qPCR result analyses both within and between laboratories is considered of high importance.[5]

In order to assess its capabilities to reduce the cost of qPCR (both in terms of analysis time and expertise required) without negatively impacting on result accuracy, Azure PCR hardware (the 'Azure PCR Node') data-analysis results were compared to those obtained using conventional methods at the West of Scotland Specialist Virology Centre (WSSVC).

This study was also designed to assess the time-to-results for patients and for this to be used as an initial marker for current cost of manual analysis. In order to best model regular clinical laboratory practice, both qualitative (yes/no) and quantitative tests were assessed.

Cycle Threshold (C_t) is used in the study to indicate the point at which the qPCR curve enters the fluorescent phase. It is also known as C_q (quantification cycle) or Crossing Point (C_p). Azure PCRs' technology automates analysis of qPCR data and has been evaluated for use in infectious disease testing. The product integrates with existing laboratory systems and is capable of importing data from most of the popular cyclers available on the market. Accuracy of over 99% has been achieved in previous retrospective studies for over 120,000 gene targets with the remainder identified as ambiguous.

In this study, Azure PCR hardware was used on-site at WSSVC to analyse data produced in both qualitative and quantitative qPCR-based infectious testing. Azure PCR results were compared to those delivered as test results to clinicians.

Methods

Diagnostic Assays

In common with many diagnostic laboratories around the world, WSSVC scientists use their own lab-developed qPCR assays for a variety of tests[7,8,9,10]:

- a quantitative assay for the Hepatitis Virus C (HCV) with Equine Arteritis Virus (EAV) used an internal control (IC);
- a multiplex qualitative assay to detect Enterovirus and Parechovirus (ENT/PEV).The ENT/PEV assay tested for two sequences;
- a multiplex qualitative assay to detect Herpes Simplex Virus (type 1 and 2) and Treponemapallidum (syphilis) (HSV1, HSV2, SYPH). This assay tested for three sequences;
- a multiplex qualitative assay to detect Herpes Simplex Virus (type 1 and 2) and Varicella Zoster Virus (HSV1, HSV2, VZV). This assay tested for three sequences;
- a multiplex qualitative assay to detect Herpes Simplex Virus (type 1 and 2) Virus, Adeno Virus, Chlamydia trachomatis and Varicella Zoster Virus (HSV1, HSV2, VZV, CT, Adeno FAM). This assay tested for four sequences;

Clinical specimens (RNA and DNA) for all assays were extracted using Abbott Molecular m2000sp or Qiagen MDX and run on the Applied Biosystems 7500 Real-Time PCR cycler (ABI 7500).

Analysis

ABI 7500 Output data were analysed using two methods:

- Manual Interpretation by WSSVC Biomedical and Senior Scientists using ABI's SDS software version 1.3.1 (SDS software);
- The Azure PCR node which produces reports and data ready for upload to clinical systems automatically.

Both methods followed the relevant WSSVC standard operating procedures (SOPs) for each assay. Interpretation rules for quantitative assay are presented in Table 1. The qualitative assays followed simpler protocols; if the sample had a C_t it was a positive result. Samples were rejected if certain controls exceeded a known amount based on the control type. Details for these precise rules can be found in the SOPs referenced to the right.

Table 1: Example interpretation rules for a WSSVC diagnostic assay[7]

Ct	Result from quantitative standard	Interpretation
None	Any	Negative
>40	Any	Negative
<40	<50 IU/ml	Positive (<50 IU/ml detectable)
<40	<50 but <1,000 IU/ml	Positive (<1,000 IU/ml detectable)
<40	>1,000 IU/ml	Positive and quantified

Quantification:

Due to the importance of quantitation accuracy in HCV testing, clinical scientists at WSSVC calibrate their standard curve according to the known 1000 IU/ml when there is significant difference between the expected (based on the standard curve) and actual C_t of this control[7] (see figure 1). This is not done for each run as it can be time consuming. The Azure PCR Node does this calibration automatically for all runs to ensure quantitative accuracy.

It is not possible to directly compare C_ts produced by the two methods of analysis as there can be constant differences between the analysis results (i.e. across the entire run). In other words, one method may produce a C_t that is always higher by, for example, two C_ts, but since this fixed difference will also affect standards and dilution series it will have no effect on quantity determined. Therefore, the known standard was used as a reference and delta-C_ts were calculated between this and the sample C_t for each result. These normalised delta-C_ts were compared to one another and derived quantities were also compared.

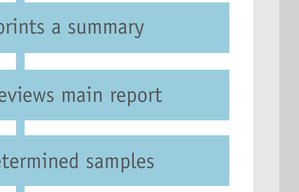


Fig.1: Example demonstration of calibration of a standard curve using known 1000 IU/ml standard.

Comparison of current (manual) steps to use of Azure PCR equipment for data analysis

MANUAL PROCESS

- Biomedical scientist (BMS): Conducts noise filtering and curve fitting of raw data using SDS software
- BMS: Sets threshold, copy number (for quantitative assays)
- BMS: Creates subset of non-determined results
- BMS: Prints a report using SDS software with main analysis data. Non-determined results also detailed.
- BMS: Creates and prints a summary
- Senior scientist: Reviews main report
- BMS: Re-runs undetermined samples
- BMS: Results ready for entry into Laboratory Information system

AZURE PCR PROCESS

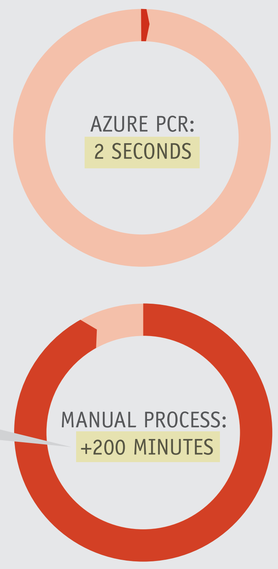
- Completed cycler runs automatically detected by 'Azure PCR widget' and sent to Azure PCR Node
- Results and coversheet created by Azure PCR node
- Results ready for entry into Laboratory Information system

average time for a Junior Scientist: 123 minutes

average time for a Senior Scientist: 94 minutes

Total average time: 217 minutes

TIME REQUIRED TO ANALYSE 96 WELL PLATE



ALL +2500 RESULTS MARKED AS CONCLUSIVE BY AZURE PCR WERE CLINICALLY ACCURATE

Results

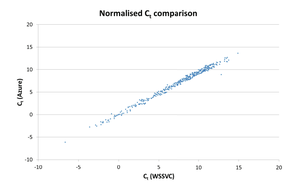
Time taken

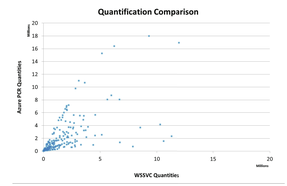
The average amount of time taken for all runs was in excess of 210 minutes, of which 123 minutes was time taken by a Junior BMS and 94 minutes by a Senior BMS. This amounts to a total cost of £56, based on publicly available NHS salary data[11]. The Azure PCR Node pricing is delivered individually to customers typically resulting in savings of 75% against their current costs. WSSVC timing measurements indicate that the key factor in time taken for manual analysis length was the number of different targets present on the plate. In common with typical clinical laboratory practice, WSSVC often run a variety of assays on the same plate for all but the most common tests, such as HCV. Therefore, if four triplex assays are run, the BMS would need to interpret twelve sets of results for the run independently and then re-combine them to produce a report. Another critical factor shown to affect how long it took for patients and clinicians to receive results was what time the data became available; for example, if before a lunch break or at the end of the day, reporting was dramatically affected. Finally, due to the need for a Senior BMS to review results, additional time and costs were incurred. In contrast, the Azure PCR Node was affected by none of these limitations as it is capable of delivering results automatically and near-instantaneously.

Accuracy

After outliers were excluded, manual analysis results for 2,835 curves were compared to those obtained using the Azure PCR Node. No discrepancies between the results were observed for Positive/Negative calls. Azure PCR analysis achieved at least equivalent accuracy to manual analysis (using the SDS software) without any user-intervention.

Quantitative analyses were based on WSSVC's HCV assay. There are no other assays run on an 'HCV plate' in routine practice as a result of desire to avoid any potential sources of inhibition (inhibition has a greater effect on derived quantity than on a qualitative call) and due to larger volumes of these tests being run on a regular basis. Thus analysis time was continually less than that for the qualitative assays. There were no runs where the SDS software was able to achieve a significantly higher r^2 than the Azure PCR Node for the standard curve, however, there was one run (out of twenty) which could not be quantified using the SDS software (the standard curve gradient did not obtain an acceptable gradient according to SOP rules). The results obtained using the Azure PCR Node were acceptable when the same rules were applied. Furthermore, it can be seen that while normalised C_ts were relatively similar, applying rules regarding calibration of dilution series had a significant impact on RNA quantities obtained.





As a result of the normalising of the dilution series, the delta-C_ts are closely correlated but the quantification values (which are not calibrated by the WSSVC method) are different. Since the C_ts are closely matched, in the case of divergent quantities the Azure results are considered to be more accurate due to the higher number of valid gradients of the standard curves and the constant normalisation to the calibration standard.

The graphs show that the delta-C_ts are closely correlated, but as a result of the normalising of dilution series to known standards the quantification values are very different.

Since the C_ts are closely matched, in the case of divergent quantities, the Azure results are considered to be more accurate due to the higher number of valid gradients of the standard curves and the constant normalisation to the calibration standard.

Limitations of the study

This research measured the time lapse between run completion and delivery of results to the clinician but not that of 'hands-on' time using the SDS software. Therefore, the results do not directly show the cost of analysis, however they are clear indicators of some the inefficiencies that affect doctors and patients. While the average cost of manual analysis was calculated using data obtained, this would be more useful if hands-on data was also available (this is expected to be the subject of further studies).

For quantitative testing, automating the 'quantification calibration' demonstrated increased accuracy. Further examination is required to determine if this improvement has clinical significance. It is also worth noting that the 'post-analysis' quantification calibration performed by Azure PCR could equally be done with SDS results using off-the-shelf software such as Microsoft Excel, however, this would increase time-taken and add a potential source of error. As a result calibration is presently only done in cases where significant discrepancies between the known standard and the standard curve are observed.

Conclusion

The Azure PCR Node demonstrated its ability to obtain both qualitative and quantitative results with the same or higher levels of accuracy and greater efficiency (measured in terms of time-taken) when compared to current established methods. Data analysis using the Azure PCR Node requires no manual intervention and does not manipulate raw data. Implementation of this automated process removes the need for manual intervention and provides more rapid data analysis, delivering cost reductions and higher throughput. This increase in speed should also yield benefits for clinicians and patients as sample-to-answer time is reduced.

The partners intend to continue their collaboration, including running controlled experiments to further assess accuracy and the effects of inhibition on both manual and Azure PCR analyses. Further, this testing will be run on different qPCR cyclers to the ABI 7500, for example, the Roche Lightcycler and Qiagen Rotorgene models. Finally, robust methods for determining hands-on time to be used together with sample-to-answer time are planned to be implemented, enabling accurate determination of costs of manual analysis.

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References
1. S, M. & Vandesomple, J., & Kubista, M. (2009). Real Time PCR: Current Technology and Applications. Caister Academic Press, p66,71,81,147.
2. Applied Biosystems (2011). Applied Biosystems ViiA7 Real-Time PCR System Getting Started Guides, part number, Rev. C, p43,45; Applied Biosystems (2010). 7500/7500 Fast Real-Time PCR System Standard Curve Experiments, Part number 4387779 Rev. C, p78,87; Applied Biosystems (2010). Relative Quantification Using Comparative Ct Getting Started Guide, part number 4364-16 Rev. D, p55-57; Bio-Rad (2008). Bio-Rad CFX96/384 Real-Time PCR Detection Systems Instruction Manual, Rev. C, p74; Corbett Research (2006).RotorGene 600 Operator Manual 1.7.87, p68; Roche (2008).LightCycler 480 Software Version 1.5, version 3.0, p171,174.
3. Murphy, J. &Bustin, S.A. (2009). Reliability of real-time reverse-transcription PCR in clinical diagnostics: gold standard or substandard? Expert Review Molecular Diagnostics, 9, p192.
4. Gunson, R.N., Collins, T.C., & Carmen, W.F. (2006) Practical experience of high-throughput real-time PCR in the routine diagnostic virology setting. Journal of Clinical Virology, 35, p365.
5. Bustin, S.A., Benes, V., Garson, J.A., et al. (2009). The MIQE Guidelines: Minimum Information for for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55(4), p611-622.
6. Gunson R.N., Collins T.C., Carman W.F. (2006) Practical experience of high throughput real time PCR in the routine diagnostic virology setting.Journal of Clinical Virology 35; 355–367
7. MacLean, A (2010), HCV Quantitative In House Real Time PCR SOP
8. MacLean, A (2010), Herpes simplex virus type 1 and 2 (HSV-1 and 2), Varicella Zoster Virus (VZV) and internal control Real Time PCR SOP
9. MacLean, A (2010), Eye PCR: Herpes simplex virus type 1 and 2 (HSV-1 and 2), Varicella Zoster Virus (VZV), Adenovirus and Chlamydia trachomatis Real Time PCR
10. MacLean, A (2010), Herpes simplex virus type 1 and 2 (HSV-1 and 2), Treponemapallidum (syphilis) and internal control Real Time PCR SOP