Novel, Fully Automated Method Allows Efficient Analysis of qPCR Data for Qualitative Calling Based on Comparative Cq

Collaborative research by Pioneer Hi-Bred (a DuPont company)
and Azure PCR Limited

Objective

Assessment to ascertain if a method for analysis of qPCR data dependent on manual intervention can be replaced by automated analysis using the AzurePCR™ method.

Introduction

qPCR is extensively applied to determine the identity of genetically modified (GM) seeds and plants. Analysis and interpretation of qPCR data for any application is limited by sample variability[1], poor assay performance[1] and arbitrarily set thresholds[2] which can all lead to ambiguous and subjective result calling that is reliant upon the expertise and experience of the scientist interpreting the assay. Processing of raw data output from thermal cyclers using analysis software, to provide data more easily interpretable by the human eye, can lead to loss of data[3] and error prone sample calling[4,5]. In this study we retrospectively examined data produced in qPCR-based plant genotyping tests at Pioneer Hi-Bred to assess if patent-pending AzurePCR automated analysis produced similar results without the need for manual input of analysis parameters or manipulation of raw data.

1. Bustin, S.A. et al. (2009) Minimum Information for Publication of Quantitative Real-Time PCR Experiments Clinical Chemistry, 55(4), p611,612
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. Pfaffl, M.; Vandesomple, J.; & Kubista, M. (2009) Real Time PCR: Current Technology and Applications. Caister Academic Press, p66,71,81,147
4. 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
5. 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

Methods

Pioneer Hi-Bred used proprietary qPCR assays to detect two target DNA sequences: the transgenic DNA and an endogenous control used to validate target marker result. In addition to the unknown samples that were tested, the following controls were tested: transgene-positive control, transgene-negative control and no template control. These were run on a Roche LightCycler 480 real-time PCR instrument. Parameters such as baseline and thresholds for detection of DNA amplification were set pre-analysis using the LightCycler 480 software version 1.5.0.39.[6]

Samples were originally analysed by Pioneer Hi-Bred's laboratory scientists using the following methodology:

1. Raw data underwent curve smoothing and fitting by the Roche LightCycler software.
2. A corresponding Cq value was assigned for all targets using the fit-points method available in the Light-Cycler 480 software. The second derivative maximum method which is also available in the software was not used due to its propensity for false positive calling.[7]
3. The Cq values obtained in step 2 were used for a modification of the Applied Biosystems (ABI) comparative Cq method[8]. This relative quantification method does not rely on a standard curve but rather on an arithmetic formula, $2^{-(ddCq)}$. Delta-delta-Cq is the difference between the delta-Cq of an endogenous control and the delta-Cq of its corresponding target ($dCq_{target} - dCt_{endogenous\ control}$).
4. In-house qualitative calling rules were applied to the values obtained in step 3 and each sample was classified either as positive, negative or low DNA (outlier result).

Following this, raw, unprocessed data was exported from the LightCycler 480 for automated analysis using the AzurePCR method. This three-stage method is based upon machine learning principles in which the software evolves in an iterative manner enabling accurate result interpretation based solely on the data provided:

1. Each curve is automatically analysed with parameters extracted to form a 'fingerprint' for each sample and target. Parameters from this fingerprint are also used to calculate Cq.
2. Data from controls are assessed by the system to form an initial classification database.
3. Samples are analysed as a group to ascertain similarities and differences and form positive and negative clusters.

In this study, 4,092 samples were subject to automated data analysis using the AzurePCR method, followed by steps 3 and 4 of Pioneer Hi-Bred's methodology. Azure PCR's qualitative calling for each sample was then compared with the calls previously obtained at Pioneer Hi-Bred, as described above.

6. Roche (2008) LightCycler 480 Software Version 1.5, version 3.0, p171,173
7. Roche (2008) LightCycler 480 Software Version 1.5, version 3.0, p168
8. Applied BioSystems (2001) User Bulletin #2, ABI PRISM 7700 Sequence Detection System, p11-13

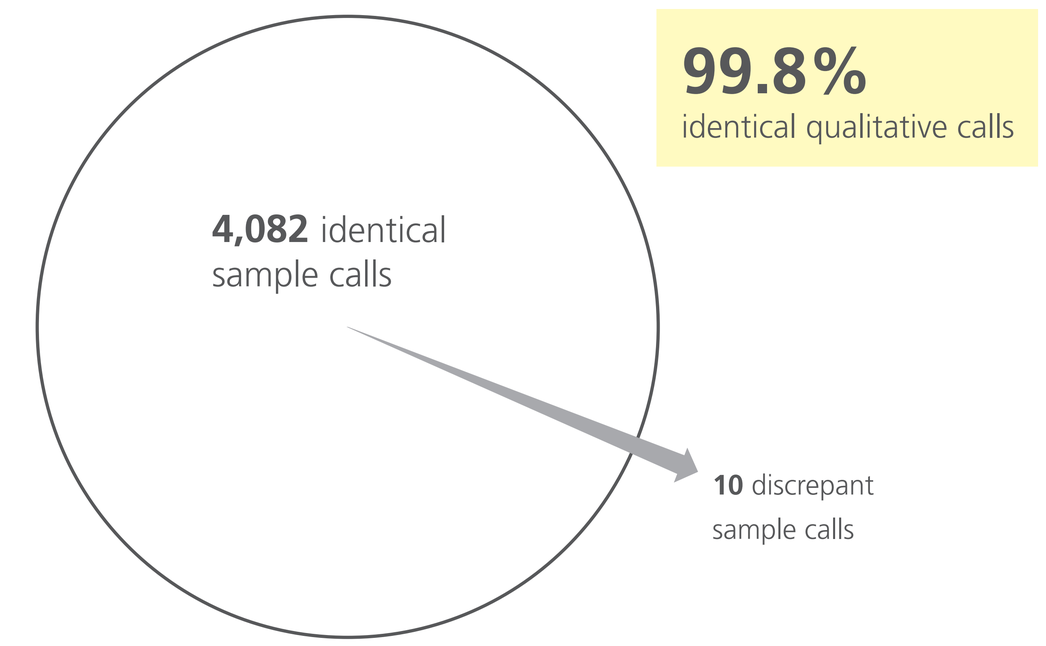
Conclusion

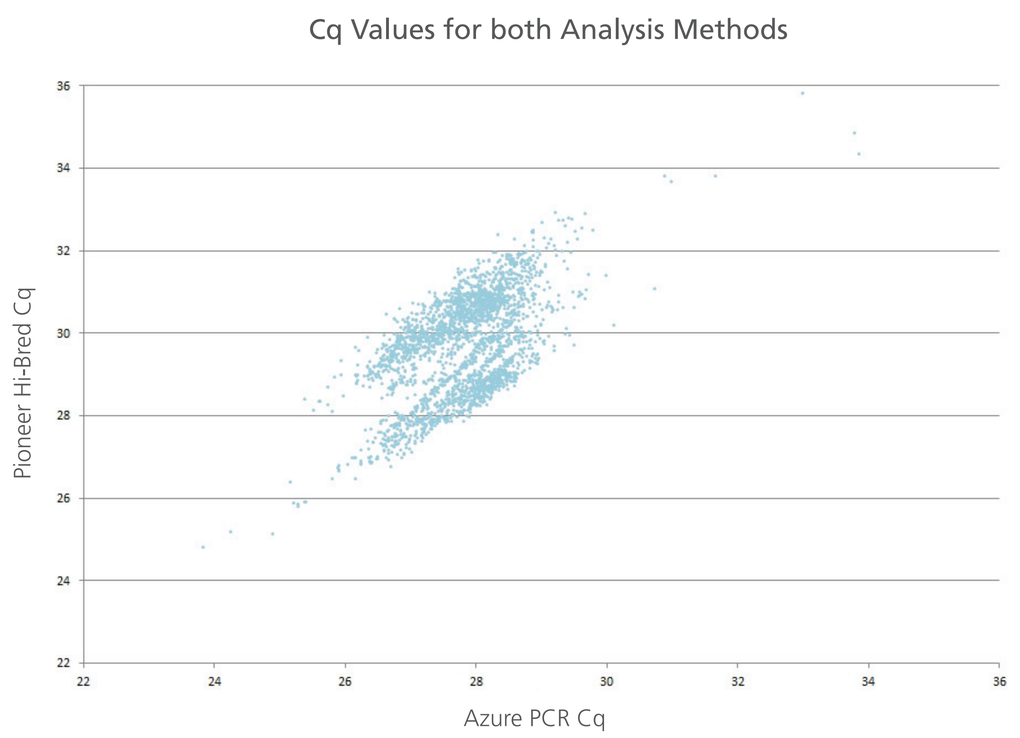
The automated AzurePCR method was demonstrated to generate qualitative calls accurately and efficiently, when compared to established methodology. Data analysis using the AzurePCR method requires no manual intervention, and does not manipulate the raw data, thus preventing potential loss of valuable data for analysis. Implementation of this automated process would remove the need for manual intervention, which should deliver reduced costs and higher throughput of sample processing.

Pioneer Hi-Bred and Azure PCR aim to follow up with a comprehensive study assessing both the qualitative and quantitative performances of the AzurePCR method.

Results

Out of 4,092 samples which were analysed by both methods – Pioneer Hi-Bred and Azure PCR's – 10 resulted in discrepant qualitative calls.





Acknowledgments

Azure PCR would like to thank Aron Cohen for providing logistical support during the study and to Rea Lavi for managing poster content.

Pioneer Hi-Bred would like to thank the management and staff of the Nucleic Acids Analysis Lab for data evaluation

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More validation studies

For validation studies of the AzurePCR method on human diagnostic data,
see www.azurepcr.com/validation-studies or email zeev@azurepcr.com