Branching Out into New Technology for Nucleic Acid Analysis

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1 Introduction

Various Bioanalytical techniques are available for the detection of RNA species. In the context of the regulated space the default choice tends to be RT-qPCR as it is an established methodology with accepted criteria for assay design and performance (1). The RT-qPCR system does however have some established limitations. The performance of the assay is dependent upon the purity and chemistry of the analyte. Sequential enzyme modification and amplification of the target is required and while the level of inhibition can be quantified for some analyte matrix combinations it may compromise the analysis. While low concentrations of enzyme inhibitors, either as a co-purified contaminants or modifications within the analyte, can compromise RT-qPCR methods they are unlikely to interfere with hybridisation based methodologies. In contrast Nucleic acid hybridisation kinetics are principally determined by the free salt concentration and temperature. A principal limitation of hybridisation platforms is their sensitivity.

Branch Chain signal amplification approaches offer a way to improve the sensitivity of hybridisation based assays. In this system there is no enzyme modification but a two fold hybridization; the first to capture the analyte, and the second to detect it and provide a backbone for the signal amplification step (figure 1). This platform is available commercially from eBioscience (Quantigene).

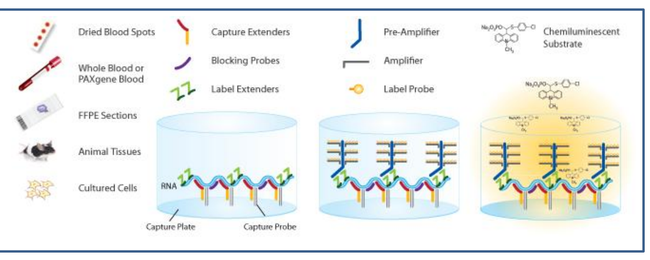


Figure 1. Manufacturers outline of the Quantigene Assay System.
An overnight hybridysation step captures the mRNA and allows the label extenders (detection probe to bind). Sequential addition of detection enzyme, mediates the signal amplificatoin

The platform offers some theoretical benefits over RT-qPCR, as it does not require high purity nucleic acid and the mass of matrix should have a lower impact on hybridisation versus enzyme kinetics. However non-specific hybridisation events will result in high background due to the Signal Amplification methodology. In order to compare these two platforms we utilised a defined analyte, Luciferase, and matrix. We examined sensitivity, reproducibility, accuracy and impact of matrix mass.

2 Experimental Approach

While a principal claimed benefit of the Quantigene system is the absence of any requirement to prepare high quality nucleic acid this is dependent upon two assumptions;

- That all targets show equivalent hybridisation kinetics under the conditions used (when multiplexing).
- That the total mass of nucleic acid has minimal impact on the end point of the hybridisation.

As a first step to determining if the Quantigene assay is a viable alternative to established RT-qPCR methods we focused on the second assumption. While the kinetics of hybridisation are dependent on the concentration of target and probe, at the end point the assay should offer a linear response with increasing matrix, and analyte, providing that the increase in total mass of material does not result in an increase in non-specific signal. Any increase in non-specific probe binding would negate the benefits of increased matrix mass by raising the noise level.

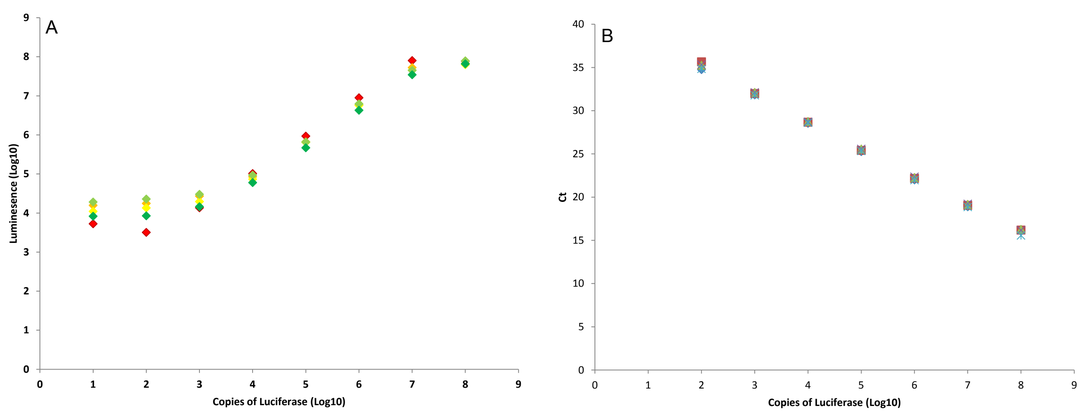
To test the performance of the two platforms we generated a series of standard curves composed of Luciferase mRNA and a fixed mass of matrix. These were used to determine reproducibility, linearity and sensitivity. To test the impact of matrix concentration a further series of samples containing a fixed number of Luciferase copies but increasing matrix concentration was prepared.

It should be noted that this study utilised high purity nucleic acid, as the focus was on the performance of the hybridisation/detection steps in an ideal situation. When used in conjunction with the on step lysis/hybridisation buffer there will be additional considerations. For example as the mass of tissue increases the concentration of protein, lipid, salt and DNA concentration within the sample will also increase – the impact these components have on hybridisation were not assessed in the current study.

Materials and Methods. Luciferase mRNA was obtained from TriLink BioTechnologies. Control Matrix, human brain total RNA, was obtained from Life Technologies. Primers for Luciferase were designed in house, with the RT-qPCR performed in a one step format using the Qiagen Quantitect Probe RT-PCR kit. For the Quantigene assay the probe set for the detection of Luciferase and all solutions and reagents were obtained from ebioscience. All methods were performed according to manufacturers recomended guidelines with minor optimisation.
Parametric statistics where performed on the normally distributed data. Calculations for comparison of sensitivity of the two assays where based on an assumption of 30pg total RNA per cell, of which 5% is mRNA.). RT-qPCR and Quantigene assays were performed in parallel for each series to minimise any variation.

3 Results

Figure 2



Our initial experiments addressed the linearity, sensitivity and reproducibility of each assay system (Figure 2). Five Independent dilution series were prepared, each containing a fixed mass of Control Matrix (20ng) and a varying copy number of Luciferase mRNA. The Quantigene assay (2A) performed in line with manufacturers claims, with a linear range and lower limit close to claimed sensitivity of 3000 copies. Similarly the RT-qPCR assay (2B) performed with a range over 7 orders of magnitude and a lower limit of 100 copies. It should be noted that the titration series was 10 fold and not designed, in either case, to formally identify the LLOD, LLOQ. For both assays the linear region gave a R2 >0.995 for all series. Both assays showed good reproducibility and low variation; for both assays the CV on the triplicate samples less than 5% in all cases and generally <1%. However, under these assay conditions RT-qPCR is 30-50 fold more sensitive than the Quantigene assay. Increasing the sensitivity of the Quantigene assay requires increasing the total matrix concentration to compensate for a lower absolute sensitivity. As a first test we examined the effect of increasing matrix concentrations on background signal (Table 1).

Table 1 Effect of Control Matrix on Signal in Absence of Analyte

Matrix Mass	A (mean, SD)	B (mean, SD)	C (mean, SD)
10ng	4.14, 0.24	4.20, 0.05	3.76, 0.11
50ng	4.39, 0.44	4.22, 0.03	3.62, 0.02
100ng	4.12, 0.17	4.18, 0.05	3.69, 0.06
200ng	4.00, 0.2	4.21, 0.03	3.62, 0.03
400ng	3.99, 0.03	4.16, 0.02	3.65, 0.07
800ng	3.97, 0.02	4.30, 0.26	3.65, 0.11
2000ng			3.71, 0.08

The data in Table 1 indicates that background signal is not highly impacted by the mass of RNA within each sample. Samples were analysed in triplicate and the Log(10) of the Luciferase signal compared. No significant difference was observed in the background signal when data from either individual experiments or the pooled data from all experiments was statistically analysed.

As a final comparison we examined the impact of Matrix mass on the back calculated number of Luciferase copies. Two Luciferase mRNA copy numbers were selected: $5x10^3$ and $5x10^5$. As before a standard matrix mass of 20ng was used per point on the standard curve, so any impact of Matrix Mass on assay performance would therefore result in shifts in the back calculated copy number. Summary data from these experiments are presented in Table 2.

Table 2 Effect of Control Matrix on Back Calculated Luciferase mRNA copies (as % of Input).

	RT-qPCR						Quantigene					
[Matrix]	$5x10^3$ Copies			$5x10^5$ Copies			$5x10^3$ Copies			$5x10^5$ Copies		
10ng	85.8	103.5	120.7	77.4	81.5	112.4	88.4	101.4	n.d.	78.2	77.6	99.4
50ng	60.0	101.5	115.3	70.5	74.6	100.4	93.1	127.1	n.d.	71.0	85.4	97.1
100ng	67.5	97.8	83.5	66.2	77.1	99.3	91.5	116.6	n.d.	76.9	75.4	104.7
200ng	61.2	84.8	66.1	54.0	69.4	87.5	94.6	104.5	n.d.	77.9	79.6	106.3
400ng	47.0	72.1	46.0	44.4	59.3	73.7	81.4	70.4	n.d.	78.9	76.6	115.3
800ng	37.2	55.5	39.1	28.7	43.4	47.1	68.8	73.4	n.d.	72.3	72.3	104.0
2000ng									n.d.			103.3

Data was returned for all three independent series, with the exception of the final Quantigene 5 x 103 series (which fell below plate background).

These data indicate a lower requirement for accurate determination of Matrix mass when performing the Quantigene Assay. While the impact of matrix mass on the RT-qPCR is as expected. Although the assay is not independent of input Nucleic Acid concentration a input range over 1 – 2 orders of magnitude appears within the tolerated range, although this should be established independently for each probe set. These data would support the ability of the assay to be used in multiplex formats with a similar range of target expression. In relation to the absolute sensitivity of the assay a rough back calculation would indicate that for the Quantigene Assay to detect an analyte present at1 mRNA per cell at an input RNA mass of 3mg would be required and a LLOQ of 5000 copies. The comparable numbers for RT-qPCR assay of similar sensitivity would be 60ng and a LLOQ of 100 copies.

4 Conclusions

Our data suggests that the Quantigene Assay system could replace RT-qPCR in some Bio-Analytical contexts. In particular in situations where a large number of samples are to be assayed for a single analyte the reduced sample processing and initial quantification requirement could be advantageous. However the validation/qualification requirements of the assay raise some challenges particularly in the context of multiplexing. Further while the sample preparation steps for the Quantigene assay are quicker that for RT-qPCR the assay itself requires significantly more intervention, negating a large part of this advantage. Finally while the Quantigene assay can reach equivalent sensitivity to RT-qPCR this is achieved by using a larger total mass of nucleic acid. This may be useful in some contexts but in situations where the sample is limiting it is a major disadvantage.

References
(1) Bustin SA et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 55(4):611-22.

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