Application Note A09-002A

Linear dynamic range of amplification

PrimePro 48

Introduction

Whether determining unknowns by absolute or relative quantification, the samples may vary greatly in concentration, so it is essential that the qPCR instrument used can detect samples over a wide linear dynamic range to allow accurate quantification. It is important to determine the linear dynamic range of an assay as part of the optimisation procedure since this will indicate the efficiency of the reaction and also the limit of quantification for the assay in question, thereby determining the range of sample concentration that can be accurately quantified.

In this application note we demonstrate a detection range of greater than 10 log orders of concentration using the Techne Prime Pro 48. Further analysis of the data is performed to determine the optimal linear dynamic range of the assay.

Method

To facilitate the testing of a large range of template concentrations, a 100 base ssDNA template was synthesized based on the genomic sequence of Enterobacteria phage lambda (Figure 1). The primers were designed to amplify a 76 base pair product within the template.

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16361: gcatggggaa acgtcttggt gcggcggatg tggataaatg ggcgctgtat gtcatcggcc

16421: agtactgcga ccagtcagtg ccggacggct ttggcggcac
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Figure 1: *Sequences of the lambda DNA template and primers. The 100 base template was based on the sequence of bases 16361 to 16460 inclusive (accession number J02459.1). Primers were designed using NCBI Primer-BLAST and are shown in blue and green.*

An estimate of copy number was based on the molecular weight, yield and quantity of the template as provided by the synthesis company (biomers.net GmbH, Germany). The template was diluted first to a concentration of $1x10^{11}$ copies/µl then serial diluted in 10-fold dilutions to 10 copies/µl. Reactions were set up using the intercalating dye reagent GoTaq® QPCR Master Mix (2x) from Promega (part code A6001) with primers at a final concentration of 0.2µM in 10µl reactions. Four replicates were prepared at each template concentration plus four no template controls (NTC). The plate was sealed with a Pro adhesive seal and centrifuged for 1 minute at 1200rpm. The plate was then cycled for 45 cycles in the Prime Pro 48 followed by a melt to check the products amplified. The entire run took less than 50 minutes to complete. The results were analysed using the ProStudy software to determine the Cq values and standard curve.

Results

Figure 2 shows the baseline corrected amplification plot for the standard dilutions. The threshold line was placed so that Cq values were obtained for all samples. The results show that the highest concentration sample ($1x10^{11}$ copies per reaction) started to amplify almost immediately and replicates were very close in Cq value throughout until the sample was diluted to approximately 100 copies per reaction. At 10 copies per reaction, Cq was more variable and coincided with amplification in the NTC samples. Inspection of the melting curves for the NTC and 10 copy samples indicated that the amplification was generally due to non-specific amplification for three out of the four NTC replicates with the remaining sample showing a very slight contamination. In comparison, amplification of the 10 copy samples was a combination of some non-specific amplification and some true product (based on the T_m values of the melting peaks, data not shown). This type of behaviour at low copy numbers indicates the limit of

quantification (LOQ) for the assay in question, suggesting that it cannot be used to quantify less than 10 copies per reaction.

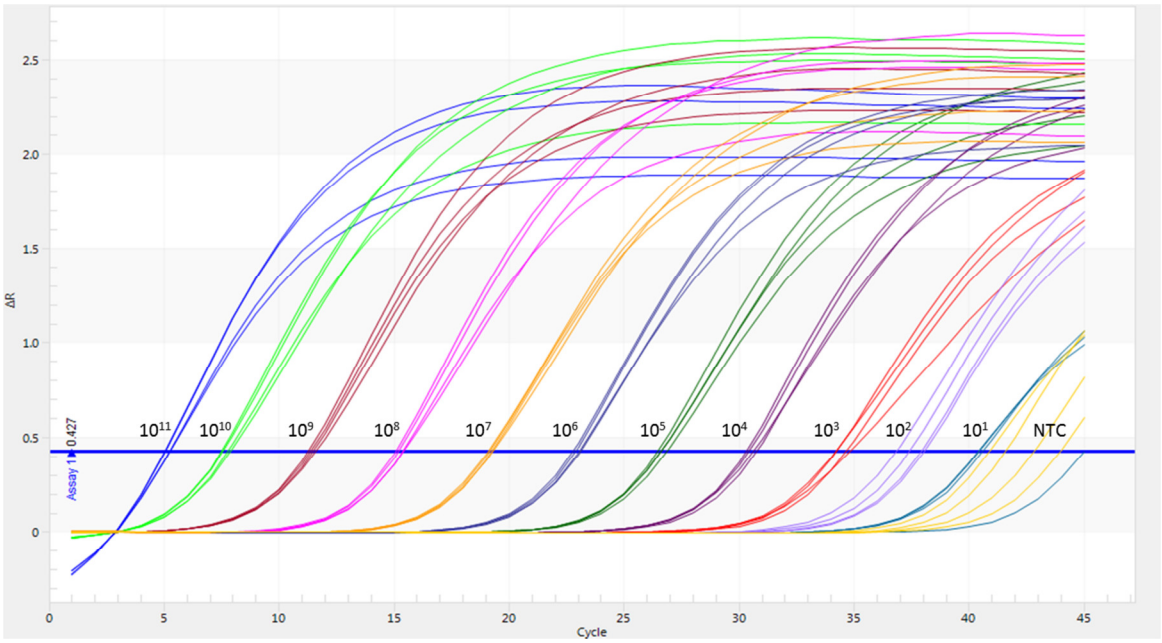

Figure 2: *Amplification curves for each of the eleven standards and NTC. Samples were amplified for 45 cycles (95°C, 2 min; 45 cycles of 95°C, 10s; 60°C, 30s) followed by a melt from 75°C to 95° (not shown). Fluorescence data was collected at the end of the 60°C step using the Green channel.*

To determine the linear dynamic range of the assay, the Cq values for each standard were plotted against the log of the concentration. Figure 3 shows the standard curve for the full range of samples.

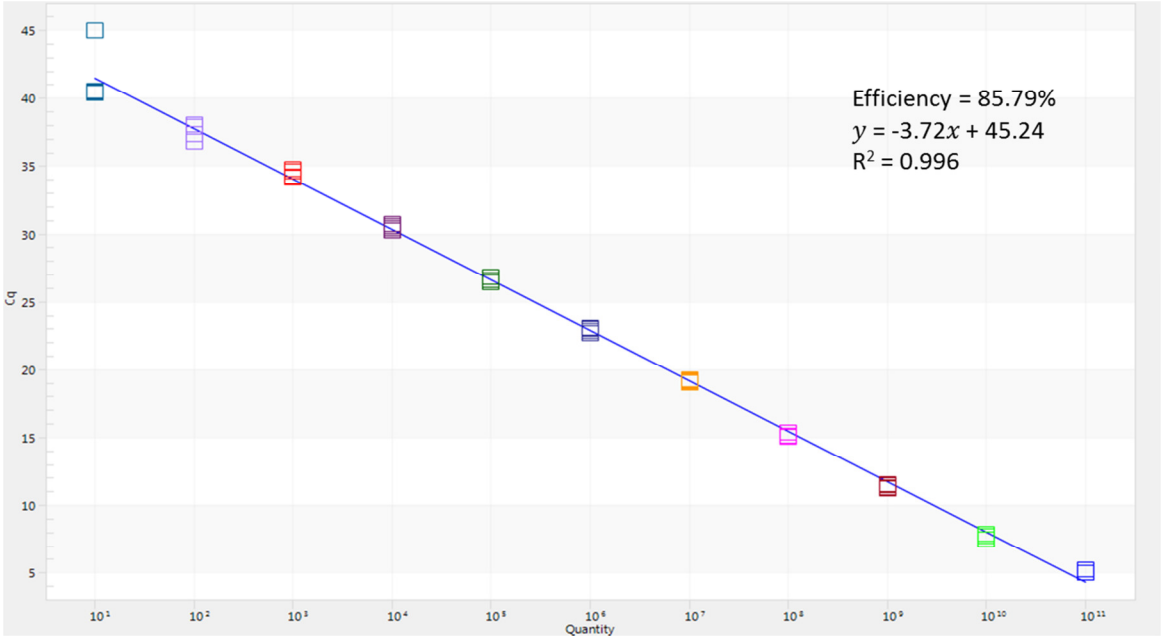

Figure 3: *Standard curve showing all 11 standard dilutions. The efficiency (E) of the reaction is calculated as* $E = 10^{(-1/slope)} - 1$.

Ideally a qPCR should have an efficiency of between 90 to 105%, which corresponds to a slope of between -3.58 and -3.21. The efficiency of the assay described is slightly low and could potentially be increased by improved primer design and assay optimisation; however the important point to note is that the standard curve is linear through a very wide concentration range.

The LOQ can be defined as the concentration at which the standard curve is no longer linear[(1)]. Considering the standard curve plot in Figure 3, the highest and lowest standards fall slightly off the slope. Therefore the upper and lower limits of the linear dynamic range in this particular assay can be defined as $1x10^{10}$ and $1x10^{2}$ copies respectively, or 9 orders of magnitude.

A low copy number sample which still gives a signal but is no longer in the linear range cannot be accurately quantified but can be determined as a positive reaction if it is within the limit of detection (LOD) of the assay. To place numerical values on the limitations, the LOD can be considered as 3 x SD of NTC replicates and the LOQ as 10 x SD of NTC replicates. For the assay described here, this equates to a value of 4.1 copies for the LOD and 13.5 copies for the LOQ and correlates with the observed results.

Conclusions

The main advantage of real-time PCR over conventional PCR methods is that real-time PCR allows accurate determination of starting template copy number over a very wide dynamic range. We have demonstrated here that the Techne Prime Pro 48 is able to detect a concentration range of greater than 10 orders of magnitude with a linear dynamic range of 9 log orders. Not only does this demonstrate the accurate thermal control of the block (within ±0.1°C) but also the unique system of fluorescence acquisition which allows the instrument to handle such a wide range of fluorescence. Adaptive LED Control (ALC) ensures that the detector is never saturated by reducing LED exposure to high emission wells and also increasing exposure to wells with very low fluorescence. This expands the linear range of detection and also ensures that wells with low levels of fluorescence are not affected by 'blooming' from adjacent wells which are highly fluorescent.

In conclusion, when selecting a new qPCR instrument, two of the most important factors to consider from a technical viewpoint are a high level of sensitivity and wide dynamic range, resulting in accurate quantitation. The Prime Pro 48 is demonstrated to be more than capable of accurate quantification over a very wide range of template concentration due to high thermal uniformity and ALC which minimises cross talk between adjacent wells.

References

(1) http://www.gene-quantification.com/miqe-webinar-short-transcript.pdf