The Effect of Amplicon Characteristics on the Success of Fast QPCR

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Introduction

Employing fast QPCR cycling protocols is a simple and effective way of maximising throughput by reducing run durations. Fast cycling protocols can easily be achieved by reducing the temperature step dwell times.

It has been reported[1] that the use of fast QPCR protocols may compromise assay sensitivity and precision. It is therefore important to realise that speeding up cycling protocols can be detrimental to the quality of results, but not in all cases. The factors which determine whether an assay can be successfully employed using fast cycling conditions are poorly understood.

The effect of three target amplicon characteristics on the success of fast QPCR was investigated using a panel of characterised assays. The amplicon properties focused on in particular were length, GC content and minimum ΔG at 60°C, the latter being a measure of the occurrence and stability of secondary structure at the annealing temperature.

Assay	Length (bp)	Min ΔG at 60°C	%GC
RAC	59	1.49	57.60
ACTB 60	60	-0.66	61.70
TBP	60	0.43	60.00
18s	68	-1	60.30
BSCL2	71	1.23	56.30
APOB	74	-2.54	55.40
UBC	76	-1.18	60.50
GUSB	80	-0.08	46.20
ABCF1	93	0.13	53.80
IGF2	96	0.13	53.80
ACTB 101	101	-0.18	57.40
ALB	104	0.8	44.20
PGK1	121	0.2	55.20
B2M	138	-0.53	47.80
ACTB 166	166	-2.32	56.60
GAPDH	226	-2.83	47.80
ALP	250	-3.79	56.00
HMBS	338	-0.17	52.70

Table 1: The eighteen hydrolysis probe assays studied and their amplicon characteristics. Values are given for amplicon length (bp), minimum ΔG at 60°C and %GC

Method

QPCR Assay Comparison

Eighteen hydrolysis probe assays (Table 1) were compared in QPCR experiments employing three 45-cycle protocols; Standard, Intermediate and Fast (see Table 2). ΔC_P values for the fast and intermediate protocols were calculated using the standard protocol mean C_P values as a baseline. ΔC_P was then used as a metric to assess changes in sensitivity. Cycling and detection were carried out in a 384-well format, on the Roche LightCycler® 480 Real-Time PCR Instrument.

Each 20 µL reaction contained 10 µL of 2x Thermo Scientific ABsolute™ Fast QPCR Mix (AB-4325)[2], 5 µL of oligonucleotide mix at 4x working concentration and 5 µL of cDNA template, equivalent to 25 ng of input total RNA per reaction. Five replicate reactions were produced for each assay and each protocol was repeated to ensure reproducibility of results.

Minimum ΔG Calculation

Amplicon minimum ΔG values were calculated using the DNA mfold web server (mfold version 3.2)[3]. The amplicon sequences were entered as linear, and the folding conditions were set at 60°C, 60 mM monovalent cations and 5.5 mM Mg^{2+}.

	Temperature (°C)	Step Durations		
		Standard	Intermediate	Fast
Enzyme Activation	95	5 minutes	5 minutes	5 minutes
Denaturation	95	15 seconds	1 second	1 second
Annealing/ Extension	60	60 seconds	40 seconds	20 seconds

Table 2: Cycling protocols employed in the comparison

Results

- In the majority of assays ΔC_P values increased (as demonstrated by $\Delta C_P > 0$ in Figure 1 a,b,c) when moving to an intermediate protocol and further increased when employing a fast protocol, indicating that faster cycling could reduce the sensitivity of an assay.
- As amplicon size increases there appeared to be a trend towards a greater ΔC_P (Figure 1a), indicating that assays with longer amplicons are more likely to suffer a higher loss in sensitivity than assays with shorter amplicons.
- As the minimum ΔG decreases (representing an increase in secondary structure) there appears to be a tendency toward greater ΔC_P (Figure 1b)
- No correlation was observed between ΔC_P and GC content (Figure 1c), possibly due to the narrow range used. Design of assays with GC content beyond the 40-70% optimal range is challenging due to parameter limitations of current assay design software.
- The weak correlations suggest that an assay's ability to produce robust results using fast protocols is multifactorial, and may also be affected in part by assay characteristics not included in this study. Data point distribution is incomplete, and requires improvement to provide a clearer relationship.

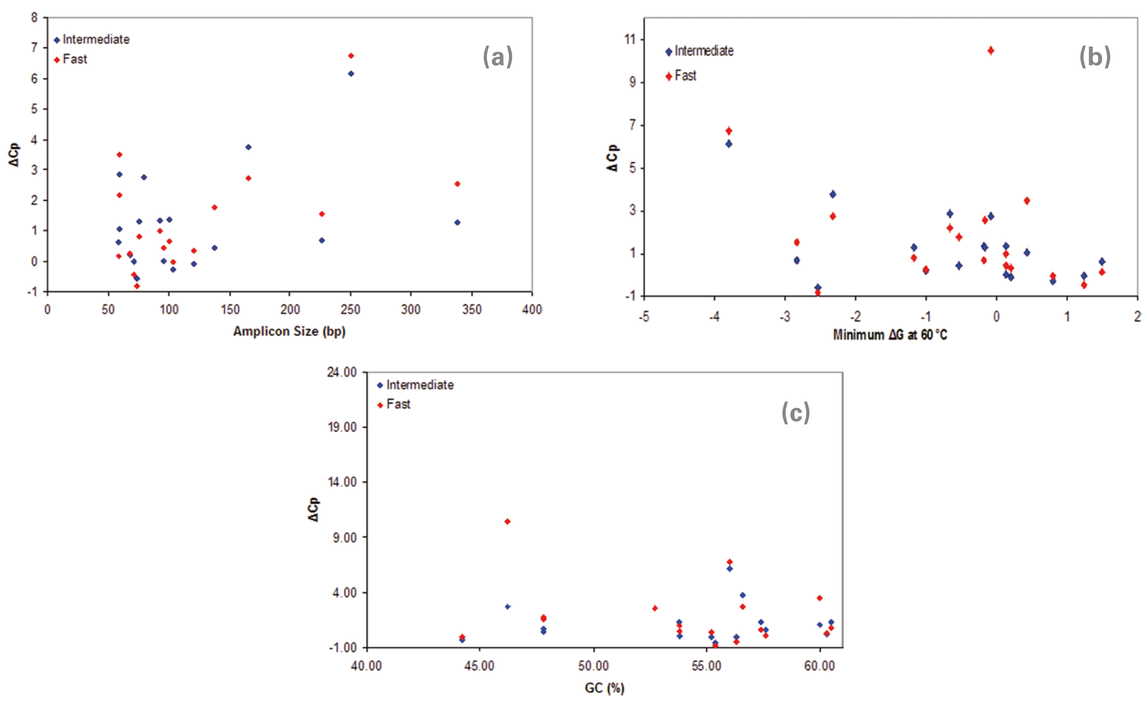

Figure 1: Correlation between ΔC_P and amplicon length (a) minimum ΔG at 60°C (b) and %GC (c) using fast (red) and intermediate (blue) cycling protocols of 45 cycles. Reactions contained cDNA equivalent to 25 ng of input RNA. ΔC_P values are mean values obtained from 2 runs of five replicates, and are determined in comparison to mean C_P values using the standard protocol.

Conclusion

- Amplicon length and severity of amplicon secondary structure (as measured by minimum ΔG) appear to be factors affecting the success of fast QPCR.
- The performance of an assay in a fast QPCR is likely to be dependant on a combination of assay-associated factors.
- Fast QPCR can be achieved with the majority of assays, but care should be taken when reducing the dwell times, so that the assay remains sensitive and the data robust.
- Further work will include a larger data set to include a wider distribution of %GC and a greater number of 150-350 bp amplicons.

References

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3. M. Zuker (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31 (13) 3406-15.

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