Quantification of siRNA by a novel competitive-qPCR method

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Abstract

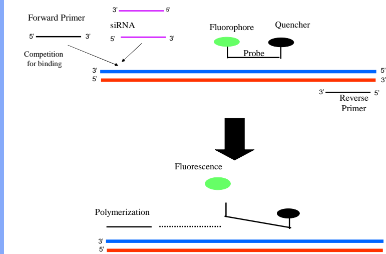
The utilization of siRNA for therapeutic purposes requires improved vectors, development of which necessitates accurate assessment of target cell uptake. Existing methods of quantification including fluorescence or radioactivity-based methods have limitations. Traditional quantitative PCR is not possible since at 19-25 nt siRNA molecules are too small to generate amplicons. We have therefore developed a competitive qPCR (cqPCR) method in which siRNA competes with a homologous forward primer to bind template DNA, giving siRNA concentration dependent inhibition.

Quantitative PCR primers and probe were design to amplify a 93 bp region of the human papillomavirus 16 E6 gene (HPV16 E6). Using genomic DNA extracted from HPV 16-positive CaSki cells we confirmed that siRNA targeting E6 mRNA could not itself function as a primer for polymerization. To maximize sensitivity of siRNA detection forward primer concentration was reduced to 6.25 nM and a 50°C annealing/extension phase temperature employed. Under these conditions target gene amplification remained efficient over 0.1-50ng of template DNA concentrations.

The addition of E6-siRNA to cqPCR led to inhibition of amplification in a linear concentration-dependent manner, with as little as 200pg of siRNA capable of being detected. Irrelevant siRNA had no effect on amplification confirming specificity.

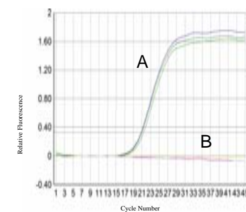
Having shown proof of principle in a cell free system attention is now focussed on detection of siRNA in cell lysate. Initial studies confirm that cqPCR remains functional in the presence of cell extracts. Detection of siRNA is currently limited by efficiency of extraction and degradation of siRNA over time within cells. Studies are ongoing to improve siRNA stability and extraction.

Quantitative siRNA assay design



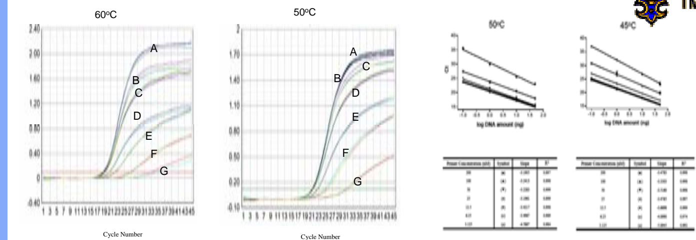
Conventional qPCR Taqman probes rely on the 5'-3' nuclease activity of Taq DNA polymerase to cleave a dual labelled probe during hybridization to the complementary target sequence. Our modified cqPCR method relies on the use of a primer competing with siRNA to bind to a complementary region of template DNA resulting in decreased PCR product and increased Ct value. For diagrammatic purposes template DNA strands are shown in opposite orientation to convention.

siRNA does not function as a primer



siRNA cannot mediate polymerization in the absence of forward primer. Amplification profile A from quantitative PCR using 100 ng template DNA extracted from CaSki cells, 200 nM forward and reverse primers and 100 nM probe. Profile B indicates reactions lacking forward primer but containing 200 nM E6-siRNA, which binds to the same region of DNA as the forward primer. Reactions were performed in triplicate.

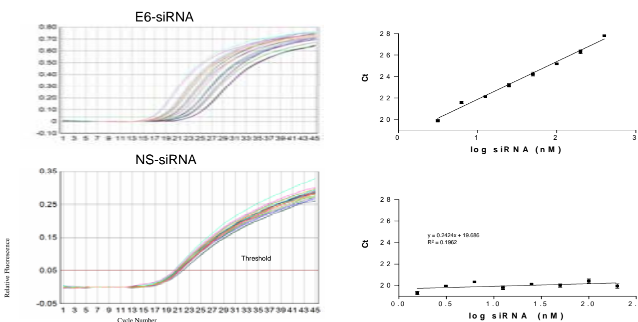
Optimization of cqPCR amplification



Effects of forward primer concentration and the annealing/extension phase temperature on Ct value. Reactions were performed using (A) 200 nM, (B) 100 nM, (C) 50 nM, (D) 25 nM, (E) 12.5nM, (F) 6.25 nM or (G) 3.125 nM forward primer, with 200 nM reverse primer and 100 nM probe in each case. Reactions were performed using a 60°C (left panel), 50°C (right panel) or 45°C (data not shown) annealing/extension phase.

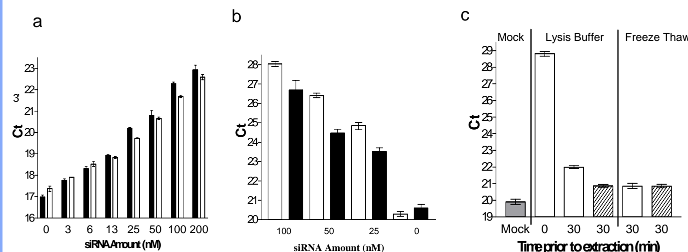
Effect of primer concentration on amplification efficiency. Reactions were performed using either a 50 ^{o}C or 45°C annealing/extension phase and forward primer concentrations ranging from 3-200 nM. The amount of template DNA ranged from 0.1-50 ng. The efficiency of amplification is indicated by the slope and degree of linearity illustrated by the R^2 value. All reactions were performed in triplicate.

Sensitivity and Specificity



The presence of siRNA inhibits amplification of template DNA in a sequence-specific and concentration dependent manner. Each reaction contains 45 ng template DNA, 6.25 nM forward primer, 200 nM reverse primer and 100 nM probe. Various amounts of E6 or non-specific (NS) siRNA ranging from 1.5-400 nM were added. Each reaction was performed in triplicate.

Measure siRNA in a cell-free and cell-based system



a) Addition of siRNA to cellular RNA extracts does not significantly affect its action as a competitor. siRNA at the indicated concentration was added directly to reactions (black bars) or in total RNA extracted from cell lysate using the mirVana kit (white bars). Each reaction contained 6.25 nM forward primer, 200 nM reverse primer and 100 nM probe. b) Comparison of siRNA spiked into cell lysate and extracted immediately with the mirVana kit (black bars) and siRNA added to RNA extracts (white bars). c) Comparison of siRNA (white bars) or siRNA/oligofectamine (hatched bars) added to cell lysate for 30 min prior to extraction.

Conclusion

1) siRNA does not act as a primer in qPCR amplification.

2) Forward primer concentration can be reduced to 6.25nM and still generate efficient amplification with a 50 ˚C annealing/extension phase.

3) siRNA is able to compete with the forward primer annealing to a homologous region on the target DNA template reducing the number of PCR products.

4) siRNA can compete with homologous primers in a sequence specific and concentration-dependent manner that permits siRNA quantification.

5) Although cell-free based data has proven cqPCR can successfully measure siRNA up to 200 pg level, cell-based assay fails to detect siRNA as efficiently as in either cell-free system or spiked buffer system. The reason may be the degradation of siRNA and the low efficiency of siRNA extraction.