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Quantification of siRNA using competitive qPCR

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ABSTRACT

We have developed a PCR-based short interfering RNA (siRNA) quantification method based on competition between siRNA and a homologous DNA primer for annealing to template DNA, avoiding the requirement for prior conversion of RNA to cDNA. Primers and probe were designed to amplify regions of the human papillomavirus E6 or enhanced green fluorescent protein genes. Having confirmed siRNA could not act as primer for amplicon generation, the lowest competing primer concentration yielding a linear relationship between template DNA amount (0.1–50 ng) and cycle of threshold (Ct) was determined (6.25 nM). Under these conditions addition of sequence-specific siRNA to the competitive quantitative PCR (cqPCR), resulted in a dose-dependent linear increase in Ct value. 2′-*O*-methyl ribose-modified siRNA retained an ability to inhibit template amplification in serum, unlike unmodified siRNAs that were susceptible to endonucleases. Mismatch-bearing or truncated siRNAs failed to inhibit template amplification confirming sequence specificity and an ability to discriminate between degraded and non-degraded siRNA sequences. Following delivery of E6 siRNA to C33-A cells using oligofectamine or His6 reducible polymers, siRNA uptake was quantified by cqPCR, revealing dose-dependent uptake. We anticipate that cqPCR will allow accurate determination of siRNA pharmacokinetics following *in vivo* delivery, greatly facilitating development of therapeutic siRNA delivery strategies.

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved process involved in physiological regulation and pathogen defence, in which double-stranded RNA induces sequence-specific gene silencing through chromatin remodelling, inhibition of protein translation or direct mRNA degradation (1–3). RNAi is facilitating our understanding of functional genomics and offers potential for therapy by targeting disease-associated genes (4,5). The simplest way to induce an RNAi response is to deliver short interfering RNA (siRNA) directly into target cells utilizing the endogenous silencing machinery of the microRNA (miRNA) pathway. In order to improve therapeutic siRNA pharmacokinetics, through chemical modifications or improved vector design, there is a requirement to obtain a detailed assessment of uptake into various tissues following *in vivo* delivery.

Methods of siRNA detection within cells, based on liquid hybridization with a ^{32}P-labelled probe followed by nuclease protection, are highly sensitive capable of detecting in the order of 10–100 attomol of siRNA (6). However, this approach is indirect and not all laboratories are set up to deal with radioactive methodologies. Fluorescence-based methods while useful for detection of siRNA uptake into cells are not amenable for quantification (7,8). Furthermore detection methods which rely on indirect labelling technology may be misleading due to differential rates of catabolism of label and siRNA. It is therefore desirable to develop alternative specific, sensitive non-radioactive quantification methods, ideally able to measure only intact functional siRNA.

Real time or quantitative PCR (qPCR) through cyclical amplification of template enables quantification of specific nucleic acid sequences with a high degree of precision. Sequence-specific Taqman probes carrying a fluorophore and quencher dye at each end are cleaved by the 5′- to 3′-exonuclease activity of Taq DNA polymerase during the annealing/extension phase of PCR, releasing a fluorescent signal proportional to the amount of accumulated PCR product (9). A variety of oligonucleotides can be quantified, however detection of siRNA by conventional qPCR is not feasible, since molecules commonly 19–25 nt in length are too small to anneal with primers and generate amplicons. Instead alternative PCR-based methods have been developed including primer extension (PE) (10), the invader assay (11), stem–loop RT-PCR (12), ligation assay (13) and crook hairpins (14) (Figure 1a). Stratford *et al.* (15) compared PE RT-PCR, ligation qPCR and stem–loop RT-PCR methodologies to detect

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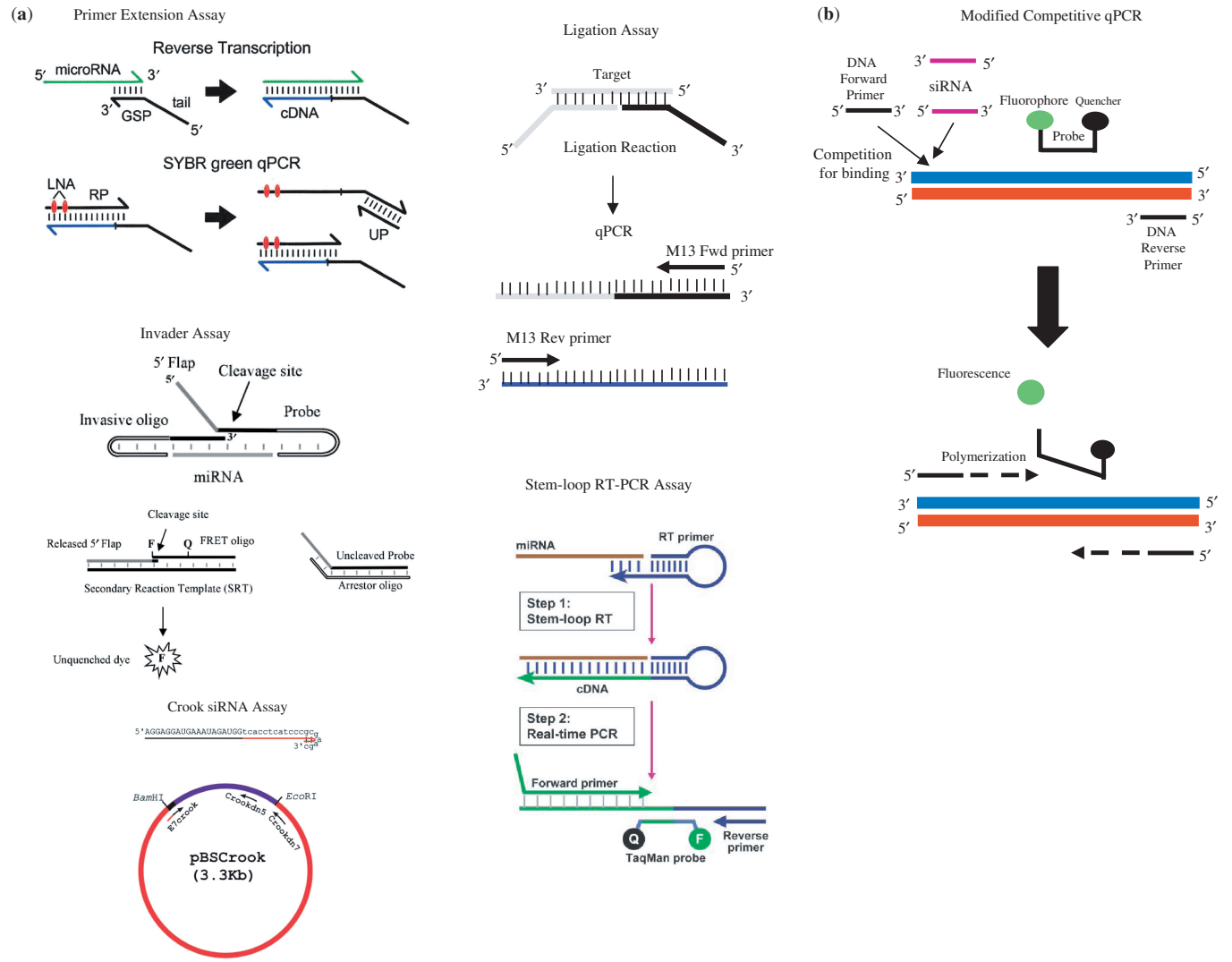


Figure 1. PCR-based methods of siRNA and miRNA quantification. (**a**) The PE assay relies on two reactions; the first involves a gene-specific primer to convert RNA to cDNA, introduces a universal PCR binding site to one end and extends the length of the cDNA. In the second reaction, cDNA is amplified when combined with a reverse primer containing locked nucleic acids (LNA) possessing a 2′-O,4′-C-methylene bridge to stabilize the sugar group and increase hybridization affinity of oligonucleotides and a generic universal primer common to all assays; amplification monitored by SYBR green fluorescence (10). The invader assay is dependent on the formation of a complex between a target nucleic acid hybridizing to both a probe and an invasive oligonucleotide in an overlapping manner. The probe then undergoes enzymatic cleavage by a structure-specific nuclease, cleavase to release the 5′-flap. In a second reaction the released flap hybridizes to a secondary reaction template which also hybridizes an overlapping FRET oligo, cleavage of which generates a fluorescent signal. An arrestor oligo added to the reaction sequesters any unreacted probe preventing binding to the secondary reaction template (11). Jiang *et al.* (14) utilized a bi-functional siRNA construct in which the 3′-end of the sense strand of siRNA was modified with a nuclease-resistant DNA hairpin. The 'crook siRNA' acts both as a primer for PCR amplification of an engineered DNA template whilst retaining silencing activity (14). The ligation assay uses ligation of 5′- and 3′-oligos containing sites for the M13 forward and reverse primers followed by qPCR amplification with M13 primers (13). The stem–loop RT-PCR assay of Chen *et al.* (12) employs a stem–loop primer binding to the 3′-end of the siRNA. Reverse transcriptase then extends the cDNA (12). Amplification occurs using a gene-specific forward primer, universal reverse primer and specific probe. Stratford *et al.* (15) adapted the assay by using a universal probe to reduce time and costs. (**b**) 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 the siRNA sense strand 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. Dotted arrow indicates the direction of polymerization which is inhibited in the presence of the competing siRNA molecule.

chemically modified siRNA in mouse plasma and tissue. They concluded that the ligation and PE RT-PCR methods required lengthy optimization and were poor at discriminating truncated (representing degraded) siRNAs, while the stem–loop RT-PCR had lower background and could effectively discriminate between full length and truncated sequences. Furthermore, they adapted the method of Chen *et al.* (12) which employed a sequence-specific probe, by using a universal Taqman probe, saving development time and costs. However, these methods rely on the use of two-stage reactions, with expensive and time-consuming reverse-transcription steps required to create

the cDNA for quantification. A more straightforward approach would be to quantify all unmodified siRNAs in a single existing qPCR reaction.

Competitive qPCR (cqPCR), like conventional qPCR, is used to estimate target gene concentration in a sample by comparison with standard curves constructed from amplification of serially diluted DNA standards. For cqPCR, an internal competitor DNA is added at known concentration to serially diluted standards and the unknown samples. Following co-amplification the ratio of internal competitor and target PCR products are calculated for both the standards and the unknown samples, and a standard curve generated plotting competitor–target PCR ratios against initial target DNA concentration of the standard dilutions (16–18). We wished to determine whether the cqPCR method could be modified, so that siRNA competes with the forward primer to anneal to a homologous region on target DNA (Figure 1b). In conventional qPCR, the primers and probe are present in excess, however in our modified cqPCR method the concentration of forward primer was carefully regulated to be at limiting concentration, so that competition by siRNA binding to the template would proportionally decrease the number of PCR products formed.

MATERIALS AND METHODS

Cell culture

CaSki human cervical epithelial carcinoma cells, containing approximately 600 copies of the human papillomavirus 16 (HPV16) genome, were maintained in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS), 10 mM HEPES, 50 U/ml penicillin and 50 μg/ml Streptomycin (Invitrogen). SiHa human cervical squamous cell carcinoma cells containing 1–2 integrated copies of the HPV16 genome were maintained in Dulbecco's modified eagle medium (DMEM) (PAA Laboratories GmbH, Yeovil, UK) supplemented with 10% FCS, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin. C-33A human cervical carcinoma cells lacking HPV DNA, were maintained in Minimal Essential Medium (MEM) (Eagle) (PAA Laboratories GmbH) supplemented with 10% FCS, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin. PC-3 metastatic human prostate carcinoma cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% FCS, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen).

Nucleic acids

The siRNAs targeting HPV16 E6 termed E6-siRNA-1 (sense: 5′-GAGCUGCAAACAACUAUACtt-3′, antisense: 5′-GUAUAGUUGUUUGCAGCUCtt-3′), E6-siRNA-2 (sense: 5′-GAGGUAUAUGACUUUGCUUtt-3′, antisense; 5′-AAGCAAAGUCAUAUACCACtt-3′), E6-siRNA-1-17mer (sense: 5′-UGCAAACAACUAUACtt-3′, antisense; 5′-GUAUAGUUGUUUGCAtt-3′), E6-siRNA-1-13mer (sense: 5′-AACAACUAUACtt-3′, antisense; 5′-GUAUAGUUGUUtt-3′), E6-siRNA-1-mismatch#1 (sense: 5′-GAGCU*AGC*AACAACUAUACtt-3′, antisense; 5′-GUAUAGUUGUU*GCU*AGCUCtt-3′), E6-siRNA-1-mismatch#2 (sense: 5′-GA*C*CUGCAAA*G*AACUAU*U*Ctt-3′, antisense; 5′-G*A*AUAGUU*C*UUUGCAG*G*UCtt-3′), mismatches shown in italics; enhanced green fluorescent protein (EGFP) (sense: 5′-GCGCUAUGACGGCAAUAAAtt-3′, antisense; 5′-UUUAUUGCCGUCAUAGCGCtt-3′) and firefly luciferase (GL-3) (sense: 5′-CUUACGCUGAGUACUUCGAtt-3′, antisense: 5′-UCGAAGUACUCAGCGUAAGtt-3′) were synthesized by Applied Biosystems (Warrington, UK). 2′-*O*-methyl chemically modified siRNA targeting HPV16 E6 termed 2′-*O*-Me-E6-siRNA-1 (sense: 5′-mGAmGCmUGmCAmAAmCAmACmUAmUAmCtt-3′, antisense; 5′-GmUAmUAmGUmUGmUUmUGmCAmGCmUCtt-3′) was also synthesized by Applied Biosystems.

Genomic DNA was extracted from CaSki cells using the QiaAMP DNA mini kit (Qiagen, Crawley, UK) in the absence of RNase according to the manufacturer's instructions. A commercially available plasmid, pEGFPC1 (Clontech, Whitchurch, UK) was grown in *Escherichia coli* HB101 competent bacteria and extracted using an EndoFree Plasmid Mega kit (Qiagen) in the absence of RNase according to the manufacturer's instructions.

qPCR and cqPCR

The qPCR was performed using various primer/probe sets. For assays involving E6-siRNA-1, E6-siRNA-2, E6-siRNA-1-17mer, E6-siRNA-1-13mer, E6-siRNA-1-mismatch#1, E6-siRNA-1-mismatch#2 and 2′-*O*-Me-E6-siRNA-1, the E6-forward primer: 5′-gagctgcaaacaactatactt-3′, E6-reverse primer: 5′-cccgaaaagcaaagtcatatacct-3′ and FAM-labelled probe: 5′-tcgcagtaactgttgcttgcagtacacacat-3′ were used (Sigma-Genesis, Wimborne, UK). For assays involving EGFP-siRNA, the forward primer: 5′-gcgctatgacggcaataaa-3′, reverse primer: 5′-aaccccgcgtttatgaaca-3′ and FAM-labelled probe: 5′-aataaaacgcacggtgttgggtc-3′ were used (Sigma-Genesis). In each instance the underlined residues in the primers represent thymidine residues, substituted for uracil residues in the corresponding competing siRNAs. Template DNA containing the HPV16 E6 ORF from CaSki cell extracts was amplified using the standard thermal cycling conditions; 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. Variations to the E6 standard reaction included lowering the annealing/extension phase temperature from 60°C to 50°C or 45°C. The reaction conditions were generated in an Applied Biosystems 7000 Sequence Detection System and subsequent analysis performed using ABI Prism 7000 SDS software.

The standard amplification reaction contained Platinum qPCR supermix (which includes 60 U/ml Taq DNA Polymerase, 40 mM Tris–HCl (pH 8.4), 6 mM $MgCl_2$, 400 μM dGTP, 400 μM dATP, 400 μM dCTP, 800 μM dUTP, 40 U/ml uracyl DNA glycosylase and 1 μM ROX passive reference dye) (Invitrogen), to which was added 200 nM forward primer, 200 nM reverse primer and 100 nM probe. The amount of template DNA from

CaSki cells varied from 1 pg to 100 ng, while 10 pg of pEGFP was used.

For development of cqPCR, the standard reaction was subsequently modified to contain between 3.125 nM and 200 nM forward primer, while the amount of E6-containing template DNA was varied from 0.1 ng to 50 ng. Unless otherwise stated competition assays were performed using 6.25 nM forward primer, 200 nM reverse primer and 100 nM probe; or 200 nM forward primer, 6.25 nM reverse primer and 100 nM probe. siRNA was added at concentrations ranging from 3.125 nM to 400 nM (208 pg to 26.6 ng) by dilution with nuclease free water. The temperature of the annealing/extension phase of the reaction was 50°C for E6-based assays and 55°C for EGFP.

Cell-based cqPCR assays

For studies in the presence of cell lysate, 3×10^6 C-33A cells were lysed with 300 µl of 20 mM Tris–HCl pH 8.5, 0.5% NP-40, 20 µg/ml tRNA buffer for 15 min at 80°C and centrifuged to remove debris. E6-siRNA was serially diluted in lysis buffer and spiked into the C-33A cell lysate (normalized for total protein), such that the siRNA concentration ranged from 3.125 nM to 200 nM. Five microlitres of lysate was then added to cqPCRs containing between 6.25 nM and 75 nM forward primer and 200 nM reverse primer. Reactions containing 50 nM forward primer and 400 nM reverse primer were also examined.

To quantify the uptake of E6-siRNA when complexed with Oligofectamine (Invitrogen) or His6 reducible polycations prepared as previously described (19,20), 10^6 C-33A cells were seeded into the wells of a 6-well plate 24 h prior to delivery. Three microlitres of Oligofectamine was added to 12 µl of Opti-MEM I medium (Gibco, Paisley, UK) in one vial and 3 µl of 20 µM E6-siRNA-1 added to 50 µl of Opti-MEM I medium in a second vial. The siRNA and Oligofectamine were then mixed and incubated for 20 min, prior to the addition of 32 µl of Opti-MEM I medium. His6 RPC/siRNA polyplexes were prepared at w/w 40 (N:P, 15:1). E6-siRNA complexes were then added to cells in wells containing 500 µl of serum-free culture medium to give a final siRNA concentration of 25 nM, 50 nM, 100 nM or 200 nM. As an additional control E6-siRNA-1/Oligofectamine complexes were added to wells lacking cells at a concentration of 25 nM or 200 nM. After 4 h the cells or 'no cell' wells were washed with PBS and lysis buffer added as described above. The siRNA was quantified by the addition of 5 µl of cell lysate to cqPCR and correlation with a standard curve generated by spiking mock-treated lysed cells with known amounts of siRNA. The assay was repeated three times using C33-A cells and once using PC-3 cells. Further controls were performed with E6-siRNA-1 complexed with Oligofectamine added directly to cqPCRs (containing 6.25 nM forward primer, 200 nM reverse primer and 100 nM probe) at 3.125–200 nM final concentration for comparison with uncomplexed siRNA.

EGFP transfection assay

SiHa cells were plated at 10^5 cells per well (24-well plate) 24 h prior to transfection. pEGFPC1 was mixed with siGFP or siLUC and added to DOTAP liposomal transfection reagent (Avanti Polar Lipids, Alabaster, AL, USA) at w/w 5:1 (DOTAP:nucleic acids) according to the manufacturer's instructions. Complexes were added to cells such that each well received 200 ng DNA and 10 nM siRNA. Control wells were transfected with pEGFPC1 alone or mock transfected. EGFP expression was analysed after 24 h by flow cytometry using a Becton Dickinson FACSCalibur and CellQuest Pro software.

RESULTS

siRNA does not function as a primer

We chose to explore the competitive assay system through amplification of the human papillomavirus 16 E6 (HPV16 E6) and EGFP genes. E6 is a viral oncogene that leads to p53-mediated degradation and is implicated in cervical neoplasia. We have previous designed siRNA to target this gene (E6-siRNA-1) for treating cervical carcinoma (Liu W.L. *et al*., submitted for publication). Template target DNA was obtained from the CaSki human cervical carcinoma cell line containing approximately 600 copies of the HPV 16 genome. For EGFP template DNA, we used the EGFP expression plasmid pEGFPC1 (Clontech).

qPCR primers and probe were designed to amplify a 93-bp region of the HPV16 E6 gene. The forward primer was designed to be in competition with the sense strand of an E6-targeting siRNA (E6-siRNA-1), to hybridize to a homologous region of the template DNA. The E6 forward primer contains three thymidine residues which are replaced by three uracil residues in the sense strand of the E6 siRNA. For siRNA to function as a competitor molecule, it is important it cannot itself perform as a primer for template polymerization. Amplification of the E6 gene from a CaSki cell genomic DNA template was compared using conventional qPCR containing excess forward primer (200 nM), with a reaction lacking forward primer but containing 200 nM E6-siRNA-1. As expected only reactions containing sequence-specific forward primer-generated PCR products with those containing siRNA fail to amplify the template after 45 cycles (Figure 2).

Optimization of cqPCR

Prior to performing cqPCR studies it was necessary to optimize, the amount of template DNA, the concentration of the forward primer and the temperature of the annealing/extension phase of the reaction. Using conventional qPCR containing excess primers and probe, there was a linear relationship between the amount of template DNA, ranging from 1 pg to 100 ng, and the threshold of cycle (Ct) value (Figure 3a). For competition studies, Ct values should be less than 25 to provide scope for an increase in Ct in the presence of competing siRNA. A Ct value of 25 related to 1 ng of template CaSki genomic DNA, while the use of 100 ng DNA generated a large

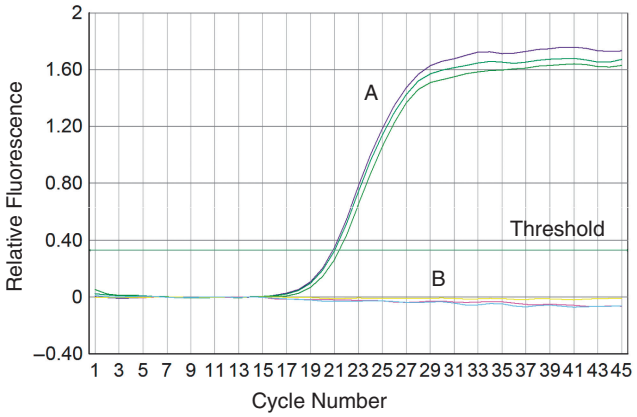


Figure 2. siRNA cannot mediate polymerization in the absence of forward primer. Amplification profile A from qPCR containing 100 ng template DNA extracted from CaSki cells, 200 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. Profile B indicates reactions prepared as in A except for the lack of forward primer, but containing 200 nM E6-siRNA-1, which binds to the same region of DNA as the forward primer. Reactions were performed in triplicate.

amount of PCR product, resulting in re-association of complementary product strands (21) manifested as a decrease in product towards the end of the reaction (Figure 3a). Consequently, the amount of template CaSki genomic DNA for competition studies should be >1 ng but <100 ng.

To maximize the changes in Ct value on addition of competing siRNA, it was necessary to determine the lowest concentration of forward primer permissive for efficient gene amplification. Reactions were tested containing between 3.125 nM and 200 nM forward primer, with reverse primer maintained at 200 nM in each case. At an annealing/extension temperature of 60°C all reactions generated PCR products, however the amount of PCR product fell as the concentration of the forward primer became limiting (Figure 3b, left panel). When the annealing/extension temperature was reduced to 50°C or 45°C, the amount of PCR products at low primer concentrations increased.

To determine that the modified reaction would still generate a linear relationship between the amount of CaSki genomic DNA template and Ct value, efficiency of amplification was further examined using a range of forward primer concentrations and between 0.1 ng and 50 ng template DNA (Figure 3c). A slope of −3.322 indicates 100% PCR efficiency, where the amount of PCR product doubles every cycle. At 50°C, amplification efficiency decreased sharply below 6.25 nM forward primer, while at 45°C, the amplification efficiency dropped below 12.5 nM. Based on these findings, for subsequent competition assays we employed a 50°C annealing/extension temperature, a 6.25 nM forward primer concentration and 45 ng of template CaSki genomic DNA.

siRNA competes with primers facilitating quantification

The effect of adding E6-siRNA-1 on E6 amplification was examined by cqPCR. Increasing siRNA concentration resulted in an increase in Ct value in a linear manner (Figure 4a) confirming inhibition of amplification. The lowest siRNA concentration tested was 3.125 nM corresponding to 208 pg. In contrast, a non-specific siRNA control with no homology to the HPV16 E6 forward primer, had no effect on E6 amplification (Figure 4b). We then designed and tested a second siRNA targeting HPV16 E6 mRNA (E6-siRNA-2), but this time with homology to the reverse primer. Various amounts of E6-siRNA-2 were added to reactions containing 6.25 nM reverse primer and 200 nM forward primer, again resulting in a proportional increase in Ct value with siRNA concentration (Figure 4c).

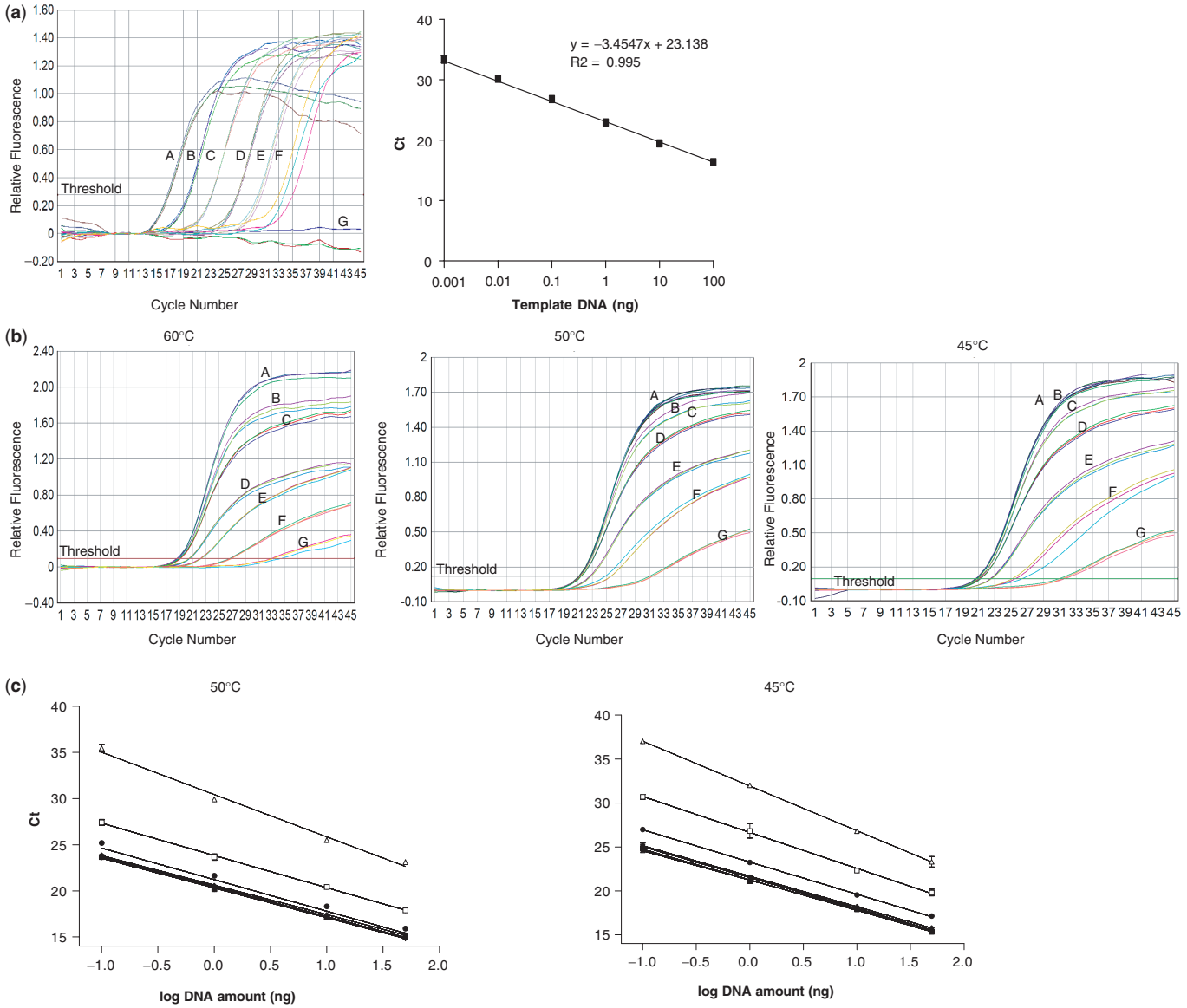
To confirm the widespread application of this technique, we designed a new set of primers and probe to amplify an 89-bp region of the EGFP gene. The plasmid nature of the template resulted in employing 4500-fold less DNA in the reaction, compared to the genomic DNA template. In order to generate a linear relationship between plasmid template amount and Ct value, the annealing temperature of the reaction was raised to 55°C, whilst the other conditions were maintained as described above. We then designed a siRNA with homology to the forward EGFP primer, obtaining 70% knockdown in EGFP expression following transient transfection of SiHa cells (data not shown). The addition of various amounts of EGFP-siRNA to the cqPCR reaction containing homologous forward primer, once again resulted in a linear relationship between Ct value and siRNA concentration (Figure 4d).

Since therapeutically active siRNA molecules require chemical modification to provide protection from serum endonucleases, we tested whether E6-siRNA-1 modified with the 2′-*O*-methyl ribose modification retained an ability to compete with a homologous primer. The data showed there was a linear relationship between chemically modified siRNA concentration and Ct value (Figure 4e). When the siRNA was mixed with 10% FCS prior to addition to cqPCRs, the unmodified siRNA failed to inhibit template amplification presumably due to degradation by serum endonucleases (Figure 4f). However, the siRNA carrying the 2′-*O*-methyl ribose modifications generated a linear increase in Ct value with siRNA dose, confirming protection from endonuclease digestion and an ability to be quantified in a clinically relevant biological sample.

To confirm specificity of the competition reaction, we tested truncated or mismatched siRNAs. Both a 13mer and 17mer E6-siRNA-1 (both truncated at the 5′-end) lost the ability to compete with the forward primer (Figure 4g), suggesting that the cqPCR technique can discriminate between intact and degraded siRNAs. Finally two E6-siRNAs, each possessing three mismatches with the forward primer, also failed to compete with the forward primer (Figure 4h) confirming the specificity of the cqPCR. These results confirm that siRNA can compete with homologous primers in a sequence-specific and concentration-dependent manner that permits siRNA quantification.

Quantification of siRNA from spiked cell lysate

Having shown that the cqPCR was functional in a cell-free system, we tested whether siRNA could be quantified in



Primer Concentration (nM)	Symbol	Slope	R^2
200	(■)	−3.1905	0.997
100	(▲)	−3.2413	0.998
50	(▼)	−3.2283	0.999
25	(◆)	−3.1961	0.999
12.5	(●)	−3.4217	0.998
6.25	(□)	−3.4967	0.989
3.125	(△)	−4.5867	0.984

Primer Concentration (nM)	Symbol	Slope	R^2
200	(■)	−3.4783	0.998
100	(▲)	−3.3303	0.998
50	(▼)	−3.5160	0.998
25	(◆)	−3.4765	0.997
12.5	(●)	−3.6698	0.999
6.25	(□)	−4.0990	0.974
3.125	(△)	−5.0945	0.992

Figure 3. Optimization of qPCR components prior to competition assays. (**a**) Linear relationship between template DNA amount and Ct value. Left panel illustrates amplification profiles from qPCR performed using (i) 100 ng, (ii) 10 ng, (iii) 1 ng, (iv) 0.1 ng, (v) 0.01 ng or (vi) 0.001 ng template CaSki cell genomic DNA; profile (G) indicates water blank. All reactions contained 200 nM E6 forward and reverse primers and 100 nM E6 probe. Right panel shows the linear relationship between the cycle at which the threshold was crossed (Ct) and the amount of template DNA. The slope of the line indicates the efficiency of the reaction. (**b**) Effect of forward primer concentration and the annealing/extension phase temperature on Ct value. Reactions were performed using (i) 200 nM, (ii) 100 nM, (iii) 50 nM, (iv) 25 nM, (v) 12.5 nM, (vi) 6.25 nM or (vii) 3.125 nM E6 forward primer, with 200 nM E6 reverse primer and 100 nM E6 probe in each case. Reactions were performed using a 60°C (left panel), 50°C (middle panel) or 45°C (right panel) annealing/extension phase. (**c**) Effect of primer concentration on amplification efficiency. Reactions were performed using either a 50°C or 45°C annealing/extension phase and E6 forward primer concentrations ranging from 3 nM to 200 nM. The amount of template DNA ranged from 0.1 ng to 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.

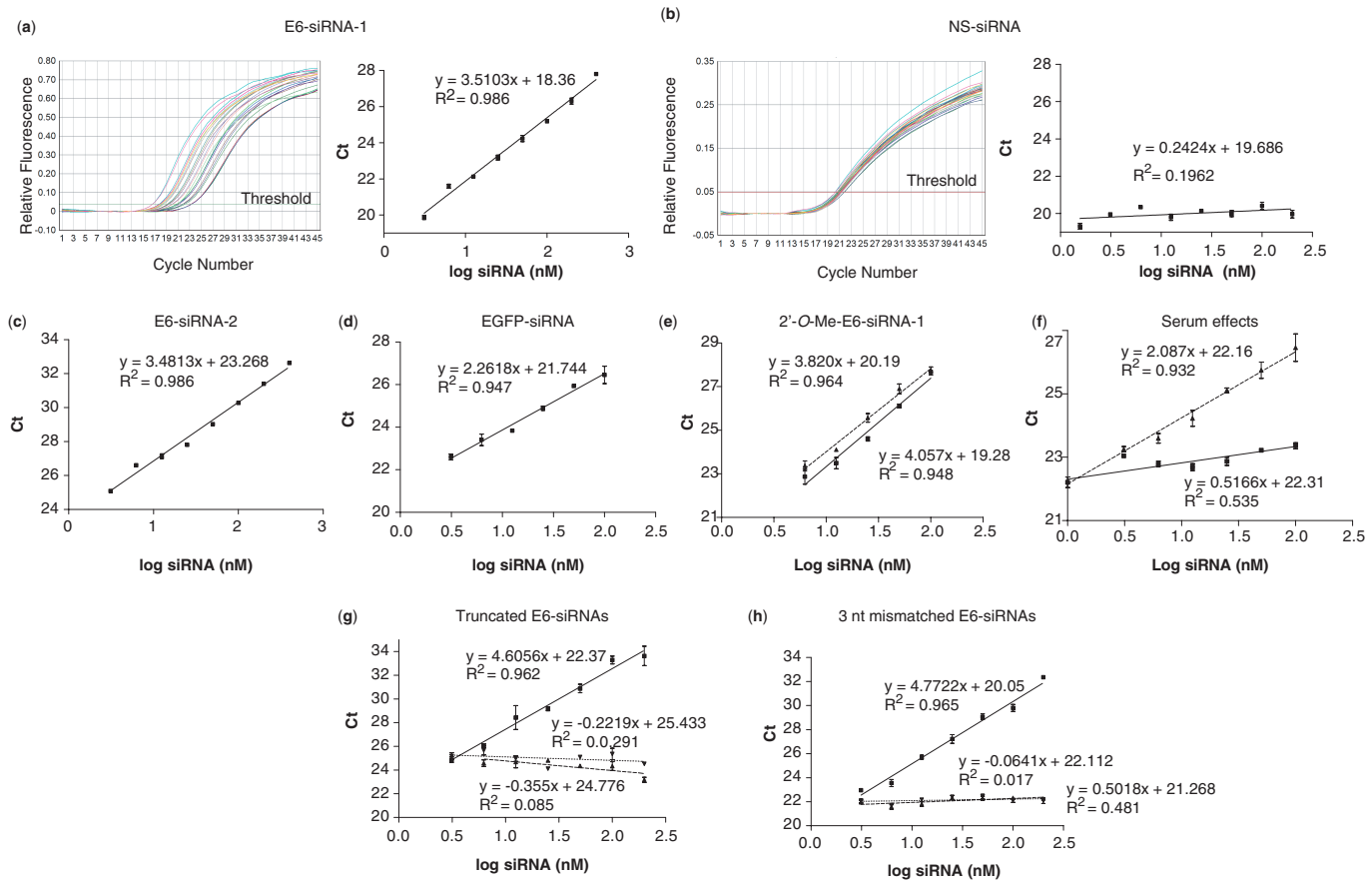


Figure 4. The presence of siRNA inhibits amplification of template DNA in a sequence-specific and concentration-dependent manner. Various amounts of (**a**) E6-siRNA-1 from 3.125 nM to 400 nM or (**b**) non-specific luciferase siRNA from 1.56 nM to 200 nM were added to cqPCR containing 6.25 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. (**c**) 3.125 nM–400 nM E6-siRNA-2 was added to cqPCR containing 200 nM E6 forward primer, 6.25 nM E6 reverse primer and 100 nM E6 probe. (**d**) 3.125 nM–100 nM EGFP-siRNA was added to cqPCR containing 6.25 nM EGFP forward primer, 200 nM EGFP reverse primer and 100 nM EGFP probe. (**e**) 3.125 nM–200 nM E6-siRNA-1 (square symbols, solid line) or 2′-O-methyl-E6-siRNA-1 (triangle symbols, dashed line) was added to cqPCR containing 6.25 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. (**f**) 3.125 nM–200 nM E6-siRNA-1 (square symbols, solid line) or 2′-O-methyl-E6-siRNA-1 (triangle symbols, dashed line) was added 10% FCS prior to addition to cqPCR containing 6.25 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. (**g**) 3.125 nM–200 nM E6-siRNA-1 (solid line), E6-siRNA-1-17mer (dashed line) or E6-siRNA-1-13mer (dotted line) were added to cqPCR containing 6.25 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. (**h**) 3.125 nM–200 nM E6-siRNA-1 (solid line), E6-siRNA-1-mismatch#1 (dashed line) or E6-siRNA-1-mismatch#2 (dotted line) were added to cqPCR containing 6.25 nM E6 forward primer, 200 nM E6 reverse primer and 100 nM E6 probe. All reactions were performed in triplicate.

C-33A cell lysate as a prelude to measuring levels of siRNA entry into mammalian cells. Known amounts of E6-siRNA-1 were spiked into cell lysate and added to a cqPCR reaction containing 6.25 nM forward primer concentration and 25 ng template. Template amplification was attenuated in the presence of cell lysate [Figure 5a, compare panels (i) and (iv)]. Raising the concentration of the forward primer from 6.25 nM to 75 nM resulted in an increase in template amplification (Figure 5a, panels iv–viii). Reactions containing 50 nM or 75 nM forward primer generated the greatest range of Ct values between the lowest and highest competing siRNA concentrations. Increasing the concentration of reverse primer from 200 nM to 400 nM, with 50 nM forward primer, resulted in an acceptable linear relationship between Ct value and log 10 siRNA concentrations [Figure 5a, panel (ix) and Figure 5b].

Quantification of siRNA from lysates of transfected cells

Finally, we delivered E6-siRNA to C-33A cells using either the commercially available lipid Oligofectamine, or a reducible polymer based on $CH_6K_3H_6C$ monomers (20). Following a 4 h incubation, the cells were washed and lysed, and the siRNA uptake quantified by cqPCR using a standard curve prepared by spiking cell lysate with known amounts of E6-siRNA. The experiment was performed on three separate occasions, the results shown in Figure 6 and Table 1 typical of one such experiment. For both Oligofectamine and His6 RPC the amount of siRNA detected inside cells increased with the dose administered (Figure 6 and Table 1). For Oligofectamine this ranged from 10% to 30% of input dose, while for His6 RPC it was 13–100% (Table 1), delivery being more efficient at the highest vector dose. All siRNA

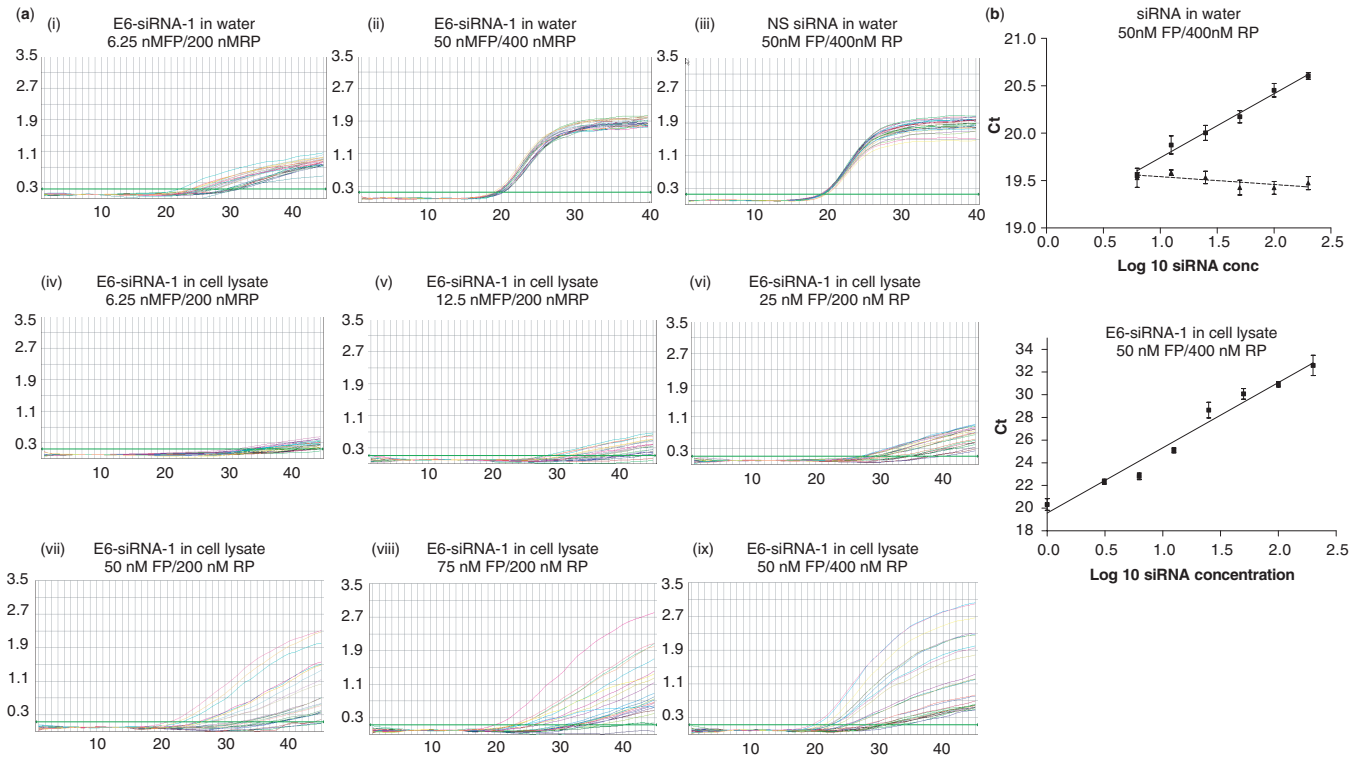


Figure 5. siRNA can inhibit template amplification in the presence of cell lysate. (**a**) siRNA was diluted in C-33A cell lysate or water and added at a final concentration of 3.125–200 nM to cqPCRs containing various concentrations of forward primer (FP) and reverse primer (RP) as indicated. In each case the *x*-axis represents number of cycles and the *y*-axis the relative level of fluorescence. (**b**) Linear relationship between Ct value and log 10 of E6-siRNA-1 concentration for reactions containing 50 nM forward primer/400 nM reverse primer in the absence (top graph) or presence (bottom graph) of cell lysate. Square symbols and solid lines, E6-siRNA-1; triangle symbols and dashed line, luciferase siRNA.

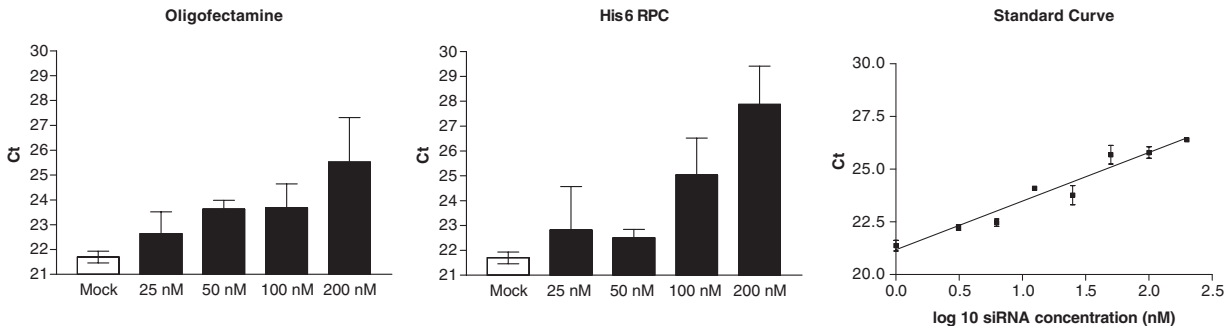


Figure 6. Quantification of E6-siRNA uptake into C-33A cells when complexed with Oligofectamine or His6 reducible polycations. E6-siRNA complexes were added to cells at 25 nM, 50 nM, 100 nM or 200 nM final concentration in triplicate as indicated. Using a standard curve generated by spiking cell lysate with known amounts of E6-siRNA and then added to cqPCR, the amount of siRNA taken up by the cells was determined. The amount of siRNA in complex with Oligofectamine that was delivered to cells was assessed in three independent experiments, the results of one such experiment is shown here.

detected were specifically associated with cells and not merely stuck to the plastic, since blank wells without cells which were spiked with 25 nM or 200 nM E6-siRNA-1 in complex with Oligofectamine-generated Ct values that were not significantly different from cqPCRs lacking siRNA (data not shown). The ability of siRNA to inhibit DNA template amplification was not affected by complexation with Oligofectamine *per se*, since the addition of E6-siRNA-1 alone or in complex to cqPCRs in the absence of cell lysate generated virtually the same slope and R^2 values (data not shown). Delivery of E6 siRNA using Oligofectamine to C33-A cells was performed on three separate occasions and once to PC-3 cells (data not shown). The results shown in Figure 6 and Table 1 are illustrative of one experiment involving C33-A cells, however in each study there was a dose-dependent increase in the amount of internalized siRNA confirming the reproducibility of the assay. The level of variability in Ct value (approximately three Ct units) appeared greater at the highest (200 nM) siRNA concentration, with lower concentrations generating more reproducible results.

Table 1. Quantification of E6-siRNA uptake into C-33A cells when complexed with Oligofectamine or His6 reducible polycations

Vector	siRNA concentration added (nM) (amount in pmol)	Mean Ct value (SD)	log 10 siRNA concentration	siRNA concentration detected (nM) in cells (amount in pmol)
Oligofectamine	25 (15)	22.64 (1.52)	0.831	6.8 (4.1)
Oligofectamine	50 (30)	23.63 (0.61)	0.985	9.7 (5.8)
Oligofectamine	100 (60)	23.68 (1.66)	0.994	9.9 (5.9)
Oligofectamine	200 (120)	25.52 (3.10)	1.667	46.5 (27.9)
His6 RPC	25 (15)	22.82 (3.03)	0.862	7.3 (4.4)
His6 RPC	50 (30)	22.51 (0.59)	0.805	6.4 (3.8)
His6 RPC	100 (60)	25.03 (2.6)	1.597	39.5 (23.7)
His6 RPC	200 (120)	27.87 (2.7)	>2.301	>200 (>120)

E6-siRNA complexes were added to cells at 25 nM, 50 nM, 100 nM or 200 nM final concentration (amount in pmol shown in brackets) in triplicate as indicated. The amount of siRNA taken up by the cells shown in Figure 6 was determined by comparing the mean Ct value [standard deviation (SD) shown in brackets] with a standard curve generated by spiking cell lysate with known amounts of E6-siRNA added to cqPCR.

DISCUSSION

The ability to accurately determine siRNA pharmacokinetics following *in vivo* delivery is highly desirable for the development of therapeutic siRNA vectors. Indeed, the interpretation of any siRNA silencing assay would be facilitated by quantification of uptake. In this manner it would be possible to study tissue distribution in order to evaluate tissue targeting strategies and siRNA stability. Our novel cqPCR method based on siRNA directly competing with a homologous DNA primer at limited concentration, and thus interfering with template DNA amplification, results in a linear relationship between siRNA concentration and Ct value.

In order for the cqPCR method to function it was important to demonstrate that the heat denatured siRNA strands cannot function as primers for DNA template amplification. Shibata *et al.* (22) showed that the ability of RNA to act as a primer in PCR is dependent upon the DNA polymerase employed. They reported Sequenase v. 2.0 DNA polymerase (Amersham) and r*Tth* DNA polymerase (Perkin-Elmer Cetus) mediated chain elongation from an RNA primer, whereas Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) did not. Stump *et al.* (23) showed that the ability of 2′-*O*-Me RNA oligonucleotides to prime DNA synthesis using *Taq* polymerase was dependent on the inclusion of a minimum of six DNA residues at the 3′-end. Therefore, our results demonstrating lack of template amplification from a siRNA primer using *Taq* polymerase are in agreement with these observations.

To enable siRNA quantification it was necessary that the competing DNA primer concentration, present in excess in conventional qPCR, was reduced. However, the primer concentration must be sufficient to maintain amplification efficiency. Reduction of the forward primer to a concentration of 6.25 nM, using a 50°C annealing/extension phase, maintained a linear relationship between DNA template amount and Ct value ($R^2 = 0.989$), with a PCR efficiency of 93.18% [$\%\,\text{eff} = (10^{-1/-3.4967}) - 1 \times 100\%$]. Following addition of varying amounts of E6-siRNA-1 to reactions containing 45 ng template DNA, we obtained an acceptable relationship between Ct value and siRNA concentration (E6-siRNA-1, R^2 value = 0.986). The robustness of the cqPCR assay was confirmed by using different siRNAs to compete with different primers (E6-siRNA-2, R^2 value = 0.986; EGFP-siRNA, R^2 value = 0.947). The standard cqPCR conditions (including the forward and reverse primer and probe concentrations), between the three siRNAs tested in the two template systems (E6 and EGFP) were the same, requiring only a change in temperature for the annealing/extension phase of the reaction based on the melting temperature of the components involved, and the amount of template DNA employed, dependent on whether plasmid or genomic DNA was used. As a result the amount of optimization required between different siRNAs is minimal. The sequence-specific nature of the assay was confirmed using irrelevant siRNA targeting luciferase or E6 targeting siRNAs bearing three mismatches, since on each occasion the Ct value was unaffected by the presence of the siRNA.

We demonstrated that both sense (E6-siRNA-1) and antisense (E6-siRNA-2) siRNA strands can be detected. It is not clear whether association of siRNA with RNAi-induced silencing complex (RISC) prevents binding to template DNA during cqPCR. If so, although not examined here, it could be possible by designing suitable primers and probes to determine the relevant amount of sense and antisense strands; differences reflecting differential loading of strands into the RISC, thus allowing evaluation of RNAi kinetics.

Chemical modifications to the siRNA are necessary for protection of nuclease degradation present in the serum during *in vivo* delivery. The commonly employed 2′-*O*-methyl ribose modification, when applied to the E6-siRNA-1, was compatible with quantification by the cqPCR technique. Furthermore unlike the unmodified siRNA, the chemically stable siRNA retained an ability to inhibit template amplification in the presence of serum in a linear manner.

We attempted to determine the amount of siRNA inside cells initially by extracting the siRNA along with other small RNA species. However, the extraction yield in our hands proved highly variable and was inefficient at low input doses (data not shown). We therefore attempted to detect siRNA directly in cell lysate using a simple cell lysis solution previously employed for the Invader assay (11). The qPCR assay, irrespective of siRNA content, required modification consisting of increased forward and reverse

primer concentrations to maintain efficient template amplification. This was due to the components of the cell lysate and not the invader assay buffer itself, since lysis buffer alone did not affect the Ct values obtained (data not shown). Cellular proteins binding to the primers and probe may be accountable for this effect. However, despite this, the ability of siRNA to inhibit amplification in a sequence-specific manner was maintained. It is possible that some loss of assay sensitivity occurs in the presence of cell lysate due to enzymatic or chemical modification of the siRNA or the presence of RNA-binding proteins. However, the lowest concentration of siRNA (E6-siRNA-1) spiked into lysate that we tested was 3.125 nM, (equivalent to ~200 pg/μl or 16 fmol/μl) which resulted in a Ct value of 22.34; a value clearly discriminated from background (Ct = 20.32). Due to the dilution factor created by adding the siRNA to the reaction this equates to a 625 pM spike-in signal, which is comparable to the 200 pM spike-in signal reported to be detectable by the PE-PCR assay (10). Soutschek *et al.* (4) reported that chemically modified siRNA reduced apolipoprotein B (apoB) expression in liver and jejunum, resulting in a 68% decrease in the plasma level of apoB protein and reduced total cholesterol. Administration of 50 mg/kg siRNA by tail vein resulted in the accumulation of 100–200 ng/g tissue in liver and jejunum. Assuming a liver weight of 1.5 g, the lysis and re-suspension of tissue at ~1 g/ml would enable siRNA detection using our cqPCR approach. Therefore, the cqPCR assay could be employed to monitor therapeutically meaningful siRNA concentrations.

siRNA therapeutics are now being commercially and clinically developed for a host of targets. However, assays for siRNA measurement (e.g. for pharmacokinetic studies) are usually reliant on labelled versions (e.g. radioactive siRNA tracers) which are rapidly degraded. The ability of the cqPCR to discriminate between truncated siRNAs suggests that the assay will allow detection of intact (and therefore functional molecules) and not partially degraded siRNAs, a problem for label-based assays. Our cqPCR method has advantages over existing PCR-based methodologies in that the reaction is performed in a single step without the need for expensive and time-consuming conversion of RNA into cDNA prior to quantification. Although a standard curve is required adding a few extra samples per run, the overall cost of the procedure is reduced compared to two-stage qPCR-based methods since there is no requirement for reverse transcriptase. However, the exact time and cost benefit of the cqPCR assay will vary depending upon the number of samples being tested, with larger sample numbers generating greater savings.

The cqPCR assay has been tested here using synthesized siRNA molecules, however we envisage that the technique could be adapted to analyse siRNAs generated from shRNA expression vectors. Furthermore, since the reaction involves heat denaturation and therefore avoidance of problems associated with secondary structure, it should be possible to quantify miRNAs in a similar manner to that achieved by the PCR-based assays, such as the PE-PCR assay (10) and stem–loop RT-PCR (12).

In summary cqPCR enables; accurate measurement of small amounts of siRNA in complex biological samples, a simple, one-step procedure, an ability to measure only intact siRNA thus avoiding degradation artefacts, and is suitable for high throughput, using conventional qPCR hardware and software (e.g. 96 or 384 well plate-based). In addition, cqPCR avoids the minimum size constraints for conventional qPCR template amplification by utilizing competition for primer binding as the quantified component, rather than the number of template copies *per se*. This is a new way of amplifying a signal by PCR, and will help to fill a gap in the repertoire of methods available for understanding siRNA biology with regard to quantification.

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