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Notes & Tips

# Quantitation of full-size small interfering RNA by tailing with terminal deoxynucleotidyl transferase and reverse transcription-polymerase chain reaction analysis

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ABSTRACT

Accurate estimation of small interfering RNA (siRNA) concentration in cells and blood is increasingly important for pharmacokinetic studies required to develop siRNA drugs. We report a method that detects siRNA having 3′-terminal deoxynucleotide overhangs, such as 3′-dTdT, present in most chemically synthesized siRNAs. Short overhangs were elongated to oligo-dG by incubation with terminal deoxynucleotidyl transferase and dGTP and were used as priming sites for reverse transcription of siRNA to complementary DNA (cDNA). The resultant cDNA was used as a template for quantitation by polymerase chain reaction. This method was reliable for determining the pharmacokinetics of siRNA in blood of injected mice.



RNA interference (RNAi)[1] is a sequence-specific posttranscriptional gene silencing event that permits degradation of specific messenger RNAs (mRNAs); thus, small interfering RNA (siRNA) consisting of 21- to 25-mer double-stranded RNA has been used broadly as a powerful tool for cellular gene knockout experiments [1]. With this highly target-specific RNAi function, siRNA has also been considered as a candidate for therapeutic drugs. siRNA promises several advantages over traditional drugs, offering new types of medicines that have strict target selectivity and are effective at low doses (nanomolar or subnanomolar concentrations) with limited toxicity [2–4].

Development of siRNA drugs, however, has been hampered by several issues, including poor intracellular uptake due to an intrinsic negatively charged structure having a relatively high molecular weight (~ 13 kDa) and a limited stability in circulating blood. The absence of a reliable method of monitoring small amounts of siRNA is another issue that makes pharmacokinetics and RNA metabolism studies difficult to pursue in preclinical studies with experimental animals and clinical trials with humans.

Radioactive siRNA or fluorescent compound-tagged siRNAs are not appropriate to administer to volunteers in a clinical study; thus, development of siRNA of natural form or of minor modification is anticipated as the first generation of siRNA drugs. However, such siRNA of natural form does not have a specific label to be used for monitoring siRNA that exists at very low concentrations in circulating blood or in organs. Ultraviolet light absorption by siRNA is insufficient for monitoring with conventional column chromatographic procedures.

Several methods to determine microRNA (miRNA) by amplification with polymerase chain reaction (PCR) after converting the sequence to complementary DNAs (cDNAs) have been described [5–8]. All of these methods primarily require prior 3′ tailing such as by addition of polyA (by incubation with polyA polymerase and ATP) and by ligation of oligoA (by RNA ligase). These methods can be used to detect siRNA. However, 3′ tailing with these methods not only occurs in siRNA (or miRNA) but also is in competition with other cellular RNA species that have a 3′ free hydroxyl ribose end and are abundant in cytoplasm of cells. Cellular RNAs, including transfer RNAs (tRNAs), 5SRNA, ribosomal RNA, mRNA, and their degradation products, can also be nonspecifically 3′-tailed and may disturb downstream reactions required for accurate quantitation of full-size siRNA drugs.

In this article, we describe a method that can detect siRNA molecules that have 3′-terminal deoxynucleotide overhangs. Most chemically synthesized siRNAs published so far have 3′-terminal overhangs consisting of deoxyribodinucleoside monophosphate such as 3′-dTdT. For example, RecQL1–siRNA induces mitotic cell death in a wide range of cancer cells (by silencing RecQL1–DNA helicase participating in DNA repair during the cell cycle), and this RecQL1–siRNA contains 3′-dTdT overhangs [9,10]. Taking advantage of this 3′-terminal deoxyribonucleotide residue that distinguishes siRNA from other RNA species and ensures 3′-terminal

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[1] *Abbreviations used:* RNAi, RNA interference; mRNA, messenger RNA; siRNA, small interfering RNA; miRNA, microRNA; PCR, polymerase chain reaction; cDNA, complementary DNA; tRNA, transfer RNA; TDTase, terminal deoxynucleotidyl transferase; RT-PCR, reverse transcription-polymerase chain reaction; M-MLV, Moloney murine leukemia virus.

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integrity of siRNA, we developed a new method that uses unique 3′-oligo-dG tailing by terminal deoxynucleotidyl transferase (TDTase, EC 2.7.7.31, TaKaRa Bio, Shiga, Japan). The method converts dG-tailed siRNA to cDNA, which is then used as the template for quantitative PCR so that the original copy number of siRNA is calculated accurately.

Fig. 1 shows the overall steps of this method that may be referred to as TDTase/reverse transcription-PCR (RT-PCR) analysis. The method consists of three steps: (i) 3′-dG tailing by TDTase, (ii) conversion to cDNA, and (iii) quantitative PCR with specific primers. The dG-tailed siRNA is reverse-transcribed to cDNA by Moloney murine leukemia virus (M-MLV) reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) using synthetic deoxyoligonucleotide anchor primer (36-mer) consisting of 5′-GGCCACGCGTCGACTAGTA (called the universal amplification primer (19-mer) because it contains a unique sequence of 19 bases in the human genomic sequence [11]) and 3′ proximal oligo-dC (17-mer) as a primer. The resulting cDNA is used as the template for quantitative PCR that uses the universal amplification primer (19-mer) and a deoxyoligonucleotide antisense primer (19-mer) that contains an identical 5′ proximal sequence to the antisense strand as PCR primers. Because the method includes the use of both 5′ and 3′ terminal sequences of siRNA as essential tags, it permits quantitation of only full-size siRNA, which we believe is important for strict pharmacokinetic studies.

To show the feasibility of this method, the pharmacokinetics of RecQL1 siRNA formulated with cationic liposome LIC-101 [12,13] and injected intravenously into mice was measured by detecting siRNA in circulating blood and organs. All siRNAs used in the experiment consisted of 19-bp RNA duplexes and had two overhanging 3′-dTdT at the 3′ termini. RecQL1–siRNA contained 5′-GUUCAGACCACUUCAGCUUdTdT-3′ (antisense strand corresponding to the 273–291 position in RecQL1 mRNA) and 5′-AAGCUGAAGUGGUCUGAACdTdT-3′ (sense strand). As the internal control of this feasibility study, GL3–siRNA, representing the sequence of part of the firefly luciferase gene (GL3, Promega, Madison, WI, USA), was used. GL3–siRNA contained sequences 5′-CUUACGCUGAGUACUUCGAdTdT-3′ (antisense strand) and 5′-UCGAAGUACUCAGCGUAAGdTdT-3′ (sense strand). When GL3–siRNA in increasing amounts was analyzed by using the TDTase/ RT-PCR method, a standard curve of perfect linearity was obtained (Fig. 2B), suggesting that the method can detect siRNA molecules in a range of 5 to 500 fmol. The standard curve was identical for siRNAs having different internal sequences such as RecQL1–siRNA (data not shown).

In in vivo experiments with the RecQL1–siRNA/LIC-101 complex, ICR mice ($n = 3$) were injected intravenously with 50 μg of RecQL1–siRNA/LIC-101 into the tail vein. Mouse blood (15 μl) was taken from the tail vein at 30 and 60 min and 3, 6, 12, and 24 h after administration, the blood was diluted 10-fold by mixing with 135 μl of phosphate-buffered saline containing 50 mM ethylenediaminetetraacetate, and the siRNA was extracted by using an miRNA Isolation Kit (Favorgen, Taiwan).

The siRNA sample was first 3′-dG-tailed by incubation at 37 °C for 30 min with 1 mM dGTP and TDTase. This process permits the specific addition of oligo-dG of approximately 10 bases to the deoxynucleotidyl 3′ terminus of siRNA. As an alternative to 3′-dG tailing, poly-dC tailing was tested by a reaction with TDTase and dCTP, but the results of siRNA quantitation were not as consistent as those with poly-dG tailing (data not shown). The 3′ overhang structure seems to facilitate 3′-dG tailing by TDTase.

After 3′-dG tailing, part of the reaction mixture was diluted with water and mixed with anchor primer (36-mer). The mixture was denatured by heat at 70 °C for 10 min to disrupt the duplex structure of dG-tailed siRNA and then was cooled on ice to let the dG-tailed single-stranded siRNA anneal with the oligo-dC containing anchor primer (36-mer) (Fig. 1). A mixture of 1 mM dNTP (where N was A, C, G, or T) and reverse transcriptase was added to the mixture, which was incubated for 1 h at 42 °C to produce the cDNA copy of full-size siRNA.

To determine the amount of siRNA, quantitative PCR was done with the cDNA template by using antisense primer (19-mer) having the antisense sequence of siRNA but lacking 3′-dTdT and universal amplification primer (19-mer) as PCR primers. The amount of siRNA was estimated based on the standard curve made with GL3–siRNA by using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). These three steps (i–iii above) can be completed in less than 3 h, and multiple samples can be processed simultaneously.

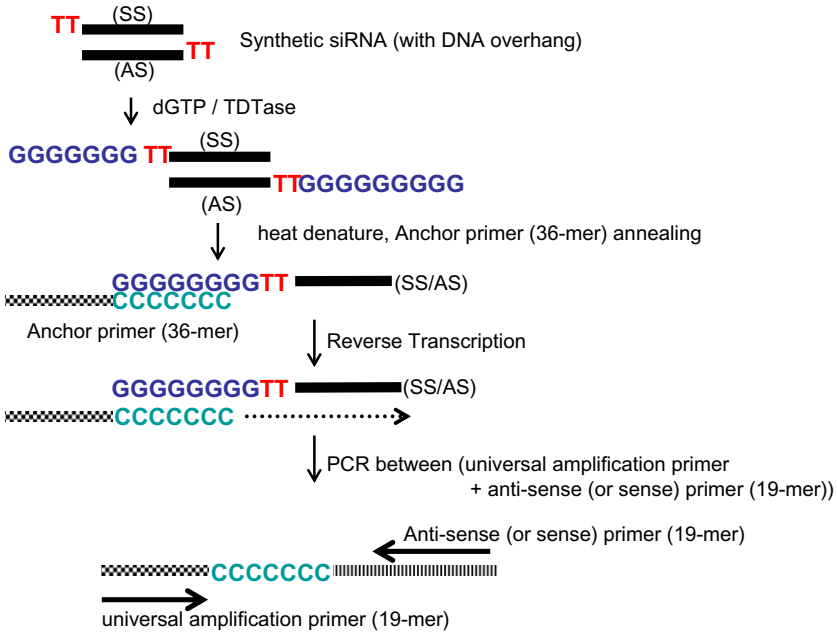


**Fig. 1.** Schematic representation of the TDTase/ RT-PCR method that permits quantitation of full-size siRNA by tailing with TDTase and RT-PCR analysis. Quantitative analysis of siRNA is estimated from the amplification curve using SYBR Green PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

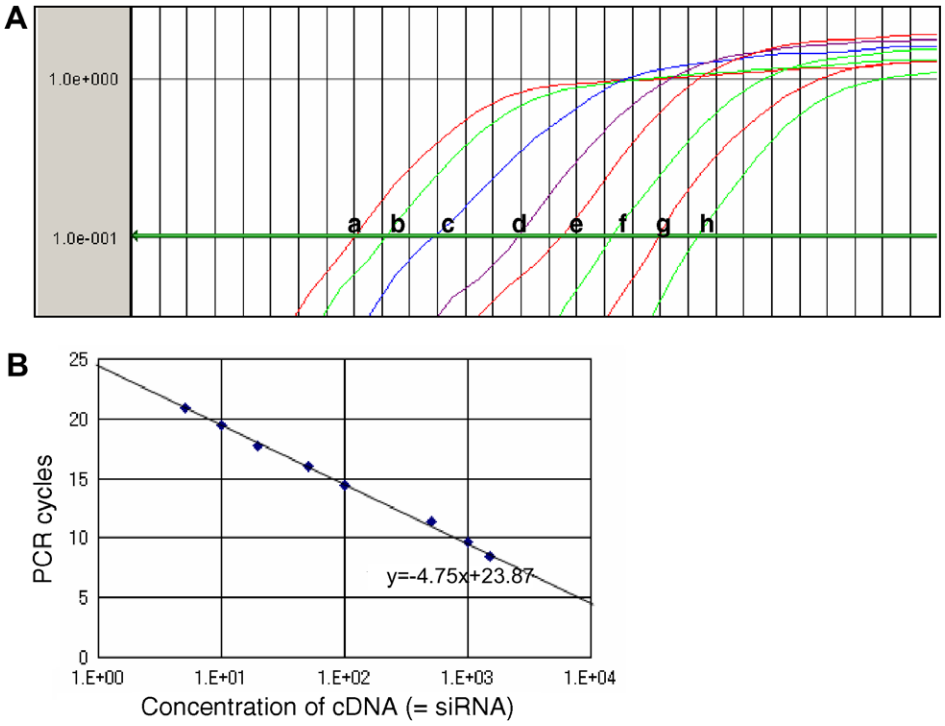


**Fig. 2.** (A) Amplification curves measuring various amounts of siRNA that had been processed through steps of the TDTase/ RT-PCR method: (a) 1.5 pmol; (b) 1.0 pmol; (c) 0.5 pmol; (d) 0.1 pmol; (e) 50 fmol (0.05 pmol); (f) 20 fmol; (g) 10 fmol; (h) 5 fmol. (B) Standard curve representing the amounts of siRNA.

As expected from the reaction based on the deoxyribonucleotidyl terminus and DNA-specific TDTase as well as from in vitro cell biological experiments using siRNA extracted from transfected cells, the TDTase/ RT-PCR method estimated siRNA accurately and reproducibly without interference from the presence of various types of cellular RNAs. The method helped our study to determine the efficiency of siRNA transfection to cells and to investigate the fate of full-size siRNA in transfected cells. The data showed that approximately 20% of siRNA was incorporated into cells when the cells were treated with 40 nM siRNA overnight at 37 °C [9]. From the in vivo experiments, RecQL1–siRNA/LIC-101 complex injected into mice was delivered preferentially to the liver; approximately 80% of the complex disappeared from the circulating blood at 30 min after administration, but it was detected by using the TDTase/ RT-PCR method for extracted RNA from the liver [10].

From our siRNA drug development viewpoint, the TDTase/RT-PCR method placed emphasis on quantitating synthetic siRNA having deoxynucleotides at the 3′ termini and siRNA molecules containing full-size structures after extraction from specimens in preclinical or clinical studies. However, the method can also be used to understand various fundamental problems of RNAi, for example, how and where the transfected siRNA molecules in cells are metabolized. We believe that this method will facilitate pharmacokinetic studies on siRNA and drug delivery studies, thereby expediting the development of siRNA medicines.

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