TRANSGENOMIC

Optimized Purification of siRNA Oligonucleotides Using the WAVE® Oligo System

Synthetic siRNA oligonucleotides represent powerful tools for RNAi-mediated gene silencing studies. Highly pure siRNA oligonucleotides can result in increased delivery efficiencies and reduce the risk of toxic effects from contaminants, thereby improving the success rates and reproducibility of gene silencing experiments. Here we describe an optimized siRNA oligonucleotide purification protocol using the OligoSep™ Prep HC Cartridge and WAVE Optimized® HA Buffers specifically developed for oligonucleotide purification applications on the WAVE Oligo System.

Introduction

RNA interference, or RNAi, is a post transcriptional gene silencing mechanism present in plants and animals (1). In nature, the pathway is activated by double-stranded RNA (dsRNA) in a two-step process. In the first step, dsRNAs are cleaved by a ribonuclease III enzyme called Dicer into 21-23 bp small interfering RNAs (siRNAs). In the second step, the siRNAs are incorporated into the RNA-induced silencing complex (RISC), which cleaves RNAs complementary to the siRNAs. Elbashir et al. (2) demonstrated that the RNAi pathway can be activated directly in cells using synthetic siRNAs, thereby bypassing the Dicer step, and also bypassing the non-specific dsRNA-dependent interferon pathway present in mammalian cells (3). Elbashir et al. further demonstrated that synthetic siRNAs could readily be designed to inhibit the expression of endogenous genes. Such synthetic siRNAs have proven to be powerful tools for silencing genes in vertebrates, and are now widely used to interrogate gene function (4, 5).

Use of high quality synthetic siRNAs is critical for accomplishing successful gene silencing experiments. siRNA preparations containing impurities carried over from synthesis can lead to inefficient delivery, reduced potencies and undesirable side effects or toxicity. Application of highly pure siRNA reagents can increase confidence that results obtained from gene silencing experiments are due to the biology of the target gene, and not due to non-specific effects from impurities inadvertently introduced into the experiment.

Here we report an optimized protocol for purifying siRNA oligonucleotides using the WAVE Oligo System and the OligoSep Prep HC Cartridge. In this protocol, n-hexylammonium is used as the ion-pairing agent in the WAVE Optimized HA Buffers specifically developed for this application. This ion-pairing agent transforms the nonpolar surface of the OligoSep Prep HC Cartridge into a dynamic anion exchanger where oligomers are separated primarily by length, with a minimal contribution from base composition. n-Hexylammonium also has the advantage of being volatile, and thus is more readily removed from the purified RNA relative to other counter-ions used in reverse-phase chromatography. In addition, the organic solvents described in the procedure reduce the risk of RNase contamination during purification, and also avoid the use of corrosive salts normally used in ion exchange chromatography, thereby reducing instrument wear and maintenance.

Use of the WAVE Oligo System and the WAVE Optimized HA Buffers described here represents a powerful and automated technique for separating full-length siRNA oligonucleotides from synthesis failures and other byproducts typically present in crude oligonucleotide preparations. This "RNA friendly" protocol exhibits high resolution, short run times and simple post-column recovery of RNA fragments, and therefore should be readily applicable to automated, high throughput purification of siRNA and other synthetic oligonucleotides.

APPLICATION NOTE 120

BIOSYSTEMS

Materials and Methods

Oligonucleotide Synthesis

Sequences of the Lamin A/C and firefly luciferase siRNA oligonucleotides used in this study were published previously (2) and are shown in Table 1.

The oligonucleotides were synthesized at a 1 µmol scale on an ABI 394 synthesizer (Applied Biosystems, Inc., Foster City, CA) using standard TBDMS/phosphoramidite chemistry on the Transgenomic T lcaa CPG support. The oligonucleotides were cleaved from the solid support and the exocyclic amino protecting groups were removed by incubation in 1 ml of 30% ammonium hydroxide/ethanol (3:1) for 5 h at 55°C. The solvents were then evaporated to dryness and the silyl protecting groups on the ribose sugars were removed by incubation in 0.75 ml triethylamine/trihydrofluoride for 1 h at 60°C. Crude material was neutralized with 1.8 ml of 3 M potassium hydroxide.

siRNA Oligonucleotide Purification

Full-length product was purified from approximately 30 ODs of crude material on the WAVE Oligo System using the protocol described in Table 2.

The peak containing the full-length product was collected in approximately 1 ml of eluent using the Transgenomic FCW 180 Fragment Collector operated in the absorbance threshold mode.

Post-column Preparation

Purified oligonucleotides were dried, resuspended in 0.5 ml of 1 M sodium chloride and applied to Oligo-MP Cartridges pre-conditioned with 5 ml of 100% acetonitrile, 5 ml of RNase free water, and finally with 5 ml of 1 M sodium chloride. The columns were washed with 20 ml of RNase-free water. Purified oligonucleotides were eluted from the columns in 2 ml of 50% acetonitrile and dried.

Table 1

Sequences of siRNA Oligonucleotides

Strand	Lamin A/C	Luciferase
Sense Strand	CUGGACUUCCAGAAGAACAdTdT	CGUACGCGGAAUACUUCGAdTdT
Antisense Strand	UGUUCUUCUGGAAGUCCAGdTdT	UCGAAGUAUUCCGCGUACGdTdT

Table 2

Purification Gradient Method

Step	Time (min)	Percent WAVE Optimized HA Buffer A	Percent WAVE Optimized HA Buffer B
Loading	0	80	20
Start Gradient	0.5	70	30
Stop Gradient	16.5	40	60
Start Clean	16.6	0	100
Stop Clean	17.6	0	100
Start Equilibrate	17.7	80	20
Stop Equilibrate	17.8	80	20

Column: OligoSep Prep HC Cartridge, 7.8 x 50 mm
WAVE Optimized HA Buffer A: 100 mM n-Hexylammonium Acetate/10% Acetonitrile
WAVE Optimized HA Buffer B: 100 mM n-Hexylammonium Acetate/50% Acetonitrile
Flow rate: 1.2 ml/min
Temperature: 80°C
Sample Loop: 500 µl
Sample Injection: 100 µl
Dead Time: 2.7 min
Detection: UV at 300 nm in preparative mode, 260 nm in analytical mode

Table 3

Percent Full-Length Material Following Purification on the WAVE Oligo System

siRNA Oligonucleotide	Percent Full-Length Crude	Percent Full-Length Purified
Lamin A/C Sense	36	>95
Lamin A/C Antisense	50	>95
Luciferase Sense	36	>95
Luciferase Antisense	48	>95

Results

Four siRNA oligonucleotides were synthesized using standard phosphoramidite chemistry. The fraction of full-length material in the crude preparations ranged from 36-50% as determined by ion exchange HPLC (see Figure 1 for an example chromatogram, Table 3 for data summary).

Full-length oligonucleotide was then purified on the WAVE Oligo System using the OligoSep Prep HC Cartridge and the WAVE Optimized HA Buffers as described in the *Materials and Methods* section. Figure 2 shows an example chromatogram of crude material analyzed with this optimized method. Note that the separation protocol using the reverse-phase ion-pairing method described here resulted in good separation of full-length material from synthesis failures, in particular the n-1 species that represents failure during the last synthesis cycle and typically is the most difficult species to remove during purification. This enhanced separation of full-length product from synthesis failures allowed for efficient capture of the full-length material (Figure 3).

Further, the retention times of the full-length oligonucleotides were about 13 minutes (Figure 2), with overall turn-around times between injections of less than 20 minutes (Table 2). Since all of the siRNAs purified here were of the same length, and the n-hexylammonium ion-pairing agent separates primarily based on length rather than base composition, the siRNAs eluted within a 45 second time window. This narrow time window simplifies automation issues related to fragment collection. Overall, full-length material was enriched to greater than 95% for all four siRNA oligonucleotides (Table 3).

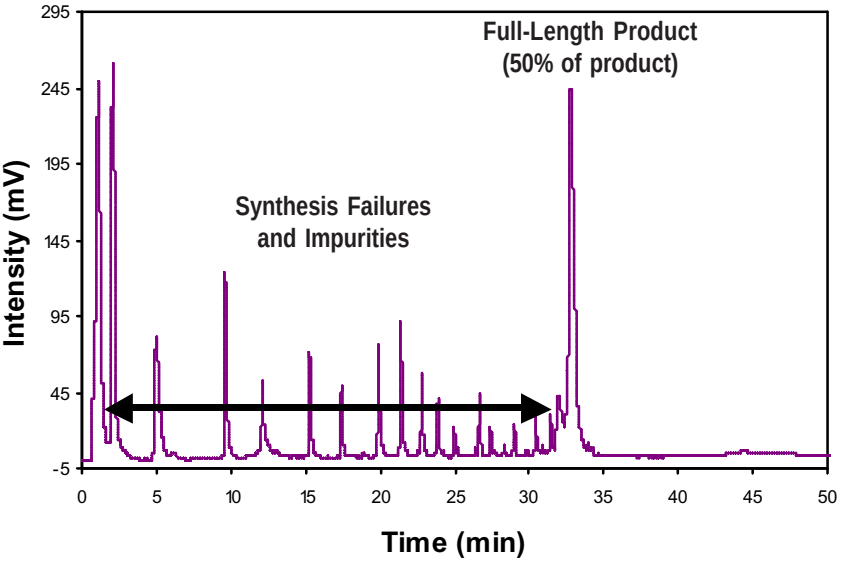

Figure 1. Ion exchange HPLC analysis of crude siRNA oligonucleotide prior to purification (siRNA oligonucleotide: Luciferase Antisense).

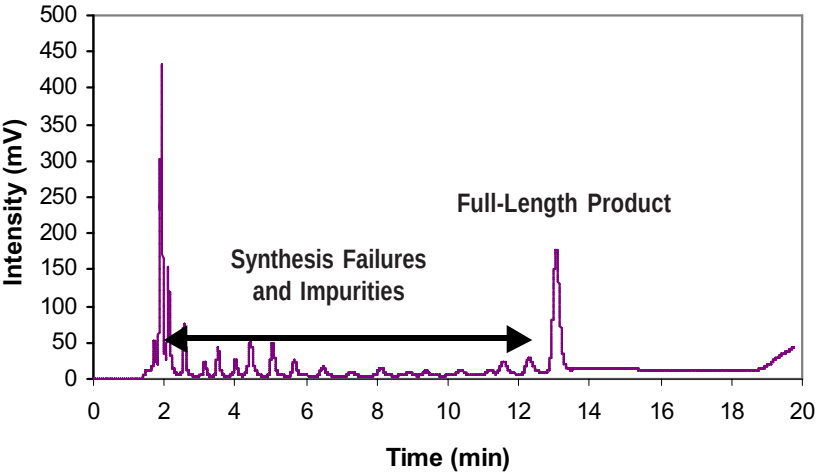

Figure 2. Example chromatogram using the optimized siRNA reverse-phase denaturing HPLC purification protocol described here for the WAVE Oligo System (siRNA oligonucleotide: Luciferase Antisense).

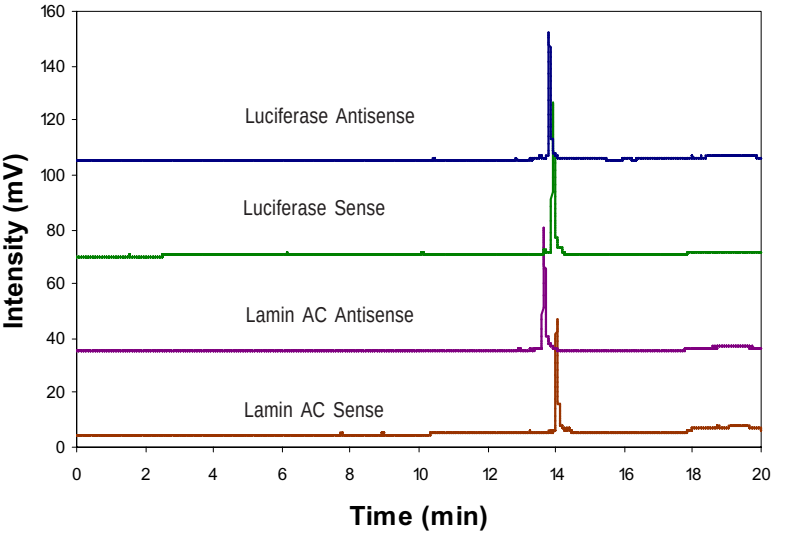

Figure 3. Analytical ion exchange HPLC analyses of siRNA oligonucleotides following purification on the WAVE Oligo System.

Discussion

Impurities in siRNA oligonucleotide preparations can lead to lower potencies of the reagents, reduced delivery efficiency and increased risk of toxic effects. Furthermore, variability in the levels of such impurities has the potential to impact the reproducibility of gene silencing experiments. Use of the optimized siRNA purification method described here on the WAVE Oligo System resulted in oligonucleotide preparations consisting of greater than 95% full-length product when starting with crude material comprising 36-50% full-length product. Following purification on the WAVE Oligo System, oligonucleotides can be prepared in less than 24 hours either for shipping or for direct application in gene silencing experiments using the simple Oligo-MP Cartridge method described in the *Materials and Methods* section.

Denaturing HPLC on the WAVE Oligo System offers several advantages over ion exchange chromatography to purify RNA oligonucleotides like siRNAs. The organic solvents used in the reverse-phase protocol described here have a lower risk of contamination, thereby reducing the chances of introducing RNases into the instrument. Furthermore, any RNases inadvertently introduced into the system during a single purification run are removed readily from the instrument during the column cleaning step, thereby ensuring the integrity of siRNA oligonucleotides purified in subsequent injections, and avoiding long down-times associated with instrument cleaning. The method described here is automated, rapid and reliable, and thus can increase the efficiency and throughput of siRNA production. Use of the "RNA friendly" siRNA purification protocol described here should improve the success rates of siRNA-mediated gene silencing experiments.

References

1. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806-811.

2. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411:494-498.

3. Jacobs, B.L. and Langland, J.O. (1996). When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology* 219:339-349.

4. Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002). Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26:199-213.

5. McManus, M.T. and Sharp, P.A. (2002). Gene silencing in mammals by small interfering RNAs. *Nature Reviews Genetics* 3: 737-747.

Application Note Product List

Product	Catalog Number
WAVE Oligo System	WAV-99-0004A
Oligo Sep Prep HC Cartridge	NUC-99-3860
Oligo-MP Cartridge (10 pack)	21-8560-86
Oligo-MP Cartridge (100 pack)	21-8560-90
WAVE Optimized HA Buffer A	553415
WAVE Optimized HA Buffer B	553416
T Icaa CPG Support	19-7640-00

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