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Application Note 121

Purification of Duplex siRNAs Directly from Crude, Annealed Oligonucleotides

We have developed a method to purify duplex siRNAs from a mixture of crude single-stranded oligonucleotides annealed directly in the deprotection milieu following synthesis. Purification is accomplished under non-denaturing conditions using the WAVE Oligo System, the OligoSep™ Prep HC Cartridge and WAVE OptimizedHA Buffers. This optimized method eliminates the need to purify and characterize sense and antisense siRNA oligonucleotides prior to annealing. Furthermore, since intact siRNA duplexes are directly purified, this method reduces uncertainty associated with the annealing efficiencies of different siRNAs. Purifying duplex siRNAs directly from crude synthesis products can more than double the throughput and productivity of siRNA purification. The high quality siRNA duplexes generated can improve the success rate and reproducibility of gene silencing experiments.

Introduction

Synthetic siRNAs have proven to be powerful tools for silencing genes in a number of species and are now widely used to interrogate gene function (1,2). Typical synthetic siRNAs are 19 bp double-stranded (duplex) RNA molecules with 2-nt 3' overhangs that mimic naturally occurring degradation products of the ribonuclease III-type endonuclease, Dicer (3). siRNAs activate the RNA interference (RNAi) gene silencing pathway present in plants and animals via the RNA-induced silencing complex (RISC), which cleaves RNAs complementary to the siRNAs (3,4).

Typically, synthetic siRNAs are produced by generating the individual sense and antisense strands, either by chemical synthesis or by *in vitro* transcription. The individual oligonucleotides are purified and characterized independently, and then annealed to generate the active duplex siRNA. Separate purification of sense and antisense siRNA oligonucleotides is not only time consuming and laborious, but also requires that each strand be carefully characterized prior to the annealing step to ensure that equimolar amounts are combined. The extra labor involved contributes significantly to the cost of obtaining purified siRNA duplexes from commercial vendors compared to merely purchasing unpurified siRNA oligonucleotides. Furthermore, annealing efficiencies can vary between different siRNA molecules, and this introduces uncertainty regarding the quality, and thus the potency, of the resulting siRNA duplexes.

Here we report a method to purify duplex siRNAs directly from annealed, crude sense and antisense siRNA oligonucleotides. Separation is accomplished by *non-denaturing*, ion-pairing, reverse-phase HPLC using the WAVE Oligo System, the OligoSep Prep HC Cartridge and WAVE Optimized HA Buffers. Previously, we described the use of WAVE Optimized HA Buffers to purify single-stranded oligonucleotides under fully-denaturing conditions (*Application Note 120*, reference 5).

The ability to purify siRNA duplexes efficiently from single strands and synthesis failures not only eliminates the need to pre-purify individual sense and antisense strands prior to annealing, but also increases confidence that the final product consists of siRNAs in the active, duplex form. Use of this optimized method can more than double the throughput of siRNA purification, and the higher quality of the resulting siRNA duplexes can improve the success rate of gene silencing experiments.

Materials and Methods

Oligonucleotide Synthesis and Deprotection

siRNA oligonucleotides targeting Lamin A/C and fire-fly luciferase (see ref. 1 for sequences) were synthesized at a 1 µmole scale on an ABI 394 synthesizer (Applied Biosystems, Inc., Foster City, CA) using standard TBDMS/phosphoramidite chemistry. Following synthesis, the CPG solid support with the synthesis products was transferred to a 2-ml screw-cap glass vial. The oligonucleotide was cleaved from the support by adding 1 ml of an aqueous solution of methylamine ($MeNH_2$ 40% vol/vol) and heating the suspension to 65°C for 15 min. The suspension was cooled to room temperature, passed through a 0.45-µm filter and the filtrate containing the cleaved oligonucleotide was collected in a 15-ml polypropylene tube. The glass vial was washed an additional three times with 1 ml ethanol/water (1:1, vol/vol) to collect any residual CPG and oligonucleotide synthesis products remaining in the vial, and these washes were also passed through the filter. The combined filtrates were evaporated to dryness by centrifugation under vacuum for 1 h at 60°C. The protecting groups were removed by dissolving the dried synthesis product in 2 ml 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF), heating to 50°C for 10 min with occasional vortexing, and then incubating overnight at room temperature. The following day, 200 µl 2 M triethylammonium acetate (TEAA) was added to buffer the solution, followed by 3 ml RNase-free water to avoid subsequent evaporation, and the THF was removed by centrifugation for 1 h under vaccum at 60°C. The above deprotection protocol can be scaled appropriately to accommodate different synthesis scales.

Formation of siRNA Duplexes

Crude sense and antisense siRNA oligonucleotides in the above deprotection milieu were combined, heated to 90°C for 1 min and cooled to 37°C over 10 min to promote annealing of the complementary strands.

Purification of siRNA Duplexes Under Non-denaturing Conditions

Duplex siRNAs were purified directly from the deprotection milieu using the WAVE Oligo System equipped with the L-7300 Plus oven according to the protocol in Table 1.

Fractions were collected at 0.3-minute intervals starting at 8.5 min, analyzed by fully-denaturing HPLC as described in Table 3, and fractions containing full-length oligonucleotides were pooled. Typically, fractions collected between 9.0–10.5 min contained full-length duplexes.

Post-column Preparation

The ion-pairing agent present in the WAVE Optimized HA Buffers was removed from the duplexes as follows. Most of the volatile ion-pairing agent was removed during drying of the duplexes by centrifugation under vaccum at 60°C. Any residual ion-pairing agent was eliminated by dissolving the dried duplexes in 0.5 ml 1 M sodium chloride and applying the resuspended material to Oligo-MP Cartridges preconditioned with 5 ml 100% acetonitrile, 5 ml RNase free water, and finally with 5 ml 1 M sodium chloride. The columns were washed with 20 ml RNase-free water, and purified duplex siRNAs were eluted from the columns in 2 ml 30% acetonitrile and dried by vacuum centrifugation at 60°C.

HPLC Analysis of Purified siRNA Duplexes

Following purification, siRNAs were analyzed using the WAVE System 3500HT, WAVE Optimized TEAA Buffers and the OligoSep Cartridge. WAVE Optimized TEAA Buffers were used for analytical purposes because separation with these eluents is dependent on both size and sequence composition. In contrast, separation with the WAVE Optimized HA Buffers used for purification is dependent primarily on the length of the oligonucleotide. The integrity of duplex siRNAs was analyzed under non-denaturing conditions (40°C was found to be optimal when using WAVE Optimized TEAA Buffers) as described in Table 2. Individual siRNA sense and antisense strands of the siRNA duplexes were resolved and analyzed under fully-denaturing conditions (80°C) following the protocol described in Table 3.

Table 1

Duplex siRNA Purification Protocol

Step	Time (min)	% WAVE Optimized HA Buffer A	% WAVE Optimized HA Buffer B
Loading	0	70	30
Start Gradient	0.1	35	65
Stop Gradient	13.1	5	95
Start Clean	13.2	0*	0
Stop Clean	13.7	0	0
Start Equilibrate	13.8	70	30
Stop Equilibrate	14.5	70	30

Instrument: WAVE Oligo System
Column: OligoSep Prep HC Cartridge, 7.8 x 50 mm
Flow Rate: 1.5 ml/min
Temperature: 30°C
Sample Loop: 2 ml
Sample Injection: 0.1–2 ml
Run Time: 15.6 min
Detection: UV at 300 nm

*WAVE Oligo System is performing an active clean at this step using 100% WAVE Optimized Solution D.

Table 2

Analysis of siRNA Duplexes Under Non-denaturing Conditions			
Step	**Time (min)**	**%WAVE Optimized TEAA Buffer A**	**%WAVE Optimized TEAA Buffer B**
Loading	0	90	10
Start Gradient	0.1	90	10
Stop Gradient	10.0	60	40
Start Clean	10.5	40	60
Stop Clean	11.0	90	10
Start Equilibrate	11.2	90	10
Stop Equilibrate	12.0	90	10

Instrument: WAVE System 3500HT
Column: OligoSep Cartridge
Flow rate: 1.5 ml/min
Temperature: 40°C
Sample Loop: 500 µl
Sample Injection: 20 µl
Run Time: 13.1 min
Detection: UV at 260 nm

Table 3

Analysis of siRNA Duplexes Under Fully-denaturing Conditions			
Step	**Time (min)**	**%WAVE Optimized TEAA Buffer A**	**%WAVE Optimized TEAA Buffer B**
Loading	0	90	10
Start Gradient	0.1	90	10
Stop Gradient	10.0	75	25
Start Clean	10.5	40	60
Stop Clean	11.0	90	10
Start Equilibrate	11.2	90	10
Stop Equilibrate	12.0	90	10

Instrument: WAVE System 3500HT
Column: OligoSep Cartridge
Flow rate: 1.5 ml/min
Temperature: 80°C
Sample Loop: 500 µl
Sample Injection: 20 µl
Run Time: 13.1 min
Detection: UV at 260 nm

Results

Separation of Duplexes from Single Strands Under Non-denaturing Conditions

Active siRNAs are double-stranded molecules, and therefore must be purified under non-denaturing conditions in order to preserve the duplex structure. To evaluate the separation characteristics of siRNA oligonucleotides under non-denaturing conditions (30°C), we analyzed both the individual strands of the Lamin A/C-targeted siRNA, and the same oligonucleotides annealed directly in the deprotection milieu, using the optimized duplex siRNA purification protocol described in Table 1. The peak intensities of the single-stranded oligonucleotides under non-denaturing conditions (Figure 1) were not as prominent as peaks observed under fully-denaturing conditions (data not shown). Upon further analysis, we found full-length sense and antisense oligonucleotides in other fractions besides the main peaks marked by arrows in Figure 1 (data not shown). These results suggest that under non-denaturing conditions (30°C), the single-stranded oligonucleotides adopted a variety of secondary structures that exhibited different retention times.

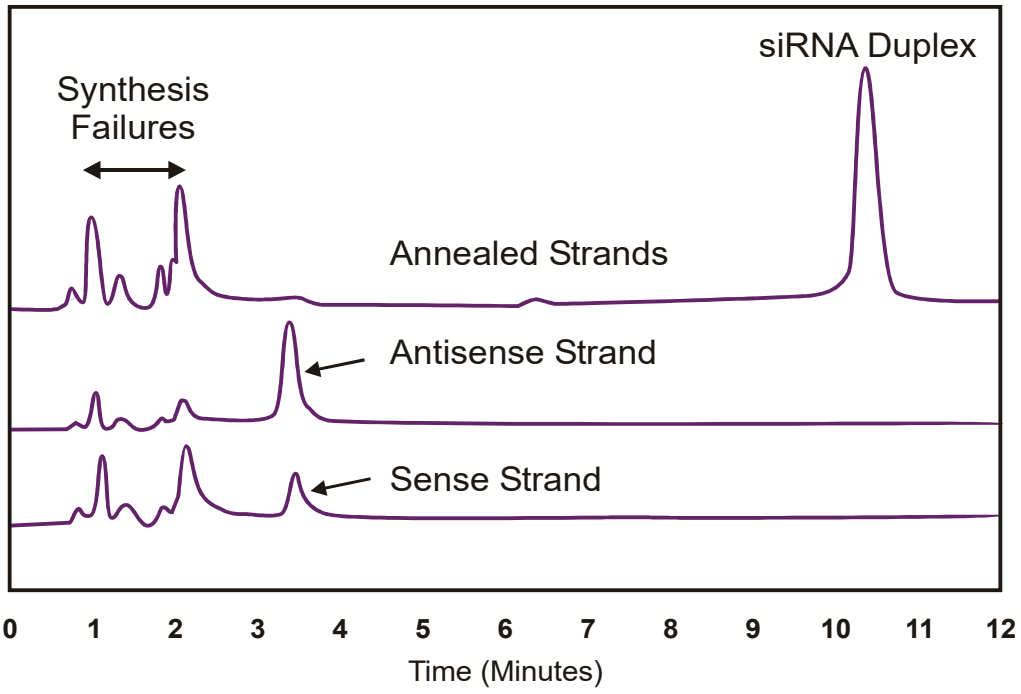

Figure 1. Chromatograms of crude sense, antisense and annealed Lamin A/C siRNA oligonucleotides generated under non-denaturing conditions (30°C) using the optimized duplex siRNA purification protocol (Table 1). Five OD units of each crude oligonucleotide were analyzed either individually, or after annealing, on the OligoSep Prep HC Cartridge using WAVE Optimized HA Buffers. Similar separation profiles were observed for the fire-fly luciferase siRNA oligonucleotides (data not shown).

The results support our previous recommendation to purify single-stranded oligonucleotides under fully-denaturing (80°C) conditions in order to melt out secondary structure, thus resolving the single-stranded oligonucleotides into single peaks (5). The major peaks corresponding to the single strands were not apparent in the chromatogram generated with the annealed siRNA oligonucleotides (Figure 1), indicating that the single strands were converted efficiently to the duplex form directly in the deprotection milieu. Relative to the single-stranded oligonucleotides, the duplexes resolved into a more prominent and symmetrical peak. This is probably due to the more rigid and uniform conformation of the duplex siRNA under non-denaturing conditions.

Analysis of Annealed siRNA Oligonucleotides Under Non-denaturing Conditions

Figure 2 shows HPLC analyses of fractions collected during separation of the annealed sense and antisense oligonucleotides. Analysis was accomplished on the WAVE System 3500HT using WAVE Optimized TEAA Buffers and the OligoSep Cartridge. OligoSep Cartridges were used for analysis because the matrix in these cartridges is optimized for greater resolution compared to the greater loading capacity of the OligoSep Prep HC Cartridge. Furthermore, WAVE Optimized TEAA Buffers were used because separation with these eluents, as opposed to the WAVE Optimized HA Buffers, is dependent on both length and sequence composition. The use of these eluents under fully-denaturing conditions (Table 3) therefore provided a means to denature the duplex siRNAs and to resolve the individual sense and antisense strands as shown in Figure 3.

Fractions 1–3 (Figure 2B), collected prior to elution of the duplex peak, contained the antisense strand, but not the sense strand, indicating that excess antisense oligonucleotide was present in the annealing reaction. An excess of one strand is expected when annealing uncharacterized crude oligonucleotide synthesis products. Synthesis failures from the sense strand, in particular n-1 species, were detected in fractions 2–5. Non-denaturing analytical HPLC and MALDI-TOF mass spectrometry showed that the sense strand synthesis failures were present as duplexes with the full-length antisense strand (data not shown). Such synthesis failures were not detected in fraction 6 demonstrating that full-length duplexes were effectively separated from duplexes containing synthesis failures. Single strands were not detected in fraction 6 collected from the duplex peak, indicating that the majority of the material in this fraction was in the duplex form.

Purification of siRNA Duplexes Under Non-denaturing Conditions

Table 4 summarizes results obtained during purification of Lamin A/C and fire-fly luciferase siRNA duplexes following annealing of 50 OD units each of the crude sense and antisense oligonucleotides. This amount of material corresponds to the yield of crude oligonucleotide typically generated from 0.2 µmole-scale syntheses using standard TBDMS chemistry. The purity of the individual oligonucleotides prior to annealing varied from 82–93% full-length product. After annealing in the deprotection milieu, 73–87% of the crude material was converted to the duplex form as determined by non-denaturing HPLC analysis. A total of 68–80 OD units of oligonucleotide was recovered during separation. The apparent reduction in OD units recovered after annealing and HPLC separation is probably due to the reduced absorbance of oligonucleotides in the duplex form relative to the single-stranded form. Pooling of fractions containing only full-length siRNA oligonucleotides resulted in the recovery of 32–46 OD units of full-length siRNA duplexes, which corresponded to a 65% recovery of duplex material. The duplexes were more than 95% pure (Table 4) as determined by non-denaturing and fully-denaturing HPLC using WAVE Optimized TEAA Buffers and the OligoSep Cartridge (Figure 3).

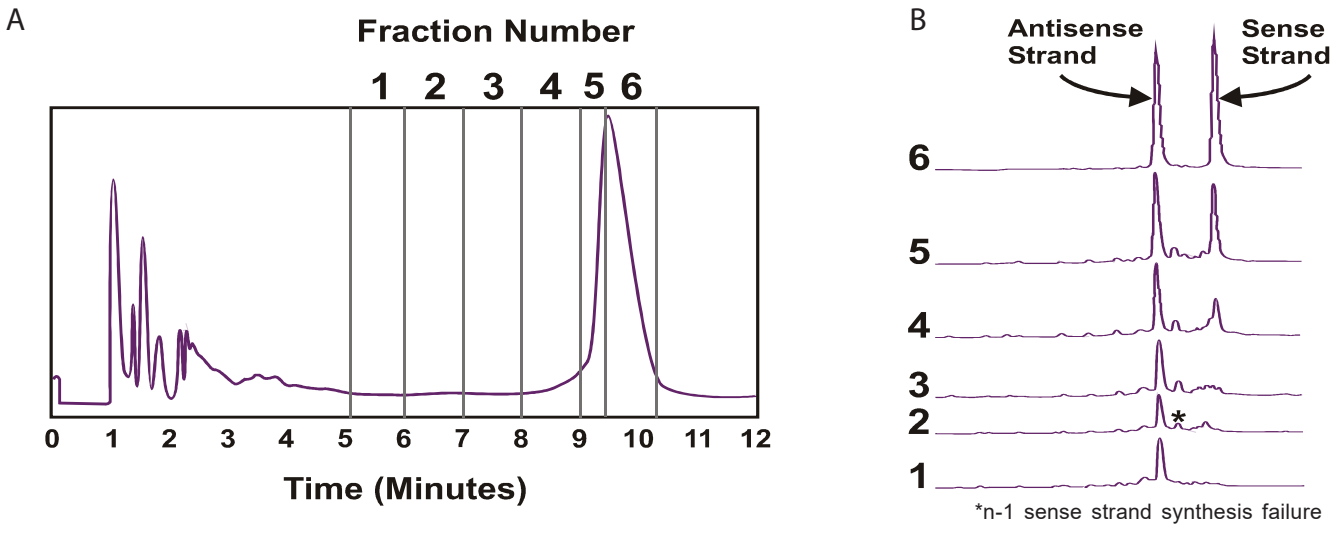

Figure 2. Analysis of fractions collected during separation of annealed Lamin A/C siRNA oligonucleotides under non-denaturing conditions. (**A**) Lamin A/C siRNA oligonucleotides (10 OD units total, 5 OD units of each crude oligonucleotide) were annealed in deprotection milieu as described in the *Materials and Methods* section and separated on the OligoSep Prep HC Cartridge using WAVE Optimized HA Buffers (Table 1). Individual fractions were collected at the times shown, dried and resuspended in water. (**B**) Equal amounts (1 µg) of oligonucleotide from each fraction were analyzed under fully-denaturing conditions with the WAVE System 3500HT, OligoSep Cartridge and WAVE Optimized TEAA Buffers (Table 3).

Table 4

Purification Yields from 100 OD Units* Annealed, Crude Oligonucleotides		
	Lamin A/C siRNA	**Luciferase siRNA**
% FLP** crude sense oligonucleotide	89%	92%
% FLP crude antisense oligonucleotide	82%	93%
% Converted to duplex after annealing	73%	87%
Total OD units recovered after HPLC	68 OD Units	80 OD Units
Fractions containing full-length duplex	9–11.1 min	8.6–11.3 min
Full-length duplex recovered	32 OD Units	46 OD Units
Duplex purity	>95%	>95%

*50 OD units of each crude oligonucleotide
** FLP = full-length product

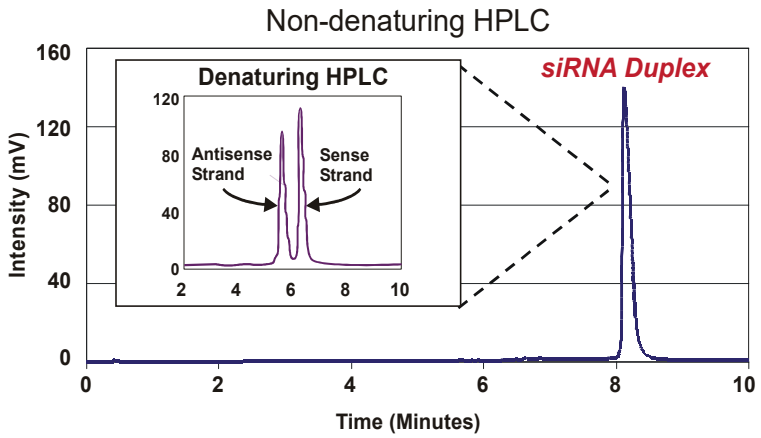

Figure 3. Analysis of duplex siRNA purified from 50 OD units of crude, annealed oligonucleotides (100 OD units total). Crude sense and antisense fire-fly luciferase siRNA oligonucleotides were annealed as described in the *Materials and Methods* section, and duplex siRNAs were purified using the optimized siRNA purification protocol (Table 1). Purified duplex siRNA was dried, resuspended in water and 1 µg was analyzed on the WAVE System 3500HT using the OligoSep Cartridge and WAVE Optimized TEAA Buffers under non-denaturing conditions (Table 2) to evaluate duplex purity, and under fully-denaturing conditions (Table 3) to confirm absence of synthesis failures in purified duplexes.

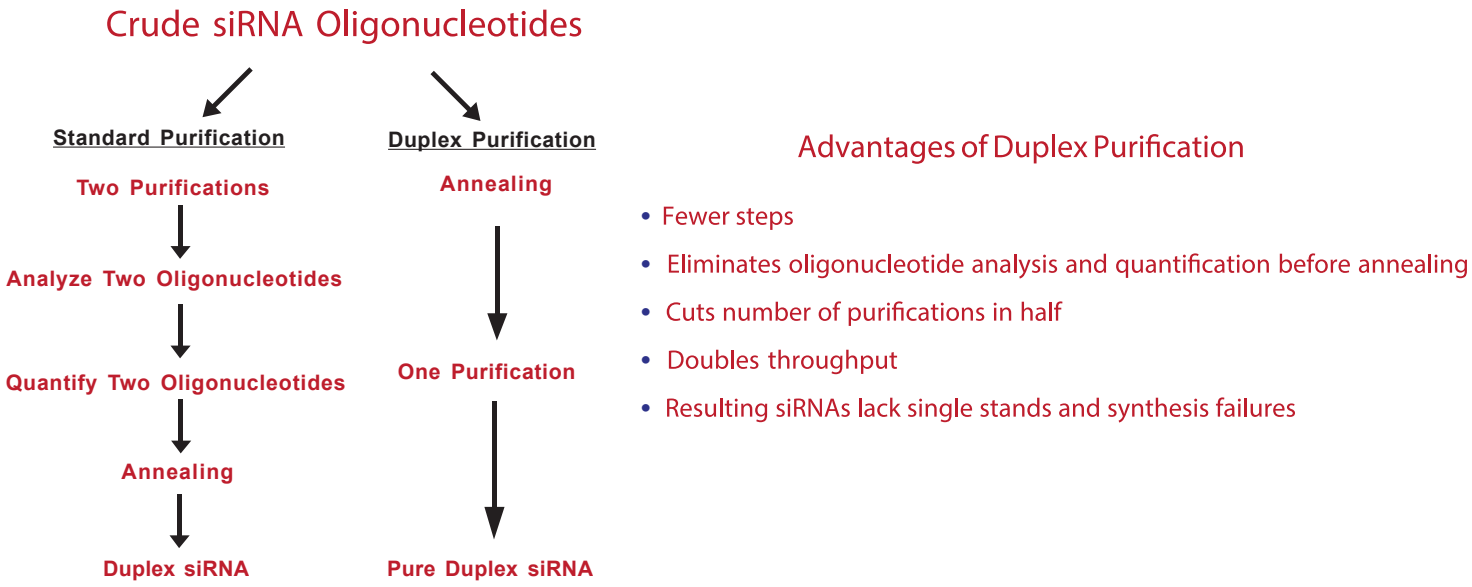

Figure 4. Flow diagram comparing the standard single-strand purification/annealing protocol to the duplex siRNA purification method described here.

Discussion

Impurities in siRNA oligonucleotide preparations can reduce the potency and delivery efficiency of siRNAs, and can increase the risk of toxicity in gene silencing experiments (6). Furthermore, variability in the levels of such impurities can affect the reproducibility of gene function studies. The method described here enables the purification of duplex siRNAs directly from crude, annealed synthesis products. This eliminates the need to purify and characterize the individual sense and antisense siRNA oligonucleotides prior to annealing (Figure 4), and reduces uncertainty associated with annealing efficiencies of different siRNAs. The high quality siRNA duplexes generated by this method will lead to improved success rates and reproducibility of gene silencing experiments.

Use of the duplex siRNA purification method described here can more than double the throughput and productivity of siRNA purification, resulting in the production of higher quality siRNA reagents (Figure 4). Purification costs often comprise a significant portion of the total cost of siRNA oligonucleotides purchased from commercial vendors. Consequently, researchers performing experiments involving large numbers of siRNAs may benefit by obtaining less-costly unpurified oligonucleotides and accomplishing purification in-house.

References

1. 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.

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4. 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.

5. Application Note 120: *Optimized Purification of siRNA Oligonucleotides Using the WAVE® Oligo System.* <http://www.transgenomic.com>

6. Cao, L., Ayers, D., McKenzie, T., Ayers, D. and Thompson, J. (2003). Purity of siRNA oligonucleotides: effect on potency and toxicity in cell culture. *Poster #10 from Applications of RNA Interference, IBC Conference, San Diego, CA.*

Application Note Product List

Product	Catalog Number	Quantity
WAVE Oligo System	WAV-99-0004A	1
WAVE System 3500HT	NAV-99-3500HT	1
OligoSep Cartridge	NUC-99-3550	1
OligoSep Prep HC Cartridge	NUC-99-3860	1
Oligo-MP Cartridge	21-8560-86	10
Oligo-MP Cartridge	21-8560-90	100
WAVE Optimized Solution D	553404	1 L
WAVE Optimized Solution D	553408	Case of 4 x 2.5 L
WAVE Optimized HA Buffer A	553415	Case of 4 x 2.5 L
WAVE Optimized HA Buffer B	553416	Case of 4 x 2.5 L
WAVE Optimized TEAA Buffer A	553401	Case of 4 x 2.5 L
WAVE Optimized TEAA Buffer B	553402	Case of 4 x 2.5 L
T Icaa CPG Support	19-7640-00	1 g

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