**Supplementary Material**

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## Materials and Methods for Supplementary Figures 1-2

Trecovirsen, also known as GEM91® was acquired from Integrated DNA Technologies, Inc. (Coralville, IA). Trecovirsen has an average mass of 7771 g/mol and a sequence of 5' d(CTC TCG CAC CCA TCT CTC TCC TTC T) 3'. To include a fluorophore, it was modified with a 5' amino terminus (5AmMC6) to link Pacific Blue. LC-MS grade methanol (MeOH) and acetic acid were purchased from Thermo Fisher Scientific (Waltham, MA), and MilliQ water was used. Triethylamine (TEA) was purchased from Millipore Sigma (Burlington, MA) and 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) was purchased from Acros Organics (Fair Lawn, NJ).

Duplicate sets of conventional LC systems (ACQUITY UPLC H-Class Bio chromatograph and ACQUITY UPLC TUV detector) and 2.1 x 50 mm stainless steel columns packed with 130 Å, 1.7 µm BEH C18 stationary phase were compared against columns and LC systems in the same formats, but employing hybrid surfaces at Waters Corporation in Milford, MA. All flow paths and columns were new and previously unexposed to oligonucleotide samples. Three separations of Pacific Blue labelled trecovirsen were performed using an ion-pairing mobile phase system comprised of 15 mM triethylamine, 400 mM 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) in water (mobile phase A) and a 50:50 solution of mobile phase A and methanol (mobile phase B). Samples were injected at a mass load of 0.51 pmol (4 ng) using an injection volume of 1 µL and run at a temperature of 60 °C, flow rate of 0.2 mL/min, and gradient from 0.5-40% B in 12 min, followed by 40-80% B in 2 min. Analyses were performed with UV detection at 260 nm using MassLynx 4.1 for data acquisition and UNIFI 1.8 for data analysis.

Fractions containing the oligonucleotide were collected during the third injection of each set, and the resultant recovery was determined through offline spectrofluorometric quantitation using calibration curve data generated offline of the LC-UV system. Offline calibration curves were produced using Pacific Blue labelled trecovirsen, which was serially diluted to a final concentration of 1.29 fmol/µL (0.01 µg/mL) and 12.87 fmol/µL (0.1 µg/mL) using a 5 to 95 percent solution of mobile phase A to mobile phase B to reflect the estimated solvent composition of the eluant in the fraction collected from LC-UV. Calibration curves with a linear dynamic range of 1.29 fmol/µL (0.01 µg/mL) to 10.29 fmol/ µL (0.08 µg/mL) in concentration were generated using a Gemini XPS spectrofluorometer from Molecular Devices (San Jose, CA). Analyses were performed with excitation and emission wavelengths of 410 nm and 455 nm using SoftMax Pro 6.5.1 for data acquisition and analysis. All previous runs of Pacific Blue labelled trecovirsen were assigned a percent recovery value based on ratios of UV peak area and the concentration determined for the fraction collected from the third injection/separation to observe any trends in recovery over the three injections.

## Materials and Methods for Supplementary Figure 4

An equimolar mixture of lyophilized 15, 20, 25, 30, and 35-mer oligodeoxythymidines was acquired from Waters Corporation (Milford, MA) in the form of the MassPREP OST Standard and reconstituted to a 5 pmol/µL concentration. 39-mer oligodeoxythymidine was acquired from Integrated DNA Technologies, Inc. (Coralville, IA). LC-MS grade acetonitrile (ACN) and acetic acid were purchased from Thermo Fisher Scientific (Waltham, MA), and MilliQ water was used. Hexylamine (HA) was purchased from Millipore Sigma (Burlington, MA).

30-mer oligodeoxythymidine was analyzed by LC-UV with an ACQUITY UPLC H-Class Bio system that had been modified to include hybrid surfaces in the fluidic path. Separations were performed on unused conventional 2.1 x 50 mm stainless steel columns or HST columns packed with a 130 Å, 1.7 µm BEH C18 stationary phase. An ion-pairing mobile phase system comprised of 25 mM hexylammonium acetate (mobile phase A) and a 50:50 solution of mobile phase A and acetonitrile (mobile phase B) at a pH of 6, 7, or 8.5 (only aqueous mobile phase A was pH adjusted). ). Samples were injected at an injection volume of 2 µL, or a mass load of 10 pmol per oligonucleotide, and run at a temperature of 60 °C, flow rate of 0.4 mL/min, and gradient from 50-86% B in 12 min. Chromatograms were recorded with an ACQUITY UPLC PDA detector equipped with a 5 µL titanium flow cell at 260 nm using chromatography software Empower 3.0. 100% recovery was considered to be the peak signal obtained after column conditioning with single or several 500 nmol injections of 39 mer oligonucleotide.

## Results and Discussion for Supplementary Figures 1-2

A quantitative experiment was performed to confirm that the signal observed with the hybrid surface column in Figure 1 was indeed representative of the high recovery of trecovirsen and not from a plateau of signal using LC-UV detection. To determine this, we used a separate, offline spectrofluorometer to generate calibration curves from neat solutions of fluorescently labelled trecovirsen. These curves were generated independently from the LC-UV system and thus can act as a “baseline” or reference point to the expected recovery of trecovirsen, which we can then use to directly quantify fractions from LC-UV using conventional or hybrid surface technologies.

Supplementary Figure 1 depicts the offline generated calibration curves and the representative chromatographic separations of trecovirsen. Percent recoveries from the third injection, as collected from the separation and quantified offline from the LC system, are also marked on each standard curve. The average oligonucleotide recoveries from conventional and hybrid surface setups are plotted in Supplementary Figure 4. With stainless steel columns and a conventional LC system, the average recovery of trecovirsen was only 55% for the third injection. Using hybrid surface columns and LC systems gave an average recovery that was 88%, an increase of 73% over that of the conventional setups.

Interestingly, the linearity in recovery is consistent across the three injections for both setups (Supplementary Figure 2). These recoveries do not vary much from injection to injection, suggesting that the sample conditioning that might occur from one injection to another was minimal. The average recoveries of trecovirsen using hybrid surfaces ranged from 84% to 88% across the three injections (Supplementary Figure 2). The conventional LC and column surfaces gave lower recoveries ranging from 51% to 55%. This suggests that if these calibration curves were generated with online LC-UV or MS detection, the slope of curves using hybrid surface columns and LC systems would be steeper than those with stainless steel and conventional technologies. This is confirmed in Figure 3 of the main text.



**Supplementary Figure 1. (A) Representative UV chromatograms of the third injection of trecovirsen as obtained using unused columns and LC systems constructed from conventional or hybrid surfaces. (B) Calibration curve and quantitative measurement of trecovirsen as generated through offline spectrofluorometric analyses. Calibration curves were generated using neat solutions of trecovirsen from 1.29 fmol/µL to 10.29 fmol/µL.**



**Supplementary Figure 2. Average percent recoveries across the three injections of trecovirsen as obtained using unused columns and LC systems constructed from conventional or hybrid surfaces.**

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**Supplementary Figure 3. UV chromatograms of the first injection (before conditioning) and fourth injection (after conditioning) of an equimolar mixture of 15, 20, 25, 30, and 35-mer oligodeoxythymidines obtained using an unused 2.1 x 150 mm (A) stainless steel or (B) hybrid surface column at a mobile phase pH of 7.**



**Supplementary Figure 4. Recovery of 30-mer oligodeoxythymidine in the first injections on new conventional or hybrid surface columns at pH 6, 7, and 8.5.**



**Supplementary Figure 5. A plot of the peak area of the internal standard (IS) as collected through multiple reaction monitoring (MRM) from unused stainless steel and hybrid surface columns.**