Supporting Information for:

Characterization of antisense oligonucleotide impurities by ion-pairing reversed-phase and anion exchange chromatography coupled to HILIC/MS using a versatile 2D-LC setup

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Figure S1: AEX profiles of the phosphorothioate ASO obtained with mobile phases containing different buffers (A), amounts of acetonitrile (B), and various column temperatures (C).
AEX experiments were performed using a DNAPAC PA200 column (2.0 x 250 mm, 8 µm). The mobile phase flow rate was set to 0.4 mL/min for and the injection volume was 1 µL for all experiments.

(A) Weak and strong mobile phases either contained 10 mM sodium hydroxide (pH 12.0), a combination of 20 mM MES and 20 mM HEPES (pH 8.0) or 40 mM Tris (pH 8.0) while 1.5 M of sodium bromide was added in the eluting mobile phase. A pH-mediated salt gradient was applied with the MES and HEPES buffers by adjusting the pH of mobile phase B to 6.0. Mobile phase B composition was increased from 0% to 100% in 45 min with the sodium hydroxide (green trace) and Tris (blue trace) additives. With the combination of MES and HEPES, the eluting mobile phase composition was increased from 10% to 80% in 45 min. Column temperature was set to 40°C, except at pH 12.0 where the temperature was 30°C in accordance with the column manufacturer recommendations under basic conditions.

(B) Weak mobile phase (A) and strong mobile phase (B) contained 40 mM Tris, pH 8.0 and 1.5 M of sodium bromide was added in mobile phase B. Acetonitrile was added in mobile phase C. Column temperature was set to 20°C. Mobile phase B composition was increased from 0% B to 100%B in the absence of acetonitrile while it was increased from 10% to 90% with 10% acetonitrile and from 20% to 80% B with 20% acetonitrile.

(C) The effect of column temperature was studied with both mobile phases containing 40 mM Tris, pH adjusted to 8.0 and containing 10% acetonitrile (v/v). 1.5 M sodium bromide was added in the eluting mobile phase. The eluting mobile phase composition was increased from 10% to 90% in 45 min.
Effect of column volume on the ASO peak shape was studied in the IPRP-HILIC 2D-LC heart-cutting mode. The ASO was transferred from the first to the second dimension within the same chromatographic run by switching the 2 positions/6 port valve during ASO elution. The loop volume was 20 µL. Composition of 1D IPRP and 2D HILIC mobile phases are provided in the main manuscript. The gradient slope was modified depending on column dimensions. With the 2.1 mm I.D. x 150 mm column, the eluting mobile phase composition was increased from 20% to 100% in 9 min at a 0.4 mL/min flow rate. With the 3.0 mm I.D. x 150 mm column, the eluting mobile phase composition was increased from 20% to 100% in 14 min at a 0.8 mL/min flow rate the first 4 min and it was then decreased to 0.4 mL/min when eluting the ASO-related species to allow higher MS ionization.
AEX experiments were performed with a weak mobile phase containing 40 mM Tris (pH 8.0) in water mixed with acetonitrile (80:20, v/v). 1.5 M of sodium bromide was added in the eluting mobile phase. The eluting mobile phase composition was increased from 20% to 60% B in 20 min. The flow rate was set to 0.5 mL/min, column temperature was maintained at 50° C and injection volume was 2.5 µL.
Figure S4: IPRP (A) and AEX (B) profiles throughout the injection sequence

The IPRP and AEX conditions are listed in the 2D-LC experimental section of the main manuscript. The injection sequence was performed with i) a first blank injection to wash the valves and the five different sample loops; ii) the injection of the ASO sample and fractionation; and iii) the subsequent analysis of the five different fractions collected.
Figure S5: 2D HILIC profiles repeatability throughout injections. (I) 2D HILIC analysis of the IPRP fraction #2 collected. (II) High-resolution mass spectrometry (HRMS) spectra of the main peak separated on the HILIC column.

The IPRP-HILIC 2D-LC conditions are listed in the experimental section of the main manuscript. The injection sequence was performed with i) a first blank injection to wash the valves and the five different sample loops; ii) the injection of the ASO sample and fractionation; and iii) the subsequent analysis of the five different fractions collected.