

CLAIMS

What is claimed is:

1. A method of characterizing a sample comprising a population of oligonucleotides of interest, comprising:
 - a. providing a sample comprising a population of oligonucleotides of interest of identical sequence;
 - b. subjecting the sample to liquid chromatography and mass spectrometry, thereby generating at least one mass spectrogram corresponding to the population of oligonucleotides of interest; and
 - c. determining a percentage of total oligonucleotides in the sample corresponding to the population of oligonucleotides of interest.
2. The method of claim 1, wherein the sample further comprises at least one impurity comprising at least one additional population of oligonucleotides.
3. The method of claim 2, wherein the additional population of oligonucleotides comprises a fragmentation product of or synthesis byproduct of the oligonucleotides of interest.
4. The method of claim 2 or 3, further comprising generating a mass spectrogram corresponding to the at least one additional population of oligonucleotides and determining the percentage of total oligonucleotides in the sample corresponding to the at least one additional population of oligonucleotides.
5. The method of any one of claims 1-4, wherein the oligonucleotides of interest are deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or DNA-RNA hybrids.
6. The method of any one of claims 1-5, wherein the oligonucleotides of interest are single stranded.
7. The method of any one of claims 1-5, wherein the oligonucleotides of interest are double stranded.
8. The methods of any one of claims 1-5, wherein the oligonucleotides of interest comprise a hairpin or stem-loop structure.
9. The methods of any one of claims 1-8, wherein the oligonucleotides of interest are between 15 and 100 nucleotides in length.
10. The method of any one of claims 1-9, wherein the oligonucleotides of interest are therapeutic oligonucleotides.
11. The method of claim 10, wherein the therapeutic oligonucleotides comprise antisense oligonucleotides (ASO), dsRNAs, siRNAs, aptamers or microRNAs.

12. The method of any one of claims 1-11, wherein the oligonucleotides of interest comprise at least one modification.
13. The method of claim 12, wherein the at least one modification is at the 5' end, the 3' end, or both, of the oligonucleotide of interest.
14. The method of claim 12 or 13, wherein the at least one modification comprises a modification to at least one internal nucleobase of the oligonucleotides of interest.
15. The method of any one of claims 12-14, wherein the at least one modification affects binding affinity, binding specificity, stability, pharmacokinetics or toxicity of the oligonucleotides of interest.
16. The method of any one of claims 12-15, wherein the at least one modification comprises a locked nucleic acid (LNA), a phosphorothioate (PS) linkage, a terminal 5' or 3' phosphate (PO), a 5' methyl (5-Me) modification, a 2'-O-Methyl (2'-O-Me) modification, a 2'-O-methoxyethyl (2'-MOE) modification, a constrained ethyl (cET) nucleoside analog, polyethylene glycol (PEG) or a combination thereof.
17. The method of any one of claims 1-16, wherein the liquid chromatography comprises hydrophilic interaction liquid chromatography (HILIC) or Ion-pairing Reversed-Phase Liquid Chromatography (IP-RPLC).
18. The method of claim 17, wherein the HILIC comprises a mobile phase buffer comprising ammonium acetate.
19. The method of claim 18, wherein the mobile phase comprises a first buffer comprising 15 mM ammonium acetate in 70% acetonitrile (ACN) and a second buffer comprising 15 mM ammonium acetate in 30% (ACN).
20. The method of claim 17, wherein the HILIC comprises a mobile phase buffer comprising ammonium formate.
21. The method of claim 20, wherein the mobile phase comprises a first buffer comprising 15 mM ammonium formate in 70% acetonitrile (ACN) and a second buffer comprising 15 mM ammonium formate in 30% (ACN).
22. The method of any one of claims 17-21, wherein HILIC separation comprises a column temperature of between 23 and 50 °C.
23. The method of any one of claims 17-21, wherein HILIC separation comprises a column temperature of 30 °C.
24. The method of any one of claims 17-23, wherein the HILIC comprises a column with a solid phase with a mean nominal particle size of 3 µm, a median particle pore size of 200 Å, a 2 mm inner diameter, and a 150 mm length column.

25. The method of claim 17, wherein the IP-RPLC comprises a mobile phase buffer comprising Hexafluoroisopropanol (HFIP) and 5 mM N,N-Diisopropylethylamine (DIEA).
26. The method of claim 25, wherein the mobile phase comprises a first buffer comprising 50 mM HFIP and 5 mM DIEA in water and a second buffer comprising 50 mM HFIP and 5 mM DIEA in acetonitrile.
27. The method of any one of claims 17, 25 or 26, wherein the IP-RPLC comprises a column with a mean nominal particle size of 1.7 μm , a median particle pore size of 130 $\text{\AA}$, a column length 100 mm and a 2.1 mm inner diameter.
28. The method of any one of claims 17, 25 or 26, wherein the IP-RPLC comprises a 1.7 μm , Oligo-XT 100 $\text{\AA}$, 50 x 2.1 mm column.
29. The method of any one of claims 1-28, wherein the mass spectrometry comprises electrospray ionization (ESI).
30. The method of claim 29, wherein the ESI comprises nano-flow ESI.
31. The method of any one of claims 1-30, wherein the liquid chromatography further comprises ultraviolet (UV) detection of the sample.
32. The method of any one of claims 1-31, wherein the mass spectrometry is tandem mass spectrometry (MS/MS).
33. The method of claim 32, wherein the MS/MS comprises Data Dependent Acquisition (DDA).
34. The method of claim 32 or 33, wherein the MS/MS comprises fragmentation of the population of oligonucleotides of interest, the at least additional population of oligonucleotides, or a combination thereof.
35. The method of claim 34, wherein the fragmentation comprises higher-energy collisional dissociation (HCD).
36. The method of claim 35, wherein the HCD comprises a normalized collisional energy (NCE) of 15% to 35%.
37. The method of claim 35, wherein the HCD comprises a normalized collisional energy (NCE) of 20%.
38. The method of any one of claims 1-37, wherein step (c) comprises determining the intact mass of the oligonucleotides of interest.
39. The method of any one of claims 2-38, wherein step (c) further comprises determining the intact mass of the at least one additional population of oligonucleotides.

40. The method of any one of claims 1-39, wherein step (c) comprises determining the structure of the oligonucleotides of interest using mass spectrometry.
41. The method of any one of claims 2-40, wherein step (c) further comprises determining the structure of the at least one additional population of oligonucleotides.
42. The method of claim 40 or 41, wherein the structure includes nucleotide sequence, modification or a combination thereof.
43. A method of making a composition comprising an oligonucleotide of interest comprising:
 - a. synthesizing the oligonucleotide of interest; and
 - b. characterizing the oligonucleotide of interest using the methods of any one of claims 1-42.
44. The method of claim 43, wherein at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 98.5%, at least 99%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8% or at least 99.9% of the total oligonucleotides in the composition are the oligonucleotide of interest.
45. The method of claim 43 or 44, wherein the method further comprises adding a pharmaceutically acceptable carrier, diluent or excipient.
46. A composition, comprising oligonucleotides of interest made by the methods of any one of claims 43-45.
47. A method of treating a subject in need thereof, comprising administering the composition of claim 46.
48. The composition of claim 46 for use in a method of treating a subject in need thereof.
49. The composition of claim 46 for use in the manufacture of a medicament for treating a subject in need thereof.
50. A method of characterizing a sample, comprising:
 - a. providing a sample comprising a population of oligonucleotides of interest of identical sequence and/or modification, and at least one impurity comprising an additional population of oligonucleotides;
 - b. subjecting the sample to liquid chromatography and tandem mass spectrometry (MS/MS),
wherein the liquid chromatography comprises:
 - i. hydrophilic interaction liquid chromatography (HILIC) comprising a mobile phase, wherein a first buffer comprises 15 mM ammonium formate or ammonium acetate in 70%

- acetonitrile (ACN), and a second buffer comprises 15 mM ammonium formate or ammonium acetate in 30% ACN, or
- ii. Ion-pairing Reversed-Phase Liquid Chromatography (IP-RPLC) comprising a mobile phase, wherein a first buffer comprises 50 mM Hexafluoroisopropanol (HFIP) and 5 mM N,N-Diisopropylethylamine (DIEA) in water and a second buffer comprises 50 mM HFIP and 5 mM DIEA in acetonitrile;

wherein the MS/MS comprises fragmentation of the population of oligonucleotides of interest and the additional population of oligonucleotides in the sample using higher-energy collisional dissociation (HCD) comprising a normalized collisional energy (NCE) of 15% to 35%,

thereby generating at least one mass spectrogram corresponding to the population oligonucleotides of interest and the additional population of oligonucleotides; and

- c. determining a percentage of total oligonucleotides in the sample corresponding to the population of oligonucleotides of interest.

51. The method of claim 50, wherein the additional population of oligonucleotides comprises a fragmentation product of or synthesis byproduct of the oligonucleotides of interest.
52. The method of claim 50 or 51, wherein the oligonucleotides of interest are deoxyribonucleic acids (DNA), ribonucleic acids (RNA), or DNA-RNA hybrids.
53. The method of any one of claims 50-52, wherein the oligonucleotides of interest are single stranded, double stranded, or a combination thereof.
54. The methods of any one of claims 50-53, wherein the oligonucleotides of interest comprise a hairpin or stem-loop structure.
55. The methods of any one of claims 50-54, wherein the oligonucleotides of interest are between 15 and 100 nucleotides in length.
56. The method of any one of claims 50-55, wherein the oligonucleotides of interest are therapeutic oligonucleotides.
57. The method of claim 56, wherein the therapeutic oligonucleotides comprise antisense oligonucleotides (ASO), dsRNAs, siRNAs, aptamers or microRNAs.
58. The method of any one of claims 50-57, wherein the oligonucleotides of interest comprise at least one modification.
59. The method of claim 58, wherein the at least one modification is at the 5' end, the 3' end, or both, of the oligonucleotide of interest.

60. The method of claim 58 or 59, wherein the at least one modification comprises a modification to at least one internal nucleobase of the oligonucleotides of interest.
61. The method of any one of claims 58-60, wherein the at least one modification affects binding affinity, binding specificity, stability, pharmacokinetics or toxicity of the oligonucleotides of interest.
62. The method of any one of claims 58-61, wherein the at least one modification comprises a locked nucleic acid (LNA), a phosphorothioate (PS) linkage, a terminal 5' or 3' phosphate (PO), a 5' methyl (5-Me) modification, a 2'-O-Methyl (2'-O-Me) modification, a 2'-O-methoxyethyl (2'-MOE) modification, a constrained ethyl (cET) nucleoside analog, polyethylene glycol (PEG) or a combination thereof.
63. The method of any one of claims 50-62, wherein the HILIC chromatography comprises a column temperature of 30 °C.
64. The method of any one of claims 50-63, wherein the HILIC chromatography comprises a column with a solid phase with a mean nominal particle size of 3 µm, a median particle pore size of 200 Å, a 2 mm inner diameter, and a 150 mm length column.
65. The method of any one of claims 50-62, wherein the IP-RPLC comprises a 1.7 µm, Oligo-XT 100 Å, 50 x 2.1 mm column.
66. The method of any one of claims 50-65, wherein the MS/MS comprises electrospray ionization (ESI).
67. The method of claim 66, wherein the ESI comprises nano-flow ESI.
68. The method of any one of claims 50-67, wherein the liquid chromatography further comprises ultraviolet (UV) detection of the sample.
69. The method of claim 50-68, wherein the MS/MS comprises Data Dependent Acquisition (DDA).
70. The method of any one of claims 50-69, wherein step (c) comprises determining the intact mass and/or structure of the population of oligonucleotides of interest and the additional population of oligonucleotides.
71. A method of making a composition comprising an oligonucleotide of interest comprising:
 - a. synthesizing the oligonucleotides of interest thereby generating a sample comprising a population of the oligonucleotides of interest; and
 - b. characterizing the sample using the methods of any one of claims 50-70.