Claims

1. A method of determining epigenetic modification status, e.g. methylation status of at least one nucleotide of interest (NOI) in a non-modified target nucleic acid sequence of interest, wherein said target nucleic acid sequence comprises a target anchor sequence comprising said NOI, said method comprising the steps of
   a. Providing an oligonucleotide comprising an anchor sequence (An), wherein the anchor sequence is a sequence at least 50% complementary to said target anchor sequence, wherein the anchor sequence comprises at least one hydrophobic nucleotide (H) positioned between the nucleotide complementary to said NOI and the nucleotide immediately 5' thereof;
   b. Incubating said oligonucleotide with said target nucleic acid of interest at a temperature which is higher than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is unmodified, such as unmethylated,
   c. Detecting whether said oligonucleotide anneals to said target nucleic acid of interest,

   thereby determining the epigenetic modification status, e.g. the methylation status, wherein
      i. said hydrophobic nucleotide (H) has the structure

         X-Y-Q,

         wherein

         X is a nucleotide or nucleotide analogue or a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue;

         Q is an intercalator which is not taking part in Watson-Crick hydrogen bonding; and

         Y is a linker moiety linking said nucleotide or nucleotide analogue or backbone monomer unit and said intercalator; and
      ii. said oligonucleotide has the structure 5'-An-Lp-St-3', wherein

         An is the anchor sequence;

         Lp is a loop sequence, which is not complementary to the target nucleic acid sequence of interest, wherein the loop sequence consists of a single nucleic acid sequence capable of forming a protruding structure, such as a loop structure or a stem structure, or consists of two or more nucleic acid sequences capable of hybridising at least partly to one another to form a complex which is capable of forming a protruding structure, such as a loop structure or a stem structure;

St is a starter sequence, capable of hybridizing to a target starter sequence, wherein the target starter sequence is a sequence of the target nucleic acid sequence positioned 5' to the target anchor sequence.

2. The method according to claim 1, wherein the epigenetic modification is methylation.

~~2.~~3. The method according to ~~claim 1~~any one of the preceding claims, wherein the target nucleic acid of interest is DNA or RNA.

~~3. The method according to claim 1, wherein the target nucleic acid of interest is RNA.~~

4. The method according to any one of the preceding claims, wherein the non-modified nucleic acid sequence of interest has not been subjected to treatment comprising bisulfite conversion, restriction enzyme digestion or TET enzymatic conversion prior to performing the method~~,~~.

5. The method according to any one of the preceding claims, wherein the NOI is a cytosine.

6. The method according to any one of the preceding claims, wherein the difference between
   A. the absolute difference between the melting temperatures of
      i. the oligonucleotide comprising said hydrophobic nucleotide(s) when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises at least one methylated NOI, such as at least one methylated cytosine; and
      ii. the oligonucleotide comprising said hydrophobic nucleotide(s) when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises no methylated NOIs, such as no methylated cytosines; and
   B. the absolute difference between the melting temperatures of
      i. an oligonucleotide identical to the oligonucleotide of A., however which does not comprise said hydrophobic nucleotide(s), when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises the same number of methylated NOIs as in A. i., such as the same number of methylated cytosines as in A. i.; and

ii. an oligonucleotide identical to the oligonucleotide of A., however which does not comprise said hydrophobic nucleotide(s), when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises no methylated NOIs, such as no methylated cytosines,

is of at least 0.50 °C, such as at least 0.75 °C, preferably at least 1.0 °C per NOI, such as per cytosine, that is methylated instead of unmethylated in said target nucleic acid sequence of interest, wherein the difference is A-B and wherein the difference is a positive difference, and wherein the melting temperature is measured in TM buffer comprising 0.02 M Na_2HPO_4, 0.02 M NaCl, and 2 mM EDTA.

~~7. The method according to any one of the preceding claims, wherein the absolute difference between the melting temperatures of~~

~~i. the oligonucleotide comprising said hydrophobic nucleotide when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises at least one methylated NOI, such as at least one methylated cytosine; and~~

~~ii. the oligonucleotide comprising said hydrophobic nucleotide when hybridized to said target nucleic acid sequence of interest, wherein said nucleic acid sequence of interest comprises no methylated NOIs, such as no methylated cytosines,~~

~~is at least 1.6 °C, such as at least 1.8 °C, such as at least 2.0 °C, such as at least 2.2 °C, preferably at least 2.4 °C per NOI, such as per cytosine, that is methylated instead of unmethylated in said target nucleic acid sequence of interest, wherein the melting temperature is measured in TM buffer comprising 0.02 M Na_2HPO_4, 0.02 M NaCl, and 2 mM EDTA.~~

~~8.~~7. The method according to any one of the preceding claims, wherein the anchor sequence comprises at least ~~the~~one ~~sequence~~ -N-H-G- sequence, wherein each -G- is complementary to ~~the~~ a cytosine of interest and each -N- individually is selected from the group consisting of C, G, A and T and is complementary to the neighbouring nucleotide of said cytosine of interest.

~~9. The method according to any one of claims 5 to 8, wherein said cytosine is located in a CpG site, and wherein the anchor sequence comprises the sequence -C-H-G-.~~

~~10. The method according to any one of the preceding claims, wherein the anchor sequence comprises at least 2, such as at least 3, for example at least 4, such as in the range of 1 to 10, for example in the range of 2 to 10, such as in the range of 3 to 10, for example in the range of 4 to 10 hydrophobic nucleotides (H).~~

~~11. The method according to any one of the preceding claims, wherein the anchor sequence comprises at least 2, such as at least 3, for example at least 4, such as in the range of 2 to 10, for example in the range of 2 to 5, for example in the range of 3 to 5, such as in the range of 4 to 5 -N-H-G- sequences, wherein each N individually is selected from the group consisting of C, G, A and T, and each sequence –N-G- is complementary to a cytosine of interest and its neighbouring nucleotide.~~

~~12. The method according to any one of the preceding claims, wherein 2 or more NOIs are positioned in a CpG site and the anchor sequence comprises at least 2, such as at least 3, for example at least 4, such as in the range of 2 to 5, for example in the range of 3 to 5, such as in the range of 4 to 5 –C-H-G- sequences.~~

~~13.~~8. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a PCR, wherein said temperature is used as the annealing temperature in one or more cycles of said PCR.

~~14. The method according to any one of the preceding claims, wherein said oligonucleotide is encoded as two or more separate oligonucleotides, such as 2, 3 or more separate oligonucleotides.~~

~~15. The method according to any one of claims 1 to 13, wherein said oligonucleotide is encoded as a single oligonucleotide.~~

~~16.~~9. The method according to any one of the preceding claims ~~1 to 14~~, wherein said oligonucleotide consists of a first and a second nucleic acid sequences, wherein the first nucleic acid sequence comprises the anchor sequence (An) complementary to the target DNA and a first part of a loop sequence not complementary to the target nucleic sequence of interest, and the second nucleic acid sequence comprises a second part of the loop sequence, capable of hybridising at least partly to the first part of the loop sequence, said second nucleic acid sequence further comprising the starter sequence (St), and wherein

the first and the second nucleic acids once hybridized are capable of forming the protruding structure.

~~17. The method according to any one of the preceding claims, wherein the anchor sequence (An) consists of in the range of 8 to 30, for example in the range of 8 to 20, such as in the range of 10 to 20, for example in the range of 15 to 20 nucleotides, wherein one or more of said nucleotides are hydrophobic nucleotide(s).~~

~~18. The method according to any one of the preceding claims, wherein the anchor sequence (An) is at least 85% complementary to the target anchor sequence.~~

~~19. The method according to any one of the preceding claims, wherein the starter sequence (St) is 100% complementary to target starter sequence.~~

~~20.~~10. The method according to any one of the preceding claims, wherein the target anchor sequence and the target starter sequence are positioned within 10, for example within 8, such as within 5 nucleotides from each other.

~~21.~~11. The method according to any one of the preceding claims, wherein Lp and St are selected such that an oligonucleotide consisting of Lp-St has a melting temperature with its complementary sequence of at least 50°C, for example at least 55°C, such as at least 65°C.

~~22. The method according to any one of the preceding claims, wherein Lp and St together consists of at least 15 nucleotides, for example at least 17 nucleotides, such as in the range of 15 to 45 nucleotide, for example in the range of 17 to 35 nucleotides, such as in the range of 17 to 25 nucleotides.~~

~~23. The method according to any one of the preceding claims, wherein Lp does not comprise a hydrophobic nucleotide.~~

~~24.~~12. The method according to any one of the preceding claims ~~1 to 22~~, wherein Lp consists of two or more nucleic acid sequences, and wherein the nucleic acid sequence(s) not covalently linked to the St sequence comprise(s) one or more hydrophobic nucleotides.

~~25.~~13. The method according to any one of the preceding claims, wherein St does not comprise a hydrophobic nucleotide.

~~26.~~14. The method according to any one of the preceding claims, wherein the difference in melting temperature between said oligonucleotide and the methylated target nucleic acid sequence of interest is at least 5°C higher, for example at least 6°C higher, such as in the range of 6 to 15°C higher, for example in the range of 6 to 12°C higher than the difference in melting temperature between said oligonucleotide and the unmethylated target nucleic acid sequence of interest.

~~27.~~15. The method according to any one of the preceding claims, wherein the difference in melting temperature between said oligonucleotide and the methylated target nucleic acid sequence compared to an unmethylated target nucleic acid sequence of interest is at least 1°C higher than the difference in melting temperature between an oligonucleotide of identical sequence except lacking the hydrophobic nucleotides and the methylated target nucleic acid sequence compared to an unmethylated target nucleic acid sequence.

~~28. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a real-time PCR.~~

~~29. The method according to claim 28, wherein said nucleic acid of interest is considered to be methylated if the Ct is lower than the Ct of a control PCR performed using unmethylated target nucleic acid sequence as template.~~

~~30. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a PCR, wherein at least the first cycle of said PCR uses an annealing temperature, which is higher than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is unmethylated.~~

~~31. The method according to any one of the preceding claims, wherein said temperature of step b) is at least 2°C higher than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is unmethylated.~~

~~32. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a PCR, wherein at least the first cycle of said PCR uses an annealing temperature, which is higher than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is unmethylated, and which is at the most 4°C higher, such as at the most 2°C higher, the same or lower than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is methylated.~~

~~33. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a PCR, wherein at least the first cycle of said PCR uses an annealing temperature, which is higher than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is unmethylated, and which is at the most 4°C higher to at the most 4°C lower than the melting temperature between said oligonucleotide and the target nucleic acid sequence of interest when said NOI is methylated.~~

~~34. The method according to any one of the preceding claims, wherein steps b) and c) together comprises performing a PCR, wherein at least the first cycle of said PCR uses an annealing temperature of in the range of 55 to 80 °C.~~

~~35. The method according to claim 34, wherein at least the first cycle of said PCR comprises incubation at the annealing temperature for in the range 30 to 120 sec.~~

~~36. The method according to any of the preceding claims, wherein the concentration of the oligonucleotide during step b) is in the range of 300 to 700 nM.~~

~~37.~~16. The method according to ~~any one of~~ claim~~s~~ 8~~13 to 36~~, wherein said PCR comprises one or more methyl-specific amplification cycles and one or more general amplification cycles, wherein the methyl specific amplification cycle(s) comprise the steps of

a. Melting nucleic acids
b. Annealing and extension under methyl specific conditions

and the general amplification cycles comprise the steps of
a. Melting nucleic acids

b. Annealing and extension under general conditions

wherein the melting comprises incubation at a temperature of at least 90 °C, and wherein the annealing and extension under methyl specific conditions is performed at a higher temperature than annealing and extension under general conditions.

~~38. The method according to any one of claims 34 to 37, wherein annealing is performed in the range of 45 to 90 sec, such as for in the range of 45 to 75 sec, for example for approximately 60 sec.~~

~~39. The method according to any one of claims 37 to 38, wherein said method comprises in the range of 1 to 100 methyl-specific amplification cycles.~~

~~40.~~17. The method according to any one of claims ~~13 to 39~~8 to 16, wherein said PCR is performed using said oligonucleotide as forward primer, wherein said PCR further comprises use of a reverse primer at least 90% identical, for example 100% identical to a sequence downstream of said target nucleic acid sequence of interest.

~~41. The method according to any one of claims 13 to 40, wherein said PCR comprises use of a detectable probe for detection of PCR products, wherein said probe comprises an oligonucleotide at least 90% complementary to a product of said PCR reaction and a detectable label.~~

~~42. The method according to claim 41, wherein said probe comprises one or more hydrophobic nucleotides.~~

~~43.~~18. The method according to any of the preceding claims, wherein target nucleic acid sequence of interest is comprised in DNA purified from a sample~~.~~

~~44. The method according to claim 43, wherein said sample is a sample~~ obtained from an individual suffering from or at risk of having a clinical condition associated with methylation in the nucleic acid of interest.

~~45. The method according to any one of the preceding claims, wherein the method does not comprise a step of pre-treatment of nucleic acid of interest to convert methylated nucleotides to detectable moieties.~~

~~46. The method according to any one of the preceding claims, wherein the method does not comprise a step of bisulfite conversion.~~

~~47.~~19. A method for determining the risk of whether an individual suffers from a clinical condition, or the risk of an individual to contract a clinical condition, or determining the likelihood of effect of a treatment of a clinical condition in an individual, wherein the clinical condition is associated with the methylation status of a NOI in a nucleic acid of interest, said method comprising

a. Providing a sample obtained from said individual;
b. Determining the methylation status of said NOI in said sample by performing the method according to any one of the preceding claims, wherein said methylation status is indicative of the presence of or risk of contracting said clinical condition or of the effects of different treatment regimens on said clinical condition.

~~48. A method for determining the likelihood of effect of a treatment of a clinical condition in an individual in need thereof, wherein the effect of said treatment of said clinical condition is associated with the methylation status of a NOI in a nucleic acid of interest, said method comprising~~

~~a. Providing a sample obtained from said individual;~~
~~b. Determining the methylation status of said NOI in said sample by performing the method according to any one of claims 1 to 43, wherein said methylation status is indicative of the effects of different treatment regimens on said clinical condition.~~

~~49. The method according to any one of claims 47 to 48, wherein the clinical condition is cancer.~~

~~50. The method according to claim 49, wherein the cancer is glioblastoma.~~

~~51.~~20. An oligonucleotide comprising or consisting of the following general structure:

$5'(N)_n\text{-HG-}(N)_m\text{-HG-}(N)_p\text{-HG-}(N)_q\text{-}3'$,

wherein N is any nucleotide or nucleotide analogue; and

G is the nucleotide guanine; and
n is an integer ≥0; and
m is an integer ≥1; and
p is an integer ≥1; and
q is an integer ≥0; and
H is a hydrophobic nucleotide
or
5'$(N)_n$-CGH-$(N)_m$-CGH-$(N)_p$-3',
wherein N is any nucleotide or nucleotide analogue; and
C is the nucleotide cytosine; and
G is the nucleotide guanine; and
n is an integer ≥0; and
m is an integer ≥1; and
p is an integer ≥0; and
H is a hydrophobic nucleotide

wherein said hydrophobic nucleotide has the structure
X-Y-Q
wherein
X is a nucleotide or nucleotide analogue or a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,
Q is an intercalator which is not taking part in Watson-Crick hydrogen bonding; and

Y is a linker moiety linking said nucleotide or nucleotide analogue or backbone monomer unit and said intercalator.

~~52.~~21. The oligonucleotide according to claim ~~51~~20~~-~~, wherein the oligonucleotide comprises or consists of the following general structure:

5'$(N)_n$-HG-$(N)_m$-HG-$(N)_p$-HG-$(N)_q$-HG-$(N)_u$-3', wherein p is an integer ≥1
or
5'$(N)_n$-CGH-$(N)_m$-CGH-$(N)_p$-CGH-$(N)_q$-CGH-$(N)_u$-3', wherein p is an integer ≥0;

wherein
q is an integer ≥1; and
u is an integer ≥0.

~~53. The oligonucleotide according to any one of claims 51 to 52, wherein n is an integer in the range of 0 to 4, for example n is 1.~~

~~54. The oligonucleotide according to any one of claims 51 to 53, wherein m is an integer in the range of 1 to 8, for example in the range of 1 to 6.~~

~~55. The oligonucleotide according to any one of claims 51 to 54, wherein p is an integer in the range of 1 to 50, such as in the range of 1 to 30, for example in the range of 1 to 10, for example in the range of 1 to 6.~~

~~56. The oligonucleotide according to any one of claims 51 to 55, wherein q is an integer in the range of 1 to 50, such as in the range of 1 to 30, for example in the range of 1 to 10, for example in the range of 1 to 6.~~

~~57. The oligonucleotide according to any one of claims 51 to 56, wherein u is an integer in the range of 1 to 50, such as in the range of 1 to 30, for example in the range of 1 to 10, for example in the range of 1 to 6.~~

~~58.~~22. The oligonucleotide according to any of claims ~~51 to 57~~20 to 21, wherein the nucleotide located immediately 5' to at least one H is a cytosine (C).

~~59. The oligonucleotide according to any of claims 51 to 58, wherein all nucleotides located immediately 5' to a H is a cytosine (C).~~

~~60. The method or the oligonucleotide according to any one of the preceding claims, wherein at least one, such as all intercalators, Q, are selected from the group consisting of polyaromates and heteropolyaromates optionally substituted with one or more selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, alkynyl, nitro, amino, alkoxyl and amido.~~

~~61. The method or the oligonucleotide according to any one of the preceding claims, wherein said polyaromates or heteropolyaromates consists of at least 3 rings, for example 4 rings.~~

62. The method or the oligonucleotide according to any one of the preceding claims, wherein the intercalators are selected from the group consisting of benzene, pentalene, indene, naphthalene, azulene, *as*-indacene, *s*-indacene, biphenylene, acenaphthylene, Phenalene, heptalene, phenanthrane, fluoranthene, phenanthroline, phenazine, phenanthridine, anthraquinone, pyrene, anthracene, napthene, phenanthrene, flurene, picene, chrysene, naphtacene, acridones, benzanthracenes, stilbenes, oxalo-pyridocarbazoles, azidobenzenes, porphyrins and psoralens and derivatives thereof.

63. The method or the oligonucleotide according to any one of the preceding claims, wherein at least one, such as all intercalators, Q are comprising or consisting of pyrene or pyrido[3',2':4,5]thieno[3,2-d]pyrimidin-4(1H)-one or 7,9-dimethyl-pyrido[3',2',4,5]thieno[3,2-d]pyrimidin-4(3H)-one.

64. The method or the oligonucleotide according to any one of the preceding claims, wherein at least one, such as all backbone monomer units are selected from the group consisting of phosphoramidites.

65. The method according to any one of claims 1 to 50, wherein the oligonucleotide is an oligonucleotide according to any one of claims 43 to 59.

66. An oligonucleotide comprising or consisting of the following general structure:

5'$(N)_n$-CGH-$(N)_m$-CGH-$(N)_p$-3',

wherein N is any nucleotide or nucleotide analogue; and

C is the nucleotide cytosine; and

G is the nucleotide guanine; and

n is an integer ≥0; and

m is an integer ≥1; and

p is an integer ≥0; and

H is a hydrophobic nucleotide

wherein said hydrophobic nucleotide has the structure

X-Y-Q

wherein

X is a nucleotide or nucleotide analogue or a backbone monomer unit capable of being incorporated into the backbone of a nucleic acid or nucleic acid analogue,

Q is an intercalator which is not taking part in Watson-Crick hydrogen bonding; and

Y is a linker moiety linking said nucleotide or nucleotide analogue or backbone monomer unit and said intercalator.

67. The oligonucleotide according to claim 66, wherein the oligonucleotide comprises or consists of the following general structure:

$5'(N)_n$-CGH-$(N)_m$-CGH-$(N)_p$-CGH-$(N)_q$-3',

wherein

p is ≥1; and

q is an integer ≥0.

68. The oligonucleotide according to any of claims 66 to 67, wherein the oligonucleotide comprises or consists of the following general structure:

$5'(N)_n$-CGH-$(N)_m$-CGH-$(N)_p$-CGH-$(N)_q$-CGH-$(N)_u$-3',

wherein

q is an integer ≥1; and

u is an integer ≥0.

69. The oligonucleotide according to any one of claims 66 to 68, wherein n, m, p, q and u are as defined in any one of claims 53 to 57.

70. The oligonucleotide according to any one of claims 66 to 69, wherein Q and X are as defined in any one of claims 60 to 64.