Claims

1. A multiplex method for detecting different analytes and different subgroups/variations of an analyte in a sample comprising:

(A) contacting the sample with at least twenty (20) different sets of analyte-specific probes for encoding of at least 20 different analytes, each set of analyte-specific probes interacting with a different analyte, wherein when the analyte is a nucleic acid each set of analyte-specific probes comprises at least five (5) analyte-specific probes which specifically interact with different sub-structures of the same analyte, each analyte-specific probe comprising

(aa) a binding element (S) that specifically interacts with one of the different analytes to be encoded, and

(bb) an identifier element (T) having a unique identifier sequence comprising a nucleotide sequence which is unique to the analyte to be encoded (,

wherein the analyte-specific probes of a particular set of analyte-specific probes differ from the analyte-specific probes of another set of analyte-specific probes in the nucleotide sequence of the identifier element (T),

wherein the analyte-specific probes in each set of analyte-specific probes binds to the same analyte and comprises the same nucleotide sequence of the identifier element (T) which is unique to said analyte; and

contacting the sample with at least two different sets of analyte-specific probes for at least one analyte and a variation thereof,

wherein the analyte-specific probes comprised in these different sets interacting with the same analyte, but specifically interact with different sub-structures of the same analyte,

wherein the analyte-specific probes of the first set of analyte-specific probes interacts with a sub-structure which is comprised in all variations of an analyte,

wherein the analyte-specific probes of the second set of analyte-specific probes (subgroup-specific probes) interacts with a sub-structure which is comprised only in a specific variation of the analyte,

wherein the analyte-specific probes of the first set of analyte-specific probes comprise the same identifier element (T) comprising a nucleotide sequence which is unique to the analyte to be encoded (unique identifier sequence), and

wherein the analyte-specific probes of the second set of analyte-specific probes comprise the same identifier element (T) comprising a nucleotide sequence which is unique to the analyte to be encoded (unique identifier sequence),

wherein the identifier elements (T) of the analyte-specific probes of the first set of analyte-specific probes and the identifier elements (T) of the analyte-specific probes of the second set of analyte-specific probes are different for binding different decoding oligonucleotides and/or non-signal decoding oligonucleotides.

(B) contacting the sample with at least one set of decoding oligonucleotides per analyte, wherein in each set of decoding oligonucleotides for an individual analyte each decoding oligonucleotide comprises:

(aa) an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and

(bb) a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;

wherein the decoding oligonucleotides of a set for an individual analyte differ from the decoding oligonucleotides of another set for a different analyte in the first connect element (t); and

(C) contacting the sample with at least a set of signal oligonucleotides, each signal oligonucleotide comprising:

(aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of a translator element (c) comprised in a decoding oligonucleotide, and

(bb) a signal element.

(D) Detecting the signal caused by the signal element;

(E) selectively removing the decoding oligonucleotides and signal oligonucleotides from the sample, thereby essentially maintaining the specific binding of the analyte-specific probes to the analytes to be encoded;

(F) Performing at least three (3) further cycles comprising steps B) to E) to generate an encoding scheme with a code word per analyte,

(G) Performing at least one (1) further cycle comprising steps B) to E) to identify the subgroup-specific probes, wherein in particular the cycle may stop with step (D).

2. The method according to claim 1, wherein the set of analyte-specific probes comprises at least five (5) subgroup-specific probes which specifically interact with different sub-structures of the same variation of an analyte.

3. The method according to any one of claims 1 to 2, wherein all steps are automated, in particular wherein steps B) to G) are automated, in particular by using a robotic system.

4. The method according to any one of claims 1 to 3, wherein each analyte is associated with a specific code word, wherein said code word comprise a number of positions, and wherein each position corresponds to one cycle resulting in a plurality of distinguishable encoding schemes with the plurality of code words.

5. The method according to any one of claims 1 to 4 wherein the code words obtained for the individual analytes in the performed cycles comprise the detected signals and additionally at least one element corresponding to no detected signal.

6. The method according to of any one of claims 1 to 5, wherein the code word zero (0) is generated by using no decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte.

7. The method according to of any one of claims 1 to 6, wherein if at least for one individual analyte a position of the code word is zero (0) in this cycle no corresponding decoding oligonucleotides having an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of a corresponding analyte-specific probe for an individual analyte are used.

8. The method according to of any one of claims 1 to 7, wherein the sample is contacted with at least two different sets of signal oligonucleotides, wherein the signal oligonucleotides in each set comprise a different signal element and comprise a different connector element (C).

9. The method according to of any one of claims 1 to 8, wherein the sample is contacted with

at least a set of non-signal decoding oligonucleotides for binding to a particular identifier element (T) of analyte-specific probes, wherein the decoding oligonucleotides in the same set of non-signal decoding oligonucleotides interacting with the same different identifier element (T),

wherein each non-signal decoding oligonucleotide comprises an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of a unique identifier sequence, and does not comprise a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide.

10. The method according to any one of claims 1 to 9, wherein the sample is contacted with

a set of non-signal oligonucleotides, each non-signal oligonucleotide comprising:

(aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of the translator element (c), and

(bb) a quencher (Q), a signal element and a quencher (Q), or does not comprise a signal element.

11. A kit for multiplex analyte encoding, comprising

(A) at least twenty (20) different sets of analyte-specific probes for encoding of at least 20 different analytes, each set of analyte-specific probes interacting with a different analyte, wherein when the analyte is a nucleic acid each set of analyte-specific probes comprises at least five (5) analyte-specific probes which specifically interact with different sub-structures of the same analyte, each analyte-specific probe comprising

(aa) a binding element (S) that specifically interacts with one of the different analytes to be encoded, and

(bb) an identifier element (T) having a unique identifier sequence comprising a nucleotide sequence which is unique to the analyte to be encoded ,

wherein the analyte-specific probes of a particular set of analyte-specific probes differ from the analyte-specific probes of another set of analyte-specific probes in the nucleotide sequence of the identifier element (T),

wherein the analyte-specific probes in each set of analyte-specific probes binds to the same analyte and comprises the same nucleotide sequence of the identifier element (T) which is unique to said analyte; and

(B) at least one set of decoding oligonucleotides per analyte, wherein in each set of decoding oligonucleotides for an individual analyte each decoding oligonucleotide comprises:

(aa) an identifier connector element (t) comprising a nucleotide sequence which is essentially complementary to at least a section of the unique identifier sequence of the identifier element (T) of the corresponding analyte-specific probe set, and

(bb) a translator element (c) comprising a nucleotide sequence allowing a specific hybridization of a signal oligonucleotide;

wherein the decoding oligonucleotides of a set for an individual analyte differ from the decoding oligonucleotides of another set for a different analyte in the identifier connect element (t); and

(C) a set of signal oligonucleotides, each signal oligonucleotide comprising:

(aa) a translator connector element (C) comprising a nucleotide sequence which is essentially complementary to at least a section of the nucleotide sequence of a translator element (c) comprised in a decoding oligonucleotide, and

(bb) a signal element.

12. An *in vitro* method for diagnosis of a disease selected from the group comprising cancer, neuronal diseases, cardiovascular diseases, inflammatory diseases, autoimmune diseases, diseases due to a viral or bacterial infection, skin diseases, skeletal muscle diseases, dental diseases and prenatal diseases comprising the use of the multiplex method according to any one of claims 1 to 10.

13. An *in vitro* method for diagnosis of a disease in plants selected from the group comprising: diseases caused by biotic stress, preferably by infectious and/or parasitic origin, or diseases caused by abiotic stress, preferably caused by nutritional deficiencies and/or unfavorable environment, said method comprising the use of the multiplex method according to any one of claims 1 to 10.

14. An optical multiplexing system configured to perform for the method according to any one of claims 1 to 10 comprising at least:
    - one reaction vessel configured to contain the kits or part of the kits according to claim 11;
    - a detection unit comprising a microscope, in particular a fluorescence microscope
    - a camera
    - a liquid handling device.

15. An *in vitro* method for screening, identifying and/or testing a substance and/or drug comprising:
    (a) contacting a test sample comprising a sample with a substance and/or drug
    (b) detecting different analytes in a sample by sequential signal-encoding of said analytes with a method according to any one of claims 1 to 10.