

CLAIMS

1. A method for detection of nucleic acids by in situ hybridization of an fixed cellular specimen comprising a hybridization step which is performed in a formamide-free,
5 hybridization buffer that comprise a chaotropic component selected from the group of urea, salts of guanidinium or guanidine and a mixture of two or more members of the group, wherein the nucleic acids are small, non-coding RNA molecules.
2. The method according to any of the preceding claims, wherein the cellular specimen is a
10 standard formalin-fixed and paraffin-embedded (FFPE) tissue section.
3. The method according to any of the preceding claims, wherein the chaotropic component is urea.
- 15 4. The method according to any of the preceding claims, wherein the in situ hybridization probe contains one or more LNA monomers.
5. The method according to any of the preceding claims, wherein the method comprise a step of hybridization wherein said cellular specimen is contacted with a hybridization-
20 solution comprising:
 - at least one non-radioactive labelled probe comprising 7 – 22 nucleotides which are capable of hybridizing to a specific RNA sequence and which comprise one or more LNA monomers.
 - a hybrid stabilizing agent selected form the group of salts of mono- and di-valent
25 cations.
 - urea in a concentration between 0,5 and 5 M;
6. The method according to any of the preceding claims, wherein the hybridization probe is
30 labeled in both 3' and 5'end with digoxigenin.
7. The method according to any of the preceding claims, wherein the method further comprise a step wherein the hybridization signal is visualized by formation of the dark-blue

NBT-formazan precipitate, the specimens are optionally counterstained with nuclear fast red, and further comprise a quantification comprising the steps of:

- taking a number, such as 8-17, random images from within the tumor area,
- checking that said random images contain evident cancer cells, excluding those images which do not, also excluding images with tissue and staining artifacts,
- employing a supervised segmentation based on Bayesian classification trained to recognize blue pixels (i.e. NBT-formazan precipitate) and, if stained with nuclear fast red, red pixels (i.e. pixels colored with nuclear fast red) and purple pixels (i.e. pixels colored with both NBT-formazan and nuclear fast red) to estimate the blue areas (B) and, if stained with nuclear fast red, the red areas (R) and purple areas (P),
- quantify the relative level of the RNA by estimating either the total blue area ($TB = B+P$) and/or, if stained with nuclear fast red, the relative total blue area ($TBR = TB/TR$, wherein total red area $TR = R+P$) and use TB and/or TBR as a measurement of the specific RNA level in the sample.

8. An improved, formamide-free, hybridization buffer for the use of detecting small non-coding RNAs in standard formalin-fixed and paraffin-embedded (FFPE) tissue sections with LNA-probes by in situ hybridization, the buffer comprise:

- a buffer comprising a hybrid stabilizing agent selected from the group of salts of mono- and di-valent cations,
- 0.5 to 5 M of a chaotropic component selected from the group comprised of urea and salts of guanidinium (or guanidine) or a mixture of two or more members of the group,
- Denhardt's solution in an amount of 0 - 2 x, and
- a carrier RNA, such as yeast t-RNA, in an amount of 0- 0.5 mg/ml.

9. The hybridization buffer according to claim 8, wherein the chaotropic component or the mixture of chaotropic components are selected from the group of urea and guanidine hydrochloride.

10. The hybridization buffer according to any of claims 8 or 9, wherein the chaotropic component is urea.

11. The hybridization buffer according to any of claims 8 to 10 wherein the buffer further comprise:

- 2.5 x SSC,
- 2M urea;

- 1 x Denhardt's Solution; and
- 0.25 mg/ml yeast t-RNA.

5 12. A kit for detection of at least one small non-coding RNAs in standard formalin-fixed and paraffin-embedded (FFPE) tissue by in situ hybridization, said kit comprise the improved, formamide-free, hybridization buffer of any of claims 8 to 11 and least one LNA-probe optimized for the specific detection of said one small non-coding RNA.

10 13. The kit according to claim 12 wherein the small non-coding RNA is a microRNA.

14. The kit according to claim 12 or 13 wherein the LNA-probe is labeled both in its 3' and 5'-end with digoxigenin.

15 15. A method of predicting the disease-free survival of a stage II colon cancer patient comprising:

- a) determining the relative level of miR-21 in at least one representative tissue section from a stage II colon cancer of said patient determined by the method of claim 5,
- b) comparing the level of miR-21 in the patient to a set of relative levels of miR-21
20 obtained by the method of claim 5 from a reference panel of stage II colon cancer samples obtained from a reference panel of patients with known disease history,
- c) grouping the reference panel in tertiles according to the relative level of miR-21 determined by the method of claim 5; and
- d) taking the miR-21 level of said at least one representative tissue section from a
25 stage II colon tumor from said patient that falls within the miR-21 level of the upper (high expressing) tertile as indicative of an increased likelihood of short disease-free survival, and taking a miR-21 level of said at least one representative tissue section from a stage II colon tumor from said patient that falls within the miR-21 level of the lower (low expressing) tertile as indicative of an increased likelihood of
30 long disease-free survival.

CLAIMS

1. A method for detection of nucleic acids by in situ hybridization of an fixed cellular specimen comprising a hybridization step which is performed in a formamide-free,
5 hybridization buffer that comprise a chaotropic component selected from the group of urea, salts of guanidinium or guanidine and a mixture of two or more members of the group, wherein the nucleic acids are small, non-coding RNA molecules.
- 10 2. The method according to any of the preceding claims, wherein the cellular specimen is a standard formalin-fixed and paraffin-embedded (FFPE) tissue section.
3. The method according to any of the preceding claims, wherein the chaotropic
15 component is urea.
4. The method according to any of the preceding claims, wherein the in situ hybridization probe contains one or more LNA monomers.
- 20 5. The method according to any of the preceding claims, wherein the method comprise a step of hybridization wherein said cellular specimen is contacted with a hybridization-solution comprising:
 - at least one non-radioactive labelled probe comprising 7 – 22 nucleotides which are capable of hybridizing to a specific RNA sequence and which comprise one or more
25 LNA monomers.
 - a hybrid stabilizing agent selected form the group of salts of mono- and di-valent cations.
 - urea in a concentration between 0,5 and 5 M;
- 30 6. The method according to any of the preceding claims, wherein the hybridization probe is labeled in both 3' and 5'end with digoxigenin.
7. The method according to any of the preceding claims, wherein the method further comprise a step wherein the hybridization signal is visualized by formation of the dark-blue

NBT-formazan precipitate, the specimens are optionally counterstained with nuclear fast red, and further comprise a quantification comprising the steps of:

- taking a number, such as 8-17, random images from within the tumor area,
- checking that said random images contain evident cancer cells, excluding those images which do not, also excluding images with tissue and staining artifacts,
- employing a supervised segmentation based on Bayesian classification trained to recognize blue pixels (i.e. NBT-formazan precipitate) and, if stained with nuclear fast red, red pixels (i.e. pixels colored with nuclear fast red) and purple pixels (i.e. pixels colored with both NBT-formazan and nuclear fast red) to estimate the blue areas (B) and, if stained with nuclear fast red, the red areas (R) and purple areas (P),
- quantify the relative level of the RNA by estimating either the total blue area ($TB = B + P$) and/or, if stained with nuclear fast red, the relative total blue area ($TBR = TB/TR$, wherein total red area $TR = R + P$) and use TB and/or TBR as a measurement of the specific RNA level in the sample.

8. An improved, formamide-free, hybridization buffer for the use of detecting small non-coding RNAs in standard formalin-fixed and paraffin-embedded (FFPE) tissue sections with LNA-probes by in situ hybridization, the buffer comprise 0.5 to 5 M of a chaotropic component selected from the group comprised of urea and salts of guanidinium (or guanidine) or a mixture of two or more members of the group.

9. The hybridization buffer according to claim 8, wherein the chaotropic component or the mixture of chaotropic components are selected from the group of urea and guanidine hydrochloride.

10. The hybridization buffer according to any of claims 8 or 9, wherein the chaotropic component is urea.

11. The hybridization buffer according to any of claims 8 to 10 wherein the buffer further comprise:

- a hybrid stabilizing agent selected from the group of salts of mono- and di-valent cations.
- urea in a concentration between 0,5 and 5 M;
- Denhardt's Solution in an amount of 0 – 2 x; and
- a carrier RNA, such as yeast t-RNA, in an amount of 0 – 0.5 mg/ml.

12. A kit for detection of at least one small non-coding RNAs in standard formalin-fixed and paraffin-embedded (FFPE) tissue by in situ hybridization, said kit comprise the improved, formamide-free, hybridization buffer of any of claims 8 to 11 and least one LNA-probe optimized for the specific detection of said one small non-coding RNA.

5

13. The kit according to claim 12 wherein the small non-coding RNA is a microRNA.

14. The kit according to claim 12 or 13 wherein the LNA-probe is labeled both in its 3' and 5'-end with digoxigenin.

10

15. A method of predicting the disease-free survival of a stage II colon cancer patient comprising:

15

a) determining the relative level of miR-21 in at least one representative tissue section from a stage II colon cancer of said patient determined by the method of claim 5,

b) comparing the level of miR-21 in the patient to a set of relative levels of miR-21 obtained by the method of claim 5 from a reference panel of stage II colon cancer samples obtained from a reference panel of patients with known disease history,

20

c) grouping the reference panel in tertiles according to the relative level of miR-21 determined by the method of claim 5; and

25

d) taking the miR-21 level of said at least one representative tissue section from a stage II colon tumor from said patient that falls within the miR-21 level of the upper (high expressing) tertile as indicative of an increased likelihood of short disease-free survival, and taking a miR-21 level of said at least one representative tissue section from a stage II colon tumor from said patient that falls within the miR-21 level of the lower (low expressing) tertile as indicative of an increased likelihood of long disease-free survival.