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(54) Titre : MICRORESEAU POUR L'ANALYSE DE SEQUENCES NUCLEOTIDIQUES
(54) Title: MICROARRAY FOR THE ANALYSIS OF NUCLEOTIDE SEQUENCES

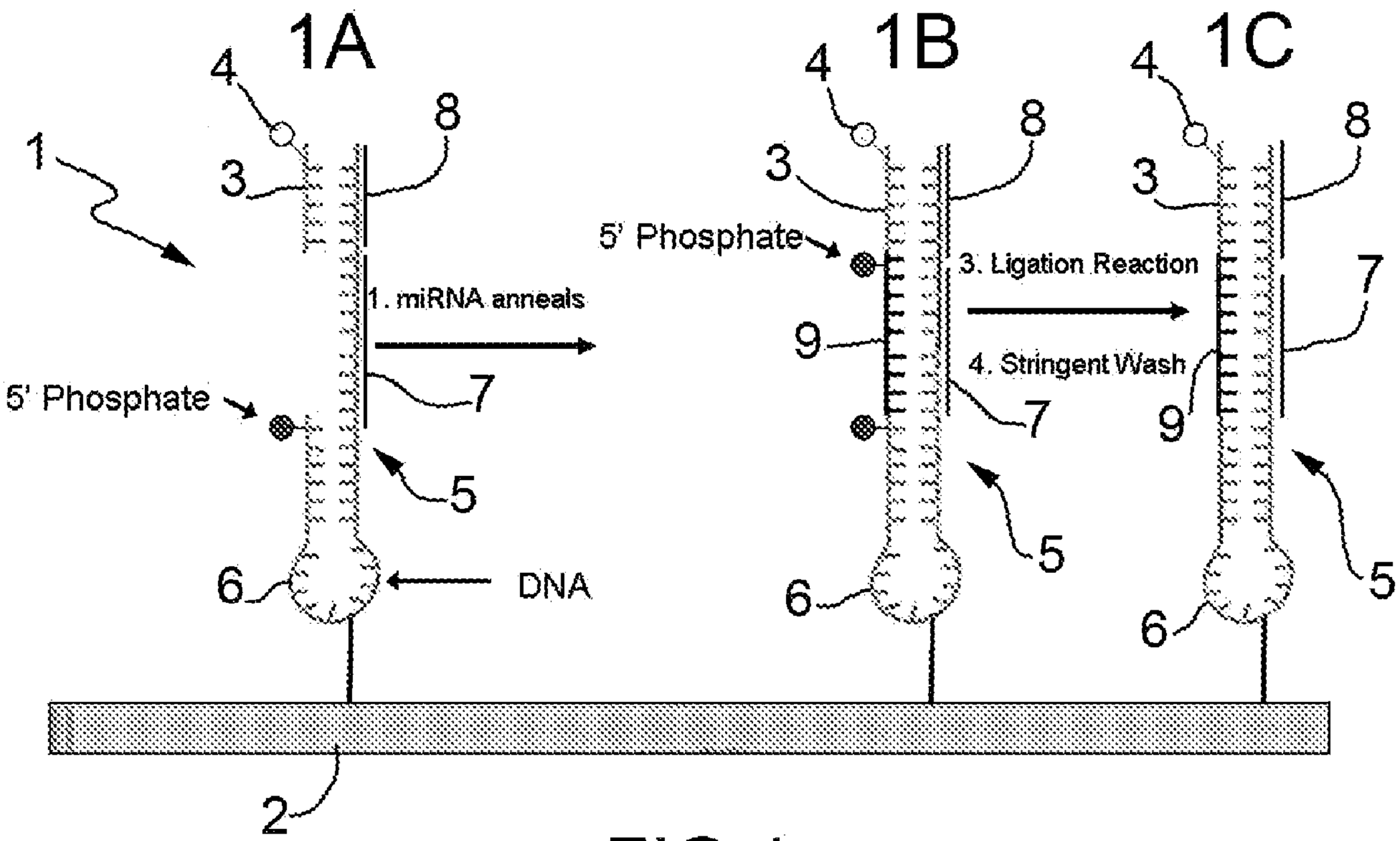

FIG.1

(57) Abrégé/Abstract:
A microarray for the analysis of single-stranded nucleotide sequences, in particular miRNAs, comprising a plurality of probe strands (5) bound to a substrate (2) and adapted to form respective probe hybrids (1) with a labelling segment (3) bound to a fluorophore

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(13) A1

(57) Abrégé(suite)/Abstract(continued):
moiety (4) and with a ligating segment. Each of the probe strands (5) consists of a complementary ligating sequence hybridised in use to the ligating segment, a complementary labelling sequence (8) hybridised in use to the labelling segment (3), and a probe sequence (7) positioned between the complementary ligating sequence and the complementary labelling sequence (8) and adapted to selectively ligate a target single-stranded nucleotide sequence.

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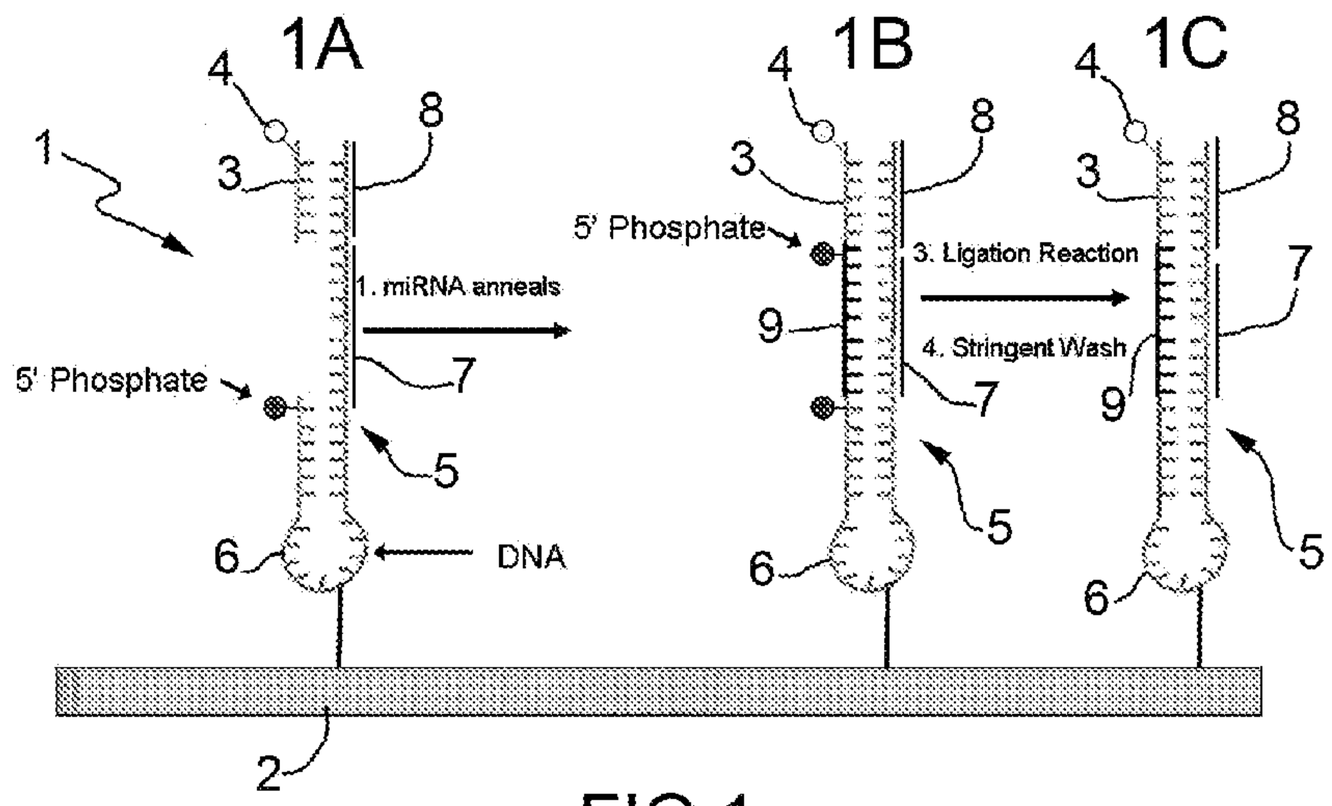

FIG.1

(57) **Abstract**: A microarray for the analysis of single-stranded nucleotide sequences, in particular miRNAs, comprising a plurality of probe strands (5) bound to a substrate (2) and adapted to form respective probe hybrids (1) with a labelling segment (3) bound to a fluorophore moiety (4) and with a ligating segment. Each of the probe strands (5) consists of a complementary ligating sequence hybridised in use to the ligating segment, a complementary labelling sequence (8) hybridised in use to the labelling segment (3), and a probe sequence (7) positioned between the complementary ligating sequence and the complementary labelling sequence (8) and adapted to selectively ligate a target single-stranded nucleotide sequence.

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MICROARRAY FOR THE ANALYSIS OF NUCLEOTIDE SEQUENCES

TECHNICAL FIELD

The present invention relates to a microarray for the analysis of nucleotide sequences.

BACKGROUND ART

Hereinafter the expression “probe strand” indicates an oligonucleotide which is covalently or non-covalently attached to a substrate for the formation of microarrays and comprises a probe sequence which is complementary to a specific nucleotide sequence to be analysed.

Hereinafter the expression “labelling segment” indicates an oligonucleotide, to which a fluorophore moiety is bound and which is adapted to hybridise to a complementary sequence of the probe strand.

Hereinafter the expression “ligating segment” indicates an oligonucleotide adapted to hybridise to a complementary sequence of the probe strand and to ligate an end thereof to a nucleotide sequence hybridised or partially hybridised to the probe strand through a ligating step.

Hereinafter the expression “probe hybrid” indicates a complex formed by a probe strand and at least one labelling sequence hybridised one to the other.

Hereinafter the expression “template strand” indicates

a nucleotide strand used to generate a probe strand. The microarray formed by immobilised template strands is defined as microarray template.

The probe strands are transferred to a surface by means of the Supramolecular Nano Stamping (SuNS) technique. SuNS is a molecular stamping technique for generating microarrays, which is disclosed and claimed in PCT patent application WO2006112815 herein incorporated by reference. In particular, the method consists in the generation of nucleotide strands by replicating template strands, and in the subsequent stamping of these molecules on a new substrate by means of the SuNS technique.

MicroRNAs (miRNA) belong to a class of small regulatory single-stranded RNA molecules, ranging from 21 to 23 nucleotides in length, the roles of which in development and disease are increasingly acknowledged. They act as gene expression down-regulators, altering the stability or translational efficiency of messenger RNAs (mRNAs) with which they share partial sequence complementarity, and are predicted to affect up to one-third of all human genes.

miRNA molecules are encoded by genes, from the DNA of which they are transcribed but not translated into protein (non-coding RNA): each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a mature and functional miRNA.

Currently, microarray based analysis is the most widely effective and employed tool to detect and compare miRNA expression profiles from different tissue or cell samples.

miRNA analyses by means of microarray techniques have the drawback resulting from the different melting temperatures (hereinafter referred to as Tms) and therefore the problem of the thermal stability of the hybrids which are formed. This situation is the result of the high variability of the CG content of the miRNAs. As may be apparent, this problem may compromise the overall analysis technique, as it does not provide the required selectivity criteria.

Another drawback occurring in analyses performed by means of microarray techniques is labelling these small RNA molecules. It has experimentally been shown that common labelling of these small targets necessarily implies an uncertainty in the analytical evaluation of the labelling response.

Although methods currently exist allowing the analysis of miRNAs by microarray, these methods have however not satisfactorily solved the above mentioned drawbacks.

DISCLOSURE OF INVENTION

It is the object of the present invention to provide microarrays, the technical features of which allow to

provide nucleotide sequence analyses without needing to label the nucleotide sequences themselves and without the method of analysis being conditioned by the Tms of the hybrids formed thereby.

It is an object of the present invention to provide a microarray for the analysis of single-stranded nucleotide sequences, the essential features of which are claimed in claim 1, and the preferred and/or auxiliary features of which are claimed in claims 2 and 5.

It is a further object of the present invention to provide a method for generating microarrays for the analysis of single-stranded nucleotide sequences, the essential features of which are claimed in claim 6, and the preferred and/or auxiliary features of which are claimed in claims 7-9.

It is still another object of the present invention to provide a process for the analysis through microarrays for the analysis of single-stranded nucleotide sequences according to claim 10.

BRIEF DESCRIPTION OF THE DRAWINGS

The following example is provided by way of non-limitative illustration for a better understanding of the invention with the aid of the figures in the accompanying drawing, in which:

Figure 1 and Figure 2 show the operation sequences of

the microarray which is the object of the present invention respectively with a target miRNA and a miRNA other than the target miRNA;

Figure 3 shows the replicating step for generating the probe strand;

Figure 4 shows the probe strand bound to the substrate for generating the microarray which is the object of the present invention; and

Figure 5 shows the secondary structure of the hairpin structure of a probe strand of the microarray which is the object of the present invention.

BEST MODE FOR CARRYING OUT THE INVENTION

In Figures 1 and 2 numeral 1 indicates a probe hybrid bound to a substrate 2. The probe hybrid consists of a labelling segment 3 which has a fluorophore moiety 4 bound thereto, and of a probe strand 5 bound to substrate 2. Probe strand 5 consists of a hairpin structure 6, a probe sequence 7 adapted to selectively bind the miRNA to be analysed and occurring as a single strand, and a complementary labelling sequence 8 hybridised in use to labelling segment 3.

In Figure 1A probe hybrid 1 is bound to substrate 2 to generate the microarray which is the object of the present invention. In Figure 1B probe hybrid 1 specifically binds target miRNA 9 by means of probe sequence 7. At this point,

a ligating step is performed in order to ligate target miRNA 9 to labelling segment 3 and to the 5'phosphate end of hairpin structure 6. Subsequently, a washing step is performed to dehybridise labelling segment 3 from complementary labelling sequence 8. As shown in Figure 1C, if target miRNA 9 has specifically bound to probe sequence 7, the ligating step successfully ligates labelling segment 3 to target miRNA 9. Labelling segment 3 thereby remains bound to probe strand 5 even following the step of washing and may therefore be detected by means of the common fluorescence techniques in virtue of the presence of fluorophore moiety 4.

Alternatively, as shown in Figures 2A-2C, if the miRNA bound to probe sequence 7 is not target miRNA 9 but instead a different miRNA 9a, the latter will have regions X which are not complementary to probe sequence 7. The presence of these non-complementary regions X impairs the action of the ligating reaction and therefore ligation of labelling segment 3 and miRNA 9a. The absence of this ligation results in labelling segment 3 not remaining bound to probe strand 7 following the step of washing and therefore not being detected as fluorophore moiety 4 is missing.

If the non-complementary region X between miRNA 9a and probe sequence 7 is at the centre of miRNA 9a and therefore external to the area sensitive to ligase, an enzyme may be used, such as for instance T7 Endonuclease, which may

introduce a single or double cut of the strand in the area of non-complementarity. After the action of the enzyme, the fluorophore moiety will be removed thus obtaining the desired analytic result.

A variant (not shown in the Figures) of the probe strand of the present invention relates to a strand that does not have hairpin structure 6, but instead comprises a complementary ligating sequence hybridised in use to a ligating segment. In other terms, the hybridisation between the complementary ligating sequence and the ligating segment replaces hairpin structure 6.

In this case, the ligating step acts on the phosphorylated end of the ligating segment in order to ligate it to the miRNA.

Figure 3 shows a template strand 10 which is formed by a first sequence 11a complementary to a promoter sequence disclosed hereafter and attached to a substrate 12, a hairpin sequence 11b, a sequence 11c identical to target miRNA 9 and a sequence 11d identical to labelling sequence 3. A replication is performed on template strand 10 (Figures 3A and 3B) from a promoter sequence 13, which has a free 3′-phosphate end and comprises a portion 14 complementary to hairpin sequence 11b to subsequently generate hairpin structure 6. Portion 14 complementary to hairpin sequence 11b ends with a free 5′phosphate end and is bound to a functional moiety 15 to allow stamping

according to the SuNS technique.

Once stamping according to the SuNS technique is completed, promoter sequence 13 hybridises by its 5'phosphate portion to hairpin sequence 11b in order to generate hairpin structure 6. Probe strands 5 shown in Figure 4 and forming the microarray object of the present invention together with labelling segments 3, are generated in this manner.

If the aim is to generate the probe strand without hairpin structure 6, the promoter strand does not comprise portion 14, whereas the sequence disclosed as hairpin sequence 6 is a sequence identical to that of the ligating segment that will hybridise to the strand generated by replication.

Furthermore, stamping is not necessarily performed according to the SuNS technique.

EXPERIMENTAL PART

The Applicant has found that for the probes to have the best efficacy for the detection of the miRNA, there must be a structure designed with the following characteristics:

- Hairpin of length between 20-40 nucleotides, allowing to create a rigid probe structure on the microarray surface; Tm between 75-85°C and GC content between 70-90%: the described structure must be stable over a wide range of temperatures (20-90°C), in order to avoid

any, even if partial, denaturation, allowing for a specific sample hybridization; the hairpin structure must be the only possible structure for the described nucleotide sequence.

- Detector sequence of length between 15-20 nucleotides. It was designed with a Tm of about 45-55°C and 50-60% GC content. Such characteristics were found to allow for better simultaneous hybridization of both miRNA sample and detector, without being limited by their respective Tms. Finally, and very importantly, the selected range of Tms for the universal detector sequence allows for its easy removal using a stringent wash at a temperature which does not destabilize the hairpin structure.

- SuNS ARRAY PREPARATION -

Generated probe 5 has a hairpin sequence 6 (SEQ ID NO:1) having a Tm of 78.9°C and a GC content of 81.8% and a complementary detector sequence 8 (SEQ ID NO:2) having a Tm of 48.4°C and a GC content of 57.1%, to be hybridised to a universal detector sequence (SEQ ID NO:3). As is shown in Figure 4, a specific miRNA binding sequence 7 is located between hairpin sequence 6 and complementary detector sequence 8.

SuNS template 10 used to generate the above mentioned probe 5 has a primer binding sequence 11a (SEQ ID NO:4) having a Tm of 49.3°C and a GC content of 60%, a hairpin sequence 11b (SEQ ID NO:5) and a detector sequence 11d (SEQ

ID NO:3). As is shown in Figure 3a, a specific miRNA sequence 11c is located between hairpin sequence 11b and detector sequence 11d.

The hairpin secondary structure at 37°C is shown in Figure 5.

SEQ ID NO:1: GCCCGGGCGCCGTTCAAGAGACGGCGCCCGGGC

SEQ ID NO:2: CATGTGTCGTGCCT

SEQ ID NO:3: AGGCACGACACATG

SEQ ID NO:4: CCGTCTCTTGAACGG

SEQ ID NO:5: GCCCGGGCG

- miRNA DETECTION -

The experimental procedure used for miRNA detection is as follows.

Sample Hybridization procedure:

An equimolar aliquot of purified and 5'phosporilated miRNA (or total RNA) sample and universal labeled-detector were prepared in 1X hybridization buffer (final concentration: 1 µM).

The sample was denatured at 90°C for 2 min and hybridization was carried on a Tecan HS Pro hybridization station, using the following protocol:

- Slide wash at 75°C, with SSC 0.1X, 20 sec soak + 20 sec flow;
- Sample injection at 75°C;
- Sample hybridization at 45°C for 1 hour, with constant agitation;

- Slide wash at 35°C with SSC 2X-SDS 0.1%, 30 sec soak + 30 sec flow, 3 cycles;
- Slide wash at 30°C with SSC 2X, 1 min soak + 1 min flow, 2 cycles;
- Slide wash at 25°C with SSC 1X, 1 min soak + 1 min flow, 2 cycles;
- Slide wash at 20°C with SSC 0.1X, 20 sec soak + 20 sec flow, 2 cycles;
- Slide rinse at 20°C with ddWater, 10 sec soak + 10 sec flow, 1 cycle;
- Slide drying with nitrogen.

The sample hybridization was followed by a double ligation reaction using T4-ligase. The reaction mix was prepared as follows:

T4-ligase: 0.3 U/µl

T4-ligase buffer: 1X

Final volume: 150 µl.

The ligase-reaction was carried out on a Tecan hybridization station, using the following protocol:

- Slide wash at 30°C, with SSC 0.1X, 20 sec soak + 20 sec flow;
- Sample injection at 28°C;
- Sample hybridization at 28°C for 16 hours, with constant agitation;
- Slide wash at 70°C with SSC 0.2X-SDS 0.1%, 30 sec soak + 30 sec flow, 5 cycles;

- Slide wash at 50°C with SSC 2X, 1 min soak + 1 min flow, 2 cycles;
- Slide wash at 30°C with SSC 1X, 1 min soak + 1 min flow, 2 cycles;
- Slide wash at 25°C with SSC 0.1X, 20 sec soak + 20 sec flow, 2 cycles;
- Slide rinse at 20°C with ddWater, 10 sec soak + 10 sec flow, 1 cycle;
- Slide drying with nitrogen.

The described protocol includes the stringent wash at 70°C that allows for labeled-detector removal in case of a mismatch introduced by an incorrect miRNA hybridization.

As is apparent from the above, the analysis technique for miRNAs with microarrays, which is the object of the present invention, does not provide for the labelling of the target miRNA, which is usually the most critical step during RNA manipulation, and does not depend on the Tm of the miRNA hybrids, as dehybridisation concerns the hybrid formed by labelling segment 3.

Furthermore, such a method of detection has the advantages relating to the use of total RNA without first having to purify miRNAs, and to the fact that the ligation reaction introduces a level of specificity that cannot be achieved by hybridization alone.

As may appear obvious to a person skilled in the art and as may be inferred from the claims, the microarray

object of the present invention may be applied not only to the analysis of miRNA but also to any nucleotide sequence for the most various purposes, for instance also for genotyping DNA.

As a matter a fact, the advantages related to the absence of labelling of the target sequences and to the non-dependence on the Tms may be exploited for the analyses of many other nucleotide sequences.

CLAIMS

1. A microarray for the analysis of single-stranded nucleotide sequences characterised in that it comprises a plurality of probe strands (5) bound to a substrate (2) and adapted to form respective probe hybrids (1) with a labelling segment (3) bound to a fluorophore moiety (4) and to a ligating segment; each of said probe strands (5) consisting of a complementary ligating sequence hybridised in use to the ligating segment, a complementary labelling sequence (8) hybridised in use to the labelling segment (3), and a probe sequence (7) positioned between said complementary ligating sequence and said complementary labelling sequence (8) and adapted to selectively ligate a target single-stranded nucleotide sequence.

2. The microarray for the analysis of single-stranded nucleotide sequences according to claim 1, characterised in that the complementary ligating sequence and the ligating segment are connected to one another so as to form a hairpin structure (6).

3. The microarray for the analysis of single-stranded nucleotide sequences according to claim 1, characterised in that said single-stranded nucleotide sequence is a miRNA.

4. The microarray according to claim 3, characterised in that said hairpin (6) has a length of 20 to 40 nucleotides, a Tm of 75 to 85°C and a GC content of 70 to 90%.

5. The microarray according to claim 4, characterised in that said detector sequence (3) has a length of 15 to 20 nucleotides, a Tm of 45 to 55°C and a GC content 50 to 60%.

6. A method for generating microarrays for the analysis of single-stranded nucleotide sequences, said method being characterised in that it comprises a step of replicating template strands (10) from a promoter sequence (13) for generating probe strands (5), and a subsequent step of stamping probe strands (5) generated by replication from said promoter sequence (13) on a substrate; said method being characterised in that said template strands (10) sequentially comprise a sequence (11a) adapted to bind the promoter sequence and bound to a substrate (12) of a support of a template microarray, a ligating sequence (11b) identical to a sequence of a ligating segment, which is adapted to be hybridised to the molecules generated in the replicating step, a sequence (11c) identical to a target single-stranded nucleotide sequence, and a labelling sequence (11d) identical to a sequence of a labelling segment bound to a fluorophore moiety and adapted to be hybridised to the molecules generated in the replicating step.

7. The method according to claim 6, characterised in that said promoter sequence (13) comprises a portion (14), the sequence of which is identical to that of said ligating segment and therefore complementary to the sequence

generated by replicating the ligating sequence (11b) in order to generate a hairpin structure (6) following dehybridisation from the template strand.

8. The method according to claim 7, characterised in that said portion (14) is bound to a functional moiety (15) to allow stamping according to the SuNS technique.

9. The method according to claim 8, characterised in that said promoter sequence (13) has a length of 20 to 45 nucleotides, a Tm of 45 to 60°C, and a GC content of 50 to 60%, and in that it forms no stable secondary structure at the second strand synthesis annealing temperature.

10. A process of analysis by means of microarrays according to one of claims 1 to 5, characterised in that it comprises the steps of:

- phosphorylating the strand to be analysed;

- simultaneously hybridising said probe strands (5) to the nucleotide sequences to be analysed, to the labelling segments (3) and also to the ligating segments when the hairpin structure (6) is not provided.

- promoting a ligation reaction between the ligating segment or the hairpin structure (6), the single small single-stranded molecule of RNA (9, 9a) and the labelling segment (3);

- stringently washing; and

- detecting the fluorophore moieties still present in the microarray.

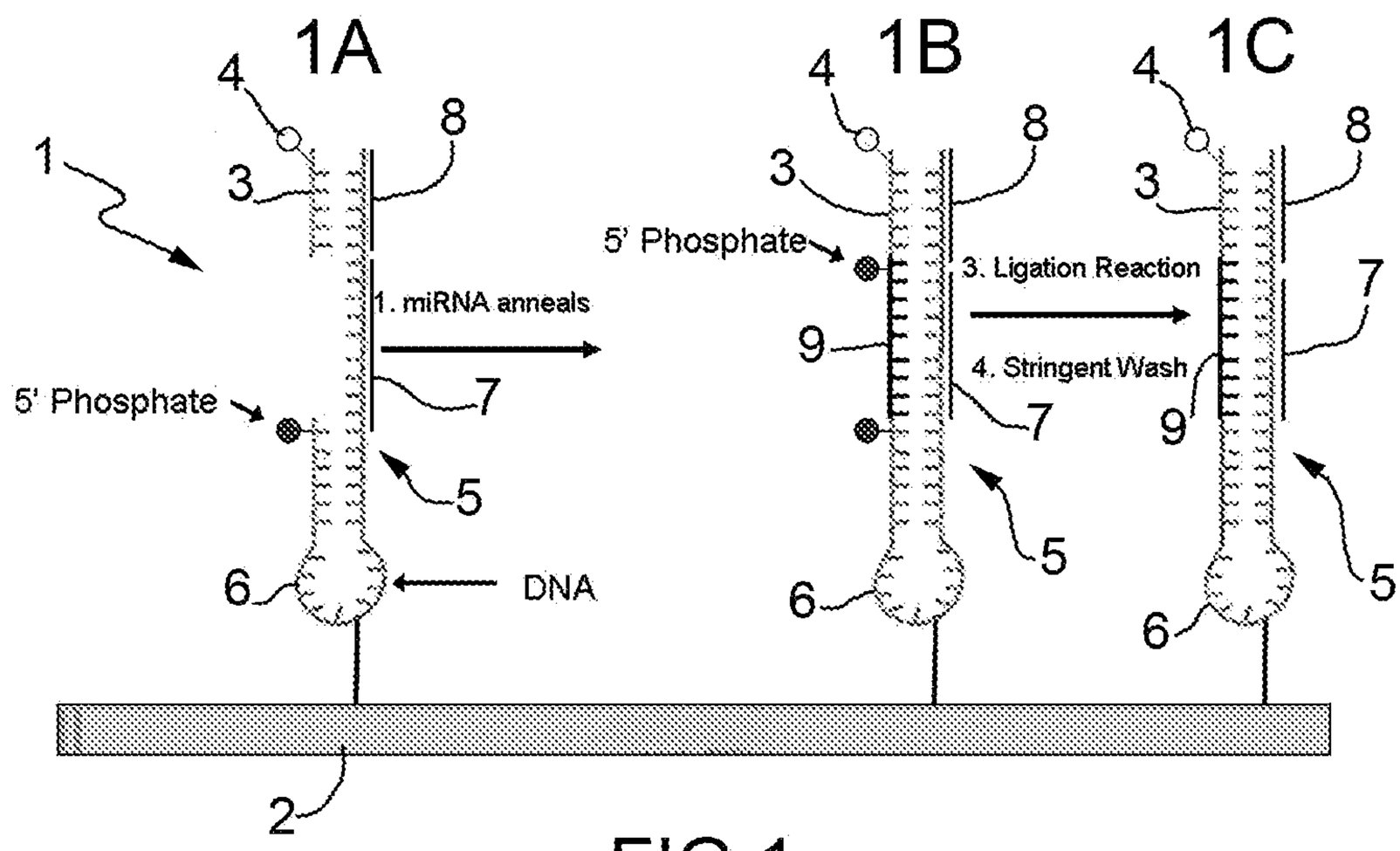

1A
1B
1C
4
8
3
1
5' Phosphate
1. miRNA anneals
7
5
6
DNA
2
9
3. Ligation Reaction
4. Stringent Wash

FIG.1

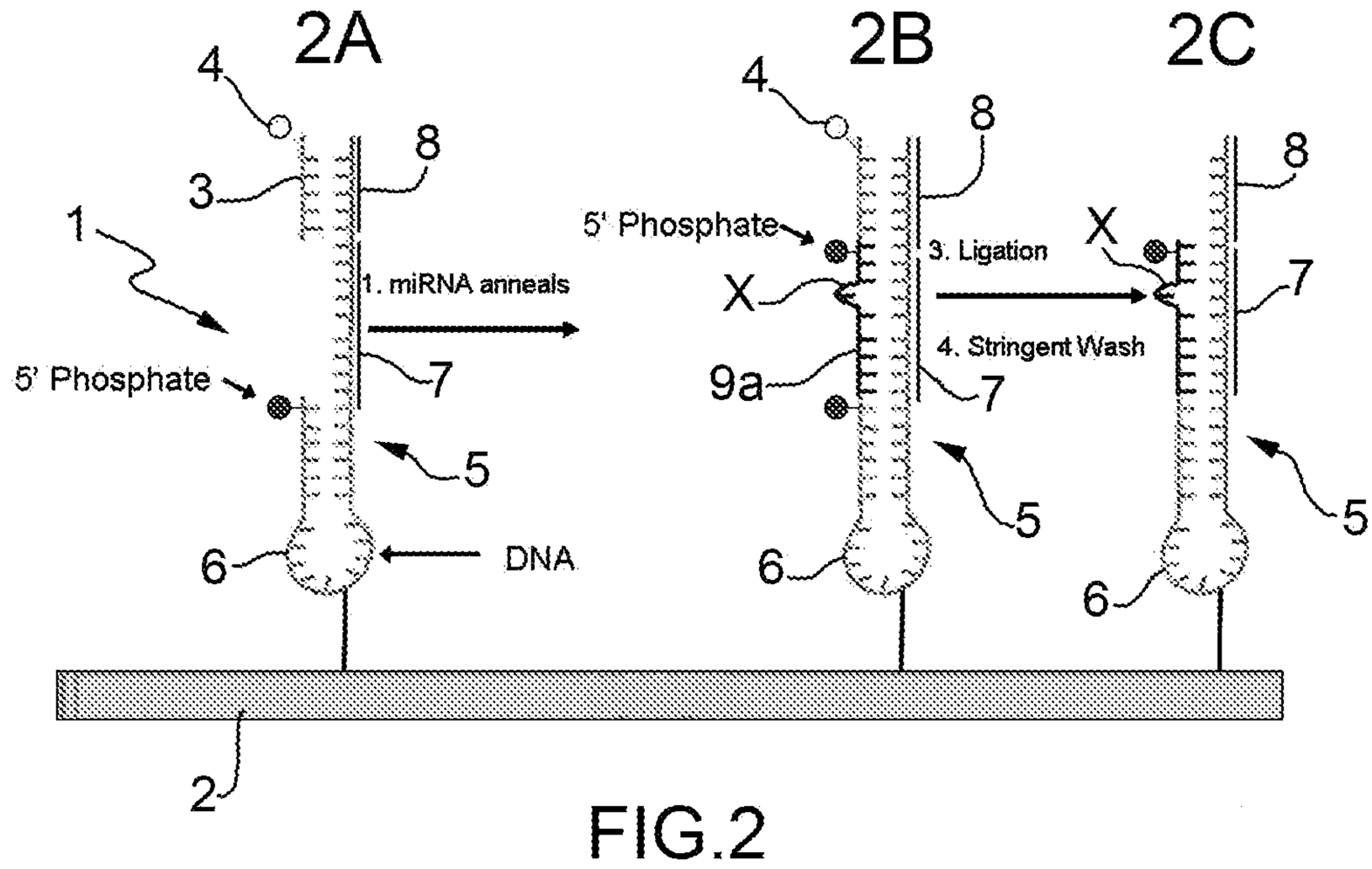

2A
2B
2C
4
8
3
1
5' Phosphate
1. miRNA anneals
7
5
6
DNA
X
9a
3. Ligation
4. Stringent Wash
2

FIG.2

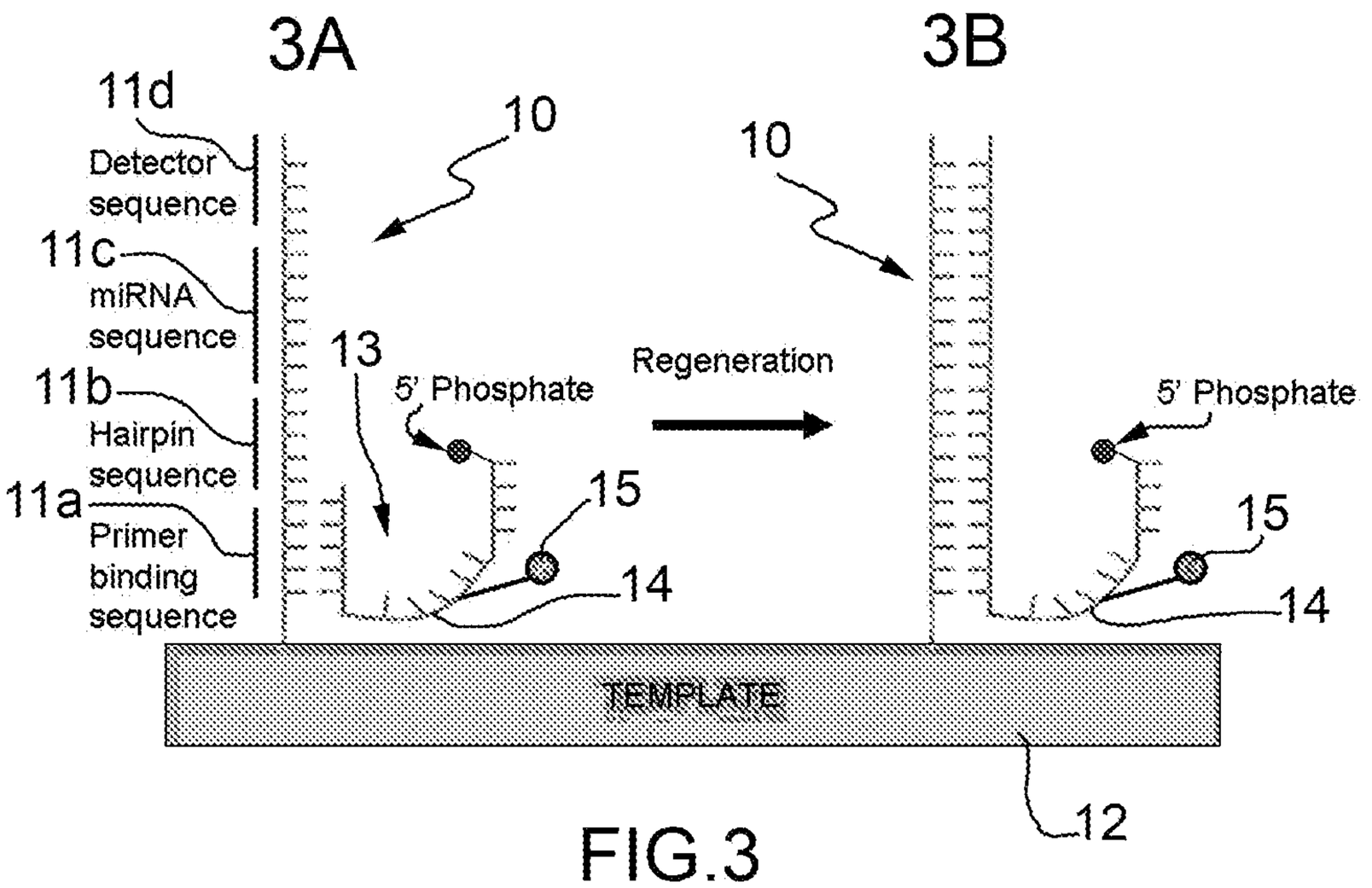

3A
3B
11d
Detector sequence
11c
miRNA sequence
11b
Hairpin sequence
11a
Primer binding sequence
10
10
13
5' Phosphate
Regeneration
15
14
5' Phosphate
15
14
TEMPLATE
12

FIG.3

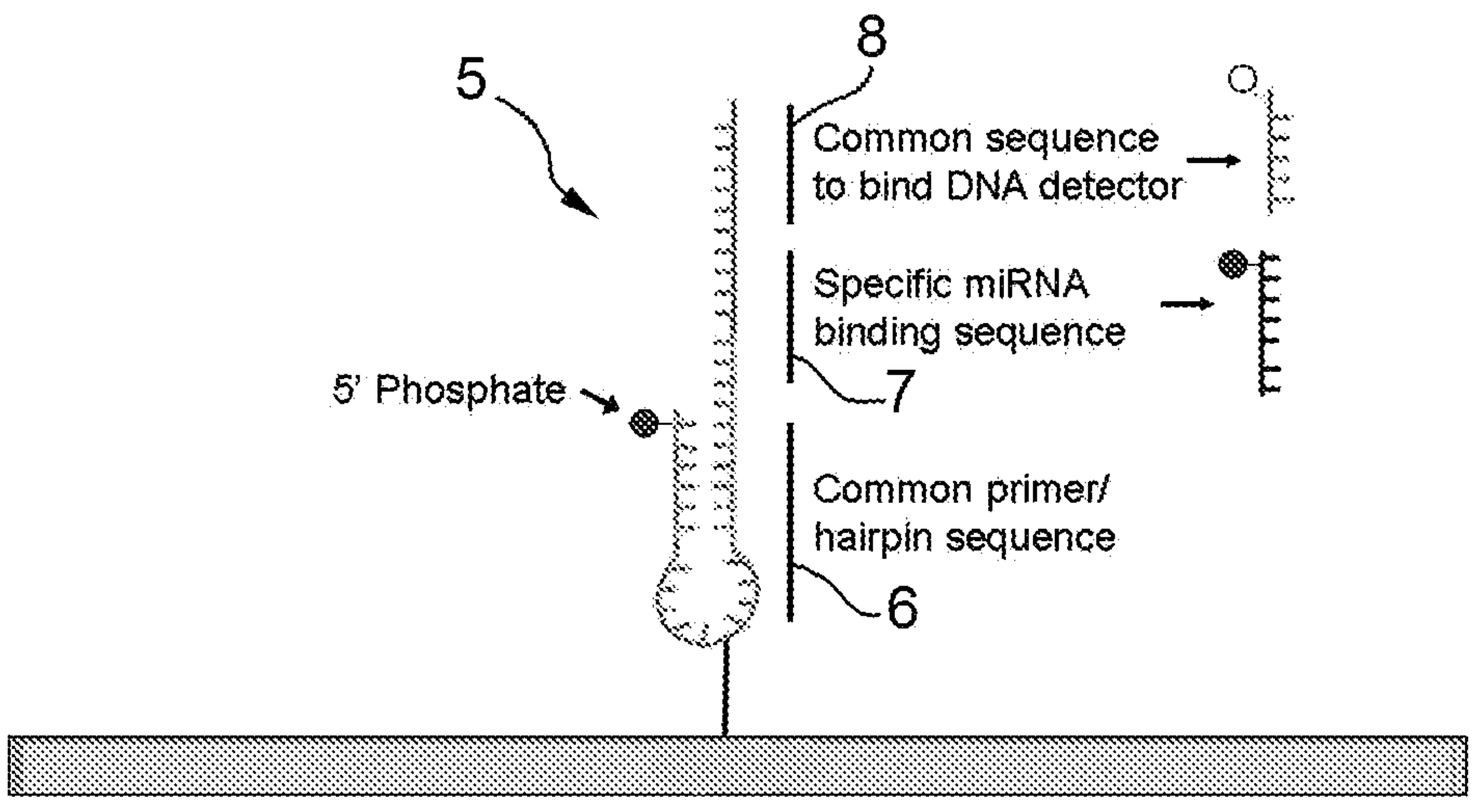

5
8
Common sequence to bind DNA detector
Specific miRNA binding sequence
7
5' Phosphate
Common primer/ hairpin sequence
6

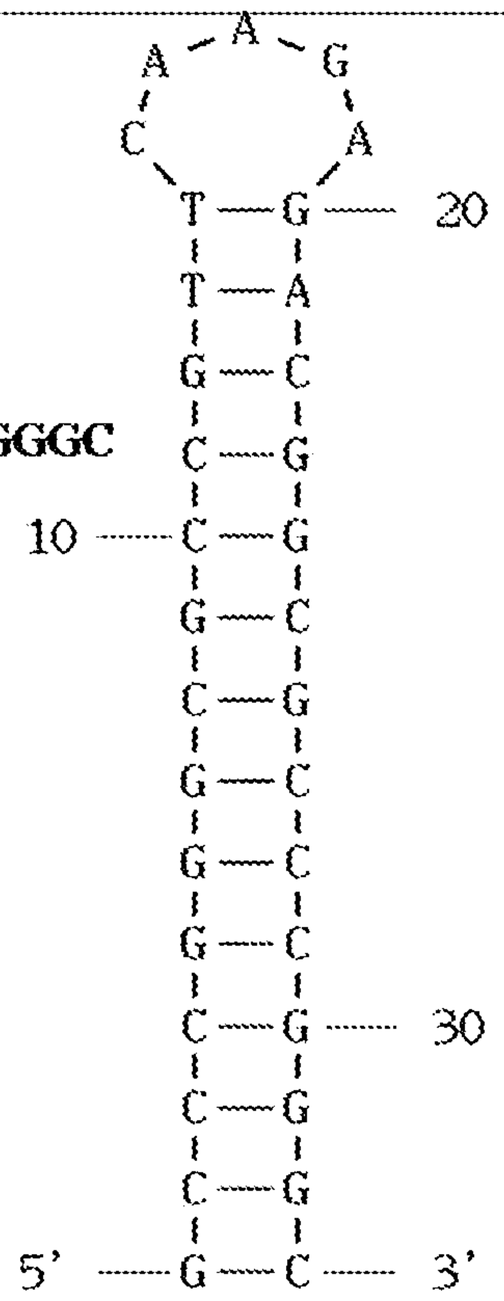
FIG.4

HAIRPIN (S1-L-S2):
GCCCGGGCGCCGTTCAAGAGACGGCGCCCGGGC

Tm (°C):
78.9

GC CONTENT (%):
81.8

LENGTH:
33



Hairpin secondary structure at 37°C

FIG.5

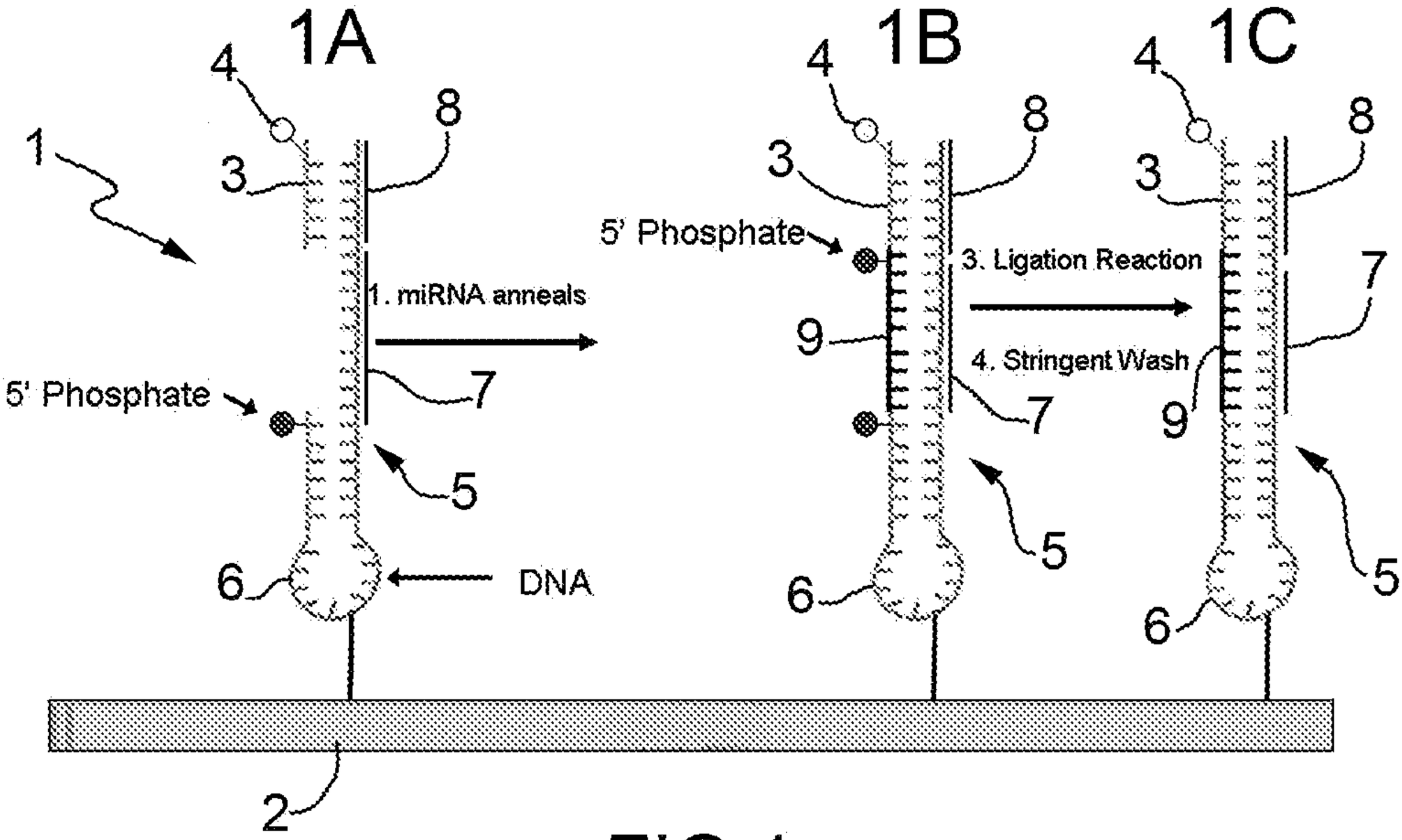

1A
1B
1C
4
8
3
1
1. miRNA anneals
5' Phosphate
7
5
6
DNA
2
5' Phosphate
9
3. Ligation Reaction
4. Stringent Wash

FIG.1