



Solid phase isothermal amplification

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(54) Title: SOLID PHASE ISOTHERMAL AMPLIFICATION

(57) Abstract: The present invention relates to an amplification technology for multiple, rapid, sensitive, specific detection of nucleic acids in samples originating from the environment, water, food, animals, plants, microorganisms, cell cultures, or humans. In particular, the present invention relates to isothermal amplification and detection of nucleic acids using a method called Solid Phase Loop Mediated Amplification (SP-LAMP) including a solid support amplification step. The invention also relates to kits and uses of the method.

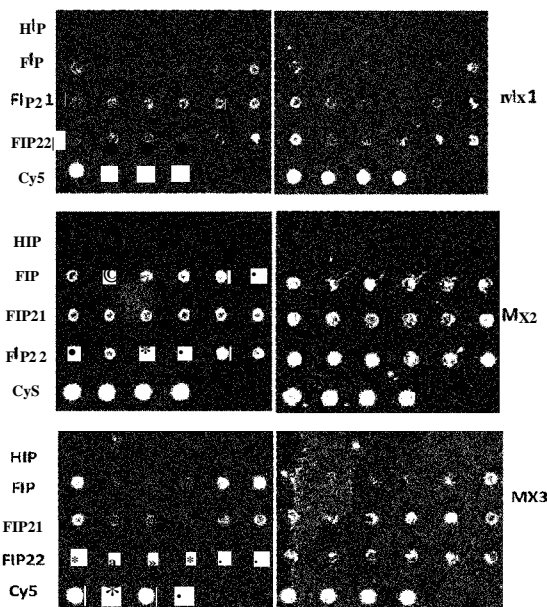


Fig. 12

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SOLID PHASE ISOTHERMAL AMPLIFICATION

Technical field of the invention

The present invention relates to an amplification technology for multiple, rapid,
5 sensitive, specific detection of nucleic acids in samples originating from the
environment, water, food, animals, plants, microorganisms, cell cultures, or
humans. In particular, the present invention relates to isothermal amplification
and detection of nucleic acids using a method called Solid Phase Loop Mediated
Amplification (SP-LAMP) including a solid support amplification step. The invention
10 also relates to kits and uses of the method.

Background of the invention

Nucleic acid amplification technologies are presently utilized in a variety of very
different disciplines such as medicine, forensic science, research applications and
15 disease diagnostics. Disease may originate and spread from many different
habitats, including plants, microorganisms, animals, humans or food. As an
example, more than 200 known diseases are transmitted through foods or food
products. Despite recent advancement in food safety, developing countries are
still experiencing the burden of foodborne pathogenesis. It is recognized that non-
20 typhoidal *Salmonella spp*, *Campylobacter jejuni*, verotoxin producing *Escherichia*
coli O157: 1-17, *Listeria monocytogenes* and *Shigella dysenteriae* are the most
common foodborne pathogens. The globalized agro-food production is becoming
more and more elongated. Food moves across borders and continents in shorter
time frames. Consequently, contaminated foods have often been consumed before
25 authorities can react. Conventional culture methods or molecular-based methods
have been the main workhorses for food safety control; they are expensive, time-
consuming, require sample enrichment, sample preparation, target purification,
target amplification and comes with the inability for online testing.

30 Food safety is a major public health threat. For example, Salmonellosis, an
infectious disease caused by *Salmonella spp*. is one of the most common food-
borne diseases worldwide. It is estimated that there are at least 100,000
Salmonellosis cases per year in the EU countries, and approximately 1 million

cases in the United States. To date, the conventional bacterial culture method is still used as gold standard for food safety control. The methods involve several steps (pre-enrichment, selective enrichment, isolation in selective agar and serological and biochemical confirmation) to obtain the result. The whole process usually takes 5 to 7 days to be completed.

In the last decade, molecular diagnostic methods such as polymerase chain reaction (PCR) have been developed for the detection of pathogens (*e.g. Salmonella*) in much shorter time. Although there are a number of effective nucleic acid-based detection assays currently commercial available, many of them often require tedious, costly, and time-consuming procedures. Furthermore, a large number of these assays require multiple handling steps, which can adversely affect the reliability of the results due to cross-contamination problems. Frequently, low concentrations of the target nucleic acid molecule of interest contribute to the inability to detect the target nucleic acid molecule in the sample. The development of the polymerase chain reaction (PCR) as a method for amplifying nucleic acids in samples has revolutionized modern life sciences research, and has improved the ability to develop sensitive and reliable nucleic acid detection assays. However, the use of PCR is limited by the thermal constraints. The PCR process needs an electrically powered thermal cycler with precise three stages temperature control and a fast transition between stages, which is usually accomplished by a bulky and power-intensive apparatus that is not suitable for online and at point of need applications.

An alternative approach is the so-called isothermal amplification that has been developed to overcome the drawbacks of PCR. For this type of amplification, the use of other polymerases allows nucleic acid amplification at constant and low temperature. There are a number of different isothermal amplification techniques, and among them, Loop-Mediated Isothermal Amplification (LAMP) (T. Notomi et al., *Nucleic Acid Res.* (2000), Vol. 28, No. 12 e63 and WO00028082) has attracted considerable interest due to its rapid amplification, simple operation, and high sensitivity and specificity. LAMP is characterized by the use of four to six different primers, specifically designed to recognize six distinct regions on the target gene, which makes it highly specific for its target even in the presence of high concentration of non-target DNA. The process is performed at a constant

temperature {e.g. 60-65°C) using a strand displacement reaction, thus obviating the demand for sophisticated thermal control. Fast and efficient amplification can be achieved since there is no time required for temperature ramping during the LAMP process. Owing to these advantages, the LAMP technology has been widely
5 applied for rapid detection of different pathogens such as *Salmonella spp.*, *Campylobacter spp.*, *Escherichia coli*, *Staphylococcus aureus*, etc. (L. Niessen et al. Food Microbiol. (2013), 36, 191-206).

In order to comply with the demands from consumers for safe, pathogen-free
10 food, there is an urgent need for development of a rapid and reliable method for online or at site *Salmonella* detection. Adapting LAMP to Lab-on-a-chip (LOC) systems seems to be a very promising approach for monitoring pathogen at point-of-care (POC).

15 Hence, an improved method for establishing traceability of foodborne pathogens in the entire food chain would be advantageous.

Summary of the invention

The Solid Phase Loop Mediated Amplification (SP-LAMP) method according to the
20 present invention provides a solid phase isothermal amplification technology, for multiple, rapid, sensitive, specific detection of nucleic acid targets. Examples of targets are foodborne pathogens, infectious diseases e.g. *Salmonella spp.*, *Campylobacter spp.*, *Listeria monocytogenes* and *Norovirus* as well as cancer markers or other diseases marker etc.). The targets may originate from e.g. from
25 the environment, food, animals or humans. The technique also poses a great potential for easily integration into different format of Lab on-a-chip systems for point of care. The novel SP-LAMP technique will open a great potential alternative to the existing techniques for online rapid diagnostic, for not only detection and identification of foodborne pathogens in food and animal production chains, but
30 also for health care, environmental, and many other fields with feasibility and field applicability.

Thus, an object of the present invention relates to the provision of an alternative LAMP amplification process involving a solid support amplification step.

In particular, it is an object of the present invention to provide a method that solves the above-mentioned problems of the prior art with point-of-care detection in e.g. a lab-on-a-chip format.

5 Thus, one aspect of the invention relates to a loop mediated isothermal amplification (LAMP) method for detecting an oligonucleotide target sequence comprising:

a) providing a sample suspected of comprising an oligonucleotide target sequence, said oligonucleotide target sequence comprising in the 3' to 5'
10 direction:

- i. an F3C region, complementary to an F3 region;
- ii. an F2C region, complementary to an F2 region;
- iii. an F1C region, complementary to an F1 region;
- iv. a spacer region;
- 15 v. a B1 region, complementary to a B1C region;
- vi. a B2 region, complementary to a B2C region; and
- vii. a B3 region complementary to a B3C region;

b) providing a primer A (modFIP) comprising in the 5' to 3' direction :

- 20 i. a PI region, complementary to a PIC region;
- ii. an F1C region, complementary to the F1 region; and
- iii. an F2 region, complementary to the F2C region;

and/or

providing a primer B (FIP) comprising in the 5' to 3' direction:

- 25 iv. an F1C region, complementary to the F1 region; and
- v. an F2 region, complementary to the F2C region;

c) providing a primer C (BIP) comprising in the 5' to 3' direction:

- i. a detection label;
- 30 ii. a B1C region, complementary to the B1 region; and
- iii. a B2 region, complementary to the B2C region;

and/or

providing a primer D (modBIP) comprising in the 5' to 3' direction :

- iv. a detection label;
- 35 v. a blocking region;

- vi. a B1C region, complementary to the B1 region; and
vii. a B2 region, complementary to the B2C region;
with the proviso that if both primer C (BIP) and primer D (modBIP) are
provided, then only one of primer C (BIP) and primer D (modBIP)
5 comprises a detection label according to step (c)(i) and step (c)(iv),
respectively;
- d) providing a primer E (F3), complementary to the F3C region;
- 10 e) providing a primer F (B3), complementary to the B3C region;
- f) providing a solid phase coupled primer G (SP-primer) comprising a free 3'-
end and having an immobilization region being complementary to a region
of the oligonucleotide target sequence or a LAMP product arising from the
15 oligonucleotide target sequences;
- g) mixing the sample according to step a) and primers according to step b)-f)
with a LAMP amplification mixture, thereby providing an amplified LAMP
product coupled to the solid support if a target sequence is present in the
20 sample;
- h) optionally, washing the solid support; and
- i) detecting the amplified product coupled to the solid support, by detecting
25 the detection label, e.g. by fluorescence.

Another aspect of the present invention relates to a kit comprising :

- Primers according to the present invention;
- An amplification mixture according to the present invention; and
- 30 - Optionally, instructions for detecting the presence or absence of a
defined target sequence using the primer set and amplification
mixture of the kit.

Yet another aspect of the present invention relates to the use of a kit according to
35 the present invention for the detection of a target sequence.

Brief description of the figures

Figure 1 shows the design of the primers in the scenario when F3, B3, FIP and BIP primers are used together with modFIP.

5 Figure 2-9 show the liquid phase (LP) steps of the present invention.

Figure 2A-C show conversion of LP-1A to LP-2A and LP-3A.

Figure 3A-D show conversion of LP-2A to LP-4A and LP-5A.

10

Figure 4A-G show conversion of LP-3A to LP-3G, LP-6A, LP-7A and LP-8A.

Figure 5A-H show conversion of LP-6A to LP-6C1, LP-6G, LP-9A, LP-10A and LP-11A.

15

Figure 6 show conversion of LP-7A to LP-2A and LP-7B.

Figure 7A-B show conversion of LP-8A to LP-4A, LP-8B and LP-12B.

20 Figure 8A-B show conversion of LP-9A to LP-2A and LP-9C

Figure 9A-D show conversion of LP-6C1 to LP-4A, LP-6C3 and LP-13C

Figure 10 shows a summary of the nucleic acid amplification in the liquid phase
25 (LP). The amplification products termed LP-4A is further amplified on the solid phase (SP).

Figure 11A-B show the solid phase (SP) steps of the present invention for the
amplification product termed LP-4A. The F2C region of LP-4A anneal to the F2
30 region of SP-1 to form SP-2. The strand is extended (SP-3) to form the final
amplification product SP-4.

Figure 12 shows detection of *Salmonella Spp.* utilizing the SP-LAMP method.

Lanes termed *FIP*, *FIP21* and *FIP22* contains target solid phase (SP) primers which
35 are designed to target products in the liquid phase. The FIP solid phase primer

(F2=20 bp) is the same as the FIP primer except for the 10T10C linker. FIP 21 has a longer binding region (F2 +15bp extended). FIP 22 has a much longer binding region (F2+29 bp extended). Primers in the liquid phase are designed based on a conserved region of *hilA* gene sequences of *Salmonella* Enteritidis and 5 was used to target *Salmonella* species. All solid phase primers (HIP, FIP, FIP21 and FIP22) and Cy5 control probes were attached on the COC surface and then the LAMP master-mix (MX1, MX2 or MX3) was added. After amplification, the COC slide was washed and scanned. The Cy5 control probe is an oligo 10T IOC which was labelled with Cy5 at 5' end and served as positive control both in the spotting 10 process and the SP-LAMP reaction. The HIP solid phase primer is designed based on the *hipO* gene sequences of *Campylobacter jejuni* - unrelated to *Salmonella spp.* The HIP solid phase primer was used as negative control in the SP-LAMP.

Figure 13A-B show (A) a schematic representation of the experimental setup. Cy5 15 represents a Cy5 control probe, which served as positive control. HIP is a solid phase primer targeting *Campylobacter jejuni*. It is used in this experiment as negative control. SP1, SP2 and SP3 are solid phase primers of different lengths, which are designed to target *Listeria monocytogenes*. (B) shows arrayed locations in which solid phase amplification as fluorescent dots.

20

Figure 14A-B show (A) gel electrophoresis visualization of the SP-LAMP amplification products of AIV of different subtypes as sampled from embryonated chicken eggs. AIV subtype is denoted in the top of each lane. Location and date of virus collection is denoted in the bottom of each lane. NDV is an abbreviation for 25 Newcastle Disease Virus - a non-AIV sample used as a control. CN is a control negative test. (B) shows gel electrophoresis visualization of the SP-LAMP (left) or RT-PCR (right) amplification products of AIV in infected wild-bird samples collected in Denmark.

30 The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined :

5

Sample

In the present context, the term "sample" refers to any biological solution, entity or subject. This can include, but is not limited to, solutions from the environment or food as well as organisms, biological fluids, tissues, organs, *e.g.* humans or
10 mammals and all of their body parts. The sample may contain organisms or a specific cell type within an organism, *e.g.* a cell overexpressing a gene of interest. An example of such a sample may be a sample derived from food containing an organism of interest that bears a characteristic gene sequence or oligonucleotide sequence.

15

Oligonucleotide

In the present context, the term "oligonucleotide" refers to a sequence of DNA or RNA (including micro RNA (miRNA) and messenger RNA (mRNA)) nucleotides residues or any nucleotide analogue thereof that form a molecule.

20 Oligonucleotides can bind their complementary sequences to form duplexes (double-stranded fragments) or even fragments of a higher order.

Oligonucleotides can be on a linear form, but also exist as circular oligonucleotide molecules, such as single stranded circular RNAs or single-stranded circular DNAs.

25

When referring the length of a sequence, reference may be made to the number of nucleotide units or to the number of bases.

Furthermore, the typical DNA or RNA nucleotides may be replaced by nucleotides analogues such as 2'-O-Me-RNA monomers, 2'-O-alkyl-RNA monomers, 2'-amino-DNA monomers, 2'-fluoro-DNA monomers, locked nucleic acid (LNA) monomers, arabino nucleic acid (ANA) monomers, 2'-fluoro-ANA monomers, 1,5-anhydrohexitol nucleic acid (HNA) monomers, peptide nucleic acid (PNA), and morpholinos.

35

Target sequence

In the present context, the term "target sequence" refers to an oligonucleotide sequence of interest that is desired to detect. The target sequence is typically representative of an organism of interest and may be detected by amplification
5 using the method of the present invention, which result in large amounts of the target sequence labelled with a detection label, such as a fluorophore.

The target sequence may comprise regions (F3C, F2C, FIC, B1, B2 and B3) complementary to the primers according to the method of the invention. The
10 primers used in the method of the present invention may be designed specifically to detect a target sequence of interest. Theoretically, it is therefore possible to detect any target sequence with a known sequence.

Typically, the target sequence is comprised of RNA or DNA residues and originates
15 from an organism of interest, such as a pathogenic organism.

Primer

In the present context, the term "primer" refers to a nucleic acid sequence that serves as a starting point for DNA synthesis. It is required for DNA replication
20 because the enzymes that catalyse this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand.

In the present context, primers are specifically designed to target a target
25 sequence of interest and to work according to the method of the present invention. Thus depending on the function of the primer, the length of the primer may vary from 10 nucleotides to as much as 100 nucleotides.

Spacer region

30 In the present context, the term "spacer region" refers to an oligonucleotide region of the target sequence that is located in between the F3C, F2C, FIC regions and the B1, B2, B3 regions of the target sequence. Thus, the spacer region is located downstream from F3C, F2C, FIC regions and upstream from B1, B2, B3 regions when reading in the 3' to 5' direction.

The spacer region may be comprised of any sequence of oligonucleotides, such as a non-functional part of a genome, an entire gene or a part of a gene.

Blocking region

- 5 In the present context, the term "blocking region" refers to an oligonucleotide region of the modBIP primer according to the present invention. The purpose of the blocking region is to prevent self-primed DNA synthesis activity of the strand, so the product LP-4A can be stable immobilized on the solid surface.
- 10 Thus, the blocking region of the modBIP primer catalyzes the generation of stem loop primers (LP-4A) without self-primed DNA synthesis activity in the liquid phase.

The blocking region may comprise any sequence of nucleotides that prevent self-primed DNA synthesis activity. Thus, the blocking region may be designed specifically to be suitable for use with any target sequence.

Solid phase/solid support

In the present context, the terms "solid phase" and "solid support" are used interchangeably and relates to a solid material. This solid material may be formed from one or more insoluble or a substantially insoluble materials, which materials may be inorganic or organic, which materials are chemically inert (or substantially chemically inert) with respect to the reagents and conditions used in the method of the present invention.

25

Examples of inorganic materials include, but are not limited to glass, silica, porous glass, aluminosilicate, borosilicate and metal oxides (for example, aluminium oxide, nickel oxide, and iron oxide).

30 The solid support may also be formed from an organic polymeric material, which polymeric material is optionally crosslinked. Examples of organic polymeric materials include, but are not limited to, cellulose (from different sources, e.g. plants, insects, bacteria *etc.*), polysaccharide, crosslinked polysaccharides, polystyrene, crosslinked polystyrene, polyacryloylmorpholide, polyamide resin, polyacryloyl pyrrolidone, polyethylene, polyethylene glycol, crosslinked

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polyethylene glycol, poly(vinyl acetate), poly(methyl methacrylate), poly(dimethylsiloxane), poly(methacrylic acid), polycarbonate, polyethylene terephthalate, polypropylene, poly(ethylenoxide), poly(lactic acid), polypropylene copolymer, polyacrylonitrile, cyclic olefin copolymer, cyclic olefin polymer, 5 polyethylene glycol-polystyrene, polystyrene, cross-linked dextran, and/or cross-linked agarose.

The solid support may be in any suitable form, including but not limited to resins, particles, beads, fibres and films.

10

In the present invention, the solid phase primer (SP-primer) may be coupled to the solid support by *e.g.* A) physical absorption (*e.g.* on amine, nitrocellulose, poly(l-lysine, PAAH, or diazonium ion surface); B) chemisorption (*e.g.* thiols-gold); C) covalent immobilization (*e.g.* amines, amino, or hydrazide-modified DNA 15 oligo nucleotides on carboxyl (with carbodiimide), aldehyde, isothiocyanate, or epoxide modified surfaces, hydrazide disulphide coupling, thiols-maleimide, thiol-mercaptopilane, thiols-acrylamide *etc.*); or D) affinity-binding (*e.g.* avidin-biotin, streptavidin-biotin). Amongst option C) is coupling via a poly(T)10-poly(C)10 tail (TC tag). Other lengths and ratios of T and C combinations are also applicable. TC 20 tags may be linked onto a solid support by UV light irradiation. The coupling of the SP-primer onto the solid support enables amplification on the solid support.

The present solid-phase amplification is a method in which molecules are bound on a solid support and amplification is performed in a reactant solution. Compared 25 with normal amplification in a liquid state, it is easier to remove excess reactant or by-product from the product.

Detection label

In the present context, the term "detection label" refers to a molecular entity, 30 which may be used to detect the amplified target sequence following amplification according to the method of the present invention.

The readout of the detection label may be based on a technique including, but not limited to, fluorescence, chemiluminescence, radioactivity, magnetism, turbidity,

phosphorescence, colorimetric, chemical labelling (*e.g.* silver), Surface Plasmon Resonance (SPR), and refractive index.

The detection label may be detected by a method selected from the group
5 consisting of microscopy, (micro) array scanners, fluorescence microscopy, fluorescence spectroscopy, Fluorescence-activated cell sorting (FACS), NMR, mass spectroscopy, optical detectors, CCD cameras and scanners.

In the present invention, it is preferred that the detection label is selected from
10 the group consisting of a fluorophore or a radioisotope. Fluorophores include, but are not limited to, synthetic fluorophores, fluorescent proteins, quantum dots, intercalating dyes and scorpion dyes. In the present context, the terms "fluorophore" and "dye" are used interchangeably.

15 As described above, an object of the present invention relates to the provision of an alternative LAMP amplification process involving a solid support amplification step.

Thus, an aspect of the present invention relates to a loop mediated isothermal
20 amplification (LAMP) method for detecting an oligonucleotide target sequence comprising:

- a) providing a sample suspected of comprising an oligonucleotide target sequence, said oligonucleotide target sequence comprising in the 3' to 5' direction:
 - 25 i. an F3C region, complementary to an F3 region;
 - ii. an F2C region, complementary to an F2 region;
 - iii. an F1C region, complementary to an F1 region;
 - iv. a spacer region;
 - v. a B1 region, complementary to a B1C region;
 - 30 vi. a B2 region, complementary to a B2C region; and
 - vii. a B3 region complementary to a B3C region;

- b) providing a primer A (modFIP) comprising in the 5' to 3' direction:
 - i. a PI region, complementary to a PIC region;
 - 35 ii. an F1C region, complementary to the F1 region; and

- iii. an F2 region, complementary to the F2C region;
and/or
providing a primer B (FIP) comprising in the 5' to 3' direction:
- 5 iv. an F1C region, complementary to the F1 region; and
 v. an F2 region, complementary to the F2C region;
- c) providing a primer C (BIP) comprising in the 5' to 3' direction:
- 10 i. a detection label;
 ii. a B1C region, complementary to the B1 region; and
 iii. a B2 region, complementary to the B2C region;
and/or
providing a primer D (modBIP) comprising in the 5' to 3' direction :
- 15 iv. a detection label;
 v. a blocking region;
 vi. a B1C region, complementary to the B1 region; and
 vii. a B2 region, complementary to the B2C region;
- with the proviso that if both primer C (BIP) and primer D (modBIP) are
provided, then only one of primer C (BIP) and primer D (modBIP)
comprises a detection label according to step (c)(i) and step (c)(iv),
20 respectively;
- d) providing a primer E (F3), complementary to the F3C region;
- e) providing a primer F (B3), complementary to the B3C region;
- 25 f) providing a solid phase coupled primer G (SP-primer) comprising a free 3'-
end and having an immobilization region being complementary to a region
of the oligonucleotide target sequence or a LAMP product arising from the
oligonucleotide target sequences;
- 30 g) mixing the sample according to step a) and primers according to step b)-f)
with a LAMP amplification mixture, thereby providing an amplified LAMP
product coupled to the solid support if a target sequence is present in the
sample;
- 35

- h) optionally, washing the solid support; and
- i) detecting the amplified product coupled to the solid support, by detecting the detection label, e.g. by fluorescence.

5

The SP-LAMP method provides a rapid and reliable method for online or at site detection of any target sequence. It is especially advantageous that the solid-phase amplification is a method in which molecules are bound on a solid support because amplification products can be easily purified by removal of excess reactants or by-products from the product. This purification is more tedious using a liquid state amplification setup.

The method is outlined in example 1 and figures 1-11, which illustrates the liquid phase and solid phase amplification of the SP-LAMP process using the primers F3, B3, FIP, BIP, modFIP and SP-primer. The SP-LAMP is also applicable using other combinations of primers. The F3 and B3 are always present. However, the SP-LAMP method may be carried out with other combinations as given below:

- a) with only normal primers FIP and BIP *{i.e. without modified primers, see MX1 data in example 2}*
- 20 b) with only modified primers *{i.e. with modFIP and modBIP}*
- c) with all "normal" and modified primers *{i.e. with FIP, BIP, modFIP and modBIP, see MX3 data in example 2}*
- d) with "cross-combinations" of "normal" and modified primers, *i.e. ;*
 - i. with FIP and modBIP
 - 25 ii. with FIP, modFIP and modBIP
 - iii. with FIP, BIP, modFIP (as outlined in example 1, see MX2 data in example 2)
 - iv. with FIP, BIP, modBIP
 - v. with BIP and modFIP
 - 30 vi. with BIP, modBIP and modFIP

The method as described herein functions also in a multiplex setup for detection of multiple target sequences out of a single sample. For multiplex detection, a solid surface comprising an array of a multitude of different solid phase coupled primers is provided and the corresponding primer sets according to the method is

35

mixed with a sample and added to the solid surface. In the multiplex setup, solid phase primers and the corresponding primer set (e.g. combination of F3, B3, FIP, BIP, modFIP and modBIP) is designed specifically for the target sequence of interest. Therefore, the method can be used for identifying a multiple of different
5 infectious species out of a single sample. Thus, in an embodiment of the invention, the sample is added to a surface comprising an array of a multitude of different solid phase coupled primers and mixed with primer sets corresponding to the target sequences of interest.

10 Therefore, the method as described herein may be used to detect simultaneously, in a single sample, target sequences originating from a multitude of different species, including, but not limited to, *Salmonella spp*, *Campylobacter jejuni*, verotoxin producing *Escherichia coli* 0157: 1-17, *Listeria monocytogenes*, *Shigella dysenteriae*, *Avian influenza Virus* and *NoroVirus*.

15

The target sequence of the SP-LAMP method is defined by seven regions (F3C, F2C, F1C, a spacer region, B1, B2 and B3 in the 3' to 5' direction). These regions may in theory be assigned to any oligonucleotide sequence of interest, making it a target sequence. Thus, the seven regions of the target sequence are "fictional" in
20 that they are assigned to the target sequence and the entire set of SP-LAMP primers are designed to function with the assigned regions.

The length of the each of the seven regions may vary depending on the selection of target sequence.

25

Thus, an embodiment of the present invention relates to the method as described herein, wherein the F3C region, complementary to a F3 region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

30 Another embodiment of the present invention relates to the method as described herein, wherein the F2C region, complementary to a F2 region, has a length of 10-30 nucleotides, such as 15-25 or such as 20-25.

Yet another embodiment of the present invention relates to the method as described herein, wherein the FIC region, complementary to a FI region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

- 5 The spacer region is located in between the F3C, F2C, FIC regions and the B1, B2, B3 regions of the target sequence. This spacer region may be comprised of any sequence of oligonucleotides, such as a non-functional part of a genome, an entire gene or a part of a gene.
- 10 Thus, an embodiment of the present invention relates to the method as described herein, wherein the spacer region in the target sequence, has a length of 1-500 nucleotides, such as 1-200 nucleotides, such as 1-100 nucleotides, or such as 5-100 nucleotides.
- 15 Another embodiment of the present invention relates to the method as described herein, wherein the B1 region, complementary to a B1C region, has a length of 10-30 nucleotides, such as 15-25 or such as 20-25.

A further embodiment of the present invention relates to the method as described
20 herein, wherein, the B2 region, complementary to a B2C region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

A still further embodiment of the present invention relates to the method as
25 described herein, wherein the B3 region, complementary to a B3C region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

To ensure that amplified products are immobilized on the solid support in order to
undergo solid phase LAMP, the solid phase primer (primer G) is designed to
comprise an immobilization region, which is complementary to a region of the
30 original target sequence.

Thus, an embodiment of the present invention relates to the method as described
herein, wherein the immobilization region, complementary to a region of the
oligonucleotide target sequence, has a length of 10-30 nucleotides, such as 15-25
35 or such as 20-25.

In some variants of the present invention, but not all, the LAMP master-mixture comprises a modFIP primer. The modFIP primer is defined by three regions (PI, F1C and F2 in the 5' to 3' direction). The nucleotide composition of the modFIP primer is decided upon after selection of a target sequence. Thus, the modFIP
5 primer is custom designed to, amongst other, complement the F1 and F2C regions of the target sequence. In addition to the F1C and F2 regions, the modFIP primer also comprise a PI region, which leads to the formation of liquid state amplification products, LP-4A, comprising a complementary PIC region. The PIC region of LP-4A may then in turn anneal to the solid phase coupled primer (SP
10 primer), which shares the PI region with the modFIP primer.

Since the PI region of the modFIP primer is not complementary to any region of the target sequence, the design of the PI region is independent of the selection of target sequence. Consequently, the PI region may be designed to obtain the
15 optimal conditions for efficient solid phase amplification. In any event, the length and composition of nucleotides of the PI region may vary to obtain the best interplay with the remaining SP-LAMP primer set.

Depending on the nucleotide composition of the target sequence, the total length of the modFIP and FIP primers may vary.

20

Thus, an embodiment of the present invention relates to the method as described herein, wherein the primer A (modFIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

25 Another embodiment of the present invention relates to the method as described herein, wherein the primer B (FIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

In some variants of the present invention, but not all, the LAMP master-mixture
30 comprises a modBIP primer.

The modBIP primer comprises three regions and a detection label (detection label, blocking region, B1C and B2 in the 5' to 3' direction). The purpose of the blocking region of modBIP is to prevent self-primed DNA synthesis activity and generation
35 of a stem loop primer (LP-4A) without self-primed DNA synthesis activity in the

liquid phase. The LP-4A products will then anneal to the solid phase coupled primer to initiate solid phase amplification of the target sequence.

Similar to the PI region of the modFIP primer, the blocking region of modBIP is
5 independent of the target sequence in that it is designed to be non-complementary to the target sequence. Therefore, the blocking region may comprise any sequence of nucleotides that prevent self-primed DNA synthesis activity. The length and composition of nucleotides of the blocking region may vary to obtain the best interplay with the remaining SP-LAMP primer set.

10

Therefore, an embodiment of the present invention relates to the method as described herein, wherein the blocking region in the primer D (modBIP) has a length of 5 to 25 nucleotides, or such as 5 to 15 nucleotides.

15 Depending on the nucleotide composition of the target sequence, the total length of the modBIP and BIP primers may vary.

Thus, an embodiment of the present invention relates to the method as described herein, wherein the primer C (BIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

20

Another embodiment of the present invention relates to the method as described herein, wherein the primer D (modBIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

25 The F3 and B3 primers are classical primers in the sense that they are only complementary to a single region of the target sequence with no nucleotide overhang. The design of the F3 and B3 primers is completely dependent on the target sequence. Depending on the nucleotide composition of the target sequence, the length of the F3 and B3 primers may vary.

30

Thus, an embodiment of the present invention relates to the method as described herein, wherein the primer E (F3), has a length of 10-30 nucleotides, such as 15-25.

Another embodiment of the present invention relates to the method as described herein, wherein the primer F (B3), has a length of 10-30 nucleotides, or such as 15-25.

5 The function of the solid phase coupled primer (SP primer) is three-fold (*i.e.* attachment, annealing, synthesis). First, the SP-primer must be attachable to a solid support. Second, the SP-primer must have an immobilization region being complementary to a region of the oligonucleotide target sequence or a LAMP product arising from the oligonucleotide target sequences. In one embodiment of
10 the present invention, the immobilization region of the SP-primer is a PI region to which the stem loop primer (LP-4A) without self-primed DNA synthesis activity can anneal. DNA synthesis is initiated subsequent to the stem loop primer (LP-4A) annealing to the SP-primer. Thus, the PI region of the SP-primer serves a dual purpose. As described above, the PI region of the SP-primer is identical to the PI
15 region of the modFIP primer and the length and composition of nucleotides of the PI region may vary to obtain the best interplay with the remaining SP-LAMP primer set.

Therefore, an embodiment of the present invention relates to the method as described herein, wherein the solid phase coupled primer G (SP-primer) has a
20 length of 10-200 nucleotides, such as 15-150, such as 20-100, such as 25-80 or such as 30-50 nucleotides.

In an embodiment of the present invention, the PI region of the SP-primer is replaced with an F2 region, which is complementary to the F2C region. Thus, the
25 stem loop primer (LP-4A) without self-primed DNA synthesis activity anneal to the SP-primer via the F2C region of LP-4A.

As the modBIP or BIP primers are designed to comprise a detectable label, all LP-4A products also comprise a detectable label by virtue of the method. After
30 completion of the SP-LAMP amplification of the target sequence it is therefore possible to determine the presence or absence of the target sequence.

The readout of the detection label may be based on any technique that does not interfere with the SP-LAMP method and include, but is not limited to,
35 fluorescence, radioactivity and magnetism.

Thus, an embodiment of the present invention relates to the method as described herein, wherein the detection label of the primer C (BIP) and/or the primer D (modBIP) is selected from the group consisting of a fluorophore and a radiolabel.

- 5 Fluorophores include, but are not limited to, synthetic fluorophores, fluorescent proteins, quantum dots, intercalating dyes and scorpion dyes.

For detection of the amplification product, it is possible to use fluorescent labels that suits the experimental setup with respect to photostability, excitation

- 10 wavelength, emission wavelength etc.

Fluorophores suitable as detection label for the amplification product of the present invention include, but are not limited to, the ones listed in table 1.

- 15 Table 1: List of Fluorophores with details of characteristic (Excitation wavelength, emission wavelength and visible colour)

Dye name	Excitation Wavelength (nm)	Emission wavelength (nm)	Visible colour
Hydrocoumarin	325	386	Blue
Methoxycoumarin	360	410	Blue
Alexa Fluor 350	346	442	Blue
Aminocoumarin	350	445	Blue
Cy2	490	510	Green (dark)
FAM	495	516	Green (dark)
Alexa Fluor 488	495	519	Green (light)
Fluorescein FITC	495	518	Green (light)
Alexa Fluor 430	434	539	Green (light)
Alexa Fluor 532	531	554	Green (light)
HEX	535	556	Green (light)
Cy3	550	570	Yellow
TRITC	547	572	Yellow
Alexa Fluor 546	556	573	Yellow
Alexa Fluor 555	555	565	Yellow

R-phycoerythrin (PE)	480; 565	578	Yellow
Rhodamine Red-X	560	580	Orange
Tamara	565	580	Red
Cy3.5 581	581	596	Red
Rox	575	602	Red
Alexa Fluor 568	578	603	Red
Red 613	480; 565	613	Red
Texas Red	561	594	Red
Alexa Fluor 594	590	617	Red
Alexa Fluor 633	632	647	Red
Allophycocyanin	650	660	Red
Alexa Fluor 647	650	668	Red
Cy5	650	670	Red
Alexa Fluor 660	663	690	Red
Cy5.5	675	694	Red
TruRed	490; 675	695	Red
Alexa Fluor 680	679	702	Red
Cy7	743	770	Red

Other suitable dyes include Tetrachlorofluorescein (TET), SYBR green I or II, YBR Green II, SYBR Gold, YO (Oxazole Yellow), TO (Thiazole Orange) and PG

5 (PicoGreen).

Furthermore, an embodiment of the present invention relates to the method as described herein, wherein the fluorophore is selected from the group consisting of Hydrocoumarin, Methoxycoumarin, Alexa Fluor, Aminocoumarin, Cy2, FAM, Alexa
10 Fluor 488, Fluorescein FITC, Alexa Fluor 430, Alexa Fluor 532, HEX, Cy3, TRITC, Alexa Fluor 546, Alexa Fluor 555, R-phycoerythrin (PE), Rhodamine Red-X, Tamara, Cy3.5 581, Rox, Alexa Fluor 568, Red 613, Texas Red, Alexa Fluor 594, Alexa Fluor 633, Allophycocyanin, Alexa Fluor 633, Cy5, Alexa Fluor 660, Cy5.5, TruRed, Alexa Fluor 680, Cy7, tetrachlorofluorescein (TET), SYBR green I or II,
15 YBR Green II, SYBR Gold, YO (Oxazole Yellow), TO (Thiazole Orange) and PG (PicoGreen).

A preferred embodiment of the present invention, relates to the method as described herein, wherein the fluorophore is Cy5.

The SP-primer is designed so that it may be coupled to a solid support. Such
5 coupling may be based on either covalent or non-covalent interactions between the SP-primer and the solid support. Such non-covalent interactions include, but are not limited to, hydrophobic interactions, electrostatic/ionic interactions, van der Waals interactions and the like.

10 The SP-primer may be attached to the solid support by UV treatment, wherein a covalent bond is formed between a TC tail and a plain and unmodified solid surface. Thus, an target oligo with a poly(T)10-poly(C)10 tail can be immobilised by UV irradiation at 254 nm with power of 3 mW/cm² for 10 min (Stratalinker 2400, Stratagene, CA, USA) on a plain and unmodified solid surface.

15

Alternative methods for coupling the solid phase primer (SP-primer) to the solid support include, but are not limited to A) physical absorption (*e.g.* on amine, nitrocellulose, poly(l-lysine, PAAH, or diazonium ion surface); B) chemisorption (*e.g.* thiols-gold); C) covalent immobilization (*e.g.* amines, amino, or hydrazide-
20 modified DNA oligo nucleotides on carboxyl (with carbodiimide), aldehyde, isothiocyanate, or epoxide modified surfaces, hydrazide disulphide coupling, thiols-maleimide, thiol-mercaptopilane, thiols-acrylamide *etc.*); or D) affinity-binding (*e.g.* avidin-biotin, streptavidin-biotin). Amongst option C) is coupling via a poly(T)10-poly(C)10 tail (TC tag). Other lengths and ratios of T and C
25 combinations are also applicable. TC tags may be linked onto a solid support by UV light irradiation. The coupling of the SP-primer onto the solid support enables amplification on the solid support.

Thus, an embodiment of the present invention relates to the method as described
30 herein, wherein the solid phase coupled primer G (SP-primer) comprises a 5' linker sequence, supporting coupling to the solid support.

The solid support may be comprised of any material that do not interact non-specifically with the nucleotide material. A list of suitable materials is given in the

definitions section. The solid support may consist of a single material or a combination of two or more materials.

An embodiment of the present invention relates to the method as described
5 herein, wherein the solid phase coupled primer G (SP-primer) is coupled to a solid support selected from the group consisting of a glass slide, a cover slip, COC (cyclic olefin copolymer), COP (cyclic olefin polymer), PS (polystyrene), PC (polycarbonate), PMMA (poly(methyl methacrylate)), PDMS (poly(dimethylsiloxane)), PET (poly(ethylene terephthalate)), cellulose-based
10 beads, sheet or paper, a glass bead, a polymeric bead, a nanobead, a nanoparticle, graphene, an ELISA plate, and a polymeric multiple well plate.

Furthermore, the solid support may be in any suitable form, including but not limited to resins, particles, beads, fibres, DNA structures (DNA origami) and films.
15

The SP-LAMP method includes not only a sample and suitable primers for amplification thereof, but also LAMP amplification mixture that is mixed with the sample and the primer set. The amplification mixture comprises all the building blocks necessary for nucleotide amplification (strand elongation) and suitable
20 polymerase enzymes to facilitate the amplification.

Thus, an embodiment of the present invention relates to the method as described herein, wherein the LAMP amplification mixture of step g) comprises at least one polymerase enzyme supporting LAMP amplification and dinucleotide triphosphates
25 (dNTPs).

Another embodiment of the present invention relates to the method as described herein, wherein the polymerase enzyme, has strand displacement activity.

30 The term strand displacement describes the ability to displace downstream DNA encountered during synthesis. Some polymerase enzymes has strand displacement activity and are active at moderate temperatures, around 20-37°C. Other polymerase enzymes, such as BST polymerase, has strand displacement activity and are active at elevated temperatures, around 65°C. In the present

invention, it is preferred to utilize polymerase enzymes that work at elevated temperatures.

Thus, an embodiment of the present invention relates to the method as described
5 herein, wherein the polymerase enzyme is selected from the group consisting of
Bst polymerase, Bst DNA Polymerase Large Fragment, Bsm DNA Polymerase, Bsu
DNA Polymerase Large Fragment, SD DNA Polymerase, Deep VentR DNA
Polymerase, Deep VentR™ (exo) DNA Polymerase, DNA Polymerase I Large
(Klenow) Fragment, M-MuLV Reverse Transcriptase, Phi29 DNA Polymerase, VentR
10 DNA Polymerase, VentR (exo) DNA Polymerase, StrandDisplace Thermostable DNA
Polymerase, and combinations thereof.

The SP-LAMP method also encompass amplification of RNA target sequences. In
that case, it is necessary to convert the RNA target sequence to DNA. This process
15 is called reverse transcription and is mainly associated with retroviruses. The
active enzyme in the process is called reverse transcriptase.

Therefore, an embodiment of the present invention relates to the method as
described herein, wherein the LAMP amplification mixture further comprises a
reverse transcriptase, such as an AMV reverse transcriptase.

20

An advantage of the SP-LAMP method is that it is performed at isothermal
conditions, meaning that the temperature is not cycled as in classical PCR. This
circumvents the need for an advanced electrically powered thermal cycler with
precise three stages temperature control and a fast transition between stages.

25

With the SP-LAMP method, amplification may be adapted to lab-on-a-chip (LOC)
or lateral flow systems that allows monitoring pathogens onsite with only the need
for a container capable of maintaining a constant temperature.

30 Therefore, an embodiment of the present invention relates to the method as
described herein, wherein the amplification steps are performed at a temperature
in the range between 30-75°C, such as 40-70°C, such as 50-65°C, or such as 55-
65°C.

Since the primer set may be specifically designed for any given target sequence, said target sequence may be any nucleotide sequence from double-stranded DNA to single-stranded genomic RNA.

5 Thus, an embodiment of the present invention relates to the method as described herein, wherein the target sequence is single-stranded or double-stranded.

Another embodiment of the present invention relates to the method as described herein, wherein the target sequence is DNA or RNA.

10

The sample may be selected for any source from which amplification of a target sequence is desired. In a preferred embodiment of the present invention, the sample is suspected to contain a pathogen.

15 Thus, an embodiment of the present invention relates to the method as described herein, wherein the sample is selected from the group consisting of food (liquid/solid and semi solid), feed (liquid/solid and semi solid), blood (serum, buffer coat, treated with heparin or EDTA), tissue (paraffin embedded), tumor, biopsy, sputum, swabs (from both human and animal), fecal (from both human
20 and animal), water and waste-water samples.

Swabs may be from cloacal, surface or skin of human or animal, but may also be of non-living origin such as from other solid surfaces, e.g. from tables and metal or rubber chains in production lines.

25

Target sequences may be selected from any pathogen of interest. However, the method also applies to non-pathogenic target sequences, wherein amplification is desired.

30 Therefore, an embodiment of the present invention relates to the method as described herein, wherein the target sequence is of pathogenic origin, such as of bacterial, fungi or viral origin, or from a gene involved in a disease.

Another embodiment of the present invention relates to the method as described
35 herein, wherein the target sequence originates from an organism selected from

the group consisting of *Salmonella spp*, *Campylobacter jejuni*, verotoxin producing *Escherichia coli* 0157: 1-17, *Listeria monocytogenes*, *Shigella dysenteriae*, *Avian Influenza Virus* and *Norovirus*.

5 A further embodiment of the present invention relates to the method as described herein, wherein the target sequence is a *salmonella* sequence.

The immobilization region is a region of the solid phase coupled primer, which is complementary to any region of the target sequence. In one embodiment, the
10 immobilization region preferably comprises an F2 region.

Thus, an embodiment of the present invention relates to the method as described herein, wherein the immobilization region comprises an F2 region, complementary to an F2C region.

15

Another embodiment of the present invention relates to the method as described herein, wherein the F2 region of the immobilization region has a length of at least 10 nucleotides, such as at least 20 nucleotides, such as at least 30 nucleotides, such as at least 40 nucleotides, such as at least 50 nucleotides.

20

The modFIP primer comprises a PI region, which may be used as binding partner for the immobilization region of the solid phase coupled primer.

Therefore, an embodiment of the present invention relates to the method as
25 described herein, wherein the method comprises providing a primer A (modFIP).

Another embodiment of the present invention relates to the method as described herein, wherein the immobilization region of the primer G is complementary to a PIC region.

30

In contrast to traditional LAMP amplification in solution, the method of the present invention is suitable for simultaneous detection of multiple target sequences out of a single sample.

Thus, an embodiment of the present invention relates to the method as described herein, wherein the method comprises primers for detection of multiple target sequences.

5 The constituents of the SP-LAMP method may be packed in an easy to use kit that comprise all the necessary parts {i.e. primers, amplification mixture, instructions etc.) to perform amplification of a single target sequence. In a preferred embodiment of the invention, the kit comprises parts suitable for SP-LAMP amplification of a pathogen.

10

Thus, another aspect of the present invention relates to a kit comprising:

- Primers according to the present invention;
 - An amplification mixture according to the present invention; and
 - Optionally, instructions for detecting the presence or absence of a
- 15 defined target sequence using the primer set and amplification mixture of the kit.

A kit as described herein may also contain multiple sets of primers that allow detection of multiple target sequences, e.g. from different pathogenic species.

20

Therefore, an embodiment of the present invention relates to the kit as described herein, wherein primers for detection of multiple target sequences are included.

Another embodiment of the present invention relates to the kit as described
25 herein, which is suitable for use in a lab-on-a-chip (LOC) or lateral flow format.

Still another aspect of the present invention relates to the use of a kit as described herein for the detection of a target sequence.

30 Another embodiment of the present invention relates to the use as described herein, wherein multiple targets are detected simultaneously in a single sample.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the
35 invention.

Items

Item 1. A loop mediated isothermal amplification (LAMP) method for detecting an oligonucleotide target sequence comprising :

- 5 a) providing a sample suspected of comprising an oligonucleotide target sequence, said oligonucleotide target sequence comprising in the 3' to 5' direction:
- i. an F3C region, complementary to an F3 region;
 - ii. an F2C region, complementary to an F2 region;
 - 10 iii. an F1C region, complementary to an F1 region;
 - iv. a spacer region;
 - v. a B1 region, complementary to a B1C region;
 - vi. a B2 region, complementary to a B2C region; and
 - vii. a B3 region complementary to a B3C region;
- 15 b) providing a primer A (modFIP) comprising in the 5' to 3' direction:
- i. a P1 region, complementary to a PIC region;
 - ii. an F1C region, complementary to the F1 region; and
 - iii. an F2 region, complementary to the F2C region;
- and/or
- 20 providing a primer B (FIP) comprising in the 5' to 3' direction:
- iv. an F1C region, complementary to the F1 region; and
 - v. an F2 region, complementary to the F2C region;
- c) providing a primer C (BIP) comprising in the 5' to 3' direction:
- 25 i. a detection label;
- ii. a B1C region, complementary to the B1 region; and
 - iii. a B2 region, complementary to the B2C region;
- and/or
- providing a primer D (modBIP) comprising in the 5' to 3' direction :
- 30 iv. a detection label;
- v. a blocking region;
 - vi. a B1C region, complementary to the B1 region; and
 - vii. a B2 region, complementary to the B2C region;
- with the proviso that if both primer C (BIP) and primer D (modBIP) are
- 35 provided, then only one of primer C (BIP) and primer D (modBIP)

comprises a detection label according to step (c)(i) and step (c)(iv), respectively;

d) providing a primer E (F3), complementary to the F3C region;

5

e) providing a primer F (B3), complementary to the B3C region;

f) providing a solid phase coupled primer G (SP-primer) comprising a free 3'-end and having an immobilization region being complementary to a region of the oligonucleotide target sequence or a LAMP product arising from the oligonucleotide target sequences;

10

g) mixing the sample according to step a) and primers according to step b)-f) with a LAMP amplification mixture, thereby providing an amplified LAMP product coupled to the solid support if a target sequence is present in the sample;

15

h) optionally, washing the solid support; and

i) detecting the amplified product coupled to the solid support, by detecting the detection label, e.g. by fluorescence.

20

Item 2. The method according to item 1, wherein the F3C region, complementary to an F3 region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

25

Item 3. The method according to items 1 or 2, wherein the F2C region, complementary to an F2 region, has a length of 10-30 nucleotides, such as 15-25 or such as 20-25.

30 Item 4. The method according to any one of the preceding items, wherein the FIC region, complementary to an FI region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

Item 5. The method according to any one of the preceding items, wherein the spacer region in the target sequence, has a length of 1-500 nucleotides, such as 1-200 nucleotides, such as 1-100 nucleotides, or such as 5-100 nucleotides.

- 5 Item 6. The method according to any one of the preceding items, wherein the B1 region, complementary to a B1C region, has a length of 10-30 nucleotides, such as 15-25 or such as 20-25.

Item 7. The method according to any one of the preceding items, wherein, the B2
10 region, complementary to a B2C region, has a length of 10-30 nucleotides, such as 15-25, or such as 20-25.

Item 8. The method according to any one of the preceding items, wherein the B3
15 as 15-25, or such as 20-25.

Item 9. The method according to any one of the preceding items, wherein the immobilization region, complementary to a region of the oligonucleotide target sequence, has a length of 10-30 nucleotides, such as 15-25 or such as 20-25.
20

Item 10. The method according to any one of the preceding items, wherein the primer A (modFIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

25 Item 11. The method according to any one of the preceding items, wherein the primer B (FIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

Item 12. The method according to any one of the preceding items, wherein the
30 primer C (BIP), has a length of 40-100 nucleotides, such as 40-80 nucleotides, or such as 40-70 nucleotides.

Item 13. The method according to any one of the preceding items, wherein the primer D (modBIP), has a length of 40-100 nucleotides, such as 40-80
35 nucleotides, or such as 40-70 nucleotides.

Item 14. The method according to any one of the preceding items, wherein the blocking region in the primer D (modBIP) has a length of 5 to 25 nucleotides, or such as 5 to 15 nucleotides.

- 5 Item 15. The method according to any one of the preceding items, wherein the primer E (F3), has a length of 10-30 nucleotides, such as 15-25.

Item 16. The method according to any one of the preceding items, wherein the primer F (B3), has a length of 10-30 nucleotides, or such as 15-25.

10

Item 17. The method according to any one of the preceding items, wherein the solid phase coupled primer G (SP-primer) has a length of 10-200 nucleotides, such as 15-150, such as 20-100, such as 25-80 or such as 30-50 nucleotides.

- 15 Item 18. The method according to any one of the preceding items, wherein the detection label of the primer C (BIP) and/or the primer D (modBIP) is selected from the group consisting of a fluorophore and a radiolabel.

Item 19. The method according to item 18, wherein the fluorophore is selected
20 from the group consisting of Hydrocoumarin, Methoxycoumarin, Alexa Fluor, Aminocoumarin, Cy2, FAM, Alexa Fluor 488, Fluorescein FITC, Alexa Fluor 430, Alexa Fluor 532, HEX, Cy3, TRITC, Alexa Fluor 546, Alexa Fluor 555, R-phycoerythrin (PE), Rhodamine Red-X, Tamara, Cy3.5 581, Rox, Alexa Fluor 568, Red 613, Texas Red, Alexa Fluor 594, Alexa Fluor 633, Allophycocyanin, Alexa
25 Fluor 633, Cy5, Alexa Fluor 660, Cy5.5, TruRed, Alexa Fluor 680, Cy7, tetrachlorofluorescein (TET), SYBR green I or II, YBR Green II, SYBR Gold, YO (Oxazole Yellow), TO (Thiazole Orange) and PG (PicoGreen).

Item 20. The method according to item 19, wherein the fluorophore is Cy5.

30

Item 21. The method according to any one of the preceding items, wherein the solid phase coupled primer G (SP-primer) comprises a 5' linker sequence, supporting coupling to the solid support.

Item 22. The method according to any one of the preceding items, wherein the solid phase coupled primer G (SP-primer) is coupled to a solid support selected from the group consisting of a glass slide, a cover slip, COC (cyclic olefin copolymer), COP (cyclic olefin polymer), PS (polystyrene), PC (polycarbonate),
5 PMMA (poly(methyl methacrylate)), PDMS (poly(dimethylsiloxane)), PET (poly(ethylene terephthalate)), cellulose-based beads, sheet or paper, a glass bead, a polymeric bead, a nanobead, a nanoparticle, graphene, an ELISA plate, and a polymeric multiple well plate.

10 Item 23. The method according to any one of the preceding items, wherein the LAMP amplification mixture of step g) comprises at least one polymerase enzyme supporting LAMP amplification and dinucleotide triphosphates (dNTPs).

Item 24. The method according to item 23, wherein the polymerase enzyme, has
15 strand displacement activity.

Item 25. The method according to items 23 or 24 wherein the polymerase enzyme is selected from the group consisting of Bst polymerase, Bst DNA Polymerase Large Fragment, Bsm DNA Polymerase, Bsu DNA Polymerase Large Fragment, SD
20 DNA Polymerase, Deep VentR DNA Polymerase, Deep VentR TM (exo) DNA Polymerase, DNA Polymerase I Large (Klenow) Fragment, M-MuLV Reverse Transcriptase, Phi29 DNA Polymerase, VentR DNA Polymerase, VentR (exo) DNA Polymerase, StrandDisplace Thermostable DNA Polymerase, and combinations thereof.

25

Item 26. The method according to any one of items 23-25, wherein the LAMP amplification mixture further comprises a reverse transcriptase, such as an AMV reverse transcriptase.

30 Item 27. The method according to any one of the preceding items, wherein the amplification steps are performed at a temperature in the range between 30-75°C, such as 40-70°C, such as 50-65°C, or such as 55-65°C.

Item 28. The method according to any one of the preceding items, wherein the
35 target sequence is single-stranded or double-stranded.

Item 29. The method according to any one of the preceding items, wherein the target sequence is DNA or RNA.

Item 30. The method according to any one of the preceding items, wherein the
5 sample is selected from the group consisting of food (liquid/solid and semi solid),
feed (liquid/solid and semi solid), blood (serum, buffer coat, treated with heparin
or EDTA), tissue (paraffin embedded), tumor, biopsy, sputum, swabs (from both
human and animal), fecal (from both human and animal), water and waste-water
samples.

10

Item 31. The method according to any one of the preceding items, wherein the
target sequence is of pathogenic origin, such as of bacterial, fungi or viral origin,
or from a gene involved in a disease.

15 Item 32. The method according to item 31, wherein the target sequence
originates from an organism selected from the group consisting of *Salmonella spp*,
Campylobacter jejuni, verotoxin producing *Escherichia coli* 0157: 1-17, *Listeria*
monocytogenes, *Shigella dysenteriae*, *Avian Influenza Virus* and *Norovirus*.

20 Item 33. The method according to any one of the preceding items, wherein the
target sequence is a *salmonella* sequence.

Item 34. The method according to any one of the preceding items, wherein the
immobilization region comprises an F2 region, complementary to an F2C region.

25

Item 35. The method according to item 34, wherein the F2 region of the
immobilization region has a length of at least 10 nucleotides, such as at least 20
nucleotides, such as at least 30 nucleotides, such as at least 40 nucleotides, such
as at least 50 nucleotides.

30

Item 36. The method according to any one of the preceding items, wherein the
method comprises providing a primer A (modFIP).

Item 37. The method according to item 36, wherein the immobilization region of
35 the primer G is complementary to a PIC region.

Item 38. The method according to any one of the preceding items, wherein the method comprises primers for detection of multiple target sequences.

Item 39. A kit comprising :

- 5
- Primers according to item 1;
 - An amplification mixture according to items 23-26; and
 - Optionally, instructions for detecting the presence or absence of a defined target sequence using the primer set and amplification mixture of the kit.

10

Item 40. The kit according to item 39, wherein primers for detection of multiple target sequences are included.

Item 41. The kit according to any one of items 39 or 40, suitable for use in a lab-
15 on-a-chip (LOC) or lateral flow format.

Item 42. Use of a kit according to any one of items 39-41 for the detection of a target sequence.

20 Item 43. The use according to item 42, wherein multiple targets are detected simultaneously in a single sample.

All patent and non-patent references cited in the present application, are hereby
25 incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

30 **Examples**

Example 1: Overall strategy of the Solid Phase Loop Mediated Amplification (SP-LAMP) method

Without being bound by theory, the following example illustrates how SP-LAMP is performed. Thus, the SP-LAMP method may be performed in variants related to

the setup described in the following example. In this example, the SP-LAMP technology is exemplified when using the F3, B3, FIP, BIP and modFIP primers.

The SP-LAMP technology consists of two phases of amplification - the liquid phase
5 amplification and the solid phase amplification. In the text below, we use the term "conventional LAMP" for the existing LAMP technology to differentiate the known art from the new SP-LAMP technology developed in this invention. It is noted that the liquid phase amplification and the solid phase amplification can be performed simultaneously as illustrated in e.g. claim 1 or in separate steps that may be
10 separated in time and/or space.

Primer design

For SP-LAMP, two primer pairs and one modFIP were used (F3/B3, FIP/BIP and modFIP). Two primer pairs F3/B3 and FIP/BIP were designed as conventional
15 LAMP primers, whereas the modFIP primer was equal to a FIP primer with an oligonucleotide added to the 5' end (Figure 1). The BIP primer was labelled with a Cy5 fluorophore at the 5' end. The two pairs of primers and modFIP were used in the liquid phase to produce stem loop primers labelled with Cy5 (LP-4A, see Fig 3D) and without self-primed DNA synthesis activity. These products (LP-4A)
20 served as the target for SP-primer binding and immobilisation on the solid phase. LP-4A does not exist in conventional LAMP.

A FIP SP-primer was designed to include an oligonucleotide repeat (10T10C) at the 5' end. The 10T10C sequence was used to link the SP-primer to the solid
25 phase by UV treatment. LP-4A products from the liquid phase annealed to the solid phase primer to initiate the SP-LAMP amplification.

The liquid phase (LP) steps of the amplification

In the LAMP reaction of the conventional LAMP technology, the LAMP primers
30 anneal to the complementary sequence on a double stranded target DNA (LP-IA, figure 2A) at 65°C and initiate the DNA synthesis using DNA polymerase with strand displacement activity. First, two pairs of primers (F3/B3 and FIP/BIP, figure 2A) were used to form a stem loop primer (LP-1B, figure 2A). This stem loop primer (mentioned in the conventional LAMP technology) has self-priming DNA

synthesis activity at the 3' end and continuously acts to form a double strand at the B2 loop of the stem loop. Secondly, the F2 region of modFIP anneal to the F2C region of LP-1C (figure 2B) and synthesize the complementary strand; simultaneously releasing a loop at 3' end (LP-1D, figure 2B). Self-priming DNA
5 synthesis activity at 3' end of LP-1D continues and releases LP-2A and LP-3A (figure 2C).

In LP-2A, simultaneously self-primed DNA synthesis activity at 3' end of LP-2A (Fig 3A), the B2 region of BIP labelled with Cy5 annealed to B2C of LP-2A and
10 synthesized the complementary strand (LP-2B, Fig3A). These activities led to the release of a loop at the 3' end (LP-2B2, Fig3B). The LP-2B2 does not have a self-priming DNA synthesis activity due to the blocking region (PIC) at the 3' end. Thus, the F2 region of modFIP can anneal to the F2C region at the 3' end of LP-2B2 and synthesis (LP-2C), leading to the release of a stem loop without self-
15 priming DNA synthesis activity and with a Cy5 label at the 5' end (LP-4A) and of LP-5A (Fig 3C). LP-5A is a dead product because there is not any loop/position for primer annealing to start amplification. LP-4A can be used as template for the FIP SP-primer.

20 Continuing with LP-3A, this product has a loop containing a B2C region that the B2 region of BIP-Cy5 annealed to (LP-3B, Fig 4A) and synthesized the other complementary strand, simultaneously releasing two loops (LP-3C, Fig 4B). The LP-3C has two loops with self-priming DNA synthesis activity at free 3' end. This activity leads to release LP-6A (Fig 4C). Simultaneously, the B2 region of BIP
25 anneal to the B2C region on the loop of LP-3D (Fig 4C) and synthesizes a complementary strand to form LP-3E with four loops (Fig 4D). LP-3E has two loops with B2C and F2C regions, which BIP and modFIP can anneal to and synthesize new strands (LP-3F, Fig 4E). Simultaneously with activity of the BIP and modFIP, self-priming DNA synthesis at 3' end of LP-3F continuously acts to
30 release LP-7A, LP-8A and LP-3G (Fig 4F and Fig 4G). LP-3G in Fig 4F continues working to form more and more loops.

On the LP-6A, the self-priming DNA synthesis activity and BIP act together (Fig 5A) to release LP-6C (Fig 5B) with two loops and a self-priming DNA synthesis at
35 the 3' end. modFIP anneals to a loop containing a F2C region and synthesizes a

new strand (LP-6C, Fig 5B). Simultaneously, the self-priming DNA synthesis acts and releases LP-9A, LP-6C1 and LP-6D (Fig 5C and Fig 5D). LP-6D has a loop containing a B2C region that BIP can anneal to (LP-6E, Fig 5D) and synthesize LP-6F (Fig 5E) with more loops. Primers modFIP and BIP continues annealing to those 5 loops (LP-6F, Fig 5E), simultaneously self-priming DNA synthesis activity and release LP-10A (Fig 5F), LP-11A (Fig 5G) and LP-6G (Fig 5H). LP-6G in Fig 5H continues working to form more and more loops.

Products contain a self-priming DNA synthesis at 3' end and multiple loops 10 including LP-7A (five loops), LP-8A (six loops), LP-9A (three loops), LP 6C1 (four loops), LP-10A (five loops), LP-11A (eight loops), LP-12A (three loops) and LP-13A (three loops). Those loops are positions for modFIP and BIP primers annealing and synthesis of new strands. Simultaneously self-priming DNA activity, they generate three types of products: LP-4A labeled with Cy5 (Fig 7B, Fig 9D) 15 which serve as template for solid phase amplification, dead products (without any loops) such as LP-12B (Fig 7B) and LP-13C (Fig 9D), and products with single loop or multiple loops such as LP-2A (Fig 6 and Fig 8B), LP-7B (Fig 6), LP-8B (Fig 7A), LP-9C (Fig 8B), LP-6C3 (Fig 9B). Those products continue to synthesize new strands since their loops are positions to which primers may anneal and elongate 20 strands.

The entire network of the liquid phase amplification part of the method is schematically represented in figure 10.

25 *The solid phase (SP) steps of the amplification*

The stem loop primers without self-primed DNA synthesis activity from liquid phase (LP-4A) served as starting materials for the solid phase amplification. The F2C region of the stem loop primers annealed to the F2 region of the immobilized solid phase primer (SP-1) on the surface (SP-2) (figure 11A) and synthesis of the 30 complementary strand (SP-3) lead to formation of a double strand with a Cy5-label (SP-4) (figure 11B).

The above steps are purely included for illustrative purposes and in practice other products may also be formed. The division into liquid phase and solid phase is also

only for illustrative purposes, since in practice the two phases are performed simultaneously.

Detection of amplification product

- 5 A scanner may be used to detect the presence of immobilised product (SP-4) on the solid surface through the fluorescent Cy5 label. The slides may be scanned using a BioAnalyzer 4F/4S scanner with 5000-ms shutter time (LaVision BioTec GmbH, Bielefeld, Germany).
- 10 This example outlines the SP-LAMP process using a single combination of primers {i.e. F3, B3, FIP, BIP, modFIP and SP-primer}. As described herein, the SP-LAMP process is also applicable using other combinations of primers (e.g. see example 2)
- 15 **Example 2: Detection of *Salmonella* spp by SP-LAMP with different combinations of primers**

Aim:

To verify that SP-LAMP can be used to amplify and detect small amounts of target DNA. In this example, the primers were designed base on the *hilA* gene sequence of *Salmonella* to target DNA originates from *Salmonella* spp. The PrimerExplorer V4 (Eiken Chemical Co. Ltd, Tokyo, Japan) was used to designed all the primers based on *hilA* gene sequence from *Salmonella Enteritica* subsp. Enteritica serova Newport (NCBI GenBank accession no. CP010.280.1). The primers then were aligned for the specificity on NCBI website.

25

Methods:

SP-LAMP was performed as described in example 1, with primers and solid phase primers designed according to table 2. The solid phase primers including FIP, FIP21 and FIP22 with poly(T)10-poly(C)10 tail was prepared at 60 μM concentration in 5X SSC buffer, 0.04% Triton X and spotted on the COC slide using non-contact array nano-plotter 2.1 (GeSim, Dresden, Germany) with a volume of 0.06 nL per spot. The COC slide was allowed to dry at room temperature and then solid phase primers were immobilized on the COC surface by exposing to the UV irradiation at 254 nm with power of 3 mW/cm² for 5 min

30

35

(Stratalinker 2400, Stratagene, CA, USA). During UV treatment, a covalent bond was formed between TC tail and unmodified surface. Subsequently, the COC slide was washed with 0.1X standard saline citrate (SSC) for 5 min then rinsed with deionized water and dried in room temperature. BSA (bovine serum albumin) (2.5 mg/mL) covered all spots for 30 min to block the surface. The slide then was rinsed in deionized water and dried in room temperature. A gene frame (Life Technologies Europe BV, Danmark) was used to cover all spots.

SP-LAMP was demonstrated with three different master-mixtures. All the master-
 10 mixtures contained all components for the LAMP reaction. Master-mix 1 (MX1) contained only normal primers F3, B3 FIP, and BIP. Master-mix 2 (MX2) contained normal primers (F3, B3 FIP, and BIP) and only one modification primer, modFIP. Master-mix 3 (MX3) contained normal primers (F3, B3 FIP, and BIP) and both modification primers, modFIP and modBIP. Details of MX1, MX2 and MX3 are
 15 given in tables 3-5.

25 uL of each master-mix was added to each gene frame as described above. After 60 min (for MX1 and MX2) or 90 min (for MX3) of amplification at 65 °C, the COC slide was washed for 5 min in washing solution containing 0.1X standard
 20 saline citrate (SSC) and 0.1% (w/v) sodium dodecyl sulfate (SDS) for 5 min then rinsed with deionized water for 2 min and dried in room temperature. The slide then was scanned 1000ms with Cy5 filter by a BioAnalyzer 4F/4S scanner with 5000-ms shutter time (LaVision BioTec GmbH, Bielefeld, Germany).

25 Table 2

Name	Sequences	Size bp
F3	CGC ATA CTG CGA TAA TCC CTT C (SEQ ID NO: 1)	22
B3	ATT CTG TCG GAA GAT AAA GAG C (SEQ ID NO: 2)	22
FIP	CGC CGC AAC CTA CGA CTC ATA CTG CAG ACT CTC GGA TTG AAC C (SEQ ID NO: 3)	43
BIP	GGC GGA GAC ACC ACT ACG ACC GTT ACA TTG AAA CAC TGT ACG (SEQ ID NO: 4)	42

modFIP	AGT AGG ACC TAC ACC TGT CAC GCC GCA ACC TAC GAC TCA TAC TGC AGA CTC TCG GATTGA ACC (SEQ ID NO:5)	63
modBIP	Cy5- CGG ATT GAA CGG CGG AGA CAC CAC TAC GAC CGT TAC ATT GAA ACA CTG TAC G (SEQ ID NO:6)	52
FIP SP-primer	TTT TTT TTT TCC CCC CCC CC C GCC GCA ACC TAC GAC TCA TAC TGC AGA CTC TCG GATTGA ACC (SEQ ID NO:7)	63
FIP21 SP-primer	TTT TTT TTT TCC CCC CCC CCC GCC GCA ACC TAC GAC TCA TAC TGC AGA CTC TCG GATTGA ACC TGA TCC TGC ATC TGA (SEQ ID NO:8)	78
FIP22 SP-primer	TTT TTT TTT TCC CCC CCC CCC GCC GCA ACC TAC GAC TCA TAC TGC AGA CTC TCG GATTGA ACC TGA TCC TGC ATC TGA AAA GGA AGT A (SEQ ID NO:9)	88

The components of the experiment were mixed according to tables 3-5.

Table 3

Components	Stock conc.	1x	Final conc.
F3 (μm)	20	0.25	0.2
B3 (μm)	20	0.25	0.2
FIP (μm)	20	1.875	1.5
BIP-Cy5 (μm)	50	0.7	1.4
dNTPs (mM)	12.5	2.8	1.4
Betaine (M)	2.5	5	0.5
Buffer (X)	10	2.5	1
H ₂ O		7.625	
Bst 2.0 warmstart (U)		1	8
DNA (ng/ μL)	2	2	
Total (μL)		25	

5

Master-mix 1 (MX1) without modification primers.

Table 4

Components	Stock conc.	1x	Final conc.
F3 (μm)	20	0.25	0.2
B3 (μm)	20	0.25	0.2
FIP (μm)	20	1.25	1
BIP-Cy5 (μm)	50	0.7	1.4
<i>modFIP</i> (μm)	50	0.25	0.5
dNTPs (mM)	12.5	2.8	1.4
Betaine (M)	2.5	5	0.5
Buffer (X)	10	2.5	1
H ₂ O		8	
Bst 2.0 warmstart (U)		1	8
DNA (ng/ μL)	2	2	
Total (μL)		25	

Master-mix 2 (MX2) with modification FIP primers.

5 Table 5

Components	Stock conc.	1x	Final conc.
F3 (μm)	20	0.25	0.2
B3 (μm)	20	0.25	0.2
FIP (μm)	20	1.25	1
BIP (μm)	50	0.5	1
<i>modFIP</i> (μm)	50	0.25	0.5
<i>modBIP-Cy5</i> (μm)	50	0.2	0.4
dNTPs (mM)	12.5	2.8	1.4
Betaine (M)	2.5	5	0.5
Buffer (X)	10	2.5	1
H ₂ O		8	
Bst 2.0 warmstart (U)		1	8
DNA (ng/ μL)	2	2	
Total (μL)		25	

Master-mix 3 (MX3) with both modification FIP and modification BIP primers.

Each of the master-mixtures (MX1, MX2 and MX3) according to tables 3-5, respectively, was utilized together with three different solid phase (SP) primers according to SEQ ID NOs: 7-9 (see table 2).

5 Results:

Figure 12 shows the SP-LAMP result. Cy5 represents a Cy5 control probe, which served as positive control. HIP is a solid phase primer targeting *Campylobacter jejuni*. It is used in this experiment as negative control. FIP, FIP21 and FIP22 are solid phase primers of different lengths, which are designed to target *Salmonella* spp. The result shows that all mixtures, MX 1, MX2 and MX3, containing the SP-LAMP primers yielded bright fluorescent spots indicating that solid phase amplification took place. In contrast, spots containing the control HIP solid phase primer originating from *Campylobacter jejuni* displayed no fluorescence. The data shows that the SP-LAMP reaction, as designed in this experiment, is specific for *Salmonella*. A Cy5 control probe served as a positive control.

Short conclusion:

The results show that SP-LAMP can successfully specifically amplify and detect small amounts of DNA, while ignoring non-target DNA.

20

Example 3: Detection of *Listeria monocytogenes*, *Norovirus*, *Campylobacter jejuni* and *Avian influenza virus* by SP-LAMP

Aim:

To verify that SP-LAMP can be used to amplify and detect small amounts of target DNA from a variety of distinct and unrelated species.

Methods:

SP-LAMP was performed as described in example 1, with primers and solid phase primers designed for detection of *Listeria monocytogenes*, *Norovirus*, *Campylobacter jejuni* or *Avian influenza virus*. The experiments were carried out as described in example 2. Below is given the sequences utilized for detection of *Listeria monocytogenes*. Master mixes (MX1-3) were mixed according to tables 3-5.

Table 6

Name	Sequences	Size bp
F3	AGT TGT GAA TGC AAT TTC GA (SEQ ID NO:10)	20
B3	TTT TCA TTC CAT CTT TCC ACT A (SEQ ID NO:11)	22
FIP	AGG GAG AAC ATC TGG TTG ATT TTC TCC TAA CCT ATC CAG GTG CT (SEQ ID NO:12)	44
BIP	GCA TTG ATT TGC CAG GTA TGA CTC TGC GTT GTT AAC GTT TGA (SEQ ID NO:13)	42
modFIP	AGT AGG ACC TAC ACC TGT CAA GGG AGA ACA TCT GGT TGA TTT TCT CCT AAC CTA TCC AGG TGC T (SEQ ID NO:14)	64
modBIP	Cy5-CGG ATT GAA CGC ATT GAT TTG CCA GGT ATG ACC TGC GTT GTT AAC GTT TGA (SEQ ID NO:15)	51
SP1-primer	TTT TTT TTT TCC CCC CCC CCA GGG AGA ACA TCT GGT TGA TTT TCT <u>CCT AAC CTA TCC AGG TGC</u> <u>ICT CGT AAA AGC G</u> (SEQ ID NO:16)	76
SP2-primer	TTT TTT TTT TCC CCC CCC CCA GGG AGA ACA TCT GGT TGA TTT TCT <u>CCT AAC CTA TCC AGG TGC</u> <u>ICT CGT AAA AGC GAA TTC GGA ATT AGT</u> (SEQ ID NO:17)	90
SP3-primer	TTT TTT TTT TCC CCC CCC CC-Hexethylene glycol monomer-GCA TTG ATT TGC CAG GTA TGA CTC <u>TGC GTT GTT AAC GTT TGA TTT AGT GGC</u> (SEQ ID NO:18)	71

Each of the master-mixtures (MX1, MX2 and MX3) according to tables 3-5, 5 respectively, was utilized together with three different solid phase (SP) primers (SP1-3) according to SEQ ID NOs: 16-18 (see table 6).

Furthermore, LAMP primer sets were designed for detection of Avian influenza virus (AIV). Sequences for detection of AIV are given in table 7. The solid phase 10 primer (SP primer) was spotted on a COP slide. The remaining primers were mixed according to table 8, the SP-LAMP reaction was run and the resulting

amplification products were determined by electrophoresis. The SP-LAMP results were compared to detection by standard reverse-transcriptase PCR (RT-PCR). Detection of AIV was tested in embryonated chicken eggs and wild-bird samples collected in Denmark.

5

Table 7

Name	Sequences	Size bp
F3	GCA GGT AGA TAT TGA AAG ATG AGT C (SEQ ID NO:19)	25
B3	CTC ACT GGG CAC GGT GA (SEQ ID NO:20)	17
FIP	GGC TTT GAG GGG GCC TGA TTC TAA CCG AGG TCG AAA CG (SEQ ID NO:21)	38
BIP	Cy5-CTT GAA GAT GTC TTT GCA GGG AAG AAC ATA GTC AGA GGT GAC AGG ATT GG (SEQ ID NO:22)	50
SP-primer	TTT TTT TTT TCC CCC CCC CCG GCT TTG AGG GGG CCT GAT TCT AAC CGA GGT CGA AAC G (SEQ ID NO:23)	58

Table 8

Components	Stock conc.	I x	Final conc.
F3 (Mm)	20	0.1	0.2 Mm
B3 (Mm)	20	0.1	0.2 Mm
FIP (Mm)	100	0.16	1.6 Mm
BIP-Cy5 (Mm)	100	0.16	1.6 Mm
dNTPs (mM)	12.5	1.12	1.4 mM
Betaine (M)	2.5	2	0.5 Mm
Buffer (X)	10	1	1 x
H ₂ O		2.34	
Bst 2.0 warmstart (U)		0.5	0.4 U μ I
DNA (ng/ML)	2	1	

Results:

Results similar to those obtained in example 2 (figure 12) were obtained for each of the different species {i.e. *Listeria monocytogenes*, *Norovirus*, *Campylobacter jejuni* or *Avian influenza virus*), data shown for *Listeria monocytogenes* and Avian
5 influenza virus (AIV).

Figure 13 shows the SP-LAMP result. Figure 13A shows a schematic representation of the experimental setup. Cy5 represents a Cy5 control probe, which served as positive control. HIP is a solid phase primer targeting *Campylobacter jejuni*. It is
10 used in this experiment as negative control. SP1, SP2 and SP3 are solid phase primers of different lengths, which are designed to target *Listeria monocytogenes*. Figure 13B shows that all mixtures, MX 1, MX2 and MX3, containing the SP-LAMP primers yielded bright fluorescent spots indicating that solid phase amplification took place. In contrast, spots containing the control HIP solid phase primer
15 originating from *Campylobacter jejuni* displayed no fluorescence. The data shows that the SP-LAMP reaction, as designed in this experiment, is specific for *Listeria monocytogenes*. A Cy5 control probe served as a positive control.

Figure 14A demonstrates that the SP-LAMP method successfully detects AIV
20 growth in embryonated chicken eggs. The non-AIV sample (Newcastle Disease Virus, NDV) and control negative test (CN) gives rise to no signal. The figure shows that the SP-LAMP method is capable of detecting different subtypes of AIV, see HA and NA subtype designation in figure 14A.

25 Moreover, figure 14B shows that the SP-LAMP method successfully detects AIV in infected wild birds samples collected in Denmark. The SP-LAMP method performed better than the EU standard test relying on reverse-transcriptase PCR (RT-PCR), demonstrating one of the advantages of the SP-LAMP method.

30 Short conclusion:

The results show that SP-LAMP can successfully specifically amplify and detect targets of different species. The method is therefore suitable for detection of a variety of different infectious or undesired species in a sample.

Example 4: Multiplex detection of different targetsAim:

To verify that SP-LAMP can be used to amplify and detect simultaneously small amounts of target DNA from a variety of distinct and unrelated species.

5

Methods:

SP-LAMP was performed as described in example 1. Primers and solid phase primers was designed for detection of *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Campylobacter coli* or *Avian influenza virus*. The solid phase
10 primers of these species were spotted and conducted in the same experiment. The master-mix contained primers of all of these species. This experiment was carried out as described in example 2.

Results:

15 In all cases, the Cy5 positive control showed fluorescence signal. When only *Salmonella* DNA was added, fluorescence signal was observed only in spots where the *Salmonella* SP-primer was spotted, whereas no fluorescence signal were detected for other spots in which SP-primers from other bacterial or virus were located, data not shown.

20

Similar results were obtained when DNA from the other pathogens (*Listeria monocytogenes*, *Campylobacter jejuni*, *Campylobacter coli* or *Avian influenza virus*) were added, data not shown.

25 When *Salmonella* DNA and *Listeria Monocytogenes* DNA were added simultaneously, fluorescence signal were observed in spots containing either *Salmonella* or *Listeria Monocytogenes* SP-primers, whereas no fluorescence signal were detected for other spots in which SP-primers from other bacterial or virus were located, data not shown.

30

When the master-mix contained all DNA targets of *Salmonella*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Campylobacter coli* or *Avian influenza virus*, fluorescence signal were observed in all spots where the SP-primers corresponded to any of these species, data not shown.

35

Short conclusion:

The results show that SP-LAMP can simultaneously and specifically amplify and detect multiple targets of different species.

5 References

- T. Notomi et al., Nucleic Acid Res. (2000), Vol. 28, No. 12 e63
- WO 00028082
- L. Niessen et al. Food Microbiol. (2013), 36, 191-206

Claims

1. A loop mediated isothermal amplification (LAMP) method for detecting an oligonucleotide target sequence comprising :

5 a) providing a sample suspected of comprising an oligonucleotide target sequence, said oligonucleotide target sequence comprising in the 3' to 5' direction:

- 10 i. an F3C region, complementary to an F3 region;
- ii. an F2C region, complementary to an F2 region;
- iii. an F1C region, complementary to an F1 region;
- iv. a spacer region;
- v. a B1 region, complementary to a B1C region;
- vi. a B2 region, complementary to a B2C region; and
- vii. a B3 region complementary to a B3C region;

15 b) providing a primer A (modFIP) comprising in the 5' to 3' direction:

- i. a P1 region, complementary to a PIC region;
- ii. an F1C region, complementary to the F1 region; and
- iii. an F2 region, complementary to the F2C region;

and/or

20 providing a primer B (FIP) comprising in the 5' to 3' direction:

- iv. an F1C region, complementary to the F1 region; and
- v. an F2 region, complementary to the F2C region;

c) providing a primer C (BIP) comprising in the 5' to 3' direction:

- 25 i. a detection label;
- ii. a B1C region, complementary to the B1 region; and
- iii. a B2 region, complementary to the B2C region;

and/or

30 providing a primer D (modBIP) comprising in the 5' to 3' direction :

- iv. a detection label;
- v. a blocking region;
- vi. a B1C region, complementary to the B1 region; and
- vii. a B2 region, complementary to the B2C region;

35 with the proviso that if both primer C (BIP) and primer D (modBIP) are provided, then only one of primer C (BIP) and primer D (modBIP)

comprises a detection label according to step (c)(i) and step (c)(iv), respectively;

d) providing a primer E (F3), complementary to the F3C region;

5

e) providing a primer F (B3), complementary to the B3C region;

f) providing a solid phase coupled primer G (SP-primer) comprising a free 3'-end and having an immobilization region being complementary to a region of the oligonucleotide target sequence or a LAMP product arising from the oligonucleotide target sequences;

10

g) mixing the sample according to step a) and primers according to step b)-f) with a LAMP amplification mixture, thereby providing an amplified LAMP product coupled to the solid support if a target sequence is present in the sample;

15

h) optionally, washing the solid support; and

i) detecting the amplified product coupled to the solid support, by detecting the detection label, e.g. by fluorescence.

20

2. The method according to claim 1, wherein the detection label of the primer C (BIP) and/or the primer D (modBIP) is selected from the group consisting of a fluorophore and a radiolabel.

25

3. The method according to claim 2, wherein the fluorophore is selected from the group consisting of Hydrocoumarin, Methoxycoumarin, Alexa Fluor, Aminocoumarin, Cy2, FAM, Alexa Fluor 488, Fluorescein FITC, Alexa Fluor 430, Alexa Fluor 532, HEX, Cy3, TRITC, Alexa Fluor 546, Alexa Fluor 555, R-phycoerythrin (PE), Rhodamine Red-X, Tamara, Cy3.5 581, Rox, Alexa Fluor 568, Red 613, Texas Red, Alexa Fluor 594, Alexa Fluor 633, Allophycocyanin, Alexa Fluor 633, Cy5, Alexa Fluor 660, Cy5.5, TruRed, Alexa Fluor 680, Cy7, tetrachlorofluorescein (TET), SYBR green I or II, YBR Green II, SYBR Gold, YO (Oxazole Yellow), TO (Thiazole Orange) and PG (PicoGreen).

35

4. The method according to any one of the preceding claims, wherein the solid phase coupled primer G (SP-primer) comprises a 5' linker sequence, supporting coupling to the solid support.
- 5 5. The method according to any one of the preceding claims, wherein the solid phase coupled primer G (SP-primer) is coupled to a solid support selected from the group consisting of a glass slide, a cover slip, COC (cyclic olefin copolymer), COP (cyclic olefin polymer), PS (polystyrene), PC (polycarbonate), PMMA (poly(methyl methacrylate)), PDMS (poly(dimethylsiloxane)), PET (poly(ethylene terephthalate)), cellulose-based beads, sheet or paper, a glass bead, a polymeric bead, a nanobead, a nanoparticle, graphene, an ELISA plate, and a polymeric multiple well plate.
6. The method according to any one of the preceding claims, wherein the LAMP amplification mixture of step g) comprises at least one polymerase enzyme supporting LAMP amplification and dinucleotide triphosphates (dNTPs).
7. The method according to claim 6, wherein the polymerase enzyme, has strand displacement activity.
- 20 8. The method according to claims 6 or 7, wherein the polymerase enzyme is selected from the group consisting of Bst polymerase, Bst DNA Polymerase Large Fragment, Bsm DNA Polymerase, Bsu DNA Polymerase Large Fragment, SD DNA Polymerase, Deep VentR DNA Polymerase, Deep VentR™ (exo) DNA Polymerase, DNA Polymerase I Large (Klenow) Fragment, M-MuLV Reverse Transcriptase, Phi29 DNA Polymerase, VentR DNA Polymerase, VentR (exo) DNA Polymerase, StrandDisplace Thermostable DNA Polymerase, and combinations thereof.
9. The method according to any one of claims 6-8, wherein the LAMP amplification mixture further comprises a reverse transcriptase, such as an AMV reverse transcriptase.
- 30 10. The method according to any one of the preceding claims, wherein the amplification steps are performed at a temperature in the range between 30-75°C, such as 40-70°C, such as 50-65°C, or such as 55-65°C.
- 35

11. The method according to any one of the preceding claims, wherein the target sequence is single-stranded or double-stranded.

12. The method according to any one of the preceding claims, wherein the target
5 sequence is DNA or RNA.

13. The method according to any one of the preceding claims, wherein the sample is selected from the group consisting of food (liquid/solid and semi solid), feed (liquid/solid and semi solid), blood (serum, buffer coat, treated with heparin or
10 EDTA), tissue (paraffin embedded), tumor, biopsy, sputum, swabs (from both human and animal), fecal (from both human and animal), water and waste-water samples.

14. The method according to any one of the preceding claims, wherein the target
15 sequence is of pathogenic origin, such as of bacterial, fungi or viral origin, or from a gene involved in a disease.

15. The method according to claim 14, wherein the target sequence originates from an organism selected from the group consisting of *Salmonella spp*,
20 *Campylobacter jejuni*, verotoxin producing *Escherichia coli* 0157: 1-17, *Listeria monocytogenes*, *Shigella dysenteriae*, *Avian Influenza Virus* and *Norovirus*.

16. The method according to any one of the preceding claims, wherein the target sequence is a *salmonella* sequence.

25

17. The method according to any one of the preceding claims, wherein the immobilization region comprises an F2 region, complementary to an F2C region.

18. The method according to any one of the preceding claims, wherein the
30 method comprises providing a primer A (modFIP).

19. The method according to claim 18, wherein the immobilization region of the primer G is complementary to a PIC region.

20. The method according to any one of the preceding claims, wherein the method comprises primers for detection of multiple target sequences.

21. A kit comprising:

- 5
- Primers according to claim 1;
 - An amplification mixture according to claim 6; and
 - Optionally, instructions for detecting the presence or absence of a defined target sequence using the primer set and amplification mixture of the kit.

10

22. The kit according to claim 21, wherein primers for detection of multiple target sequences are included.

23. The kit according to any one of claims 21 or 22, suitable for use in a lab-on-a-
15 chip (LOC) or lateral flow format.

24. Use of a kit according to any one of claims 21-23 for the detection of a target sequence.

20 25. The use according to claim 24, wherein multiple targets are detected simultaneously in a single sample.

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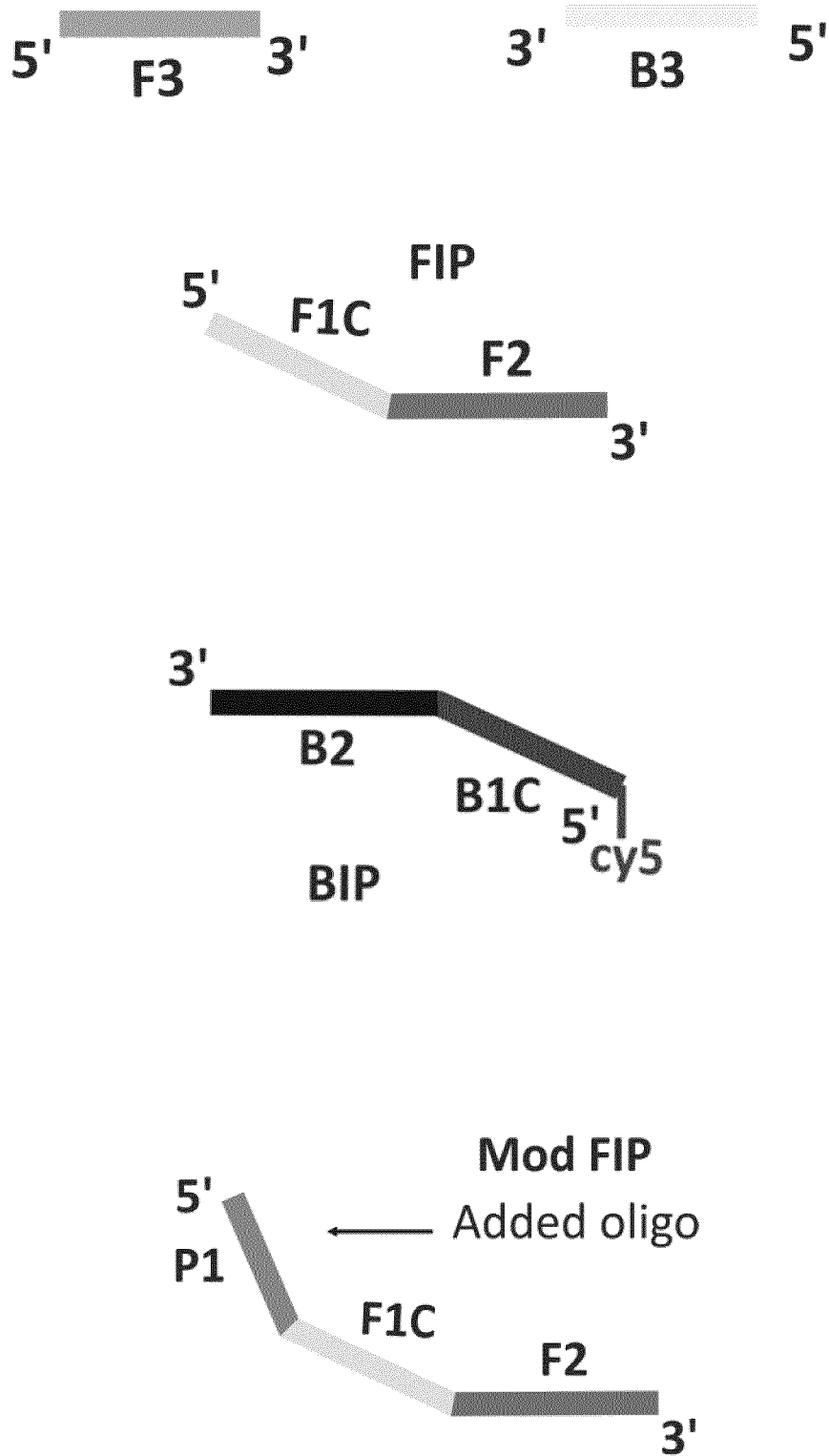


Fig. 1

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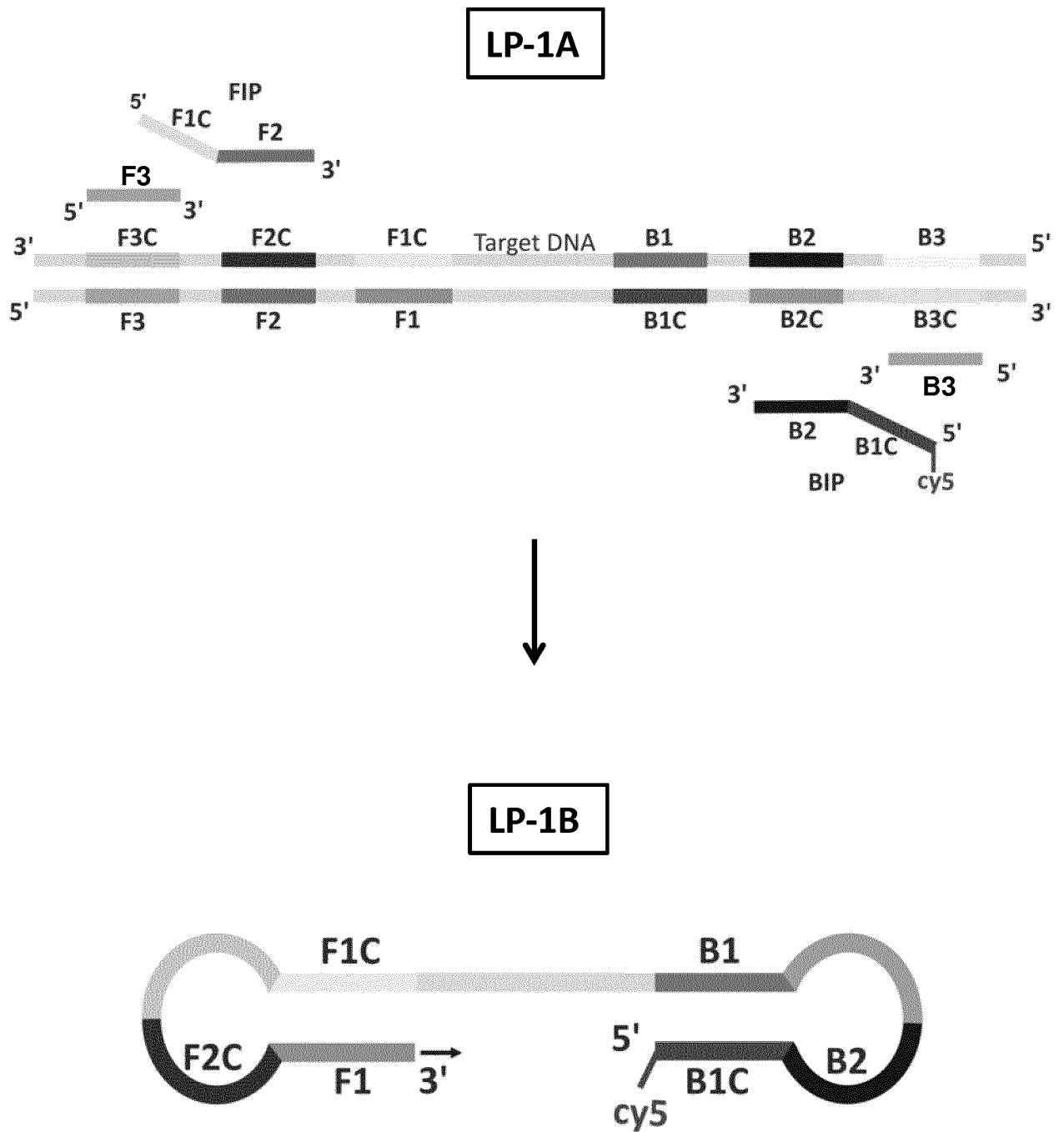
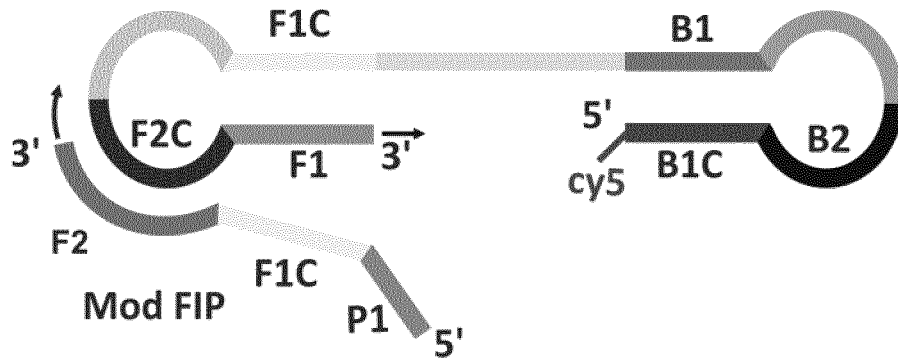


Fig. 2A

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LP-1C



LP-1D

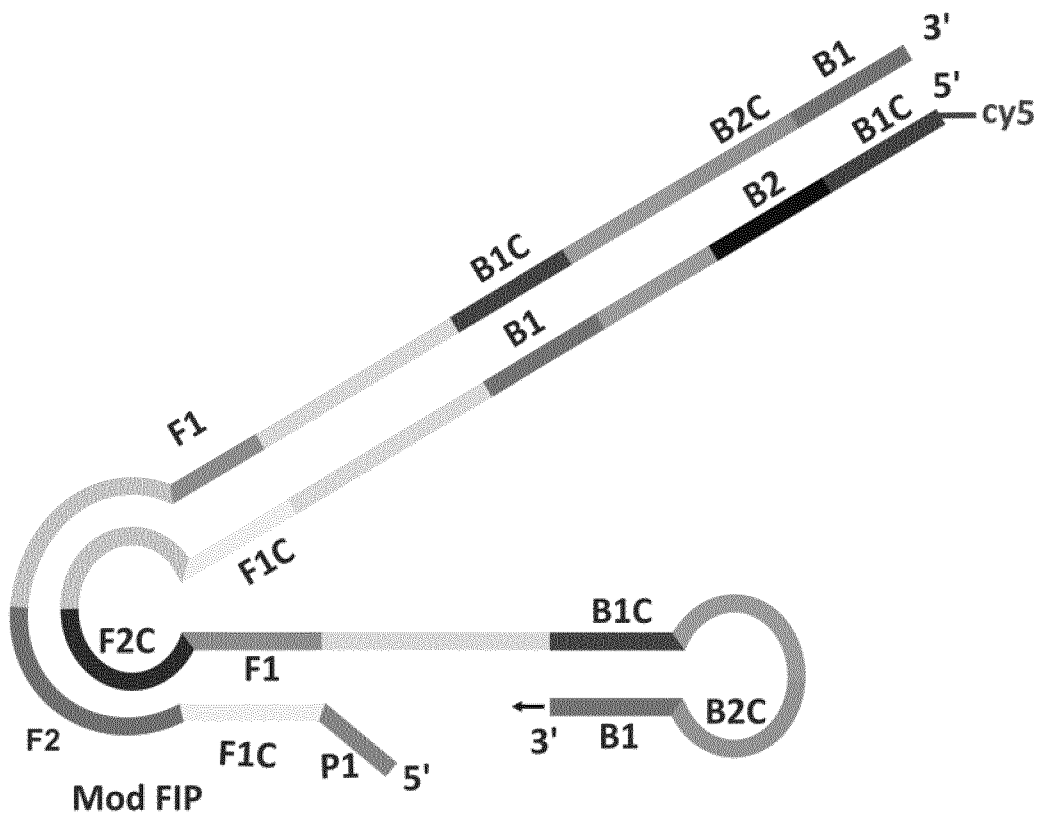


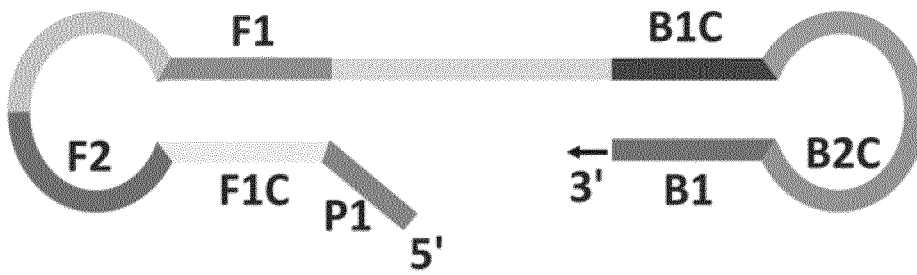
Fig. 2B

4/39



From LP-1D

LP-2A



+

LP-3A

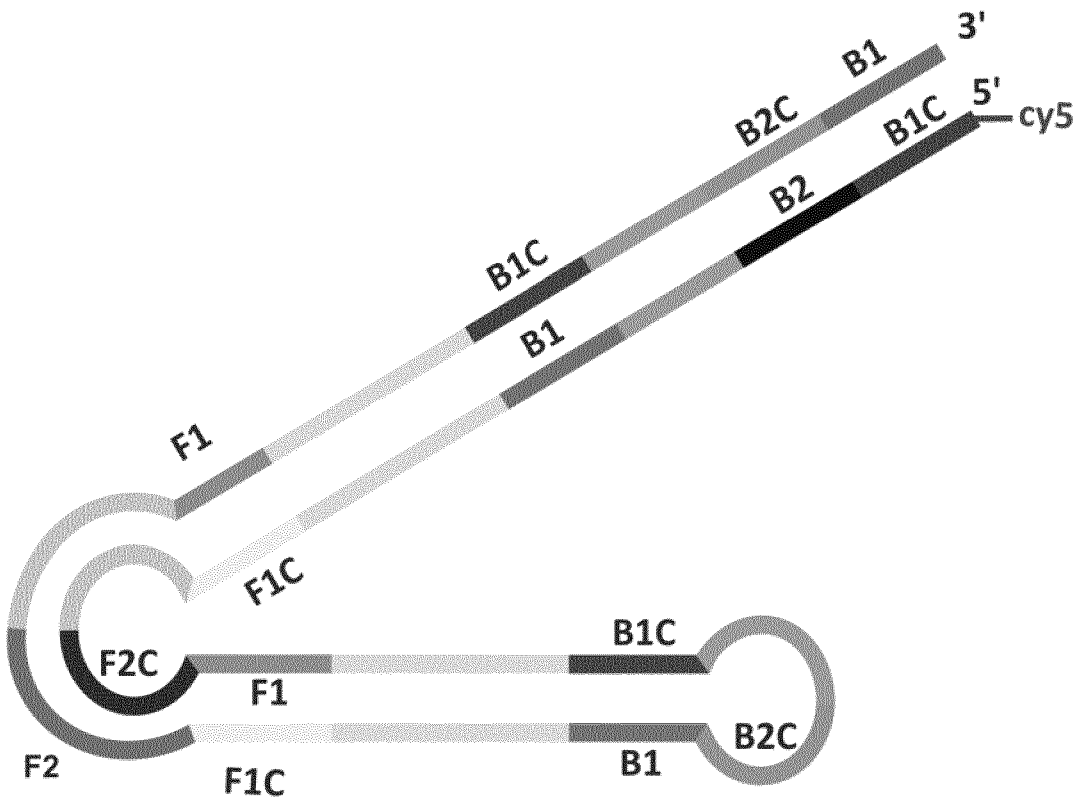


Fig. 2C

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LP-2A continued

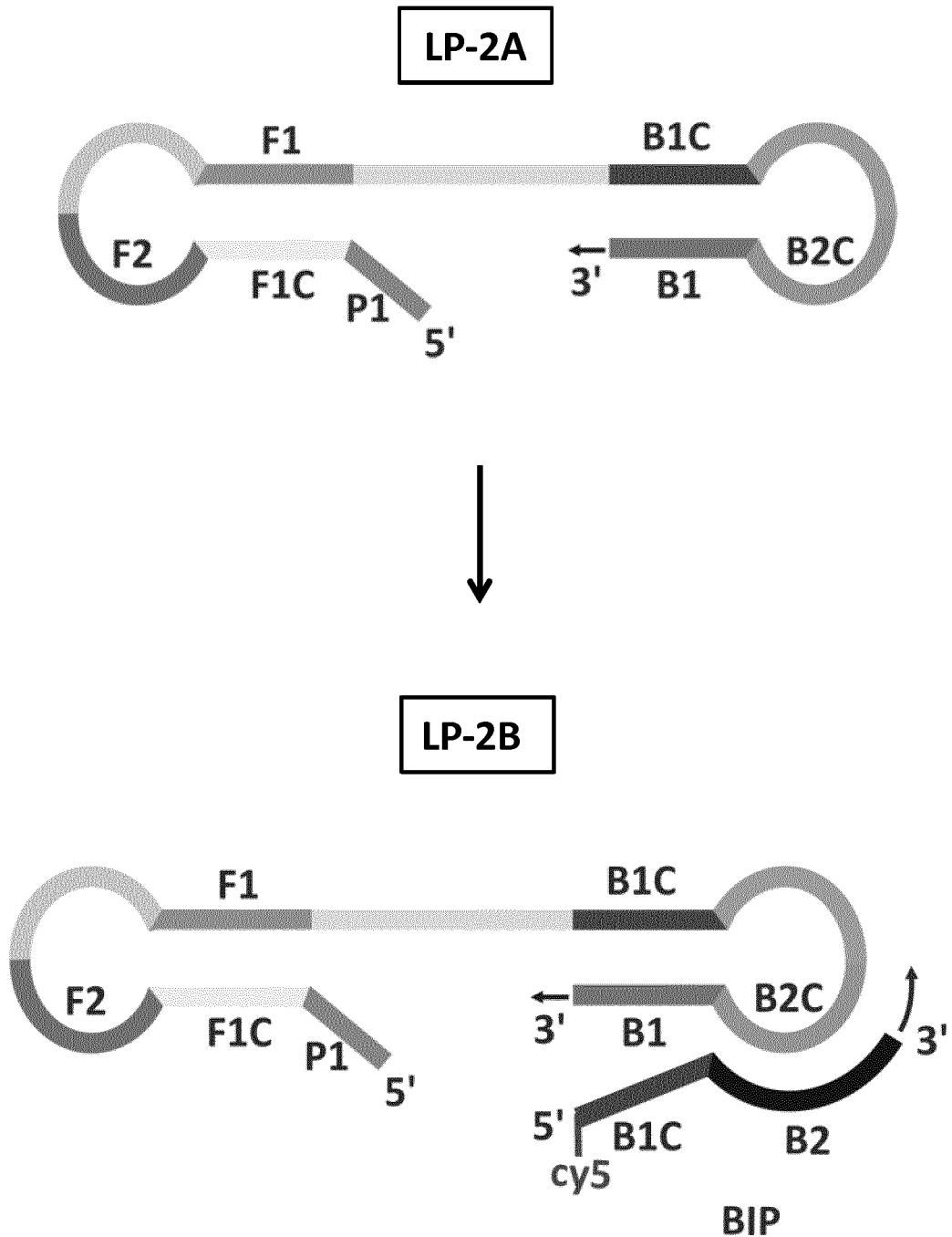
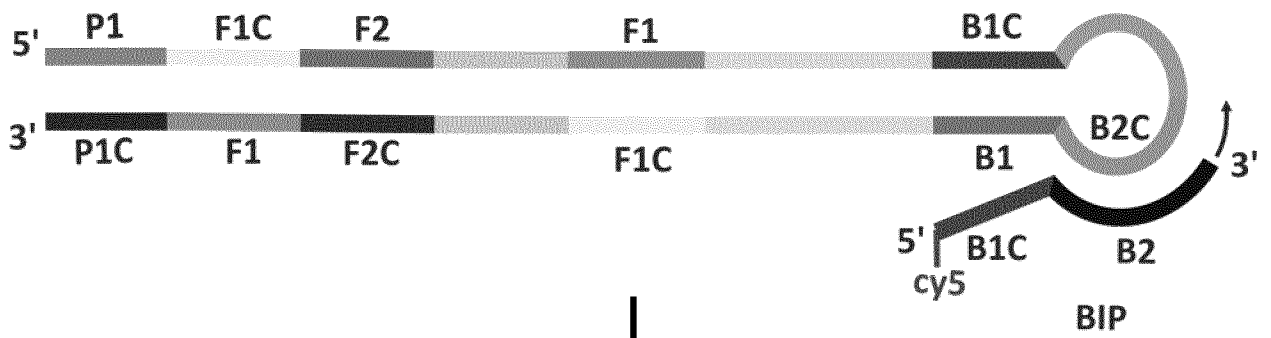


Fig. 3A

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From LP-2B

LP-2B1



LP-2B2

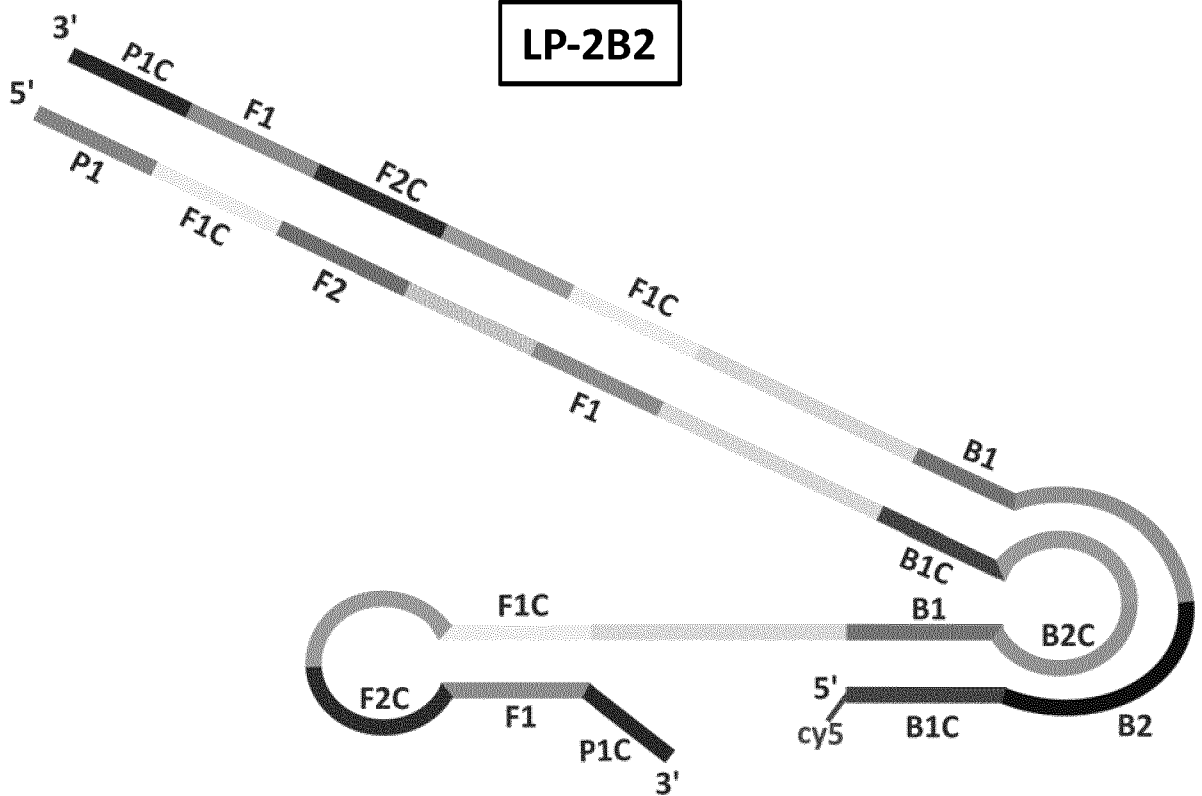


Fig. 3B

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From LP-2B2

LP-2C

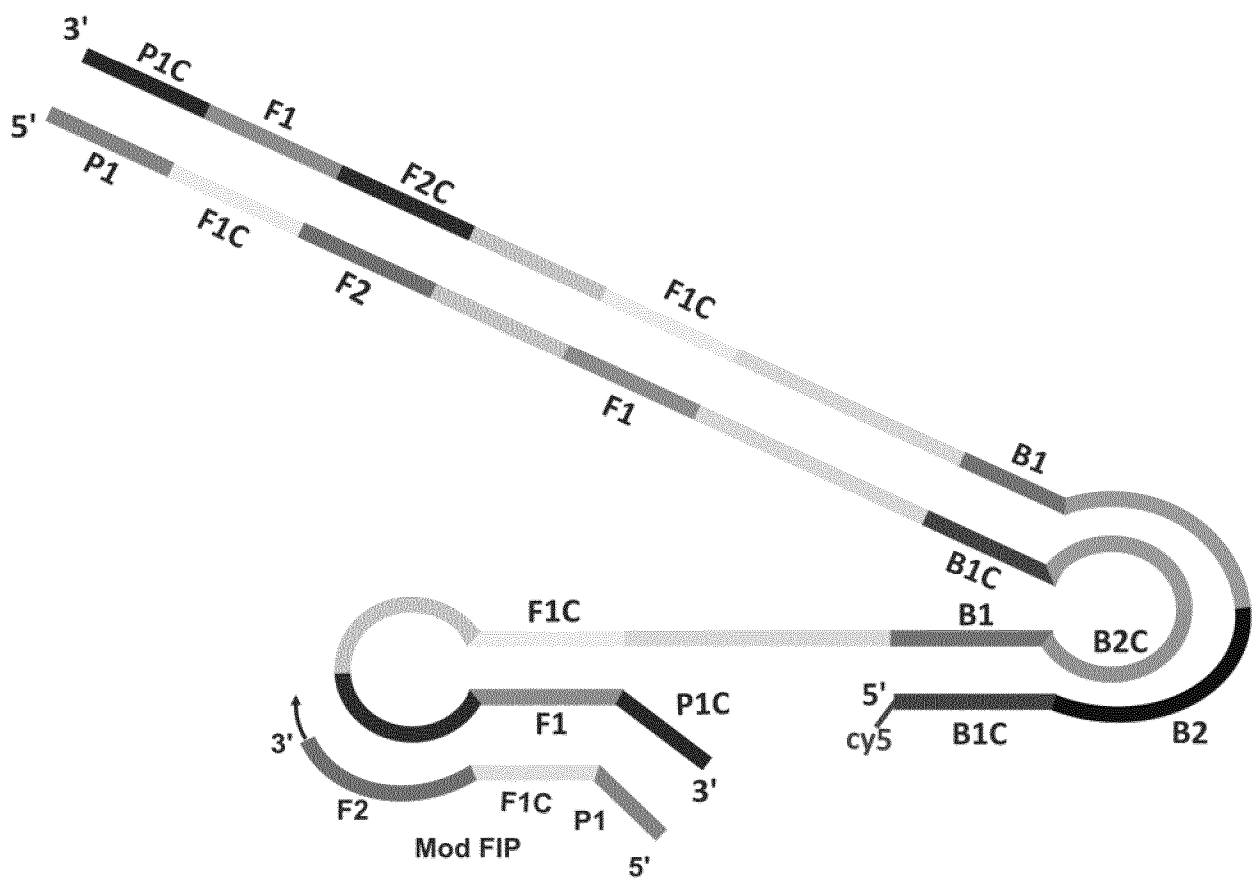
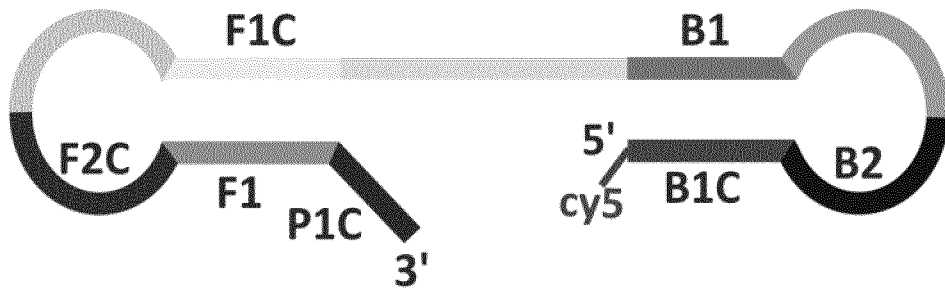


Fig. 3C

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From LP-2C

LP-4A



+

LP-5A

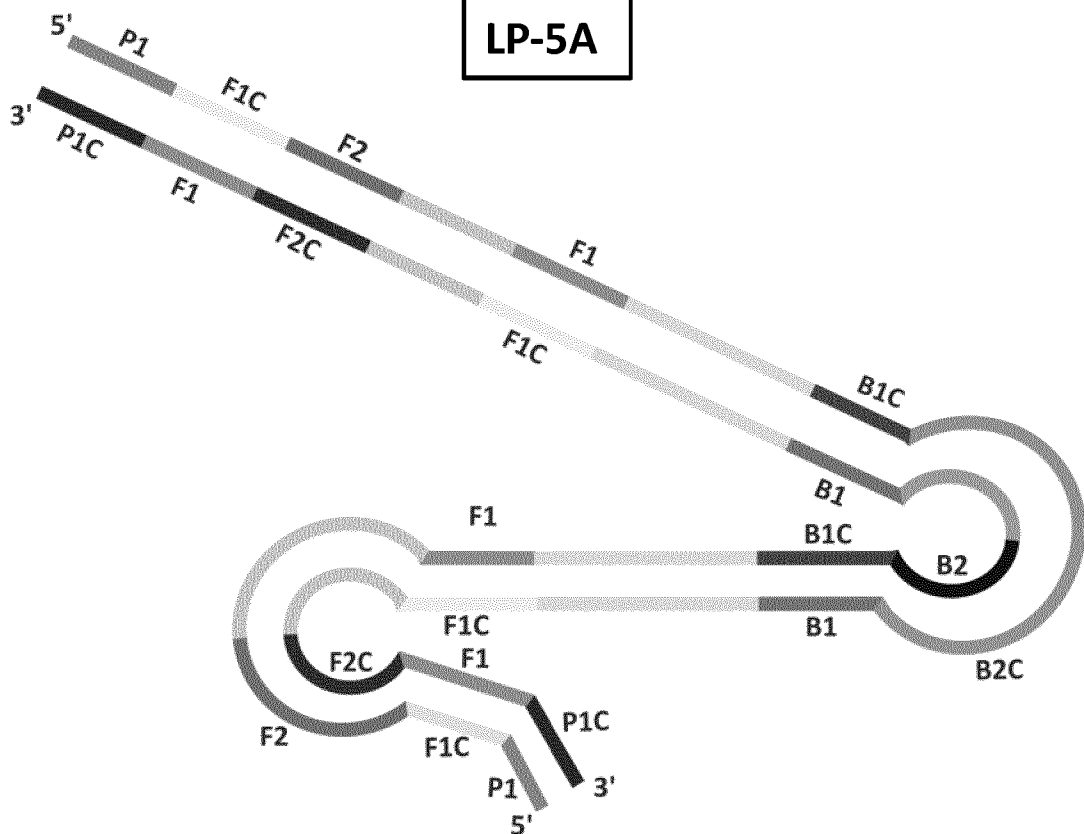


Fig. 3D

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LP-3A continued

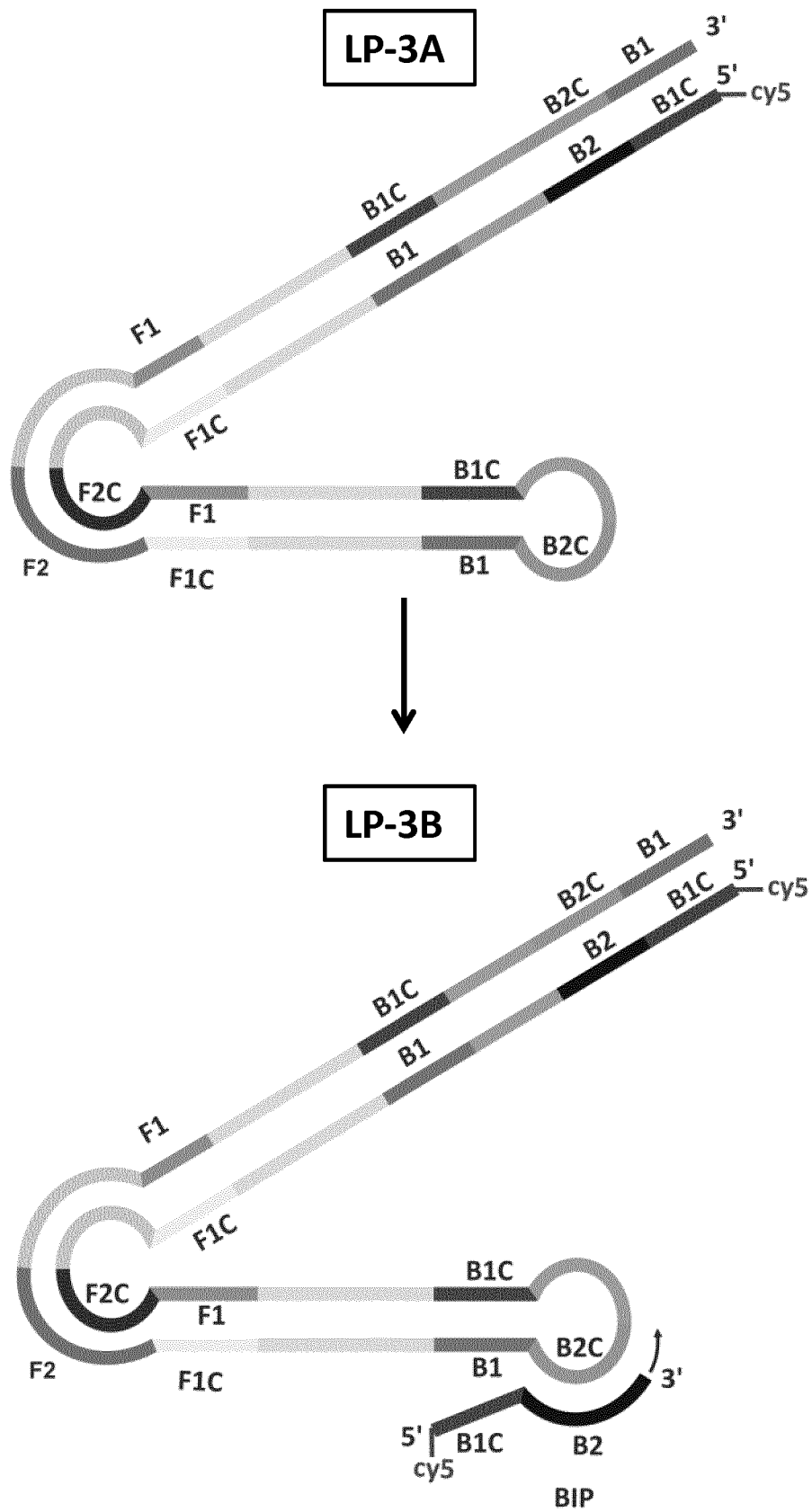


Fig. 4A

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From LP-3B

LP-3C

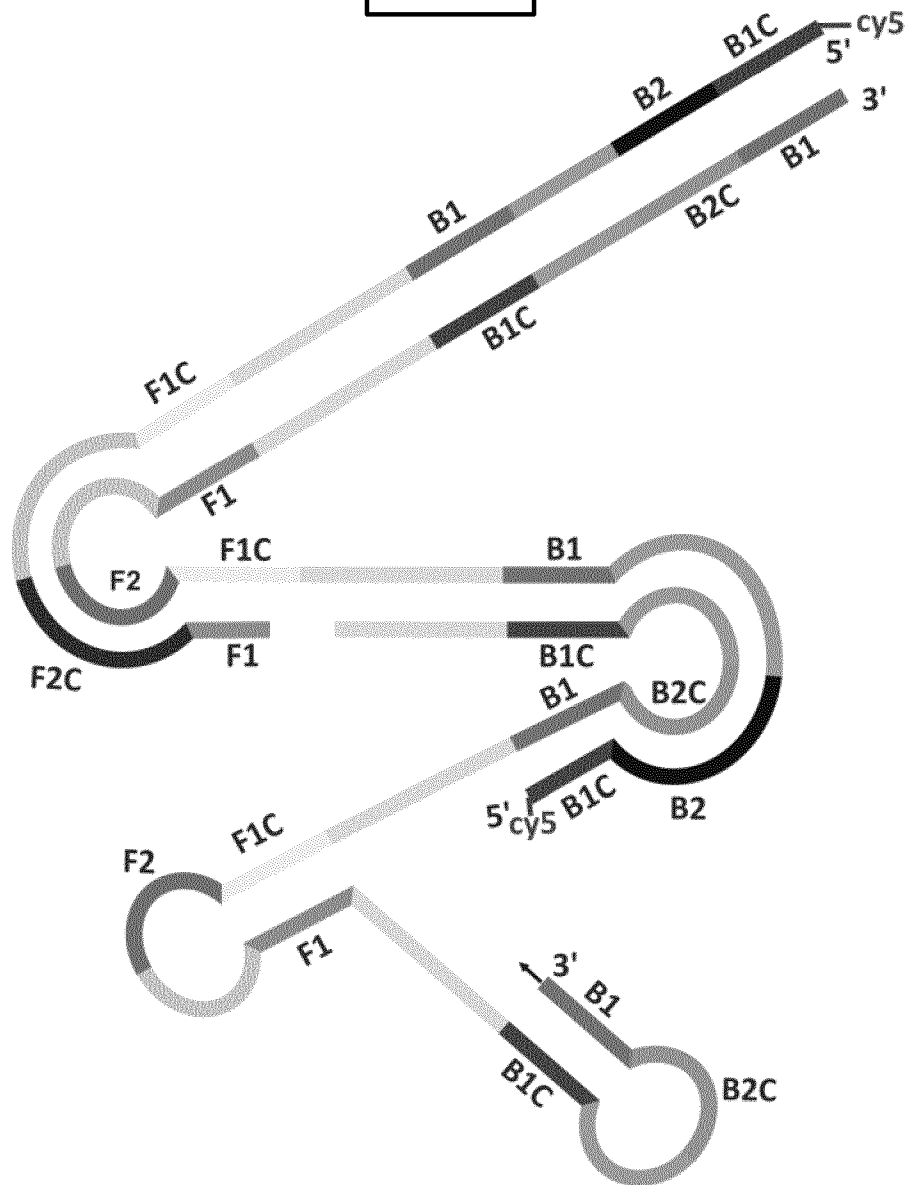
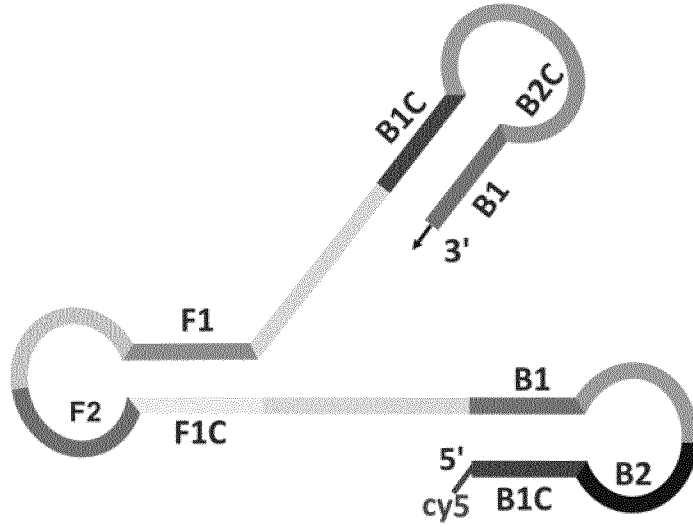


Fig. 4B

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LP-6A

→
From
LP-3C



LP-3D

→
From
LP-3C

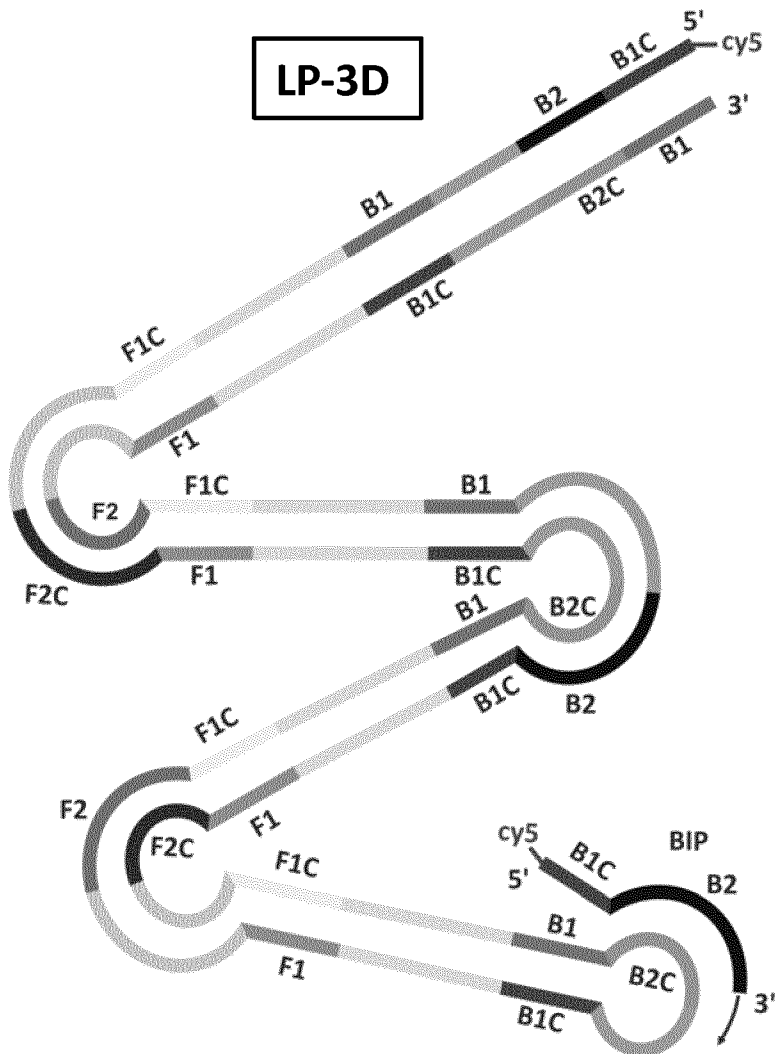


Fig. 4C

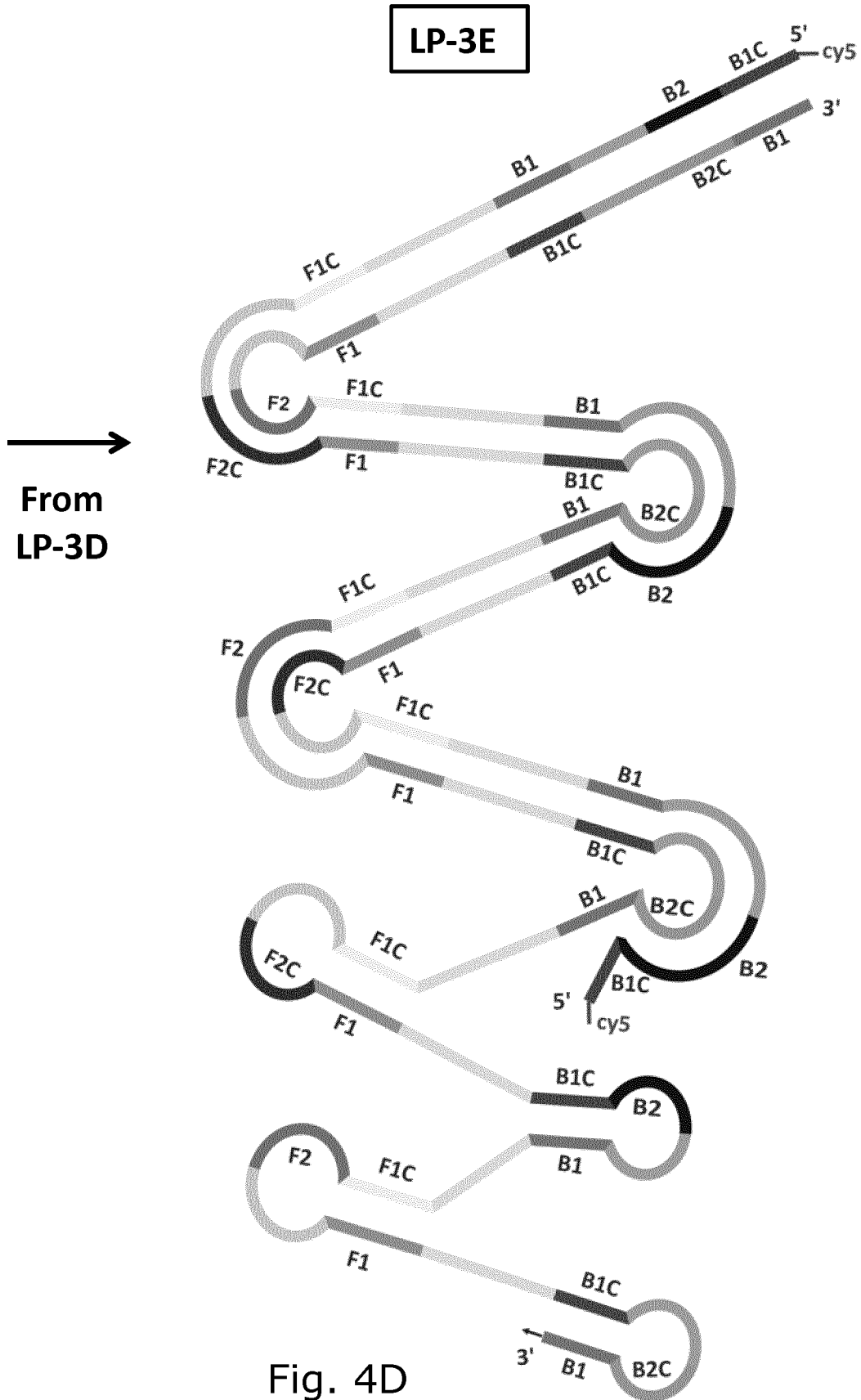


Fig. 4D

LP-3F

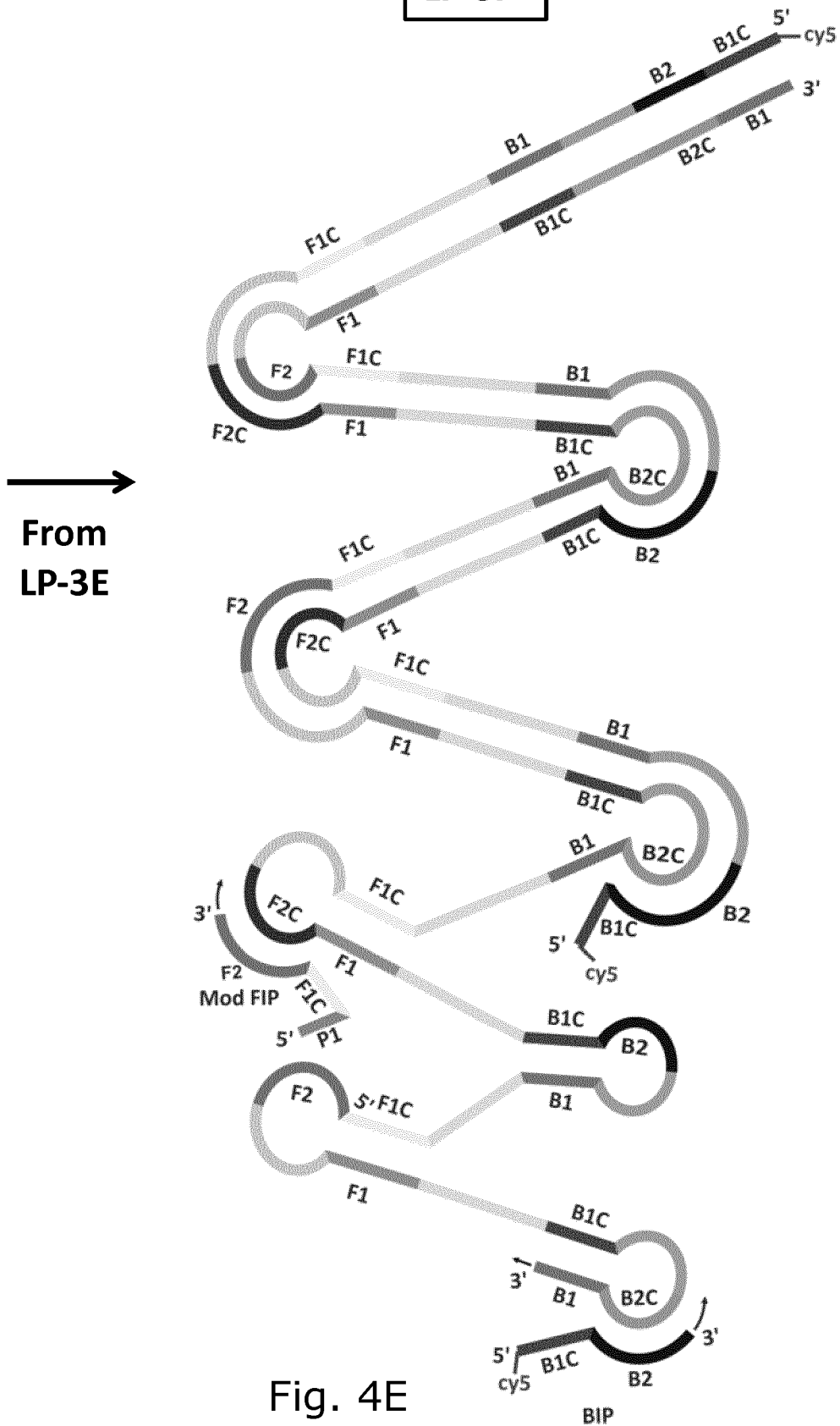
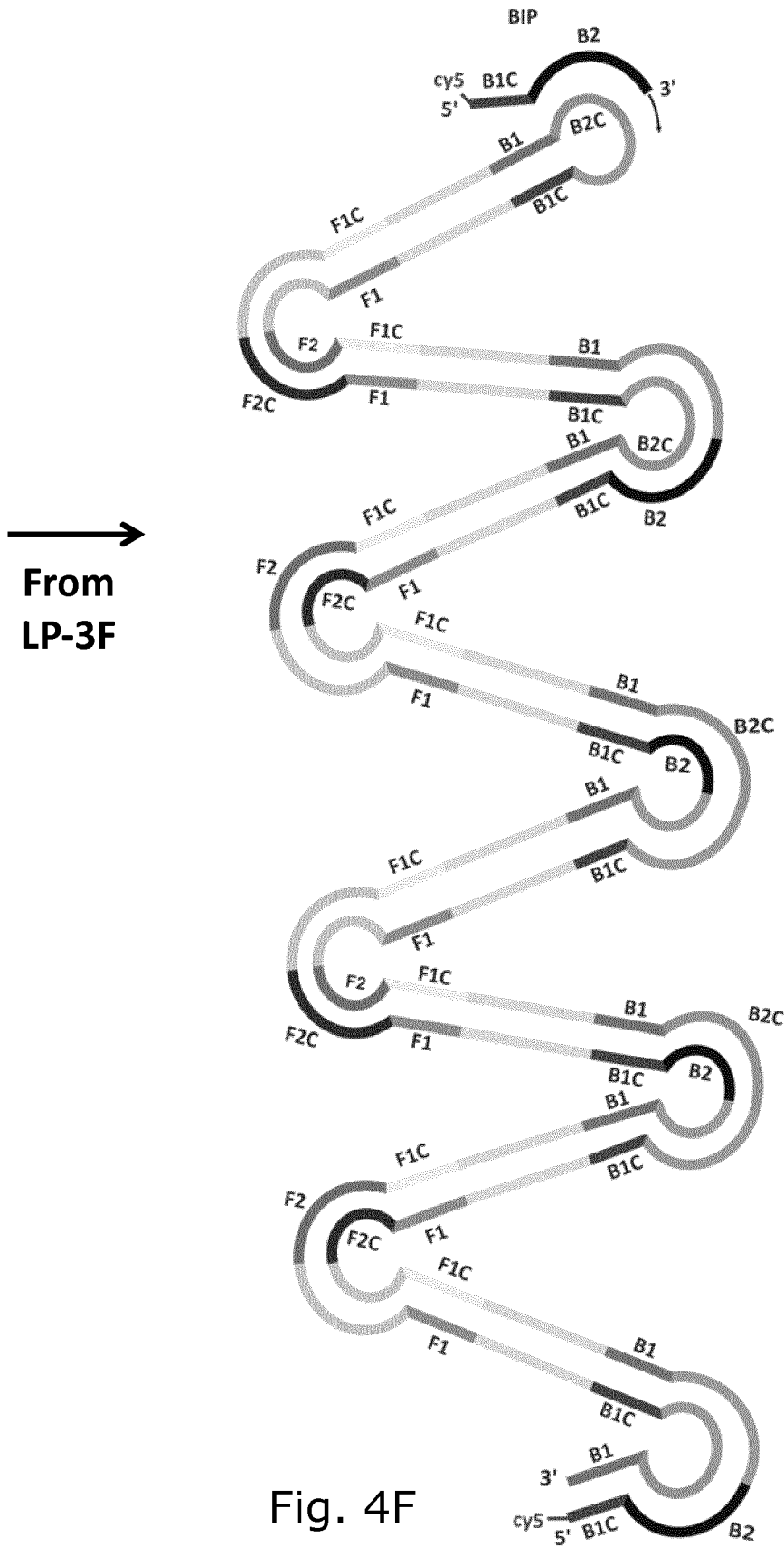


Fig. 4E

LP-3G



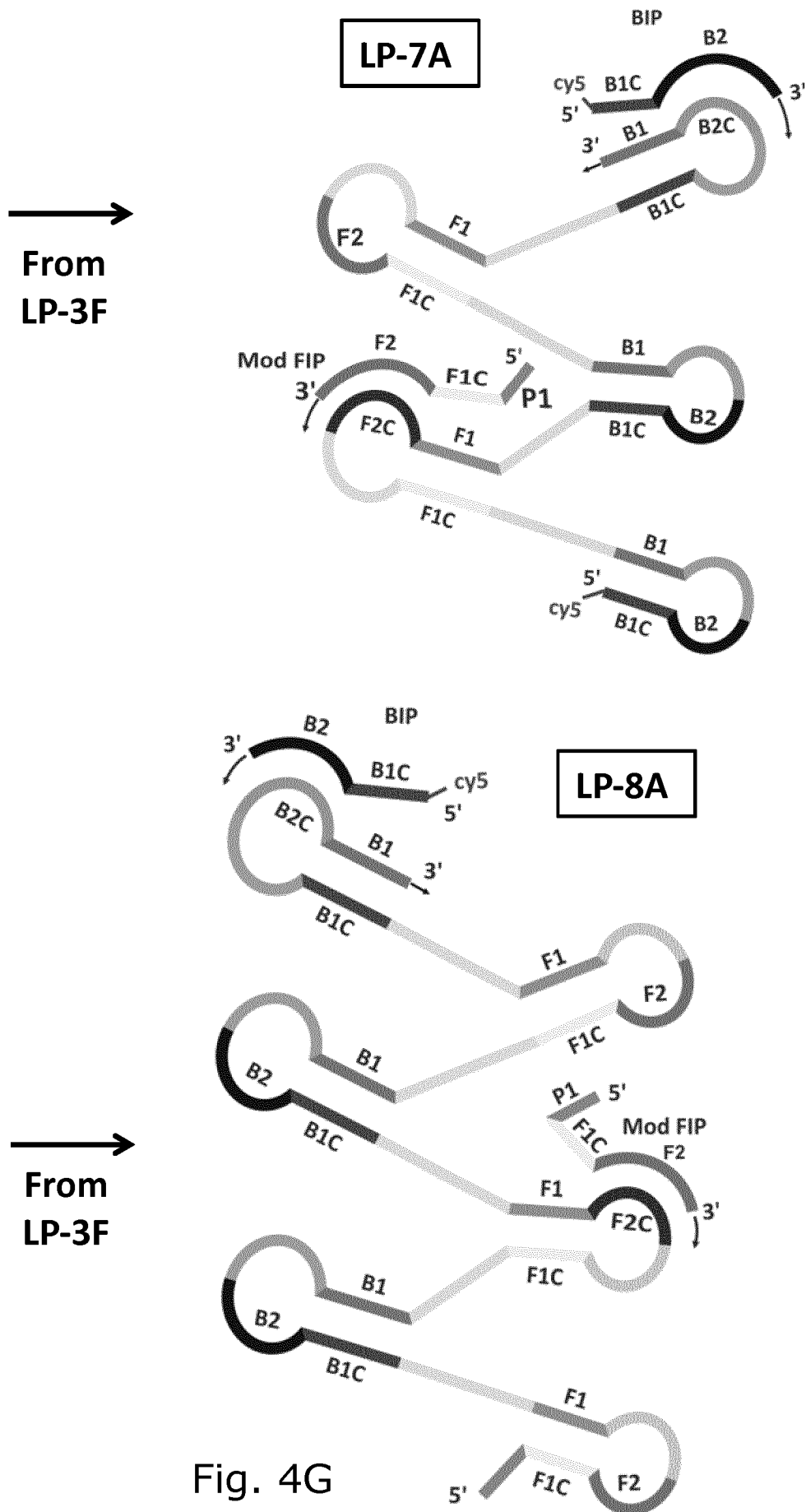
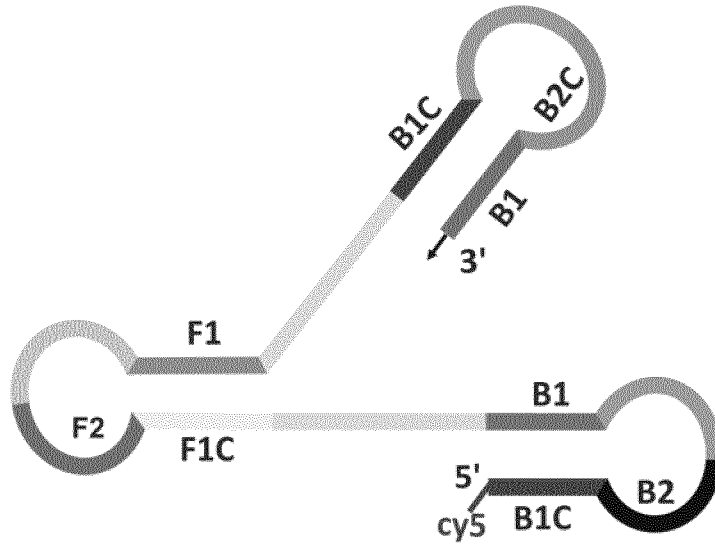


Fig. 4G

LP-6A continued

LP-6A



LP-6B

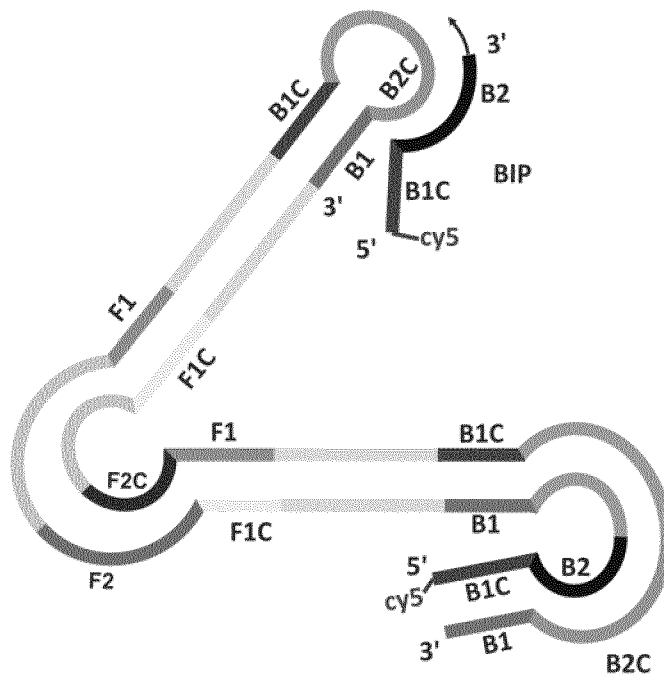


Fig. 5A

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From LP-6B

LP-6C

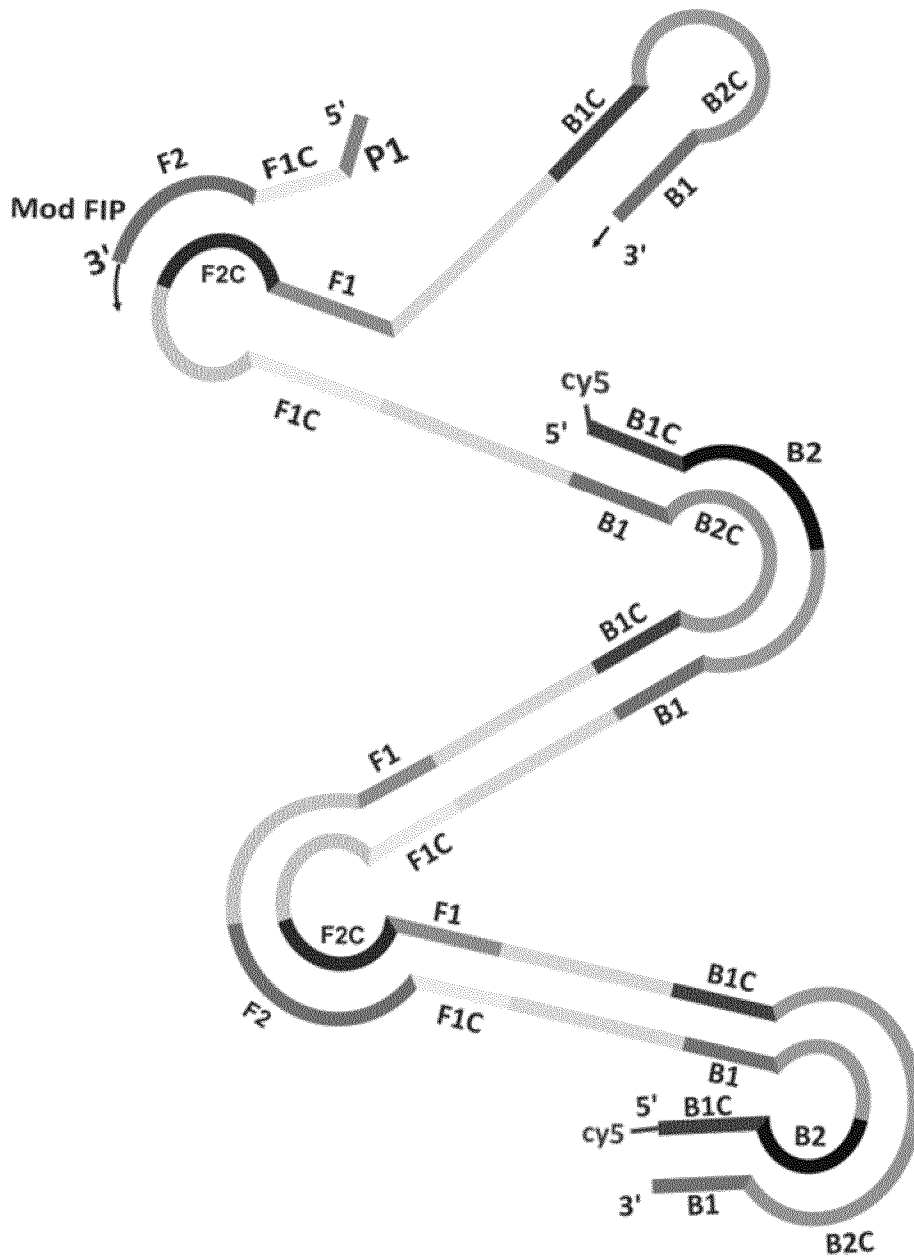
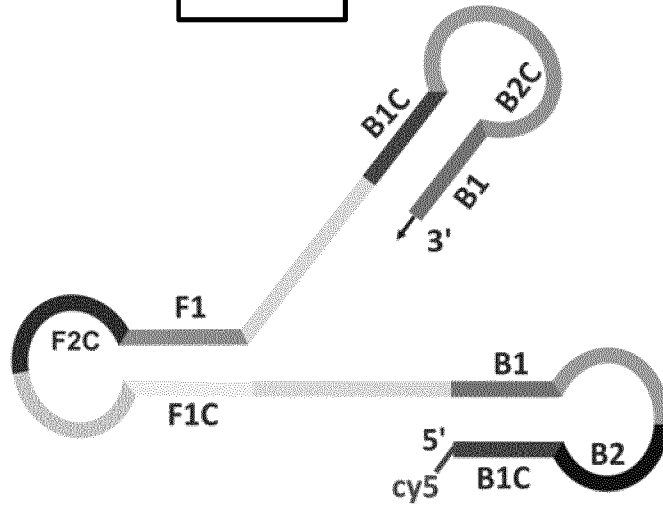


Fig. 5B

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LP-9A

→
From
LP-6C



LP-6C1

→
From
LP-6C

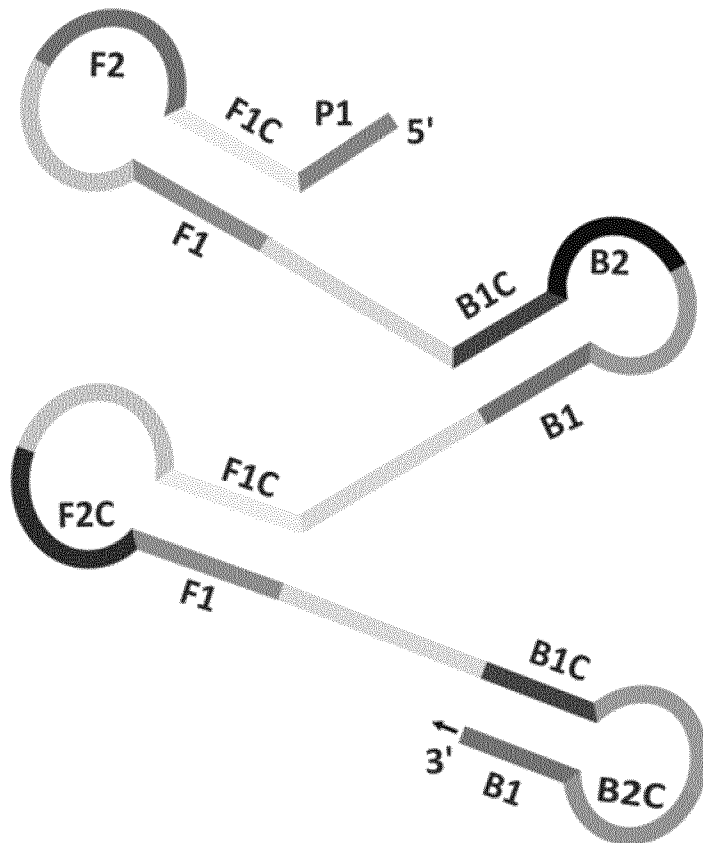
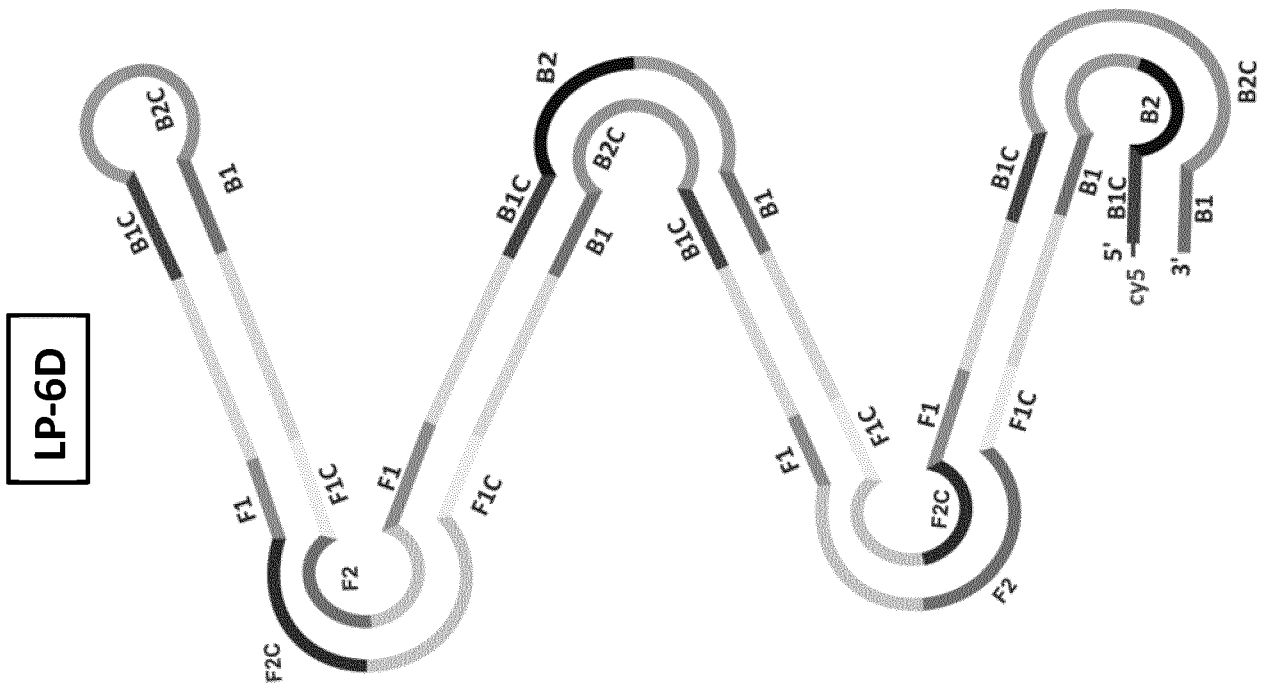
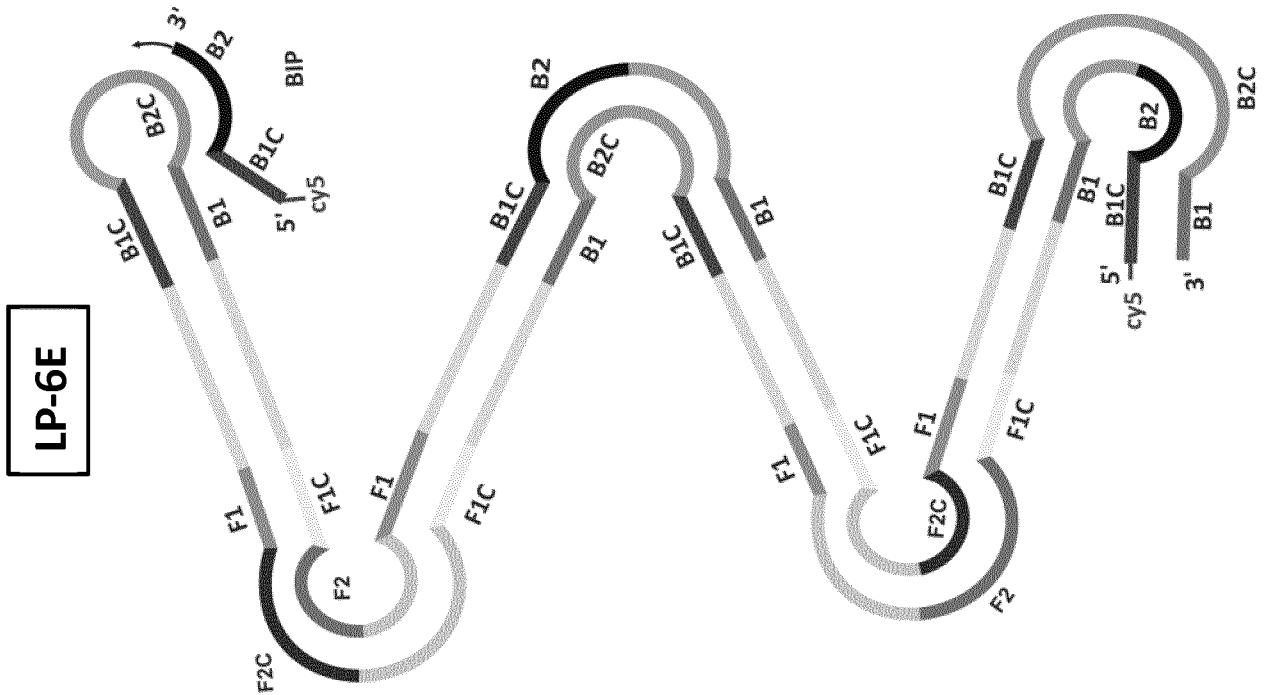


Fig. 5C



From LP-6C

Fig. 5D

LP-6F

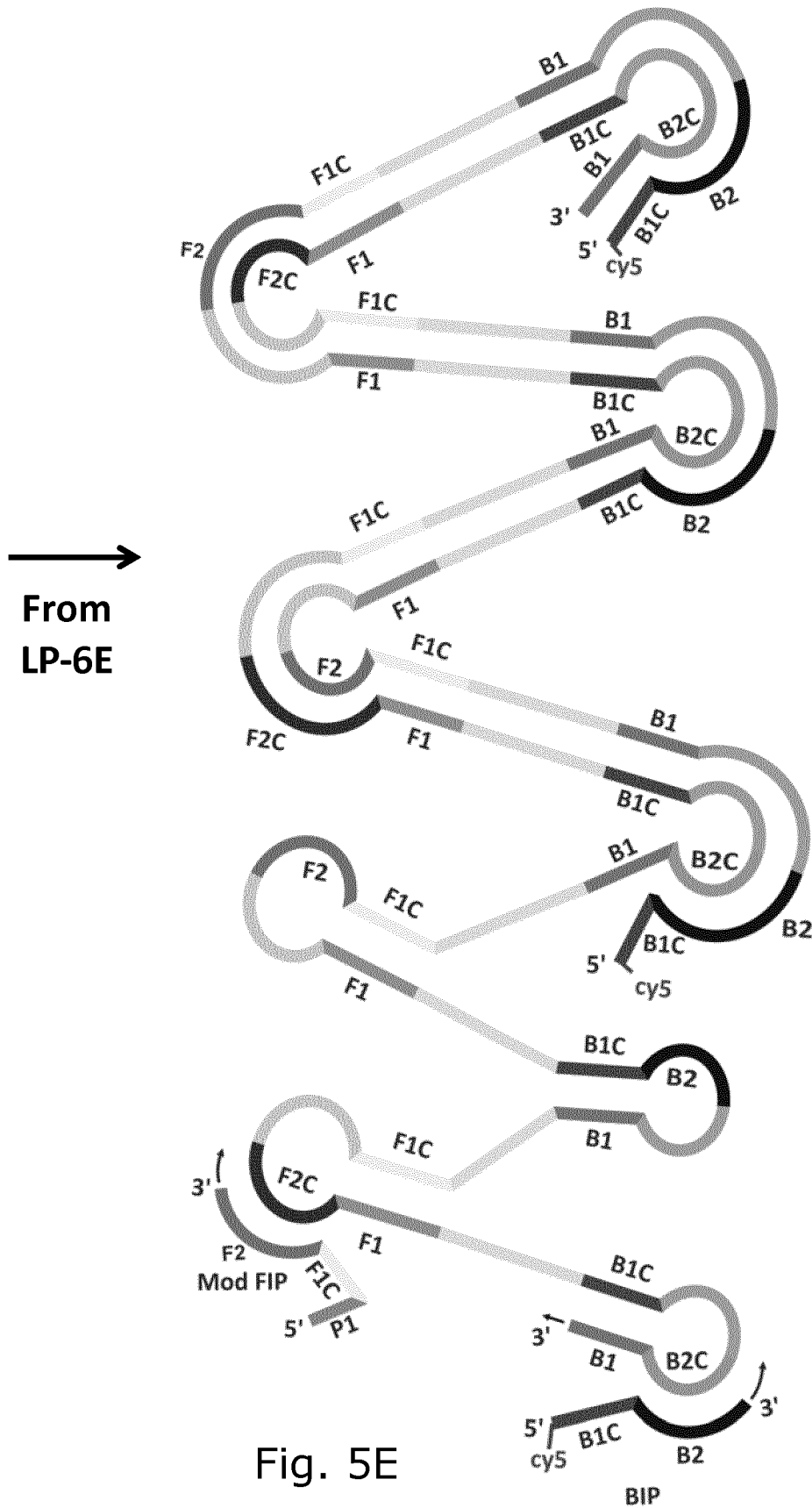


Fig. 5E

LP-10A

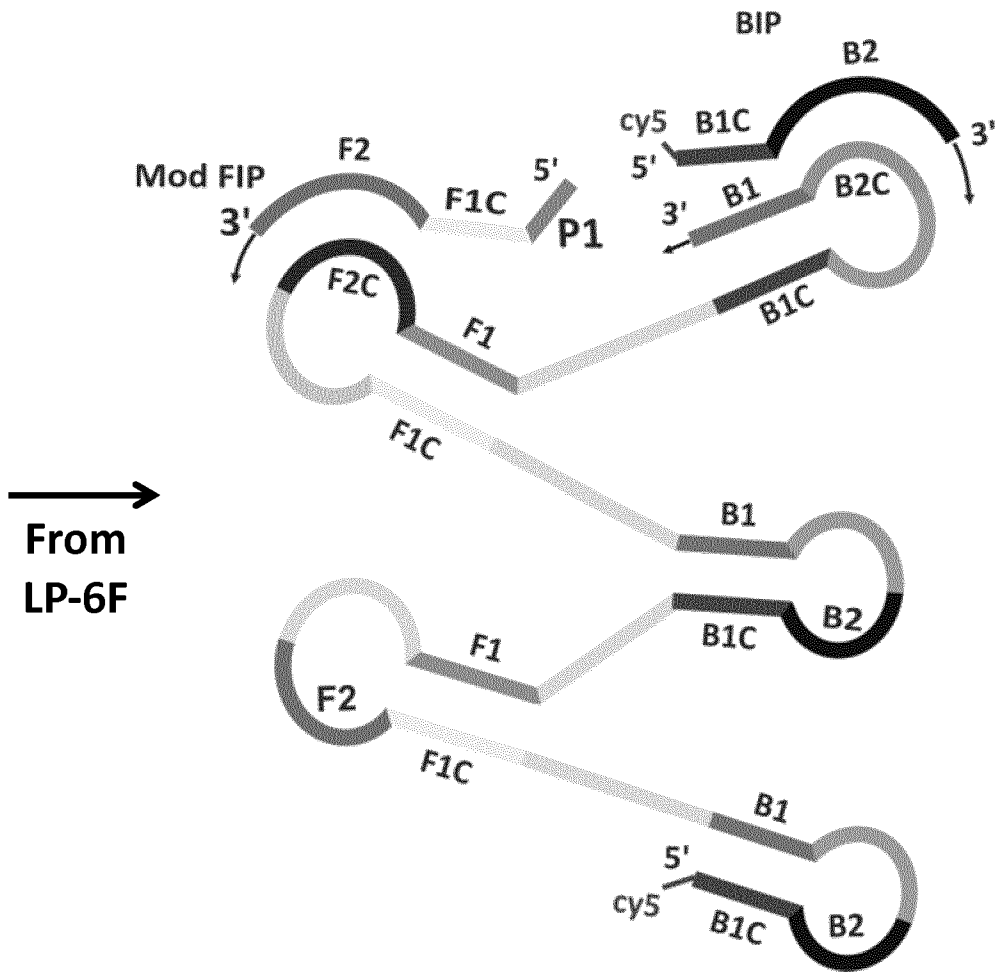


Fig. 5F

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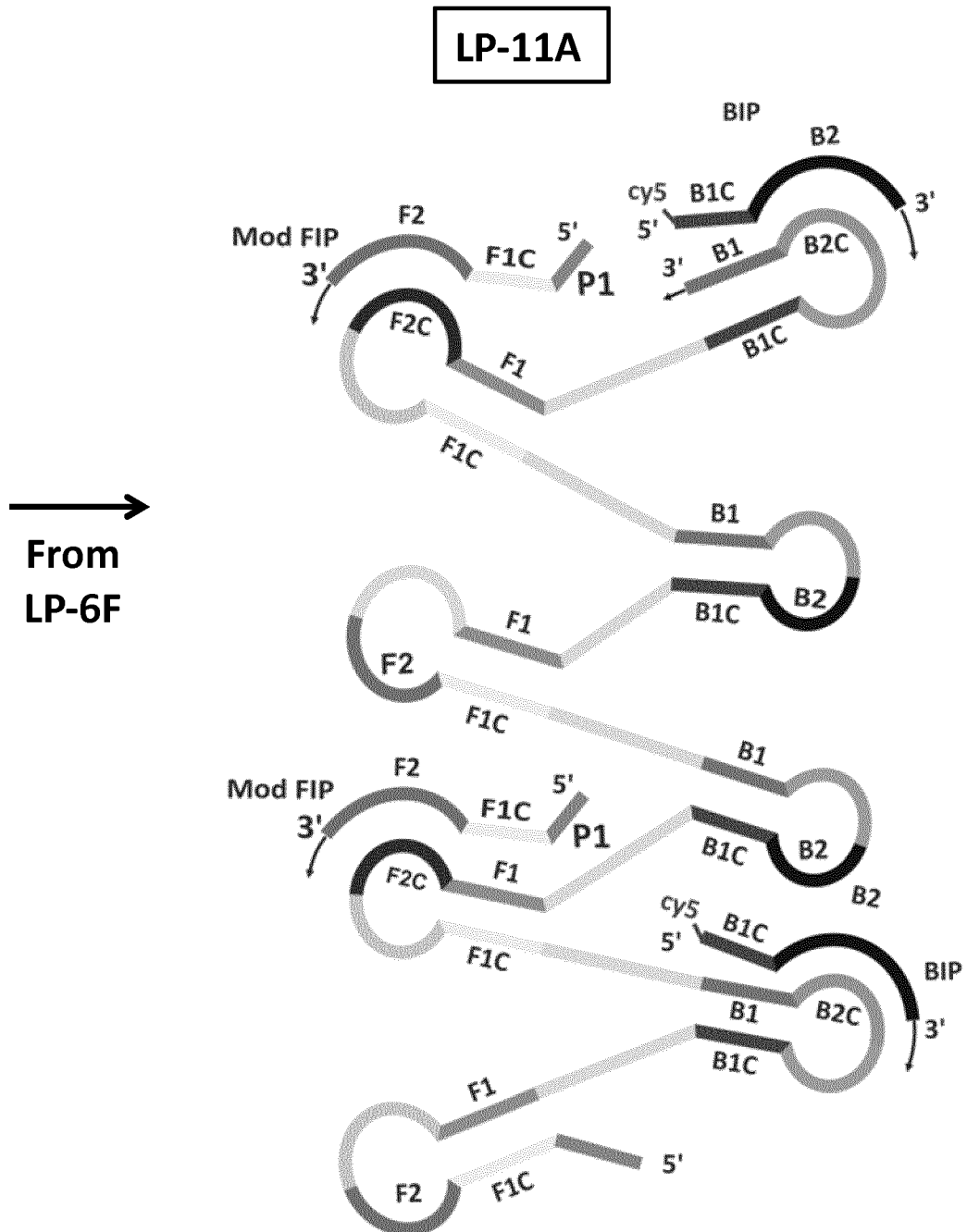


Fig. 5G

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LP-6G

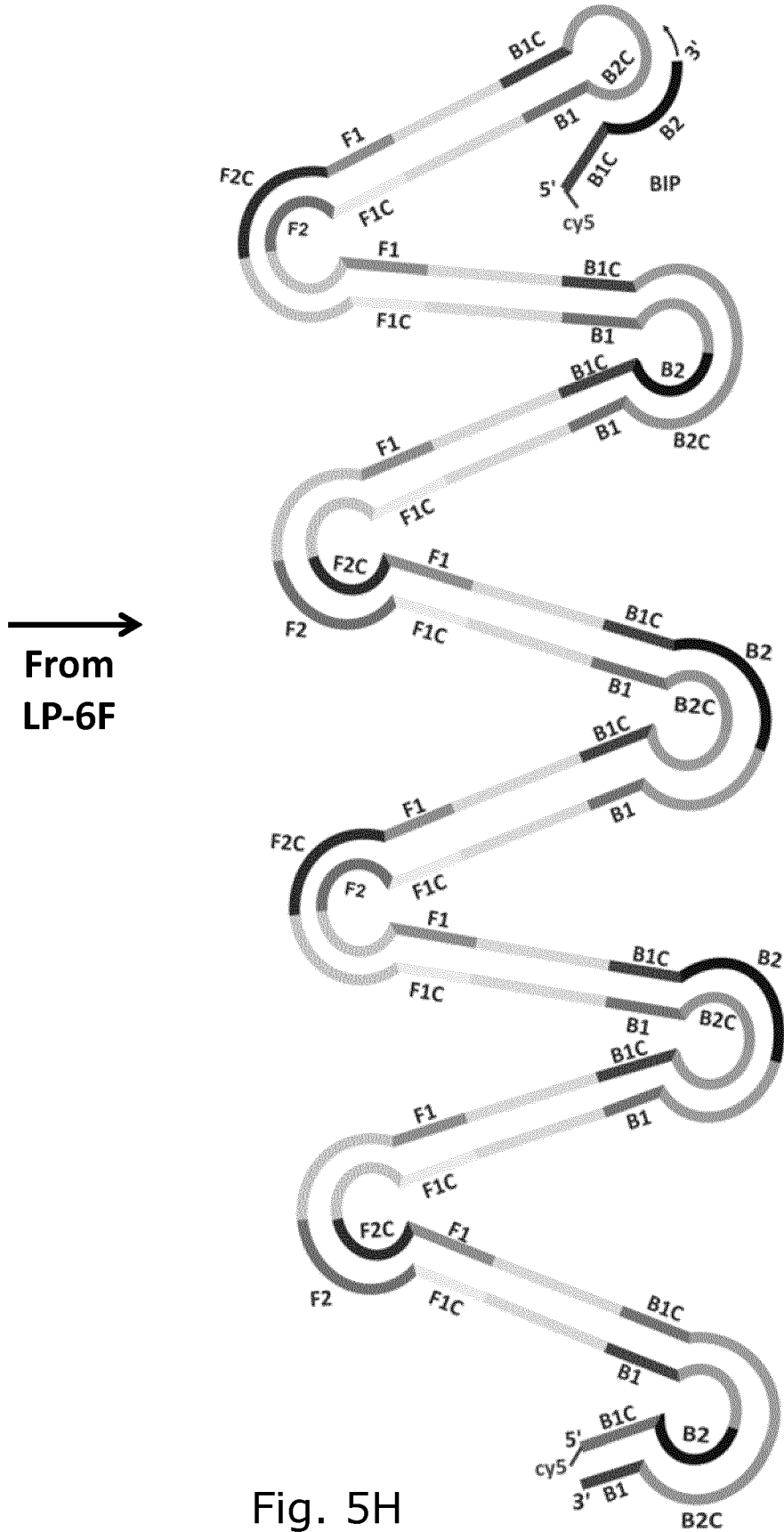
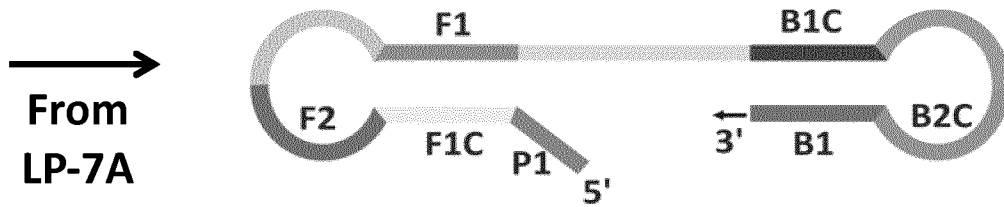


Fig. 5H

LP-7A continued

LP-2A



LP-7B

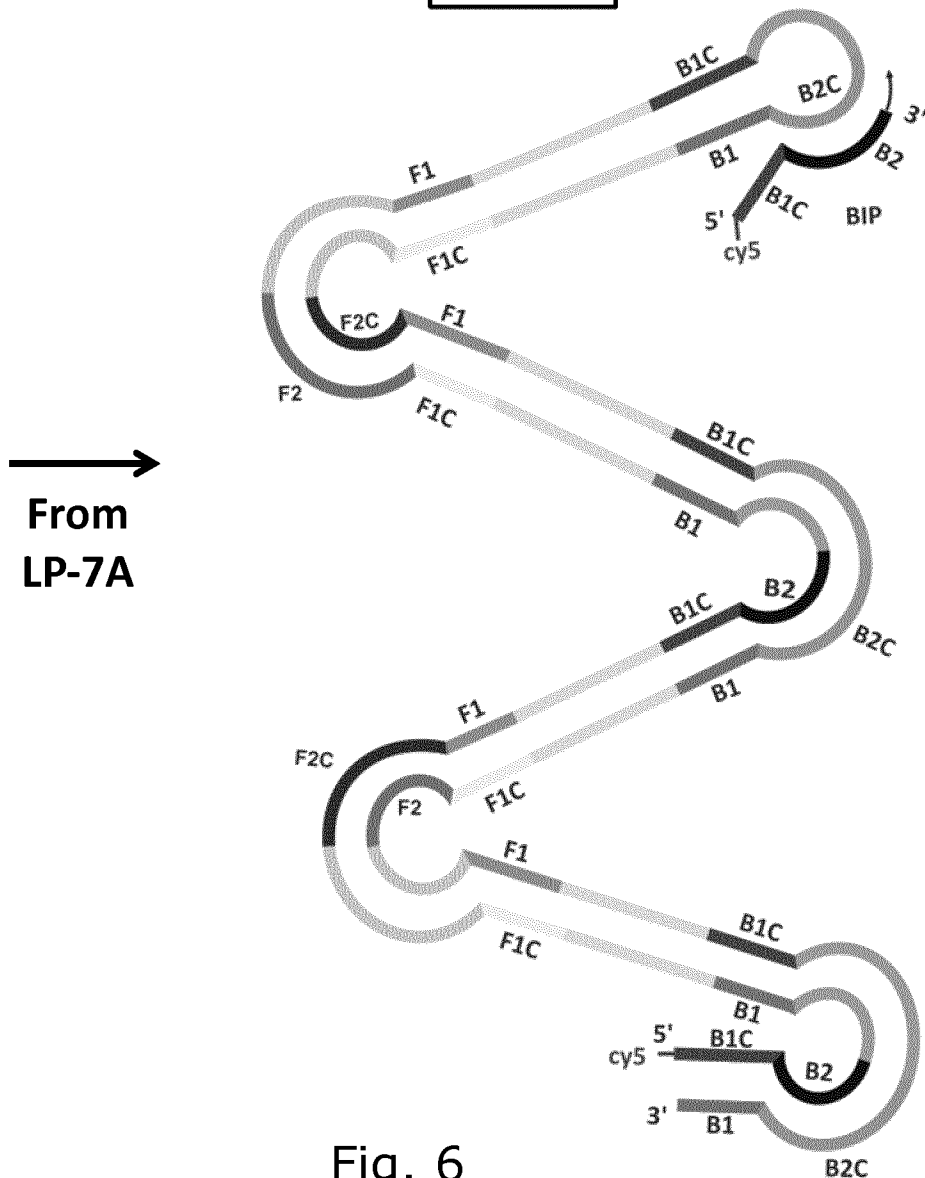
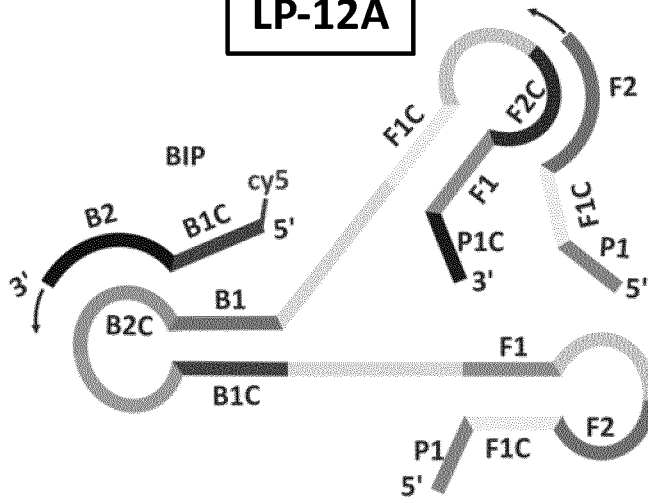


Fig. 6

LP-11A continued

LP-12A

→
From LP-8A



LP-8B

→
From LP-8A

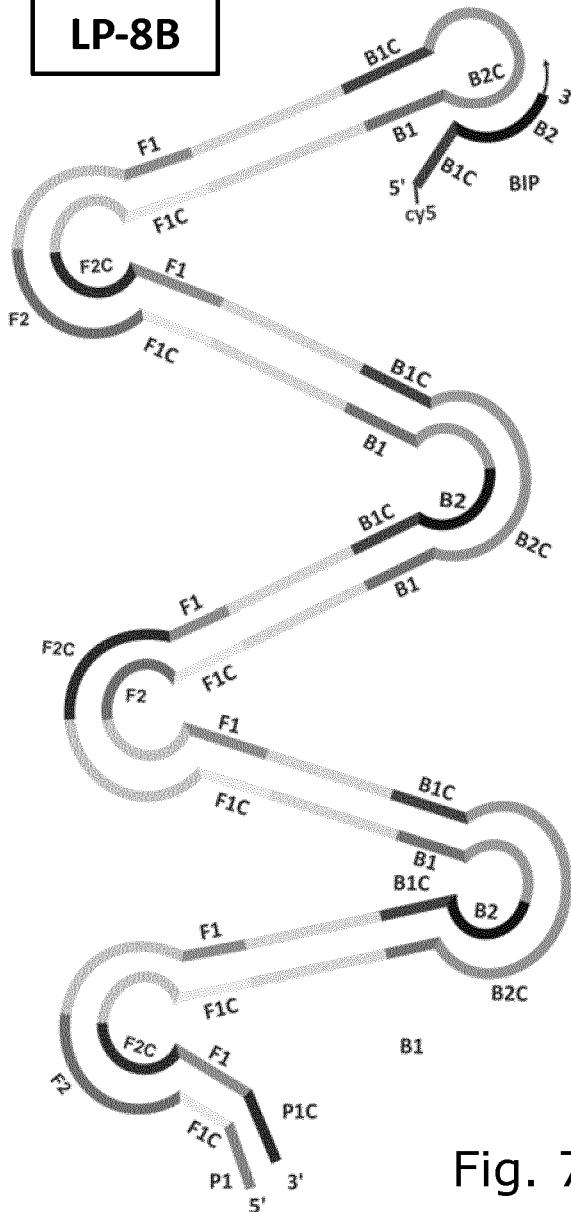
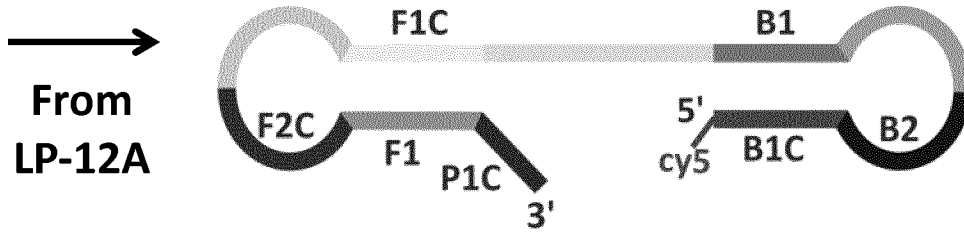


Fig. 7A

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LP-4A



LP-12B

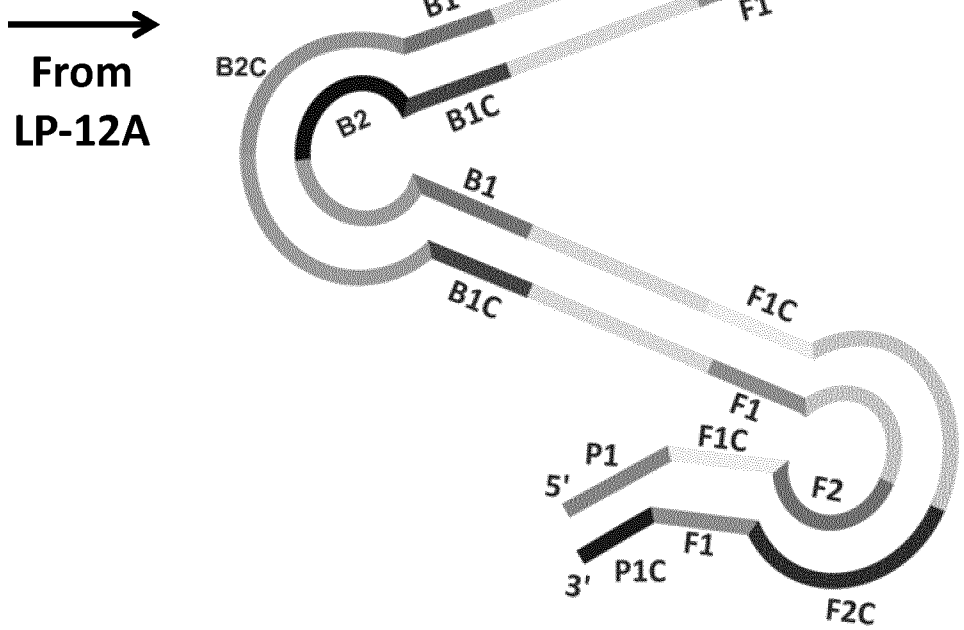
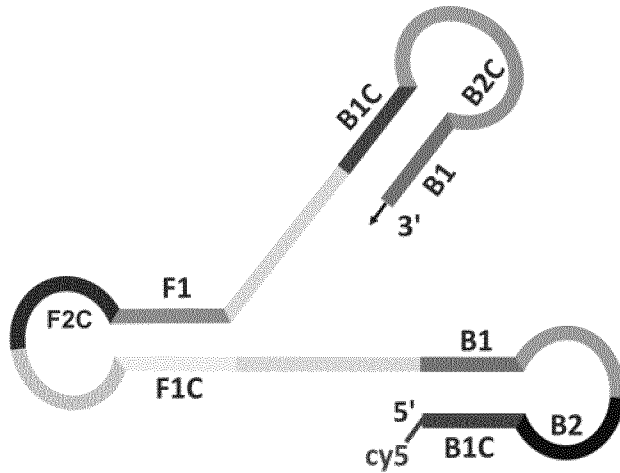


Fig. 7B

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LP-9A continued

LP-9A



LP-9B

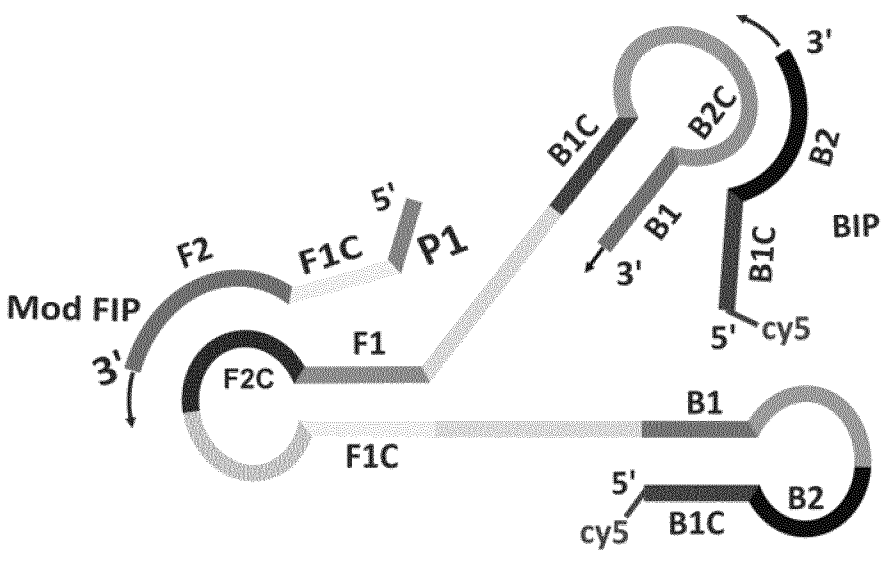
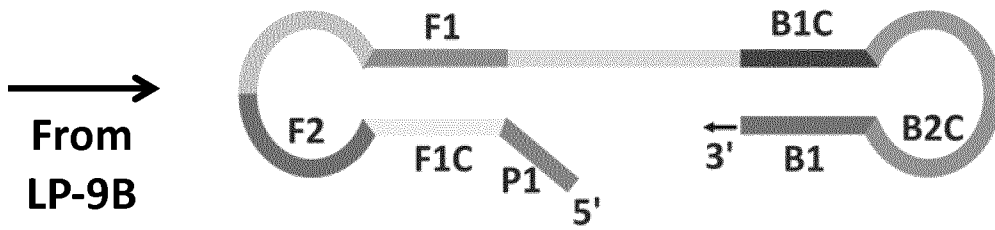


Fig. 8A

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LP-2A



LP-9C

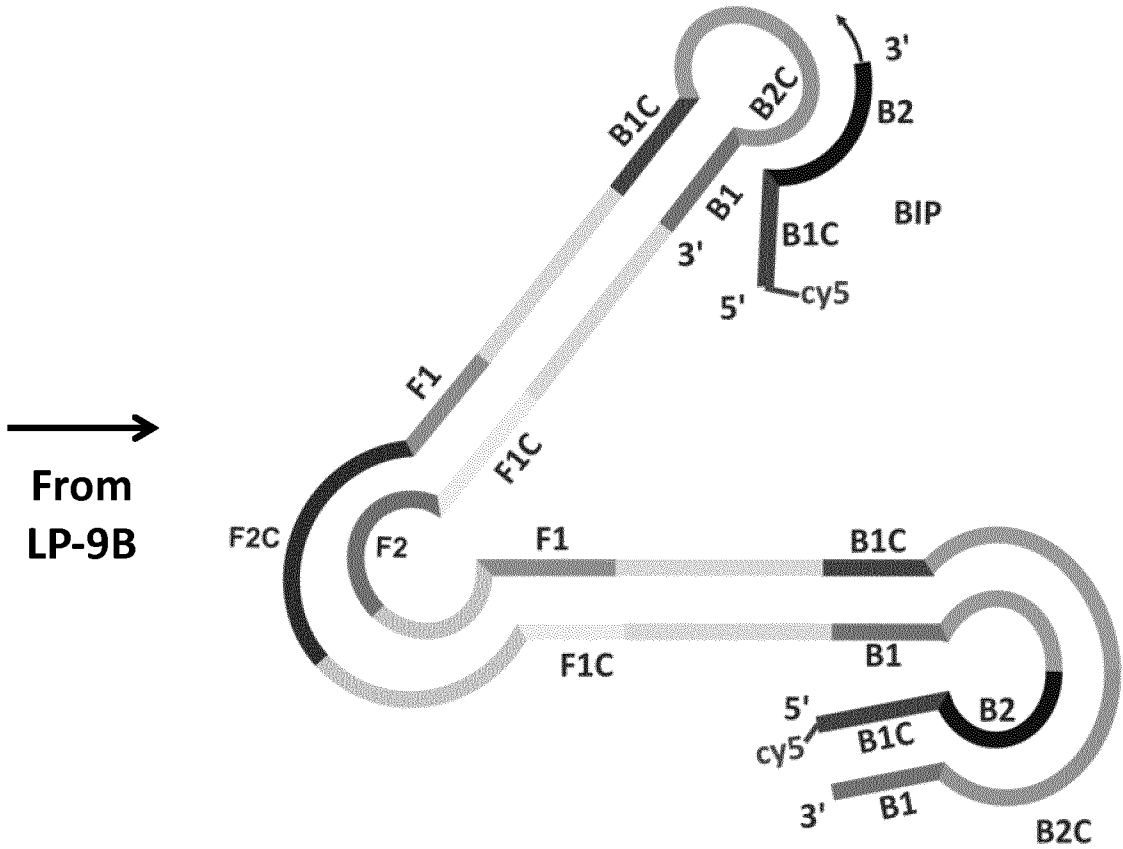


Fig. 8B

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LP-6C1 continued

LP-6C2

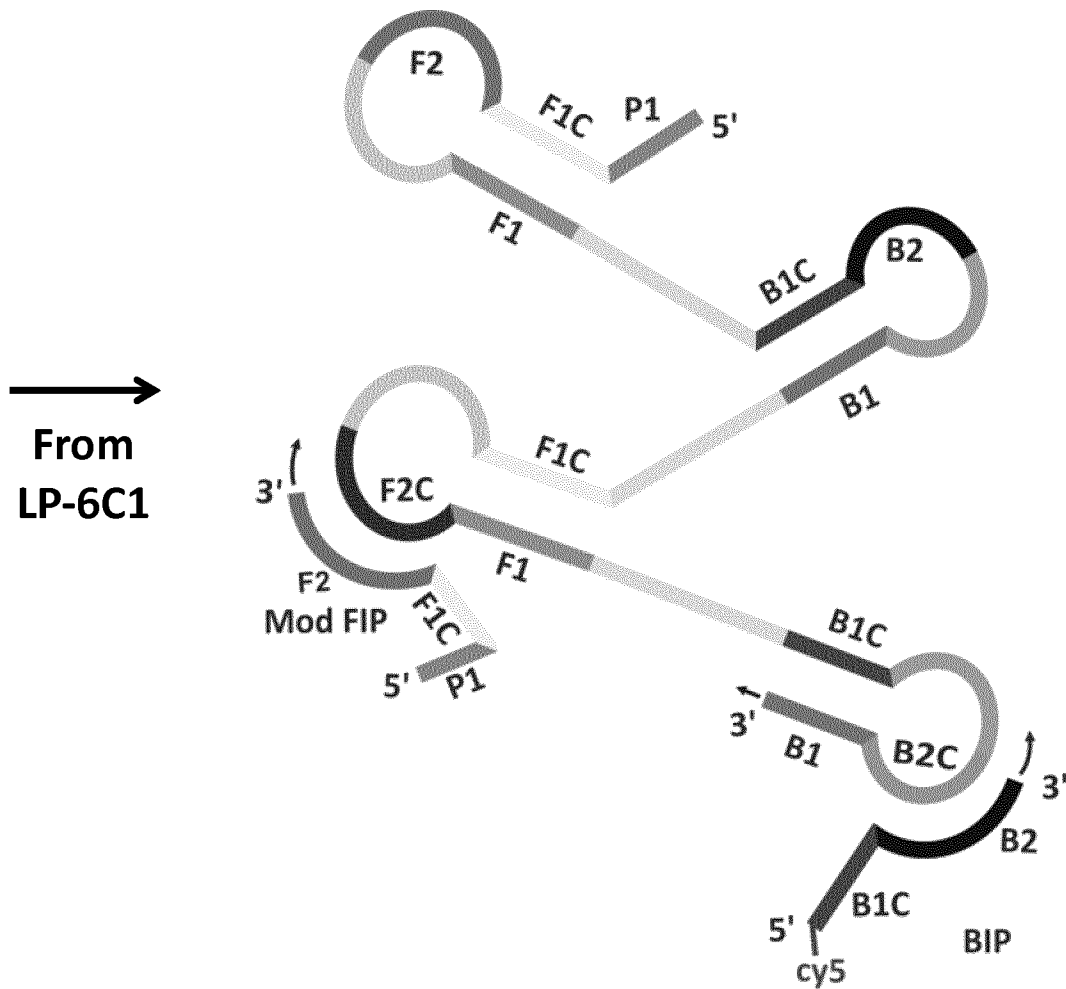
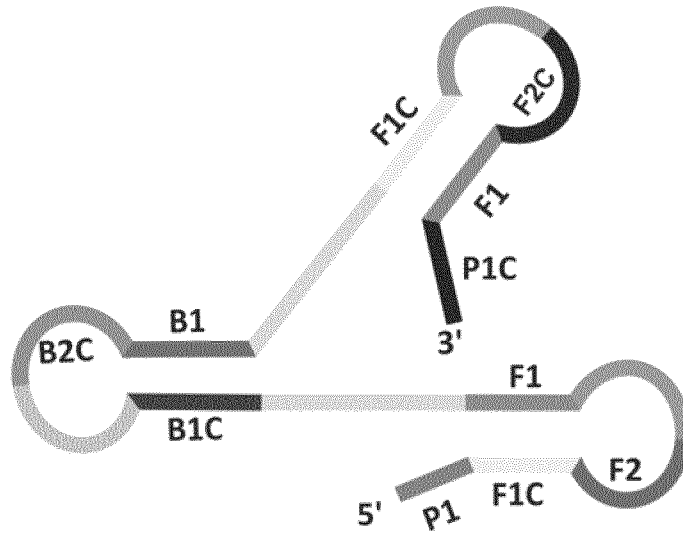


Fig. 9A

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LP-13A

→
From
LP-6C2



LP-6C3

→
From
LP-6C2

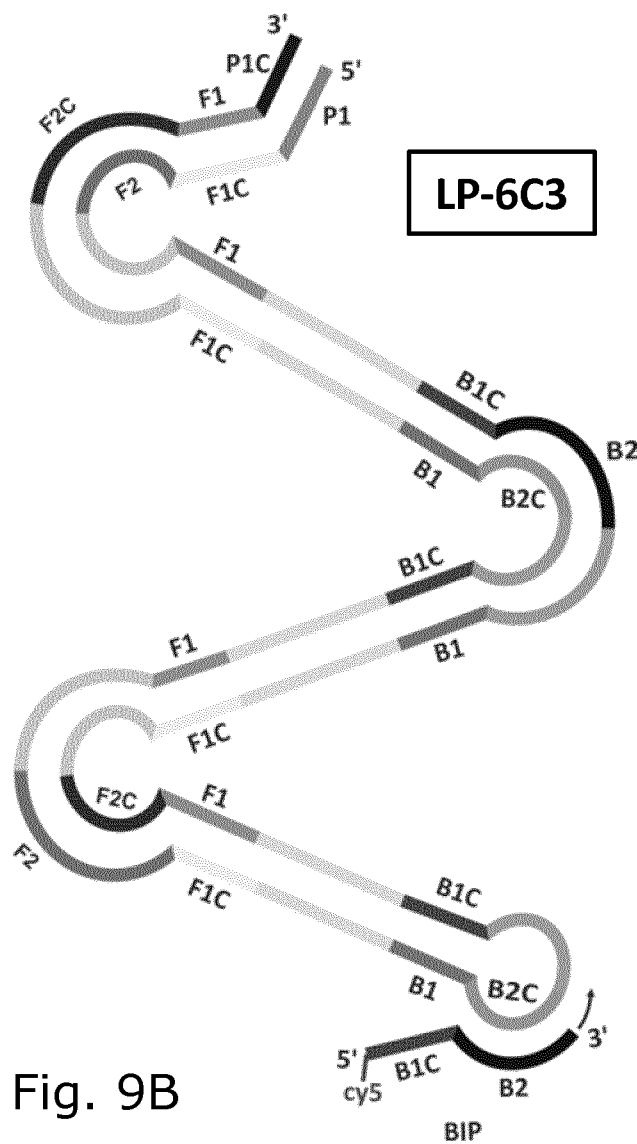


Fig. 9B

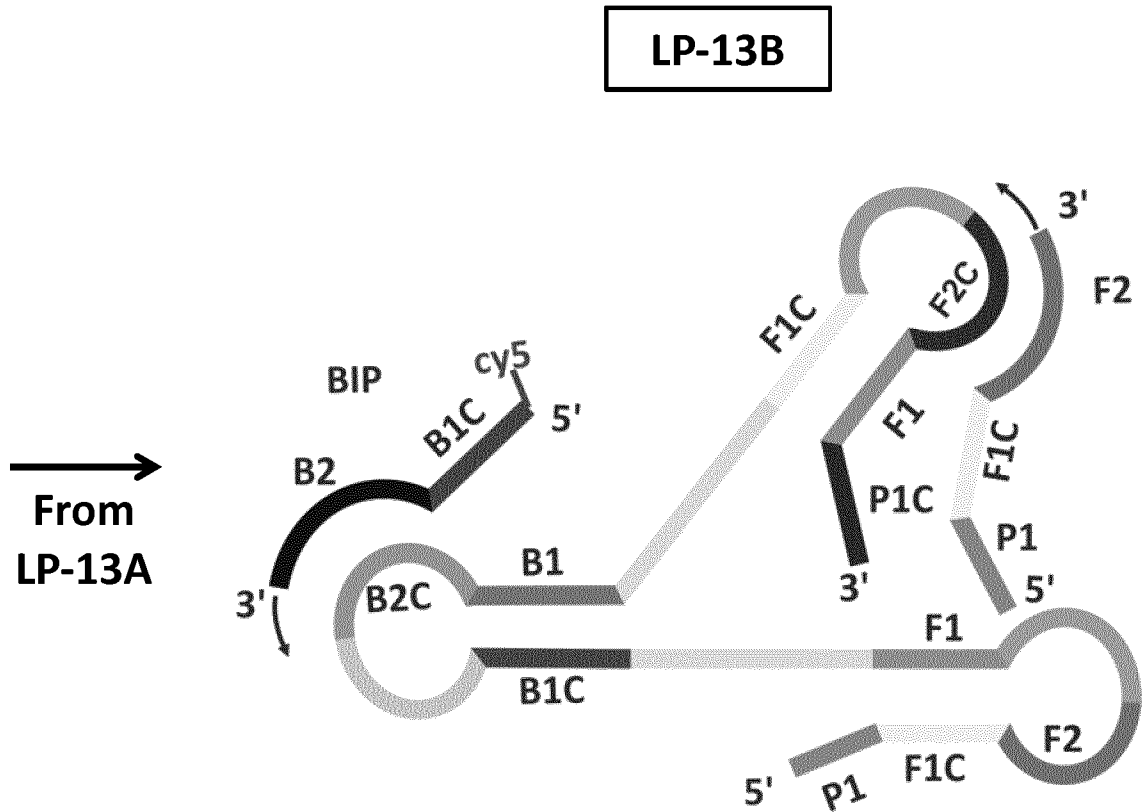
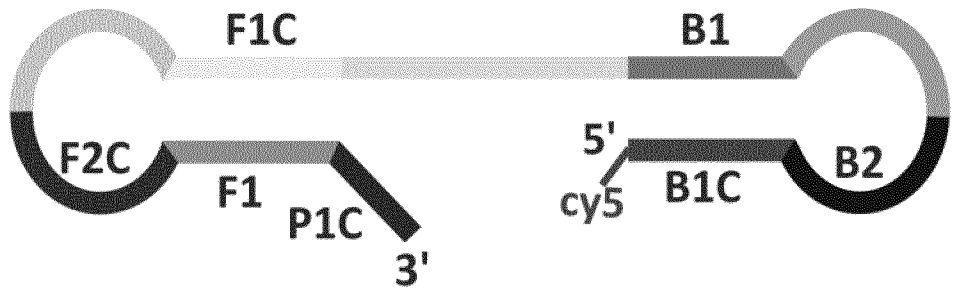


Fig. 9C

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LP-4A

From LP-13B



From LP-13B

LP-13C

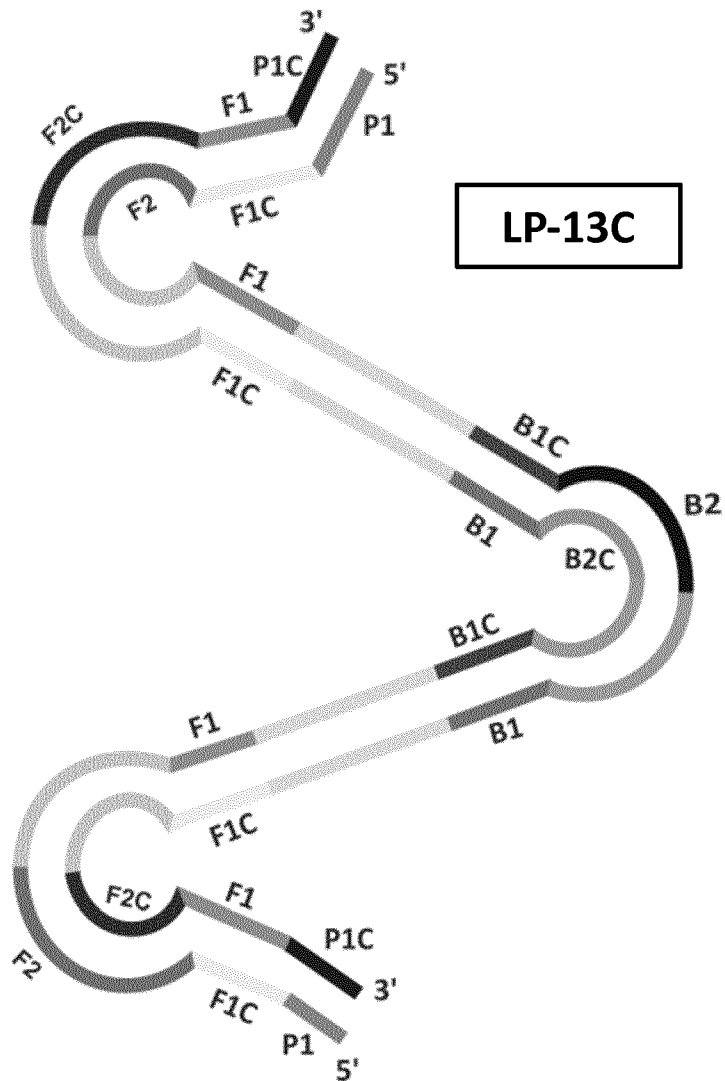


Fig. 9D

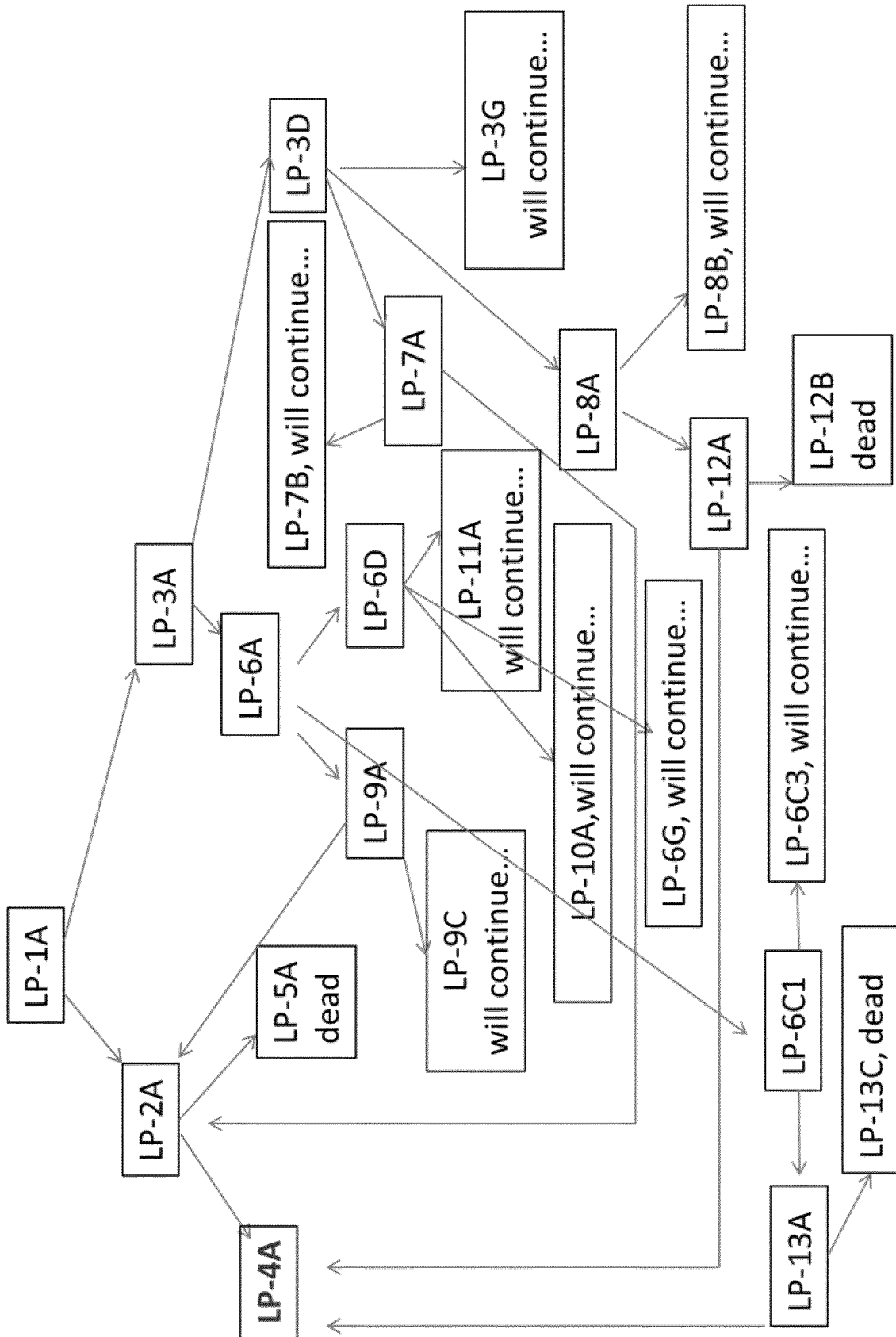


Fig. 10

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Solid phase starting from LP-4A

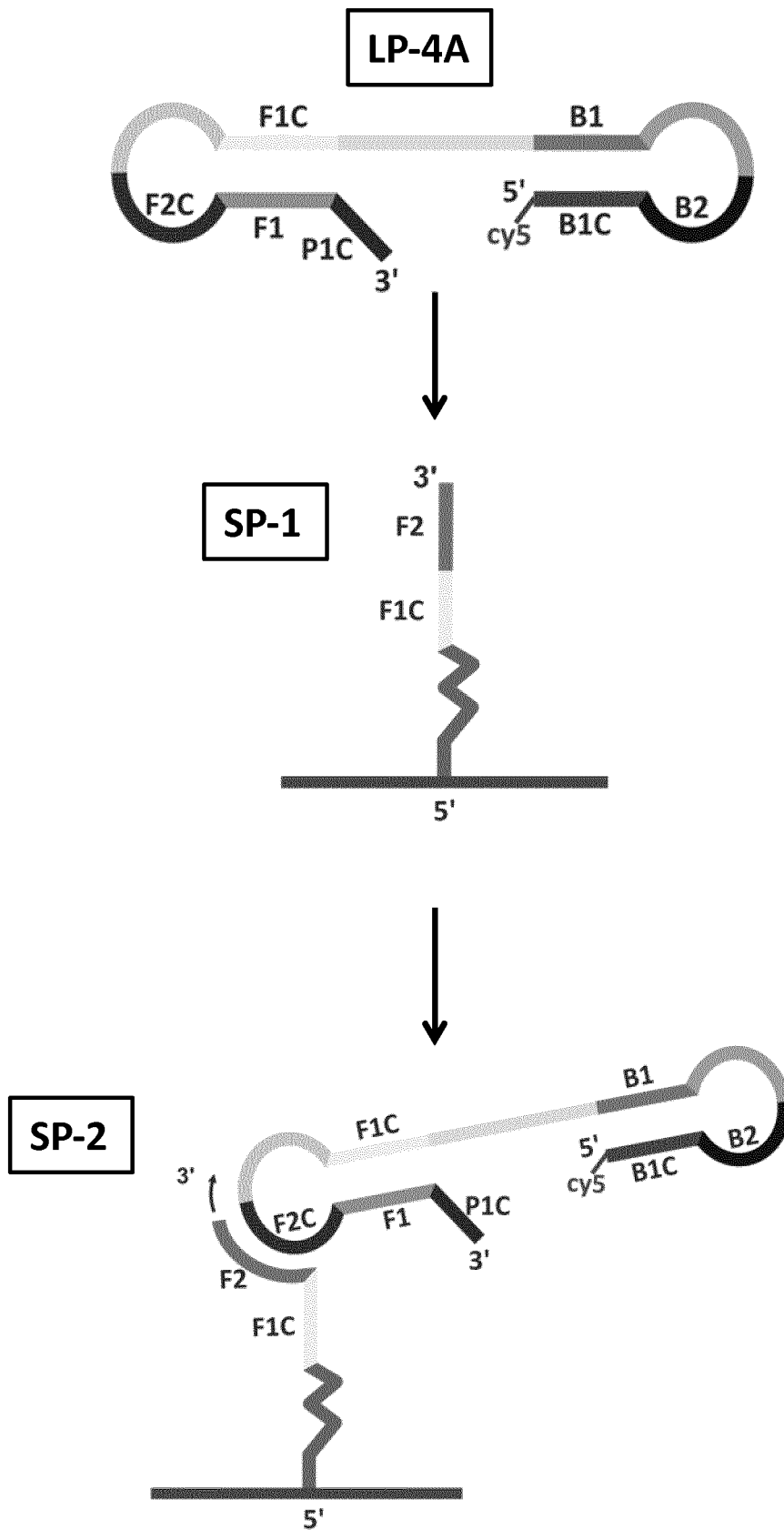


Fig. 11A

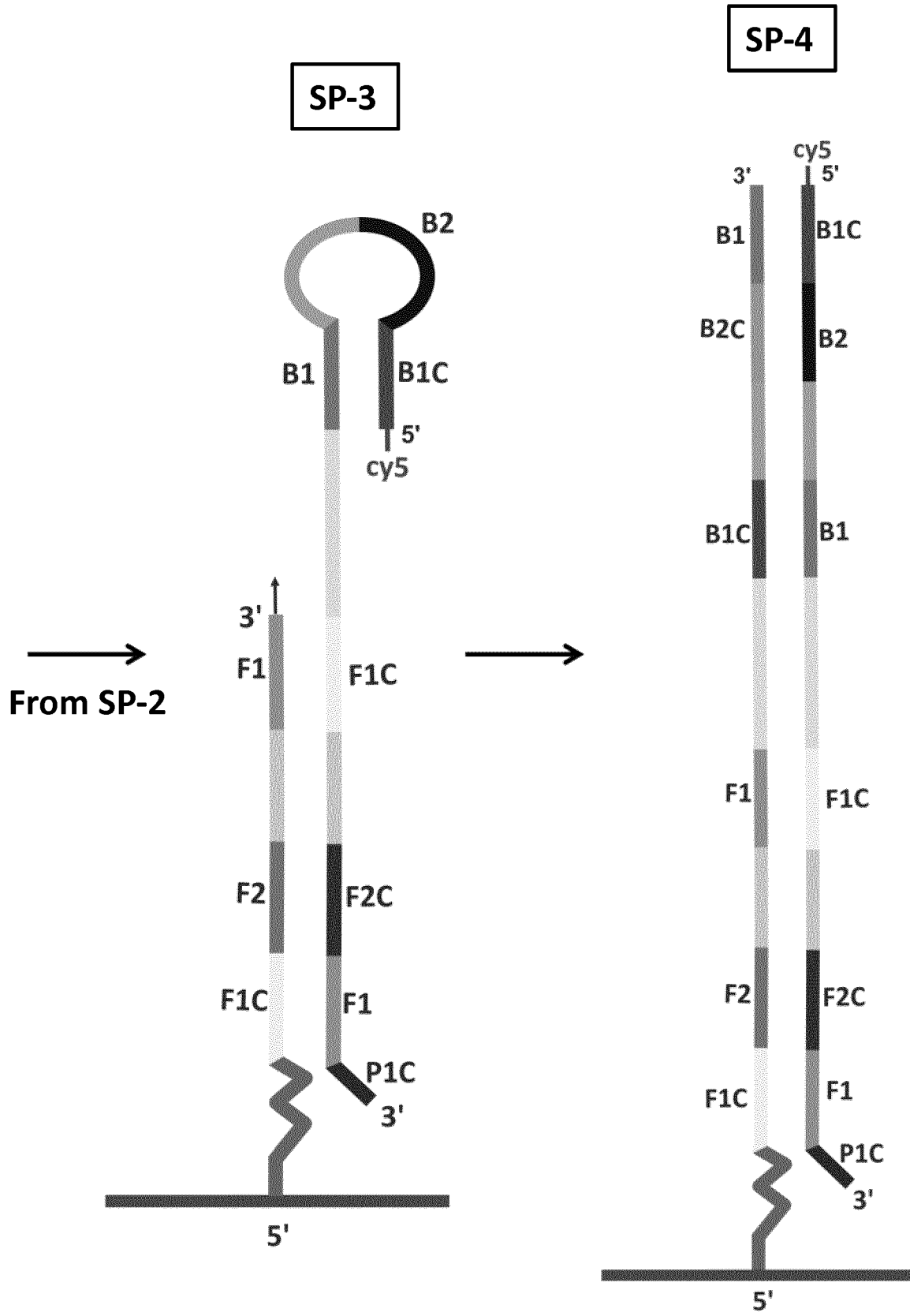


Fig. 11B

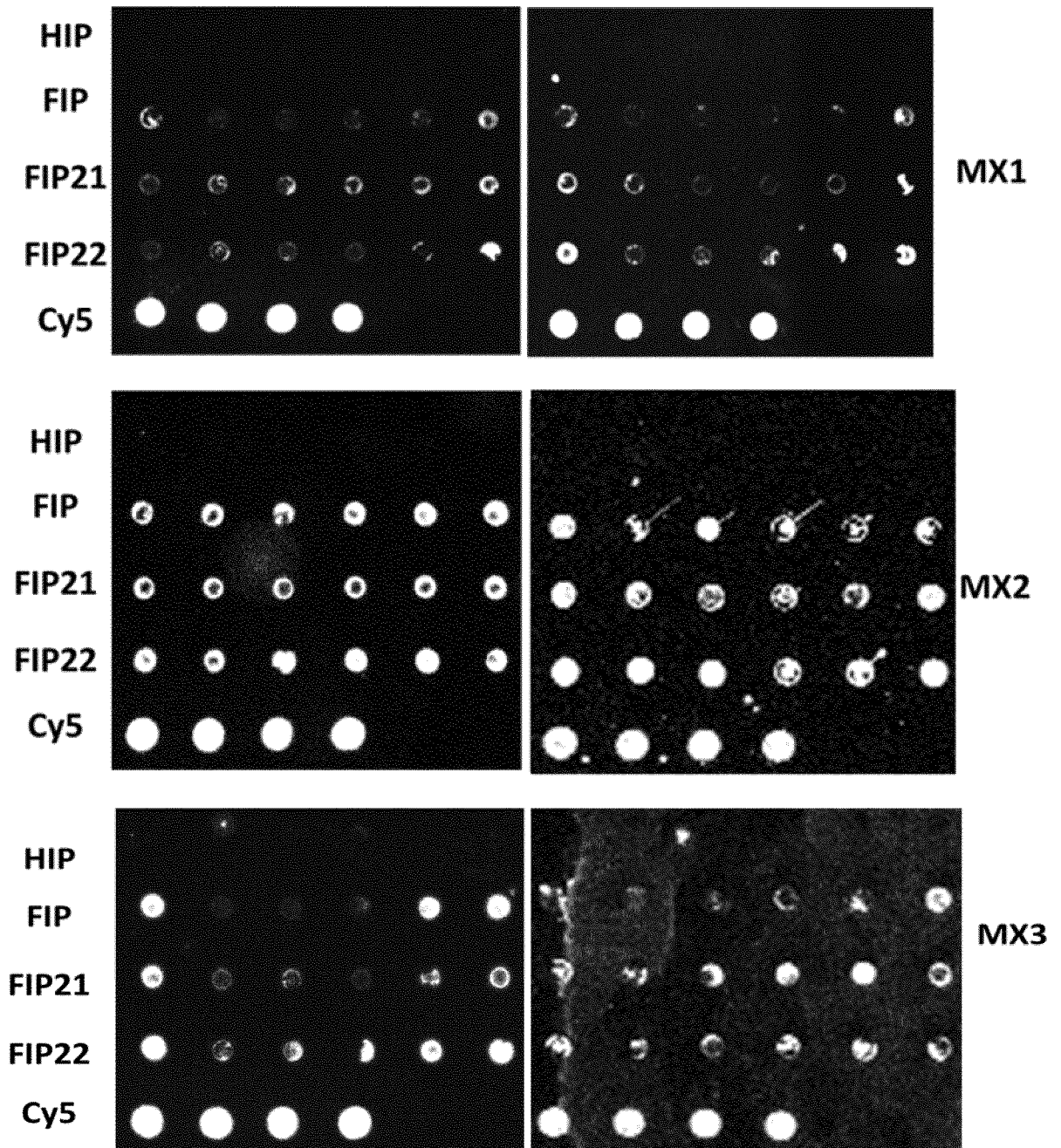


Fig. 12

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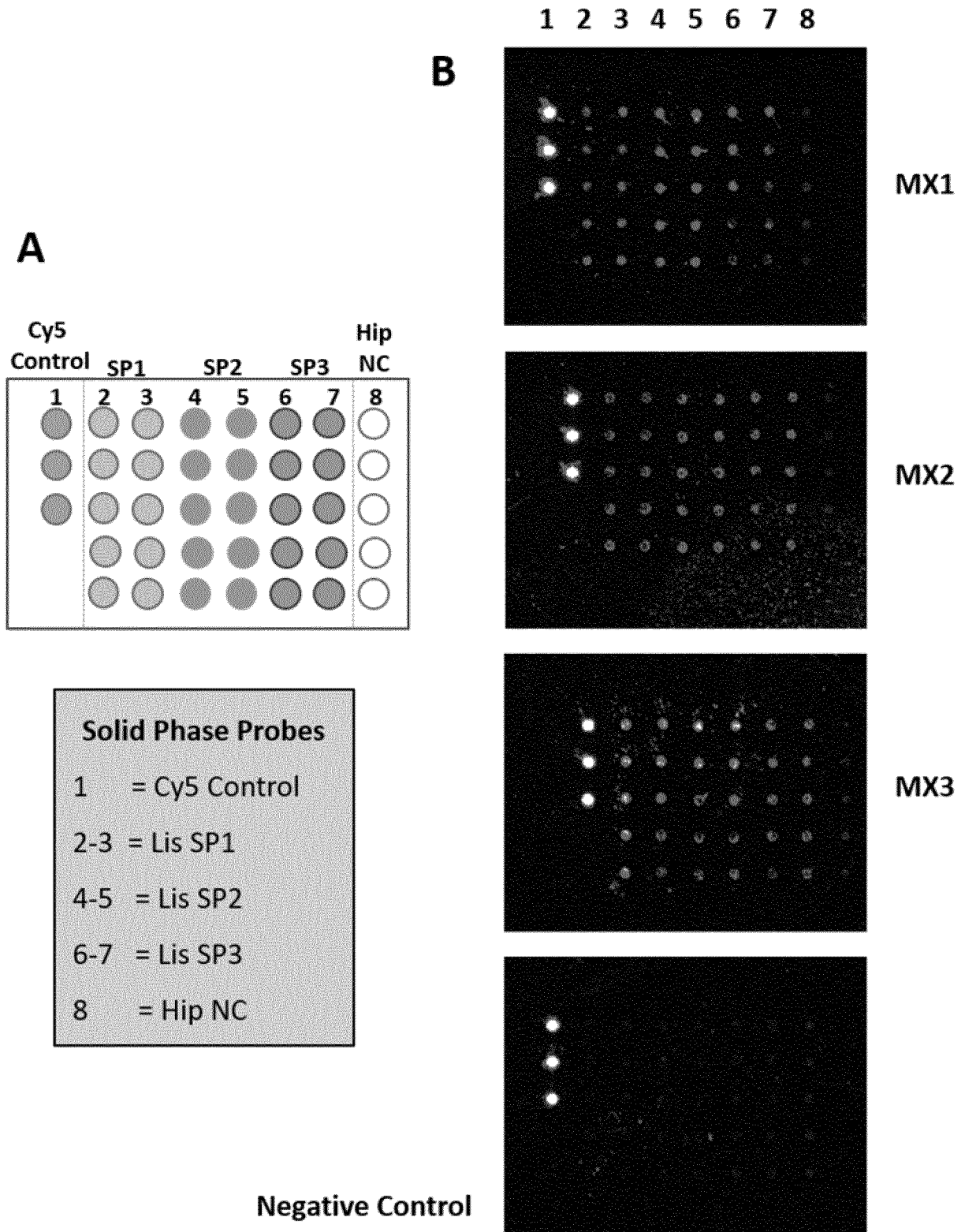
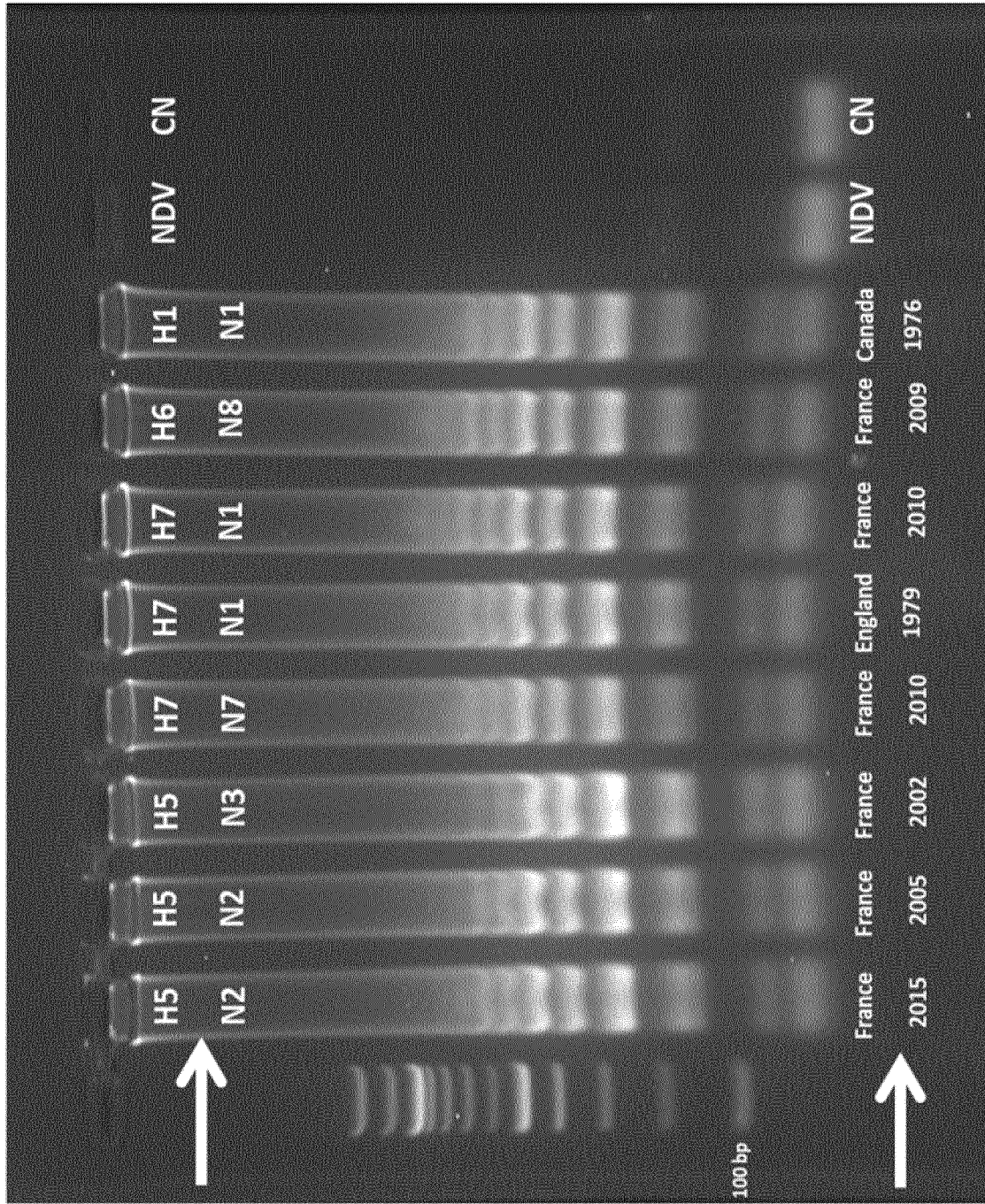


Fig. 13A-B



AIV Subtypes

Location and date of virus collection

Fig. 14A

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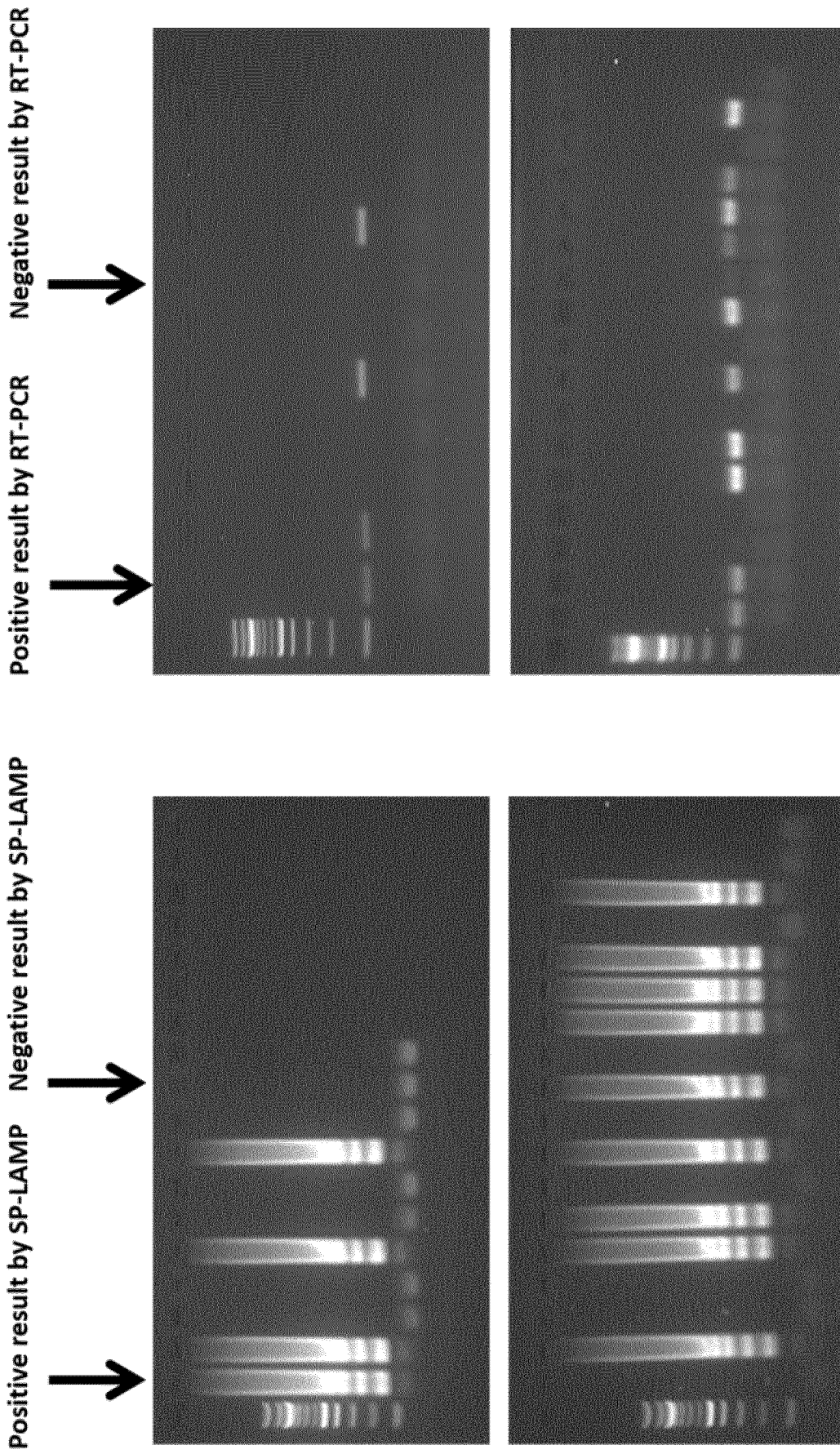


Fig. 14B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/077914

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
 ADD.
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 333 089 A1 (EI KEN CHEMICAL [JP]) 6 August 2003 (2003-08-06) abstract paragraph [0007] claim 9 ----- -/- .	1-25

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 26 November 2018	Date of mailing of the international search report 07/12/2018
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Barz , Wolfgang
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/077914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NAKAMURA N ET AL: "Detecti on of Si x Singl e-Nucl eoti de Polymorphi sms Associ ated with Rheumatoi d Arthri tis by a Loop-Medi ated Isothermal Amplifi cati on Method and an Electrochemi cal DNA Chi p", JOURNAL OF ANALYTICAL CHEMIS, CONSULTANTS BUREAU. NEW YORK, US, vol . 79, no. 24, 7 November 2007 (2007-11-07) , pages 9484-9493 , XP007905271 , ISSN: 1061-9348, DOI: 10. 1021/AC0715468 abstract; figure 3 page 9486, right-hand col umn page 9488 concl usi on</p>	1-25
A	<p>GRAMMOUSTIANOU ARISTEA ET AL: "Sol id-Phase Isothermal DNA Amplifi cati on and Detecti on on Quartz Crystal Mi crobal ance Usi ng Li posomes and Di ssi pati on Moni tori ng" , IEEE SENSORS LETTERS, IEEE, vol . 1, no. 5, 1 October 2017 (2017-10-01) , pages 1-4, XP011661421 , DOI: 10. 1109/LENS. 2017 .2739803 the whol e document</p>	1-25
A	<p>XUEEN FANG ET AL: "Loop-Medi ated Isothermal Amplifi cati on Integrated on Mi crofl uidi c Chi ps for Poi nt-of-Care Quanti tati ve Detecti on of Pathogens" , ANALYTICAL CHEMISTRY, vol . 82, no. 7, 1 Apri l 2010 (2010-04-01) , pages 3002-3006, XP055058165 , ISSN: 0003-2700, DOI: 10. 1021/ac1000652 the whol e document</p>	1-25
A	<p>PETER J. ASI ELLO ET AL: "Mi ni aturi zed i sothermal nucl eic aci d amplifi cati on, a revi ew", LAB ON A CHI P, vol . 11, no. 8, 1 January 2011 (2011-01-01) , page 1420, XP055117080, ISSN: 1473-0197 , DOI: 10. 1039/c0I c00666a the whol e document</p>	1-25
	----- -/-	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/077914

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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