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(54) Title: METHOD FOR DETECTION OF A PCR PRODUCT

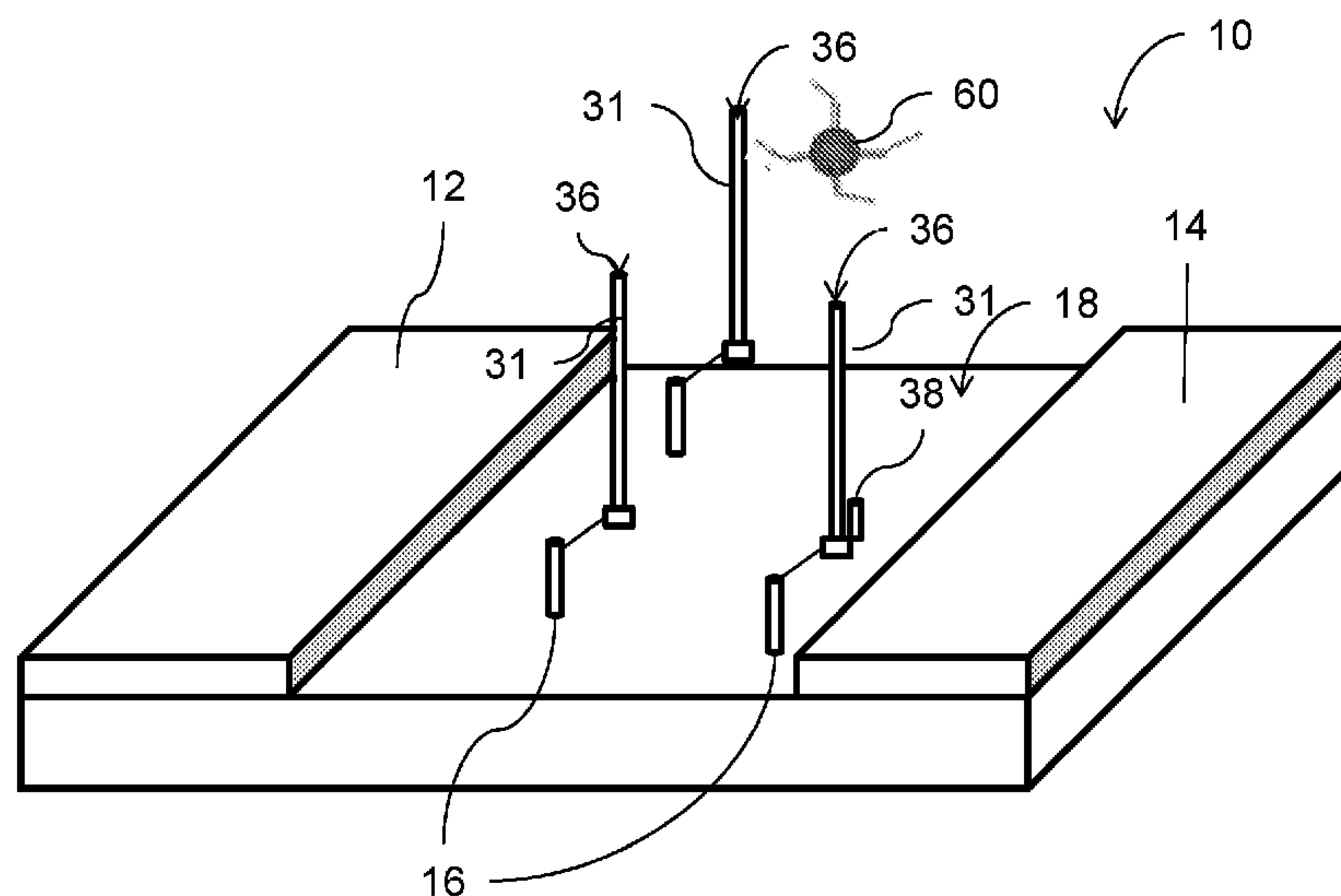


FIG. 6

(57) **Abrégé/Abstract:**

A method for detecting a nucleic acid molecule in a biological sample includes amplifying a nucleic acid molecule to generate an amplicon having a single 5'-tail and coupling the 5'-tail to one of a plurality of capture probes on a surface of a sensor. The amplicon is converted to a single strand molecule and a target-specific catalyst cluster is bound to the single strand molecule. The catalyst cluster is subjected to metallization in order to detect the target nucleic acid.

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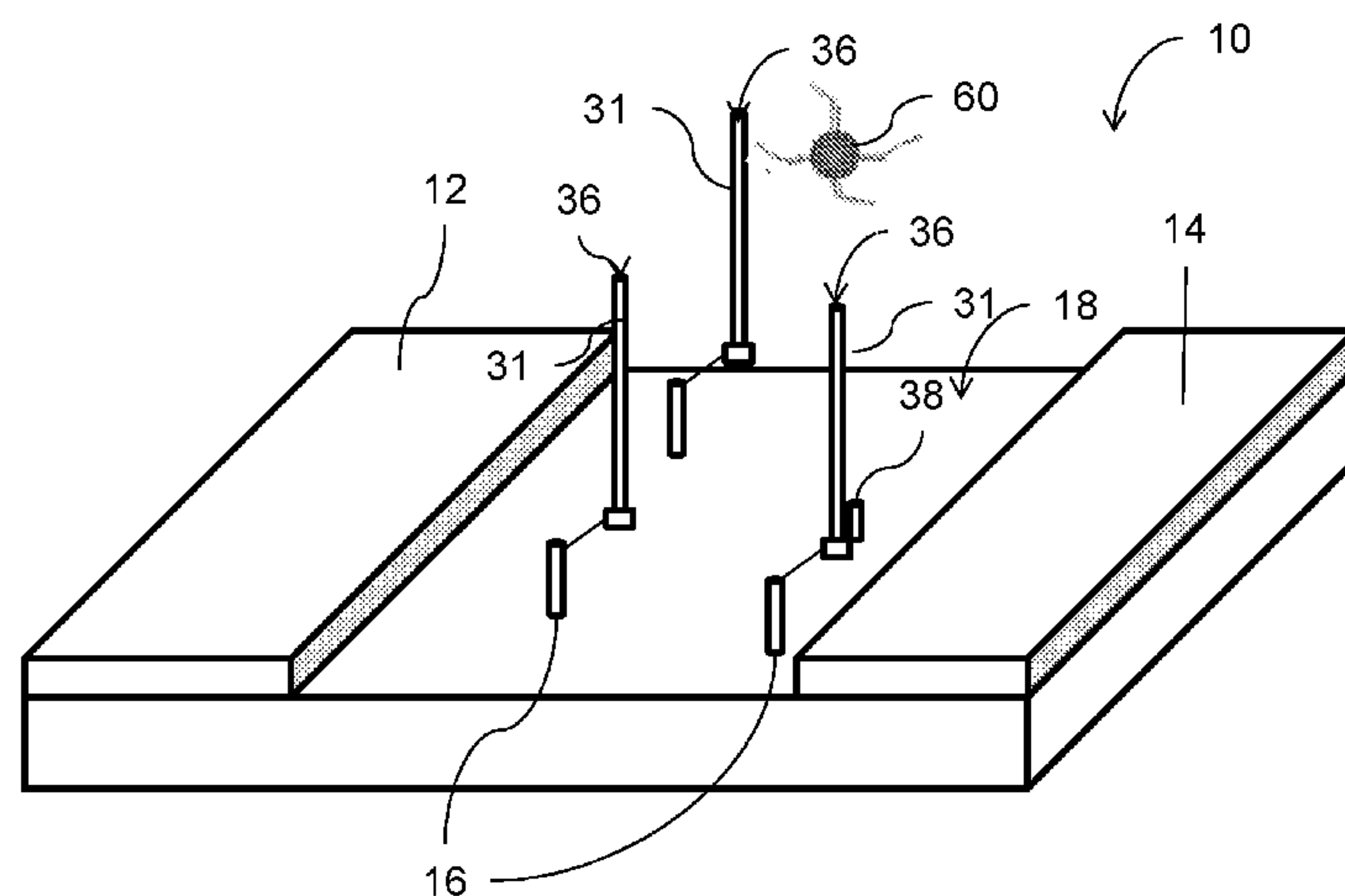


FIG. 6

(57) Abstract: A method for detecting a nucleic acid molecule in a biological sample includes amplifying a nucleic acid molecule to generate an amplicon having a single 5'-tail and coupling the 5'-tail to one of a plurality of capture probes on a surface of a sensor. The amplicon is converted to a single strand molecule and a target-specific catalyst cluster is bound to the single strand molecule. The catalyst cluster is subjected to metallization in order to detect the target nucleic acid.

[Continued on next page]

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METHOD FOR DETECTION OF A PCR PRODUCT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application Serial No. 62/337,917, filed May 18, 2016 and entitled “NESTED DETECTION OF PCR PRODUCT,” the entirety of which is incorporated herein by reference.

BACKGROUND

[0002] The subject matter disclosed herein relates to a polymerase chain reaction (PCR) process and, more particularly, to a method for nested detection of a PCR product.

[0003] PCR is a technique that allows for replicating and amplifying trace amounts of DNA fragments into quantities that are sufficient for analysis. As such, PCR can be used in a variety of applications, such as DNA sequencing and detecting DNA fragment in samples, such as for detection of pathogens in samples.

[0004] In operation, PCR involves the use of a series of repeated temperature changes or cycles that cause the DNA to melt or denature, yielding two single-stranded DNA molecules that then act as templates. Primers, short DNA fragments, containing sequences complementary to a target region of DNA along with a DNA polymerase, are used to selectively repeat amplification for a particular DNA region or sequence. Typically, two primers are included in a reaction mixture. The primers are single-stranded sequences, but are shorter than the length of the target region of DNA. The primers bind to a complementary part of the DNA strand and the DNA polymerase binds to the primer-DNA hybrid and begins DNA formation of a new DNA strand complementary to the DNA template strand. The process is repeated until multiple copies of the DNA strands have been created.

[0005] However, in some instances, the primers can be subject to hetero-dimerization, in which sequences of the primer bind to each other, rather than to the DNA, resulting in short chains of dimers or artifact amplification products, known as primer dimers. These artifact products can form in the early stages of PCR and subsequently be amplified.

[0006] An electronic sensor for detection of specific target nucleic acid molecules can include capture probes immobilized on a sensor surface between a set of paired electrodes. An example of a system and method for detecting target nucleic acid molecules is described in U.S. Patent No. 7,645,574, the entirety of which is herein incorporated by reference. Following PCR, amplified products or amplicons derived from targeted pathogen sequences are captured by the probes via a 5' single-stranded tail, which was incorporated in the molecules during the amplification process by the use of primers made with an internal replication block. Nano-gold clusters, functionalized with a second capture oligonucleotide having a complementary sequence to a universal 5'-tail tagged onto the other end of the amplified product, are used for localized hybridization to only sensor sites having captured amplification products. Subsequently, using a short treatment with a gold developer reagent, the nano-gold clusters serve as catalytic nucleation sites for metallization, which cascades into the development of a fully conductive film. The presence of the gold film shorts the gap between the electrodes and is measured by a drop in resistance, allowing the presence of the captured amplification products can then be measured. However, primer-only artifact products or possible amplicons derived from spurious nucleic acid molecules, with both primers having the requisite 5' tails, can react with such sensors in the same way a DNA target would and can result in false positive results.

SUMMARY

[0007] A method for detecting a nucleic acid molecule in a biological sample includes amplifying a nucleic acid molecule to generate an amplicon having a single 5'-tail and hybridizing the 5'-tail to one of a plurality of capture probes on a surface of a sensor. The amplicon is converted to a single strand molecule and a target-specific catalyst cluster is bound to the single strand molecule. The catalyst cluster is subjected to metallization in order to detect a target nucleic acid.

[0008] In an embodiment, a method for detecting a target nucleic acid molecule in a sample with a sensor is disclosed. The sensor includes a first electrode and a second electrode coupled to a sensor surface in a spaced apart arrangement and a plurality of

capture probes coupled to the sensor surface between the first electrode and the second electrode. The method includes performing nucleic acid molecule amplification via polymerase chain reaction (PCR) using a first primer having a 5'-tail and a second primer having no 5'-tail to form a plurality of double-stranded amplicons having a first strand with a 5'-tail and a second strand with no tail and hybridizing the plurality of amplicons to the plurality of capture probes. The method further includes converting the plurality of amplicons to a plurality of single strand molecules and binding a catalyst cluster to an interior section of each of the plurality of single strand molecules. In addition, the method includes contacting the plurality of single strand molecules having a catalyst cluster bound thereon with a metal or metal alloy to deposit the metal or metal alloy on the catalyst cluster and determining if an electrical current can be carried between the electrodes. The electrical current between the electrodes indicates the presence of the target nucleic acid molecule in the sample.

[0009] In another embodiment, a method for preparing a nucleic acid molecule detector is disclosed. The nucleic acid molecule detector includes a first electrode and a second electrode coupled to a sensor surface in a spaced apart arrangement and a plurality of capture probes coupled to the sensor surface between the first electrode and the second electrode. The method includes receiving a biological sample, amplifying a nucleic acid molecule within the biological sample to generate an amplicon having a single 5'-tail, and binding the 5'-tail of the amplicon to one of the plurality of capture probes. The method additionally includes employing an exonuclease to digest one strand of the amplicon to convert the amplicon to a single strand molecule and synthesizing a target-specific catalyst cluster. The method further includes contacting the catalyst cluster with the single strand molecule of the amplicon to bind the target-specific catalyst cluster to an interior region of the single strand molecule.

[0010] An advantage that may be realized in the practice of some disclosed embodiments is reduction or elimination of false positives due to the formation of primer-dimer artifacts or other unintended amplification products.

[0011] The above embodiments are exemplary only. Other embodiments are within the scope of the disclosed subject matter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] So that the manner in which the features of the invention can be understood, a detailed description of the invention may be had by reference to certain embodiment, some of which are illustrated in the accompanying drawings. It is to be noted, however, that the drawings illustrate only certain embodiments of this invention and are therefore not to be considered limiting of its scope, for the scope of the disclosed subject matter encompasses other embodiments as well. The drawings are not necessarily to scale, emphasis generally being placed upon illustrating the features of certain embodiments of the invention. In the drawings, like numerals are used to indicate like parts throughout the various views.

[0013] FIG. 1 is an illustration of an embodiment of a nucleic acid molecule sensor surface;

[0014] FIG. 2 is a flowchart illustrating a method of detecting nucleic acid molecules;

[0015] FIG. 3 is an illustration of an embodiment of an amplicon having a single 5' tail;

[0016] FIG. 4 is an illustration of the sensor surface of FIG. 1 having the amplicon of FIG. 3 coupled thereto;

[0017] FIG. 5 is an illustration of the sensor surface of FIG. 4 with the amplicon converted to a single strand molecule;

[0018] FIG. 6 is an illustration of the sensor surface of FIG. 5 with a catalyst cluster coupled to the single strand molecule;

[0019] FIG. 7A is an illustration of an embodiment of a method of forming a catalyst cluster;

[0020] FIG. 7B is an illustration of an embodiment of another method of forming a catalyst cluster;

[0021] FIG. 8A is an illustration of an embodiment of a method of forming a multiplexed catalyst cluster;

[0022] FIG. 8B is an illustration of an embodiment of a method of mixing multiplexed catalyst clusters;

[0023] FIG. 8C is an illustration of an embodiment of a method of multiple site nesting of multiplexed catalyst clusters on single strand amplicon molecules;

[0024] FIG. 9 is an illustration of a portion of a ribosomal gene used for testing;

[0025] FIG. 10 is a photograph of microchip surfaces resulting from testing the effects of time on exonuclease digestion of amplicon strands;

[0026] FIG. 11 is a photograph of microchip surfaces resulting from testing the effects of varying exonuclease concentration;

[0027] FIG. 12 is a photograph of microchip surfaces comparing the results of two different catalyst cluster reagents;

[0028] FIG. 13A is a photograph of a gel analysis of replicates of RT-PCR for dengue viral RNA in multiplexed reaction using pan-flavivirus primers mixed with Cal/Bun primers; and

[0029] FIG. 13B is a photograph of a gel analysis of replicates of RT-PCR for LaCrosse RNA in multiplexed reaction using pan-flavivirus primers mixed with Cal/Bun virus primers.

DETAILED DESCRIPTION

[0030] FIG. 1 illustrates an embodiment of a detector sensor microchip 10. In this embodiment, the microchip 10 includes a first electrode 12 and a second electrode 14 positioned so that the first 12 and second 14 electrode do not contact each other, with a plurality of capture probes 16 in the form of a functionalized oxide surface allowing attachment and immobilization of capture probe molecules 16 on the sensor surface 18 between the first electrode 12 and the second electrode 14. The capture probes 16 are designed to capture PCR amplified products via interaction with 5' tails incorporated during the amplification process.

[0031] FIG. 2 illustrates an embodiment of a method 20 for detection of target nucleic acid molecules. At block 22, target nucleic acid molecules collected from a biological sample are amplified via PCR. The biological sample could be any suitable type of

material, such as blood, mucous, and skin, among others. It is to be understood that any suitable type of PCR methodology can be employed. In this embodiment, two primers are used during the PCR process. The first primer is synthesized as a 5'-tailed oligonucleotide with an internal replication block and the second primer is a non-tailed oligonucleotide. In an example, the second primer has a 5' phosphate group. Multiple cycles are performed until a plurality of double-stranded amplicons 30 (replications of the target nucleic acid molecules) are formed having a first strand 31 with a 5' tail 32 and a second strand 33 extended from the non-tailed primer, as illustrated in FIG. 3. In an example, illustrated in FIG. 3, the second strand 33 has a 5'-phosphate group 34. In another example, not illustrated, the second strand 33 does not have a 5'-phosphate group. Returning to FIG. 2, at block 24, the amplicons 30 are hybridized to the capture probes 16 on the surface of the detector microchip 10, illustrated in FIG. 1. In particular, the 5' tails of the amplicons 30 bind to the capture probes 16, as illustrated in FIG. 4.

[0032] At block 26, the hybridized amplicons 30 are converted to single strand molecules 36, as illustrated in FIG. 5. In an example, rather than employing heat, which would denature the amplicons 30 off of the capture probes 16, a 5'-to-3' directional helicase is used to convert the amplicons 30 to single strand molecules 36. In another example, an exonuclease is employed to convert the amplicons 30 to single strand molecules 36. In this example, the exonuclease has 5' to 3' directionality and preferentially digests the strand 33 extended from the non-tail primer. Because the tailed strand 31 is bound to the capture probe 16, the exonuclease cannot access the 5' end of the strand 31, and therefore cannot digest the strand 31, preventing degradation of the tailed strand 31. In an example, depending on nuclease processivity or steric hindrance at a particular distance from the sensor surface 18, the strand 33 may experience incomplete digestion, resulting in a remaining portion 38 of the digested strand 33. In an example, the strand 33 extended from the non-tail primer is synthesized with a 5' phosphate and the exonuclease is a lambda exonuclease.

[0033] Digestion of the strand 33 exposes the internal sequence region of the tailed strand 31. At block 28 of the method 20 (FIG. 2), a catalyst reagent, such as a gold

catalyst reagent, is directly hybridized to the tailed strand 31, as illustrated in FIG. 6. In an embodiment, the catalyst reagent is in the form of catalyst clusters. In an embodiment, a single catalyst cluster 60 binds to the tailed strand 31. In another embodiment, and depending on the length of the tailed strand 31, a plurality of catalyst clusters 60 bind to each tailed strand 31.

[0034] Since a generic oligonucleotide is not suitable for binding to internal sequences within the amplicons, the catalyst clusters are target-specific, i.e., the catalyst clusters bind to specific target sequences in the strand 31. Because the primer-dimer artifacts do not include these target sequences, the catalyst clusters 60 do not bind to primer-dimer artifacts, thus avoiding potential false positive measurements. As illustrated in FIG. 7A, in one example, a thiol-modified oligonucleotide 40 can be reacted with a catalytic cluster 42 to form a catalyst cluster 44 functionalized with at least 20 oligonucleotides. In another example, illustrated in FIG. 7B, a universal oligonucleotide 46 that possesses a universal cluster binding sequence 45 and amplicon-specific bind sequence 47 can be hybridized with a base generic cluster 48 to form a catalyst cluster 50. As a result, each new oligonucleotide is concatenated at the 3'-end with an adaptor specific sequence complementary to the cluster generic oligonucleotide. While the resulting catalyst cluster 50 in FIG. 7B is depicted as having four hybridized adaptor oligonucleotides 52, it is to be understood that the actual number of adaptors will depend on cluster size, which limits the number of capture oligonucleotides on the base cluster, and the stoichiometry of adaptor oligonucleotides added to the cluster.

[0035] As illustrated in FIG. 8A, the base generic clusters 48 can be prepared with mixtures of probe oligonucleotides A, B, C, D to form a catalyst cluster 54 with multiplexing capabilities. Depending on cluster size, which determines the surface area available for functionalization, it is possible to load multiple different probes onto a single cluster. In order to support efficient metallization reactions, the ratio for the number of each probe type per cluster may require optimization. As illustrated in FIG. 8B, tailored mixtures of multiplexed clusters 54 can be created. The mixtures of

multiplexed clusters 54 can both multiplex for a large number of targeted amplicons and populate each targeted amplicon with multiple clusters, as illustrated in FIG. 8C.

[0036] Returning to FIG. 2, at block 30, metallization of the catalyst clusters 60, which serve as catalytic nucleation sites, can be performed to form a conductive film and resistance between the electrodes 12, 14 (FIG. 1) can be measured to detect target nucleic acid molecules at block 32. In an example, the catalyst clusters 60 are gold clusters and a gold developer reagent is applied to the catalyst clusters 60 to cascade into the development of the conductive film, which in this example is a gold film. In this example, the presence of the gold film electrically shorts the gap between the electrodes 12, 14 and is measured by a drop in resistance. In this example, a negative sensor has a resistance of more than one million ohms and a positive sensor has a resistance of about one thousand ohms.

EXAMPLE:

[0037] An existing test developed for plasmodium falciparum was used to test the method 20 described above. FIG. 9 is a screenshot taken from the INVITROGENTM program showing a portion of the 18S ribosomal gene to which primers (shown in bold lettering) are designed. Typically, the two primers, forward primer 60 and reverse primer 62, used in PCR for this method each have a sensor and catalyst binding 5'-tail. However, using the method 20 described above, a new reverse primer 64 located downstream of the original reverse primer 62 was identified. Synthesis of the new reverse primer 64 omitted the 5' tail for catalyst binding while including a 5'-phosphorol group to facilitate exonuclease degradation.

[0038] Because the 5'-tail on the original reverse primer 62 hybridizes to a universal catalyst reagent, modification of the catalyst cluster for use with the method 20 was accomplished by pre-hybridization of the original reverse primer oligonucleotide onto a universal cluster to form target-specific catalyst gold clusters. Preparation of these target-specific catalyst gold clusters included a heated incubation with 1000 fold molar excess of the primer 62, cooling to room temperature, and removal of unbound excess oligonucleotide by washing, repeated twice, via high-speed centrifugation and

resuspension with the final reagent buffer. Similarly, a second catalyst reagent 66 with specificity towards a different sequence element located upstream of the first cluster binding sequence was prepared. The ability of this second cluster to effect metallization and detection served as only a preliminary attempt to assess the processivity of the exonuclease in the digestion of sensor-bound amplicons. With this particular derived amplicon, the nuclease must have digested to within at least 95 nucleotides of completely degrading the extraneous DNA strand, leaving a single-stranded tract of about eighty nucleotides available for cluster binding.

[0039] Select 5' to 3' exonucleases with suitable properties to perform a digestion as per the method 20 were assessed. Two suitable commercially available exonucleases, Lambda and T7, were identified. Lambda was used in this example. FIGs. 10 and 11 illustrate the results of this experiment.

[0040] FIG. 10 illustrates the results of time coarse experiments of Lambda exonuclease treatments on derived amplicons hybridized to microchips. All microchips in Panel A and Panel B were hybridized for five minutes at 45 degrees Celsius with 100 ng of the amplicon, washed twice by dipping into a 10 mL volume of hybridization buffer, and dried under a nitrogen gas stream. In a humidified petri dish held in a 37 degree Celsius incubator, the microchips were spotted with 25 μ L Lambda reaction solution containing one unit of the exonuclease. One unit of exonuclease is defined as the amount of enzyme required to produce 10 nmol of acid-soluble deoxyribonucleotide from a double-stranded substrate in a total reaction volume of 50 μ L in 30 minutes at 37 degrees Celsius in 1X lambda Exonuclease Reaction Buffer with 1 μ g sonicated duplex [3 H]-DNA. After incubation for a designated time, indicated above each microchip, the reactions were quenched by transferring the microchips into a petri dish with 20 mL of hybridization buffer. Subsequently, for metallization, catalyst hybridization with clusters modified with the primer oligonucleotide was performed for five minutes at 45 degrees Celsius, two washes were performed by swirling the chips in petri dishes containing hybridization buffer, and then gold development was performed for four minutes at 55 degrees Celsius with 25 μ L of developer reagent placed on the chip surface. FIG. 11 illustrates the effect

of titration of the Lambda exonuclease on the gold development of target amplicons that were hybridized on the surface of the microchips. The microchips were hybridized as described above with regard to FIG. 10. However, the units of Lambda exonuclease used on each microchip illustrated in FIG. 11 was varied as indicated above each microchip.

[0041] One finding of these experiments was that the Lambda exonuclease could not digest the 5'-tail primer extended strand, which would have caused a loss in the capacity to capture the catalyst reagent. This finding is supported by the longer time coarse digestion experiment illustrated in FIG. 10, which shows there is no loss of chip metallization, even with the most extensive Lambda incubation time of 300 minutes.

[0042] The ability of the adaptor modified clusters to hybridize to a more internal site within the 5'-tailed strand were investigated by preparation of the second cluster reagent, described above. This second cluster was formed to bind about thirty nucleotides proximal of the hybridization site of the first cluster. The results of this investigation of illustrated in FIG. 12, which compares development of a first microchip 70 processed with the first cluster reagent and a second microchip 72 processed with the second cluster reagent. Comparison of the first microchip 70 and second microchip 72 shows that both clusters performed equally well yielding comparable gold spots on the respective treated microchips. In addition, the results established that, on a majority of the hybridized amplicons, the Lambda exonuclease digested a minimum of ninety bases and was not inhibited while approaching the microchip surface. In addition, the longer stretch of single-stranded DNA with which the second cluster interacted did not appear to affect bind of the second cluster relative to binding of the first cluster.

[0043] Using the method described above for *Plasmodium falciparum* (*P.f.*), fifty test cartridge runs were performed. Negative and positive samples consisted respectively of either 10 μ L of water or a 1 μ L blood culture of *P.f.* (10^5) cells diluted in a buffer to 10 μ L. After pipetting and sealing a sample into the test cartridge, a fully automated assay, including sample preparation, PCR amplification, and microchip hybridization, nuclease digestion, and metallization reactions, was carried out. Post assay electrical measurements were performed by removal of each microchip board from the cartridge

after each test run and visually inspecting each microchip before placing the microchip on a probe station for collection of electrical results. Table 1 presents the raw data from the fifty assays. As indicated by “NO TEST” in the “results” column of Table 1, certain assays were excluded due to machine failure or operator mistakes, as further indicated in the “Notes” column in Table 1. Table 2 indicates the percentages of correct true positives and negatives obtained. As illustrated in Table 2, using a modified, single-tailed amplicons resulted in 100% correct negative measurements and 88% correct positive measurements.

TABLE 1:

E-test # of shorted sensors						
Date	TEST	TARGET	CTL	NCOMP	RESULT	Notes
3/14/16	POS	10	0	0	CORRECT	
3/14/16	POS	10	0	0	CORRECT	
3/14/16	NEG	0	0	0	CORRECT	
3/14/16	NEG	0	0	0	CORRECT	
3/14/16	NEG	0	0	0	CORRECT	
3/15/16	POS	0	0	0	INCORRECT	No sign of target development - Benchtop development indicates SP-PCR ok
3/15/16	POS	0	0	0	NO TEST	Cartridge leaked. Benchtop development of hyb buffer indicates SP-PCR ok
3/15/16	NEG				NO TEST	PCR Temp Timeout
3/15/16	NEG	0	0	0	CORRECT	
3/15/16	NEG	0	0	0	CORRECT	
3/15/16	NEG	0	0	0	CORRECT	
3/16/16	POS	10	0	0	CORRECT	
3/16/16	POS	10	0	0	CORRECT	
3/16/16	NEG	0	0	0	CORRECT	
3/17/16	POS	10	0	0	CORRECT	
3/17/16	POS	10	0	0	CORRECT	
3/17/16	NEG	0	0	0	CORRECT	
3/17/16	NEG	0	0	0	CORRECT	
3/18/16	POS	10	0	0	CORRECT	
3/18/16	POS	10	0	0	CORRECT	
3/18/16	NEG	0	0	0	CORRECT	
3/18/16	NEG	0	0	0	CORRECT	
3/18/16	NEG	0	0	0	CORRECT	
3/21/16	POS	10	0	0	CORRECT	
3/21/16	POS	10	0	0	CORRECT	
3/21/16	NEG	0	0	0	NO TEST	Developer misloaded - turned out ok when completed on benchtop
3/21/16	NEG	0	0	0	CORRECT	
3/21/16	NEG	0	0	0	CORRECT	
3/22/16	POS	10	0	0	CORRECT	
3/22/16	POS	10	0	0	CORRECT	
3/22/16	NEG	0	0	0	CORRECT	
3/22/16	NEG	0	0	0	CORRECT	
3/22/16	NEG	0	0	0	CORRECT	
3/23/16	POS	9	0	0	CORRECT	
3/23/16	POS	0	0	0	NO TEST	Weak development - machine ran unusually slow (SD card issues)
3/23/16	POS	10	0	0	CORRECT	
3/23/16	NEG	0	0	0	CORRECT	
3/23/16	NEG	0	0	0	CORRECT	
3/23/16	POS	10	0	0	CORRECT	
3/23/16	POS	10	0	0	CORRECT	
3/28/16	POS	0	0	0	INCORRECT	No development
3/28/16	POS	0	0	0	INCORRECT	No development
3/28/16	POS				NO TEST	Motor COM error, Motor timeout
3/28/16	POS	10	0	0	CORRECT	
3/28/16	NEG				NO TEST	Syringe timeout error
3/31/16	POS	10	0	0	CORRECT	
3/31/16	POS	10	0	0	CORRECT	
3/31/16	POS	6	0	0	CORRECT	Light development
3/31/16	POS	10	0	0	CORRECT	

TABLE 2:

50	Total runs
32	Positives
23	Negatives
4	Runs discarded for reader failures
92.0%	Mechanical performance
1	Runs excluded for operator reagent misload
1	Positive run excluded for mechanical leak

19	19	Correct Negatives : Negative Runs	100.0%
22	25	Correct Positives : Positive Runs	88.0%

41	44	Aggregate	93.2%
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EXAMPLE 2:

[0044] Various combinations of multiplexed reverse transcription PCR (RT-PCR) were assessed using primer sets designed for development of a pan-flavivirus/pan-alphavirus/pan-bunyavirus test. A total of nine primers were used in this assay, with all assayed materials derived from the homogenization, using ultrasonically driven bead-beating, of pooled six to eight mosquitoes that were spiked or non spiked with a virus, VEE-TC83, Dengue, or LaCrosse Virus. Nucleic acid material was isolated from the homogenates using magnetic particle purification, desalted by gel-filtration, and used in the RT-PCR amplification with the appropriate multiplexed primer sets, either pan-flavivirus plus bunyavirus primers or alpha primers, to generate single-stranded 5'-tailed amplicons. The findings indicate that the primer sets for the pan-alpha and pan-flavivirus tests inhibit one another during PCR amplification. To address this problem, the test cartridge provides two separate PCR chambers allowing for interfering primer sets to be run separately and mixed prior to hybridization on the sensor chip. FIG. 13A illustrates gel analysis of gene replicates of RT-PCR for dengue viral RNA (lanes 1-4), purified from spike mosquitoes, in multiplexed reaction using pan-flavivirus primers mixed with the Cal/Bun primers. Lane 5 is a negative control with no RNA input. FIG. 13B illustrates gel analysis of RT-PCR for LaCrosse RNA (lanes 1-5), purified from spiked

mosquitoes, in multiplexed reaction using pan-flavivirus primers mixed with the Cal/Bun primers. Lane 6 is a negative control, no RNA input. The findings indicate that combinations of pan-bunyavirus and pan-flavivirus primers are compatible in PCR reactions.

[0045] Possible advantages of the above described method include reduction or elimination of false positive measurements due to primer-dimer artifact formation.

[0046] While the present invention has been particularly shown and described with reference to certain exemplary embodiments, it will be understood by one skilled in the art that various changes in detail may be effected therein without departing from the spirit and scope of the invention that can be supported by the written description and drawings. Further, where exemplary embodiments are described with reference to a certain number of elements it will be understood that the exemplary embodiments can be practiced utilizing either less than or more than the certain number of elements.

[0047] The patentable scope of the invention is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal language of the claims.

[0048] To the extent that the claims recite the phrase “at least one of” in reference to a plurality of elements, this recitation is intended to mean at least one or more of the listed elements, and is not limited to at least one of each element. For example, “at least one of an element A, element B, and element C,” is intended to indicate element A alone, or element B alone, or element C alone, or any combination thereof. “At least one of element A, element B, and element C” is not intended to be limited to at least one of an element A, at least one of an element B, and at least one of an element C.

AMENDED CLAIMS

received by the International Bureau on 31 October 2017 (31.10.2017)

What is claimed is:

1. A method for detecting a target nucleic acid molecule in a sample with a sensor comprising a first electrode and a second electrode coupled to a sensor surface in a spaced apart arrangement and a plurality of capture probes coupled to the sensor surface between the first electrode and the second electrode, the method comprising:

performing nucleic acid molecule amplification via polymerase chain reaction (PCR) using a first primer having a 5'-tail and a second primer having no 5'-tail to form a plurality of double-stranded amplicons having a first strand with a 5'-tail and a second strand with no tail;

hybridizing the plurality of amplicons to the plurality of capture probes;

converting the plurality of amplicons to a plurality of single strand molecules;

binding a catalyst cluster to an interior section of each of the plurality of single strand molecules;

contacting the plurality of single strand molecules having a catalyst cluster bound thereon with a metal or metal alloy to deposit the metal or metal alloy on the catalyst cluster; and

determining if an electrical current can be carried between the electrodes, the electrical current between the electrodes indicating presence of the target nucleic acid molecule in the sample.

2. The method of claim 1, wherein converting the plurality of amplicons to the plurality of single strand molecules comprises employing an exonuclease to digest the second strand with no tail.

3. The method of claim 1, wherein the catalyst cluster comprises a catalyst gold cluster and wherein the metal comprises gold.

4. The method of claim 1, further comprising forming a target specific catalyst cluster configured to bind to an interior region of the first strand of the amplicon.

5. The method of claim 1, wherein the catalyst cluster is a generic cluster having an adaptor oligonucleotide coupled thereto.
6. The method of claim 5, wherein the generic cluster has a plurality of oligonucleotides coupled thereto, each oligonucleotide configured to target a different amplicon.
7. The method of claim 1, wherein binding the catalyst cluster comprises binding a plurality of catalyst clusters to interior sections of each of the single strand molecules.
8. A method for preparing a nucleic acid molecule detector comprising a first electrode and a second electrode coupled to a sensor surface in a spaced apart arrangement and a plurality of capture probes coupled to the sensor surface between the first electrode and the second electrode, the method comprising:
 - receiving a biological sample;
 - amplifying a nucleic acid molecule within the biological sample to generate an amplicon having a single 5'-tail;
 - coupling the 5'-tail of the amplicon to one of the plurality of capture probes;
 - employing an exonuclease to digest one strand of the amplicon to convert the amplicon to a single strand molecule;
 - synthesizing a target-specific catalyst cluster; and
 - contacting the catalyst cluster with the single strand molecule of the amplicon to bind the target-specific catalyst cluster to an interior region of the single strand molecule.
9. The method of claim 8, wherein the catalyst cluster comprises a catalyst gold cluster.
10. The method of claim 8, wherein synthesizing the target-specific catalyst cluster comprises hybridizing a base generic cluster with an adaptor oligonucleotide to form a catalyst cluster having a plurality of adaptor oligonucleotides coupled thereto.
11. The method of claim 10, wherein the adaptor oligonucleotide has a cluster binding sequence and an amplicon-specific binding sequence.

12. The method of claim 10, wherein hybridizing the base generic cluster comprises hybridizing the generic cluster with a plurality of adaptor oligonucleotides, each adaptor oligonucleotide configured to target a different amplicon.

13. The method of claim 8, wherein amplifying the nucleic acid molecule comprises performing polymerase chain reaction (PCR) using a first primer having a 5'-tail and a second primer having no 5'-tail.

14. The method of claim 8, wherein each of the plurality of capture probes comprises a functionalized oxide surface configured to immobilize molecules to the sensor surface.

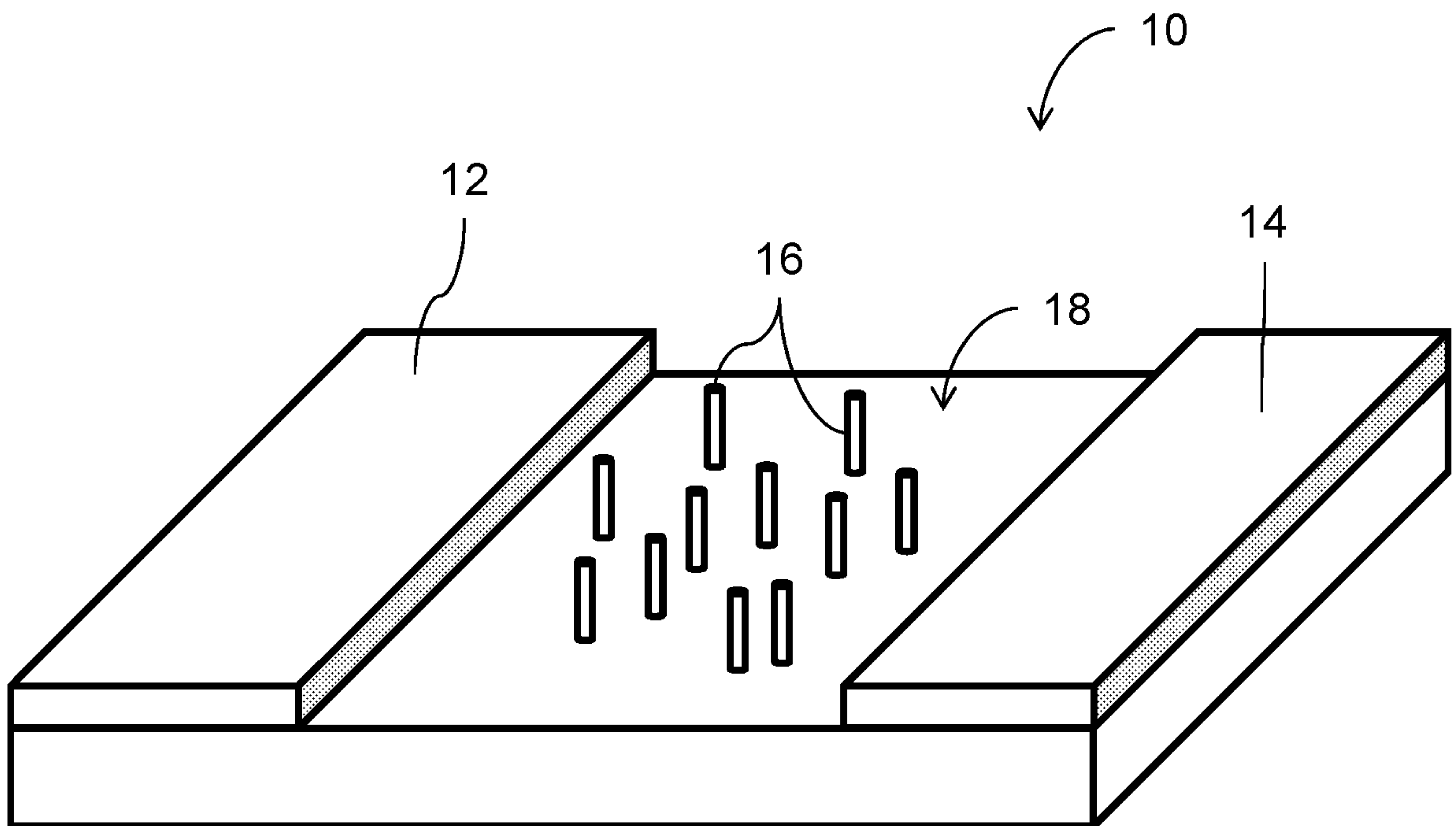


FIG. 1

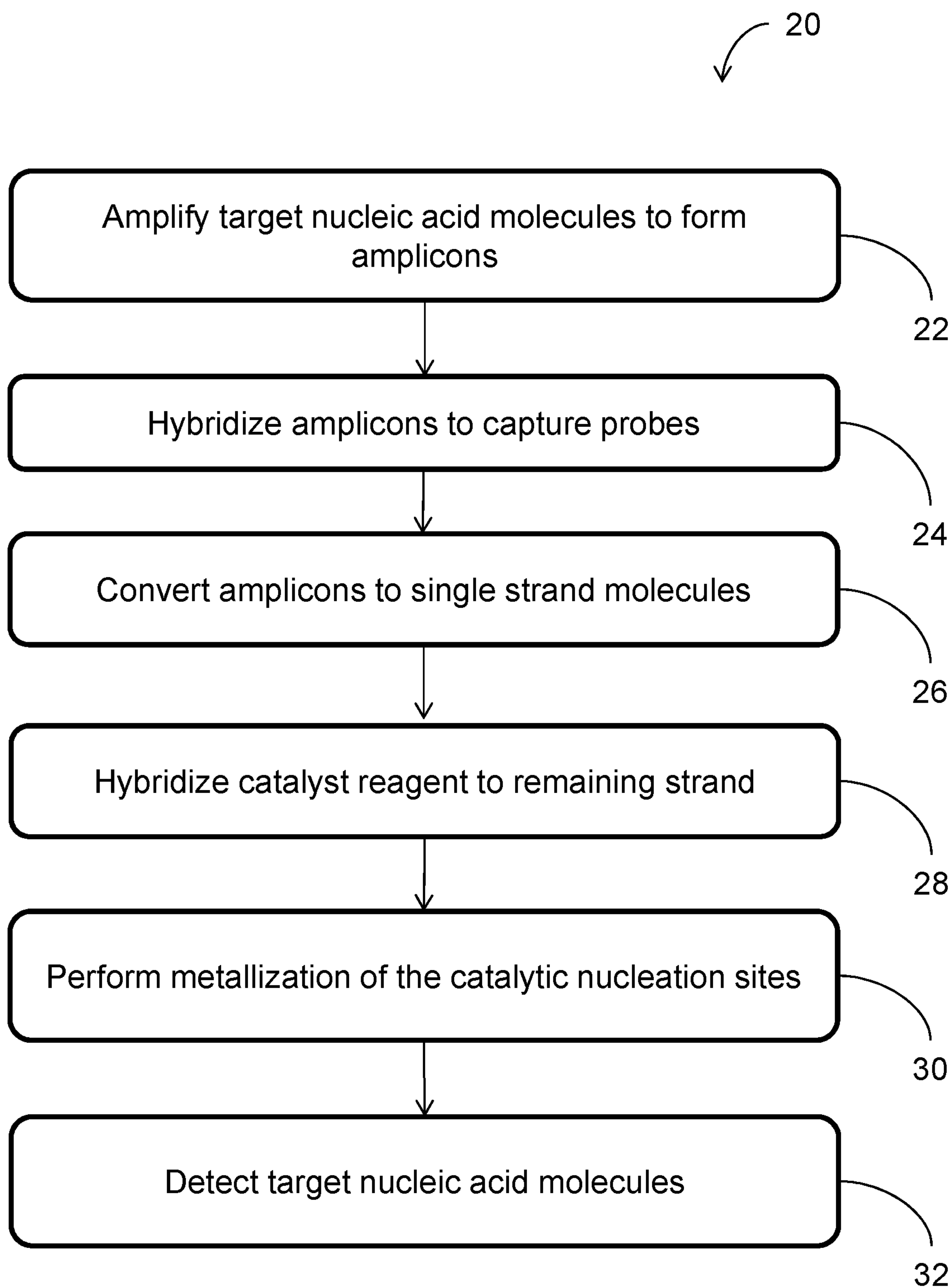


FIG. 2

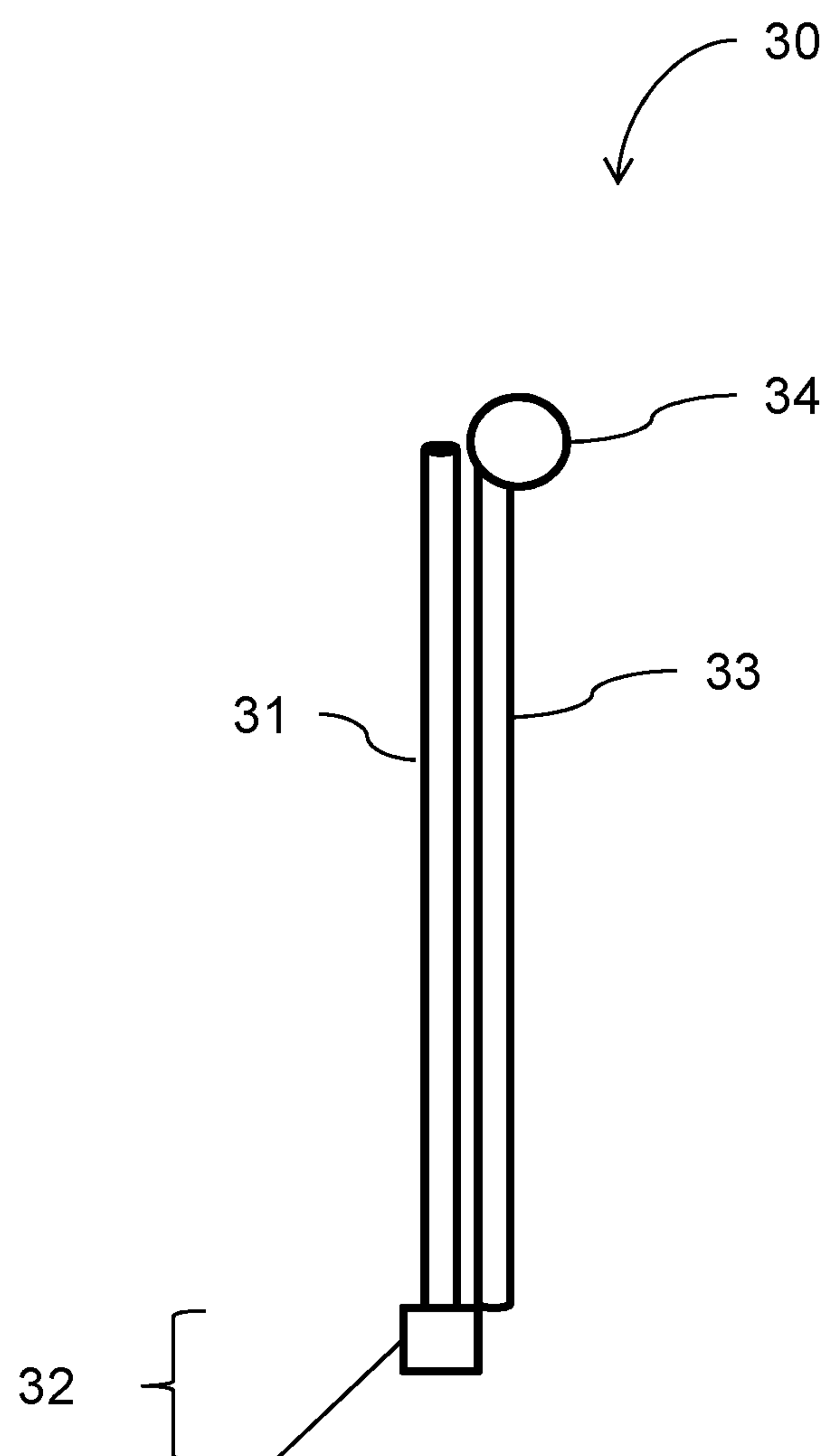


FIG. 3

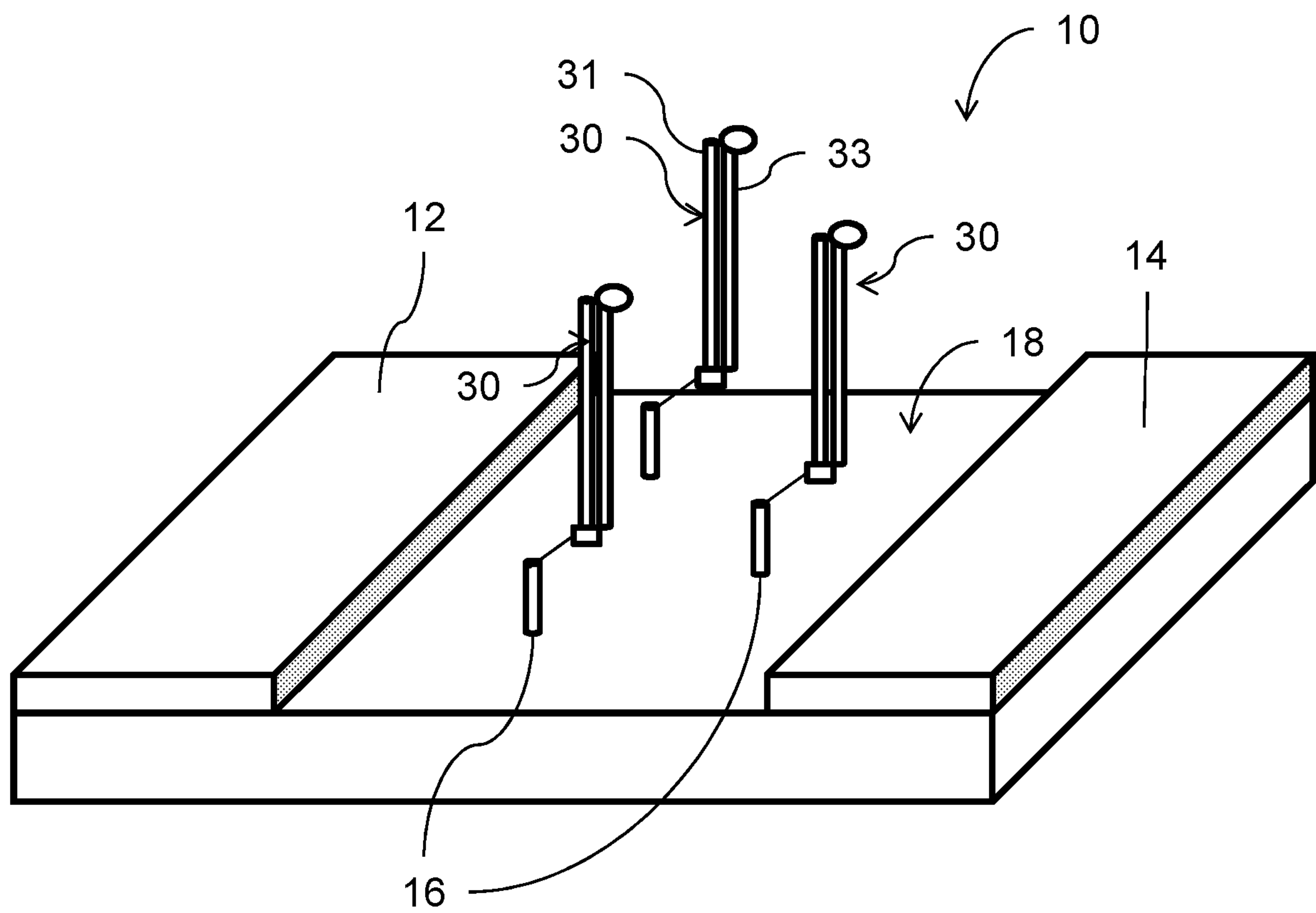


FIG. 4

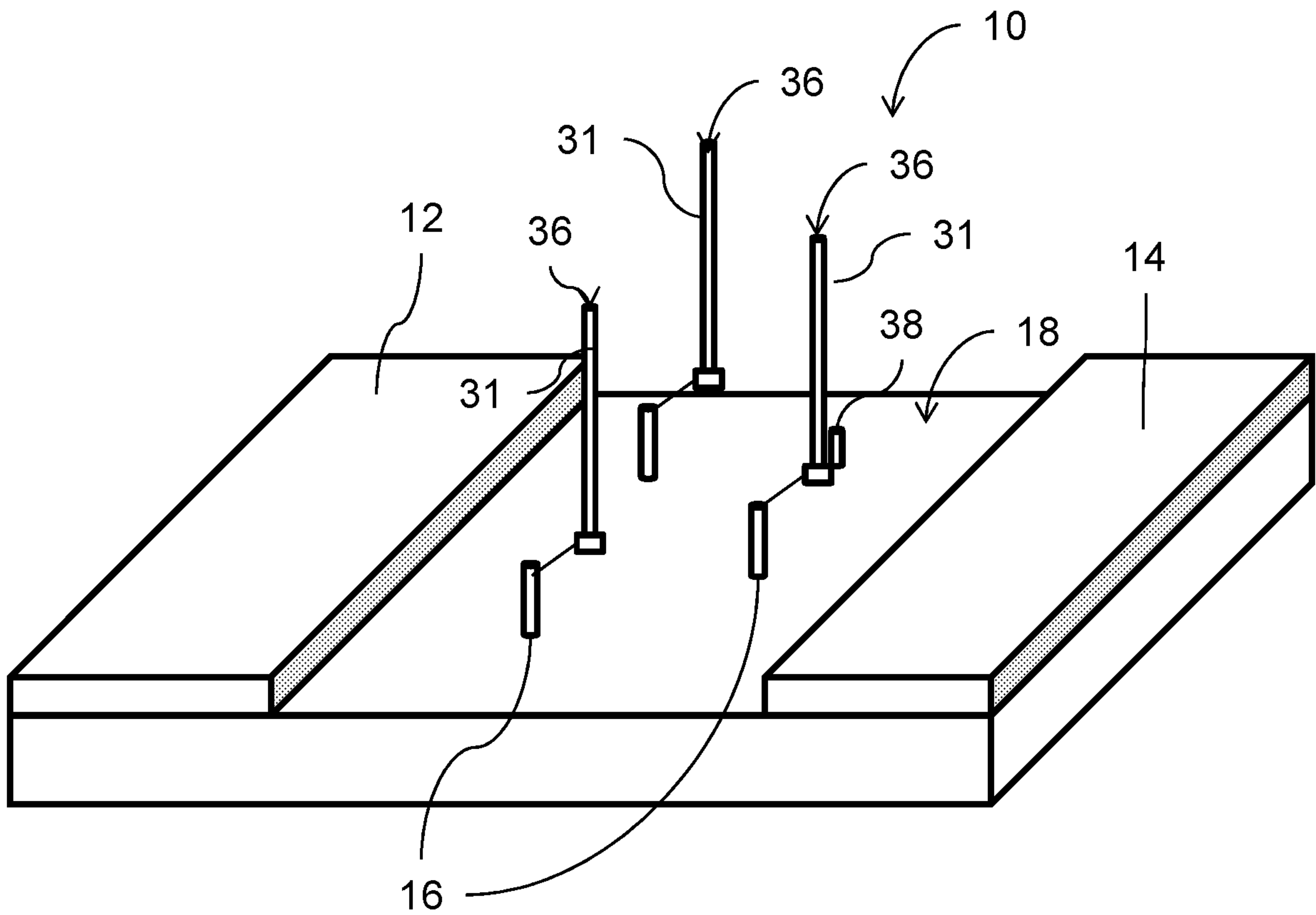


FIG. 5

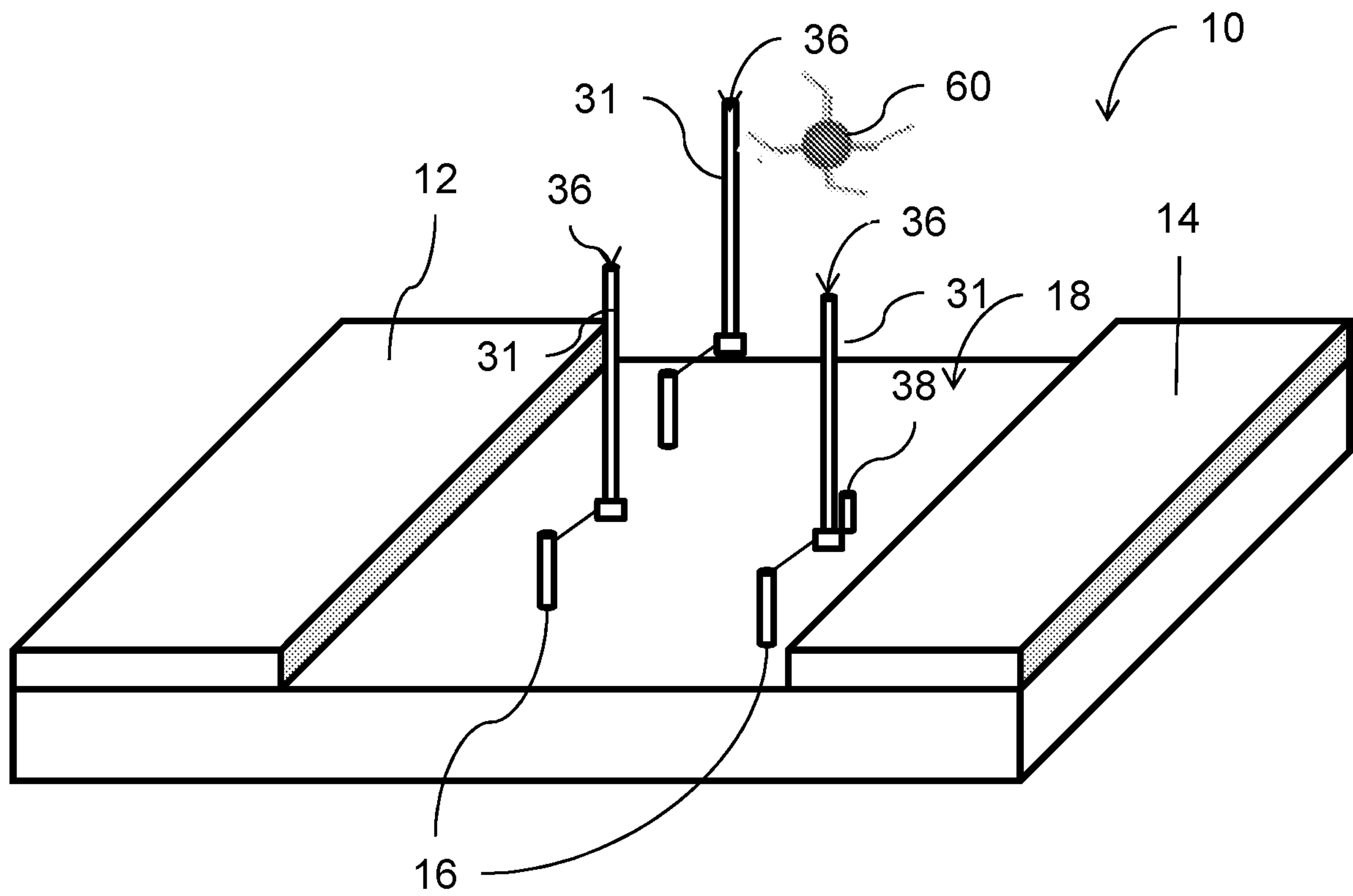


FIG. 6

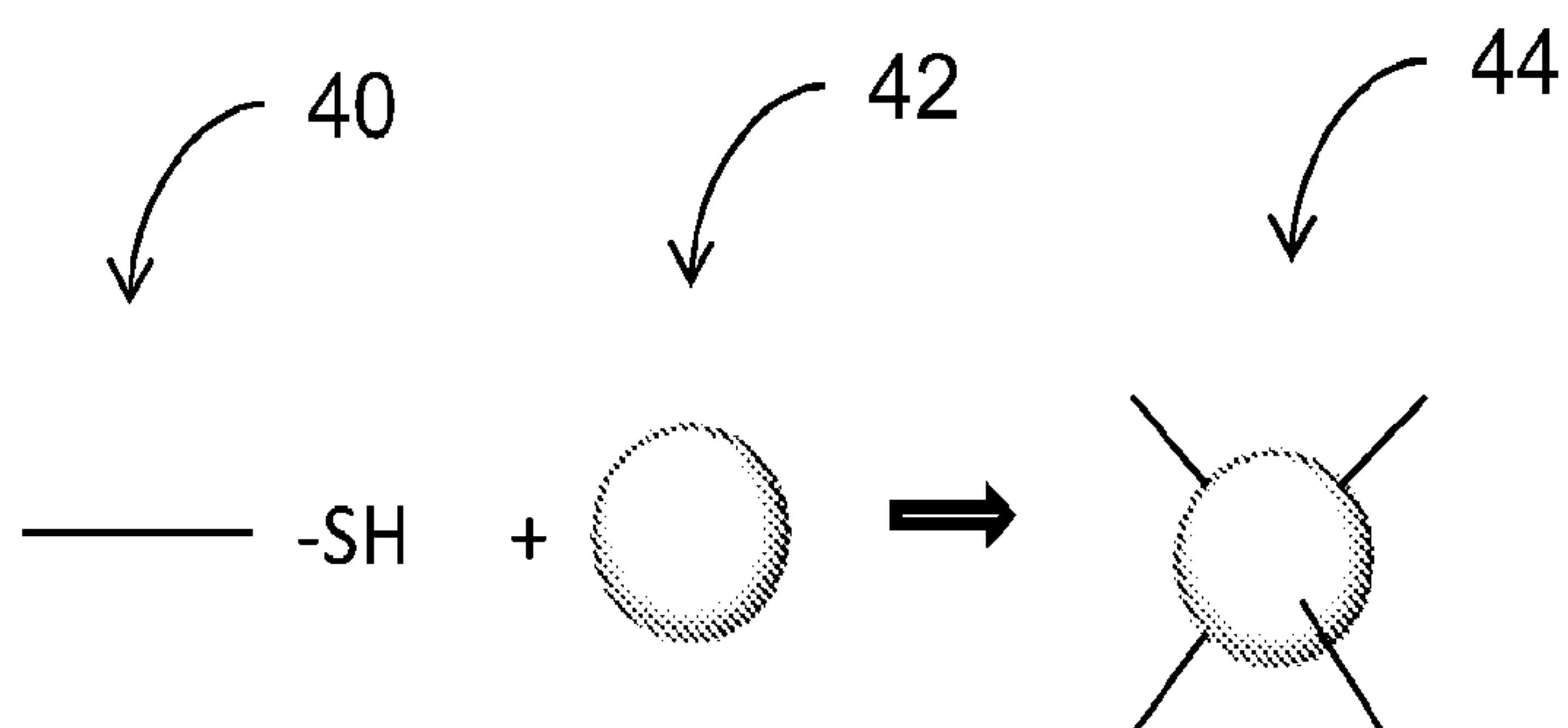


FIG. 7A

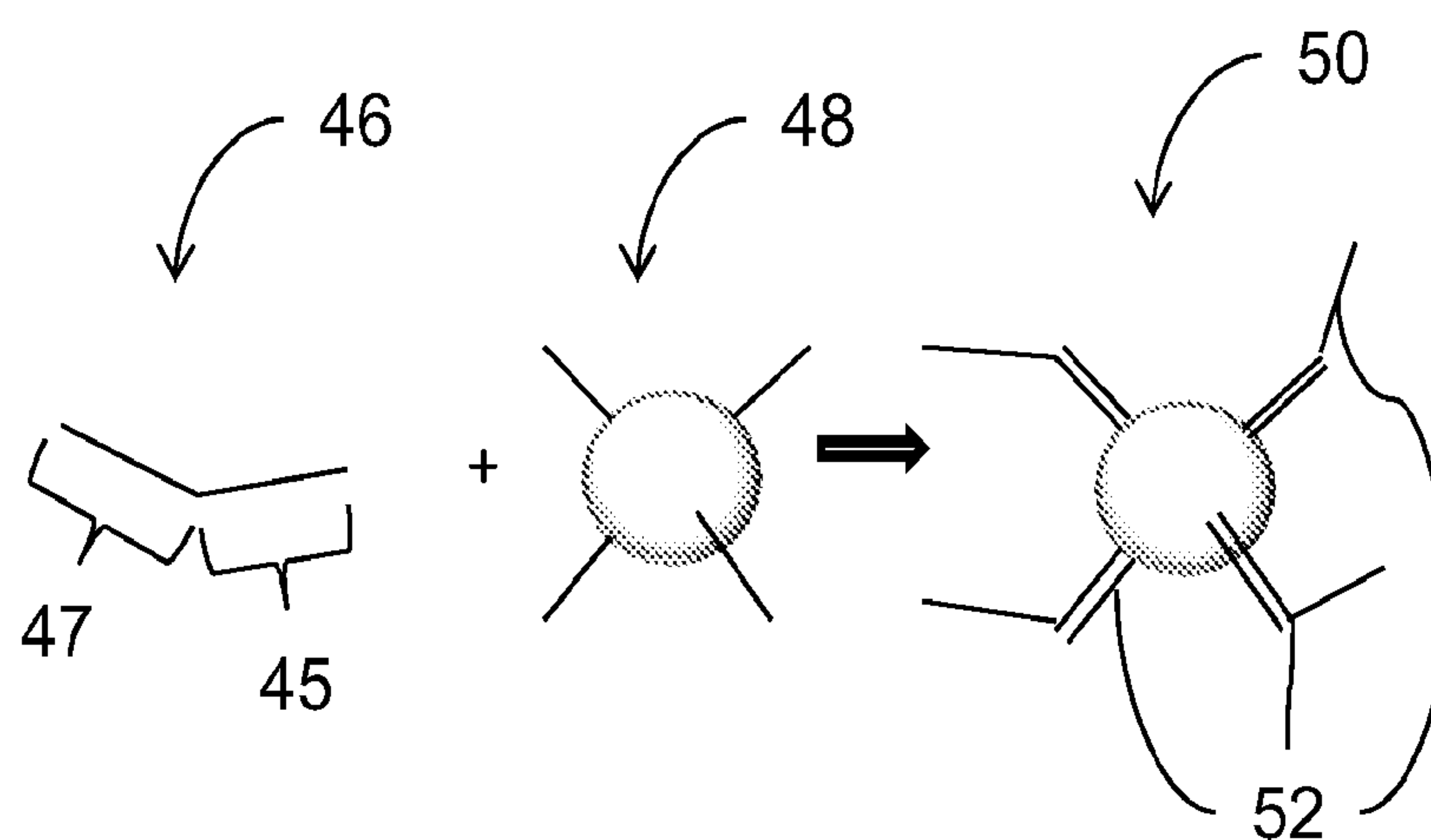


FIG. 7B

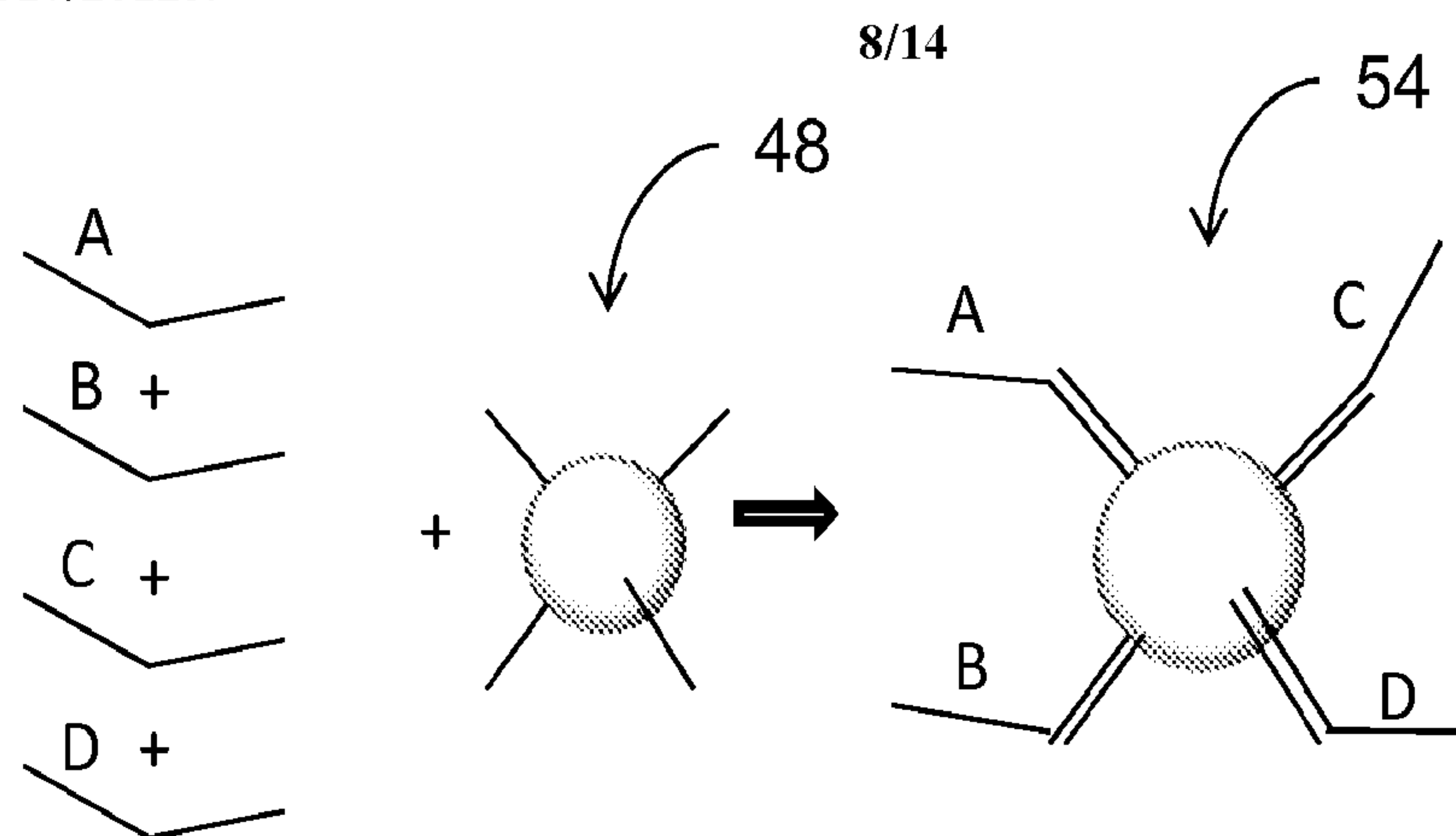


FIG. 8A

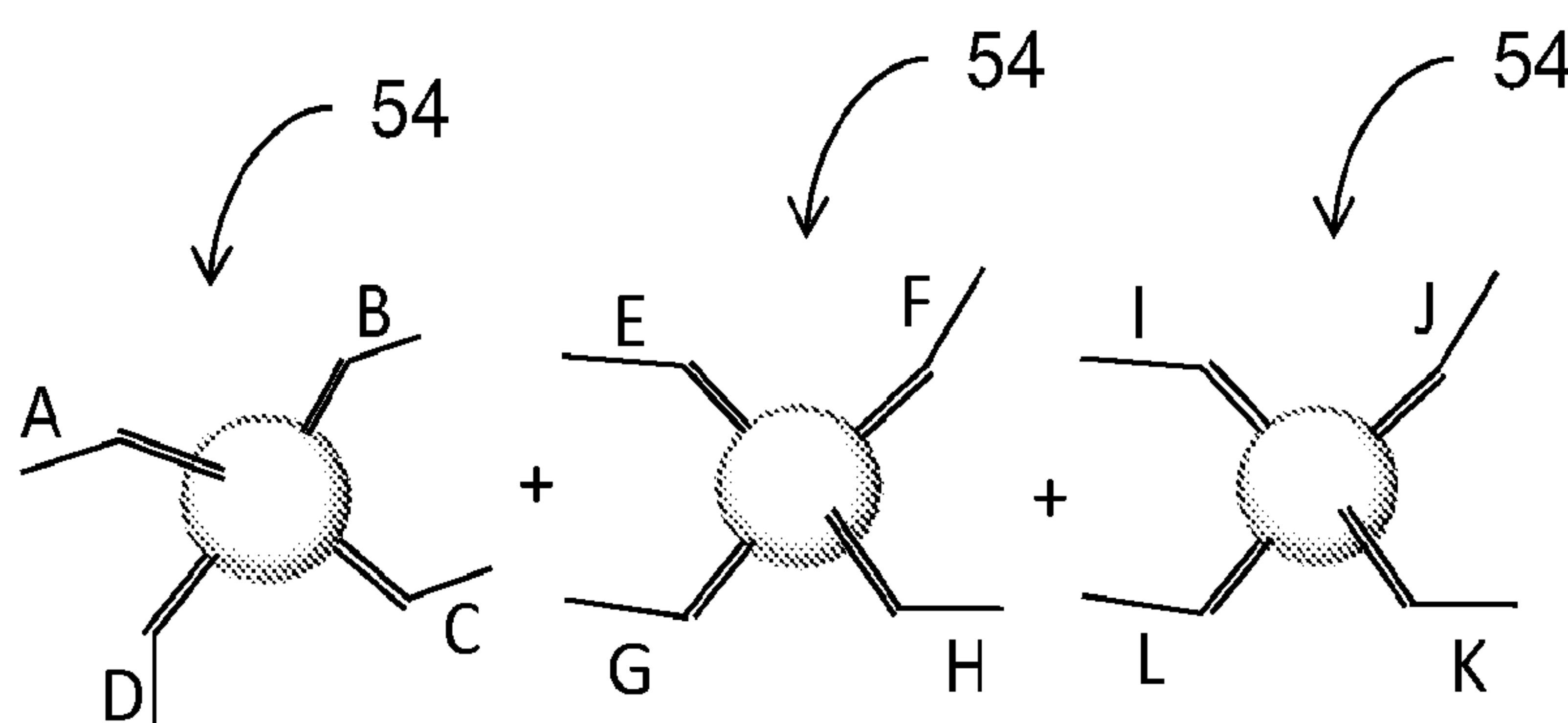


FIG. 8B

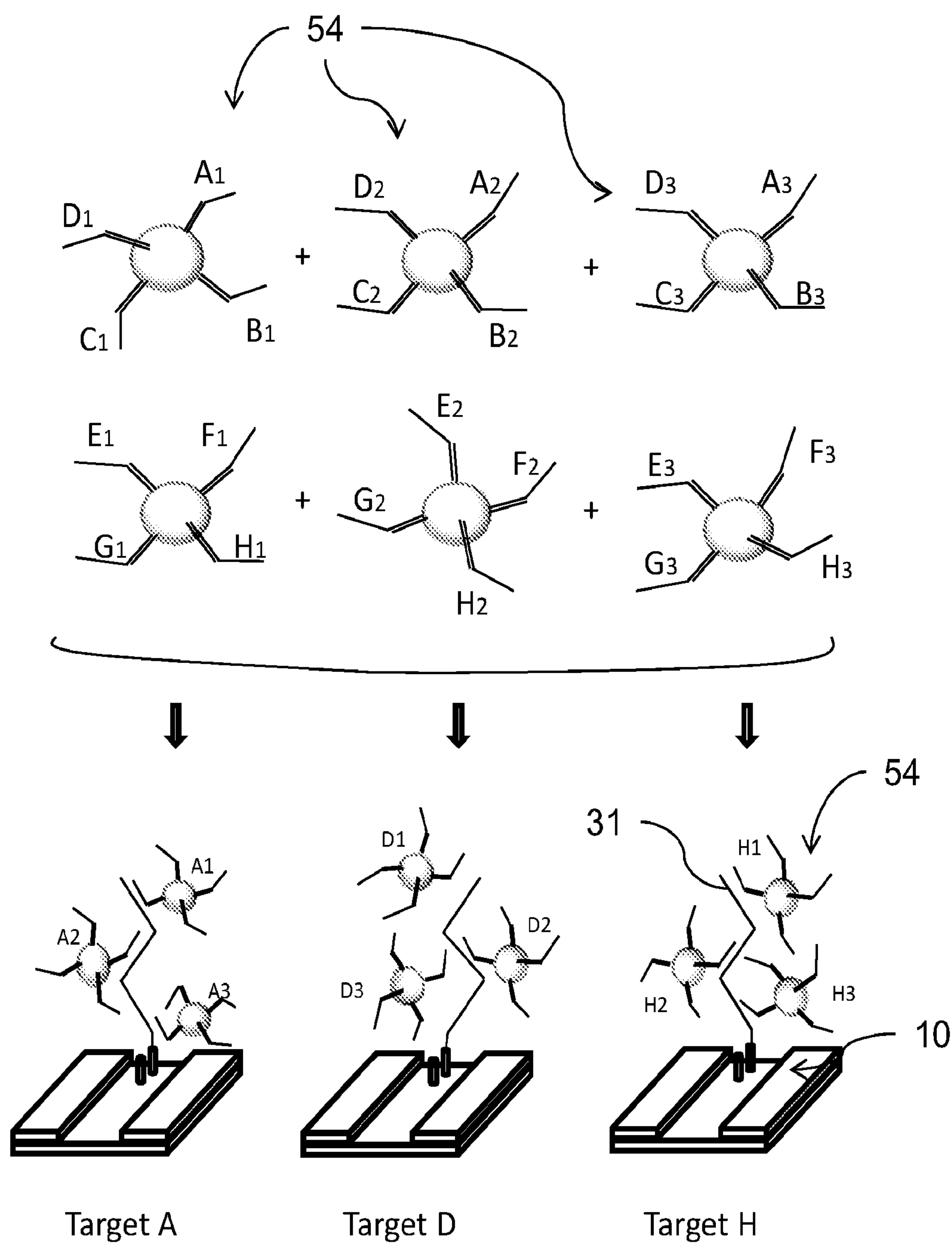
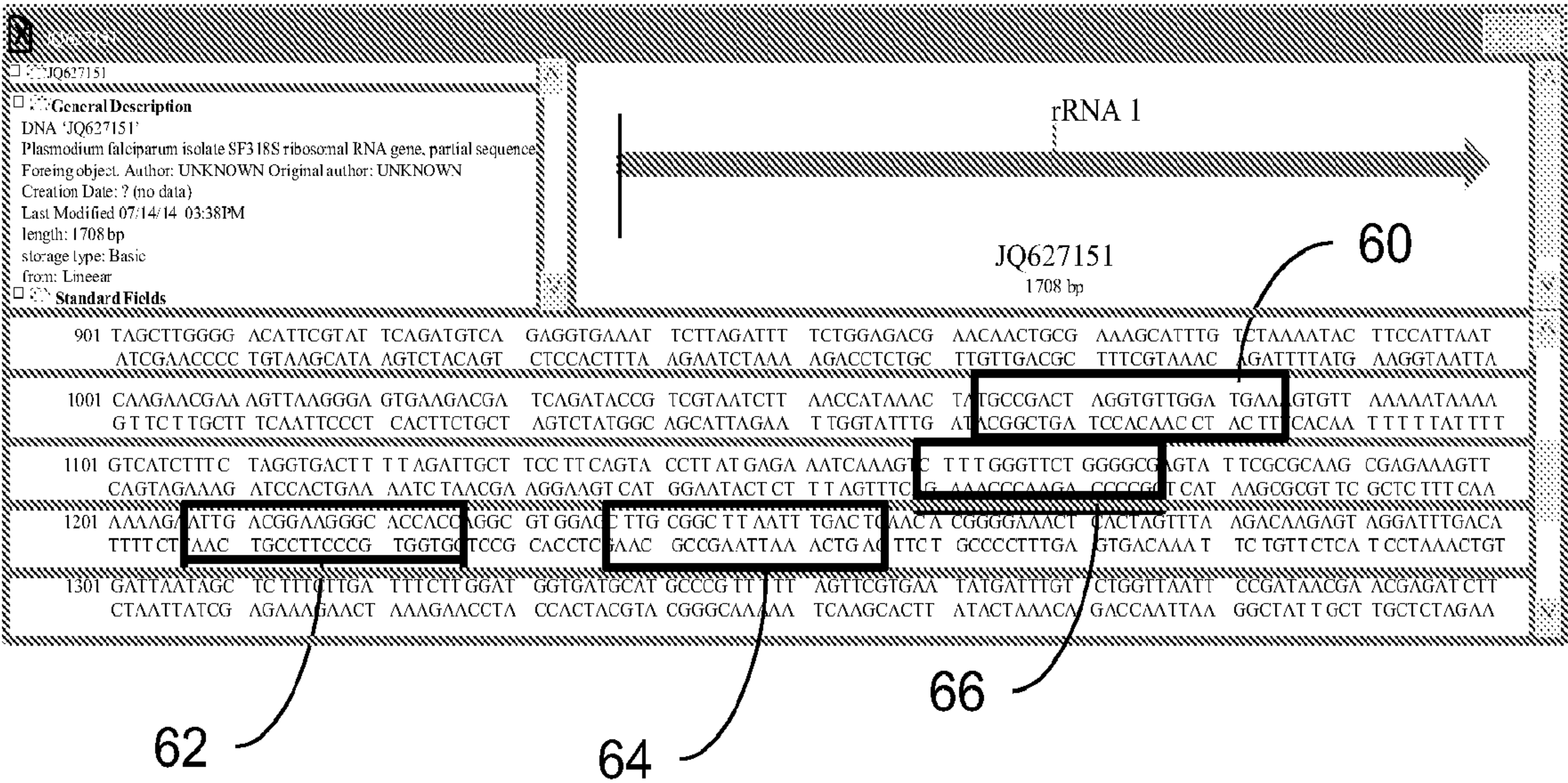


FIG. 8C



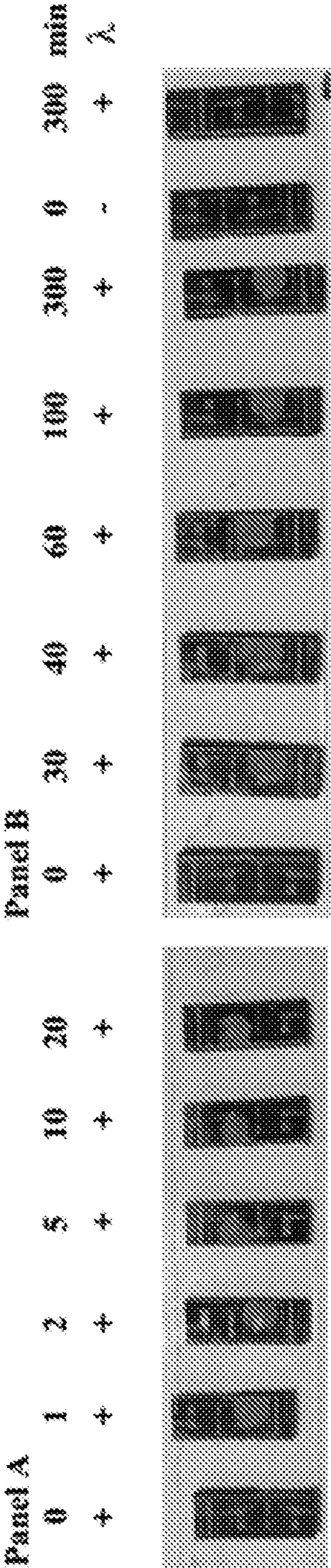


FIG. 10

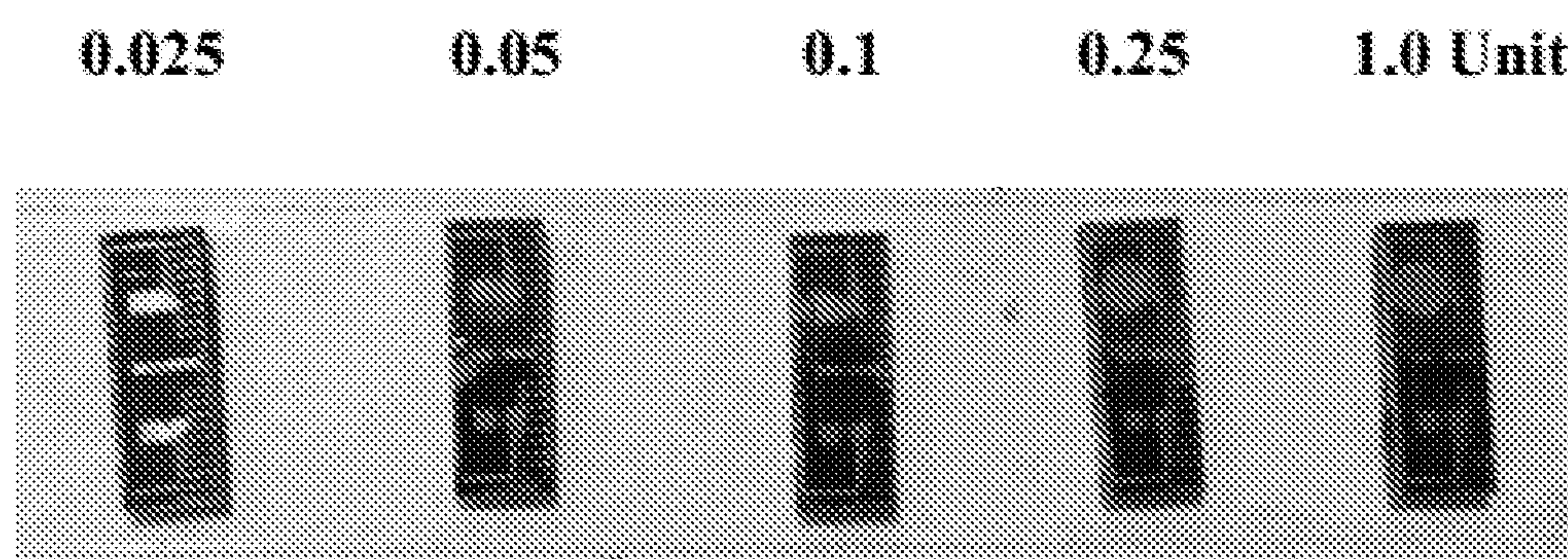


FIG. 11

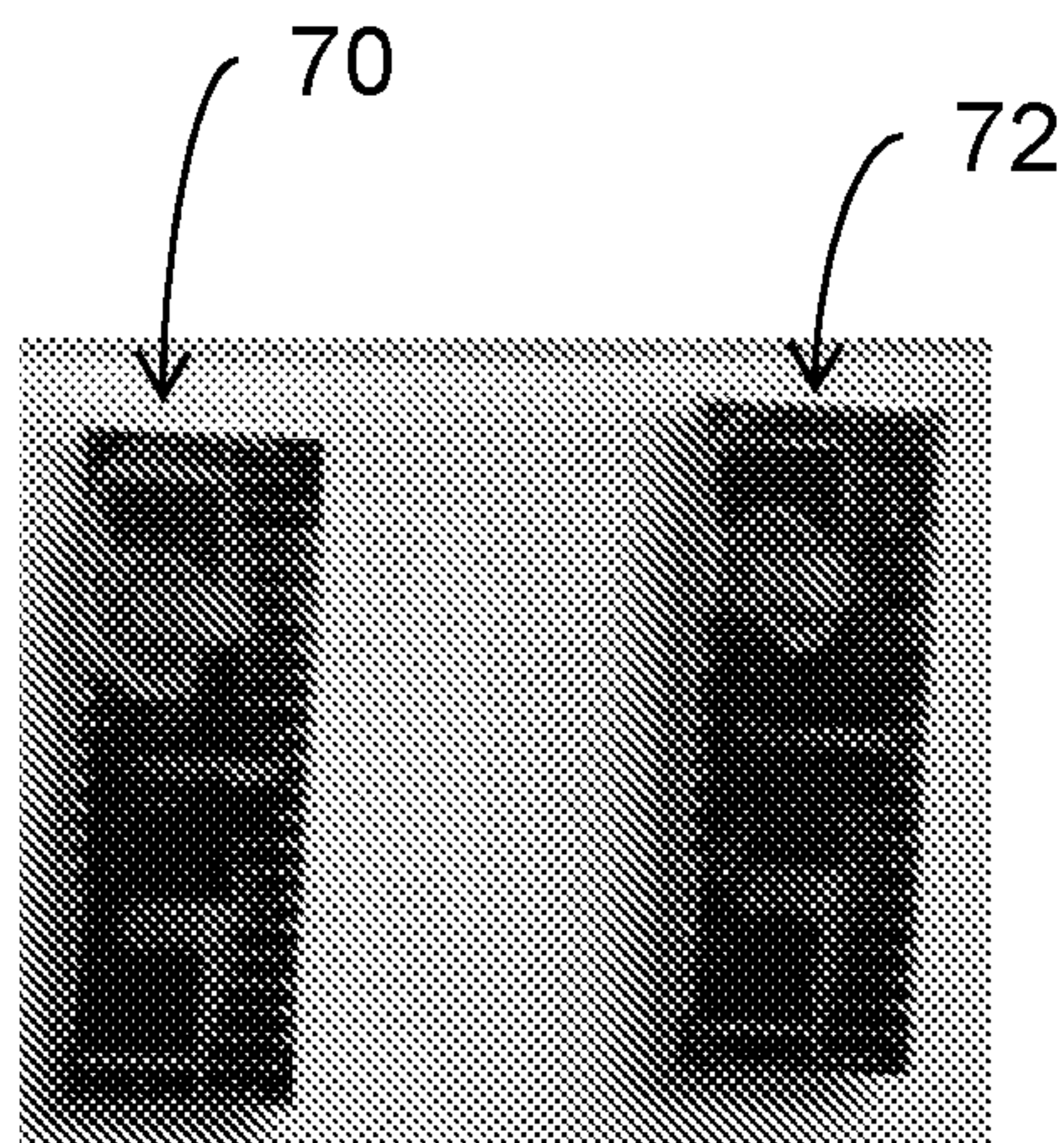


FIG. 12

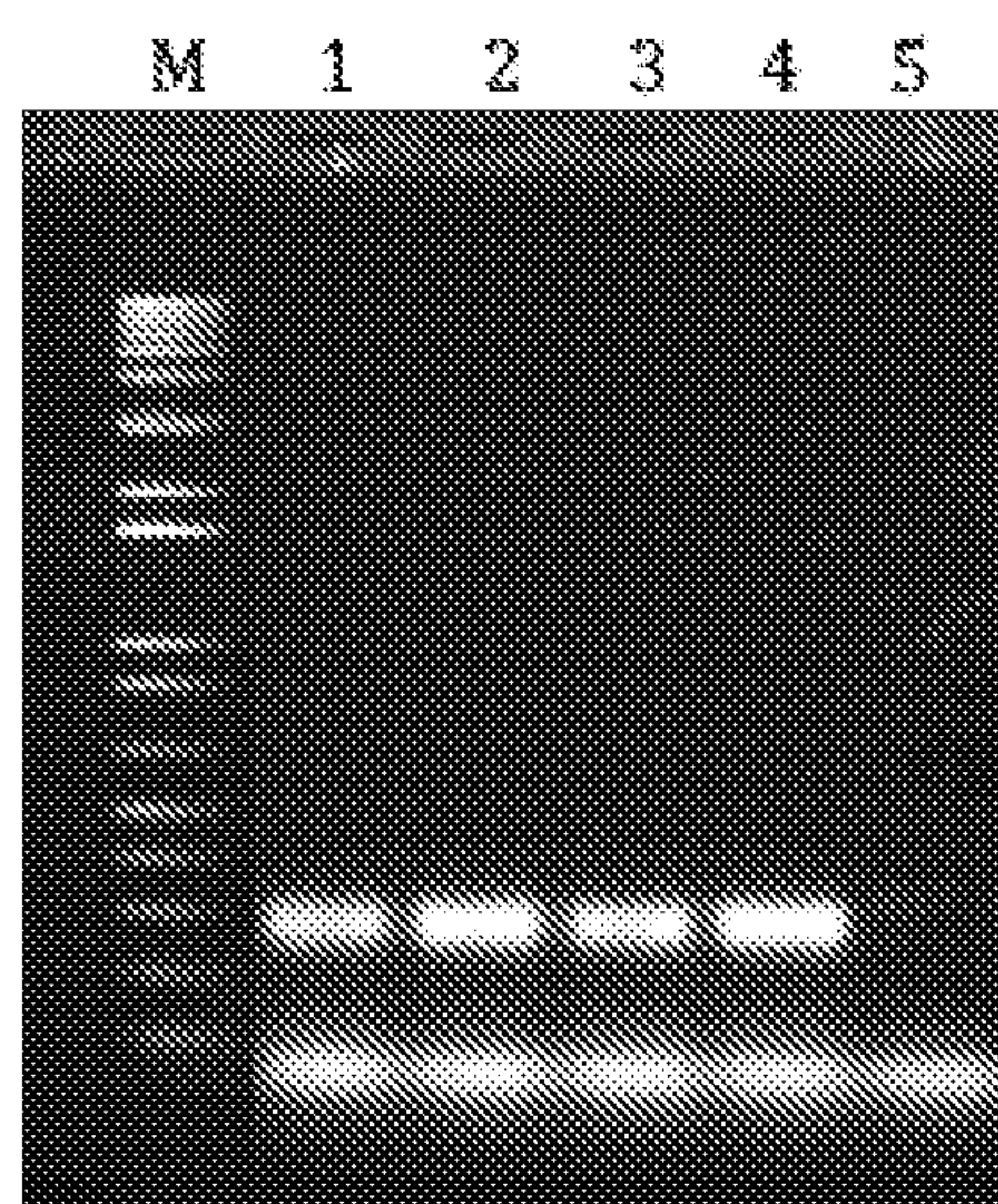


FIG. 13A

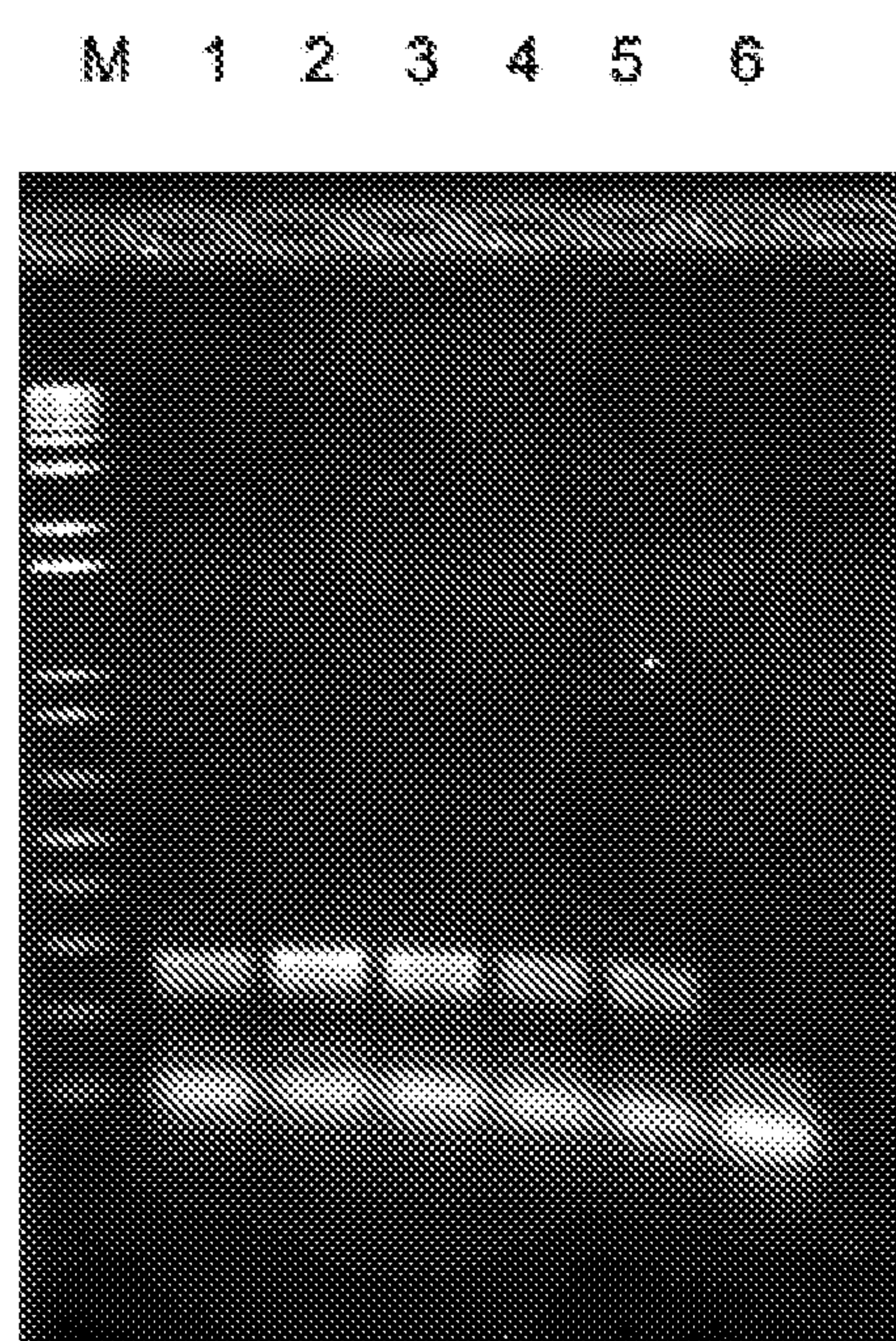


FIG. 13B

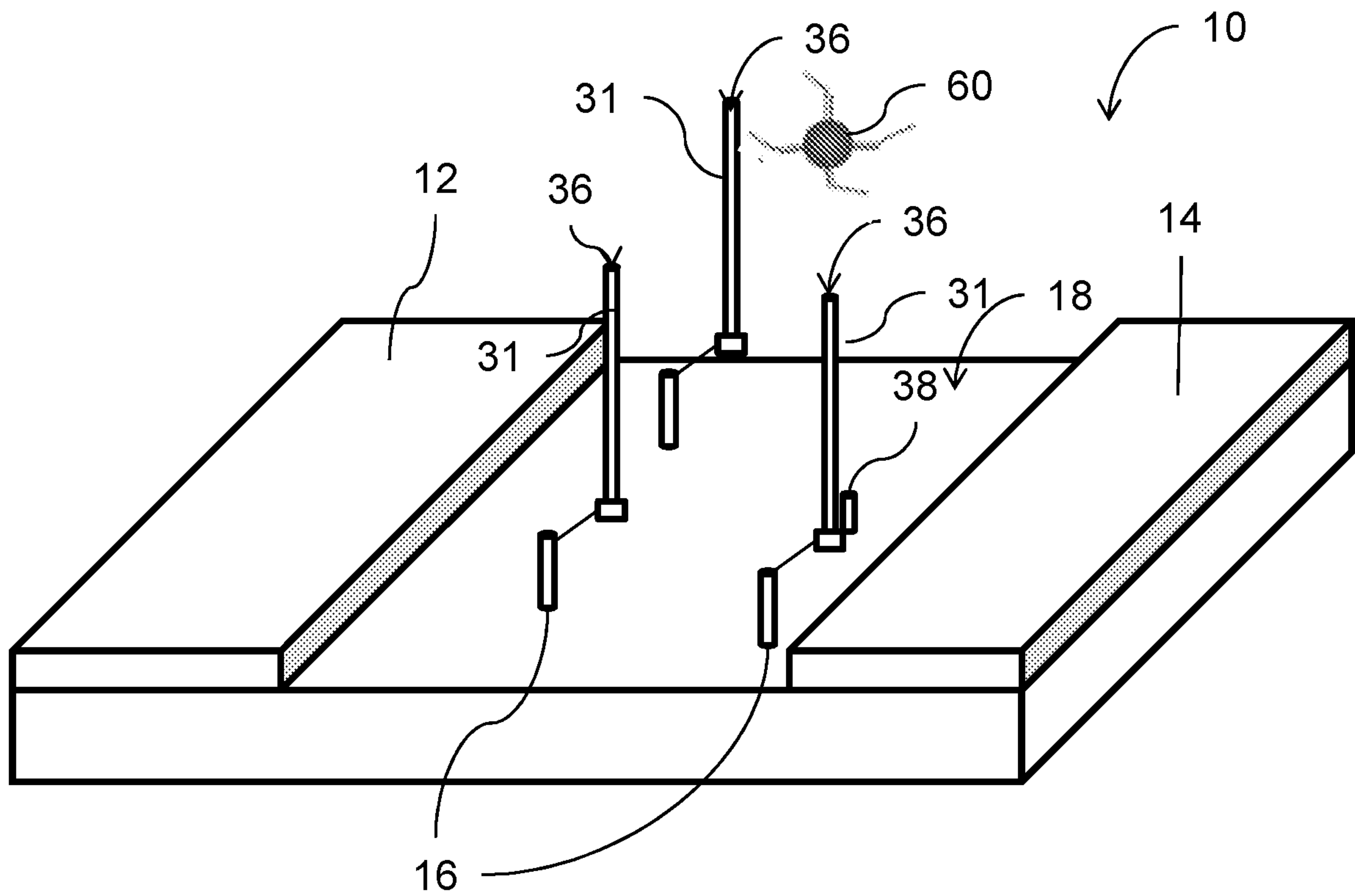


FIG. 6