orientation. It is about the same number as in the art. To use information about RNA orientation the search was performed twice (for reads mapped in the forward direction and then for reads mapped in the reverse direction), yielding in 549 novel gene candidates in forward orientation and 512 - in reverse. The whole genome was split into 50 bp windows (non-sliding). A "new gene" was defined as a joined group of more than 2 consecutive windows, with at least 2 sequence reads (of the same direction) mapped per window. The gap between "new genes" should be at least 50 bp, the gap between "new gene" and an annotated gene (with the same transcription direction as the "new gene") - at least 100 bp.

With the RNA-Seq procedure it is possible to determine the 3'-boundaries of genes using those sequencing reads, which overlap with 3'-borders of genes. These reads may be mapped to the reference genome only after removal of oligo(dT) tail. The inventive method has the advantage of reduced noise in comparison with the RNA-Seq protocol because only one orientation of the homopolymeric stretch is allowed.

Example 2: Transcriptome of the brain of *Mus musculus*

In this example a further application of the method according to the invention is presented. Strand-specific sequencing of the whole mouse brain transcriptome is performed. Subsequently, the sequence of each clone is determined and matched with already existing DNA sequence of the mouse genome. Methods are similar to those used in Example 1. Therefore only steps which differ from Example 1 are indicated.

RNA isolation:

Two eleven weeks old female mice (C57B1/6J) were dissected and the whole brain was taken for RNA preparation. Total RNA was extracted using the Trizol method.

Data analysis:

The resulting sequencing reads were aligned to the *M. musculus* (UCSC mm9) genomes using modification of Eland software (Gerald module v.1.27, Illumina).

Example 3: Comparison of strand-specific and non-strand-specific amplification of polynucleotides

To demonstrate that second-strand synthesis with deoxyuridine does not disturb the transcriptional landscape both strand-specific (according to the inventive method) and non-strand-specific transcriptome (RNA-Seq) sequencing for the same RNA

sample was performed (mouse liver transcriptome). The resulting scatter plot (Fig. 7A) shows that both RNA-Seq and the inventive method produce identical transcription patterns.

Example 4: Reproducibiliity of the inventive method and comparison with previously published results from another laboratory

In order to test the reproducibility of the inventive method independent analyses of whole brain mouse (Fig. 7B) transcriptomes were carried out under identical conditions. A high reproducibility can be seen in the respective scatter plots. A comparison of the data with previously published results also demonstrates good correlation (Fig. 7C).

Example 5: Stability of the inventive method

The inventive method results in a high degree of certainty in identifying transcript polarity. In theory, one uridine base in a molecule of a sequencing library is enough to prevent the appearance of a sequencing read from the false strand. Even if UNG occasionally does not remove the uridine base, the molecule still would not be amplified, since uridine-containing template strongly suppresses Phusion DNA polymerase used for library amplification. Apparently, most second cDNA strands in the library contain more than one uridine base, because a 75%/25% dUTP/dTTP mixture in the second strand synthesis reaction gives essentially the same results as 100% dUTP (Fig. 8A and 8B).

Example 6: Influence of actinomycin D

To prevent spurious second-strand cDNA synthesis, which was shown to be the major source of artifactual antisense transcripts. Antisense artifacts in transcriptome microarray experiments are resolved by actinomycin D. Actinomycin D was included in the reverse transcription reaction. Actinomycin D specifically inhibits DNA-dependent, but not RNA-dependent, DNA synthesis. The presence of actinomycin D in the reaction practically did not influence the level of antisense transcription (Fig. 9A and 9B). Actinomycin D is included in the protocol to make the reaction more reproducible.

Example 7: Transcriptome of the liver of *Mus musculus*

In this example a further application of the method according to the invention is presented. Strand-specific sequencing of the whole mouse liver transcriptome is performed. Subsequently, the sequence of each clone is determined and matched with already existing DNA sequence of the mouse genome. Methods are similar to those used in Example 1. Therefore only steps which differ from Example 1 are indicated.

ds cDNA synthesis:

dTTP was substituted by dUTP during first strand cDNA synthesis. For the second strand cDNA synthesis, the mixture of standard dNTPs was used.

Data analysis:

The resulting sequencing reads were aligned to the *M. musculus* (UCSC mm9) genomes using modification of Eland software (Gerald module v.1.27, Illumina).

Example 8: Transcriptome of the human samples from blood and tumor (melanoma)

In this example a further application of the method according to the invention is presented. Strand-specific sequencing of the human transcriptome is performed. Subsequently, the sequence of each clone is determined and matched with already existing DNA sequence of the human genome (hg18, NCBI build 36.1). Methods are similar to those used in Example 1. Therefore only steps which differ from Example 1 are indicated.

ds cDNA preparation:

Poly A RNA was chemically fragmented prior to ds cDNA synthesis. Compared to ultrasound shearing after ds cDNA synthesis this approach resulted in a more even distribution of reads along the transcript (no 3' bias).

Example 9: Transcriptome of E.coli

In this example a further application of the method according to the invention is presented. Strand-specific sequencing of the E.coli transcriptome is performed. Subsequently, the sequence of each clone is determined and matched with already existing DNA sequence of the E.coli (K12 reference genome (NC_000913).

Methods are similar to those used in Example 1. Therefore only steps which differ from Example 1 are indicated.

ds cDNA preparation:
Instead of dUTP, biotinylated dTTP was used for the second strand cDNA synthesis.

Sequencing library preparation.
After ligation of polarity-specific Y-shaped adapters, biotinylated strand was separated from non-biotinylated using streptavidine coated magnetic beads. Biotinylated strand was further used for amplification.

Data analysis:
The resulting sequencing reads were aligned to K12 reference genome (NC_000913) using the Eland program (Gerald module v.1.27 of the Illumina pipeline)).

Example 10:Preparation of single-stranded hybridisation target

The task was to prepare an array with spotted single-stranded molecules, corresponding to different genomic regions (300-500bp long). First, selected regions were amplified using genomic DNA as a template and PCR primers corresponding to the genomic DNA, where one primer in each pair was selected so, that it had at least one T within last 3 nucleotides on the 3' end of the primer. This dTTP was substituted by dUTP during the primer synthesis. Usage of such uridine-containing primer in PCR resulted in obtaining PCR product with an uridine-marked strand.
PCR products were purified. After UNG treatment and APE 1 digestion a nick appeared in the uridine-containing strand in the place of uridine. From that nick, a nick-translation reaction was performed using dNTP mix with dUTP instead of dTTP and DNA polymerase I in a corresponding buffer. Nick-translation resulted in substitution of one of the strands in a PCR product to a strand containing multiple UTPs along its whole length (Fig. 3A). After UNG treatment and heating, the uridine strand was destroyed. Resulting small fragments were washed off by purification on a silica column. Single stranded DNA molecules were spotted on Nexterion slides (Schott) according to the manufacturers instructions.

Example 11:Preparation of single-stranded hybridisation target

The task and methods were similar to those used in Example 10. The difference was that biotin-associated dTTP were used instead of dUTP in the nick-translation reaction. Biotinylated strand was further separated from non-biotinylated by immobilisation on streptavidine-coated magnetic beads. The non-biotinylated strand was further used for hybridization

Claims

1. A method for selection of a particular strand from a double stranded polynucleotide comprising the following steps:
   I) Providing polynucleotide templates, primers, buffer solution, at least one enzyme with polymerase activity, sufficient amounts of the four dNTPs dATP, dCTP, dGTP and dTTP or analogues thereof, wherein at least one of the four dNTPs is replaced by a corresponding modified dNTP used for strand-specific selection;
   II) Performing first-strand synthesis on the polynucleotide templates with unmodified dNTPs or corresponding analogues thereof;
   III) Performing second-strand synthesis wherein at least one of the four dNTPs is replaced by a corresponding modified dNTP used for strand-specific selection, so that double-stranded polynucleotides each consisting of a second strand and a matching strand are generated, wherein the secondstrand contains the at least one modified nucleotide;
   IV) Optionally, performing molecular cloning operations with double-stranded DNA; and
   V) Specific selection of at least one of the strands of the said polynucleotide duplex using different interaction of modified and unmodified nucleotides with at least one selection factor.

2. A method for selection of a particular strand from a double stranded polynucleotide comprising the following steps:
   I) Providing polynucleotide templates, primers, buffer solution, at least one enzyme with polymerase activity, sufficient amounts of the four dNTPs dATP, dCTP, dGTP and dTTP or analogues thereof, wherein at least one of the four dNTPs is replaced by a corresponding modified dNTP used for strand-specific selection;
   II) Performing first-strand synthesis on the polynucleotide templates, so that double-stranded polynucleotides each consisting of a template and a matching strand are generated, wherein the matching strand contains the at least one modified nucleotide;
   III) If said polynucleotide templates on step I) are single-stranded RNA, second-strand synthesis with unmodified dNTPs or corresponding analogues thereof is performed;
   IV) Optionally, performing molecular cloning operations with double-stranded DNA; and

V) Specific selection of at least one of the strands of the said polynucleotide duplex using different interaction of modified and unmodified nucleotides with at least one selection factor.

3. The method for selection of a particular strand from a double stranded polynucleotide according to claim 1 or 2, wherein said polynucleotide templates are selected from DNA/DNA hybrid with distinguishable adapters, single-stranded RNA, single-stranded DNA and DNA/RNA hybrid.

4. A method for selection of a particular strand from a double stranded polynucleotide according to claim 3 comprising the following steps:

I) Providing polynucleotide templates, primers, buffer solution, at least one enzyme with polymerase activity, sufficient amounts of the four dNTPs dATP, dCTP, dGTP and dTTP or analogues thereof, wherein at least one of the four dNTPs is replaced by a corresponding modified dNTP used for strand-specific selection;

If said polynucleotide templates are single-stranded DNA

IIa) Performing first-strand synthesis by primer extension reaction from either random or sequence-specific primer, where at least one of dNTP´s is a modified nucleotide further used for selection of a specific strand;

or if said polynucleotide templates are single-stranded RNA,

IIb) Performing first-strand synthesis by reverse transcription reaction using either random or oligo(dT)/oligo(dU) or sequence specific primer, where at least one of dNTPs is a modified nucleotide further used for selection of a specific strand; and

III) then performing second-strand synthesis with all four unmodified dNTPs or analogues thereof

or if said polynucleotide template are DNA/DNA hybrid with distinguishable adapters,

IIc) Performing first-strand synthesis by nick-translation or strand displacement reactions initiated either by setting a nick in one of the adapters or annealing a new primer to the adapter region where at least one of dNTPs is a modified nucleotide further used for selection of a specific strand;

or if said polynucleotide template are DNA/RNA hybrid,

IId) Performing first-strand synthesis with either the DNA or the RNA as template, where at least one of dNTPs is a modified nucleotide further used for selection of a specific strand;

IV) Optionally, performing one or more molecular cloning operations on the double-stranded DNA, wherein the molecular cloning operations are selected from shearing, restriction, digestion, end-polishing, A-tailing, G-tailing and adaptor ligation; and

V) Performing strand-specific selection by using a selection factor for selecting strands containing the at least one modified nucleotide from strands containing the corresponding unmodified nucleotide.

5. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein said selection factor for selecting strands is at least one chemical agent modifying selectively the at least one modified nucleotide or one or more chemical groups of the at least one modified nucleotide.

6. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein said selection factor for selecting strands is at least one factor displaying selective affinity to an affinity group of the at least one modified nucleotide.

7. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein said selection factor for selecting strands is one or more antibodies binding selectively to the at least one modified nucleotide.

8. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein said selection factor for selecting strands is a solid phase at which the strand containing the at least one modified nucleotide is immobilized.

9. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein said selection factor for selecting strands is a different sensitivity to nucleases of the modified and the unmodified nucleotide.

10. The method for selection of a particular strand from a double stranded polynucleotide according to claim 9, wherein said nuclease is Uracil-N-Glycosylase and said modified nucleotide is dUTP.

11. The method for selection of a particular strand from a double stranded polynucleotide according to claim 6, wherein said factor displaying selective affinity to an affinity group of the at least one modified nucleotide is streptavidin or avidin and the at least one modified nucleotide is a biotinylated nucleotide.

12. The method for selection of a particular strand from a double stranded polynucleotide according to claim 5, wherein the at least one modified nucleotide with said chemical group is Aminoallyl-dUTP.

13. The method for selection of a particular strand from a double stranded polynucleotide according to claim 7, wherein the at least one modified nucleotide recognized selectively by an antibody is 5-bromodeoxyuridine.

14. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein the ratio of the at least one modified dNTP towards the corresponding unmodified dNTP or analogues thereof is at least 1:20.

15. The method for selection of a particular strand from a double stranded polynucleotide according to any of claims 1 - 4, wherein if the polynucleotide strand is a single RNA strand the RNA is selected from mRNA, tRNA, rRNA, hnRNA, snRNA, snoRNA, scaRNA, asRNA, micro RNA, siRNA, short transcripts, synthetic RNA polynucleotides and semisynthetic RNA polynucleotides as well as physiological or externally induced modifications thereof, or if the polynucleotide strand is a DNA the DNA is selected from siDNA, single-stranded DNA copies from retroviral or hepadnaviral RNA and single-stranded DNA, single-stranded synthetic DNA polynucleotides and single-stranded semisynthetic DNA polynucleotides as well as physiological or externally induced modifications thereof.

16. A kit for ds cDNA synthesis from RNA preserving information about direction of original RNA strand comprising:

A) All four dNTPs dATP, dCTP, dGTP and dTTP and at least one modified nucleotide corresponding to at least one from dATP, ATP, dCTP, CTP, dGTP, GTP, dTTP, TTP, dUTP and UTP;

B) Primers;

C) One or more DNA polymerases with reverse transcriptase activity;

D) One or more DNA polymerases; and

E) One or more selection factors suitable to specifically interact with said at least one modified nucleotide and/or one or more selective media for removing the strand containing the modified nucleotide;

Or additionally comprising

F) Components for specific removal of at least one modified or at least one unmodified nucleotide by gel-filtration or purification on silica-column.

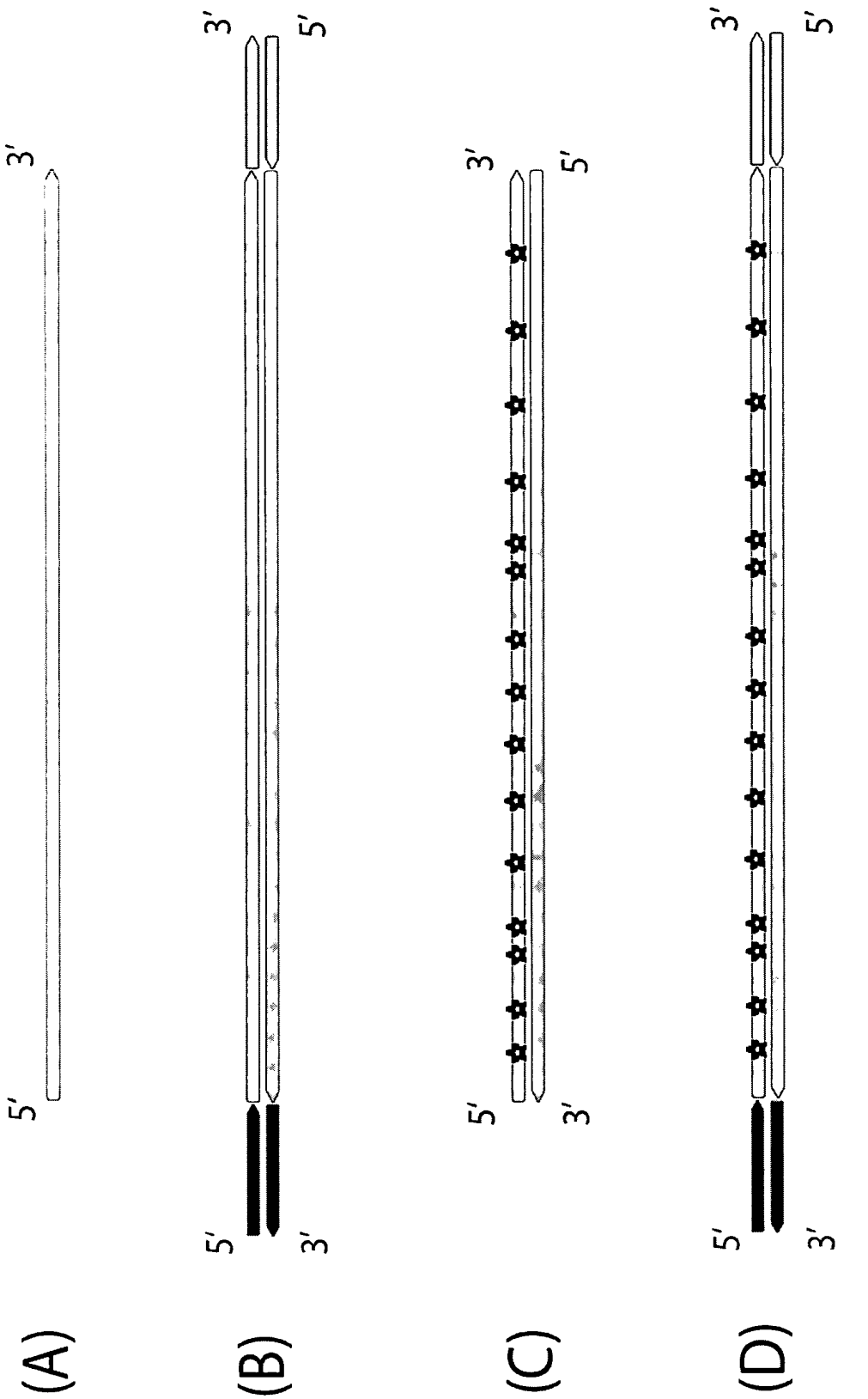


Figure 1

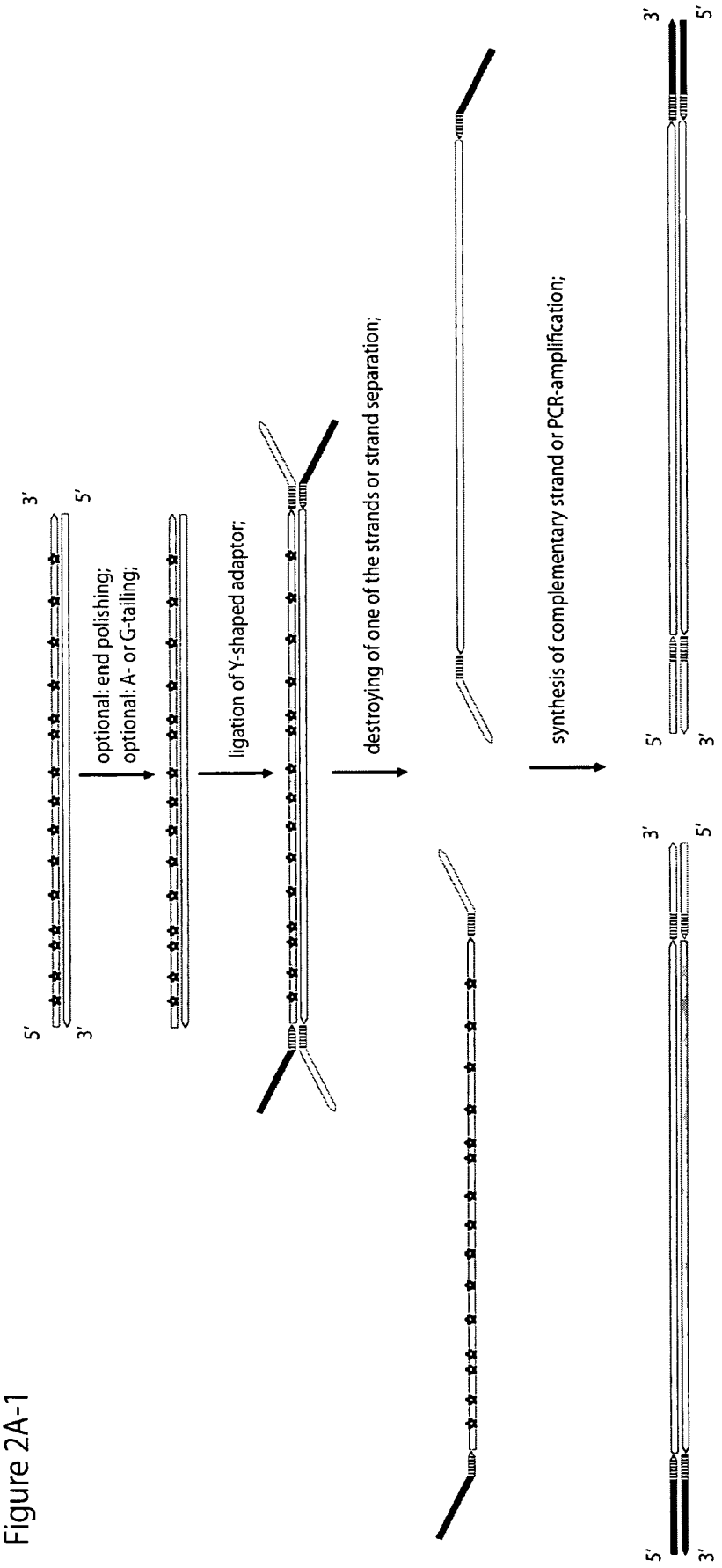

5'
3'
optional: end polishing;
optional: A- or G-tailing;
ligation of Y-shaped adaptor;
destroying of one of the strands or strand separation;
synthesis of complementary strand or PCR-amplification;
3'
5'
5'
3'
3'
5'
5'
3'

Figure 2A-1

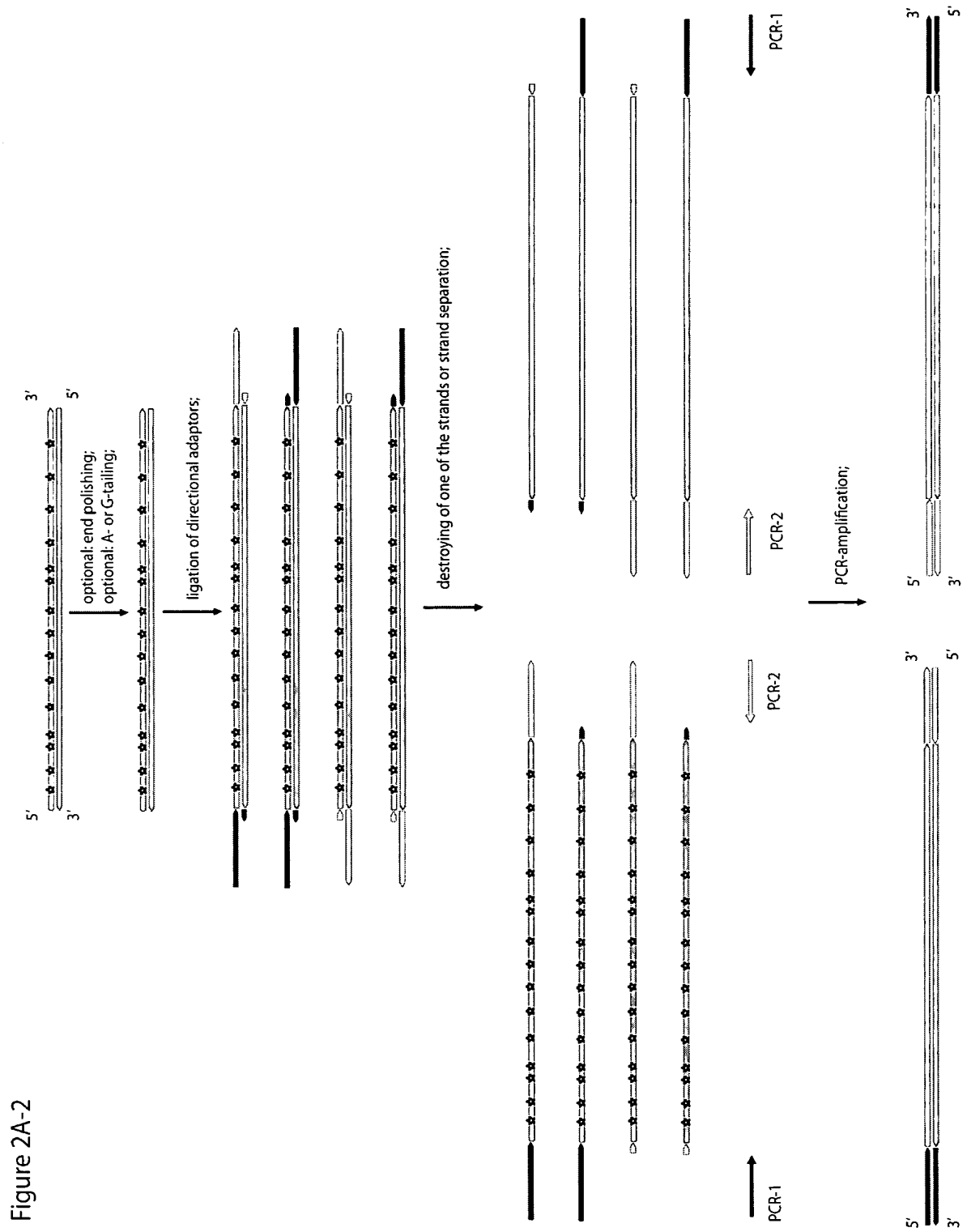

5'
3'
optional: end polishing;
optional: A- or G-tailing;
ligation of directional adaptors;
destroying of one of the strands or strand separation;
PCR-1
PCR-2
PCR-2
PCR-1
PCR-amplification;
3'
5'
5'
3'
3'
5'
5'
3'

Figure 2A-2

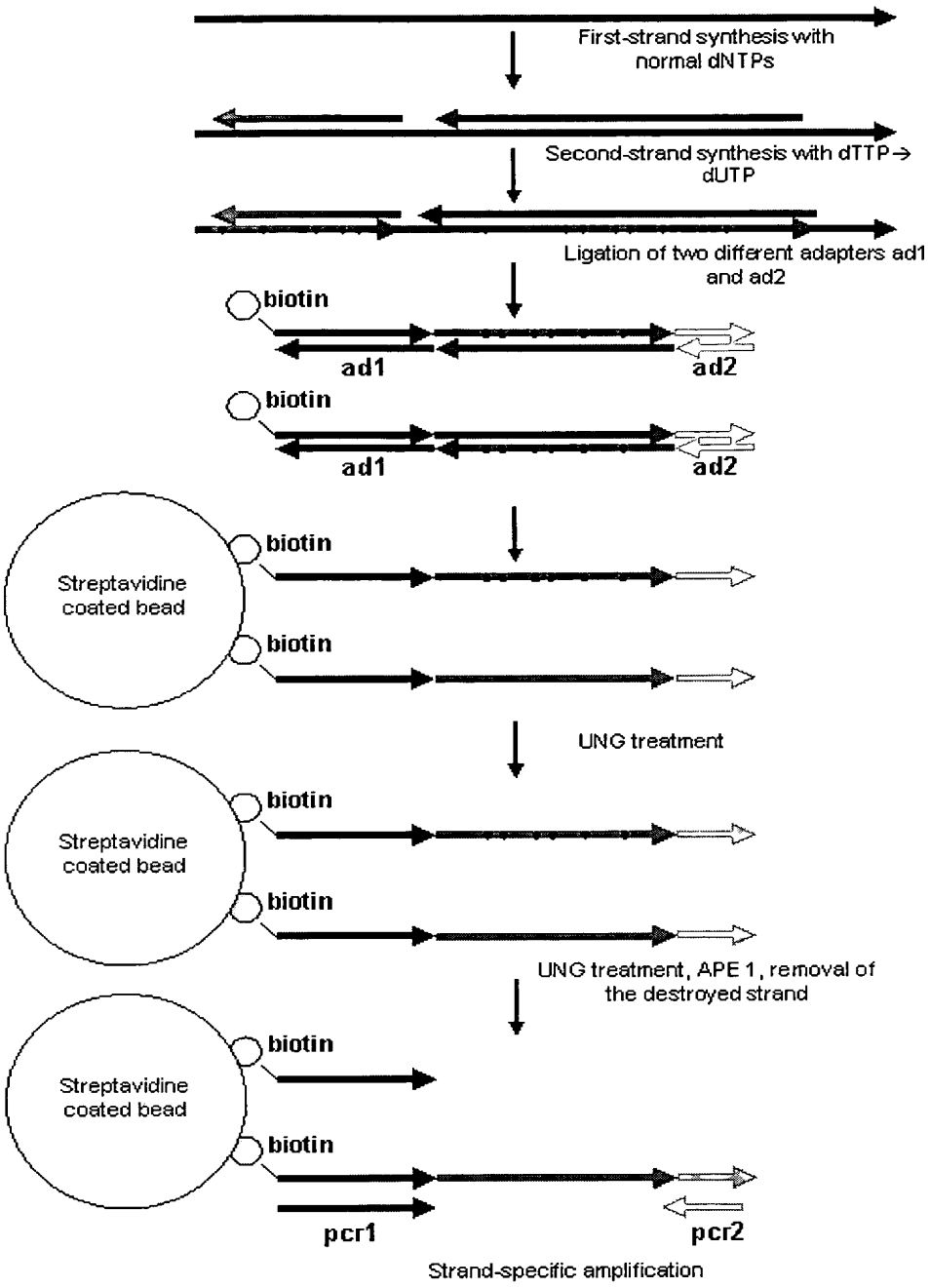

First-strand synthesis with normal dNTPs
Second-strand synthesis with dTTP→ dUTP
Ligation of two different adapters ad1 and ad2
biotin
ad1
ad2
biotin
ad1
ad2
Streptavidine coated bead
biotin
biotin
UNG treatment
Streptavidine coated bead
biotin
biotin
UNG treatment, APE 1, removal of the destroyed strand
Streptavidine coated bead
biotin
biotin
pcr1
pcr2
Strand-specific amplification

Figure 2A-3

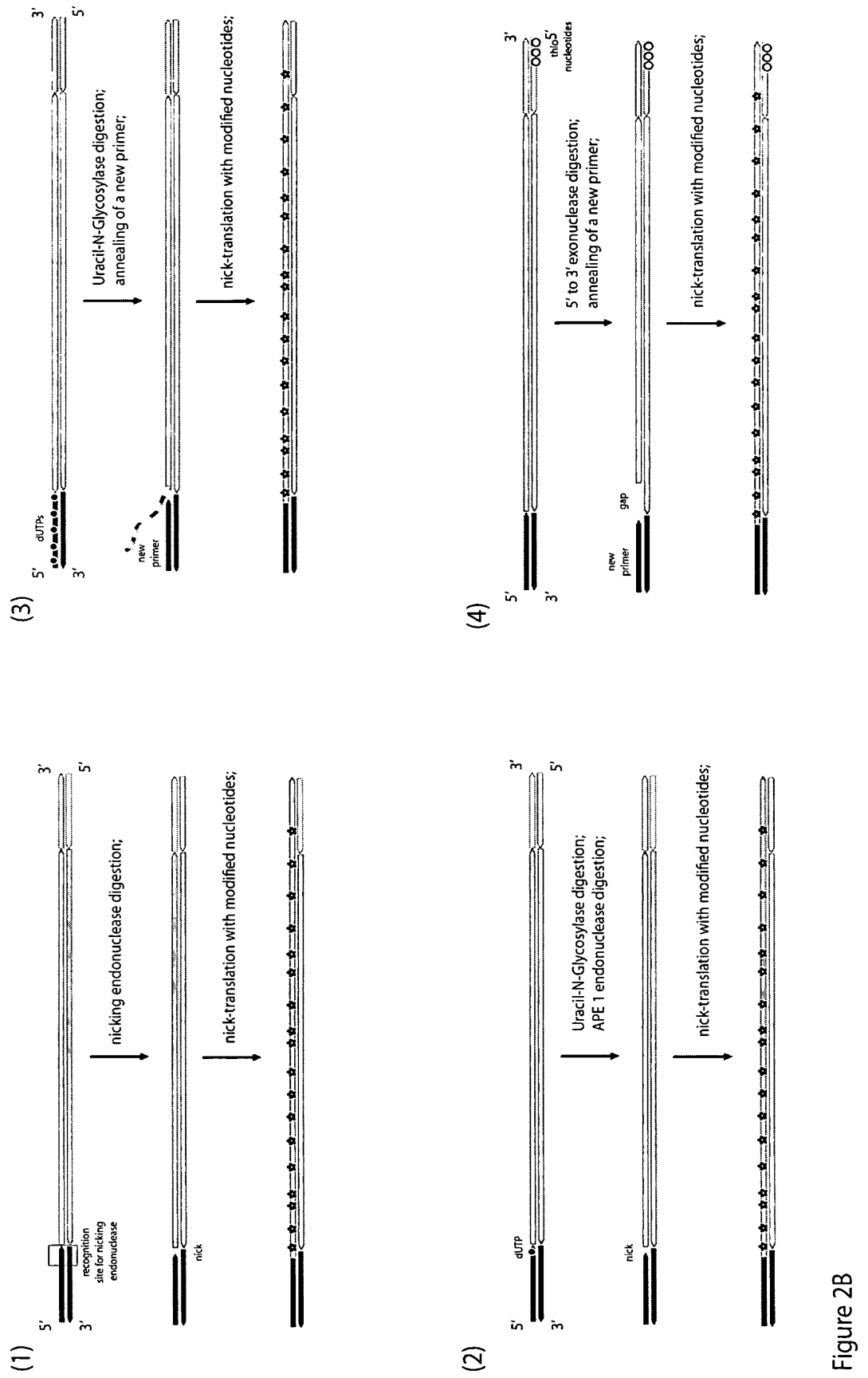

(1)
5'
3'
3'
5'
recognition site for nicking endonuclease
nicking endonuclease digestion;
nick
nick-translation with modified nucleotides;
(2)
5'
3'
dUTP
3'
5'
Uracil-N-Glycosylase digestion;
APE 1 endonuclease digestion;
nick
nick-translation with modified nucleotides;
(3)
5'
dUTPs
3'
3'
5'
Uracil-N-Glycosylase digestion;
annealing of a new primer;
new primer
nick-translation with modified nucleotides;
(4)
5'
3'
thio
5'
nucleotides
5' to 3' exonuclease digestion;
annealing of a new primer;
new primer
gap
nick-translation with modified nucleotides;

Figure 2B

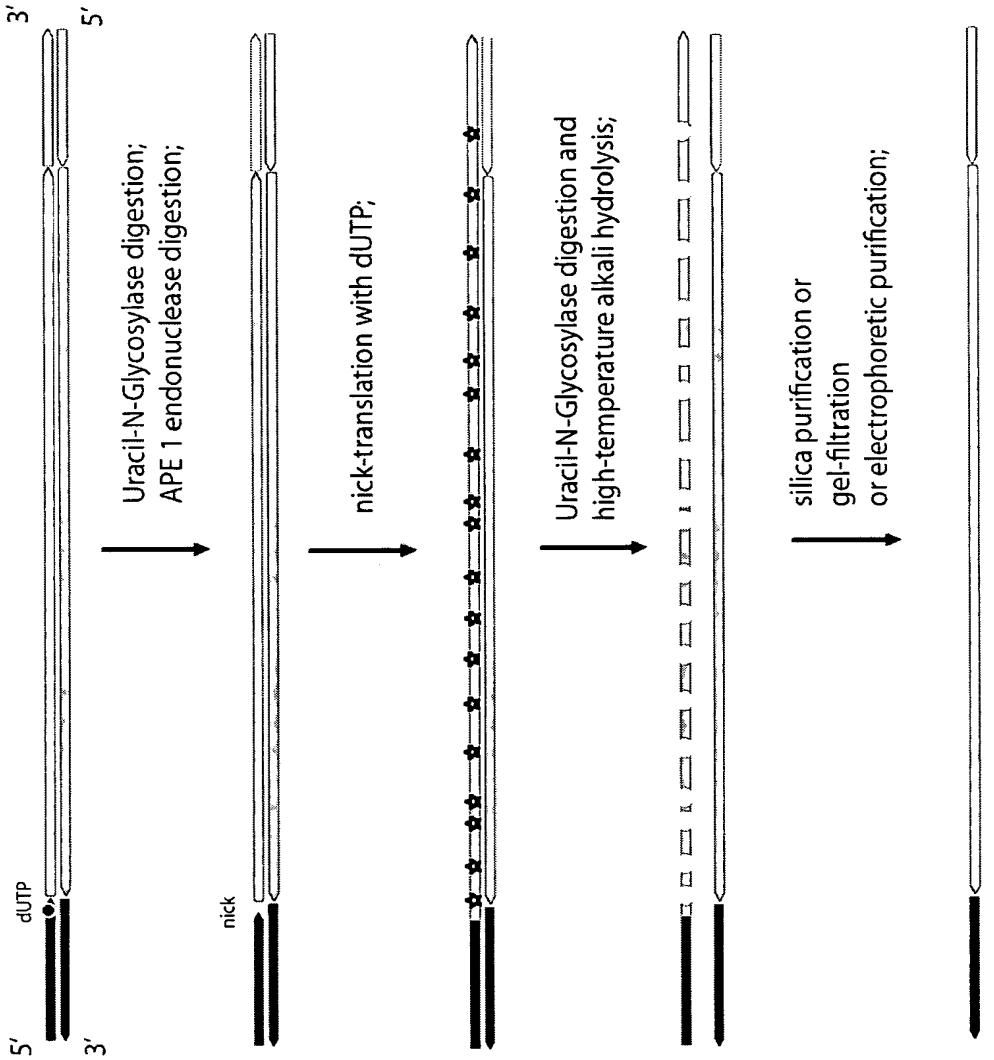

3′
5′
dUTP
5′
3′
Uracil-N-Glycosylase digestion;
APE 1 endonuclease digestion;
nick
nick-translation with dUTP;
Uracil-N-Glycosylase digestion and
high-temperature alkali hydrolysis;
silica purification or
gel-filtration
or electrophoretic purification;

Figure 3A

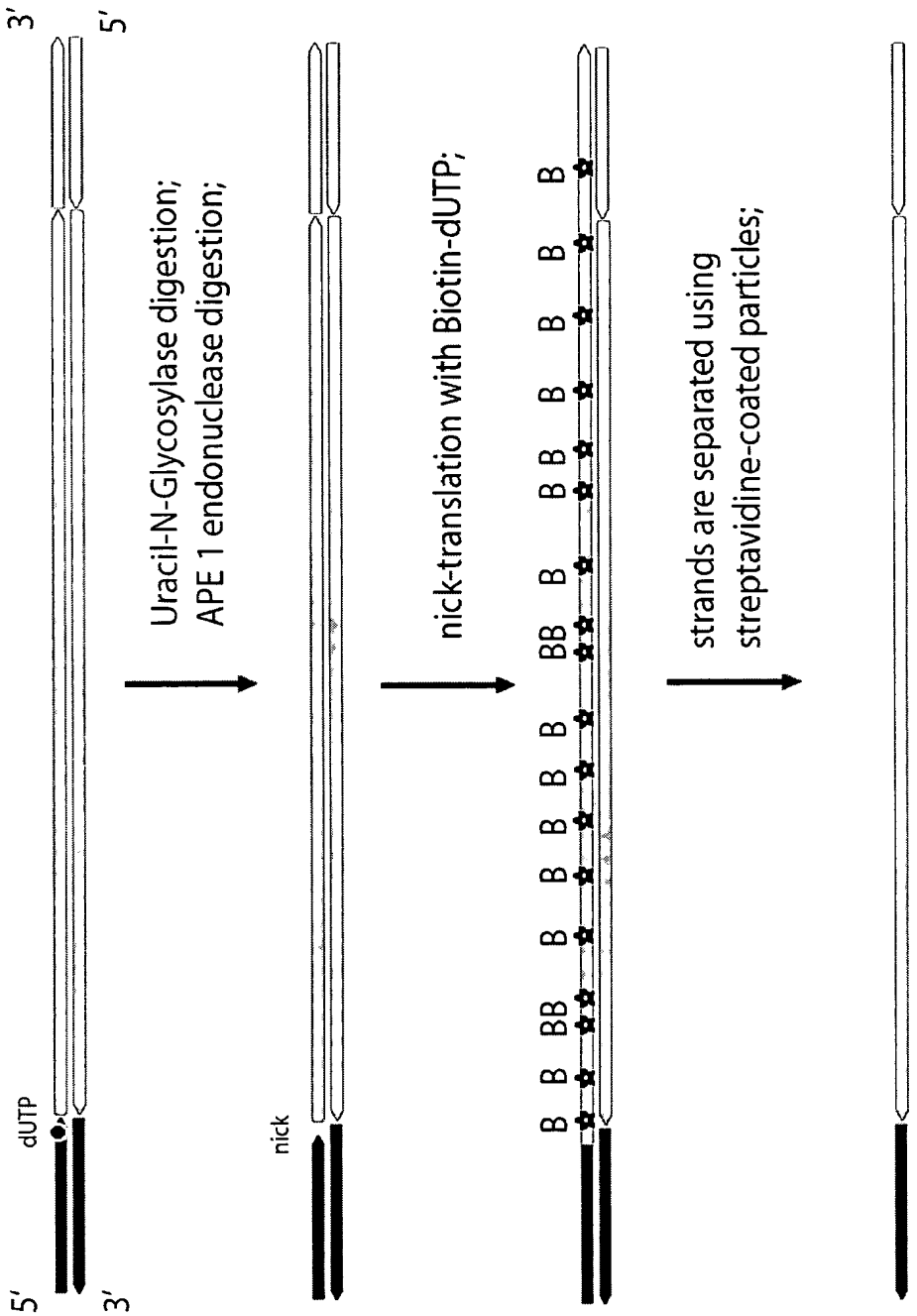

3′
5′
dUTP
5′
3′
Uracil-N-Glycosylase digestion;
APE 1 endonuclease digestion;
nick
nick-translation with Biotin-dUTP;
B B BB B B B B B BB B B B B B B
strands are separated using
streptavidine-coated particles;

Figure 3B

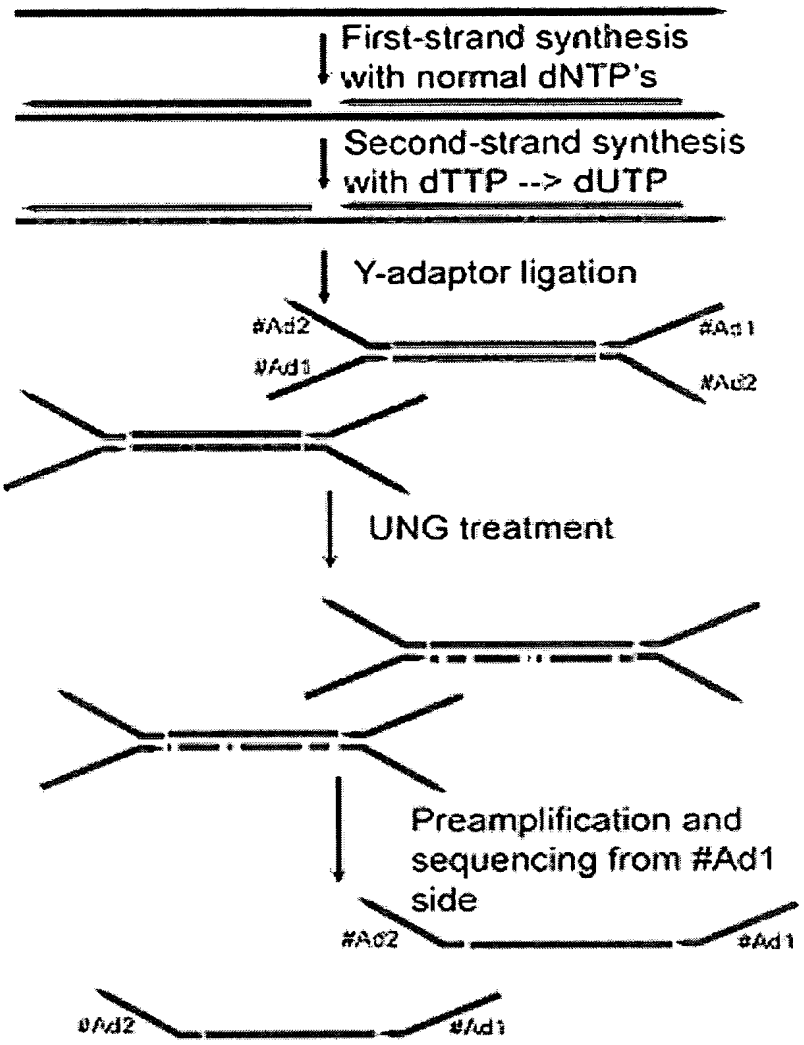
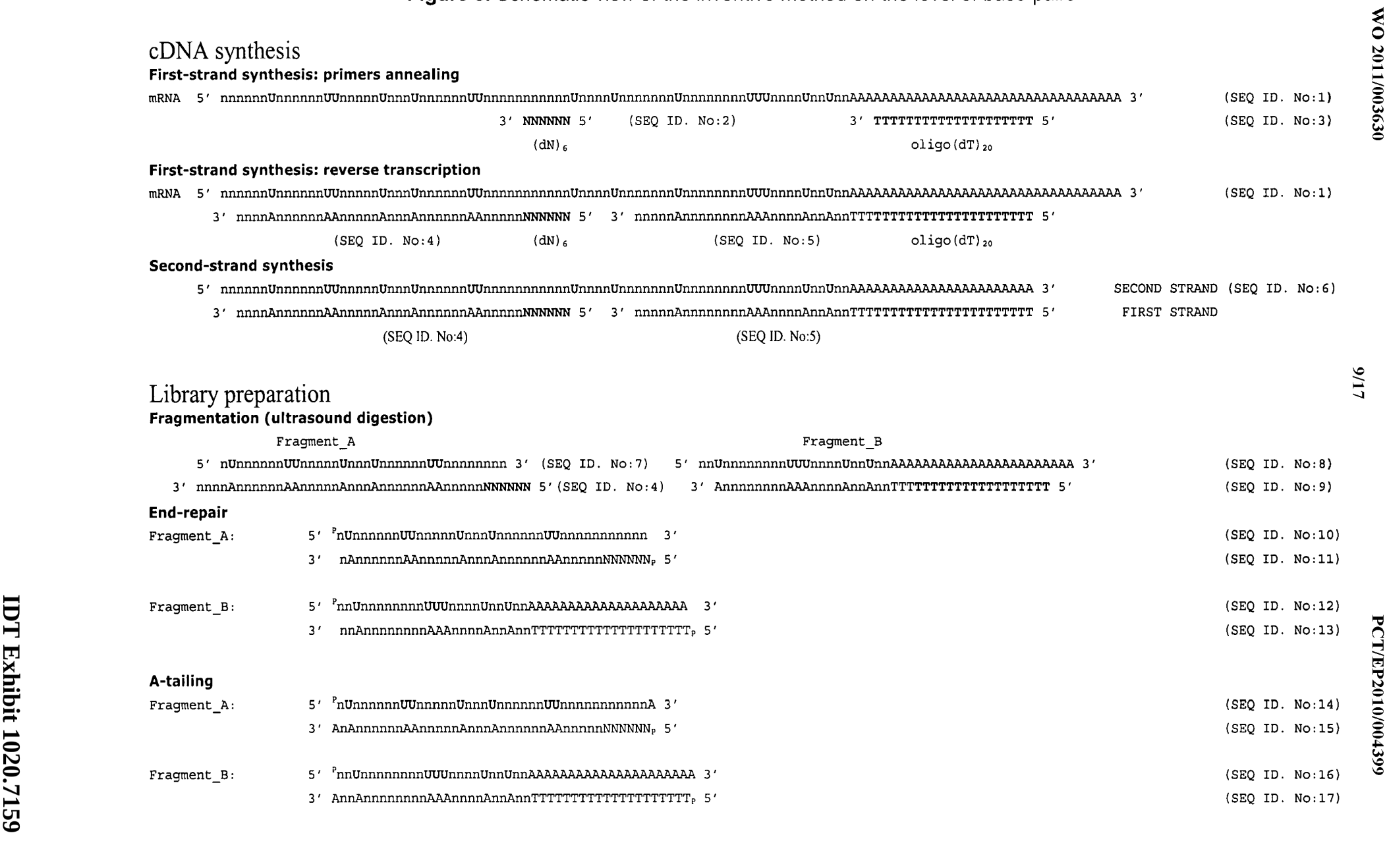

First-strand synthesis
with normal dNTP's
Second-strand synthesis
with dTTP --> dUTP
Y-adaptor ligation
#Ad2
#Ad1
#Ad1
#Ad2
UNG treatment
Preamplification and
sequencing from #Ad1
side
#Ad2
#Ad1
#Ad2
#Ad1

Figure 4

Figure 5. Schematic view of the inventive method on the level of base pairs



IDT Exhibit 1020.7159

Adaptor ligation

```
Fragment_A:
                                                                #Ad_2
5' TACACTCTTTCCCTACACGAC                                 TCGTATGCCGTCTTCTGCTTG 3'   (SEQ ID. No:18)
                GCTCTTCCGATCT nUnnnnnnUUnnnnnnUnnnUnnnnnnUUnnnnnnUnnnnA GATCGGAAGAGC
                CGAGAAGGCTAG AnAnnnnnnAAnnnnnnAnnnAnnnnnnAAnnnnnnNNNNNN TCTAGCCTTCTCG
3' GTTCGTCTTCTGCCGTATGCT                                          CAGCACATCCCTTTCTCACAT 5'   (SEQ ID. No:19)
        #Ad_2                                                              #Ad_1
Fragment_B:
                                                                        #Ad_2
5' TACACTCTTTCCCTACACGAC                                          TCGTATGCCGTCTTCTGCTTG 3'   (SEQ ID. No:20)
                GCTCTTCCGATCT nnUnnnnnnnnnUUUnnnnnUnnnUnnAAAAAAAAAAAAAAAAAAAA GATCGGAAGAGC
                CGAGAAGGCTAG AnnAnnnnnnnnnAAAnnnnnAnnAnnTTTTTTTTTTTTTTTTTTTT TCTAGCCTTCTCG
3' GTTCGTCTTCTGCCGTATGCT                                          CAGCACATCCCTTTCTCACAT 5'   (SEQ ID. No:21)
        #Ad_2                                                              #Ad_1
```

UDGase treatment

```
Fragment_A:
                                                                #Ad_2
        #Ad_1
          SEQ ID. No:22)      (SEQ ID. No:24) (SEQ ID. No:26)
5' TACACTCTTTCCCTACACGAC  (SEQ ID. No:23)(SEQ ID. No:25) (SEQ ID. No:27)      TCGTATGCCGTCTTCTGCTTG 3'
                GCTCTTCCGATCT n·nnnnnn··nnnnn·nnn·nnnnnn··nnnnnnnnnnnA GATCGGAAGAGC
                CGAGAAGGCTAG AnAnnnnnnAAnnnnnnAnnnAnnnnnnAAnnnnnnNNNNNN TCTAGCCTTCTCG
3' GTTCGTCTTCTGCCGTATGCT                                          CAGCACATCCCTTTCTCACAT 5'   (SEQ ID. No:19)
        #Ad_2                                                              #Ad_1
Fragment_B:
                                                                        #Ad_2
        #Ad_1
          (SEQ ID. No:28)      (SEQ ID. No:30)    (SEQ ID. No:32)
5' TACACTCTTTCCCTACACGAC  (SEQ ID. No:29) (SEQ ID. No:31)          TCGTATGCCGTCTTCTGCTTG 3'
                GCTCTTCCGATCT nn·nnnnnnnn···nnnn·nn·nnAAAAAAAAAAAAAAAAAAAA GATCGGAAGAGC
                CGAGAAGGCTAG AnnAnnnnnnnnnAAAnnnnnAnnAnnTTTTTTTTTTTTTTTTTTTT TCTAGCCTTCTCG
3' GTTCGTCTTCTGCCGTATGCT                                          CAGCACATCCCTTTCTCACAT 5'   (SEQ ID. No:21)
        #Ad_2                                                              #Ad_1
```

IDT Exhibit 1020.7160

Preamplification

Fragment_A:

```
                          #PCR_2                                                                                    #PCR 1
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT 3'      (SEQ ID. No:33)          3' TCTAGCCTTCTCGCAGCACATCCCTTTCTCACATCTAGAGCCACCAGCGGCATAGTAA 5' (SEQ ID. No:34)
  |<------------  34bp  ------------>|<              50-150bp            >|<------------------------  58bp  ------------------------>|
Fragment_A:
3' GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAGAnAnnnnnnnAAnnnnnAnnnAnnnnnnnAAnnnnnNNNNNNTCTAGCCTTCTCGCAGCACATCCCTTTCTCACAT 5'                    (SEQ ID. No:19
             #Ad_2                                                                                  #Ad_1
Fragment_B:
3' GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAGAnnAnnnnnnnnnAAAnnnnnAnnAnnTTTTTTTTTTTTTTTTTTTTTTCTAGCCTTCTCGCAGCACATCCCTTTCTCACAT 5'                    (SEQ ID. No:21
             #Ad_2                                                                                  #Ad_1
```

FlowCell preparation

Amplification

Fragment_A:

```
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnTnnnnnnnTTnnnnnnTnnnTnnnnnnnTTnnnnnnnnnnnnAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3' (SEQ ID. No:35)
3' GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAGAnAnnnnnnnAAnnnnnAnnnAnnnnnnnAAnnnnnNNNNNNTCTAGCCTTCTCGCAGCACATCCCTTTCTCACATCTAGAGCCACCAGCGGCATAGTAA 5' (SEQ ID. No:36)
Fragment_B:
                                                                                                                                                (SEQ ID. No:37
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnnTnnnnnnnnnTTTnnnnTnnTnnAAAAAAAAAAAAAAAAAAAAAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3'
3' GTTCGTCTTCTGCCGTATGCTCGAGAAGGCTAGAnnAnnnnnnnnnAAAnnnnnAnnAnnTTTTTTTTTTTTTTTTTTTTTTCTAGCCTTCTCGCAGCACATCCCTTTCTCACATCTAGAGCCACCAGCGGCATAGTAA 5'
                                                                                                                                                (SEQ ID. No:38
```

Linearisation

Fragment_A:

```
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnTnnnnnnnTTnnnnnnTnnnTnnnnnnnTTnnnnnnnnnnnnAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3' (SEQ ID. No:35)
Fragment_B:
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnnTnnnnnnnnnTTTnnnnTnnTnnAAAAAAAAAAAAAAAAAAAAAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3'
                                                                                                                                                (SEQ ID. No:37
```

IDT Exhibit 1020.7161

Sequencing primer annealing

Fragment_A:

```
                                    sequencing primer
                                <---- 3' TCTAGCCTTCTCGCAGCACATCCCCTTTCTCACA 5'   (SEQ ID. No:39)
                                      ||||||||||||||||||||||||||||||||||
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnTnnnnnnnTTnnnnnnTnnnTnnnnnnnnnnnnAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT 3' (SEQ ID. No:35)
Fragment_B:
                                <---- 3' TCTAGCCTTCTCGCAGCACATCCCTTTCTCACA 5'   (SEQ ID. No:39)
                                      ||||||||||||||||||||||||||||||||||
5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTnnTnnnnnnnnTTTnnnnTnnTnnTnnAAAAAAAAAAAAAAAAAAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT
                                                                                (SEQ ID. No:37)
```

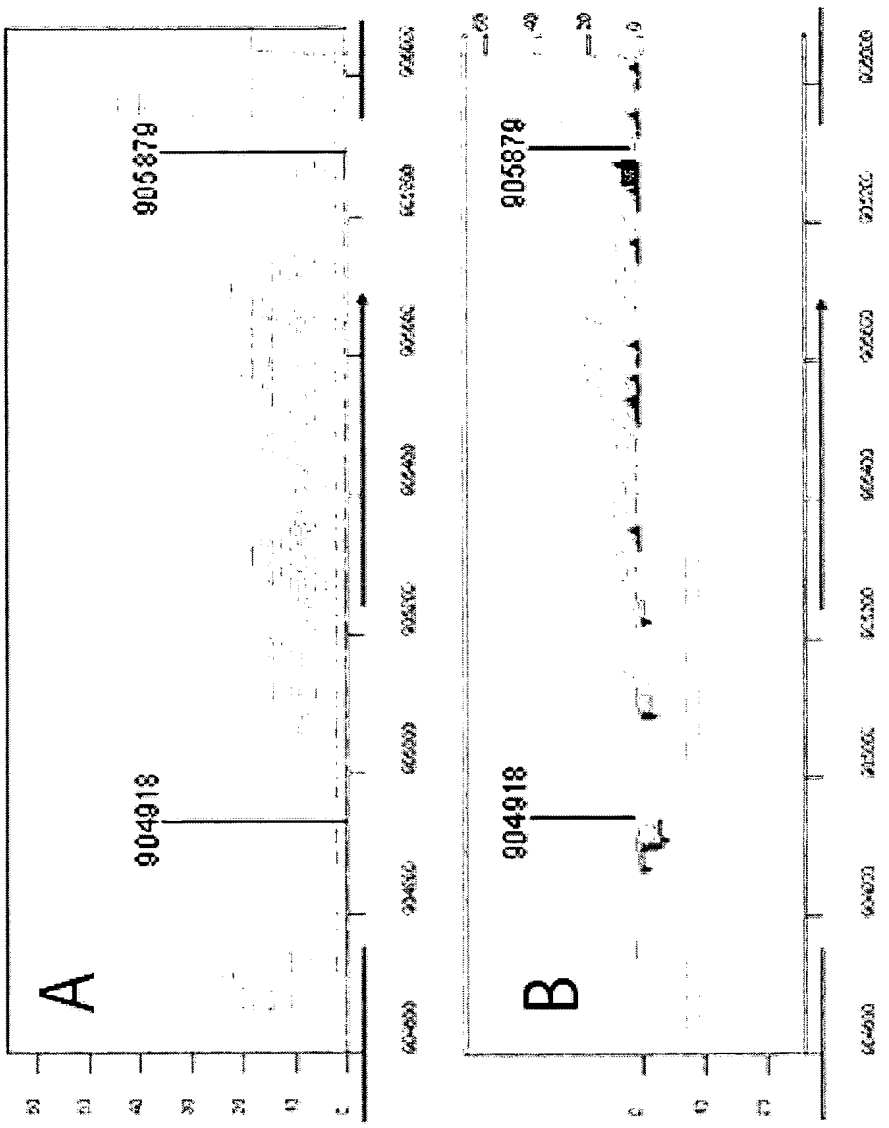


Figure 6

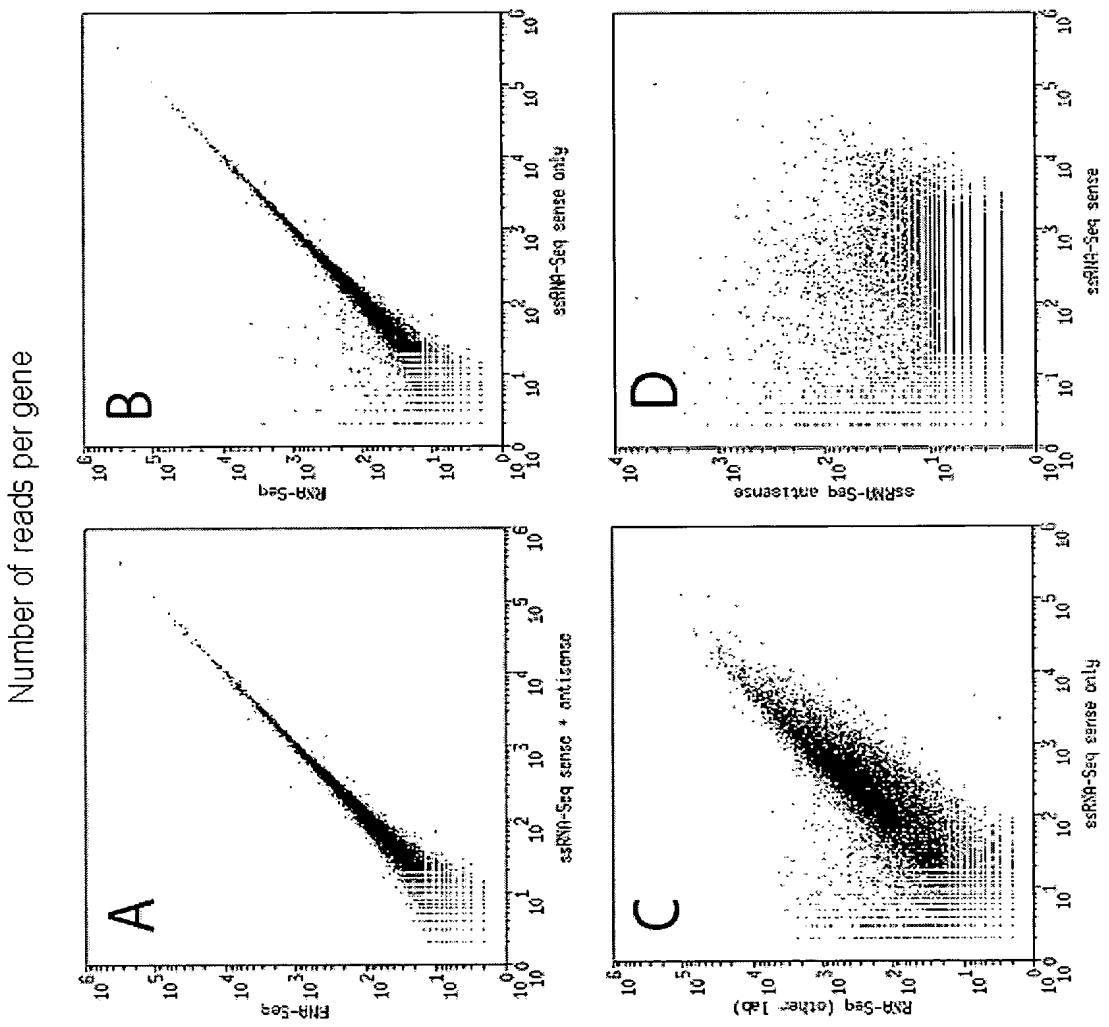

Number of reads per gene
A
RNA-Seq
ssRNA-Seq sense + antisense
B
RNA-Seq
ssRNA-Seq sense only
C
RNA-Seq (other lab)
ssRNA-Seq sense only
D
ssRNA-Seq antisense
ssRNA-Seq sense

Figure 7

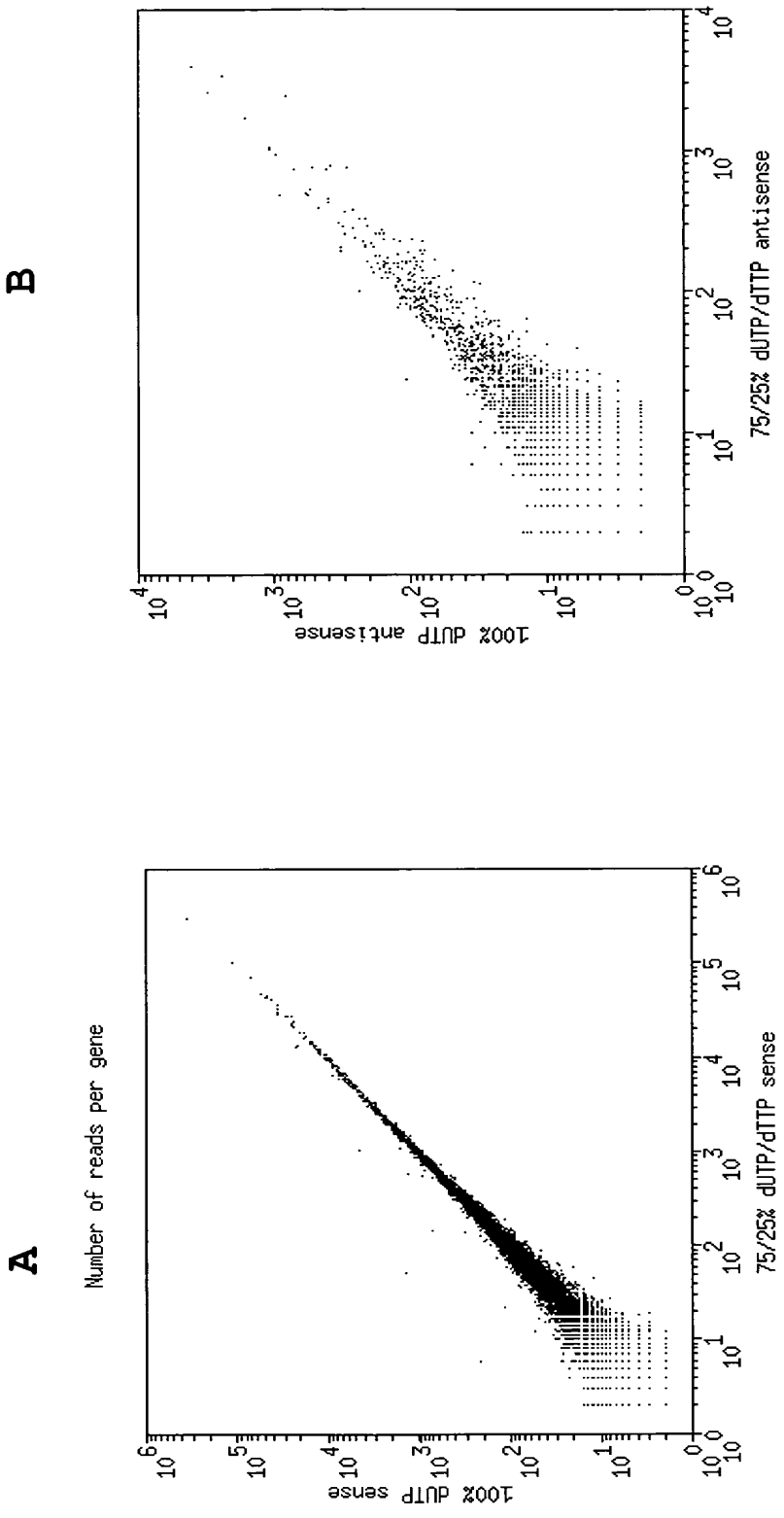

A
Number of reads per gene
100% dUTP sense
75/25% dUTP/dTTP sense
B
100% dUTP antisense
75/25% dUTP/dTTP antisense

Figure 8

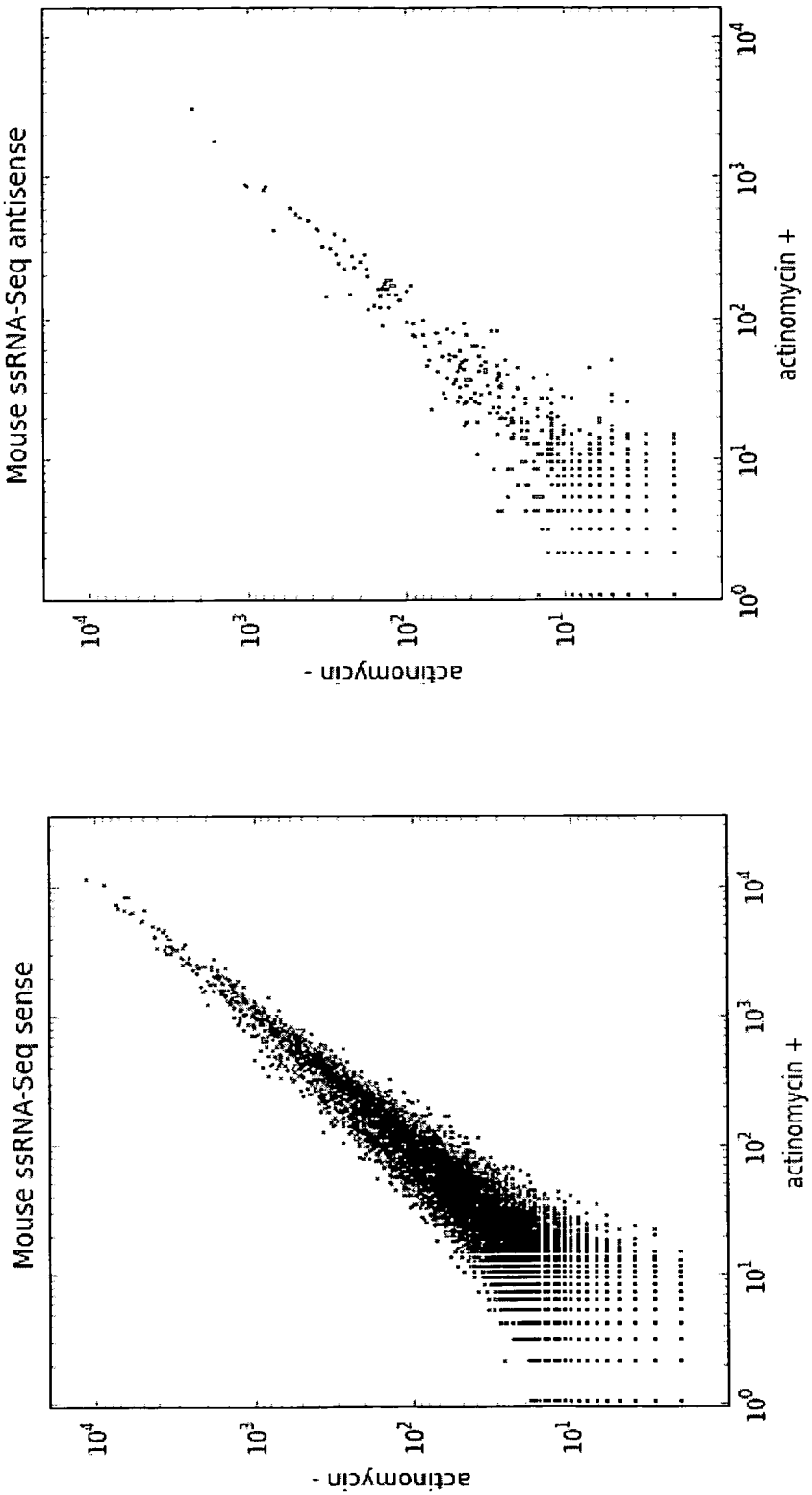

Mouse ssRNA-Seq antisense
actinomycin -
actinomycin +
Mouse ssRNA-Seq sense
actinomycin -
actinomycin +

Figure 9

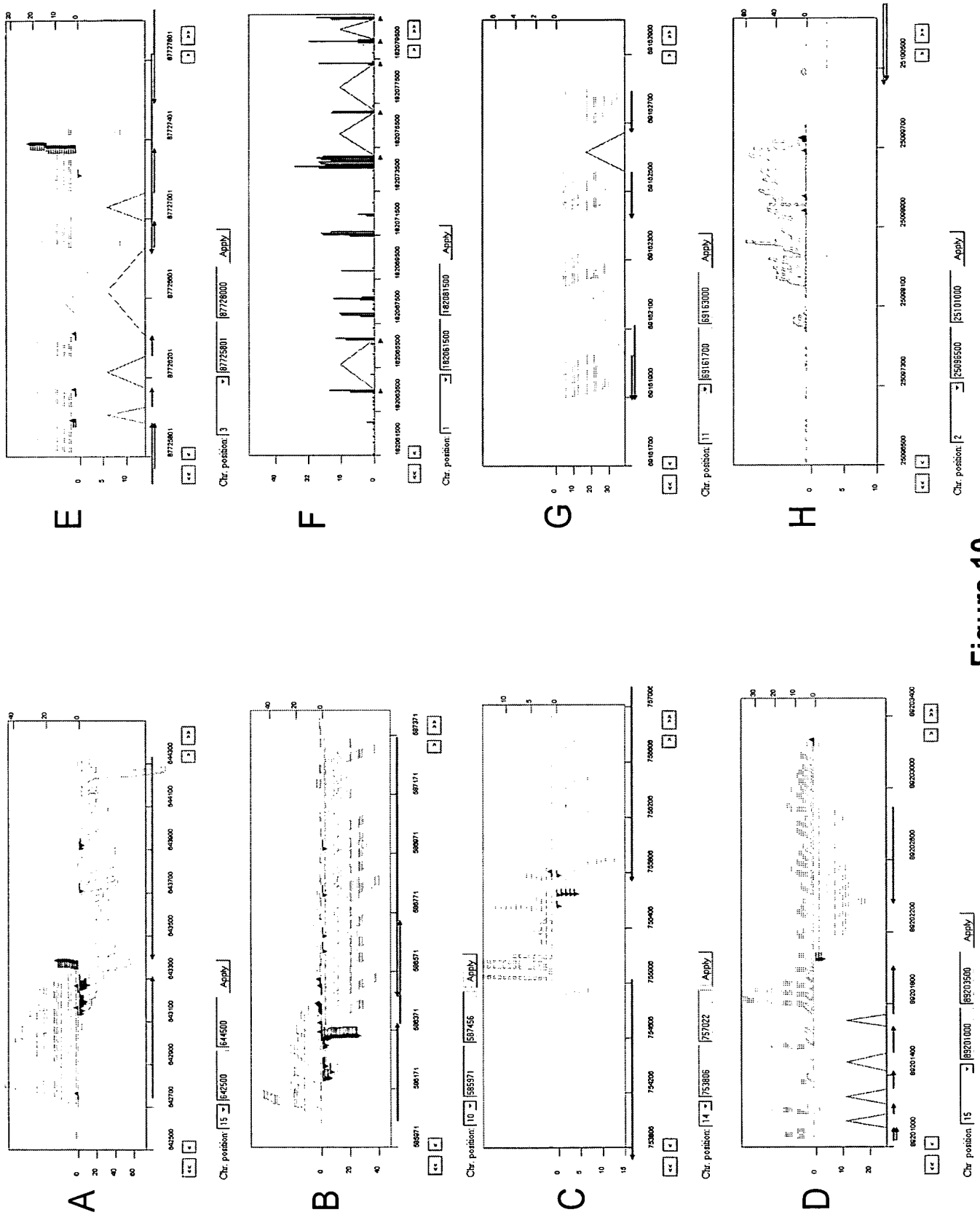


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/004399

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 practical, 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 A	US 2007/281863 A1 (RABBANI ELAZAR [US] ET AL) 6 December 2007 (2007-12-06) * abstract; claims 100-114; figure 2; examples 1,6 page 3, column 1, paragraph 4 - column 2, paragraph 1 page 6, column 2, paragraph 3; figure 2	16 1-15
A	WO 2009/006438 A2 (EPICT TECHNOLOGIES CORP [US]; DAHL GARY [US]; SOOKNANAN ROY RABINDRANA) 8 January 2009 (2009-01-08) * abstract page 60, paragraph 3	1-16
A	WO 03/008538 A2 (MOLECULAR STAGING INC [US]) 30 January 2003 (2003-01-30) * abstract; claims 26-29; figure 5; example 9	7,13

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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

"E" earlier document but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 2010	08/12/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Tilkorn, A

Form PCT/ISA/210 (second sheet) (April 2005)

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/004399

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	PARKHOMCHUK DMITRI ET AL: "Transcriptome analysis by strand-specific sequencing of complementary DNA.", NUCLEIC ACIDS RESEARCH OCT 2009 LNKD-PUBMED:19620212, vol. 37, no. 18, 20 July 2009 (2009-07-20) , page E123, XP002608410, ISSN: 1362-4962 the whole document	1-16

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/004399

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [ ] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/004399

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007281863 A1	06-12-2007	NONE	
WO 2009006438 A2	08-01-2009	CA 2707436 A1	08-01-2009
		EP 2173872 A2	14-04-2010
		US 2009053775 A1	26-02-2009
WO 03008538 A2	30-01-2003	AU 2002259196 A1	03-03-2003
		US 2004091857 A1	13-05-2004

IDT Exhibit 1020.7171

Espacenet

Bibliographic data: CN105890722 (A) — 2016-08-24

Method for measuring DNA molecular mass according to resonance principle

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Applicant(s): UNIV CHANGZHOU ± (常州大学)

Classification: **- international:** ***G01G3/16***
- cooperative: **G01G3/165 (CN)**

Application number: CN201610408614 20160613 Global Dossier

Priority number (s): CN201610408614 20160613

Abstract of CN105890722 (A)

The invention discloses a method for measuring DNA molecular mass by according to a resonance principle, and belongs to the field of detection and weighing of organic macromolecular mass. The system comprises a vibration exciter, a hinged support A, a hinged support B, a micro simply supported beam of which two ends are respectively assembled on the hinged support A and the hinged support B, a DNA molecular adsorption layer assembled on the micro simply supported beam, a DNA molecule to-be-measured, and an amplitude receiver assembled on the micro simply supported beam. The micro simply supported beam is subjected to vibration excitation so as to freely vibrate by the vibration exciter, and the amplitude receiver receives the corresponding amplitude and vibration frequency, so that the DNA molecular mass can be calculated. The method for measuring DNA molecular mass through the resonance principle disclosed by the invention is simple in principle and high in measurement accuracy.

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DESCRIPTION CN105890722A

Abstract The invention discloses a method for measuring the quality of DNA molecules using the principle of resonance, which belongs to the field of detection and weighing of organic macromolecules. It includes an exciter, a hinge support A, a hinge support B, a minimally supported beam mounted on the hinge support A and a hinge support B at both ends, and DNA molecule adsorption on the minimally supported beam Layer, DNA molecules to be measured, amplitude receiver installed on the simply supported beam; the free vibration of the simply supported beam is excited by the exciter, and the amplitude receiver receives the corresponding amplitude and vibration frequency, thereby calculating the DNA molecule's quality. The invention is a method for measuring the quality of DNA molecules by the resonance principle with simple principle and higher measurement accuracy.

Method for measuring DNA molecular mass using resonance principle

Technical field

The invention mainly relates to the field of detection and weighing of the mass of organic macromolecules, in particular to a method for measuring the quality of DNA molecules using the principle of resonance.

Background technique

DNA molecules are the main genetic material. Infections of various diseases in humans can be reflected in the corresponding changes in the structure and quality of DNA molecules. Therefore, it is of great significance to quickly or effectively weigh or detect the molecular weight of DNA. However, because the molecular mass of DNA is too small, existing static mass measurement equipment cannot accurately measure it. Therefore, it is of great significance to design a device for dynamically measuring DNA molecules.

Summary of the invention

The technical problem to be solved by the present invention is: In view of the technical problems existing in the prior art, the present invention provides a method for measuring the quality of DNA molecules using a resonance principle with a simple principle and higher measurement accuracy.

In order to solve the above problems, the solution proposed by the present invention is: a method for measuring the molecular mass of DNA using the principle of resonance, which includes a vibrator, a hinge support A, a hinge support B, and two ends respectively mounted on the hinge Miniature support beams on the support A and the hinge support B, the adsorbed layer of DNA molecules installed on the miniature support beams, DNA molecules to be measured, and the Amplitude receiver

The method for measuring the quality of DNA molecules using the principle of resonance in the present invention consists of the following steps:

Use the exciter to act on the simply supported beam to make it vibrate freely; use the amplitude receiver to receive the amplitude of the simply supported beam and record the maximum amplitude and the corresponding vibration frequency; determine the simply supported beam The natural frequency f0 without DNA molecules;

Placing the DNA molecule to be measured on the adsorption layer of the DNA molecule, using the vibrator to act on the simply supported beam to make it vibrate freely; using the amplitude receiver to receive the amplitude of the simply supported beam,

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And record the maximum amplitude and the corresponding vibration frequency; use the amplitude receiver to receive the amplitude of the simply supported beam, and record the maximum amplitude and the corresponding vibration frequency; determine the natural frequency f1 of the simply supported beam when there are DNA molecules;

The equivalent stiffness of the equivalent spring of the system is k, the equivalent damping of the equivalent damper is η, and the resonance model of the DNA molecule is established; and the formula m=(K/6.28)<2>×(1/f1-1 /f0) Calculate the mass of the DNA molecule.

Compared with the prior art, the present invention has the following advantages and beneficial effects:

(1)

The method for measuring the quality of DNA molecules using the principle of resonance in the present invention applies excitation to the simply supported beam through an exciter, and the amplitude receiver receives the maximum amplitude and corresponding frequency of the simply supported beam, thereby calculating the system before and after the action of DNA molecules Natural frequency change value.

(2)

The method for measuring the quality of DNA molecules using the resonance principle of the present invention has a simple principle, measurement accuracy, and achieves the measurement of the quality of DNA molecules through the resonance principle.

BRIEF DESCRIPTION

FIG. 1 is a schematic structural diagram of a measuring device for measuring the quality of DNA molecules using the principle of resonance according to the present invention.

2 is a schematic diagram of the mechanical model of the present invention that uses the principle of resonance to measure the quality of DNA molecules.

In the figure, 1---DNA molecule; 2---rack; 21---hinge support A; 22---hinge support B; 31---DNA adsorption layer; 32---miniature support beam; 33----equivalent spring; 34----equivalent Damper; 4-vibrator; 5-amplitude receiver; 6-bend beam axis.

detailed description

The present invention will be further described in detail below with reference to the drawings and specific embodiments.

As shown in FIG. 1, a method for measuring DNA molecular mass using the principle of resonance in the present invention includes an exciter 4, a hinge support A21, a hinge support B22, and two ends respectively installed on the hinge support A21 and the hinge support The micro support beam 32 on the base B22, the DNA molecule adsorption layer 31 mounted on the micro support beam 32, the DNA molecule to be measured 1, and the amplitude receiver 5 mounted on the micro support beam 32.

As shown in Figure 1 and Figure 2, the method of measuring the molecular mass of DNA using the principle of resonance consists of the following specific steps:

As shown in FIG. 1 and FIG. 2, the vibrator 4 acts on the micro-supported beam 32 to cause it to vibrate freely; the amplitude receiver 5 is used to receive the amplitude of the micro-supported beam 32 and record the maximum amplitude and the corresponding vibration Frequency; determine the natural frequency f0 of the simply supported beam 32 without DNA molecules;

As shown in FIGS. 1 and 2, the DNA molecule 1 to be measured is placed on the DNA molecule adsorption layer 31, and the vibrator 4 acts on the simply supported beam 32 to cause it to vibrate freely; the amplitude receiver 5 is used to receive the micro The amplitude of the simply supported beam 32, and record the maximum amplitude and the corresponding vibration frequency; use the amplitude receiver 5 to receive the amplitude of the micro simply supported beam 32, and record the maximum amplitude and the corresponding vibration frequency; The natural frequency f1 of the simply supported beam 32;

Referring to FIGS. 1 and 2, the equivalent spring 33 of the system has an equivalent stiffness of k, and the equivalent damper 34 has an equivalent damping of η, establishing a resonance model of the DNA molecule 1; and using the formula m=(K/ 6.28) <2>×(1/f1-1/f0) calculate the mass of DNA molecule 1.

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CLAIMS CN105890722A

1.

The method of measuring the molecular mass of DNA using the principle of resonance is characterized in that it includes a vibration exciter (4), a hinge support A (21), a hinge support B (22), and two ends are respectively installed on the hinge support A (21) and the minimally-supported beam (32) on the hinge support B (22), the DNA molecule adsorption layer (31) installed on the minimally-supported beam (32), and the DNA molecule to be measured (1) Amplitude receiver (5) installed on the simple support beam (32).

2.

The method for measuring the quality of DNA molecules using the principle of resonance according to claim 1, which is specifically composed of the following steps: (a) using the vibration exciter (4) to act on the simply supported beam (32), Make the simple supported beam (32) vibrate freely; adopt the amplitude receiver (5) to receive the amplitude of the simple supported beam (32), and record the maximum amplitude and the corresponding vibration frequency; determine the simple supported beam (32)) The natural frequency f0 when there is no DNA molecule; (b) Place the DNA molecule (1) to be measured on the DNA molecule adsorption layer (31), and use the vibrator (4) to act on the micro-branched support The beam (32) makes the simple supported beam (32) vibrate freely; the amplitude receiver (5) is used to receive the amplitude of the simple supported beam (32), and the maximum amplitude and the corresponding vibration frequency are recorded; The amplitude receiver (5) receives the amplitude of the simply supported beam (32) and records the maximum amplitude and the corresponding vibration frequency; the natural frequency f1 of the simply supported beam (32) when the DNA molecule (1) is present is determined; (c) The equivalent stiffness of the equivalent spring (33) of the system is k, the equivalent damping of the equivalent damper (34) is η, and the resonance model of the DNA molecule (1) is established; and the formula m = (K/6.28)<2>×(1/f1-1/f0) to calculate the mass of DNA molecule (1).

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Bibliographic data: WO03083435 (A2) — 2003-10-09

SINGLE PRIMER ISOTHERMAL NUCLEIC ACID AMPLIFICATION-ENHANCED ANALYTE DETECTION AND QUANTIFICATION

Inventor(s): KURN NURITH [US]; DAFFORN GEOFFREY A [US] ± (KURN, NURITH, ; DAFFORN, GEOFFREY, A)

Applicant(s): NUGEN TECHNOLOGIES INC [US]; KURN NURITH [US]; DAFFORN GEOFFREY A [US] ± (NUGEN TECHNOLOGIES, INC, ; KURN, NURITH, ; DAFFORN, GEOFFREY, A)

Classification: - international: ***C12N15/09; C12P21/08; C12Q1/34; C12Q1/68; G01N33/53; G01N37/00;*** (IPC1-7): G01N
- cooperative: **C12Q1/6804; C12Q1/6865** more

Application number: WO2003US10148 20030331 Global Dossier

Priority number (s): US20020368628P 20020329

Also published as: WO03083435 (A3) AU2003222178 (A1) CA2480525 (A1) EP1490514 (A2) EP1490514 (A4) more

Abstract of WO03083435 (A2)

The present invention provides novel methods of indirect analyte detection and quantification through amplification of oligonucleotide template attached to binding partners for analytes by nucleic acid amplification utilizing isothermal, single primer linear nucleic acid amplification methods. Methods of binding of binding partner that is attached to an oligonucleotide template to analyte, then amplifying at least a portion of the oligonucleotide template using a composite primer, primer extension, strand displacement, and optionally a termination sequence, are provided. Methods for amplifying sense RNA using a composite primer, primer extension, strand displacement, optionally template switching, a propromoter oligonucleotide and transcription are also provided. Methods for detecting and quantifying amplification products are also provided. The invention further provides compositions and kits for practicing said methods.

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60/368,628 29 March 2002 (29.03.2002) US

(71) Applicant *(for all designated States except US)*: **NUGEN TECHNOLOGIES, INC.** [US/US]; 821 Industrial Road, Unit A, San Carlos, CA 94070 (US).

(72) Inventors; and

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(74) Agents: **POLIZZI, Catherine M.** et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/083435 A2

(54) Title: SINGLE PRIMER ISOTHERMAL NUCLEIC ACID AMPLIFICATION-ENHANCED ANALYTE DETECTION AND QUANTIFICATION

(57) Abstract: The present invention provides novel methods of indirect analyte detection and quantification through amplification of oligonucleotide template attached to binding partners for analytes by nucleic acid amplification utilizing isothermal, single primer linear nucleic acid amplification methods. Methods of binding of binding partner that is attached to an oligonucleotide template to analyte, then amplifying at least a portion of the oligonucleotide template using a composite primer, primer extension, strand displacement, and optionally a termination sequence, are provided. Methods for amplifying sense RNA using a composite primer, primer extension, strand displacement, optionally template switching, a propromoter oligonucleotide and transcription are also provided. Methods for detecting and quantifying amplification products are also provided. The invention further provides compositions and kits for practicing said methods.

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SINGLE PRIMER ISOTHERMAL NUCLEIC ACID AMPLIFICATION-ENHANCED ANALYTE DETECTION AND QUANTIFICATION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority benefit of the provisional patent application U.S. Serial No. 60/368,628, filed March 29, 2002, which is incorporated by reference in its entirety.

TECHNICAL FIELD

The invention relates to the field of detection of analytes through amplification of polynucleotide attached to a binding partner. More particularly, the invention provides methods, compositions and kits for binding of analyte binding partners to analyte wherein the binding partner is attached to an oligonucleotide template, and for amplifying (i.e., making multiple copies) the oligonucleotide template, where the methods employ a single RNA/DNA composite primer, with the amplification optionally involving transcription, and detecting amplification products.

BACKGROUND

Advances in nucleic acid technology have had major effects on various aspects of detection technologies. Automated oligonucleotide synthesis, which incorporates the use of many modified nucleotides, both deoxyribo- and ribo-nucleotides and combinations thereof, has enabled the use of these highly diverse molecules in various applications. Moreover, the large body of knowledge on binding specificity of complementary oligonucleotides, conjugation of oligonucleotides to other molecules and surfaces, has contributed to the wide use of oligonucleotides for many applications, including their use as reporter-groups and capture agents in various analytical procedures for the detection and quantification of single and multiple non-nucleic acid analytes. The ability to amplify oligonucleotide targets in vitro, or to generate multiple copies of reporter-oligonucleotide targets, further enhances their use for enhanced detection.

The enhanced sensitivity of the detection of antibodies bound to antigen by coupling of immune recognition with DNA amplification has been documented previously (Sano, Smith and Cantor, 1992). Immuno-PCR is carried out employing unique DNA sequence tags, which are associated with a specific antigen either covalently or through streptavidin-biotin

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interactions. Antibody binding to antigens is detected by PCR amplification of the associated DNA tag. The utility of using multiple antibodies and DNA tags was demonstrated by simultaneous analysis of several antigens by immuno-PCR. Although immuno-PCR was shown to be significantly more sensitive than ELISA, the method is limited by drawbacks of PCR such as the requirement for thermal cycling and difficulties in quantification of the amplification products. These limitations have restricted the widespread adoption of immuno-PCR as an alternative to ELISA.

Other methods have been employed for detection technology wherein nucleic acid amplification is used to produce a detectable signal from low levels of analyte. These include the methods described in U.S. Patent Nos. 6,083,689; 5,985,548; 5,854,033; 5,655,539; and 5,849,478.

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods for determining presence or absence of an analyte in a sample by a) contacting the sample with a binding partner that is attached to an oligonucleotide template and that is capable of binding, directly or indirectly, to the analyte, if present, under conditions that permit binding, whereby an analyte-binding partner complex is formed if analyte is present; b) separating analyte-binding partner complex from unbound binding partner; c) amplifying a polynucleotide sequence complementary to at least a portion of the oligonucleotide template according to a method comprising: (i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) extending the composite primer with DNA polymerase, whereby a primer extension product comprising a detectable identifying characteristic is produced; (iii) cleaving RNA of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement to produce cleaved primer extension product that comprises a detectable identifying characteristic; whereby multiple copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template are produced; and whereby detection of the cleaved primer extension product comprising the detectable identifying characteristic indicates presence of the analyte in the sample. In another aspect, the invention provides methods for quantifying an analyte in a sample by performing steps a) to c) above, and also comparing the amount of copies of the polynucleotide sequence

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complementary to at least a portion of the oligonucleotide template obtained in the sample, if any, to the amount of copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template obtained in a reference comprising a known amount of the analyte obtained in a reference comprising a known amount of the analyte that is subjected to steps (a) to (c); whereby the comparison provides quantification of amount of analyte in the sample.

In another aspect, the invention provides methods for determining presence or absence of an analyte in a sample by a) contacting the sample with a binding partner that is attached to an oligonucleotide template and that is capable of binding to the analyte under conditions that permit binding, whereby an analyte-binding partner complex is formed if analyte is present; b) separating analyte-binding partner complex from unbound binding partner; c) amplifying a polynucleotide sequence complementary to at least a portion of the oligonucleotide template in the analyte-binding partner complex by (i) hybridizing a composite primer to the oligonucleotide template attached to the binding partner in the analyte-binding partner complex, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) extending the composite primer with DNA polymerase, whereby a primer extension product is produced; (iii) cleaving RNA of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement; and (iv) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced; whereby multiple copies of the RNA transcripts are produced; and whereby detection a detectable identifying characteristic of the RNA transcripts indicates presence of the analyte in the sample. In another aspect the invention provides methods for quantifying an analyte in a test sample by performing steps (a) to (c) above and also comparing amount of RNA transcripts obtained in the sample to the amount of RNA transcripts obtained in a reference comprising a known amount of the analyte that is subjected to steps (a) to (c); whereby the comparison provides quantification of amount of analyte in the sample.

In another aspect the invention provides methods for determining presence or absence of each of a plurality of analytes in a sample by: a) contacting the sample with a plurality of different binding partners, each of which is attached to an oligonucleotide template and each of which is capable of binding one of the plurality of different analytes under conditions that permit binding, whereby an analyte-binding partner complex is formed for a particular pair

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of analyte and binding partner if the analyte is present, wherein the oligonucleotide template for each different binding partner comprises a primer-binding region that is common to all of the binding partners and a primer-extension region that is unique for each binding partner; b) separating analyte-binding partner complexes from unbound binding partners; c) amplifying a polynucleotide sequence complementary to at least a portion of each oligonucleotide template present after step b) according to a method comprising: (i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) extending the composite primer with DNA polymerase, whereby a unique primer extension product comprising a unique detectable identifying characteristic is produced for each analyte-binding partner complex; (iii) cleaving RNA of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement to produce unique cleaved primer extension product for each different oligonucleotide template that comprises a unique detectable identifying characteristic; whereby multiple copies of the polynucleotide sequence complementary to at least a portion of each oligonucleotide template present after step b) are produced; and whereby detection of the unique detectable identifying characteristic of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template attached to the binding partner for an analyte indicates the presence of the analyte in the sample. In another aspect the invention provides methods for quantifying the relative amounts of each analyte in the sample by comparing the relative amounts of polynucleotide sequence complementary to at least a portion of the oligonucleotide template attached to the binding partner in each analyte-binding partner complex.

In another aspect the invention provides methods for detecting the presence or absence of an analyte in a sample comprising incubating a reaction mixture, said reaction mixture comprising: (a) a sample suspected of containing a complex of the analyte and a binding partner, wherein the binding partner is attached to an oligonucleotide template; (b) a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (c) a DNA polymerase, dNTPs, and an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit hybridization of the composite primer and the oligonucleotide template, oligonucleotide polymerization, and RNA cleavage, such that multiple copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template are produced, and wherein detection of a detectable identifying characteristic of the copies

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of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template indicates the presence of the analyte.

In another aspect the invention provides methods for detecting the presence or absence of an analyte in a sample comprising incubating a reaction mixture, said reaction mixture comprising: (a) a sample suspected of containing a complex of the analyte and a binding partner, wherein the binding partner is attached to an oligonucleotide template; (b) a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (c) a DNA polymerase, dNTPs, an enzyme that cleaves RNA from an RNA/DNA hybrid; (d) a polynucleotide comprising a propromoter and a region homologous to a region of the oligonucleotide template; RNA polymerase; and NTP's; wherein the incubation is under conditions that permit hybridization of the composite primer and the oligonucleotide template, oligonucleotide polymerization, and RNA cleavage; and hybridization of the polynucleotide comprising a propromoter and a region homologous to a region of the oligonucleotide template to cleaved primer extension product, to produce a hybridization product comprising a promoter, and transcription of the hybridization product comprising a promoter by the RNA polymerase, whereby multiple RNA transcripts are produced, and wherein detection of the RNA transcripts indicates presence of the analyte.

In another aspect the invention provides methods for generating multiple copies of and/or quantifying a polynucleotide sequence complementary to a polynucleotide sequence attached to a binding partner by: (a) hybridizing a composite primer to the oligonucleotide template attached to the binding partner, said composite primer comprising an RNA portion and a 3' DNA portion; (b) extending the composite primer with DNA polymerase; (c) cleaving RNA of the hybridized composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, whereby multiple copies of the polynucleotide sequence complementary to at least a portion of an oligonucleotide template attached to a binding partner are produced.

In another aspect the invention provides methods for generating multiple copies of RNA transcripts of oligonucleotide template attached to a binding partner by (a) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (b) extending the composite primer with DNA polymerase; (c) cleaving RNA of the hybridized composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to

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the oligonucleotide template and repeats primer extension by strand displacement to produce a displaced primer extension product; and (d) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions that allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, whereby multiple copies of the polynucleotide sequence complementary to at least a portion of an oligonucleotide template attached to a binding partner are produced.

In one aspect, the invention provides methods for determining presence or absence of an analyte in a sample by: a) contacting the sample with a binding partner capable of binding to the analyte under conditions which permit binding, whereby a complex of analyte and binding partner is formed if analyte is present; wherein the binding partner is attached to an oligonucleotide template; b) optionally, separating the complex, if present, from unbound binding partner; c) amplifying a polynucleotide sequence complementary to a portion of the oligonucleotide template attached to the binding partner in the complex according to a method comprising:

(i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) optionally, hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide template; (iii) extending the composite primer with DNA polymerase, whereby a primer extension product comprising a detectable identifying characteristic is produced; (iv) cleaving the RNA portion of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement; whereby multiple copies of the polynucleotide sequence complementary to a portion of the oligonucleotide template are produced; and whereby detection of the cleaved primer extension product comprising the detectable identifying characteristic indicates presence of the analyte in the sample.

In another aspect, the invention provides methods for determining presence or absence of an analyte in a sample by: a) contacting the sample with a binding partner capable of binding to the analyte under conditions which permit binding, whereby a complex of analyte and binding partner is formed if analyte is present; wherein the binding partner is attached to an oligonucleotide template; b) optionally, separating the complex, if present, from unbound binding partner; c) amplifying a portion of the oligonucleotide template attached to the

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binding partner in the complex according to a method comprising: (i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) optionally, hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide template;

(iii) extending the composite primer with DNA polymerase; (iv) cleaving the RNA portion of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement; (v) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts comprising a detectable identifying characteristic are produced; whereby multiple copies of a portion of the oligonucleotide template are produced; and whereby detection of the RNA transcripts comprising the detectable identifying characteristic indicates presence of the analyte in the sample.

In another aspect, the invention provides methods for quantifying an analyte in a test sample by: a) contacting the sample with a binding partner capable of binding to the analyte under conditions which permit binding, whereby a complex of analyte and binding partner is formed if analyte is present; wherein the binding partner is attached to an oligonucleotide template; b) optionally, separating the complex, if present, from unbound binding partner; c) amplifying a polynucleotide sequence complementary to a portion of the oligonucleotide template attached to the binding partner in the complex according to a method comprising: (i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide target reporter; (iii) extending the composite primer with DNA polymerase; (iv) cleaving the RNA portion of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement; whereby multiple copies of the polynucleotide sequence complementary to a portion of the oligonucleotide template are produced; and

(d) comparing amount of amplified polynucleotide sequence to amount of amplified polynucleotide sequence obtained in a reference sample comprising a known amount of the

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analyte; whereby the comparison provides quantification of amount of analyte in the test sample.

In another aspect the invention provides methods for quantifying an analyte in a test sample by: a) contacting the sample with a binding partner capable of binding to the analyte under conditions which permit binding, whereby a complex of analyte and binding partner is formed if analyte is present; wherein the binding partner is attached to an oligonucleotide template; b) separating the complex, if present, from unbound binding partner; c) amplifying a polynucleotide sequence complementary to a portion of the oligonucleotide template attached to the binding partner in the complex according to a method comprising: (i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (ii) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide target reporter; (iii) extending the composite primer with DNA polymerase; (iv) cleaving the RNA portion of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement; (v) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts comprising a detectable identifying characteristic are produced; whereby multiple copies of a portion of the oligonucleotide template are produced; and (d) comparing amount of amplified portion of the oligonucleotide template to amount of amplified portion of the oligonucleotide template obtained in a reference sample comprising a known amount of the analyte; whereby the comparison provides quantification of amount of analyte in the test sample.

In another aspect the invention provides methods for detecting the presence of an analyte in a sample comprising incubating a reaction mixture, said reaction mixture comprising: (a) a complex of the analyte and a binding partner, wherein the binding partner is attached to an oligonucleotide template; (b) a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (c) optionally, a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide template; (d) a DNA polymerase, dNTPs, and an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit

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hybridization of the composite primer and the oligonucleotide template, oligonucleotide polymerization, and RNA cleavage, such that a cleaved primer extension product comprising a detectable identifying characteristic is produced, and wherein the cleaved primer extension product is detected, whereby the presence of the analyte is detected.

In another aspect the invention provides methods for detecting the presence of an analyte in a sample comprising incubating a reaction mixture, said reaction mixture comprising: (a) a complex of the analyte and a binding partner, wherein the binding partner is attached to an oligonucleotide template; (b) a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion; (c) optionally, a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the composite primer to the oligonucleotide template; (d) a DNA polymerase, dNTPs, an enzyme that cleaves RNA from an RNA/DNA hybrid;

(e) a polynucleotide comprising a propromoter and a region homologous to a region of the oligonucleotide template; RNA polymerase; and NTP's;

wherein the incubation is under conditions that permit hybridization of the composite primer and the oligonucleotide template, oligonucleotide polymerization, and RNA cleavage; and hybridization of the polynucleotide comprising a propromoter and a region homologous to a region of the oligonucleotide template to cleaved primer extension product, to produce a hybridization product comprising a promoter, and transcription of the hybridization product comprising a promoter by the RNA polymerase, whereby multiple copies of a portion of the olignonucleotide template comprising a detectable identifying characteristic are produced, and wherein the olignonucleotide template is detected, whereby the presence of the analyte is detected.

In another aspect the invention provides methods for generating multiple copies of and/or quantifying a polynucleotide sequence complementary to a polynucleotide sequence attached to a binding partner by: (a) hybridizing a single stranded DNA template with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion, wherein the single stranded DNA template is attached to a binding partner; (b) extending the composite primer with DNA polymerase; (c) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the template and repeats primer extension by strand displacement,

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whereby multiple copies of the complementary sequence of the polynucleotide sequence attached to the binding partner are produced.

In another aspect the invention provides methods for generating multiple copies of a polynucleotide sequence attached to a binding partner by:(a) hybridizing a single stranded DNA template with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion, wherein the single stranded DNA template is attached to a binding partner; (b) extending the composite primer with DNA polymerase; (c) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the template and repeats primer extension by strand displacement to produce displaced primer extension product; (d) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, whereby multiple copies of the polynucleotide sequence attached to the binding partner are produced.

In another aspect, the invention provides methods of determining presence or absence of an analyte in a sample by extending a composite primer comprising an RNA portion and a 3' DNA portion in a complex comprising: (a) an oligonucleotide template attached to a binding partner bound to an analyte; and (b) a primer extension product produced by extension of a composite primer comrpsing an RNA portion and a 3' DNA portion, wherein RNA from the extension product has been cleaved by an enzyme that cleaves RNA from an RNA/DNA hybrid such that the composite primer can hybridze to the oliognucleotide template and be extended by DNA polymerase, whereby the cleaved primer extension product is displaced, and whereby detection of the cleaved primer extension product (which comprises a detectable identifying characteristic) indicates presence of the analyte in the sample.

In some embodiments of the foregoing methods, the analyte may be bound to an intermediate binding partner, binding of the analyte to an intermediate binding partner, wherein the intermediate binding partner binds to the binding partner that is attached to the oligonucleotide template, whereby the binding partner that is attached to an oligonucleotide template indirectly binds to analyte via the intermediate binding partner instead of by binding directly to the analyte, and whereby an analyte-binding partner complex is formed. For example, the intermediate binding partner may comprise an antibody specific to the analyte, for example, the analyte may, in some embodiments, comprise a member of the

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Botulinum toxin (BoNT) family. In some embodiments of the foregoing methods, the binding partner that has an attached oligonucleotide template may comprise secondary antibody specific to the intermediate binding partner antibody, for example, a secondary antibody to the antibody specific for a member of the BoNT family.

In some embodiments of the foregoing methods, the analyte may comprise a structure chosen from the group consisting of proteins, polypeptides, peptides, nucleic acid segments, carbohydrates, cells, microorganisms and fragments and products thereof, an organic molecule, and an inorganic molecule. The analyte may, for example, comprise a peptide, for example a member of the Botulinum toxin (BoNT) family.

In some embodiments of the foregoing methods, the RNA portion of the composite primer is 5' with respect to the 3' DNA portion, and in some of these embodiments, the 5' RNA portion is adjacent to the 3' DNA portion. In some embodiments of the foregoing mehods, the oligonucleotide template comprises a ssDNA portion, wherein the ssDNA portion has a length of about 25 to about 100 nucleotides, and in some embodiments the ssDNA portion has a length of about 25 to about 200 nucleotides.

In some embodiments of the foregoing methods, the enzyme that cleaves RNA is RNase H.

In some embodiments of the foregoing methods, the oligonucleotide template is covalently attached to the binding partner. In other embodiments of the foregoing methods, the oligonucleotide template is non-covalently attached to the binding partner.

In some embodiments of the foregoing methods, the detectable identifying characteristic is selected from the group consisting of size of the cleaved primer extension product or RNA transcript, sequence of the cleaved primer extension product or RNA transcript, and detectable signal associated with the cleaved primer extension product or RNA transcript. In some of these embodiments, the detectable identifying characteristic may comprise the sequence of the cleaved primer extension product or RNA transcript, wherein the sequence is detected by hybridizing the cleaved primer extension product or RNA transcript with a nucleic acid probe that is hybridizable to the cleaved primer extension product or RNA transcript, for example, the nucleic acid probe may comprise DNA. In some embodiments, the nucleic acid probe is provided as an array, for example wherein the array comprises the probe immobilized on a substrate fabricated from a material selected from the group consisting of paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, metal, polystyrene, and optical fiber.

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In some embodiments of the foregoing methods, the detectable signal is associated with a label on a deoxyribonucleoside triphosphate or ribonucleoside triphosphate or analog thereof that is incorporated during primer extension or during transcription.

In some embodiments of the foregoing methods, the detectable signal is associated with interaction of two labels, wherein the labels are on deoxynucleoside triphosphates or ribonucleoside triphosphates or analogs thereof, and wherein one or both of the labels is incorporated during primer extension or during RNA transcription. In some of these embodiments, one label is on a deoxyribonucleoside triphosphate or analog thereof that is incorporated during primer extension and another label is on a deoxyribonucleoside triphosphate or analog thereof located in the primer portion of the primer extension product.

In some embodiments of the foregoing methods, the analyte-binding partner complex, if present, is separated from unbound binding partner by capture of the analyte on a solid surface that comprises a capture partner specific for said analyte.

In some embodiments of the foregoing methods, the analyte(s) or analyte-binding partner complex(s) is attached to a solid surface. In other embodiments of the foregoing methods, the analyte(s) analyte-binding partner complex(s) is in solution.

As this disclosure makes clear, contacting a sample includes contacting an analyte in the sample, if present.

The invention also provides compositions (including complexes) as well as kits.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 shows a detectable complex of an analyte and a binding partner, where the binding partner is attached to an oligonucleotide template.

FIG. 2 shows capture of an analyte-binding partner complex on a solid surface.

FIG. 3 shows a complex formed by an analyte that is bound to an intermediate binding partner, which is bound to a binding partner attached to an oligonucleotide template.

FIG. 4 shows capture of the complex of FIG 3 on a solid support.

FIG. 5 shows a "multiplex" analyte (termed herein as a "multisubunit analyte" or "multiple-subunit analyte"), composed of two different subunits, bound to two different binding partners, each of which is attached to an oligonucleotide template.

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FIG. 6 shows a single analyte bound to two binding partners, each of which is attached to an oligonucleotide template.

FIG. 7 shows an analyte-binding partner complex of analyte and three binding partners, two of which are attached to oligonucleotide templates and the third of which is attached to a capture moiety (which can be, for example, a capture oligonucleotide).

FIG. 8 shows capture of the complex of FIG. 7 on a solid support by binding of the capture moiety of the analyte-binding partner complex to its corresponding capture moiety on the support.

FIG. 9A-C shows enhanced amplification by RNA transcription to produce sense RNA, utilizing single primer isothermal amplification (SPIA™) and a template switch oligonucleotide containing a propromoter to produce dsDNA for transcription.

FIG. 10 A-C shows single primer isothermal linear amplification (SPIA™) of nucleic acid to produce ssDNA that is complementary to the amplified strand, using a blocker sequence.

FIG. 11 A-D shows enhanced single primer isothermal linear amplification of nucleic acid using a propromoter template oligonucleotide and RNA polymerase to produce sense RNA.

FIGSs 12A and 12B show single primer isothermal amplification (SPIA™) of an oligonucleotide template.

MODES FOR CARRYING OUT THE INVENTION

Overview of the Invention and Its Advantages

The invention provides methods, compositions and kits for ultra sensitive detection and quantification of the interaction of two or more molecules to form a specific complex. In particular, the invention discloses novel methods, compositions and kits for ultrasensitive

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detection and/or quantification of analyte(s). The invention further provides methods, compositions and kits for detection and quantification of multiple-subunit analytes and interaction of multiple binding partners with single or multiple analytes.

The methods generally comprise using a binding partner to the analyte, where the binding partner is attached to an oligonucleotide template, and further generally comprise amplifying a portion of the oligonucleotide template using an RNA/DNA composite primer, optionally a termination sequence, and, in embodiments in which transcription is used, a propromoter oligonucleotide sequence, to produce amplification product that comprises a detectable identifying characteristic. As this disclosure makes clear, reference to amplifying "a portion" of the template refers to amplifying at least a portion of the template, and that at least a portion (but not necessarily only a portion) of the template is amplified. Reference to amplifying a sequence complementary to "a portion" of the oligonucleotide template generally refers to amplifying a sequence complementary to the oliognucleotide template, without implying that the complementary sequence corresponds to the entire oligonucleotide template.

Amplification products are characterized to determine the presence and/or quantity of the analyte in a sample. Conversely, lack of, or insignificant amounts of, amplification products indicates absence of the analyte in a sample. "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or *de minimus* levels, generally due to lack of significant accumulation of product.

As a general summary, the methods work as follows: a binding partner is provided that binds to analyte, if present, to form an analyte-binding partner complex. In some embodiments, a single analyte is detected using a single binding partner. In other embodiments, multiple sites on a single analyte are detected using multiple binding partners. In yet other embodiments, multiple subunit analyte is detected using a single or multiple binding partners. In still other embodiments, multiple analytes are detected using a single binding partner. In yet still other embodiments, multiple analytes are detected using multiple (different) binding partners. In other embodiments, analyte binds to one or more intermediate binding partners, one or more of which bind to a binding partner attached to an oligonucleotide template.

In some embodiments, after binding partner binds to analyte, unbound binding partner is removed or separated from bound binding partner. It is understood that, for purposes of this invention, removal need not be complete and absolute removal, but removal or separation sufficient to permit assessment of presence or absence of analyte (i.e., without significant

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interference or "noise" arising from amplification of oligonucleotide template attached to unbound binding partner). Alternatively, in other embodiments the two are not separated but only bound binding partner is available for amplification of its oligonucleotide template. A portion of the oligonucleotide template attached to the binding partner is then amplified.

As a general summary, the amplification methods work as follows: a composite RNA/DNA primer forms the basis for replication of at least a portion of the oligonucleotide template. In some embodiments, a termination sequence provides the basis for an endpoint for the replication by either diverting or blocking further replication along the oligonucleotide template, but this is optional. As described below, in some embodiments, the polynucleotide comprising a termination sequence is a template switch oligonucleotide (TSO), which contains sequences that are not of sufficient complementarity to hybridize to the oligonucleotide template (in addition to sequences which are of sufficient complementary to hybridize); in other embodiments, the termination sequence comprises primarily sequences that are of sufficient complementarity to hybridize to the oligonucleotide template. DNA polymerase effects copying of the portion of the oligonucleotide template from the primer. An enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNase H) cleaves (removes) RNA sequence from the hybrid, leaving sequence on the oligonucleotide template available for binding by another composite primer. Another strand is produced by DNA polymerase, which displaces the previously replicated strand, resulting in displaced extension product that is ssDNA complementary to at least a portion of the oligonucleotide template. In some embodiments, a second composite primer may bind to the displaced extension product and replicate it, resulting in a ssDNA that is identical to the original portion of the oligonucleotide template. It will be understood by those of skill in the art that "identical," in this context, includes ssDNA that contains non-identical bases introduced through normal errors in the polymerization process. Optionally, a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product (which can be, for example, a template switch oligonucleotide or propromoter template oligonucleotide) that contains sequences of sufficient complementarity to hybridize to the 3' end of the displaced extension product, binds to the displaced primer extension product. The promoter drives transcription (via DNA-dependent RNA polymerase) to produce sense RNA products.

Accordingly, the amplification methods of the invention provide methods of producing at least one copy of a portion of an oligonucleotide template (generally, methods of amplifying a portion of an oligonucleotide template) comprising combining and reacting the

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following: (a) a single-stranded oligonucleotide template (attached to a binding partner) comprising a portion to be amplified; (b) a composite primer comprising an RNA portion and a 3' DNA portion; (c) a DNA polymerase; (d) deoxyribonucleoside triphosphates or suitable analogs; (e) an enzyme, such as RNaseH, which cleaves RNA from an RNA/DNA duplex; (f) optionally, a second primer comprising an RNA portion and a 3' DNA portion; and (g) optionally, a polynucleotide comprising a termination sequence, such as any of those described herein, which comprises a portion (or region) which hybridizes to the oligonucleotide template. The termination sequence is optional, however, as the oligonucleotide template is synthetic and thus may contain, as part of the ssDNA of the template, termination sites or other sites that cause polymerization to cease. The combination is subjected to suitable conditions such that (a) the composite primer (and, optionally, a polynucleotide comprising a termination sequence) hybridizes to the oligonucleotide template; (b) primer extension occurs from the composite primer, to form a duplex; (c) RNaseH cleaves RNA of the composite primer from the RNA/DNA duplex; (d) another composite primer hybridizes to the oligonucleotide template, and another round of primer extension (mediated by DNA polymerase) occurs, displacing the strand already copied from the template; (e) optionally, the second composite primer hybridizes to the displaced primer extension product and steps (a) through (d) are repeated on the displaced primer extension product.

Optionally, the following is also included in the amplification reaction (either at the same time as those components listed above or added separately): (f) a polynucleotide comprising a propromoter sequence (which can be in any of a number of forms, as described herein) and a region which hybridizes to the displaced primer extension product; (g) ribonucleoside triphosphates or suitable analogs; and (h) RNA polymerase, under conditions such that transcription of the displaced strand can occur. Details regarding the various components of the methods of the present invention are provided below.

The amplification step of the invention provides amplification product corresponding to a portion of the oligonucleotide template that has been multiplied manyfold (e.g., 10^{12}-fold).

Detection of analyte (if present) is through detection of product formation, which may be indicated and detected in a number of ways that arise from the process of primer extension. In some embodiments, detection of analyte is through detection of a detectable identifying characteristic of the amplification product. In these embodiments, the amplification product comprises a detectable identifying characteristic. Examples of such detectable identifying characteristics are size, sequence, and label. These may be used

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singly or in combination in the detection and/or quantification of the amplification product. When size is used, various methods known in the art may be used to characterize the size of the amplification product, such as electrophoresis and other techniques described herein. When sequence is used, the amplification products may be detected by any means known in the art of detecting an oligonucleotide sequence. For example, in some embodiments the method of detection and/or quantification is hybridization to complementary oligonucleotide(s) immobilized on a solid support. Alternatively, in some embodiments, detection is accomplished by detecting pyrophosphate released during amplification of the oligonucleotide template. Thus, detection of product can include indirect detection such as detection of pyrophosphate, or other indicia of extension.

When a label is used as a detectable identifying characteristic, the optical properties of a label may be altered subsequent to attachment to the primer. For example, fluorescence polarization of fluorescent dyes attached to free nucleotide triphosphates has been shown to change upon attachment to a primer by a polymerase. When the amplification product is labeled and the method of detection is immobilization on a solid support, the immobilization of the amplification product on the solid support and detection of the label indicates the presence of the product in the reaction mixture. It is also possible to detect altered spectral properties of a label by means of energy transfer. When the primer is labeled by a donor or acceptor dye, and/or the nucleotide triphosphates, or their analogs, are labeled with acceptor or donor dyes, respectively, the incorporation of the dyes into an amplification product enables energy transfer between the donor and acceptor dyes, thus resulting in specific spectral properties of the attached dyes. Fluorescence dyes useful for this detection mode are known in the art and described herein.

The methods of the invention are further useful for multiple analysis of analyte-binding partner complexes. That is to say, various oligonucleotide templates that are attached to different binding partners that bind to different analytes, may be amplified simultaneously in a single reaction mixture. In such embodiments, the composite primer binding regions of the template oligonucleotides may be the same or different. If the same composite binding region is used, only a single composite primer need be provided, and the relative quantities of various analytes in the analyte-binding partner complexes is more accurately determined.

The methods of the invention further provide for halting the reactions at various timepoints, and the further reaction of the complexes and mixtures that are produced at each timepoint.

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The invention also provides compositions, kits, and systems useful in the methods of the invention. As described herein, the invention further provides complexes that comprise, for example, an analyte and a binding partner that is attached to an oligonucleotide template.

Other methods which use the methods and the amplified products described herein are provided below.

Advantages of the invention

The methods of the invention provide several significant advantages over other methods of analyte detection and/or quantification using nucleic acid amplification. The formation of primed oligonucleotide template, primer extension and displacement of the previously generated extension product is dependent on the cleavage of RNA of the hybridized primer by a ribonuclease activity. Thus, the primer extension product is lacking the 5'-most portion of the primer. Consequently, if a second round of replication is used, or if transcription-based methods are used, the second DNA replication product or the RNA transcription product does not contain at its 3' end the sequence complementary to this portion of the primer. Thus, the amplification products are not capable of hybridizing to the (first) primer for productive amplification, making the amplification methods of the invention resistant to non-specific amplification due to contamination with products generated by prior amplifications reactions. This feature clearly distinguishes it from other known methods of analyte detection and/or quantification that rely on non-linear nucleic acid amplification, such as PCR, NASBA and the like, and renders the methods of the invention suitable for open tube platforms commonly used in clinical laboratories, high throughput testing sites, and the like.

The methods of the invention do not require thermocycling in order to detect and/or quantify analyte, in that amplification can be performed isothermally. This feature provides numerous advantages, including facilitating automation and adaptation for high throughput analyte detection and/or quantification. Other methods that have been reported require thermal cycling for the separation of amplification products from the original sequence. The isothermal reaction is faster than that afforded by thermal cycling and is suitable for miniaturized devices.

Another advantage of the analyte detection/quantification methods of the invention is that only a single primer is required. A single primer is utilized to provide unidirectional primer extension that results in amplification of a portion of the oligonucleotide template. This obviates the numerous drawbacks associated with having to use primer pairs, for

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example cost of designing and making two sets of primers and the increased probability that amplified products are the result of non-specific priming.

The products of the amplification according to the methods of the invention are single stranded and are readily detectable by various known nucleic acid detection methods. It is understood that, generally, "detection" of a product (such as a cleaved amplification product comprising a detectable identifying characteristic) means detection of significant amounts of product arising from cycling (i.e., repeated cycles of a reaction). The cycling results in accumulated amplification product. Lack of cycling (due to, or example, absence of an analyte, and thus absence of an analyte-binding partner complex) results in *de minimus*, or insignificant amount of product which, for purposes of the methods of the invention, is not "detected".

The methods are also suitable for quantitative determinations. Quantification of the accumulated amplification product permits quantification of the analyte or analytes in the sample.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

Primers, oligonucleotides and polynucleotides employed in the present invention can be generated using standard techniques known in the art.

Definitions

"Amplification," as used herein, generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies," as used herein, means at least 2 copies. A "copy" does not necessarily mean perfect sequence complementarity or identity to the template sequence. A "copy" includes a nucleic acid sequence that is hybridizable

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(preferably complementary) to the sequence of interest; e.g., to the portion of the oligonucleotide template to be amplified. Copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template), and/or sequence errors that occur during DNA polymerization.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These

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alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.oligonucleotide

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. Oligonucleotides in the present invention include the composite primer(s), TSO, PTO and blocker sequence. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

"Oligonucleotide template," as used herein, refers to an oligonucleotide, a portion of which serves as a template for a DNA polymerase to produce a strand complementary to the portion of the template strand. Generally an oligonucleotide template is attached to a binding partner.

A "primer" is generally a short single-stranded polynucleotide (often referred to as an oligonucleotide), generally with a free 3'-OH group, that binds to a particular polynucleotide. It can be used to promote polymerization of a polynucleotide complementary to the original polynucleotide.

A "termination polynucleotide sequence" or "termination sequence," as used interchangeably herein, is a polynucleotide sequence which effects cessation of DNA replication by DNA polymerase with respect to the oligonucleotide template. A termination sequence comprises a portion (or region) that generally hybridizes to the template at a location 5' to the termination point (site). The hybridizable portion (e.g., the portion that hybridizes) may or may not encompass the entire termination sequence. Examples of suitable termination polynucleotide sequences (such as blocker sequences and TSOs) are provided herein. Termination sequences are optional in the methods of the present invention.

"Blocker sequence," or "blocking sequence" as used interchangeably herein, is an example of a termination sequence, and refers to an oligonucleotide that binds, generally with high affinity, to the oligonucleotide template nucleic acid at a location 5' to the termination site and effects cessation of DNA replication by DNA polymerase with respect

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to the template comprising the target sequence. Its 3' end may or may not be blocked for extension by DNA polymerase. Blocker sequences are optional in the methods of the present invention.

"Termination site," or "termination point," as used interchangeably herein, refers to the site, point or region of the oligonucleotide template that is last replicated by the DNA polymerase before termination of polymerization (generally, primer extension) or template switch. Optionally, for example, with respect to a TSO, it is the position or region in the oligonucleotide template that is complementary to the 3' end of the primer extension product prior to switching template from the template polynucleotide to the unhybridized portion of the TSO.

"Protopromoter sequence," and "propromoter sequence," as used herein, refer to a single-stranded DNA sequence region which, in double-stranded form is capable of mediating RNA transcription. In some contexts, "protopromoter sequence," "protopromoter," "propromoter sequence," "propromoter," "promoter sequence," and "promoter" are used interchangeably.

"Template switch oligonucleotide (TSO)," as used herein, refers to an oligonucleotide that comprises a portion (or region) that is hybridizable (e.g., that hybridizes) to a template at a location 5' to the termination site of primer extension and that is capable of effecting a template switch in the process of primer extension by a DNA polymerase. TSOs are generally known in the art. "Template switch" refers to a change in template nucleic acid, generally from the target nucleic acid to the unhybridized portion of a TSO, during the course of a single round of primer extension. The use of a TSO in the methods of the invention is optional.

"Propromoter template oligonucleotide (PTO)," as used herein, refers to an oligonucleotide that comprises a propromoter sequence and a portion, generally a 3' portion, that is hybridizable (e.g., that hybridizes) to the 3' region of a primer extension product. The propromoter sequence and the hybridizable portion may be the same, distinct or overlapping nucleotides of an oligonucleotide.

A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as is described herein, given certain components of a reaction, and the type of product(s) of the reaction, existence of a complex can be inferred. For purposes of this invention, a complex is generally an intermediate with respect to the final reaction product(s).

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A "system," as used herein, includes a device, apparatus or machinery (e.g., automated) for carrying out the methods of the invention.

A "portion" or "region," used interchangeably herein, of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a region or portion is at least about any of 3, 5, 10, 15, 20, 25 contiguous nucleotides.

A "reaction mixture" is an assemblage of components, which, under suitable conditions, react to form a complex (which may be an intermediate) and/or a product(s).

"A", "an" and "the", and the like, unless otherwise indicated include plural forms.

In accordance with a well-established principle of patent law, "comprising" means including.

Conditions that "allow" or "permit" an event to occur or conditions that are "suitable" for an event to occur, such as hybridization, primer extension, oligonucleotide ligation and the like, or "suitable" conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the nucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as hybridization, cleavage, primer extension.

"Microarray" and "array," as used interchangeably herein, refer to an arrangement of a collection of nucleotide sequences in a centralized location. Arrays can be on a solid substrate, such as a glass slide, or on a semi-solid substrate, such as nitrocellulose membrane. The nucleotide sequences can be DNA, RNA, or any permutations thereof.

The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "3'-DNA portion," "3'-DNA region," "3'-RNA portion," and "3'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 3' end of the polynucleotide or oligonucleotide, and may or may not include the 3' most nucleotide(s) or moieties attached to the 3' most nucleotide of the same polynucleotide or oligonucleotide. The "3' most nucleotide" (singular form) refers to the 3' last nucleotide of a polynucleotide or oligonucleotide. The 3' most nucleotides (plural form) includes the 3'

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most nucleotide and can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

The term "5'-DNA portion," "5'-DNA region," "5'-RNA portion," and "5'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 5' end of the polynucleotide or oligonucleotide, and may or may not include the 5' most nucleotide(s) or moieties attached to the 5' most nucleotide of the same polynucleotide or oligonucleotide. The "5' most nucleotide" (singular form) refers to the 5' first nucleotide of a polynucleotide or oligonucleotide. The 5' most nucleotides (plural form) includes the 5' most nucleotide and can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

"Hybridizable," or "capable of hybridizing" as used herein, refers to the capability and/or ability of two polynucleotide sequences to hybridize through at least some degree of complementary base pairing, under conditions used in any of the methods described herein; i.e., at the temperature, pH, ionic concentrations, and the like, used in carrying out the methods of the invention. As such, a sequence (such as a primer) which is hybridizable to another sequence (such as an oligonucleotide template) hybridizes to that sequence under suitable conditions.

"Detectable identifying characteristic," as used herein, refers to characteristics of a reaction product that indicates its presence, wherein the characteristic is detectable by methods known in the art.

"Detection" includes any means of detecting, including direct and indirect detection. For example, "detectably fewer" products may be observed directly or indirectly, and the term indicates any reduction (including no products). Similarly, "detectably more" product means any increase, whether observed directly or indirectly.

The term "analyte" as used herein refers to a substance to be detected or assayed by the methods of the present invention, for example, a compound whose properties, location, quantity, presence, absence, and/or identity is desired to be characterized. Typical analytes may include, but are not limited to proteins, polypeptides, peptides, nucleic acid segments, carbohydrates, cells, microorganisms and fragments and products thereof, organic molecules, inorganic molecules, or any substance for which attachment sites for binding partner(s) can be developed. In a related application, "analyte" also referred to a moiety that serves to indirectly detect a structure of interest, by binding (either directly or indirectly) to a structure of interest (thus, more properly, "analyte" in this context refers to an "intermediate binding partner"; for clarity, and in accordance with what is conveyed herein, former

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reference to "structure of interest" has been replaced by "analyte"; and, where intermediate binding partners are used, former reference to "analyte" has been replaced by reference to "intermediate binding partner").

It is understood that, with respect to all embodiments described herein, as generally "comprising" components or aspects, the invention also includes embodiments which "consist essentially of" these components or aspects. The invention also includes embodiments which "consist of" these components or aspects. This applies to all embodiments described herein.

Methods of the invention

I. Binding of analyte and binding partner

A. Components

1. Analyte

Generally, the first reaction in the methods of the invention is the binding of an analyte or analytes to a binding partner or binding partners to form an analyte-binding partner complex. One or more of the binding partners in the analyte-binding partner complex is attached to oligonucleotide template(s), the amplification of a portion of which provides the signal indicating the presence of the analyte.

The analyte that forms a complex with a binding partner or binding partners may be any substance or combination of substances whose characteristics are sought to be determined. Such characteristics include, but are not limited to presence, absence, quantity, state of assembly, conformational state, or binding state.

In some embodiments of the methods of the invention, the analyte includes one binding site for a binding partner (see Figure 1). In some embodiments the analyte includes multiple binding sites for multiple binding partners; in these embodiments the binding sites may be for the same or different binding partners (see Figure 6). It will be appreciated that if an analyte possesses multiple binding sites, the accessibility of the binding sites for their respective binding partners will determine the nature of the complex formed upon binding of analyte to binding partner(s), and the composition of the complex may be detected and provide information as to the state of the analyte. For example, an analyte that is a ligand may possess two or more binding sites, one or more of which is blocked upon binding of the ligand to its receptor; the presence of complexes of ligand with one or more binding partners

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may be detected, allowing one to determine presence or absence of ligand, presence or absence of binding of ligand to receptor, and relative and/or absolute quantities of bound versus unbound ligand.

In some embodiments the analyte is a single entity, and in other embodiments multiple units may be in association to form a multisubunit analyte or multiple-subunit analyte (see Figure 5). Examples of multisubunit analytes include, but are not limited to, multisubunit proteins (including multisubunit enzymes), replication and repair complexes where multiple proteins interact with a polymerase or a repair enzyme, assemblies of large and small ribosomal subunits, and other multisubunit assemblies or molecular or supramolecular compositions which may vary in structure and size. The composition and/or organization of a multisubunit analyte may change with changing conditions in the environment in which it is located. The environment may be in a non-living or living system; examples of environments in living systems include supramolecular assemblies, organelles, cytoplasm, nucleoplasm, membranes, cells, tissues, organs, and/or organisms. The methods of the invention allow one to determine the state of organization of the multisubunit analyte, e.g., the stage of assembly of a supramolecular complex. In some embodiments, the state of organization may be detected by providing multiple binding partners, each of which is specific for a different unit of the multi-subunit analyte. In some embodiments, the state of organization may be detected by providing binding partners that bind to conformations that correspond to different states of organization of the multisubunit analyte. An example of the latter is where the multi-subunit analyte is a multisubunit protein, where the subunits must be associated in order to form the site that the binding partner binds to. Thus, binding of binding partner indicates the association of the subunits into the multisubunit protein.

In some embodiments an intermediate binding partner binds to the analyte, and a second binding partner, to which oligonucleotide template is attached, binds to the intermediate binding partner (see Figures 3 and 4). It will be appreciated that there may also be any number of intermediate binding partners between analyte and the binding partner that is attached to an oligonucleotide template. It will also be appreciated that the binding partner that is attached to an oligonucleotide template in these "sandwich" embodiments may be specific for a class of intermediate binding partners rather than for a single intermediate binding partner. Thus, for example, the intermediate binding partner in a sandwich assay may be a monoclonal antibody specific for a particular hapten, while the binding partner that is attached to an oligonucleotide template may be a secondary antibody that is specific for a class of antibodies of which the intermediate binding partner antibody is a member, e.g., an

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anti-IgG antibody. Other intermediate binding partners for which the binding partner attached to an oligonucleotide template is specific may be specific for other haptens. Such sandwich methods allow the detection of any of a number of analytes using a class of intermediate binding partners, each of which is specific for each individual analyte, and a single binding partner attached to an oligonucleotide template, specific for the entire class of intermediate binding partners, obviating the necessity for attaching oligonucleotide templates to multiple binding partners specific for specific analytes.

Other combinations and permutations will be apparent to one of skill in the art.

The analyte (or analyte-binding partner complex) may be free in solution, or, in other embodiments, immobilized on a surface, e.g., as part of an array as discussed herein (see Figures 2, 4, and 8). Analyte(s) (or analyte-binding partner complex(es)) may be immobilized on a surface (substrate) fabricated from a material such as paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, polystyrene, silicon, metal, and optical fiber. Alternatively, analyte(s) (or analyte-binding partner complex(es)) may be immobilized on the surface (substrate) in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and/or cylinders. The analyte may be attached to a solid surface . The attachment may be covalent or non covalent. Means of attachment of analyte to solid surface are well-known in the art. See, e.g., U.S. Patent Nos. 6,309,843; 6,306,365; 6,280,935; 6,087,103 (and methods discussed therein).

Typical analytes may include, but are not limited to proteins, polypeptides and peptides, nucleic acid molecules or segments thereof, lipids, carbohydrates, supramolecular assemblies, organelles, cells, microorganisms and fragments and products thereof, organic molecules, inorganic molecules, or any substance for which one or more attachment sites for binding partner(s) naturally exist or can be developed.

In some embodiments of the invention the analyte is a protein, polypeptide, or peptide. In some of these embodiments, the analyte is a toxin. In some embodiments the analyte is a member of the Botulinum toxin (BoNT) family. The BoNT toxin that serves as an analyte in these embodiments may be present in any amount, including a single molecule. Strains of *C. botulinum* produce seven different BoNT, with toxin types A, B, E, and F being the main toxins that affect humans. The toxin consists of disulfide linked heavy and light chain, with three major domains. The receptor binding site is at the carboxy terminus of the heavy chain (HC or C-fragment). The binding domain mediates attachment to specific receptors on the presynaptic side of the synapse (Haberman and Dryer (1986) *Curr. Topics Microbiol.*

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Immunol. 129: 93-179). The N-terminus of the heavy chain (HN) is a channel-forming domain, which permits the light chain to cross the membrane of the endocytic vesicle. The light chain is a zinc protease, which cleaves one of several proteins on the synaptosomal complex.

2. Binding partners

Binding partners of the invention include binding partners that bind to the analyte as well as intermediate binding partners that bind to other binding partners. Some intermediate binding partners bind both to other binding partner(s) and to analyte.

The binding partner for the analyte may be any moiety that is capable of binding to the analyte with a desired degree of specificity. As described above, a binding partner may be specific for a single analyte, for multiple analytes in association (such as multisubunit analyte), or for a class of analytes, and/or an analyte may have sites for binding more than one binding partner.

Examples of analyte-binding partner pairs, as well as intermediate binding partner-binding partner pairs include, but are not limited to, receptor ligand, antibody-antigen, two or more antibodies binding to one or more antigens, enzyme-substrate, enzyme-inhibitor, enzyme-cofactor, nucleic acid-probe, subunits of a multi subunit entity, and the like. In all of these examples, as well as others that will be apparent to one of skill in the art, either one of the members of the pair may be binding partner or may be analyte.

In some embodiments, a binding partner may be an antibody, antibody fragment, or antibody derivative specific for an analyte. An antibody binding partner may be polyclonal or monoclonal, and may be human, non-human, chimeric, and/or humanized.

An antibody fragment is a fragment which contains the binding region of the antibody. Such fragments may be Fab-type fragments which are defined as fragments devoid of the Fc portion, e.g., Fab, Fab' and $F(ab')_2$ fragments, or may be "half-molecule" fragments obtained by reductive cleavage of the disulfide bonds connecting the heavy chain components of the intact antibody.

An antibody derivative is an artificial construct that is derived from the amino acid sequences of an antibody or antibodies and that retains the antigen specificity of the original antibody or antibodies. Two or more antibody derivatives may be combined in a single antibody derivative binding partner. Examples of antibody derivatives include, but are not limited to, humanized antibodies, chimeric antibodies, single chain fragment variable fragments (ScFv) and exocyclic peptide-based complementarity determining region (CDR)

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subunits. The availability of large ScFv and cyclic CDR libraries allows the creation of an antibody derivative binding partner for virtually any molecule. See Zhang et al. (2001) *PNAS*, 98: 5497-5502; Scott et al. (1999) *PNAS* 96:13638-13643; Barth et al. (2000) *J. Mol. Biol.* 301: 751-757. Other examples of antibody derivatives well-known in the art include conjugates.

As described above, the binding partner may be a "universal" binding partner that binds to a multiplicity of different analytes, such as an anti-IgG antibody.

3. Oligonucleotide template

At least one binding partner that binds directly or indirectly (e.g., via intermediate binding partner(s)) to the analyte is attached to an oligonucleotide template. A portion of the oligonucleotide template is amplified through composite primer-based, single primer isothermal amplification (SPIA™) primer extension, as described below. As used herein, a "portion" of the oligonucleotide template indicates at least a portion.

The oligonucleotide template includes a single stranded (ss) polynucleotide, such as DNA, sequence. For simplicity, DNA is exemplified herein. However, as this disclosure and the definition of oligonucleotide make clear, other nucleic acid embodiments are contemplated and included in the invention. The DNA sequence may include non-standard nucleotides as well as standard nucleotides. The DNA sequence contains at least one primer binding region that is complementary to a composite primer (discussed below) and also contains at least one primer extension region, onto which the primer is extended by a DNA polymerase. The DNA sequence may be flanked on its 3' and/or 5' ends by other, non-DNA components such as RNA or PNA, or by non-nucleic acid components such as peptide sequences. The length of the ssDNA portion of the oligonucleotide template is, at least 25, at least 30, or at least 40, or at least 50, or at least 70, or at least 100, or at least 200, or at least 400 nucleotides. The maximum length of the ssDNA portion is determined by the sequences that are included for composite primer binding, primer extension, termination, and/or other sequences that may optionally be added in order to improve the performance and/or efficiency of the amplification and detection of the template, including, but not limited to, sequences designed to bind to probe oligonucleotides, or sequences designed to bind to capture (immobilized or non-immobilized) oligonucleotides.

The oligonucleotide template is attached to the binding partner, either by its 5' end or its 3' end, although it may be attached near the 5' or 3' end. Methods of attaching a template oligonucleotide to binding partners are known in the art. See, e.g. U.S. Patent Nos.

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6,309,843; 6,306,365; 6,280,935; 6,087,103 (and methods discussed therein). The attachment may be covalent or noncovalent. An example of a noncovalent attachment is an avidin-biotin interaction; thus, the binding partner may be conjugated to streptavidin and the oligonucleotide template may be conjugated to biotin, or bis-biotin may be employed. Another example is his tags binding to chelated metals (see, e.g., Janknecht, *et al.*, 1991, *Proc. Nat. Acad. Sci. USA*, 88:8972-8976) . These methods are known in the art. See, e.g., U.S. Patent No. 6,153,442. In some embodiments, a tether or linker moiety may be employed between the oligonucleotide template and the binding partner. Such tethers and linkers are well-known in the art.

As stated, at least a portion of the oligonucleotide template serves as a primer extension template. This portion, as well as, generally, a portion of the primer and, optionally, a portion of a TSO or other blocker, serves as a basis for the formation of amplification product. Thus amplification product, discussed in more detail below, is complementary to these combined portions. The sequences of these portions may be chosen so that the amplification product has various desirable characteristics. Thus, the portion of the oligonucleotide template that serves as a primer extension template may contain sequences that, alone or in combination with sequences of these other portions, produces amplification products that hybridize to an oligonucleotide attached to a solid support for capture, and/or that hybridize to a labeled oligonucleotide for detection.

The oligonucleotide template may be synthesized or produced by methods well-known to those of skill in the art, such as recombinant methods.

B. Binding

The analyte(s) and the binding partner(s) are contacted under conditions that allow binding. Such conditions depend on the nature of the analyte and the binding partners and are well-known in the art. See, e.g., U.S. Patent Nos. 6,083,689; 5,985,548; 5,854,033; 5,665,539; 5,849,478; 6,255,060; 6,183,960; 5,328,985; 6,210,884; and Sano et al. *Science*(1992) 258: 120-122; Huang et al. *Lett Appl. Microbiol.* (2001) 32:321-325; and Zhang et al. PNAS USA (2002) 98: 5497-5502. In some embodiments, the binding of the binding partner to the analyte, or to other binding partner(s), is non-covalent, e.g., in most ligand-receptor binding. In some embodiments the binding may be covalent, e.g., when the analyte is an enzyme and the binding partner is an inhibitor that acts by forming a covalent bond at the active site.

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The interaction to be detected or quantified may be carried out using a solid surface, when analyte is immobilized, or in solution. The interaction of the analyte and binding partners, either directly or indirectly (e.g., in a sandwich assay), results in formation of a complex comprising the oligonucleotide template. One or more complexes may be formed simultaneously, each comprising one or more oligonucleotide templates. The detection of the association of specific target/reporter oligonucleotide in the complex may be carried out by separation of the complexes from the non-reacted labeled binding partner, exposing the separated components to conditions which result in generation of multiple copies of oligonucleotide molecules complementary to the target/reporter and detection and, optionally, quantification of the multiple copies. The generation of the multiple copies is carried out by combining the separated components with composite primer of the invention, and amplification reagents.

II. Separation of bound from unbound binding partner

The binding partner-analyte complex may be separated (removed) from unbound binding partner(s) by any suitable means. It is understood that, for purposes of this invention, removal need not be complete and absolute removal, but removal or separation sufficient to permit assessment of presence or absence of analyte (i.e., without significant interference or "noise" arising from amplification of oligonucleotide template attached to unbound binding partner). Such means are well-known in the art. See, e.g., U.S. Patent Nos. 6,083,689; 5,985,548; 5,854,033; 5,665,539; 5,849,478; 6,255,060; 6,183,960; 5,328,985; 6,210,884; and Sano et al. (1992) *Science* 258: 120-122; Huang et al. (2001) *Lett Appl. Microbiol.* 32:321-325; and Zhang et al. (2002) *Proc. Natl. Acad. Sci. USA* 98: 5497-5502. Figures 7 and 8 illustrate an exemplary possibility for separation of analyte-binding partner complex from unbound binding partner. Analyte binds to binding partner(s) that is/are attached to oligonucleotide template(s) for amplification (Fig. 7 shows an analyte bound to two such binding partners, but it will be understood that one such binding partner, or more than two such binding partners, may bind to the analyte). In addition, a binding partner that is attached to a capture moiety binds to the analyte. The capture moiety may be any structure that binds to another structure, e.g., an oligonucleotide that binds to its complementary oligonucleotide, or one member of a streptavidin/biotin pair. Thus, in this example, the analyte-binding partner complex includes binding partner(s) attached to capture moiety(ies), and other binding partner(s) attached to oligonucleotide template(s) for amplification. The analyte-binding partner complex binds to a solid support via interaction

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of the capture moiety attached to a binding partner in the complex with a capture moiety on the solid support (Fig. 8). For example, the capture moiety on the solid support may be the complementary oligonucleotide to an oligonucleotide attached to binding partner in the analyte-binding partner complex. Unbound binding partner may be removed by, e.g., washing.

Alternatively, bound and unbound binding partner need not be separated. In these embodiments, amplification methods are such that only, or substantially only, bound binding partner is subject to amplification and/or detection. As described above, in one embodiment the methods of the invention require a separation of the analyte-binding partner-oligonucleotide conjugate complex from uncomplexed conjugate. Otherwise, it is difficult to distinguish signal from the complex and from excess free binding partner conjugate. Most assays are also described as heterogeneous because they normally employ a two-phase, heterogeneous system for the separation step. Homogeneous assays occurring in a single phase without any separation are widely recognized as preferable whenever possible because they require less manipulation of the reaction mixture- typically only a series of reagent additions rather than the transfer and washing steps common to heterogeneous methods. Thus, they are faster, require less labor to perform, and are easier to automate. However, homogeneous assays are limited in many cases because the distinction between signal from bound and unbound labels is rarely complete. Consequently, a homogeneous assay with a given signal level will typically have a higher background signal and thus lower sensitivity than a similar heterogeneous assay.

In some embodiments, signal may be preferentially generated from an analyte-binding pair complex by taking advantage of the well-known "proximity effect": two or more functional groups, molecules, or even proteins will react more rapidly with each other when other structures hold them in close proximity to each other. The origin of this effect is well understood, arising from decreases in translational and rotational entropy on binding, and examples are known in a variety of fields. A related phenomenon, the "channeling" effect, has been exploited in the design of high-sensitivity homogeneous assays.

The present invention offers several possible ways to exploit the proximity effect by bringing reactants together. In particular, either polymerase or RNase is attached to a second binding partner for the analyte. Formation of a sandwich complex brings together a high local concentration of the enzyme and the oligonucleotide-binding partner-primer complex, allowing the enzyme to act more rapidly in either extending or digesting the primer compared to the same enzyme diffusing freely through solution. Consequently, this

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sandwich complex produces more extension product per unit time than is generated by uncomplexed product in solution. The rate enhancements depend on many factors, most notably the concentrations of the reagents in solution compared to the "local concentration" in the complex, but they can be several orders of magnitude.

This proximity effect might also be useful in conjunction with a SPIA™-enhanced heterogeneous assay (i.e., assay utilizing a separation step). In many high-sensitivity assays, detection is actually limited by background signal generated by label nonspecifically bound to surfaces. This is likely to be the case in particular with assays such as immuno-PCR where any nonspecifically bound material can be exponentially amplified. However, if a sandwich complex such as described above is formed on a surface, signal is generated only from that complex, but not from other binding partner conjugates bound nonspecifically to the surface. Thus it is possible to obtain both very high signal from the SPIA™ enhancement and very low background from a combination of washing (separation) and the proximity effect.

III. Amplification of oligonucleotide template

A. Components, reaction conditions, and procedures

1. Composite primer

The primer binding region of the oligonucleotide template hybridizes to a composite primer under suitable conditions, and the composite primer is extended along the primer extension template region of the oligonucleotide template, and optionally along other components such as a template switch oligonucleotide (see below), by a DNA polymerase to produce a primer extension product. The composite primer is composed of RNA and DNA portions. The composite design of the primer is important for subsequent displacement of the primer extension product by binding of a new (additional) composite primer and the extension of the new primer by the polymerase. In addition, cleavage of RNA of the primer extension product leads to generation of amplification product which is not a substrate for amplification by the composite primer, as described below. Composite primers, as well as the method to amplify (SPIA™) are described in, e.g., U.S. Patent No. 6,251,639.

Composite primers for use in the methods and compositions of the present invention comprise at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence of the primer binding region of the oligonucleotide template independent of hybridization of the DNA portion(s) to a sequence on the primer binding region of the oligonucleotide template; and (b) being cleaved with a ribonuclease when hybridized to the oligonucleotide

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template DNA. The composite primers bind to the primer binding region of the oligonucleotide template to form a partial heteroduplex in which only RNA of the primer is cleaved upon contact with a ribonuclease such as RNase H, while the oligonucleotide template strand remains intact, thus enabling annealing of another composite primer.

The composite primers also comprise a 3' DNA portion that is capable of hybridization to a sequence of the primer binding region of the oligonucleotide template such that its hybridization to the oligonucleotide template is favored over that of the nucleic acid strand that is displaced from the oligonucleotide template by the DNA polymerase. Such primers can be rationally designed based on well known factors that influence nucleic acid binding affinity, such as sequence length and/or identity, as well as hybridization conditions. In a preferred embodiment, hybridization of the 3' DNA portion of the composite primer to its complementary sequence in the primer binding region of the oligonucleotide template is favored over the hybridization of the homologous sequence in the 5' end of the displaced strand to the oligonucleotide template.

Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO99/42618 (and references cited therein). The composite primer comprises a combination of RNA and DNA (see definition above), with the 3'-end nucleotide being a nucleotide suitable for nucleic acid extension. The 3'-end nucleotide can be any nucleotide or analog that when present in a primer, is extendable by a DNA polymerase. Generally, the 3'-end nucleotide has a 3'-OH. Suitable primers include those that comprise at least one portion of RNA and at least one portion of DNA.

Composite primers can comprise a 5'-RNA portion and a 3'-DNA portion (in which the RNA portion is adjacent to the 3'-DNA portion); or 5'- and 3'-DNA portions with an intervening RNA portion. Accordingly, in one embodiment, the composite primer comprises a 5' RNA portion and a 3'-DNA portion, preferably wherein the RNA portion is adjacent to the 3'-DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion (i.e., an RNA portion between the two DNA portions). In yet another embodiment, the composite primer of the present invention comprises a3'-DNA portion and at least one intervening RNA portion (i.e., an RNA portion between DNA portions).

The length of an RNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 25, more preferably from about 3 to about 20, even more preferably from about 4 to about 15, and most preferably from about 5 to about 10 nucleotides. In some embodiments of a composite primer comprising a

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3'-DNA portion and an RNA portion, an RNA portion can be at least about any of 1, 2, 3, 4, 5 nucleotides, with an upper limit of about any of 10, 15, 20, 25, 30 nucleotides.

The length of the 5'-RNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be at least about any of 1, 2, 3, 5, with an upper limit of about any of 5, 6, 7, 10 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 or nucleotides.

The length of an intervening RNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. The length of an intervening RNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about

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6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. In a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 3' DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

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In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In certain embodiments of the primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion (i.e., any DNA portion other than the 3'-DNA portion) in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides. The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some

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embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. It is understood that the lengths for the various portions can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

In some embodiments, the 5'-DNA portion of a composite primer includes the 5'-most nucleotide of the primer. In some embodiments, the 5'-RNA portion of a composite primer includes the 5' most nucleotide of the primer. In other embodiments, the 3'-DNA portion of a composite primer includes the 3' most nucleotide of the primer. In other embodiments, the 3'-DNA portion is adjacent to the 5'-RNA portion and includes the 3' most nucleotide of the primer (and the 5'-RNA portion includes the 5' most nucleotide of the primer).

The total length of the composite primer can be preferably from about 10 to about 40 nucleotides, more preferably from about 15 to about 30 nucleotides, and most preferably from about 20 to about 25 nucleotides. In some embodiments, the length can be at least about any of 10, 15, 20, 25 nucleotides, with an upper limit of about any of 25, 30, 40, 50 nucleotides. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention. In some embodiments the RNA portion of the composite primer consists of, for example, about 7 to about 20 nucleotides and the DNA portion of the composite primer consists of, for example, about 5 to about 20 nucleotides. In other embodiments, the RNA portion of the composite primer consists of, for example, about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of, for example, about 7 to about 20 nucleotides.

To achieve hybridization (which, as is well known and understood in the art, depends on other factors such as, for example, ionic strength and temperature), composite primers for use in the methods and compositions of the present invention are preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the primer binding region of the oligonucleotide template. The individual DNA and RNA portions of the composite primers are preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the primer binding region of the oligonucleotide template.

The hybridization conditions chosen depend on a variety of factors known in the art, for example the length and type (e.g., RNA, DNA, PNA) of primer and primer binding region of

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the oligonucleotide template. General parameters for specific (i.e., stringent) hybridization conditions for nucleic acids are described in Sambrook (1989), supra , and in Ausubel (1987), supra. Useful hybridization conditions are also provided in, e.g., Tijessen, 1993, Hybridization With Nucleic Acid Probes, Elsevier Science Publishers B.V. and Kricka, 1992, Nonisotopic DNA Probe Techniques, Academic Press San Diego, Calif. For a given set of reaction conditions, the ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific will be the binding of the two sequences. Increased stringency is achieved by any one or more of the following: elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

One factor in designing and constructing primers is the free energy parameters of hybridization of given sequences under a given set of hybridization conditions. The free energy parameters for the formation of a given hybrid may be calculated by methods known in the art (see, e.g., Tinoco et al. *Nature* (1973) 246:40-41. and Freier et al., Proc. Natl. Acad. Sci. USA (1986) 83:9373-9377; computer programs, e.g., Oligo Primer Analysis Software from Molecular Biology Insight, and references therein), and it is possible to predict, for a given oligonucleotide template, primer sequences for which the required free energy changes for formation of various complexes will be met.

One of skill in the art will understand that other factors affect nucleic acid hybridization affinities. For example, any and all of the guanosine-cytosine content of the primer-target and primer-primer duplexes, minor groove binders, O-methylation or other modification of nucleotides, temperature, and salt are potentially important factors in constructing primers with the requisite differences in binding energies.

As described herein, one or more composite primers may be used in a reaction.

2. A polynucleotide comprising a termination polynucleotide sequence

Although it is not a necessary component of the invention, in some embodiments of the methods of the present invention, especially if transcription-based amplification is used, a polynucleotide comprising a termination sequence is optionally included, examples of which are provided. A "propromoter," or "propromoter sequence" is a sequence that is designed for formation of a double stranded promoter of an RNA polymerase. In some

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embodiments, the polynucleotide is a TSO which contains a propromoter sequence, as discussed in the section describing TSO's. Such polynucleotides are described in, e.g., U.S. Patent No. 6,251,639.

a. Template switch oligonucleotide

A second oligonucleotide that can optionally, though not necessarily, be used in the amplification methods of the invention is a template switch oligonucleotide (TSO). In one embodiment, the TSO functions as a termination sequence. In another embodiment, the TSO functions as a termination sequence and provides a propromoter sequence.

Previously described amplification methods based on template switch oligonucleotide were restricted in the concentration of this oligonucleotide due to inhibition of hybridization of the second primer, or the second hybridization step of the same primer when the method is designed to utilize a single primer species. The methods of the invention are free of this limitation. In contrast to previously described methods using TSOs, the template switch oligonucleotide can be used at high concentration for amplification according to the methods of the present invention. This feature ensures efficient hybridization of the TSO to the oligonucleotide template, and maximizes the yield of the tri molecular complex, the substrate for primer extension and template switch. An additional attribute of this feature is the efficient hybridization of the displaced primer extension product to the template switch oligonucleotide to form a substrate for the RNA polymerase, as described.

A TSO comprises a 3' portion that can hybridize to the oligonucleotide template and a 5' portion which is designed for strand switch during polymerization see Figure 9A-C. Design of a TSO that would effect strand switch is known in the art, such as was previously described in Patel et al., *Proc. Nat'l Acad. Sci.* USA 1996, 93:2969-2974. The 3' portion hybridizes to the oligonucleotide template at a location 5' to the position or region in the oligonucleotide template that is complementary to the 3' end of the primer extension product prior to switching template from the oligonucleotide template to the unhybridized portion of the TSO ("termination site").

In one embodiment, strand switch is promoted by the presence of mutually complementary short sequences in the TSO segments immediately 5' and 3' to the junction between the hybridized and non-hybridized portions of the TSO. Without intending to be bound by theory, one explanation is that in the event that the primer extension product is extended into the portion of the oligonucleotide template that is hybridized to the TSO

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(through displacement of the hybridized portion of the TSO), the 3' end of the primer extension product would comprise a short sequence that can bind to its complementary short sequence in the segment of the TSO immediately adjacent to the junction between the hybridized and non-hybridized portions of the TSO. This increases the efficiency of template switching by increasing the probability that the primer extension product would switch to the TSO tail portion as a template. The length of the short complementary sequences is preferably from about 3 to about 20 nucleotides, more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 10 nucleotides. In some embodiments, length is at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 15, 20, 25 nucleotides. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

In some embodiments, the 5' portion of the TSO comprises a sequence (hereinafter "propromoter sequence"),This embodiment of the TSO would function both as a termination sequence and to provide a promoter template. In this embodiment, the propromoter sequence of the TSO serves as a template for incorporation of a propromoter sequence (generally complementary to the propromoter sequence of the template TSO) into the primer extension product. Subsequent hybridization of a TSO comprising a propromoter sequence that is hybridizable (e.g., that hybridizes) to the propromoter sequence of the primer extension product results in formation of a double stranded promoter capable of effecting transcription by a suitable RNA polymerase. Promoter sequences that allow transcription of a template DNA are known in the art, as are methods of obtaining and/or making them. Preferably, the promoter sequence is selected to provide optimal transcriptional activity of the particular RNA polymerase used. Criteria for such selection, i.e., a particular promoter sequence particularly favored by a particular RNA polymerase, are also known in the art. For example, the sequences of the promoters for transcription by T7 DNA dependent RNA polymerase and SP6 are known in the art. The promoter sequence can be from a prokaryotic or eukaryotic source. In one embodiment, the promoter sequence is adjacent to a sequence that is designed to provide for enhanced, or more optimal, transcription by the RNA polymerase used. In some embodiments, the sequence is not related (i.e., it does not substantially hybridize) to the oligonucleotide template. More optimal transcription occurs when transcriptional activity of the polymerase from a promoter that is operatively linked to said sequence is greater than from a promoter that is not so linked. The sequence requirements for optimal transcription are generally known in the art as previously described

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for various DNA dependent RNA polymerases, such as in U.S. Pat. Nos. 5,766,849 and 5,654,142.

In a preferred embodiment, a segment of the 3' portion of the TSO (including the entire 3' portion that hybridizes to oligonucleotide template) that hybridizes to the oligonucleotide template DNA is attached to the oligonucleotide template DNA such that displacement of the TSO by the polymerase that effects primer extension is substantially, or at least sufficiently, inhibited. Suitable methods for achieving such attachment includes techniques known in the art, such as using a cytosine analog that contains a G-clamp heterocycle modification (described in Flanagan et al., Proc. Natl. Acad. Sci. USA 1999, 96(7):3513-8); and locked nucleic acids (described, e.g., in Kumar et al., Bioorg. Med. Chem Lett. 1998, 8(16):2219-22; and Wahlestedt et al., Proc. Natl. Acad. Sci. USA 2000, 97(10):5633-8). Other suitable methods include using, where appropriate, sequences with a high GC content and/or cross-linking. Any of these methods for obtaining enhanced attachment may be used alone or in combination. Displacement of the TSO is substantially or sufficiently inhibited if the polymerase switches template from the oligonucleotide template to the unhybridized portion of the TSO in at least about 25%, preferably at least about 50%, more preferably at least about 75%, and most preferably at least about 90%, of the events of primer extension. Substantially or sufficiently inhibited TSO displacement can also be empirically indicated if the amplification methods lead to a satisfactory result in terms of amount of the desired product. Generally, under a given set of conditions, the "modified" TSO binds more tightly to template as compared to a TSO not so modified.

The length of the TSO portion that hybridizes to the oligonucleotide template is preferably from about 15 to 50 nucleotides, more preferably from about 20 to 45 nucleotides, and most preferably from about 25 to 40 nucleotides. In other embodiments, the length is at least about any of the following: 10, 15, 20, 25, 30; and less than about any of the following: 35, 40, 45, 50, 55. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention. The complementarity of the TSO portion that hybridizes to the oligonucleotide template is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the oligonucleotide template.

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b. Blocker sequence

In some embodiments, the primer extension termination sequence is provided by an optional blocker sequence. The blocker sequence is a polynucleotide, usually a synthetic polynucleotide, that is single stranded and comprises a sequence that is hybridizable (e.g., that hybridizes), preferably complementary, to a segment of oligonucleotide template sequence 5' of the position in the oligonucleotide template that is complementary to the 3' end of the primer extension product ("termination site"). The blocker comprises nucleotides that bind to the target nucleic acid with an affinity, preferably a high affinity, such that the blocker sequence resists displacement by DNA polymerase in the course of primer extension, in preferably more than about 30%, more preferably more than about 50%, even more preferably more than about 75%, and most preferably more than about 90%, of primer extension events. The length and composition of the blocker polynucleotide should be such that excessive random non-specific hybridization is avoided under the conditions of the methods of the present invention. The length of the blocker polynucleotide is preferably from about 3 to about 30 nucleotides, more preferably from about 5 to about 25 nucleotides, even more preferably from about 8 to about 20 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments, the blocker polynucleotide is at least about any of the following: 3, 5, 8, 10, 15; and less than about any of the following: 20, 25, 30, 35. It is understood that the length can be greater or less as appropriate under the reaction conditions of the methods of this invention. The complementarity of the blocker polynucleotide is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the oligonucleotide template.

In one embodiment, the blocker sequence comprises a segment that hybridizes to the oligonucleotide template such that displacement of the blocker sequence by the polymerase that effects primer extension is substantially, or at least sufficiently, inhibited. Suitable means for achieving such attachment and determining substantial, or sufficient, inhibition of displacement are as described above for TSO used in the methods of the present invention.

In one embodiment, the blocker polynucleotide cannot function efficiently as a primer for nucleic acid extension (i.e., extension from the blocker sequence is reduced, or inhibited). Techniques for blocking the primer function of the blocker polynucleotide include any that prevent addition of nucleotides to the 3' end of the blocker by a DNA polymerase. Such techniques are known in the art, including, for example, substitution or modification of the 3' hydroxyl group, or incorporation of a modified nucleotide, such as a

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dideoxynucleotide, in the 3'-most position of the blocker polynucleotide that is not capable of anchoring addition of nucleotides by a DNA polymerase.

3. Polynucleotide comprising a termination sequence and further comprising a propromoter sequence

In some embodiments of methods of the invention, an optional termination sequence and a propromoter sequence are provided in a single polynucleotide. The polynucleotide comprises a portion (generally a 3' portion) that comprises a termination sequence that does not effect template switch under conditions wherein the termination sequence is hybridizable (e.g., hybridizes) to an oligonucleotide template, and a portion (generally a 5' portion) that comprises a propromoter sequence, wherein the portion that comprises a propromoter sequence generally does not hybridize to the oligonucleotide template (under conditions wherein the portion that comprises a termination sequence hybridizes to the oligonucleotide template). A termination sequence can be designed so as not to effect template switch using techniques known in the art, for example by ensuring that design characteristics that are known to promote template switch (such as described in Patel et al., Proc. Nat'l Acad. Sci. USA 1996, 93:2969-2974) are not present in the polynucleotide. The polynucleotide is hybridizable (e.g., hybridizes) to the sequence of the oligonucleotide template that is in the 5' direction with respect to the template sequence which is hybridizable (e.g., hybridizes) to the primer. The polynucleotide further comprises a sequence which is hybridizable (e.g., hybridizes) to a complementary sequence of the oligonucleotide template. The sequence that is hybridizable (e.g., hybridizes) to a complementary sequence of the oligonucleotide template may be non-overlapping, overlapping or co-extensive with the termination sequence and/or propromoter sequence of the combination polynucleotide. Generally and preferably the sequence that is hybridizable (e.g., hybridizes) to a complementary sequence of the oligonucleotide template is hybridizable (e.g., hybridizes) to a 3' portion of the complementary sequence of the oligonucleotide template. Thus, in some embodiments of methods of the invention, a polynucleotide that comprises a portion comprising a termination sequence and a portion comprising a propromoter sequence functions both to effect termination of primer extension and to provide a propromoter sequence in the same amplification reaction. Such polynucleotides are described in, e.g., U.S. Patent No. 6,251,639.

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4. Polynucleotide comprising a propromoter and a region which hybridizes to a displaced primer extension product

Some embodiments employ a polynucleotide comprising a propromoter and a region which hybridizes to a displaced primer extension product. In some embodiments, the polynucleotide is a TSO which contains a propromoter sequence, as discussed above. In other embodiments, the propromoter sequence is contained in a PTO, as described below. Such polynucleotides are described in, e.g., U.S. Patent No. 6,251,639. In some embodiments a propromoter without PTO may be used.

a. Propromoter template oligonucleotide

In some embodiments, the methods employ a promoter sequence for transcription which is provided by a propromoter template oligonucleotide (PTO).

A PTO for use in the methods and compositions of the present invention is a single-stranded polynucleotide, generally DNA, comprising a propromoter sequence that is designed for formation of a ds promoter of an RNA polymerase, and a portion capable of hybridizing to the 3' end of the primer extension product. In a preferred embodiment, the propromoter sequence is located in the 5' portion of the oligonucleotide and the hybridizing sequence is located in the 3' portion of the oligonucleotide. In one embodiment, and most typically, the promoter and hybridizing sequences are different sequences. In another embodiment, the promoter and hybridizing sequences overlap in sequence identity. In yet another embodiment, the promoter and hybridizing sequences are the same sequence, and thus are in the same location on the PTO. In the embodiments wherein hybridization of the PTO to the primer extension product results in a duplex comprising an overhang (the 5' end of the PTO that does not hybridize to the displaced primer extension product, typically comprising all or part of the propromoter sequence), DNA polymerase fills in the overhang to create a double stranded promoter capable of effecting transcription by a suitable RNA polymerase.

Promoter sequences that allow transcription of a template DNA are known in the art and have been discussed above. Preferably, the promoter sequence is selected to provide optimal transcriptional activity of the particular RNA polymerase used. Criteria for such selection, i.e., a particular promoter sequence particularly favored by a particular RNA polymerase, is also known in the art. For example, the sequences of the promoters for transcription by T7 DNA dependent RNA polymerase and SP6 are known in the art. The promoter sequence can be from a prokaryotic or eukaryotic source.

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In some embodiments, the PTO comprises an intervening sequence between a propromoter sequence and a portion capable of hybridizing to the 3' end of the primer extension product. Suitable length of the intervening sequence can be empirically determined, and can be at least about 1, 2, 4, 6, 8, 10, 12, 15 nucleotides. Suitable sequence identity of the intervening sequence can also be empirically determined, and the sequence is designed to preferably, but not necessarily, enhance degree of amplification as compared to omission of the sequence. In one embodiment, the intervening sequence is a sequence that is designed to provide for enhanced, or more optimal, transcription by the RNA polymerase used. Generally, the sequence is not related (i.e., it does not substantially hybridize) to the oligonucleotide template. More optimal transcription occurs when transcriptional activity of the polymerase from a promoter that is operatively linked to said sequence is greater than from a promoter that is not so linked. The sequence requirements for optimal transcription are generally known in the art as previously described for various DNA dependent RNA polymerases, such as in U.S. Pat. Nos. 5766849 and 5654142, and can also be empirically determined.

In another embodiment, the PTO comprises a sequence that is 5' to the propromoter sequence, i.e., the PTO comprises additional nucleotides (which may or may not be transcriptional regulatory sequences) located 5' to the propromoter sequence. Generally, but not necessarily, the sequence is not hybridizable to the primer extension product.

In one embodiment, the PTO cannot function efficiently as a primer for nucleic acid extension. Techniques for blocking the primer function of the PTO include any that prevent addition of nucleotides to the 3' end of the PTO by a DNA polymerase. Such techniques are known in the art, including, for example, substitution or modification of the 3' hydroxyl group, or incorporation of a modified nucleotide, such as a dideoxynucleotide, in the 3'-most position of the PTO that is not capable of anchoring addition of nucleotides by a DNA polymerase. It is also possible to block the 3' end using a label, or a small molecule which is a member of a specific binding pair, such as biotin.

The length of the portion of the PTO that hybridizes to the displaced primer extension product is preferably from about 5 to about 50 nucleotides, more preferably from about 10 to about 40 nucleotides, even more preferably from about 15 to about 35 nucleotides, and most preferably from about 20 to 30 nucleotides. In some embodiments, the hybridizing portion is at least about any of the following: 3, 5, 10, 15, 20; and less than about any of the following: 30, 40, 50, 60. The complementarity of the hybridizing portion is preferably at least about 25%, more preferably at least about 50%, even more preferably at

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least about 75%, and most preferably at least about 90%, to its intended binding sequence on the displaced primer extension product.

5. DNA polymerase, ribonuclease and RNA polymerase

The amplification methods of the invention employ the following enzymes: a DNA polymerase, ribonuclease such as RNase H, and, optionally a DNA dependent RNA polymerase. These components are described in, e.g., U.S. Patent No. 6,251,639.

DNA polymerases for use in the methods and compositions of the present invention are capable of effecting extension of the composite primer according to the methods of the present invention. Accordingly, a preferred polymerase is one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of deoxynucleotides. The polymerase should be able to displace a nucleic acid strand from the polynucleotide to which the to-be-displaced strand is bound. Preferably, the DNA polymerase has high affinity for binding at the 3'-end of an oligonucleotide hybridized to a nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Preferably, the polymerase has little or no 5'->3' exonuclease activity so as to minimize degradation of primer, or primer extension polynucleotides. Generally, this exonuclease activity is dependent on factors such as pH, salt concentration, whether the template is double stranded or single stranded, and so forth, all of which are familiar to one skilled in the art. Mutant DNA polymerases in which the 5'->3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Mutant DNA polymerases which lack both 5' to 3' nuclease and 3' to 5' nuclease activities have also been described , for example, exo$^{-/-}$Klenow DNA polymerase. Suitable DNA polymerases for use in the methods and compositions of the present invention include those disclosed in U.S. Pat. Nos. 5,648,211 and 5,744,312, which include exo$^-$ Vent (New England Biolabs), exo$^-$ Deep Vent (New England Biolabs), Bst (BioRad), exo$^-$ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus. The DNA polymerase displaces primer extension products from the oligonucleotide template in at least about 25%, or at least about 50%, or at least about 75%, or at least about 90%, of the incidence of contact between the polymerase and the 5' end of the primer extension product. In some embodiments, the use of thermostable DNA polymerases with strand displacement activity is preferred. Such polymerases are known in the art, such as described in U.S. Pat. No.

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5,744,312 (and references cited therein). Preferably, the DNA polymerase has little to no proofreading activity.

The agent for cleaving RNA from a RNA/DNA hybrid may be an enzyme, for example, a ribonuclease. Preferably, the ribonuclease cleaves ribonucleotides regardless of the identity and type of nucleotides adjacent to the ribonucleotide to be cleaved. It is preferred that the ribonuclease cleaves independent of sequence identity. Examples of suitable ribonucleases for the methods and compositions of the present invention are well known in the art, including ribonuclease H (RNase H).

The DNA-dependent RNA polymerase for use in the methods and compositions of the present invention are known in the art. Either eukaryotic or prokaryotic polymerases may be used. Examples include T7, T3 and SP6 RNA polymerases. Generally, the RNA polymerase selected is capable of transcribing from the promoter sequence provided by the TSO or PTO as described herein. Generally, the RNA polymerase is a DNA dependent polymerase, which is preferably capable of transcribing from a single stranded DNA template so long as the promoter region is double stranded.

In general, the enzymes used in the methods and compositions of the present invention should not produce substantial degradation of the nucleic acid components of said methods and compositions.

Single-stranded nucleic acid or DNA binding protein ("SSB") can be used to enhance the efficiency of the hybridization and the denaturation of the primer and the oligonucleotide template. Examples of SSBs suitable for use in the present invention include E. coli SSB ("EcoSSB"), T4 gene 32 protein, T7 SSB, Coliphage N4 SSB, calf thymus unwinding protein and adenovirus DNA binding protein. SSBs may decrease or remove secondary structure in ssDNA. EcoSSB is stable up to 100°C, and appears to be less sensitive to salt concentrations than SSB32. EcoSSB also has a lower tendency to aggregate than SSB32. Generally, EcoSSB, SSB32 and phage T7 SSB may improve hybridization of polynucleotides with complementary nucleic acid sequences. SSB32 may be useful for improving the specificity of hybridization, and can be used in embodiments where the presence or absence and/or quantity of a plurality of analytes is to be determined.

6. Reaction conditions

Appropriate reaction media and conditions for carrying out the methods of the present invention are those that permit binding of analyte and binding partner, and nucleic acid amplification according to the methods of the present invention. Such media and

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conditions are known to persons of skill in the art, and are described in various publications, such as U.S. Pat. Nos. 5,679,512 and 6,251,639, and PCT Pub. No. WO99/42618.

For example, a buffer may be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is preferably from about 5 to about 11, more preferably from about 6 to about 10, even more preferably from about 7 to about 9, more preferably from about 7.5 to about 8.5, and most preferably about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{2+} or Mn^{2+}, at a final concentration of free ions that is within the range of from about 0.01 to about 10 mM, and most preferably from about 1 to 5 mM. The reaction medium can also include other salts, such as KCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 100 mM, more preferably from about 0 to about 75 mM, and most preferably from about 0 to about 50 mM. The reaction mixture may also contain a ssDNA binding protein; for example, it may contain 3 ug T4gp32 (USB). The reaction medium can further include additives that could affect performance of the amplification reactions, but that are not integral to the activity of the enzyme components of the methods. Such additives include proteins such as BSA, and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT, that are capable of maintaining enzyme activities can also be included; for example, DTT may be included at a concentration of about 1 to about 5 mM. Such reagents are known in the art. Where appropriate, an RNase inhibitor (such as Rnasine) that does not inhibit the activity of the RNase employed in the method can also be included.

Any aspect of the methods of the present invention can occur at the same or varying temperatures. Preferably, the reactions are performed isothermally, which avoids the cumbersome thermocycling process. The amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer, optionally PTO, or, optionally, TSO) of the present invention to the oligonucleotide template and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, more preferably about 37°C to about 70°C, and most preferably at about 55°C. In some embodiments that include RNA transcription, the temperature for the transcription steps is lower than the temperature(s) for the preceding steps. In these embodiments, the temperature of the transcription steps can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, and most preferably about 37°C to about 70°C.

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Nucleotide and/or nucleotide analogs, such as deoxyribonucleoside triphosphates, that can be employed for synthesis of the primer extension products in the methods of the invention are provided in the amount of from preferably about 50 to about 2500 μM, more preferably about 100 to about 2000 μM, even more preferably about 500 to about 1700 μM, and most preferably about 800 to about 1500 μM. Deoxyribose nucleoside triphosphates (dNTPs) may be used at a concentration of, for example, about 250 to about 500 uM. In some embodiments, a nucleotide or nucleotide analog whose presence in the primer extension strand enhances displacement of the strand (for example, by causing base pairing that is weaker than conventional AT, CG base pairing) is included. Such nucleotide or nucleotide analogs include deoxyinosine and other modified bases, all of which are known in the art. Nucleotides and/or analogs, such as ribonucleoside triphosphates, that can be employed for synthesis of the RNA transcripts in the methods of the invention are provided in the amount of from preferably about 0.25 to about 6 mM, more preferably about 0.5 to about 5 mM, even more preferably about 0.75 to about 4 mM, and most preferably about 1 to about 3 mM.

The oligonucleotide components of the amplification reactions of the invention are generally in excess of the number of oligonucleotide template sequence to be amplified. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of oligonucleotide template. Composite primers, TSO, PTO and the blocker sequence can each be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM.

In one embodiment, the foregoing components, and others as needed to promote analyte binding to binding partner, as will be apparent to one of skill in the art, are added simultaneously at the initiation of the amplification process. In another embodiment, components are added in any order prior to or after appropriate timepoints during the analyte binding to binding partner, and during the amplification process, as required and/or permitted by the binding and/or amplification reaction. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. The enzymes used for nucleic acid amplification according to the methods of the present invention can be added to the reaction mixture either prior to the analyte-binding partner binding step, simultaneous with the analyte-binding partner binding step, following the analyte-binding partner binding step, or following hybridization of the primer and/or blocker sequence to the target DNA, as determined by considerations known to the person of skill in the art.

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7. Detectable identifying characteristics of amplification product

The product of the amplification step (or "amplification product") can be either displaced primer extension product comprising polynucleotide (typically, DNA) complementary or identical to the primer extension region and part or all of the DNA portion of the composite primer (if amplification is carried out without an RNA transcription step) or sense RNA corresponding to part or all of the DNA portion of the composite primer and the primer extension region of the oligonucleotide template. In some embodiments, the amplification product, whether DNA or RNA, possesses at least one detectable identifying characteristic. Appropriate detectable identifying characteristics to be incorporated in amplification product can be determined by one skilled in the art, in view of and based on the context of the components (such as type and/or form of the dNTP(s) provided, and/or type of label associated with the dNTPs provided and the primer). It is appreciated that one or more detectable identifying characteristics may be used to characterize an amplification product, and that characterization may be performed iteratively. It is also understood that more than one amplification product, from more than one oligonucleotide template, may be present in the reaction mixture, and that each amplification product may have its own detectable identifying characteristic. In some embodiments, detection is accomplished through detection of amplification products such as, e.g., pyrophosphate, and does not require detection of an identifiable characteristic of the DNA or RNA produced by amplification.

Examples of detectable identifying characteristics for amplification products include size of the product (since the sizes of the various components which contribute to the amplification product are known, and thus the size of the amplification product is known), sequence of the amplification product (since the sequence of the amplification product is known), and detectable signal associated with the amplification product. Detectable signal may be associated with a label on a deoxyribonucleoside triphosphate or ribonucleotide triphosphate or analog thereof that is incorporated during primer extension or RNA transcription Detectable signal may also be associated with interaction of two labels. For example, one label may be on a deoxyribonucleoside triphosphate or analog thereof that is incorporated during primer extension and another label is on a deoxyribonucleoside triphosphate or analog thereof located in the primer portion of the primer extension product, or both labels may be on deoxyribonucleoside triphosphates or analogs thereof that are incorporated during primer extension. It is understood by one skilled in the art that while the preceding discussion addresses detection of accumulated amplification products comprising

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a detectable identifying characteristic(s), absence of accumulated amplification products comprising a detectable identifying characteristic(s) is also informative, indicating absence of analyte in the sample.

In one example, characterization of amplification products based on size may be used when determining whether a plurality of analytes, and/or a plurality of binding partner binding sites on a single analyte, are present or absent in a sample. Various combinations of length of the primer extension regions, length of DNA portion of primer, and length of 5' region of TSO or of the PTO, if used, may be employed to produce amplification products of different sizes for each of the different oligonucleotides attached to different binding partners. Thus, there can be the creation of different sized amplification products for each analyte, and/or each binding area of an analyte, and/or each subunit of a multisubunit analyte.

In another example, detection and/or characterization of amplification product can be based on the sequence of the amplification product, which can be designed to be unique for all or part of each amplification product or groups of amplification products. Methods of detection based on sequence are well known in the art. Sequence-based detection methods include hybridization of the amplification product to specific oligonucleotides, for example, immobilized on an array. This method is particularly useful when a plurality of amplification products are produced.

In yet another example, characterization of amplification product can be based on detection of a signal, or lack thereof, from amplification product. Such a signal may be associated with incorporation of labeled dNTP(s), or analog(s) thereof into the product. For example, in the scenarios illustrated above, a labeled dNTP corresponding to an analyte could be incorporated into an amplification product. Detection of product with the signal generated by the label indicates the presence of that analyte.

The amplification products may be labeled by incorporation of labeled nucleotide during replication, may be labeled subsequent to replication by elongation with labeled nucleotides (end label using terminal transferase), labeled by incorporation of label using a labeled primer, or indirectly labeled by binding of sequence-specific labeled probe.

Labels suitable for use in the methods of this invention are known in the art, and include, for example, fluorescent dye labels and isotopic labels. Homogeneous detection of the amplification product can also be employed. For example, the optical properties of a label associated with a dNTP can be altered subsequent to incorporation of the labeled dNTP into an amplification product. Such a label includes fluorescent dyes that undergo

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fluorescence polarization between being attached to free dNTPs and being incorporated in a polynucleotide. See, e.g. U.S. Patent No. 6,326,142, and references cited therein.

Another example of homogeneous detection is based on alteration of spectral properties of a label by means of energy transfer. When a primer is labeled by a donor or acceptor dye, for example, and/or the dNTPs, or their analogs, are labeled with acceptor or donor dyes, respectively, incorporation of the labeled dNTPs into the product enables energy transfer between the donor-acceptor dyes, thus resulting in specific detectable spectral properties of the attached dyes. These dyes are known in the art, as described in, for example, U.S. Pat. No. 4,996,143 (e.g., fluorescein and Texas Red donor acceptor dye pair), and U.S. Pat. No. 5,688,648. Other label combinations are also possible. For example, two ligands (such as digoxigenin and biotin) each attached to different parts of an amplification product (generally, primer and non-primer portion, or for RNA transcripts, different nucleotides in the transcript) can be brought into close proximity in the context of an amplification product. Binding of the two ligands with their corresponding antibodies which are differentially labeled can be detected due to the interaction of the labels. For instance, if the two different labels are a photosensitizer and a chemiluminescent acceptor dye, the interaction of the labels can be detected by the luminescent oxygen channeling assay as described in U.S. Pat. No. 5,340,716. Other interacting label pairs useful in the present invention are known in the art, see, e.g., 5,340,716; 3,999,345; 4,174,384; and 4,261,968 (Ullman et al.); and 5,565,322 (Heller et al.); 5,709,994 (Pease et al.); and 5,925,517 (Tyagi et al.). Examples of ligands in which one member modulates the signal of another include a fluorescent label, a chemiluminescent label, and a bioluminescent label. In some embodiments, the ligands produce little or no signal when in close proximity, and a greater signal when separated. In other embodiments, the ligands may generate a signal when in close proximity and generate less or no signal when separated.

For nucleic acids, the use of light-up probes in nucleic acid analysis allows one member of a complementary pair to be labeled in such a way that binding of the two strands results in a large increase in fluorescence signal. The use of such probes is known in the art and discussed in, e.g., U.S. Patent No. 6,329,144; Svanvik et al., *Ana. Biochem.* (2000) 281:26-35.

As will be apparent to one of skill in the art, the idea of interacting labels that result in modulation of signal may be more generally applied. For example, an enzyme that catalyzes a reaction producing a detectable product may be paired with its cofactor or its inhibitor, or multiple subunits of an enzyme may be paired. It will be apparent that in the case of

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cofactor or multiple subunits, pairing of the entities in close proximity increases signal (e.g., a detectable product whose production is catalyzed by the enzyme), whereas in the case of inhibitor, pairing of the entities in close proximity decreases signal.

In addition, the amplification product can be detected by hybridizing it to a labeled oligonucleotide. In one embodiment, amplification product contains one member of a interacting label pair and the labeled oligonucleotide to which the amplification product hybridizes contains the other member. In another embodiment, one member of an interacting label pair is used in an attachment oligonucleotide to which amplified product binds and the other member is used in a third (capture) oligonucleotide. The third oligonucleotide may be immobilized on a solid surface and the hybridization of the labeled amplification product-attachment oligonucleotide hybrid to the third (capture) oligonucleotide results in an altered detectable signal due to the interaction of the labels on the amplification product and the third oligonucleotide. The immobilized oligonucleotide may be a part of an array of a plurality of oligonucleotides, each oligonucleotide being specific for a amplification product comprising a distinct sequence.

Detection of these detectable identifying characteristics can be achieved by a variety of methods evident to one skilled in the art. Methods of determining size and sequencing of polynucleotides (such as an attached oligonucleotide combination product) are known in the art. Methods of detecting detectable signals are known in the art, and are described above.

In one embodiment, the resultant amplification products in a reaction mixture are separated for analysis on a suitable matrix. Any of a number of methods can be used to effect the separation, as described in, for example, McIntosh et al., *infra*. Such methods include, but are not limited to, oligonucleotide array hybridization, mass spectrometry, flow cytometry, HPLC, FPLC, size exclusion chromatography, affinity chromatography, and gel electrophoresis.

It is appreciated that while the preceding discussion describes detection of amplification product comprising a detectable identifying characteristic(s), the absence of accumulation of amplification product comprising a detectable identifying characteristic(s) is also informative, indicating absence of analyte in the sample.

Reaction conditions, components, and other experimental parameters as well as illustrative embodiments in this section are generally as described herein.

B. Methods of amplification of the oligonucleotide template

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In one aspect of the amplification step of the methods of the present invention, a method for amplifying a nucleotide sequence complementary to a portion of the oligonucleotide template is provided. In this method, isothermal linear amplification is achieved. In another aspect, the amplified portion of the oligonucleotide template is itself amplified. In another aspect, a method for amplifying a portion of the oligonucleotide template wherein the amplified product is sense RNA is provided. The latter two aspects are sometimes referred to herein as an "enhanced" linear amplification methods.

The methods for amplifying the primer extension template region generally comprise using an RNA/DNA composite primer, optionally a termination sequence, and, in embodiments in which transcription is used (i.e., enhanced linear amplification), a propromoter oligonucleotide sequence.

The methods work as follows (see, e.g., U.S. patent 6,251,639): a composite RNA/DNA primer forms the basis for replication of the primer extension template region of the oligonucleotide template. In some optional embodiments, a termination sequence provides the basis for an endpoint for the replication by either diverting or blocking further replication along the template oligonucleotide strand. As described below, in some optional embodiments, the polynucleotide comprising a termination sequence is a template switch oligonucleotide (TSO), which contains sequences that are not of sufficient complementarity to hybridize to the template oligonucleotide strand (in addition to sequences which are of sufficient complementary to hybridize); in other optional embodiments, the termination sequence comprises primarily sequences that are of sufficient complementarity to hybridize to the template oligonucleotide strand. DNA polymerase effects copying of the primer extension region from the primer. An enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNaseH) cleaves (removes) RNA sequence from the hybrid, leaving sequence on the template oligonucleotide strand available for binding by another composite primer. Another strand is produced by DNA polymerase, which displaces the previously replicated strand, resulting in displaced extension product.

Accordingly, the linear amplification step of the methods of the invention generally comprises combining and reacting the following: (a) a single-stranded oligonucleotide template (attached to the binding partner) comprising a primer extension template region; (b) a composite primer comprising an RNA portion and a 3' DNA portion; (c) a DNA polymerase; (d) deoxyribonucleoside triphosphates or suitable analogs; (e) an enzyme, such as RNaseH, which cleaves RNA from an RNA/DNA duplex; and (f) optionally, a polynucleotide comprising a termination sequence, such as any of those described herein,

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which comprises a portion (or region) which hybridizes to the oligonucleotide template. A termination sequence is used if transcription-based amplification (i.e., enhanced linear amplification, see below) is also used. The combination is subjected to suitable conditions such that (a) the composite primer (and, optionally, a polynucleotide comprising a termination sequence) hybridizes to the oligonucleotide template; (b) primer extension occurs from the composite primer, to form a duplex; (c) RNaseH cleaves RNA of the composite primer from the RNA/DNA duplex; (d) another composite primer hybridizes to the oligonucleotide template, and another round of primer extension (mediated by DNA polymerase) occurs, displacing the strand already copied from the oligonucleotide template.

Methods of enhanced linear amplification further include either steps for a second round of composite primer-based amplification or a round of transcription-based amplification.

The composite primer-based enhanced amplification further includes the following steps: a second composite primer binds to the displaced primer extension product. The primer serves as an initiation point for DNA-polymerase-catalyzed polymerization along the displaced primer extension product. The second composite primer is cleaved by an RNA cleaving agent. The polymerization product is displaced by another round of polymerization initiated by binding of another second composite primer.

In transcription-based enhanced linear amplification, the following steps are included in addition to the linear amplification: a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product (which can be, for example, a propromoter template oligonucleotide or a template switch oligonucleotide), which contains sequences of sufficient complementarity to hybridize to the 3' end of the displaced primer extension product, binds to the displaced primer extension product. The promoter drives transcription (via DNA-dependent RNA polymerase) to produce sense RNA products.

Thus, if transcription-based enhanced linear amplification is used, the following is also included in the amplification reaction (either at the same time as those components listed above or added separately): (e) a polynucleotide comprising a propromoter sequence (which can be in any of a number of forms, as described herein) and a region which hybridizes to the displaced primer extension product; (f) ribonucleoside triphosphates or suitable analogs; and (g) RNA polymerase, under conditions such that transcription of the displaced strand can occur. Details regarding the various components of the methods of the present invention are provided below.

The following are examples of the amplification methods of the invention. It is understood that various other embodiments may be practiced, given the general description

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provided above. For example, reference to using a composite primer means that any of the composite primers described herein may be used. In one embodiment, a method of enhanced isothermal linear nucleic acid sequence amplification which is TSO-based is provided (hereinafter "Method 1"). In another embodiment, a method of enhanced isothermal linear nucleic acid sequence amplification which is blocker sequence and PTO-based is provided (hereinafter "Method 2").

1. Linear nucleic acid sequence amplification resulting in DNA amplification product

When not linked to transcription, the amplification method of the invention provides for isothermal linear amplification of a portion of the oligonucleotide template. The method utilizes a single composite primer. In one embodiment, the method also employs a termination sequence, such as a blocker sequence as described in Method 2, or a TSO, as described in Method 1. Methods 1 and 2 are described below. Insofar as the linear amplification is not linked to transcription, the components and steps leading to formation of a complex comprising a promoter sequence for a DNA dependent RNA polymerase, are not included.

The termination sequence (either TSO or blocker sequence component, if used) is added for producing a product of defined 3'-end. In some embodiments, sequence(s) within the oligonucleotide template 5' of the primer binding site inhibits nucleic acid polymerization such that termination of primer extension is achieved. Such sequences are known in the art, for example, GC rich sequences, or can be empirically determined.

When this feature is not desired, the isothermal linear amplification according to the methods of the invention can be carried out without a termination sequence. The isothermal linear amplification further utilizes two enzymes, a DNA polymerase and a ribonuclease such as RNase H. Schematic description of the linear isothermal nucleic acid amplification of the invention is shown in Figure 10 A-C and in Figures 12 A and 12B. Figures 10 A-C show the amplification with a blocker sequence present while Figures 12A and 12B show amplification without a blocker sequence.

Similar to Methods 1 and 2 as described below, the linear amplification method is designed to amplify a single stranded DNA oligonucleotide template.

As shown in Figure 10 A-C and 12A and 12B, the linear isothermal amplification method of the invention comprises steps similar to the initial steps of the enhanced linear amplification methods (Methods 1 and 2) described below and in Figures 9 A-C and 11 A-

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D. The oligonucleotide template is combined with a composite primer, DNA polymerase, a ribonuclease such as RNase H (Figure 12A) and optionally a blocker sequence component or TSO (Figure 10A), as described above. In one embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize to different oligonucleotide template sequences or different sites along the same oligonucleotide template strand. Advantages of this embodiment include multiplex detection and/or analysis of a plurality of analytes through amplification of a plurality of oligonucleotide template species in a single amplification reaction.

Figure 10 A-C illustrates an embodiment that includes a termination sequence and Figures 12A and 12B illustrate an embodiment without blocker. For simplicity, only the termination sequence (TSO or blocker sequence) embodiment will be described (Figures 10 A-C). The embodiment without termination sequence is the same except for the absence of termination sequence, which is not necessary when polymerization is terminated by steric considerations or by reaching the end of the oligonucleotide template (Figures 12A and 12B). The composite primer and the termination sequence (TSO or blocker sequence component) hybridize to the same oligonucleotide template, to form a tri molecular complex, XX (Figure 10 A). The 3'-end of the composite primer is extended along the oligonucleotide template by the polymerase, optionally up to the site of hybridizing of the TSO or blocker sequence component, to yield complex XXI (Figure 10 A-C). A ribonuclease such as RNase H cleaves the RNA, generally the 5'-RNA, portion of the extended primer of complex XXI (Figure 10 A-C) to produce complex XXII (Figure 10 A-C). A second composite primer binds to complex XXII (Figure 10 A-C) by hybridization of the RNA, generally the 5' RNA, portion to yield complex XXIII (Figure 10 A-C). The free 3' portion of the bound composite primer then displaces the 5' end of the primer extension product and hybridizes to the oligonucleotide template to form complex XXIV (Figure 10 A-C). The hybridization of the 3' end of the composite primer to the oligonucleotide template is generally favored over the hybridization of the 5' end of the primer extension product since the hybridized 3' end of the primer is a site of binding of the DNA polymerase which will then extend the 3' end of the primer along the oligonucleotide template. Primer extension results in displacement of the first primer extension product to yield complex XXV (Figure 10 A-C). The process is repeated to yield multiple single stranded DNA

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displacement products which are generally complementary to the primer extension template region of the oligonucleotide template.

As is evident from the description of the amplifications methods, displaced primer extension products can serve as templates for further amplification. A composite primer can be hybridized to displaced primer extension products (serving as DNA templates), and extended and displaced as described herein. Accordingly, in some embodiments, the invention provides methods for amplifying a portion of an oligonucleotide template comprising: (a) hybridizing a first composite primer to a single stranded DNA template comprising the complementary sequence of a portion of the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion, wherein said single stranded DNA template is generated by a method comprising: (i) hybridizing a portion of the oligonucleotide template with a second composite primer, said second composite primer comprising an RNA portion and a 3' DNA portion; (ii) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template that is 5' with respect to hybridization of the second composite primer to said oligonucleotide template; (iii) extending the second composite primer with DNA polymerase; and (iv) cleaving RNA of the annealed second composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, whereby multiple copies of a single stranded DNA template comprising the complementary sequence of a portion of the oligonucleotide template are generated; (b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the first composite primer to the template; (c) extending the first composite primer with DNA polymerase; (d) cleaving RNA of the annealed first composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the template and repeats primer extension by strand displacement; whereby multiple copies of a portion of the oligonucleotide template are produced.

The single stranded DNA (i.e., the displaced primer extension products) of the isothermal linear amplification method are readily detectable by any of many detection methods known in the art. Various homogeneous or heterogeneous detection methods suitable for the detection of single stranded nucleic acid molecules are described herein, including identification by size and/or migration properties in gel electrophoresis, or by hybridization to sequence-specific probes.

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The detection of the amplification product is indicative of the presence of the analyte. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of an analyte to the product of amplification of a reference sample that has a known quantity of the analyte, or of binding partner for the analyte, the amount of analyte in the test sample can be determined.

The production of at least 1, at least 10, at least about 100, at least about 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}, complementary or identical copies of each copy of oligonucleotide template can be expected, thus leading to at least 1, at least 10, at least 100, at least 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}-fold enhancement with respect to each copy oligonucleotide template.

2. Enhanced linear amplification based on further DNA replication from the displaced primer extension product.

As is evident from the description of the amplifications methods, displaced primer extension products can serve as templates for further amplification. Another composite primer can be hybridized to displaced primer extension products (serving as DNA templates), and extended and displaced as described herein. Accordingly, in some embodiments, the invention provides methods for amplifying a portion of an oligonucleotide template comprising: (a) hybridizing a first composite primer to a single stranded DNA template comprising the complementary sequence of a portion of the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion, wherein said single stranded DNA template is generated by a method comprising: (i) hybridizing a portion of the oligonucleotide template with a second composite primer, said second composite primer comprising an RNA portion and a 3' DNA portion; (ii) extending the second composite primer with DNA polymerase; and (iv) cleaving RNA of the annealed second composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, thus displacing the primer extension product, whereby multiple copies of a single stranded DNA template comprising the complementary sequence of a portion of the oligonucleotide template are generated; (b) extending the first composite primer with DNA polymerase; (c) cleaving RNA of the annealed first composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand

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displacement, thus displacing the primer extension product; whereby multiple copies of a portion of the oligonucleotide template are produced.

The single stranded DNA (i.e., the displaced primer extension products) of the isothermal linear amplification method are readily detectable by any of many detection methods known in the art, as described above.

The detection of the amplification product is indicative of the presence of the analyte. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of an analyte to the product of amplification of a reference sample that has a known quantity of the analyte, or a known quantity of binding partner for the analyte that is attached to the oligonucleotide template to be amplified, the amount of analyte in the test sample can be determined.

The production of at least 1, at least 10, at least about 100, at least about 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}, complementary or identical copies of each copy of oligonucleotide template can be expected, thus leading to at least 1, at least 10, at least 100, at least 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}-fold enhancement with respect to each copy oligonucleotide template.

3. Enhanced linear amplification based on transcription and resulting in sense RNA amplification product

The present invention also provides methods for amplifying a portion of an oligonucleotide template wherein the amplified product is RNA containing the sense sequence (i.e., same sequence as the portion of the oligonucleotide template). Amplification of a portion of the oligonucleotide template according to Method 1, which results in the generation of a unique intermediate amplification product comprising oligonucleotide template and template switch oligonucleotide (TSO)-related portions, provides for coupling of the linear amplification to transcription. The complex formed by the hybridization of the template switch oligonucleotide and the displaced primer extension product is a substrate for transcription by the RNA polymerase, which generates an RNA product of the same sense as the initial portion of the oligonucleotide template. Similarly, amplification of a portion of the oligonucleotide template according to Method 2 results in formation of a displaced primer extension product which when hybridized to the promoter template oligonucleotide forms a complex, which is a substrate for the RNA polymerase. As in Method 1, this process results in coupling of the linear amplification to transcription. The production of preferably at least about 1, more preferably at least about 50, even more preferably at least

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about 75, still more preferably at least about 100, and most preferably at least about 1000, RNA transcript products from each primer extension product is expected, thus leading to preferably at least about 1, more preferably at least about 50, even more preferably at least about 75, still more preferably at least about 100, and most preferably at least about 1000-fold enhancement with respect to the non-transcription linked methods of amplification.

Below are two exemplary methods.

a. Method 1-TSO-based enhanced linear nucleic acid amplification

In one embodiment, the TSO-based linear amplification method of the present invention is linked to transcription from the primer extension products to provide enhanced nucleic acid amplification. A schematic description of this novel amplification method, Method 1, is shown in Figure 9 A-C.

The TSO-based nucleic acid amplification method of the invention employs a single composite primer, as described above. A second oligonucleotide optionally used in the amplification method of the invention is a template switch oligonucleotide (TSO), also as described above. In some embodiments, a TSO is not required and instead the promoter sequence is provided by an oligonucleotide that binds to displaced template amplification product. The amplification method of the invention employs the following enzymes: a DNA polymerase, a ribonuclease such as RNase H, and a DNA dependent RNA polymerase.

The new TSO-based enhanced linear amplification method of the present invention can produce multiple copies of an RNA product that are sense to a portion of the oligonucleotide template sequence.

The oligonucleotide template is combined with the composite primer, a TSO oligonucleotide, DNA polymerase, ribonuclease such as RNase H, a DNA dependent RNA polymerase, and nucleotides, such as deoxyribonucleoside triphosphates (dNTPs) and ribonucleoside triphosphates (rNTPs), in a reaction medium suitable for nucleic acid hybridization and amplification, as known in the art. Suitable reaction medium and conditions are as described above. In one embodiment, transcription is performed at a different temperature, generally lower, than that of the preceding steps. In another embodiment, all the steps of the methods are performed isothermally.

In one embodiment, the amplification reaction mixture includes composite primers of identical sequence. In another embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize

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to different oligonucleotide templates or different sites along the same oligonucleotide template. Advantages of this embodiment include multiplex detection and/or analysis of a plurality of analytes through amplification of a plurality of oligonucleotide template species in a single amplification reaction.

In one embodiment, the TSO functions as a termination sequence and provides a propromoter sequence. In another embodiment, the TSO does not comprise a propromoter sequence. In this embodiment, a propromoter sequence is provided separately by another oligonucleotide, such as a PTO, that comprises a propromoter sequence and is hybridizable (e.g., hybridizes) to the 3' portion of the primer extension product such that transcription of the primer extension product can occur.

The single composite primer and the TSO then hybridize to the same strand of the oligonucleotide template to be amplified. Hybridization of the two oligonucleotides to the oligonucleotide template results in the formation of the tri molecular complex I (Figure 9 A-C).

A DNA polymerase carries out primer extension. The primer is extended along the oligonucleotide template of complex I (Figure 9 A-C), up to the site of TSO hybridization. Template switching from the oligonucleotide template strand to the 5' unhybridized portion of the TSO, and further primer extension along the TSO template results in the formation of the tri molecular complex II. The last comprises an oligonucleotide template, the TSO and the first primer extension product. The first primer extension product is a unique DNA comprising both an oligonucleotide template dependent portion (i.e., sequence complementary to a portion of the oligonucleotide template) and a TSO dependent portion (i.e., sequence complementary to the unhybridized portion of the TSO) .

Complex II (Figure 9 A-C) is a substrate for both an RNA polymerase and a ribonuclease such as RNase H. The DNA dependent RNA polymerase binds to the functional ds promoter of complex II and transcribes the first primer extension product to produce a sense RNA product III (Figure 9 A-C). A ribonuclease, such as RNase H, which is specific for degradation of the RNA strand of an RNA/DNA heteroduplex, degrades the 5' portion of the primer extension product in complex II to form the tri molecular complex IV.

Free composite primer hybridizes to the primer complementary site of the oligonucleotide template in complex IV (Figure 9 A-C). This hybridization results in formation of complex V (Figure 9 A-C) in which only the RNA portion, generally the 5' RNA portion, of the primer is hybridized to the oligonucleotide template. Displacement of the 5' most portion of the primer extension product by the 3' DNA portion of the partially

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hybridized primer will result in formation of complex VI (Figure 9 A-C), which is a substrate for a DNA polymerase. Extension of the primer along the oligonucleotide template (VII; Figure 9 A-C) results in displacement of the first primer extension product from the complex. Repeated primer extensions and strand displacements result in generation of multiple copies of polynucleotides that are at least substantially complementary to a portion of the oligonucleotide template.

The primer extension product generated as described above is used as a template for transcription in the embodiment wherein TSO that comprises a propromoter sequence is provided. The displaced primer extension product (VIII; Figure 9 A-C) hybridizes to free TSO oligonucleotide to form the partial duplex IX (Figure 9 A-C). Complex (duplex) IX comprises a double stranded portion at one end and two non-complementary single strands respectively derived from the primer extension product and the TSO. The double stranded portion of this partial duplex contains a fully functional double stranded promoter for the DNA dependent RNA polymerase. The last binds to the promoter of the partial duplex IX and transcribes the primer extension product to form multiple copies of a sense RNA product X (Figure 9 A-C).

The products of the amplification described above can be detected by either homogenous or heterogeneous detection methods, including identification by size and/or migration properties in gel electrophoresis, or by hybridization to sequence-specific probes. The detection of the amplification product is indicative of the presence of the analyte. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of an analyte to the product of amplification of a reference sample that has a known quantity of the analyte or its binding partner, the amount of analyte in the test sample can be determined.

b. Method 2 –Blocker sequence-based enhanced nucleic acid amplification

In another embodiment, the blocker sequence-based linear amplification method of the present invention is linked to transcription from the primer extension products to provide enhanced nucleic acid amplification. This alternative enhanced linear amplification, Method 2, which does not involve a template switch step, is shown in Figure 11 A-D.

Method 2 utilizes the single composite primer, as in Method 1, as described above, an optional blocker sequence component which is either an oligonucleotide or an oligonucleotide analog, which, as described above, is further able to hybridize to a sequence on the same oligonucleotide template as the single primer, and a third oligonucleotide, the

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promoter template (PTO), which, as described above, comprises a 3'-portion which is able to hybridize (and is preferably complementary) to the 3'-end of the displaced extension product and a 5'-portion which includes at its 5' end a sequence of a promoter for a DNA dependent RNA polymerase. As in the TSO described above, the sequence immediately adjacent to the promoter sequence is designed to provide for preferably optimal transcriptional activity by the RNA polymerase used in the amplification according to the method of the invention. The optional blocker sequence component is designed to hybridize to the oligonucleotide template at a site which is located upstream, towards the 5' end of the oligonucleotide template, relative to the site of hybridization of the single primer. Stated alternatively, and as described above, the blocker sequence hybridizes to a segment of oligonucleotide template 5' of the position in the oligonucleotide template that is complementary to the 3' end of the primer extension product. The blocker sequence binds with sufficiently high affinity so as to block primer extension at the site of blocker hybridization to the oligonucleotide template. This optional feature provides a strong stop for primer extension by the polymerase and defines the 3'-end of the primer extension product; alternatively, and more commonly, primer extension is terminated by steric factors or by runoff from the end of the oligonucleotide template, in which cases a blocker sequence is not required.

The oligonucleotide template is combined with the single composite primer, the blocker component, the propromoter template (PTO), DNA polymerase, ribonuclease such as RNase H, a DNA dependent RNA polymerase, and nucleotides, such as NTPs (e.g., dNTPs and rNTPs), as was described for Method 1. Suitable reaction medium and conditions are as described above. In one embodiment, the transcription is performed at a different temperature, generally lower, than that of the preceding steps. In another embodiment, all the steps of the methods are performed isothermally.

In one embodiment, each amplification reaction includes composite primers of one identical sequence. In another embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize to different oligonucleotide templates or different sites along the same oligonucleotide template. Advantages of this embodiment include analysis of a plurality of analytes through amplification of a plurality of oligonucleotide templates in a single amplification reaction.

The single composite primer and the blocker sequence component hybridize to the same oligonucleotide template to form a tri molecular complex. The primer is extended

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along the oligonucleotide template up to the site of hybridization of the blocker sequence, to form complex XII (Figure 11 A-D).

As in Method 1, a ribonuclease, such as RNase H, cleaves RNA, generally the 5' RNA portion, of the single composite primer of complex XII to form complex XIII (Figure 11 A-D). As described above, the enzyme is specific for cleaving the RNA strand of an RNA/DNA hybrid, and does not digest single stranded RNA. Thus, the ribonuclease does not degrade the free composite primer. The following steps, as illustrated in Figure 11 A-D, of primer hybridization (XIV), displacement of the 5' end of the primer extension product by the 3'-DNA portion of the composite primer (XV), primer extension and displacement of the first primer extension product (XVI), proceed as in Method 1, to yield multiple copies of the displaced primer extension product (XVII). Unlike the displacement product of Method 1, XVII is fully complementary to a portion of the oligonucleotide template and does not comprise a 3' end portion which is not complementary to part of oligonucleotide template. Repeated primer extensions and strand displacements result in generation of multiple copies of polynucleotides that are complementary to a portion of the oligonucleotide template.

The promoter template oligonucleotide (PTO) binds to the displaced extension product to form complex XVIII (Figure 11 A-D), by hybridization of the 3' end portion (A) of the propromoter template to the 3' end of the displaced primer extension product. As described above, the 3' end of the PTO may be blocked or not. When the 3' end of the propromoter template is not blocked, the template will be extended along the displaced primer extension product. The 3' end of the displaced product will be extended by the nucleotide (DNA) polymerase along the B portion (see Figure 11 A-D) of the hybridized propromoter template to form complex XIX, which comprises at its one end a ds promoter sequence that can be utilized by the DNA dependent RNA polymerase. Complex XIX is depicted in Figure 11 A-D as the product of hybridization of a promoter template in which the 3' end is blocked for extension by the polymerase. Alternatively, when the 3' end of the promoter template is not blocked extension of the 3' end along the displaced primer extension product results in formation of a fully double stranded complex. DNA-dependent RNA polymerase will transcribe the extended displaced primer extension product of complex XIX, in both forms (the choice of RNA polymerase must take into account its capability to transcribe from a ds and/or ss DNA template), that is to say either the partial duplex or the fully double stranded duplex forms of the complex. Multiple copies of a single stranded RNA products are produced by this transcription step.

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The products of the amplification described above can be detected by either homogenous or heterogeneous detection methods, including identification by size and/or migration properties in gel electrophoresis, or by hybridization to sequence-specific probes. The detection of the amplification product is indicative of the presence of the analyte. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of an analyte to the product of amplification of a reference sample that has a known quantity of analyte or its binding partner, the amount of analyte in the test sample can be determined.

The amplification products of the methods of the present invention can be further modified, such as through cleavage into fragments or by attachment of detectable labels, as required and/or permitted by the techniques used.

IV. Detection and/or quantification of amplified products

The amplification methods described above produce amplification products. There are various ways to measure amplification product formation (if any). Some ways are direct with respect to the product. Other ways are indirect. The most universal product of amplification, whose presence may provide a simple yes-no answer as to whether amplification has occurred, and thus as to whether analyte that has bound to a binding partner is present, is pyrophosphate, which is released during DNA polymerization, and whose levels can be measured by means known in the art. Other amplification products are more specific to the amplification method used. Linear amplification produces single stranded amplification product (usually ssDNA) that is complementary to at least a portion of the oligonucleotide template. Enhanced linear amplification using further DNA replication produces, in addition to ssDNA complementary to oligonucleotide template, also ssDNA that is identical to a portion of the oligonucleotide template. Enhanced linear amplification using RNA transcription produces, in addition to ssDNA complementary to oligonucleotide template, RNA that is sense to the original portion of the oligonucleotide template. All of these products are referred to as "amplification product(s)," or "amplified product(s)" The amplification products comprise at least one detectable identifying characteristic. Examples of detectable identifying characteristics of amplified products that may serve as the basis for detection include size, sequence, and labels. The amplification products are detected by any means available to those of skill in the art. Examples of such means are given below.

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A. Detection methods

Determination of the formation of pyrophosphate may be accomplished by standard methods in the art. See e.g. Ronaghi, M. et al., 1998 *Science* 281: 363-365; Nyren, P., et al., 1987 *Anal. Bioch.* 167:235-238.

The detectable identifying characteristics of DNA and RNA products of amplification described above can be detected by methods known in the art.

Size of a polynucleotide (amplification product) can be determined by, for example, gel electrophoresis sizing and mass spectrometry (see, for example, Monforte et al., U.S. Pat. Nos. 5,830,655 and 5,700,642).

Methods of sequencing a polynucleotide (amplification product) are well-known. Suitable sequencing methods are known in the art, and include, for example, using nucleotide triphosphates that upon incorporation into amplification product terminates nucleotide polymerization. Suitable sequencing methods also include sequencing by synthesis, which is a method known in the art, wherein nucleotide sequence is determined based on whether there is extension (polymerization) of a primer hybridized to an amplified product by a polymerase when a known dNTP type is provided in the synthesis (polymerization) reaction, wherein polymerization is indicated by the formation of pyrophosphate.

Detection of sequence in an amplification product can also be achieved by methods such as limited primer extension, which are known in the art and described in, for example, U.S. Patent Nos. 5,888,819; 6,004,744; 5,882,867; 5,710,028; 6,027,889; 6,004,745; 5,763,178; 5,011,769; 5,185,243; 4,876,187; 5,882,867; WO US88/02746; WO 99/55912; WO92/15712; WO 00/09745; WO 97/32040; WO 00/56925, and in co-pending U.S. Application Ser. No. 60/255,638, filed 13 December, 2000.

The amplified polynucleotide products, either DNA or RNA (i.e., products of any of the amplification methods described herein), can be also be analyzed using, for example, probe hybridization techniques known in the art, such as Southern and Northern blotting. In addition, the single stranded DNA and RNA products may serve as starting material for other starting material for other analytical and/or quantification methods known in the art, such as real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons,

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methods described in Kurn, U.S. Patent NO. 6,251,639, and the like. Thus, the invention includes those further analytical and/or quantification methods as applied to any of the products of the methods herein.

The labeled amplified products are particularly suitable for analysis (for example, detection and/or quantification) by contacting them with, for example, microarrays (of any suitable surface, which includes glass, chips, plastic), beads, or particles, that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify) an analyte by generating labeled polynucleotide (generally, DNA or RNA) products using amplification methods of the invention, and analyzing the labeled products. Analysis of labeled products can be performed by, for example, hybridization of the labeled amplification products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles, or probes immobilized on blots (such as a membrane), for example arrays. Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled amplification products and probes from solution. The identity of the probes provides characterization of the sequence identity of the amplified products, and thus by extrapolation the identity of the analyte present in a sample. Hybridization of the labeled products is detectable, and the amount of specific labels that are detected is proportional to the amount of the labeled amplification products of a specific analyte. This measurement is useful for, for example, measuring the relative amounts of various analytes in a sample. The amount of labeled products (as indicated by, for example, detectable signal associated with the label) hybridized at defined locations on an array can be indicative of the detection and/or quantification of the corresponding analyte in the sample.

It will be appreciated that the analysis of the amplification product(s) produced is especially adaptable to hybridization by, for example oligonucleotides or polynucleotides immobilized on a solid or semi-solid surface, such as an array. Suitable nucleic acid probes will be evident to one skilled in the art, and include, for example, probes that comprise DNA, RNA, DNA and RNA, peptide nucleic acid (PNA), or any combination of DNA, RNA and/or PNA. These probes can be provided in any suitable form, including, for example, as microarrays. Methods of specific hybridization of a polynucleotide (amplification product) to polynucleotides immobilized on an array are well known in the art. As is known in the art, a microarray refers to an assembly of distinct polynucleotides or

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oligonucleotides immobilized at defined positions on a substrate (surface). Arrays are formed on substrates fabricated with materials such as paper, glass, plastic (e.g., polypropylene, nylon), polyacrylamide, nitrocellulose, metal, silicon, optical fiber, polystyrene, or any other suitable solid or semi-solid support, and configured in a planar (e.g., glass plates, silicon chips) or three-dimensional (e.g., pins, fibers, beads, particles, microtiter wells, capillaries) configuration.

Polynucleotides or oligonucleotides forming arrays may be attached to the substrate by any number of ways including (i) *in situ* synthesis (e.g., high-density oligonucleotide arrays) using photolithographic techniques (see, Fodor et al., *Science* (1991), 251:767-773; Pease et al., *Proc. Natl. Acad. Sci. U.S.A.* (1994), 91:5022-5026; Lockhart et al., *Nature Biotechnology* (1996), 14:1675; U.S. Pat. Nos. 5,578,832; 5,556,752; and 5,510,270); (ii) spotting/printing at medium to low-density (e.g., cDNA probes) on glass, nylon or nitrocellulose (Schena et al, *Science* (1995), 270:467-470, DeRisi et al, *Nature Genetics* (1996), 14:457-460; Shalon et al., *Genome Res.* (1996), 6:639-645; and Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* (1995), 93:10539-11286); (iii) by masking (Maskos and Southern, *Nuc. Acids. Res.* (1992), 20:1679-1684) and (iv) by dot-blotting on a nylon or nitrocellulose hybridization membrane (see, e.g., Sambrook et al., Eds., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.)). Polynucleotides or oligonucleotides may also be noncovalently immobilized on the substrate by hybridization to anchors, by means of magnetic beads, or in a fluid phase such as in microtiter wells or capillaries. Arrays or microarrays of polynucleotides are generally nucleic acids such as DNA, RNA, PNA, and cDNA but may also include proteins, polypeptides, oligosaccharides, cells, tissues and any permutations thereof which can specifically bind the amplification products.

Methods of detecting detectable signals are known in the art. Signal detection may be visual or utilize a suitable instrument appropriate to the particular label used, such as a spectrometer, fluorimeter, or microscope. For example, where the label is a radioisotope, detection can be achieved using, for example, a scintillation counter, or photographic film as in autoradiography. Where a fluorescent label is used, detection may be by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence, such as by microscopy, visual inspection or photographic film. Where enzymatic labels are used, detection may be by providing appropriate substrates for the enzyme and detecting the resulting reaction product. Simple colorimetric labels can usually

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be detected by visual observation of the color associated with the label; for example, conjugated colloidal gold is often pink to reddish, and beads appear the color of the bead.

In one embodiment, the amplification products in a reaction mixture are analyzed on a suitable matrix. In some embodiments, separation of the amplification products from the reaction mixture is performed before they are contacted with the array. Any of a number of methods can be used to effect the separation, as described in, for example, McIntosh et al. (PCT Pub. No. WO98/59066). Such methods include, but are not limited to, mass spectrometry, flow cytometry, HPLC, FPLC, size exclusion chromatography, affinity chromatography, and gel electrophoresis.

Depending on the sensitivity of the detection method, the limits of detection of the methods of the invention are a minimum of 1 analyte moiety (when combined with highly sensitive detection methods, e.g., luminescent oxygen channeling and some array-based methods), or, with less sensitive detection methods, a minimum of 10, 100, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or 10^{10} analyte moieties.

B. Quantification

It is apparent that the primer extension- and transcription-based methods described herein may also be used to quantify an analyte in a sample. The amount of amplification product produced is linearly related to the amount of analyte in the sample. Thus, in some embodiments, comparison of amount of amplification product obtained in a test sample with the amount of amplification product obtained in a reference sample comprising a known amount of an analyte provides quantification of the analyte in the test sample. Methods of making such comparisons are known in the art. One of skill in the art will appreciate that it is also possible to quantify an analyte by using a reference comprising a known amount of binding partner, without analyte. As is evident, as used herein, "quantification" refers to the determination of an absolute level of an analyte (for example, amount of an analyte and/or binding partner in a sample as measured by number of copies or weight), as well as a relative levels of an analyte in a sample. In one embodiment, amount of an analyte is compared to amount of another analyte. Thus, quantification of an analyte also includes the determination of the relative level of two or more analytes, for example, of bound and unbound ligand. Comparison of the amount of amplification product containing a first detectable identifying characteristic obtained in a test sample and the amount of amplification product containing a second detectable identifying characteristic obtained in the same test sample permits quantification of the relative amounts of each analyte. It is

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further appreciated that a reference label is desirably used, for example, to normalize signal intensity for incorporated labeled dNTPs (or ddNTPs) and to control for variation in experimental and/or detection conditions. A non-limiting example of a reference dye includes LIZ (ABI).

Reaction conditions, components, and other experimental parameters as well as illustrative embodiments in this section are generally as described herein.

VII. Timepoints and Complexes

In one embodiment, the foregoing components are added simultaneously at the initiation of analyte-binding partner binding. In other embodiments, components are added in any order prior to or after appropriate timepoints during the binding, amplification, or detection process, as required and/or permitted by the respective reactions. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. The present invention encompasses complex(es) exemplified by the complexes that are present at each such timepoint.

For example, the process may be paused or halted at least at the following time points: after contacting binding partner and analyte (if present) (forming an analyte-binding partner complex and, generally, free binding partner); after separating unbound (free) binding partner from analyte-binding partner complex, if present (leaving the analyte-binding partner complex); after binding of composite primer to oligonucleotide template of the analyte-binding partner complex (forming analyte-binding partner-composite primer complex); at various timepoints in the amplification process (described in more detail below); after amplification of a portion of the oligonucleotide template (giving a mixture of analyte-binding partner-composite primer complex and amplification product); after separation of amplification product (if performed) (leaving separated amplification product suitable for detection).

Methods for stopping the reactions involved in the various steps of the methods of the invention are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity or heating the reaction mixture to a temperature that destroys an enzyme. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity or replenishing a destroyed (depleted) enzyme. In some embodiments, one or more of the components of the reactions is replenished prior to, at, or

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following the resumption of the reactions. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

The reaction can be allowed to proceed without purification of intermediate complexes, for example, to remove unbound binding partner, or to remove primer in the amplification steps. Products can be purified at various timepoints, which can be readily identified by a person of skill in the art.

Accordingly, as would be evident to one skilled in the art, the invention includes various aspects in which a complex formed in the detection and/or quantification methods of the invention serves as a starting material for detection and/or quantification according to the invention. Complexes included within the present invention include analyte-binding partner complex, wherein at least one binding partner of the complex is attached to an oligonucleotide template. In some embodiments, the complex further comprises composite primer(s) bound to the oligonucleotide template(s) of the analyte-binding partner complex. In other embodiments, the complex further comprises primer extension product(s) bound to the oligonucleotide template(s) of the analyte-binding partner complex. In other embodiments, the complex further comprises composite primer(s) and primer extension product(s) bound to the oligonucleotide template(s) of the analyte-binding partner complex.

The invention also provides methods for detecting and/or quantifying an analyte in a sample by amplifying a polynucleotide sequence complementary to an oligonucleotide template comprising: (a) hybridizing a polynucleotide comprising a termination polynucleotide sequence to an oligonucleotide template-composite primer complex, wherein said oligonucleotide template is bound to a binding partner (in some embodiments, in an analyte-binding partner complex), and wherein said complex comprises a composite primer hybridized to a single stranded oligonucleotide template comprising the primer extension region, said composite primer comprising an RNA portion and a 3' DNA portion, whereby said polynucleotide comprising a termination polynucleotide sequence is hybridized to a region of the oligonucleotide template which is 5' with respect to hybridization of the composite primer to the oligonucleotide template; (b) extending the composite primer in the oligonucleotide template-composite primer complex of step (a) with DNA polymerase; (c) cleaving RNA of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, whereby multiple copies of the complementary sequence of the oligonucleotide template are produced.

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In another embodiment, the invention provides methods for amplifying a polynucleotide sequence complementary to an oligonucleotide template that is bound to a binding partner (in some embodiments, in an analyte-binding partner complex) comprising: (a) extending a composite primer in a complex comprising (i) a single stranded oligonucleotide template comprising the primer extension region, wherein said oligonucleotide template is bound to a binding partner (in some embodiments, in an analyte-binding partner complex); and (ii) the composite primer, said composite primer comprising an RNA portion and a 3' DNA portion, wherein the composite primer is hybridized to the oligonucleotide template; (b) cleaving RNA of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, whereby multiple copies of the complementary sequence of the oligonucleotide template are produced.

In still another embodiment, the invention provides methods for amplifying a polynucleotide sequence complementary to an oligonucleotide template that is attached to a binding partner (in some embodiments, in an analyte-binding partner complex) comprising: (a) extending a composite primer in a complex comprising (i) a single stranded oligonucleotide template comprising the primer extension region, wherein said oligonucleotide template is bound to a binding partner (in some embodiments, in an analyte-binding partner complex); (ii) the composite primer, said composite primer comprising an RNA portion and a 3' DNA portion, wherein the composite primer is hybridized to the oligonucleotide template; and (iii) a polynucleotide comprising a termination polynucleotide sequence, wherein the polynucleotide comprising a termination polynucleotide sequence is hybridized to a region of the template which is 5' with respect to hybridization of the composite primer to the oligonucleotide template; (b) cleaving RNA of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement, whereby multiple copies of the complementary sequence of the oligonucleotide template are produced.

In embodiments of the invention wherein displaced primer extension product is generated, the methods can further comprise hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA

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transcripts are produced comprising sequences complementary to the displaced primer extension products; whereby multiple copies of the oligonucleotide template are produced.

For example, in yet another embodiment, the invention provides methods for amplifying an oligonucleotide template attached to a binding partner (in some of these emboiments, in an analyte-binding partner complex) comprising: hybridizing a primer extension product with a polynucleotide comprising a propromoter and a region which hybridizes to the primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the primer extension product, wherein the primer extension product is a displaced primer extension product generated by: (a) hybridizing an oligonucleotide template that is attached to a binding partner (in some embodiments, in an analyte-binding partner complex) with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion; (b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the oligonucleotide template which is 5' with respect to hybridization of the composite primer to the template; (c) extending the composite primer with DNA polymerase; (d) cleaving RNA of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement to produce displaced primer extension product; whereby multiple copies of at least a portion of the oligonucleotide template are produced.

VIII. Compositions, kits, and systems of the invention

The invention also provides compositions and kits used in the methods described herein. The compositions may be any component(s), complex, reaction mixture and/or intermediate described herein, as well as any combination thereof.

For example, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion. In another example, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a composite primer, wherein the composite primer comprises a 5'-RNA portion and a 3'-DNA portion. In one embodiment, the RNA portion is adjacent to the DNA portion. In another example, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a composite primer, wherein the composite primer comprises 5'- and 3'-DNA portions

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with at least one intervening RNA portion. In other examples, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a composite primer that is further derivatized by attachment of a moiety capable of effecting attachment of a polynucleotide comprising the composite primer to a solid substrate used in preparing nucleic acid microarrays. In some embodiments, the composite primer is further derivatized by attachment of a positively charged moiety such as an amine. In other embodiments, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a TSO (i.e., any of the TSO embodiments described herein, including TSOs containing one or more modifications which enhance binding to template). In some embodiments, the compositions comprise a composite primer and a termination sequence. In some embodiments, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a polynucleotide comprising a propromoter sequence, such as a TSO or PTO (i.e., any of those embodiments described herein), and may further comprise a composite primer and/or a blocker sequence. In some embodiments, the invention provides a composition comprising a complex of (a) a binding partner attached to an oligonucleotide template and (b) a blocker sequence (i.e., any of the embodiments described herein, including blocker sequences with modifications). Any of these complexes (and any of the complexes described in this section) can further comprise analyte(s).

In some embodiments, e.g., those employing "sandwich" methods, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion. In another example, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a composite primer, wherein the composite primer comprises a 5'-RNA portion and a 3'-DNA portion. In one embodiment, the RNA portion is adjacent to the DNA portion. In another example, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a composite primer, wherein the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In other examples, the

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invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a composite primer that is further derivatized by attachment of a moiety capable of effecting attachment of a polynucleotide comprising the composite primer to a solid substrate used in preparing nucleic acid microarrays. In some embodiments, the composite primer is further derivatized by attachment of a positively charged moiety such as an amine. In other embodiments, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a TSO (i.e., any of the TSO embodiments described herein, including TSOs containing one or more modifications which enhance binding to template). In some embodiments, the compositions comprise a composite primer and a termination sequence. In some embodiments, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a polynucleotide comprising a propromoter sequence, such as a TSO or PTO (i.e., any of those embodiments described herein), and may further comprise a composite primer and/or a blocker sequence. In some embodiments, the invention provides a composition comprising a complex of (a) an intermediate binding partner specific for an analyte; (b) a binding partner attached to an oligonucleotide template, wherein said binding partner is specific for the intermediate binding partner and (c) a blocker sequence (i.e., any of the embodiments described herein, including blocker sequences with modifications).

In other embodiments, the invention provides compositions comprising a complex of (a) a binding partner attached to an oligonucleotide template; (b) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion (in some embodiments, the RNA portion is adjacent to the DNA portion); and (c) a termination sequence. In some embodiments, the termination sequence is a TSO. In other embodiments, the termination sequence is a blocking sequence. In some embodiments, the composite primer comprises a 5'-RNA portion and a 3'-DNA portion (in certain embodiments, the RNA portion is adjacent to the DNA portion). In other embodiments, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In some embodiments, the composition comprises a complex of (a) a binding partner attached to an oligonucleotide template; (b) a composite primer; (c) a polynucleotide

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comprising a termination sequence; (d) a polynucleotide comprising a propromoter sequence. In some embodiments, the propromoter sequence is provided by a PTO. In other embodiments, the propromoter sequence is provided by a TSO. Any of the above compositions may further comprise any of the enzymes described herein (such as DNA polymerase, RNaseH, and/or RNA polymerase). The compositions are generally in aqueous form, preferably in a suitable buffer.

The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of DNA (sense or anti-sense) or RNA (sense) molecules which are copies of an oligonucleotide template attached to a binding partner, which are produced by any of the methods described herein.

The compositions are generally in a suitable medium, although they can be in lyophilized form. Suitable media include, but are not limited to, aqueous media (such as pure water or buffers).

The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for any one or more of the following uses: contacting analyte and binding partner under conditions suitable for formation of analyte-binding partner complex; amplifying a nucleotide sequence; detection of amplification products; and quantification of amplification products.

The kits of the invention comprise one or more containers comprising any combination of the components described herein, and the following are examples of such kits. A kit may comprise any of the binding partners and composite primers described herein. A kit may comprise a binding partner attached to an oligonucleotide template or have these components separately provided (generally with instructions as to how to effect attachment). For example, a kit may comprise an antibody specific to a member of the Botulinum toxin (BoNT) family. In some embodiments, a kit comprises two or more binding partners and/or two or more composite primers, which may or may not be separately packaged. In other embodiments, a kit comprises a binding partner, a composite primer and a termination sequence (any of those described herein). A kit may comprise a binding partner, a composite primer, a polynucleotide comprising a termination sequence, and a polynucleotide comprising a propromoter sequence (which may be a PTO or TSO). The composite primer may be labeled or unlabeled. Kits may also optionally include an intermediate binding partner specific for an analyte, and/or any of one or more of the enzymes described herein, as well as deoxynucleoside triphosphates and/or ribonucleoside triphosphates. Kits may also

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include one or more suitable buffers (as described herein). Kits useful for producing labeled amplification products may optionally include labeled or unlabelled nucleotides or nucleotide analogs. Kits may further provide a capture moiety and/or solid surface. One or more reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing any of the methods described herein. Each component can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit.

The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (*e.g.*, magnetic diskette or optical disk) containing instructions are also acceptable, relating to the use of components of the methods of the present invention for the intended analyte detection and/or quantification, nucleic acid amplification, and/or, as appropriate, for using the amplification products for purposes such as detection and quantification. The instructions included with the kit generally include information as to reagents (whether included or not in the kit) necessary for practicing the methods of the presentation invention, instructions on how to use the kit, and/or appropriate reaction conditions.

The component(s) of the kit may be packaged in any convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations. Where kits are provided for practicing analyte detection using the transcription-based enhanced linear amplifications methods of the present invention, the RNA polymerase (if included) is preferably provided separately from the components used in the steps prior to the transcription steps.

The relative amounts of the various components in the kits can be varied widely to provide for concentrations of the reagents that substantially optimize the reactions that need to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

The invention also provides systems for effecting the methods described herein. These systems comprise various combinations of the components discussed above. For example, in some embodiments, the invention provides a system suitable for detecting and/or quantifying an analyte comprising (a) a binding partner specific for the analyte, attached to an oligonucleotide template, (b) a composite primer (any of those described herein), (c) DNA polymerase; and (d) ribonuclease. In some embodiments, the system further comprises a polynucleotide comprising a termination sequence (any of those described

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herein). In some embodiments, the system further comprises a polynucleotide comprising a propromoter sequence (which may be a PTO or TSO) and a DNA-dependent RNA polymerase. Any of the systems embodiments may also comprise one or more intermediate binding partners, as described herein.

The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. In some embodiments, the invention provides reaction mixtures comprising (a) a binding partner, such as an antibody or antibody derviative, attached to an oligonucleotide template; (b) a composite primer comprising a 3' DNA portion and an RNA portion; and (c) DNA polymerase. As described herein, any of the composite primers may be in the reaction mixture (or a plurality of composite primers), including a composite primer comprises a 5' RNA portion which is adjacent to the 3' DNA portion. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H. A reaction mixture of the invention can also comprise any of the polynucleotides comprising termination sequences described herein. Another example of a reaction mixture is (a) a displaced primer extension product (which, as such, contains at its 5' end sequence complementary to the 3' DNA portion of the composite primer, but not sequences complementary to the RNA portion of the composite primer); (b) a polynucleotide comprising a propromoter sequence (for example, a PTO); and (c) RNA polymerase. Other reaction mixtures are described herein and are encompassed by the invention. For example, any reaction mixture may further comprise one or more analytes. A reaction mixture may also comprise one or more intermediate binding partners.

IX. Methods using the detection and quantification methods of the invention

The methods and compositions of the invention can be used for a variety of purposes. Methods of determining the presence or absence of analyte(s) and analysis and/or comparison of multiple analytes through hybridization of amplification product to microarrays have been described above. Methods of diagnosis of infectious diseases, genetic defects and cancer, as well as other types of diagnosis (e.g., ultrasensitive detection of antibodies or antigens in plasma, blood, urine, or other samples); forensics; drug testing; detection of biowarfare agents; detection of metabolic or pathological state of a cell, cells, tissue, organ, or organism by detection of presence or absence of analyte(s) corresponding to such a state, and the like, are also possible. As will be apparent from the foregoing, the "sample" in which the presence, absence, and/or quantity of analyte is detected depends on

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the application for which the methods of the invention are used. For example, samples for diagnosis or for forensics include, but are not limited to, blood, plasma, serum, skin, muscle, or other tissue or organ samples, saliva, urine, feces, semen, vaginal secretions, and any other sample useful for diagnosis or forensic analysis, as will be readily apparent to those of skill in the art . Samples for biowarfare agent detection include, but are not limited to, air, soil, clothing, building materials, transportation materials, missile components, and the like. Samples may be prepared for analysis using methods well known in the art. In some embodiments one or more isolation steps are performed to enrich concentration of analyte and/or reduce contaminants or other components which may interfere with the assay. Analyte may be modified by chemical or other means to enhance binding or other characteristics advantageous in the methods of the invention. Other applications will be readily apparent to one of skill in the art.

The methods of the invention are specially suited for integration with a miniaturized microfluidics device for the detection and identification of bio-warfare agents, such as anthrax or botulinum. Current DNA-based assays and devices require the incorporation of a miniaturized temperature cycling component for performance of PCR based sequence amplification. The ability to integrate a highly efficient isothermal amplification in these devices will greatly reduce device complexity. Moreover, the single stranded amplification product is suitable for both homogeneous and heterogeneous detection. Various antibodies employed in the detection of multiple antigens may be conjugated with defined oligonucleotides, each comprising a specific nucleotide sequence and a common sequence complementary to a common SPIA™ primer. The detection of specific threat agents, such as the BoNT, may be carried out using specific antibody pairs for capture and detection. The detection of captured antigen (toxin) is carried out by SPIA™ amplification. A common SPIA™ primer may be employed for amplification of a single or a plurality of oligonucleotide templates, which may be detected by various means. When multiple antigens are detected simultaneously, the various amplification products may be detected using an array of immobilized oligonucleotides each corresponding (e.g., complementary) to a specific primer extension portion of an oligonucleotide sequence. Various array compositions have been described in recent years, which may be integrated with the rapid detection system. The hand-held micro-device is suitable for use in field-testing by emergency personnel as well as in urgent care facilities.

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All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

The following Examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1. Single Primer Isothermal Amplification

This example illustrates the accuracy and amplification power of single primer isothermal amplification.

Various target human and bacterial (*E. coli*, M13) genomic DNA sequences as well as synthetic single-stranded control sequences were amplified using the single primer isothermal amplification (SPIA™) method. See U.S. Patent No. 6,251,639. SPIA™ was carried out as follows: A single composite primer, comprising a 3' DNA portion and a 5' RNA portion, was used for amplification of a defined sequence. Specific composite primers for the control and test nucleic acid sequences were employed. The design of the sequence specific composite primers was carried out using commercially available software programs, as used for PCR primers, and the primers were prepared by Dharmacon. The reaction was carried out in Tris buffer at pH 8.5, 0 to 50 mM KCl, 2 to 5 mM $MgCl_2$, 0.25 to 0.5 mM dNTPs, a DNA polymerase with strong strand displacement activity, such as Bca or Bst DNA polymerases, RNase H, and 1 to 5 mM DTT, 3 ug T4gp32 (USB), BSA, and Rnasine. The reaction mixtures comprising the primers, samples and/or controls, were first denatured by incubation at 95^0C for 2 to 5 min., and the primer(s) were allowed to anneal to the respective target by incubation at 55^0C for 5 min. The enzyme mixture was than added to the reaction tubes and the amplification and signal generation and detection was carried out by further incubation at this temperature for 30 min.

The amplification efficiency of various specific genomic and synthetic targets was determined by quantification of the amplification products using the Quantitative Real Time PCR method. The quantification reactions were carried out using primer pairs designed to amplify the specific products, commercially available kits for quantitative Real Time PCR and BioRad iCycler equipped with fluorescent detectors. Further characterization of SPIA™ amplification products of specific target sequences was carried out by sequence determination, using cycle sequencing and determination of the product sequence using capillary electrophoresis (ABI Prism® 370 Genetic Analyzer). An example of the

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alignment of the detected sequence of a SPIA™ amplification product of a control synthetic target (sequence 221) with the expected sequence is shown below. The amplification product used in the cycle sequencing reaction was generated by SPIA™ amplification of 10^3 molecules of the target sequence. Dotted line denotes the sites of primer binding. Perfect sequencing results were obtained for the amplification product, indicating the high fidelity and efficiency of SPIA™ sequence amplification reaction.

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Syn221 TCTCAGGTTT CAGGGATTAG GGAGATATTA TTTGGCCAAA CACACAAACG*SPIA™ prod.* ..... *GGAGATATTA TTTGGCCAAA CACACAAACG*

Syn221 GAGATGAAAA GGGAAAGATG TGCCAGATAC TGGGGAGCCT TGGAGGGTTG*SPIA™ prod* *GAGATGAAAA GGGAAAGATG TGCCAGATAC TGGGGAG*.....

Assessment of the amplification efficiency was generally carried out by quantification of the specific product using a commercially validated method, such as Real Time PCR, using primer-pairs designed to anneal to the predicted SPIA™ product sequence. In addition, the sequences of the various specific products were also determined using cycle sequencing (ABI kits and instrumentation). Amplification efficiency for a synthetic DNA control target is shown in Table 1. Similar SPIA™ amplification efficiencies were obtained for amplification of genomic sequences.

Table 1: SPIA™ efficiency of amplification of a synthetic control sequence 221, determined by Real Time PCR for quantification of SPIA™ amplification products.

Input Copy Numbers	Amplification Efficiency
2×10^8	2×10^5
2×10^7	$>2 \times 10^5$
2×10^6	$>2 \times 10^6$
2×10^5	$>2 \times 10^6$
2×10^4	$>2 \times 10^7$
2×10^3	$>2 \times 10^7$

This example illustrates the fidelity and amplification power of SPIA™ for rapid amplification and detection of specific nucleic acid sequence tags.

Example 2: SPIA™-enhanced immunoassay of TNFα

This example illustrates the detection and quantification of human TNFα, using commercially available monoclonal antibodies and a synthetic DNA template as a tag.

Oligonucleotide templates are synthesized and conjugated to specific antibodies using thiol chemistry as previously described (Wiltshire *et al*, 2000, Detection of Multiple Allergen-specific IgEs on Microarrays by Immunoassay with Rolling Circle Amplification, *Clin Chem* 46:1990-1993). Anti-human TNFα monoclonal antibodies and antigen are obtained from BD Biosciences (Pharmingen, http://www.bdbiosciences.com/ptProductList.jsp?page=14). Thiol modified synthetic target

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oligodeoxynucleotides is obtained from Operon, and is conjugated to the specific antibody using published procedures (Operon). The synthetic target DNA is designed with a 3' sequence corresponding to a well-characterized SPIA™ primer known to yield highly efficient amplification. The immune complex is formed in the reaction mixture and is captured on solid support by contacting the solution with solid surface comprising an immobilized second antibody, i.e. anti TNF monoclonal antibody, to form a tri molecular complex of the analyte bound to the two antibodies, one of which is labeled with the target nucleic acid. The free labeled complex is washed away and the solid surface is combined with the amplification reaction mixture. The amplification reaction mixture comprises a composite primer, polymerase, RNase H, dNTPs and buffer components, as described above.

Various methods for the detection and quantification of the amplification products are used. For example, detection of SPIA™ amplification products using fluorescent probe hybridization methods, such as Molecular Beacons, is used. The use of sequence specific Molecular Beacon probes for real time detection of amplification products affords sensitive detection of specific amplification products and has been described (Tyagi S and Kramer F, 1996, Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechmol.* 14:303-308). These methods are used for detection of the products of the SPIA™-enhanced immunoassay.

Example 3. SPIA™-enhanced immunoassay for BoNT.

This example demonstrates an assay system directed at the detection of multiple analytes that allows the detection of any of a group of specific toxins.

BoNT are among the most potent toxins known, with an LD_{50} in the range of 1 to 5 ng kg^{-1}. Pairs of antibodies specific for specific BoNT are obtained and tested for specificity and sensitivity of a non-enhanced ELISA assay, to select for suitable capture and detector antibody pairs. The capture antibody is the antibody immobilized on a solid surface, such as a well of a microtiter plate, or a bead. The detector antibody is the antibody conjugated to the target nucleic acid reporter. Both antibodies recognize the analyte but are not competing for the same recognition site. Affinity purified antibodies for specific toxins and the corresponding toxins are commercially available from MetaBiologics Inc. (http://www.metabiologics.com/products.htm). BoNT-specific monoclonal antibodies

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suitable for detection of the specific botulinum toxins are also used. These are provided by Dr. James Marks at the University of California, San Francisco. In addition, combinations of affinity purified polyclonal antibodies and monoclonal antibodies are assessed for maximal assay performance. The preparation of antibody- target (tag) DNA conjugates is performed as defined for the TNFα system and the performance of the SPIA™-enhanced immunoassay is performed using purified antigen. Then the method is assessed for the detection and quantification of specific BoNT in samples (e.g. soil, water, biofluids, etc.) that mimic field conditions.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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CLAIMS

What is claimed is:

1. A method for determining presence or absence of an analyte in a sample comprising:

a) contacting the sample with a binding partner that is attached to an oligonucleotide template and that is capable of binding, directly or indirectly, to the analyte, if present, under conditions that permit binding, whereby an analyte-binding partner complex is formed if analyte is present;

b) separating analyte-binding partner complex from unbound binding partner;

c) amplifying a polynucleotide sequence complementary to at least a portion of the oligonucleotide template according to a method comprising:

(i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion;

(ii) extending the composite primer with DNA polymerase, whereby a primer extension product comprising a detectable identifying characteristic is produced;

(iii) cleaving RNA of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement to produce cleaved primer extension product that comprises a detectable identifying characteristic;

whereby multiple copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template are produced; and

whereby detection of the cleaved primer extension product comprising the detectable identifying characteristic indicates presence of the analyte in the sample.

2. A method for detecting the presence or absence of an analyte in a sample comprising incubating a reaction mixture, said reaction mixture comprising:

(a) a sample suspected of containing a complex of the analyte and a binding partner, wherein the binding partner is attached to an oligonucleotide template;

(b) a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion;

(c) a DNA polymerase, dNTPs, and an enzyme that cleaves RNA from an RNA/DNA hybrid;

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wherein the incubation is under conditions that permit hybridization of the composite primer and the oligonucleotide template, oligonucleotide polymerization, and RNA cleavage, such that multiple copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template are produced, and wherein detection of a detectable identifying characteristic of the copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template indicates the presence of the analyte.

3. A method for generating multiple copies of a polynucleotide sequence complementary to at least a portion of an oligonucleotide template attached to a binding partner comprising:

(a) hybridizing a composite primer to the oligonucleotide template attached to the binding partner, said composite primer comprising an RNA portion and a 3' DNA portion;

(b) extending the composite primer with DNA polymerase;

(c) cleaving RNA of the hybridized composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement,

whereby multiple copies of the polynucleotide sequence complementary to at least a portion of an oligonucleotide template attached to a binding partner are produced.

4. A method for determining presence or absence of each of a plurality of different analytes in a sample comprising:

a) contacting the sample with a plurality of different binding partners, each of which is attached to an oligonucleotide template and each of which is capable of binding one of the plurality of different analytes under conditions that permit binding, whereby an analyte-binding partner complex is formed for a particular pair of analyte and binding partner if the analyte is present, wherein the oligonucleotide template for each different binding partner comprises a primer-binding region that is common to all of the binding partners and a primer-extension region that is unique for each binding partner;

b) separating analyte-binding partner complexes from unbound binding partners;

c) amplifying a polynucleotide sequence complementary to at least a portion of each oligonucleotide template present after step b) according to a method comprising:

(i) hybridizing a composite primer to the oligonucleotide template, said composite primer comprising an RNA portion and a 3' DNA portion;

(ii) extending the composite primer with DNA polymerase, whereby a unique

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primer extension product comprising a unique detectable identifying characteristic is produced for each analyte-binding partner complex;

(iii) cleaving RNA of the hybridized extended composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer hybridizes to the oligonucleotide template and repeats primer extension by strand displacement to produce unique cleaved primer extension product for each different oligonucleotide template that comprises a unique detectable identifying characteristic;

whereby multiple copies of the polynucleotide sequence complementary to at least a portion of each oligonucleotide template present after step b) are produced; and

whereby detection of the unique detectable identifying characteristic of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template attached to the binding partner for an analyte indicates the presence of the analyte in the sample.

5. The method of claim 4 further comprising quantifying the relative amounts of each analyte in the sample by comparing the relative amounts of polynucleotide sequence complementary to at least a portion of the oligonucleotide template attached to the binding partner in each analyte-binding partner complex.

6. The method of any of claims 1, 2, or 4, wherein said detectable identifying characteristic is selected from the group consisting of size of the cleaved primer extension product, sequence of the cleaved primer extension product, and detectable signal associated with the cleaved primer extension product.

7. The method of claim 6, wherein the detectable identifying characteristic comprises the sequence of the cleaved primer extension product, wherein the sequence is detected by hybridizing the cleaved primer extension product with a nucleic acid probe that is hybridizable to the cleaved primer extension product.

8. The method of claim 7, wherein the nucleic acid probe comprises DNA.

9. The method of claim 7, wherein the nucleic acid probe is provided as an array.

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10. The method of claim 9, wherein the array comprises the probe immobilized on a substrate fabricated from a material selected from the group consisting of paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, metal, polystyrene, and optical fiber.

11. The method of claims 1, 2, or 4 wherein said detectable signal is associated with a label on a deoxyribonucleoside triphosphate or analog thereof that is incorporated during primer extension.

12. The method of claims 1, 2, or 4 wherein said detectable signal is associated with interaction of two labels, wherein the labels are on deoxynucleoside triphosphates or analogs thereof, and wherein one or both of the labels is incorporated during primer extension.

13. The method of claim 12, wherein one label is on a deoxyribonucleoside triphosphate or analog thereof that is incorporated during primer extension and another label is on a deoxyribonucleoside triphosphate or analog thereof located in the primer portion of the primer extension product.

14. The method of claim 1 or 2 further comprising (d) quantifying the analyte in the sample by comparing amount of copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template obtained in the sample, if any, to the amount of copies of the polynucleotide sequence complementary to at least a portion of the oligonucleotide template obtained in a reference comprising a known amount of the analyte obtained in a reference comprising a known amount of the analyte that is subjected to steps (a) to (c); whereby the comparison provides quantification of amount of analyte in the sample.

15. The method of any of claims 1-4 further comprising binding of the analyte to an intermediate binding partner, wherein the intermediate binding partner binds to the binding partner that is attached to the oligonucleotide template, whereby the binding partner that is attached to an oligonucleotide template indirectly binds to analyte via the intermediate binding partner instead of by binding directly to the analyte, and whereby an analyte-binding partner complex is formed.

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16. The method of claim 15 wherein the intermediate binding partner comprises an antibody specific to the analyte.

17. The method of claim 16 wherein the binding partner that is attached to an oligonucleotide template comprises a second antibody specific to the intermediate binding partner antibody.

18. The method of any of claims 1-4, wherein the RNA portion of the composite primer that hybridizes to the oligonucleotide template is 5' with respect to the 3' DNA portion.

19. The method of claim 18, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

20. The method of any of claims 1-4 wherein the oligonucleotide template comprises a ssDNA portion, and wherein the ssDNA portion has a length of about 25 to about 100 nucleotides.

21. The method of any of claims 1-4 wherein the oligonucleotide template comprises a ssDNA portion, and wherein the ssDNA portion has a length of about 25 to about 200 nucleotides.

22. The method of any of claims 1-4, wherein the enzyme that cleaves RNA from an RNA/DNA hybrid is RNase H.

23. The method of claim 17 wherein the analyte comprises a member of the Botulinum toxin (BoNT) family.

24. The method of any of claims 1-4 wherein the analyte is selected from the group consisting of proteins, polypeptides, peptides, nucleic acid segments, carbohydrates, cells, microorganisms and fragments and products thereof, an organic molecule, and an inorganic molecule.

25. The method of claim 24 wherein the analyte comprises a peptide.

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26. The method of claim 25 wherein the analyte comprises a member of the Botulinum toxin (BoNT) family.

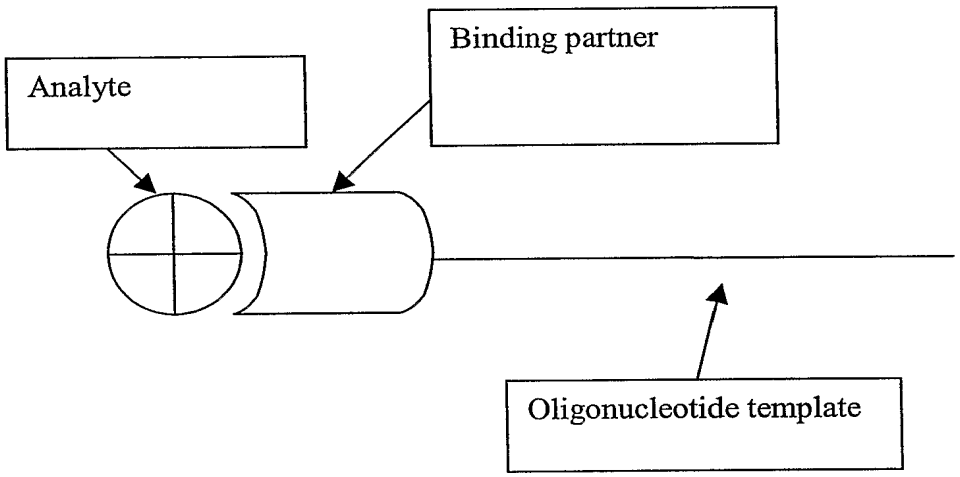
27. The method of any of claims 1-4, wherein the oligonucleotide template is covalently attached to the binding partner.

28. The method of any of claims 1-4, wherein the oligonucleotide template is non-covalently attached to the binding partner.

29. The method of any of claims 1-4 wherein the analyte-binding partner complex is immobilized on a solid surface.

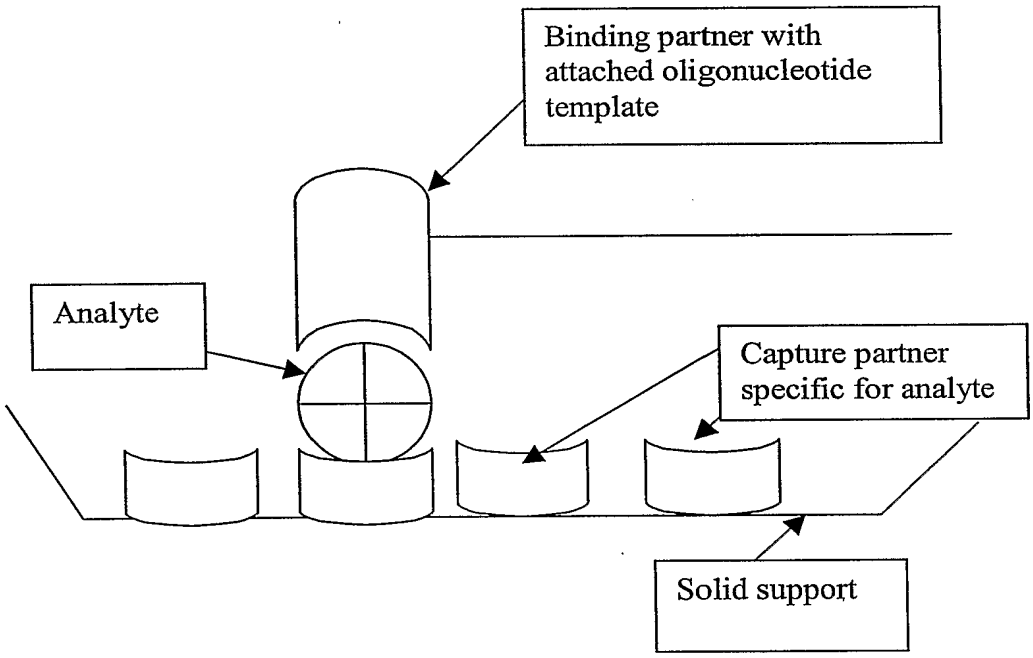
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FIGURE 1



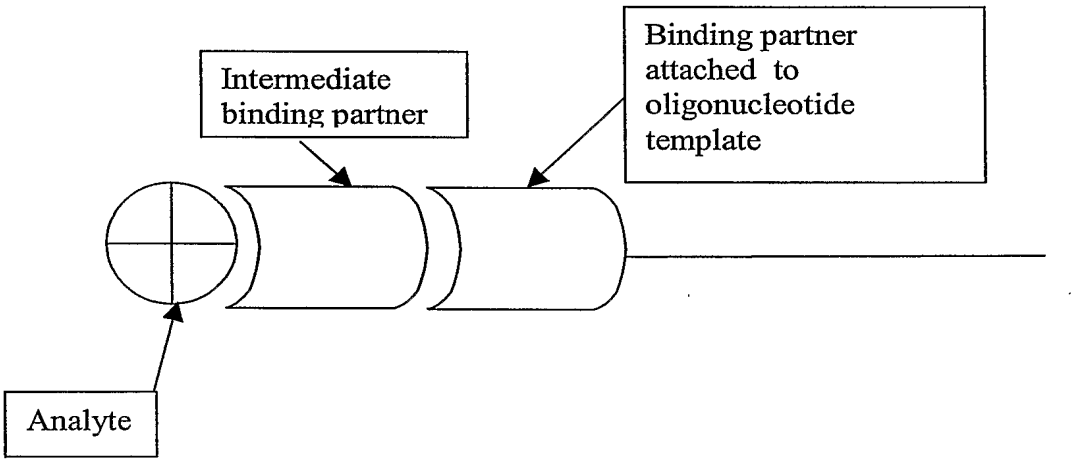
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FIGURE 2



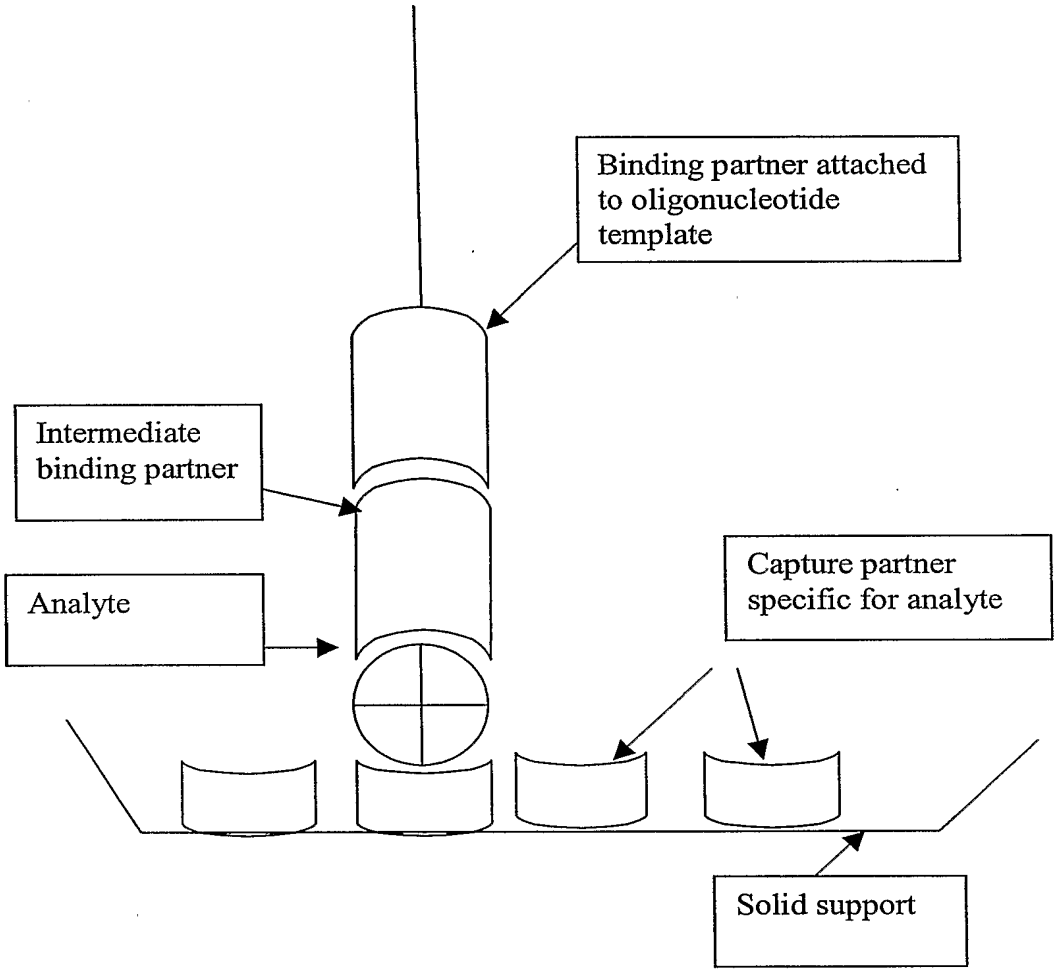
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FIGURE 3



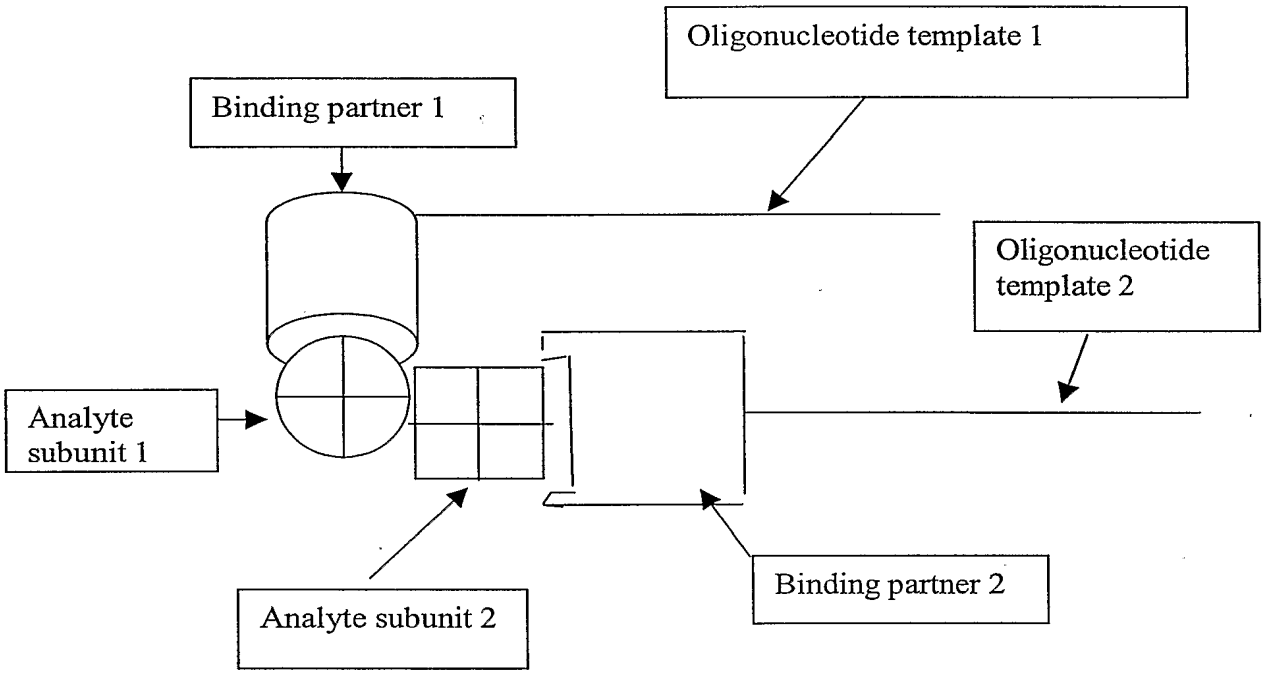
IDT Exhibit 1020.7273

FIGURE 4



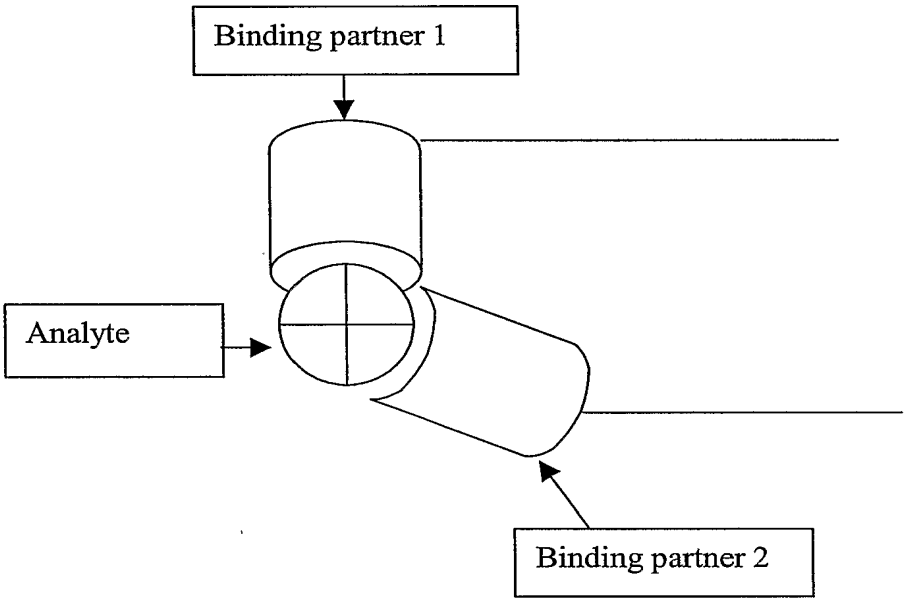
IDT Exhibit 1020.7274

FIGURE 5



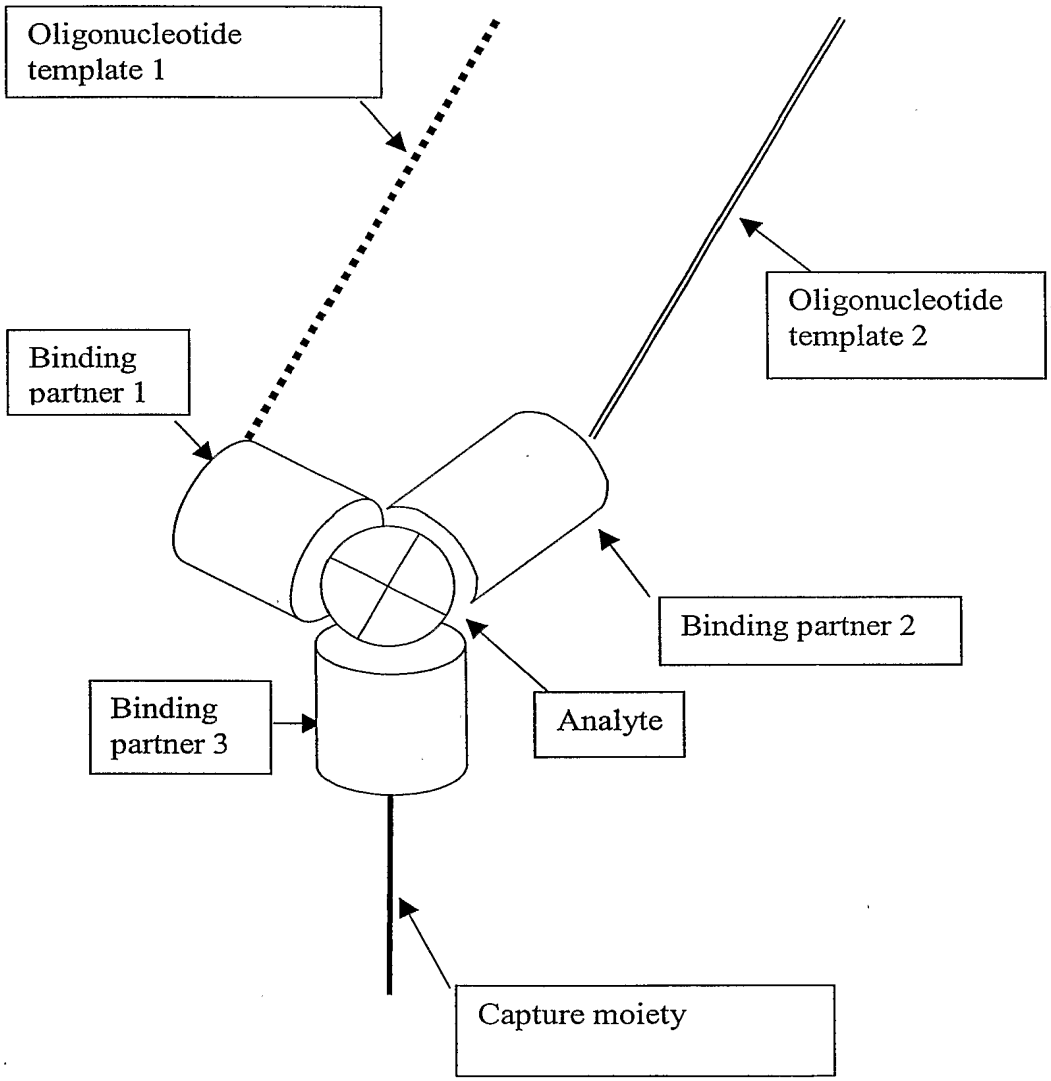
IDT Exhibit 1020.7275

FIGURE 6



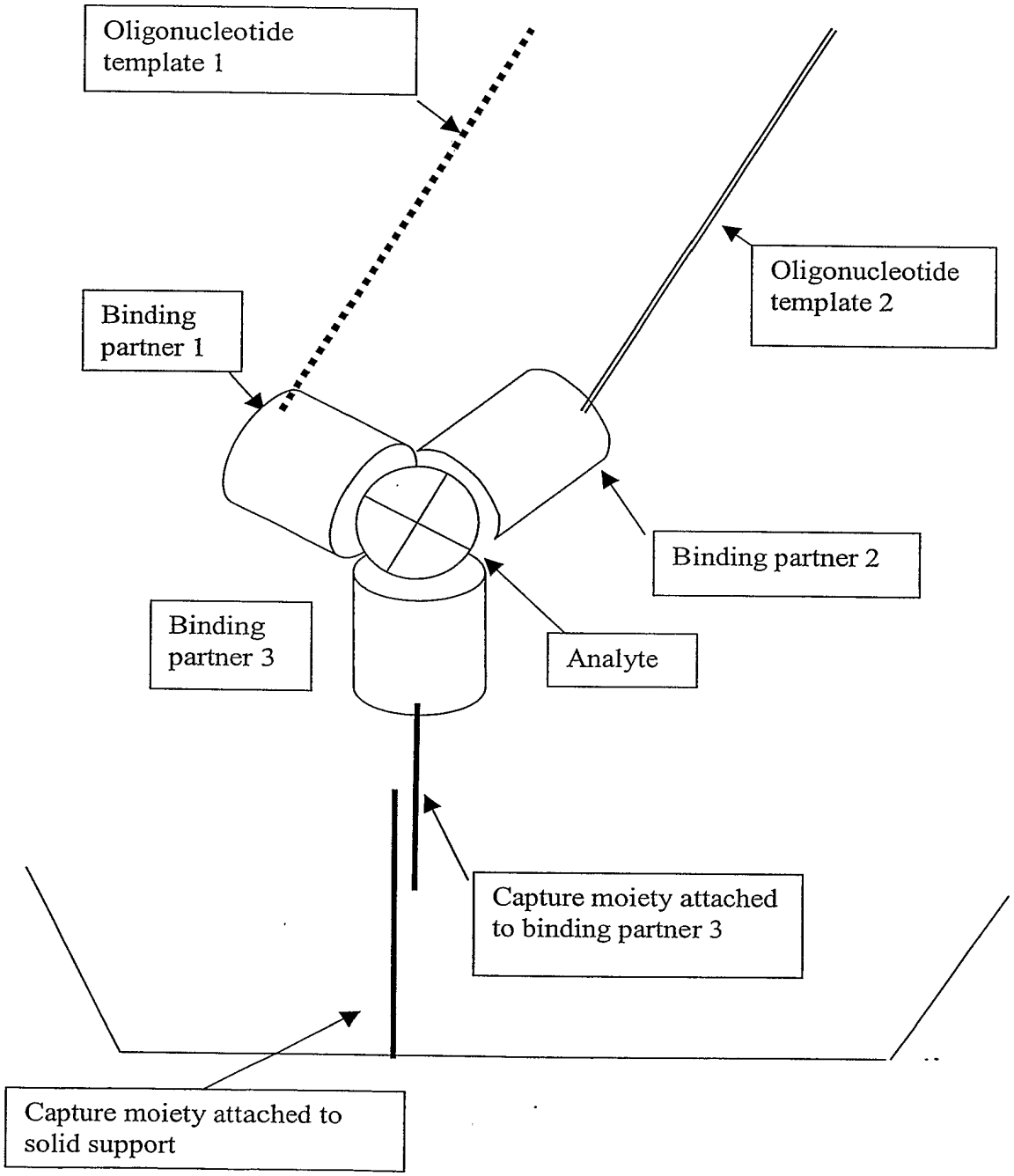
IDT Exhibit 1020.7276

FIGURE 7



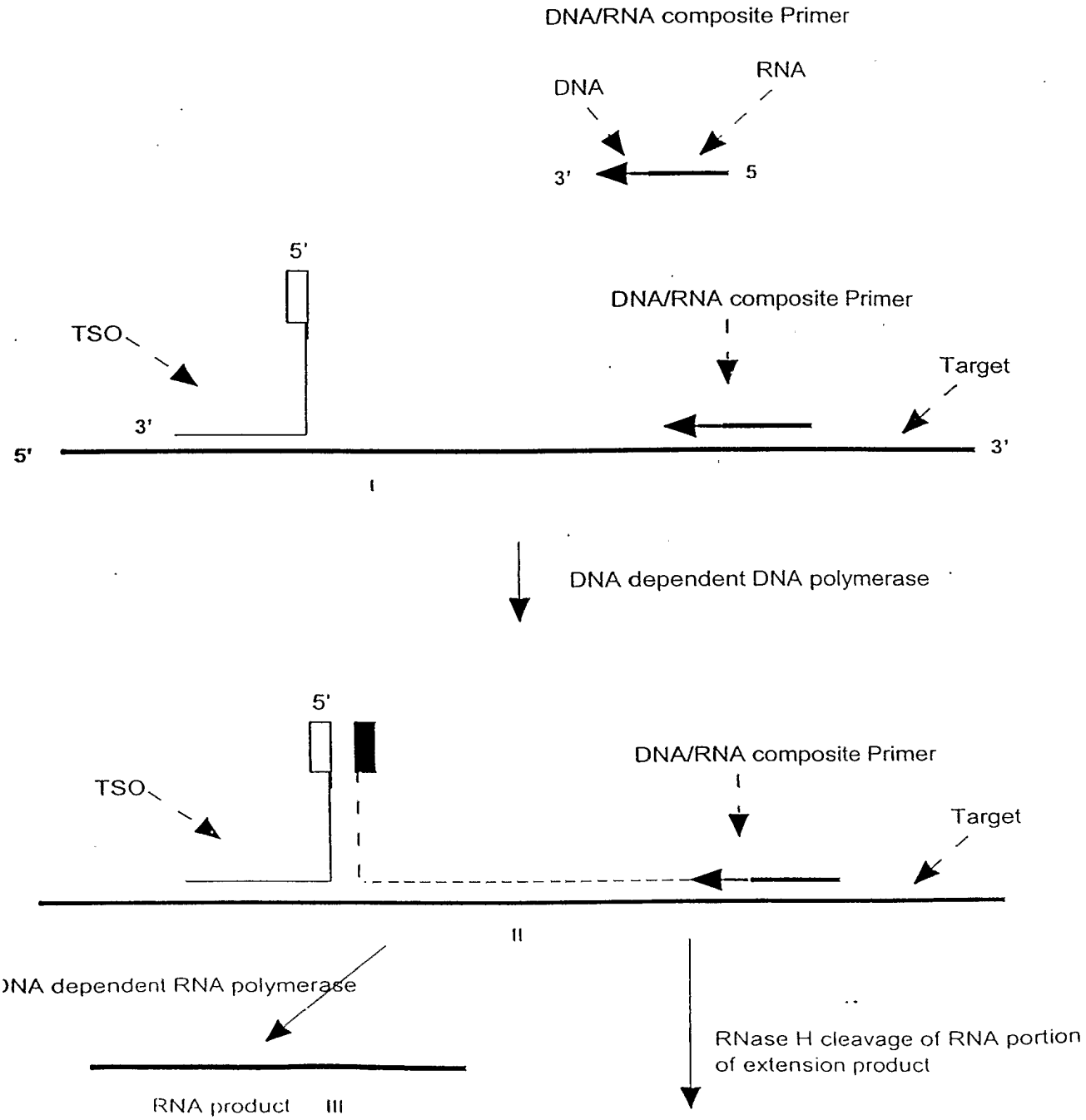
IDT Exhibit 1020.7277

FIGURE 8



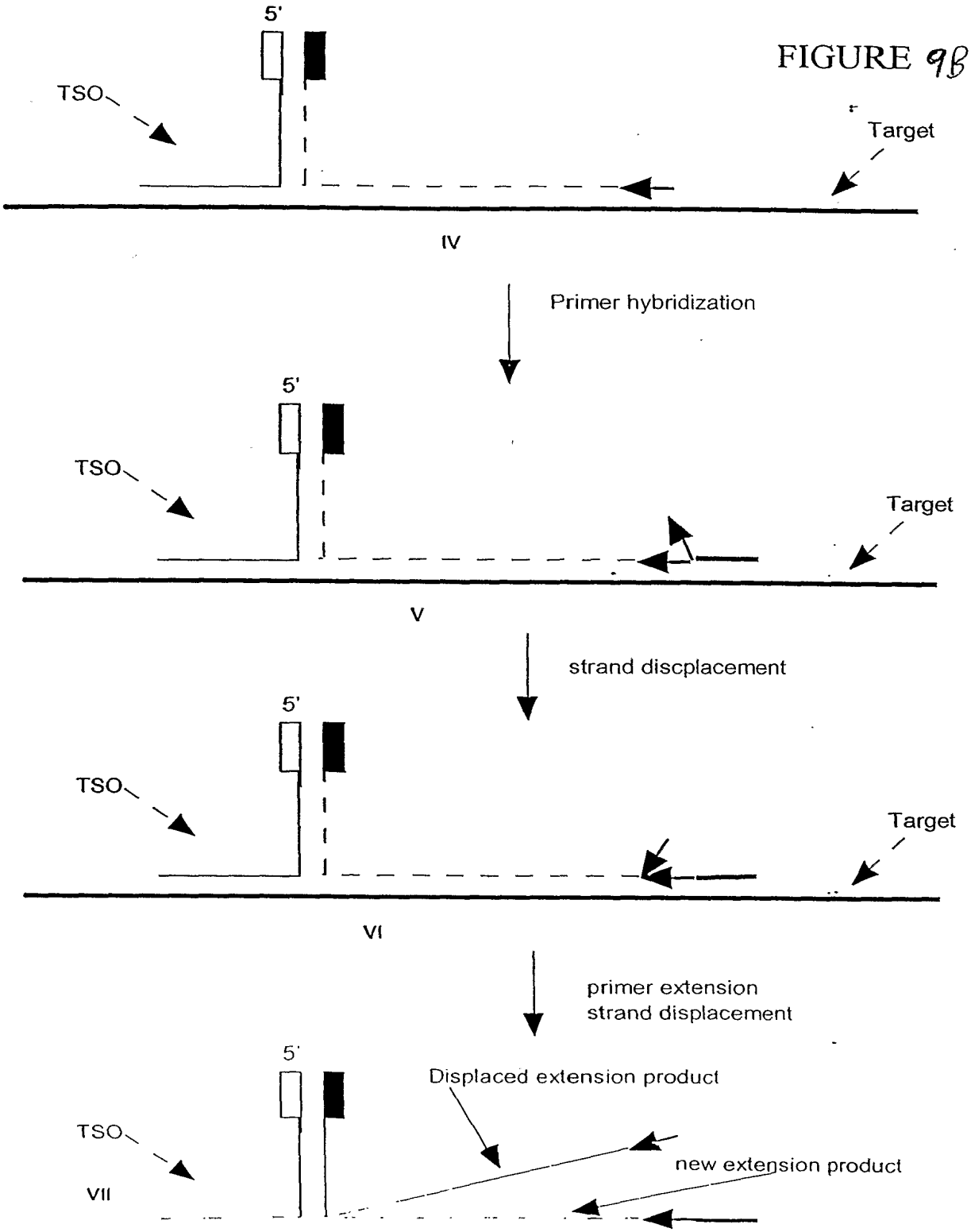
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FIGURE 9A



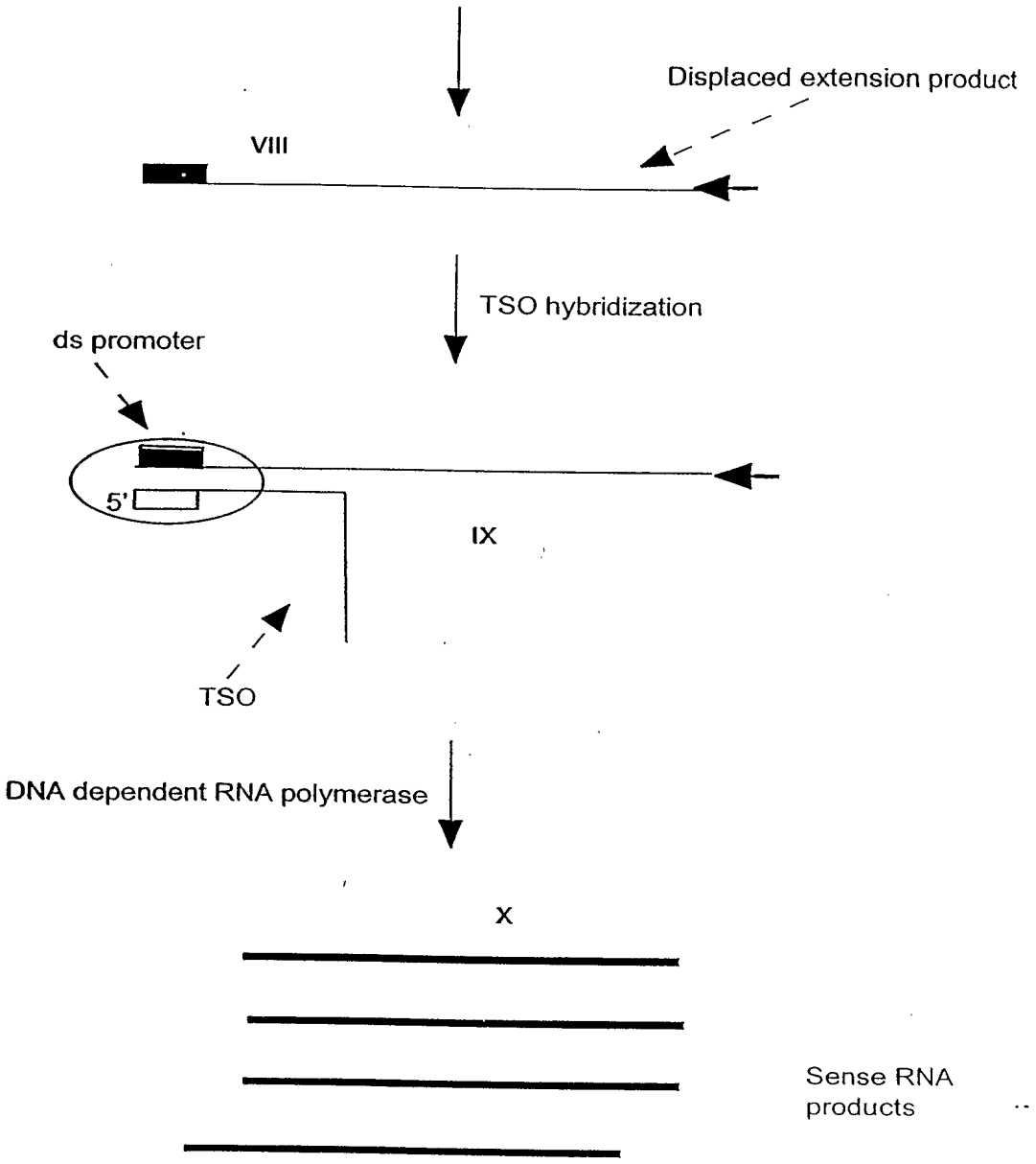
IDT Exhibit 1020.7279

FIGURE 9B



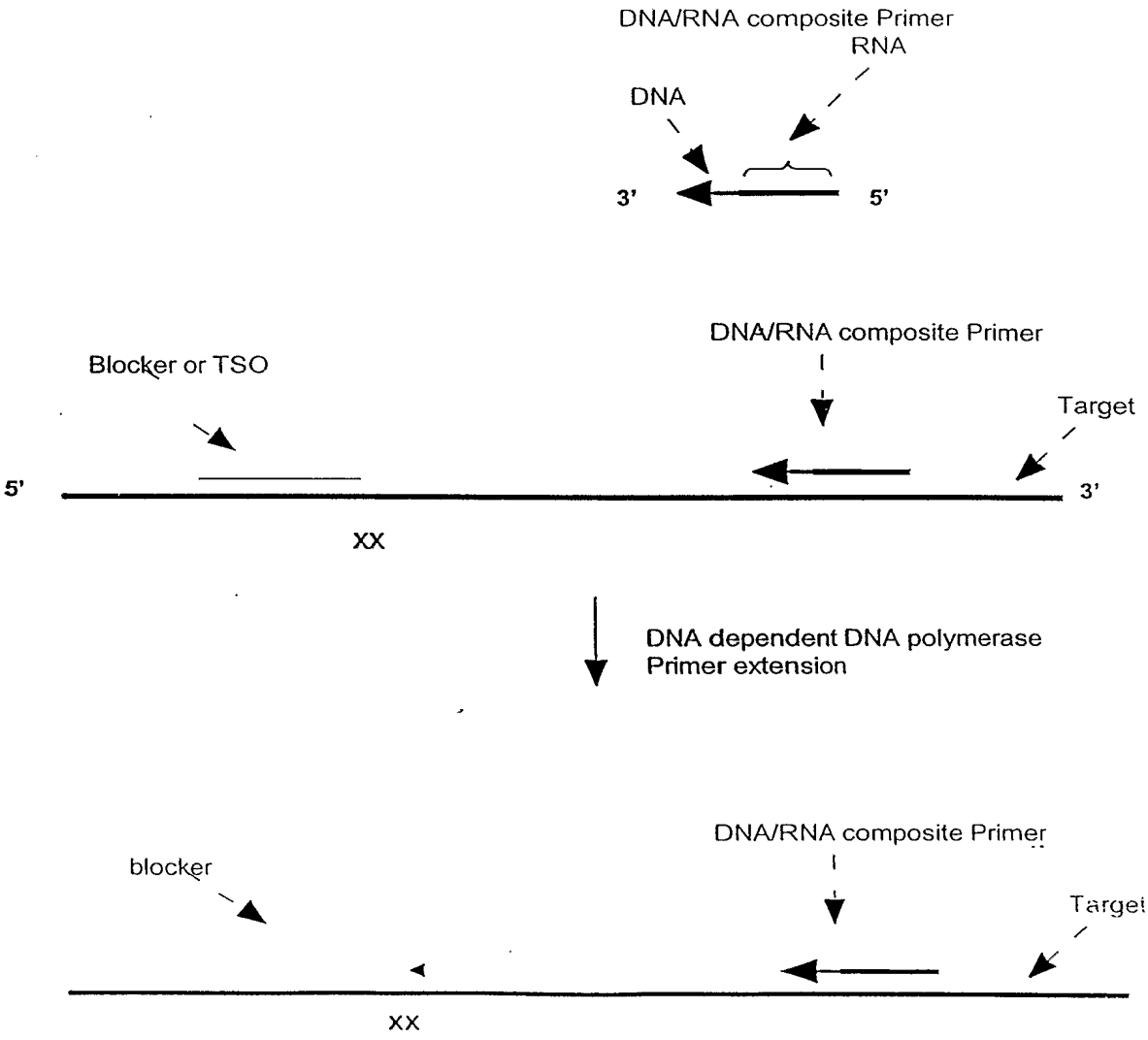
IDT Exhibit 1020.7280

FIGURE 9C



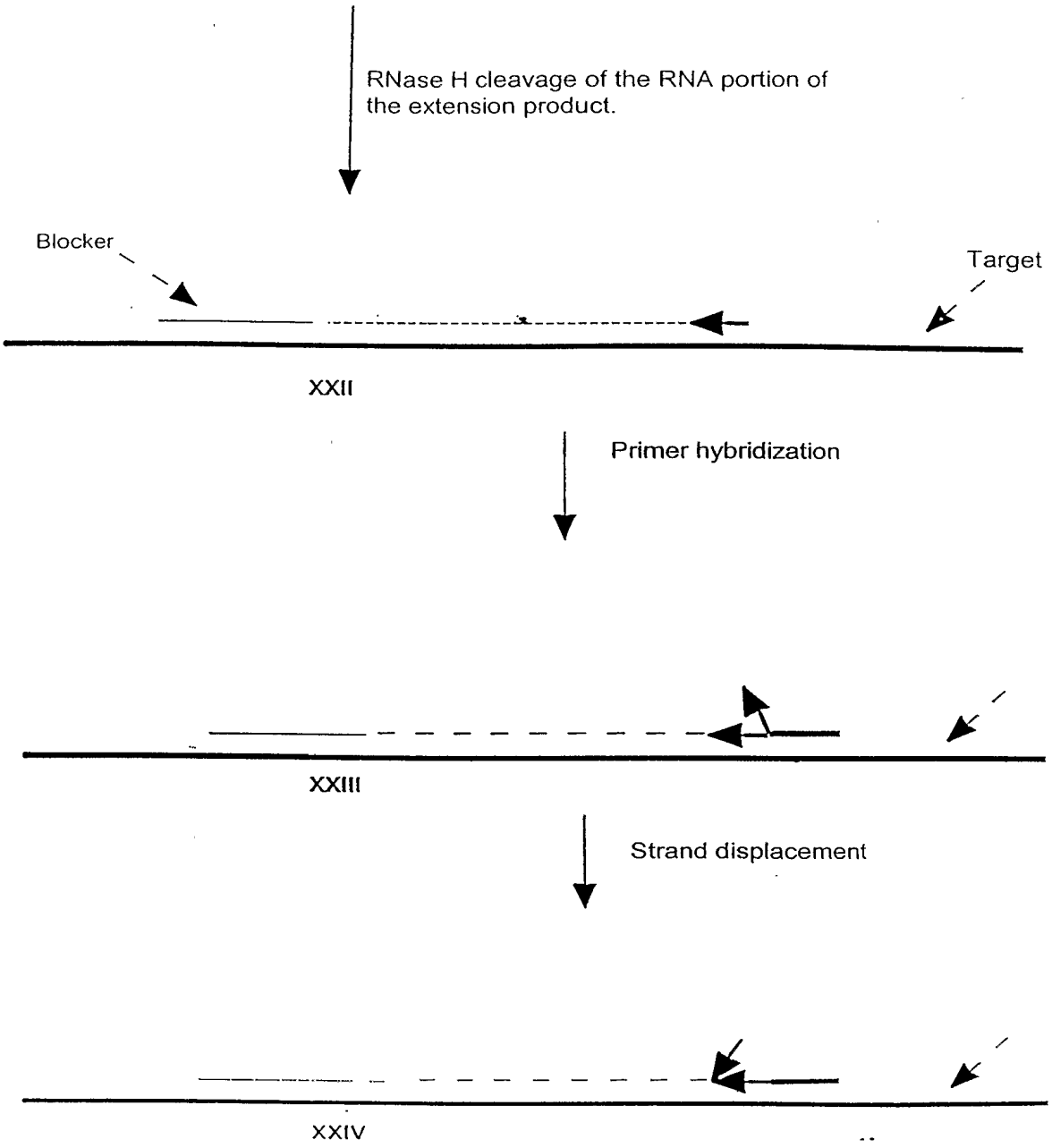
IDT Exhibit 1020.7281

FIGURE 10A



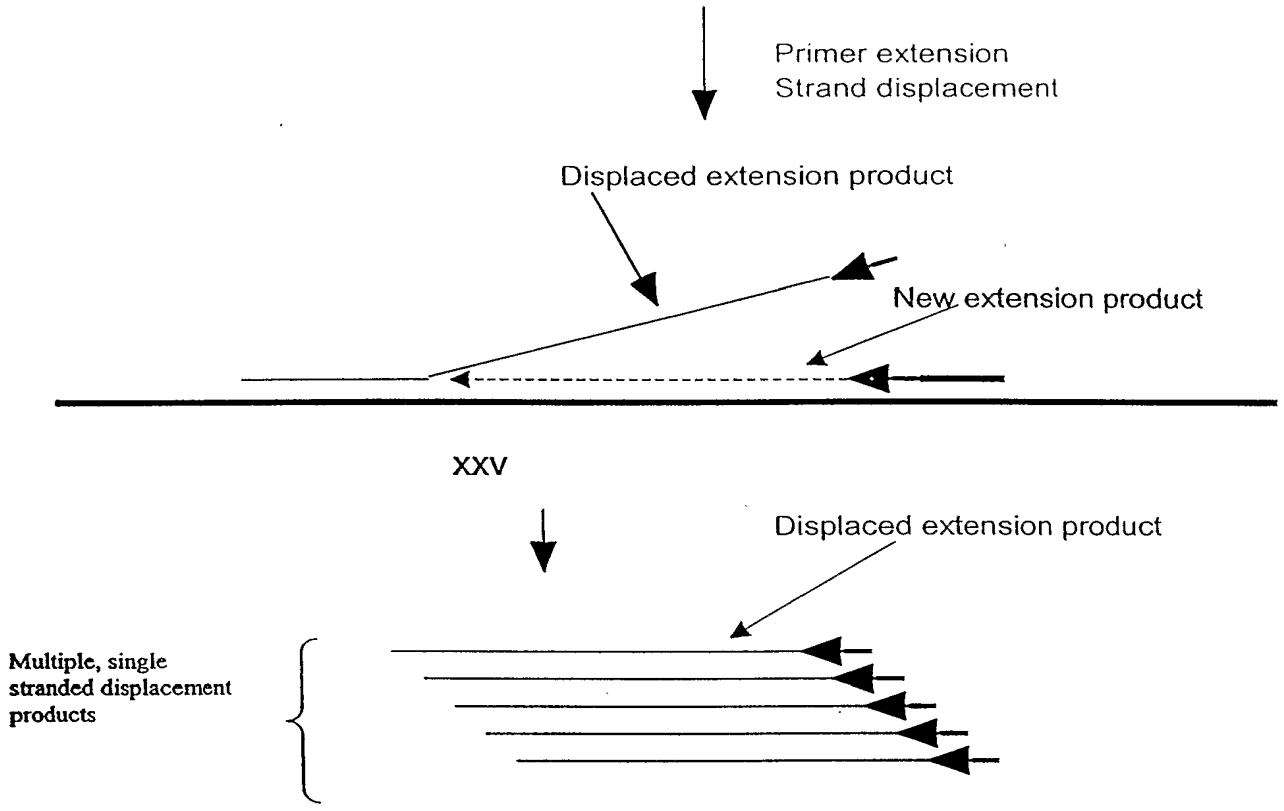
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FIGURE 10B



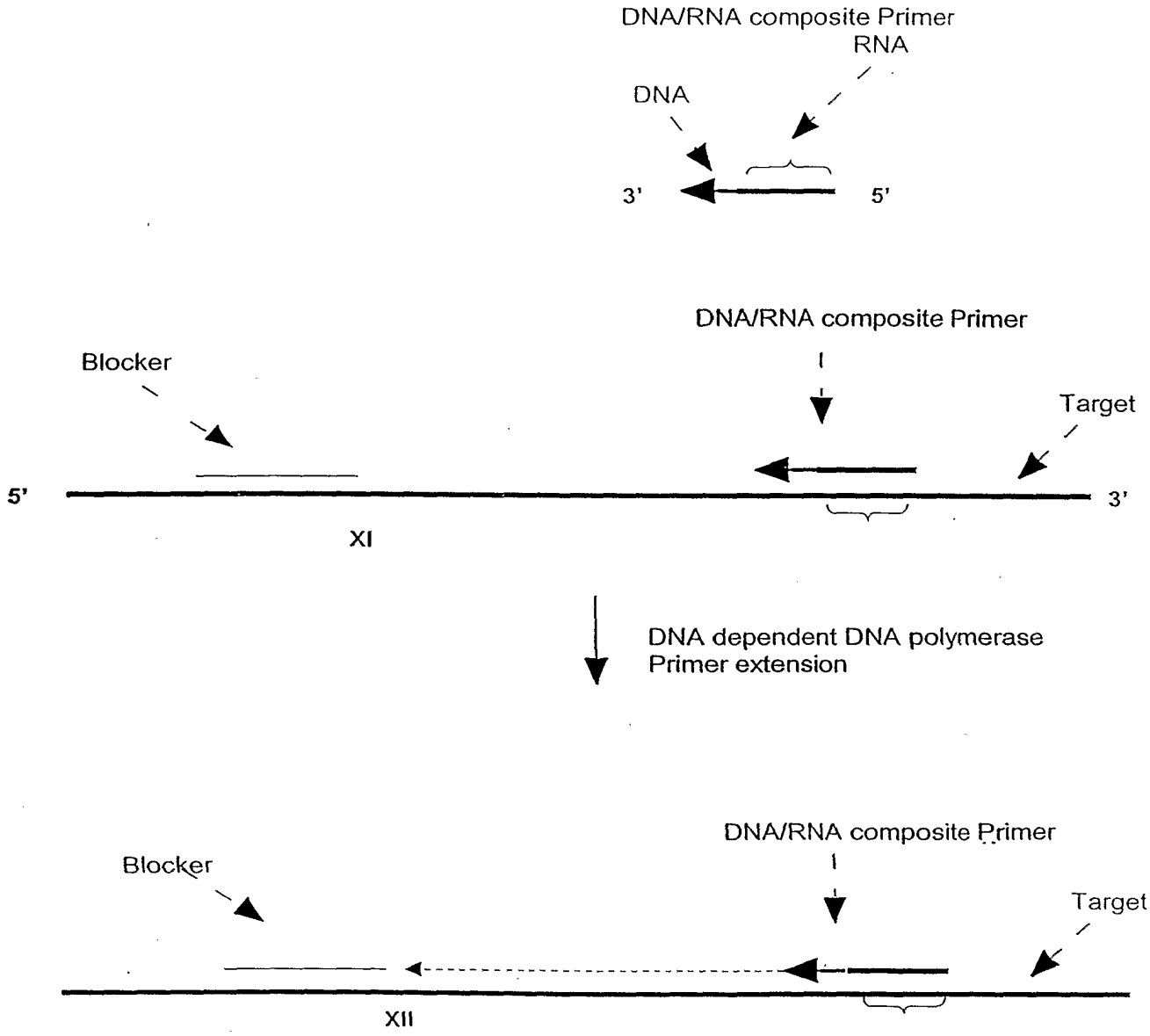
IDT Exhibit 1020.7283

FIGURE 10C



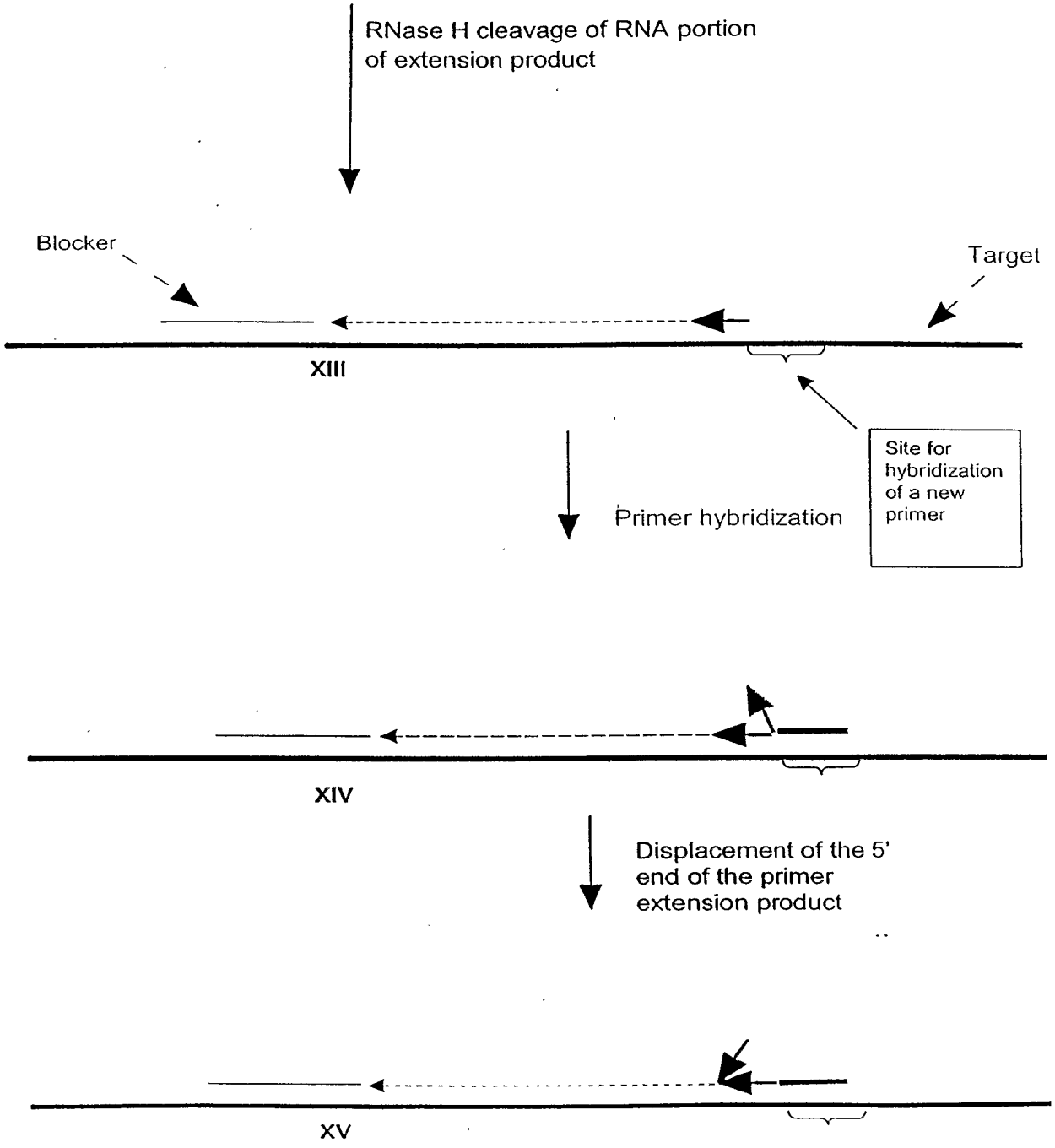
IDT Exhibit 1020.7284

FIGURE 11A



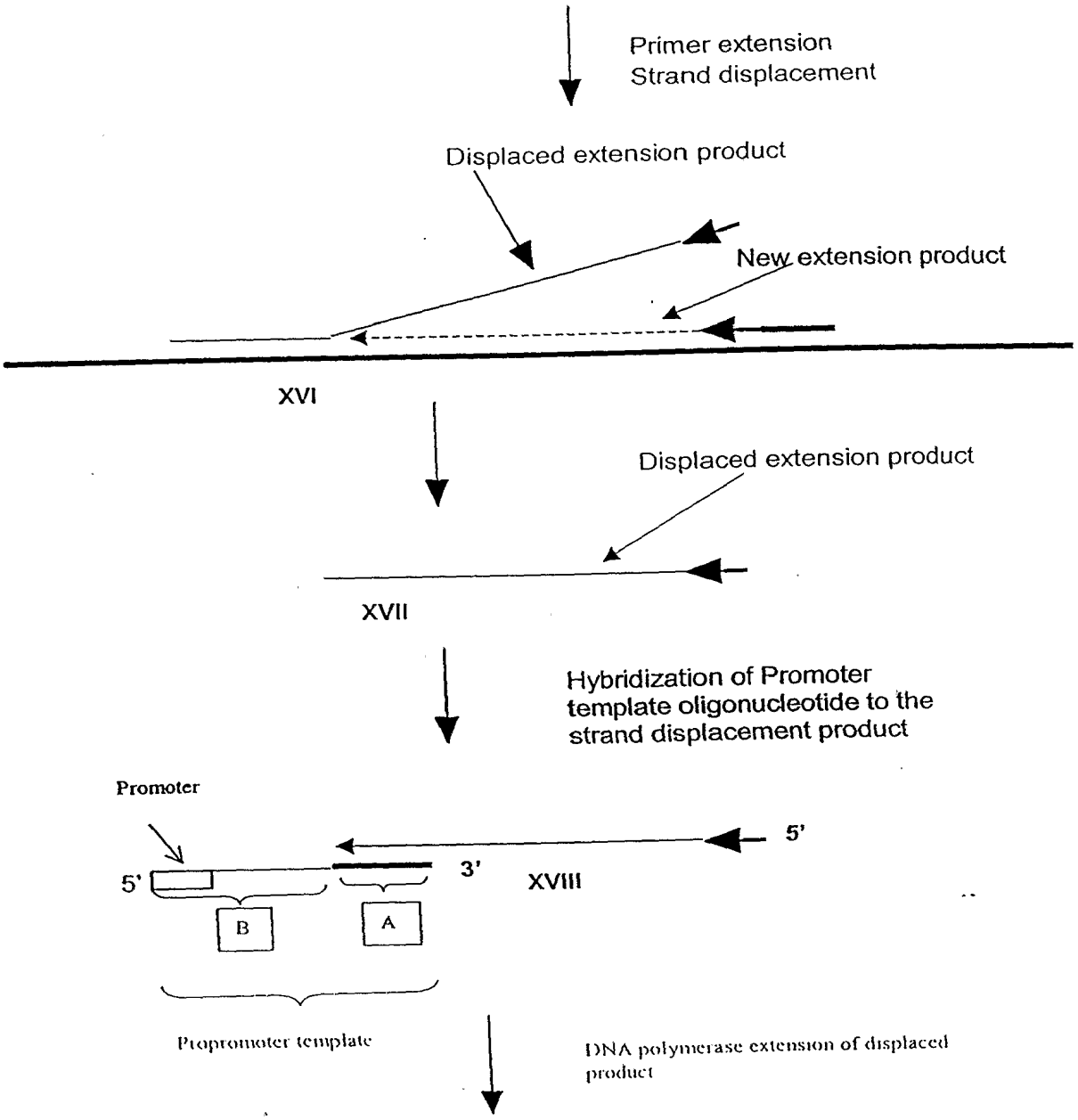
IDT Exhibit 1020.7285

FIGURE 11B



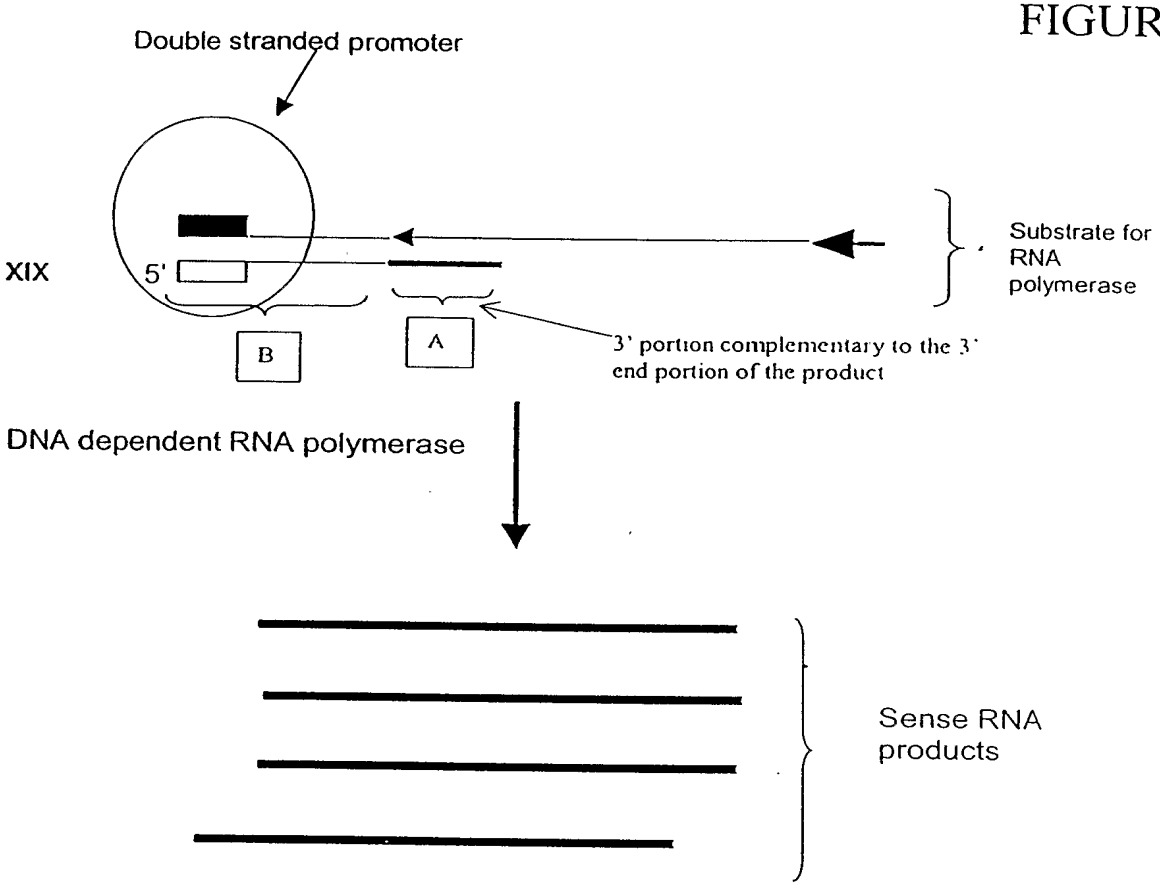
IDT Exhibit 1020.7286

FIGURE 11C



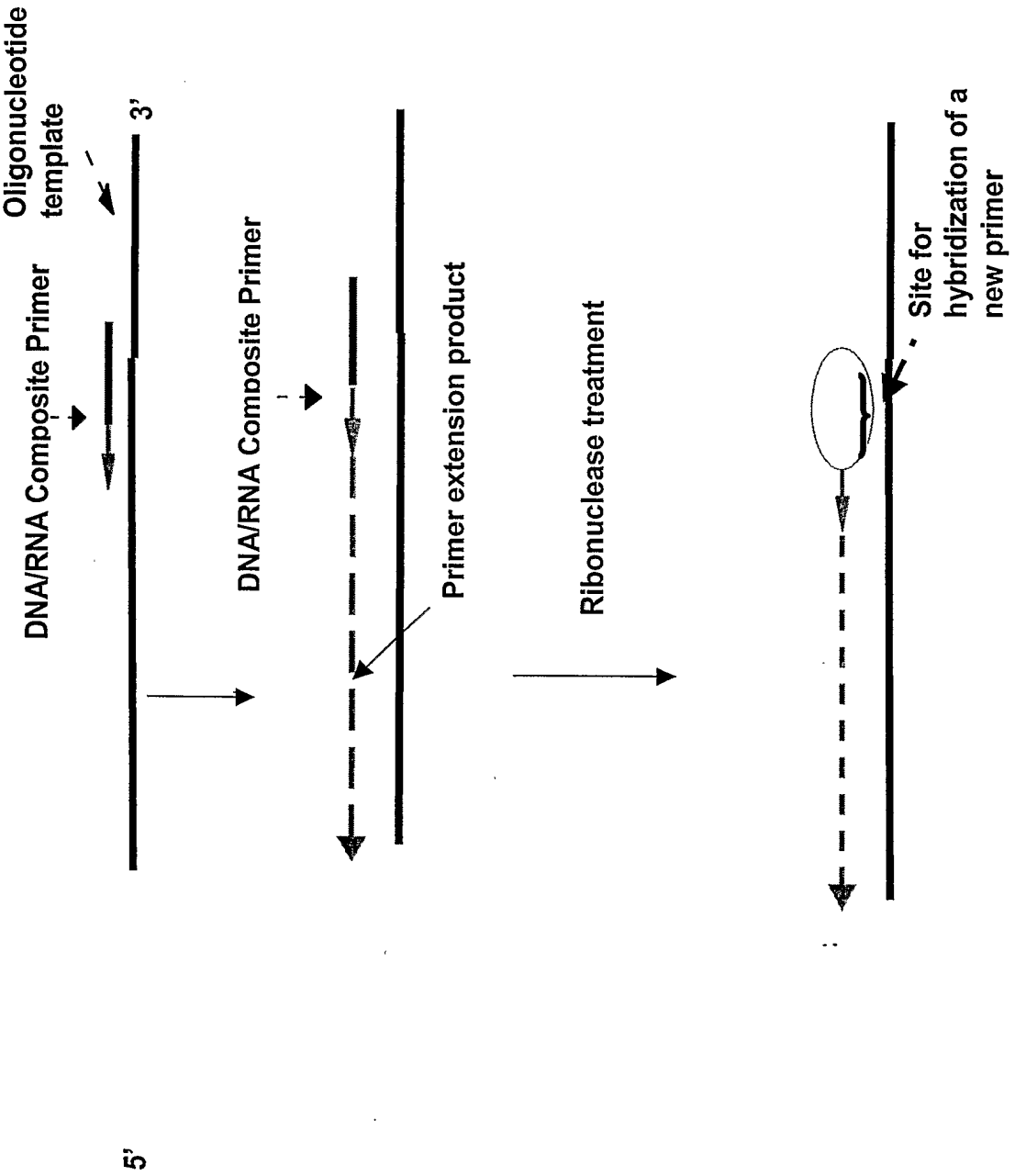
IDT Exhibit 1020.7287

FIGURE 11D



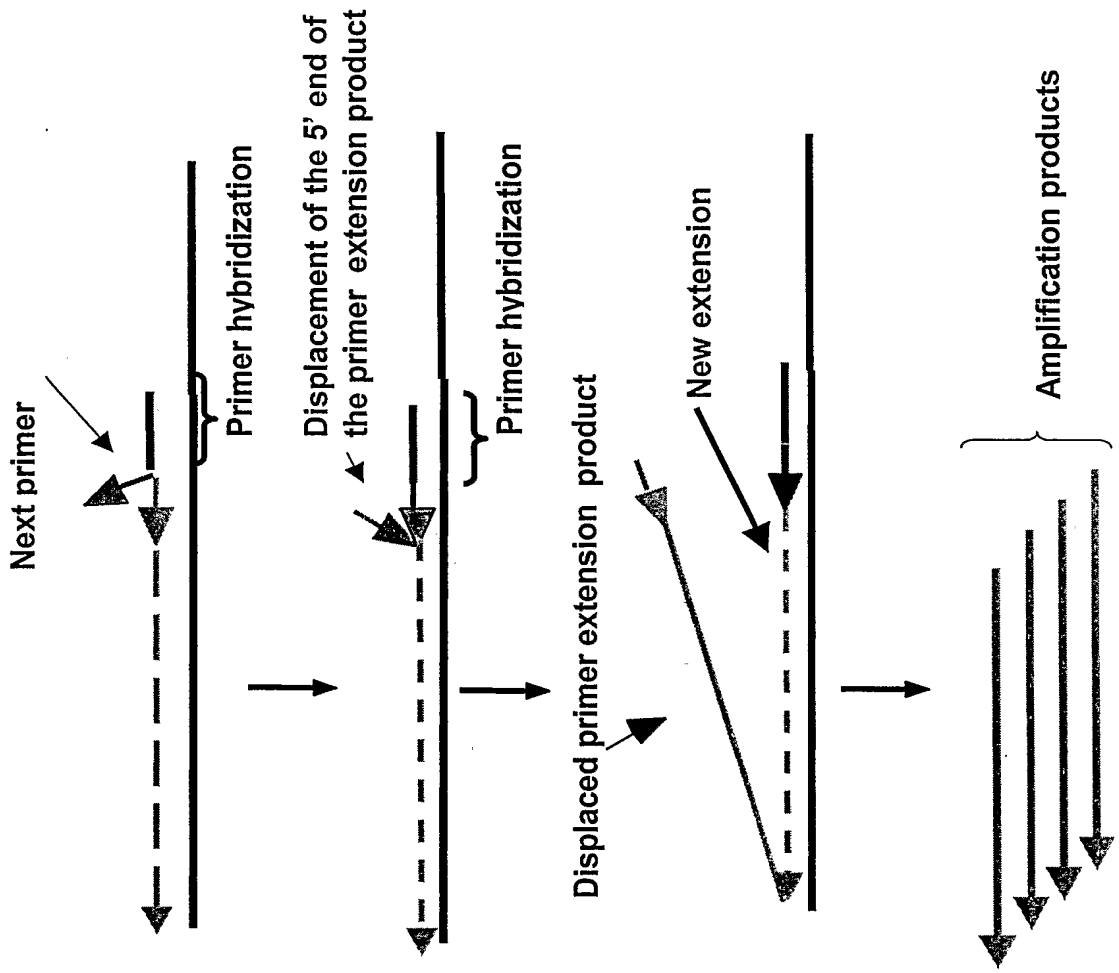
IDT Exhibit 1020.7288

12A



IDT Exhibit 1020.7289

12B



IDT Exhibit 1020.7290

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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
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[Continued on next page]

(54) Title: RAPID IDENTIFICATION OF VIRUSES OF THE UPPER RESPIRATORY TRACT INFECTION INCLUDING SARS CAUSING CORONUS VIRUSES

(57) Abstract: This invention provides a method for the detection of a pathogen through analysis of nucleic acid sequences, comprising the steps of: selecting at least one appropriate target sequence from the pathogen; screening said sequence to obtain at least one appropriate primer for amplification; selecting an appropriate probe from the sequence; immobilizing the allelic-Specific-Oligonucleotide site of each sequence onto a solid matrix suitable for capturing the target sequence; amplifying the sequence(s) wherein the steps (d) or (e) can be performing concurrently or sequentially; performing blot analyses on each sequence; and comparing the results of the blot analysis with using positive or negative controls. This invention further provides a method for amplifying the target sequence of a pathogen for hybridization comprising the steps of: selecting an appropriate primer pair; performing reverse transcription on the primer pair; and performing the first and second round of PCR in a single tube.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

RAPID IDENTIFICATION OF VIRUSES OF THE UPPER RESPIRATORY TRACT INFECTION INCLUDING SARS CAUSING CORONUS VIRUSES

This is a continuation-in-part application Of U.S. Serial No. 60/476,907, filed June 5, 2003, the content of which is incorporated into this application by reference in its entirety.

Throughout this application, various publications are referenced. Disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

FIELD OF INVENTION

The present invention relates to method of making definitive identification of upper respiratory viruses and Severe Acute Respiratory Syndrome (SARS) causing viruses such as coronus and related viruses in human and animals by DNA analysis and the device thereof.

BACKGROUND OF THE INVENTION

The Severe Acute Respiratory Syndrome (SARS) epidemic has drastically impacted the world recently, and different or new strains, genotypes or species of coronus viruses have been identified from patients with SARS. Subsequently, it has become obvious that the SARS virus has a very high rate of mutation, particularly when ribavirin or other antiviral drugs are used for treating the patients.

Coronaviridae has been known and isolated from many other species including human. Although different strains of coronus virus may cause different severity of SARS, however, not all strains or genotypes of coronus virus, such as the Human coronavirus 229E, have been implicated for causing SARS. Therefore, simply detecting the existence or presence of the coronus virus without knowing the detailed characteristics, such as the genotypes or variants, is insufficient or inadequate for the diagnosis of SARS. Even within the subclass of the coronus virus isolated from SARS patients, different variants, which caused the disease in different severity, were identified. Hence, it is imperative that when detecting the coronavirus or related viruses we must not only detect the general type within a common region but also provide an additional region(s) for verification, or better yet, the device or method deployed in the field, such as the method and device of the present invention, should be able to identify the respective genotype(s) of the given virus.

Since other viruses or pathogens may also be responsible for causing SARS, simultaneous detection of such virus(es) or pathogen(s) is imperative to save lives. In the case of SARS, other than the confirmed pathogen of coronus virus, other pathogen, such as Chlamydia, myxoviruses, and C. pneumoniae, have also been reported to be the possible causative pathogens for SARS. Although it is still not definitive whether any of these latter pathogens are indeed the causative agents for SARS, the fact that they have been identified in patients with SARS means that the detection of these pathogens may be important in prescribing an effective treatment.

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For effective management and prevention of the spread of SARS, it is extremely important to be able to differentiate the patient(s) who are infected with other viruses which cause SARS-like symptoms, such as upper respiratory track infections, from the SARS patients as early as possible. One of the reasons that the death rates in some SARS-affected regions are unusually high is the indiscriminate use of drugs to treat patients who display SARS-like symptoms but, in fact, the symptoms or illnesses of those patients are not caused by the same causative pathogens, such as coronus virus; thus, rendering the treatments ineffective. For this and other health reasons, more accurate, comprehensive early detection method or device for the causative pathogens is urgently needed.

The present invention is based on a low-density array membrane flow-through system which provides a better detection format. Using the flow-through hybridization device, the present invention not only provides a fast, simple format for identifying the coronus virus with adequate controls, but also provides simultaneous identification of the known genotypes of the causative pathogens for SARS. Moreover, if needed, additional pathogens, whether SARS related or unrelated, can also be detected in the same reaction and/or in the same membrane or matrixes simultaneously. Once the results are known, appropriate and potentially life-saving treatment regimen specific to the identified pathogen(s) can be prescribed for the patient. Hence, this invention provides a novel method of analysis for SARS which can also be used for the analyses of other gene(s) or pathogens. The method is based on our patented principle of Direct

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Flow-through DNA Hybridization (Tam JWO, US Pat. No. 5,471,547 (1998) & 6,020,187(2000)).

Direct Flow-through DNA Hybridization is the fastest annealing process that uses a very inexpensive device for accurate mutation detection, genotyping and fingerprinting analysis. The present invention, as applied and described herein, presents the scheme and data obtained from analyzing the SARS pathogen and SARS causative pathogens by RT-PCR and Allele (or gene or sequence) Specific Oligonucleotides (ASO) probes (oligo-probes) using the Flow-through format. This invention also presents an improved protocol or testing procedures to improve the detection sensitivity for the analyses and controls.

SUMMARY OF THE INVENTION

In accordance with these and other objects of the invention, a brief summary of the present invention is presented. Some simplifications and omission may be made in the following summary, which is intended to highlight and introduce some aspects of the present invention, but not to limit its scope. Detailed descriptions of a preferred exemplary embodiment adequate to allow those of ordinary skill in the art to make and use the invention concepts will follow in later sections.

Results suggested that the Allelic-Specific-Oligonucleotide Reversed-Dot- Blotting (ASO-RDB) direct Flow-through Hybridization is a good alternative for the detection of specific target pathogen(s) through the analysis of nucleic acids (DNA or RNA) sequences. Data obtained refer to the specific segments of coronus virus at multiple regions that are able to provide accurate determination of the genotypes and/or multiple species of pathogens,

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such as the upper respiratory track infectious viruses, using the fast hybridization procedures, and the ASO oligo-probes in a low-density array format of the present invention.

PCR amplification using multiple pairs of PCR primers (multiplex PCR) was used. Nucleic acids amplification can be performed using other amplification methods, such as strand displacement amplification (SDA), transcription mediated amplification (TMA), loop-mediated amplification (LAMP) and other amplification methods already established or new amplification technology to be invented in the future. The primers and oligo-probes shown in the sequence listing described herein and attached have been tested and confirmed to be useful for the upper respiratory viruses including SARS causing viruses. But these primers and oligo-probes are used as examples and, therefore, are not the only primers and oligo-probes which can be used with this invention; other primers and oligo-probes can be created by the methods set out in this invention as soon as the sequences of the viruses are known.

Using the scheme presented in Fig. 1, additional primers and oligo-probes can be selected, tested and validated for full genotyping in the future. Although PCR was used as the amplification method, any other method that can produce specific target sequences in enough quantity and/or without amplification if naturally occurring in high enough quantity for testing can also be used for the ASO-RDB flow through Hybridization determination. The detection can be by labeling of the target DNA or conjugates through any known or those developed in the future appropriate for present method of detection.

Although coronus virus and its variants and causative viruses of the upper respiratory track are being used as the practical example here, this SNP-based analysis method can be applied to other gene and sequences of any organism by following the general procedures

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as shown in Figure 1. The Flow-through Device shown in Figure 2 is one example. Hybridization devices, such as those described in U.S. Patents No. 5,741,547 or 6,020,187 or any new embodiments which are capable of carrying out the above-described flow-through hybridization process, can also be used.

DETAILED DESCRIPTION OF THE DRAWINGS

The drawings which illustrate specific embodiments of the invention should not be construed as restricting the spirit or scope of the invention in any way:

Fig. 1 is the diagram of the method for obtaining the ASO probes and PCR primers data base of the invention.

Fig. 2 is one of the examples the hybridization device already in production and registered elsewhere.

Fig. 3 is the agarose gel image showing the detection limit of SARS coronavirus down to 2 copies.

Fig. 4 shows the result of SARS coronus virus detection by flow-through hybridization. Positive signals are represented by blue dots on the pre-probed membrane.

Fig. 5 is the arrangement of the ASO probes of the membrane array.

Fig. 6 is the hybridization result of the SARS coronus virus variant (genotyping identification) detection. Table below shows three known strains or variants CA, CB, Tor (as the wide type), and the mutated sequence probes that were used to test the correspondent mutants. The probe arrangement is given along with resulted image.

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SNP position for SARS coronavirus genotyping

Nucleotide position	SNP	Forward primer	Reverse primer	Hybridization probe 1 (WT)	Hybridization probe 2 (M)
21706	G / A (CA)	CuBJ_1aF	CuBJ_1aR	CuBJ_1aWTcap	CuBJ_1aCap
22207	T / C (CB)	CuBJ_1bF	CuBJ_1bR	CuBJ_1bWTcap	CuBJ_1bCap
23220	T / G (TOR)	SIN2679F	SIN2679R	TOR2WTcap	TOR2cap

Fig. 7 is the arrangement of the viral detection array for the upper respiratory track infection.

Legend:

1. Respiratory syncytial virus (RSV)
2. Enterovirus (Entero)
3. Adenovirus (Adeno)
4. Parainfluenza virus type 1 (PIV1)
5. Parainfluenza virus type 3 (PIV 3)
6. Parainfluenza virus type 3B (PIV 3B)
7. Influenza A (Inf A)
8. Influenza B (Inf B)
9. SARS coronavirus (SARS)
10. Metapneumovirus (Meta)
11. Mycoplasma pneumoniae (M. Pneu)
12. Chlamydia pneumoniae (CP)

Fig. 8 shows the hybridization results of viral detection array after multiplex PCR: A) SARS coronavirus; B) Influenza A virus; C) Parainfluenza type 1 virus; D) Respiratory syncytical virus.

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The test specificity was evaluated with the use of nucleic acid samples and probes of 10 other respiratory tract pathogens (respiratory syncytial virus (RSV), parainfluenza virus type 1 & 3 (PIV 1 & 3), *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, enterovirus, influenza A & B, adenovirus, metapneumovirus). No cross-reaction was detected. This figure shows examples of the specific HybriMax-based hybridization of SARS coronavirus and other pathogens PCR products to SARS coronavirus probe and other specific probes respectively.

Membrane positions of probes are as follows:

1. Respiratory syncytial virus (RSV)
2. Enterovirus (Entero)
3. Adenovirus (Adeno)
4. Parainfluenza virus type 1 (PIV1)
5. Parainfluenza virus type 3 (PIV 3)
6. Parainfluenza virus type 3B (PIV 3B)
7. Influenza A (Inf A)
8. Influenza B (Inf B)
9. SARS coronavirus (SARS)
10. Metapneumovirus (Meta)
11. Mycoplasma pneumoniae (M. Pneu)
12. Chlamydia pneumoniae (CP)

Analytical sensitivity was determined by RT-PCR of serial dilution of in vitro transcribed cloned RT-PCR products. The detection limits were as follows:

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Species	No. of RNA copies
SARS coronavirus	2
Influenza A virus	200
Influenza B virus	80
Parainfluenza virus type 1	800
Parainfluenza virus type 3	6000
Respiratory syncytial virus type A	2000
Respiratory syncytial virus type B	3000

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for the detection of a pathogen through analysis of nucleic acid sequences, comprising the steps of: (a) selecting at least one appropriate target sequence from the pathogen; (b) screening said sequence to obtain at least one appropriate primer for amplification; (c) selecting an appropriate probe from the sequence; (d) immobilizing the sequence-specific-oligonucleotide site of each sequence onto a solid matrix suitable for capturing the target sequence; (e) amplifying the sequence(s) wherein the steps (d) and (e) are performed separately; (f) performing blot analyses on each sequence; and (g) comparing the results of the blot analysis with using positive or negative controls. In an embodiment, steps (d) and (e) are performed sequentially or concurrently. In another embodiment, the sequence is a gene, pathogen or allelic sequence.

IDT Exhibit 1020.7301

In an embodiment, the solid matrix is a low-density array for detecting multiple SARS variant. In another embodiment, the solid matrix is a viral detection array for detecting upper respiratory tract infection. In a further embodiment, the array is on a single membrane.

The pathogen includes but is not limited to the upper respiratory viruses. In an embodiment, the pathogen is a coronavirus. In another embodiment, the pathogen is an avian influenza virus. In another embodiment, the primer and the sequence-specific-oligonucleotide site are obtained by screening data from the GenBank or by sequencing the target sequence or in combination thereof. In a further embodiment, target sequence comprises a gene, nucleic acid or DNA sequence.

In a further embodiment, the probe is a single nucleotide polymorphism probe. In a further embodiment, the solid matrix is a membrane or nylon. In a further embodiment, a target sequence having a concentration less than 0.5 f moles is amplified by the above describe method. In further embodiment, the amplification step can be performed by PCR amplification, isothermal amplification, nested RT-PCR amplification, two-step nested RT-PCR amplification.

In a further embodiment, the upper respiratory viruses include but is not limited to a group selected from respiratory syncytial virus (RSV), parainfluenza virus type 1 (PIV1), parainfluenza virus type 3 (PIV3), parainfluenza virus type 3B (PIV3B), *Mycoplasma pneumoniae*

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(MP), *Chlamydia pneumoniae* (CP), Enterovirus (EN), Influenza A (Inf A), Influenza B (Inf B); Adenovirus (Adeno), Metapneumovirus (Meta), and SARS coronavirus (SARS).

The invention provides a method for the detection of more than one pathogen through analysis of nucleic acid sequences, comprising the steps as described above. However, more than one pair of primers can also be used. In an embodiment, the detection procedure can be completed within two hours. In another embodiment, the detection procedure is finished within minutes. In a further embodiment, the detection procedure is finished within 20 minutes using enzyme link color development. Preferably, the detection procedure can be completed within between 2 to 3 minutes with using use colloidal gold or florescence dye.

This invention further provides a method for the detection of pathogens including but not limited to SARS causative pathogens.

This invention further provides novel polymerase chain reaction (PCR) primers and allele-, gene- or sequence-specific oligonucleotides (ASO) probes or oligo-probes which can be used for detecting pathogens. This invention further provides a method for identifying novel probes and primers which can be used to detecting pathogen(s). This invention provides a kit for detection of pathogen comprising a compartment containing the primer(s) or probe(s) or a combination thereof, which are disclosed herein or developed by the methods as describe above.

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This invention provides a machine capable of detecting pathogens through analysis of nucleic acid sequences. In an embodiment, the machine can be programmed to automatically carryout the methods as described above.

This invention provides a method for amplifying the target sequence of a pathogen for hybridization comprising the steps of: selecting an appropriate primer pair; performing reverse transcription on the primer pair; and performing the first and second round of PCR in a single tube. In an embodiment, the second round of PCR is performed with a thermal cycling program having the following thermal cycling profile: (1) 95°C, 5 min; (2) 95°C, 10 sec; (3) 56°C, 10sec; (4) 72°C, 30sec; (5) 39 cycle repeats on steps 2 to 4, followed by 72°C, 10 min final extension time.

In another embodiment, the second round PCR was done using 1ul of the 1:10 dilution of the first round product in 50ul reaction volume. In a further embodiment, the reverse transcription is performed with positive control RNA of about $2x10^{-5}$ copies using the Thermoscript RT-PCR system and Platinum Taq DNA polymerase. In a further embodiment, thermal cycling profile: (1) 95°C - 3min; (2) 95°C , 10sec; (3) 60°C, 10sec (drop by 1°C/cycle); (4) 72°C, 30 sec; (5) 9 cycle repeats on steps 2 to 4; (6) 95°C, 10sec; (7) 56°C, 10sec; (8) 72°C, 30sec; (9) 9 cycle repeats on steps 6 to 8; and (10) Store at 4°C.

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The following is one of the general procedures for the present invention:

(a) Select gene segments and determine the PCR primers and the ASO sites:

1. Select the appropriate target sequences to be analyzed by either screening data from the GenBank and/or by sequencing the target genes or target DNA segments to get the SNP (or ASO) profile related to the pathogen(s).
2. From these data, determine the ASO sites to be used for genotyping based on the sequence data to evaluate if the sites are indeed unique for the genotype or variant within target species to be tested.
3. Determine the number of ASO capture probes.

(b) Perform ASO-RDB detection for genotyping:

1. Immobilizing the ASO oligonucleotides onto the membrane or any matrix for capturing the target loci.
2. Amplifying the target segments using the appropriate primers by labeling the amplified target DNA molecules for the ASO-probes for hybridization detection.
3. Performing ASO profile analyses by Flow-through Hybridization process using a device as depicted in figure

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The hybridization is done as described in pages 9-15 of the DNA HybriMax™ Owner's Manual (Hong Kong DNA Limited) of the device. The following protocol is an example:

i) Denature and drop the target DNA solution onto the membrane;

ii) Wash and develop the color for visual inspection or spectrometric measurements (The target DNA can be labeled with fluorescence dye in which case direct spectrometric determination other than color development by enzyme linked immuno-specific assay is needed. Other developing methods like colloidal Gold or magnetic bead or quantum-dot or any other conjungate labeling systems already used or developed in the future can also be used).

iii) Results are compared with known sequence data for accuracy evaluation.

iv) Modify the probes and testing conditions for accuracy. The RDB-ASO data are verified by DNA sequencing.

(c) Validation (for diagnostic kits development)

Proceed with validation using (i) control panels; (ii) random patient samples and; (iii) clinical trials.

Development scheme:

Following the scheme as in Figure 1:

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1. Select conserved region(s) for generic types (detection the **existence** of a given pathogens or genes, without detailed genetic/sequence specific).
2. Put in **Positive** and **negative** controls to ensure the validation of the test
3. Select the specific regions (for genotyping for a given species and/or different species)
4. Design the respective primers and detection probes for the test.
5. Establish complete protocols.

EXAMPLES

In examples which are intended to illustrate embodiments of the invention but which are not intended to limit the scope of the invention:

(I) Diagnosis for the SARS causative pathogen, the coronus virus and its variants

Detailed sequences of the first isolated coronus virus and subsequently the first 7 variants were submitted to the GeneBank for public knowledge. We designed the ASO probes of all 7 variants for the coronus viruses and four PCR primers for the amplification of the pathogen(s). These sequences are listed in the Appendix. In principle, the ASO or SNP probe sequences and specific primers sequences to be used can be designed appropriately for any given organism with which the nucleic sequence data is known or can be determined accurately to perform the test in the present invention. In order to have a detectable signal for the hybridization, a concentration of the target nucleic acid should be above 0.5 f moles

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for avidin-AP conjugate enzyme link color development (other labeling development system may be more sensitive). Hence if the concentration of the target molecule is low, amplification must be performed before the hybridization process. For demonstration on the validity of the present invention, the PCR amplifications were used for examples given here. Other amplification methods, such as those mentioned elsewhere in this text can also generate similar concentration and therefore should be feasible. In fact, isothermal amplification methods are preferable because the relatively expensive PCR equipment (Thermal Cycler) will not be needed, and the amplification process is much faster. A two-step nested RT-PCR protocol was used for initial development of the SARS diagnosis kit. The reversed transcription was done with the first primer pair: 5'-ATGAATTACCAAGTCAATGGTTAC-3' and 5'-CATAACCAGTCGGTACAGCTAC-3' with positive control RNA of about $2x10^{-5}$ copies using the Thermoscript RT-PCR system and Platinum Taq DNA polymerase, followed by the first round of PCR. The 2nd round PCR was done using 1ul of the 1:10 dilution of the first round product in 50ul reaction volume. The thermal cycling program used for both round was the same: (1) 95°C, 5 min; (2) 95°C, 10 sec; (3) 56°C, 10sec; (4) 72°C, 30sec (5) 39 cycle repeats on steps 2 to 4, followed by 72°C, 10 min final extension time. A unique two-stage PCR protocol (see Table I) was developed to amplify to the lowest detection limit of a single target molecule and reproducibly for 2 molecules with consistency. The nested PCR was done in one tube for the first and the second round PCR. This protocol not only simplifies the sample handling, it also saves time, and most importantly, by removing the second round sample preparation,

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the possibility of cross contamination which is the main concern for all PCR processes is reduced. Figure 3 shows the typical amplification results evaluated by agarose gel electrophoresis for such amplifications. The process was a two-stage single program nested PCR amplification in which the outside primer pair was designed to have a higher annealing temperature for the initial amplification of 10 to 20 cycles followed by a lower annealing temperature amplification cycles. As shown in Figure 3, this process has produced a much more specific (less non-specific band(s) was obtained other than the target sequence) result even at the limit of its detection.

Figure 4 showed the hybridization result for SARS. Evidently, the hybridization result was very specific and there was no cross (non-specific) reaction for the negative control. Double-blind studies were performed on a panel of 20 clinical samples, i.e. diluted samples of the archive samples that had been detected by the Real Time PCR. The results as shown in Table II indicated that 18 of the 20 samples were correct. The two positive samples that were not successfully amplified were in fact below the claimed detection limit of one copy each. Despite this result, the sensitivity and specificity achieved by the two-stage PCR amplification and flow-through hybridization was 90% and 100% respectively.

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Table I. Two-stage single program nested PCR protocol

PTC-200 Thermal cycling profile

Program	SARS
Step 1	95C - 3min
Step 2	95C - 10sec
Step 3	60C - 10sec (drop by 1°C/cycle)
Step 4	72C - 30 sec
Step 5	(go to 2 x 9 cycles)
Step 6	95C - 10sec
Step 7	56C - 10sec
Step 8	72C- 30sec
Step 9	go to 6 X 39 cycles
Step 10	4C forever

Table II. Analytical Detection of SARS Comparing the HybriMax Test to Real-time PCR

		Real-time PCR		
		Positive	Negative	Total
HybriMax Test	Positive	18	0	18
	Negative	2**	10	12
	Total	20	10	30

** The concentration of one of these two samples was only 1 copy per microliter (i.e. a maximum of 1 copy per reaction)

One-step and nested PCR were also tested (the nested PCR cycling program is given in Table III below). Based on the known sequence data of the SARS variants published in the GeneBank, a set of ASO (or SNP) oligo-probes and PCR primers, which are listed in the Appendix, were designed. In principle, ASO or SNPs probe(s) and amplification primers of any organisms with adequate sequence data can be designed to perform such genetic analysis, and can be detected by the

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flow-through hybridization method. The results of hybridization were obtained within minutes. These SNP oligo-probes were used as the low-density array for detecting multiple SARS variants on a single membrane for the SARS causing coronus viral nucleic acid diagnosis.

Figure 5 showed the array of ASO probes dotted onto the membrane for hybridization detection with target samples. Using the three archived known variant samples as a control panel, the protocol of the fast flow-through hybridization was developed successfully. The typical result is shown in Figure 6. To evaluate the accuracy of this test, 10 random clinical samples were tested and the results were confirmed by DNA sequencing. The results shown in Table IV below indicated 100% agreement.

Table III. Nested PCR Amplification Cycling Program Specifically for SARS Corona Virus

Component	volume	Final concentration
10 x PCR buffer	2.5 µl	1 x
25 mM MgCl2	1.5 µl	1.5 mM
10 mM dNTP	0.5 µl	200 µM
10 µM SARS3 primer	0.5 µl	0.2 µM
10 µM SARS4 primer	0.5 µl	0.2 µM
AmpliTaq Gold (5U/µl)	0.25 µl	
Water	17.25 µl	
First round RT-PCR product	2 µl	
Total volume	25 µl	

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Thermal cycling profile	
95°C	5 min
95°C	10 s x 30 cycles
56°C	10 s x 30 cycles
72°C	20 s x 30 cycles
72°C	10 min

Table IV. Analytical Detection of SARS Variants Comparing the HybriMax Test to DNA Sequencing

		DNA Sequencing		
		Mutant	Wide type	Total
HybriMax Test	Mutant	0	0	0
	Wide type	0	10	10
	Total	0	10	10

(II) Diagnosis of the Upper Respiratory Track infectious viruses, including SARS coronus virus

Detailed sequences of the various viruses isolated as the causative pathogens for the upper respiratory track infection have been published and the sequence data submitted to GeneBank. The ASO probes of all the 11 species listed in this invention were designed for the production of the test array (shown in Figure 7). PCR primers for the amplification of the pathogens and the probe sequences are listed in the Appendix. The multiplex RT-PCR amplification was developed using the cycling program given in Table V and the analytical sensitivities for the detection limit of each of the viruses have been evaluated using serial dilution of the in vitro transcribed products from the respective viral clones. The test specificity was evaluated with the use of nucleic acid samples and probes of

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10 other respiratory tract pathogens (respiratory syncytial virus (RSV), parainfluenza virus type 1, 3 & 3b (PIV 1, 3 & 3b), *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, enterovirus, influenza A & B, adenovirus, metapneumovirus). The flow through hybridization protocols are similar to that described above for the SARS diagnosis. Figure 8 showed the typical results of the test array with 4 of the 11 the viruses for such evaluation. This figure shows examples of the specific HybriMax-based hybridization of SARS coronavirus and other pathogens PCR products to SARS coronavirus probe and other specific probes respectively. Membrane positions of probes are as follows: 1 Respiratory syncytial virus (RSV); 2 Enterovirus (EN); 3 Adenovirus (Adeno); 4 Parainfluenza virus type 1 (PIV1); 5 Parainfluenza virus type 3 (PIV3); 6 Parainfluenza virus type 3b (PIV3b); 7 Influenza A (Inf A); 8 Influenza B (Inf B); 9 SARS coronavirus (SARS); 10 Metapneumovirus (Meta); 11 *Mycoplasma pneumoniae* (MP); 12 *Chlamydia pneumoniae* (CP).

Table V. Multiplex One-step RT-PCR Amplification Program for the Upper Respiratory Track Viral Detection

component	volume	Final concentration
2 x PCR buffer (Superscript II)	12.5 µl	1 x
6-plex primer mix*	5 µl	1 µM
Biotin-16-dUTP	0.5 µl	10 µM
Reverse transcriptase / Platinum Taq Mix	0.5 µl	
RNA template	2 µl	
Water	4.5 µl	

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Total volume	25 µl	
*6-plex primer mix contains primers for SARS coronavirus (SARS1 & SARS2), influenza A & B viruses, parainfluenza virus type 1 & 3, respiratory syncytial virus.		

Thermal cycling profile	
50°C	30 min
94°C	2 min
94°C	30 s x 40 cycles
50°C	30 s x 40 cycles
72°C	30 s x 40 cycles
72°C	7 min

Evidently the process did not produce non-specific cross reaction. To ensure the validity, this array test format was evaluated with archived clinical samples provided by hospital. In evaluating the HybriMax system, immunofluorescence, rapid antigen test or viral culture was used as the reference method to determine the relative false-positive and false-negative rates. Of the 57 samples, the proportion of immunofluorescence/rapid antigen test/viral culture negative results that were positive by HybriMax was 1/5, resulting in a relative false-positive rate of 20% and a specificity of 80%. Conversely, the proportion of immunofluorescence/rapid antigen test/viral culture positive results that were negative by HybriMax was 2/52, resulting in a relative false-negative rate of 3.8% and a sensitivity of 96.2%. The actual test data and the summary are given in Table VI & VII below. The false negative results from Samples 225433 and 225647 were due to PCR failure (not due to hybridization failure) which may have been caused by the degradation of the samples

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during storage.

The ability to simultaneously and rapidly detect the other viruses causing the SARS like syndrome is of utmost important because this will be the most effective way for triage patients for effective treatment and prevent unnecessary spread of the serious communicable infectious SARS disease. Other than identify the general species as list in Figure 7, the present invention can also be extended to include simultaneously identify variants of these viruses in one single membrane test strip to enable physicians to prescribe the most appropriate therapy to save lives. The rapid and simplicity of test which can be carried out onsite is unique to the present invention.

Table VI. Clinical evaluation of Multiple Pathogen Multiplex PCR and Hybridization System Targeting 57 Patient Samples to Determine Sensitivity and Specificity

Sample No.	Fast Test Results	HybriMax Results
225422	Influenza A	Influenza A
225423	Influenza A	Influenza A
225431	Influenza A	Influenza A
225433	Influenza A	Neg
225488	Influenza A	Influenza A
225493	Influenza A	Influenza A
225513	Influenza A	Influenza A
225515	Influenza A	Influenza A
225527	RSV	RSV
225558	RSV	RSV
225601	PIV 1	Influenza A + PIV 1
225606	Influenza A	Influenza A
225609	Influenza A	Influenza A
225640	Influenza A	Influenza A

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225641	Influenza A	Influenza A
225662	Influenza A	Influenza A
225663	Influenza A	Influenza A
225679	Influenza A	Influenza A
225684	Influenza A	Influenza A
225708	Influenza A	Influenza A
225714	Influenza A	Influenza A
225715	Influenza A	Influenza A
225716	Influenza A	Influenza A
225717	Influenza A	Influenza A
225718	Influenza A	Influenza A
235642	Influenza A	Influenza A
225763	Influenza A	Influenza A
225764	Influenza A	Influenza A
225769	Influenza A	Influenza A
225774	Influenza A	Influenza A
225775	Influenza A	Influenza A
225776	Influenza A	Influenza A
225777	Influenza A	Influenza A
225778	Influenza A	Influenza A
225783	Influenza A	Influenza A
225786	Influenza A	Influenza A
225787	Influenza A	Influenza A
225800	Influenza A	Influenza A
225819	Influenza A	Influenza A
225820	Influenza A	Influenza A
225840	Influenza A	Influenza A
225860	Influenza A	Influenza A
227408	Neg	Neg
227412	Neg	Neg
227417	Neg	Influenza A
227418	Neg	Neg
235647	Influenza A	Neg
226053	Influenza A	Influenza A
225993	Influenza A	Influenza A

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226142	Influenza A	Influenza A
225865	Influenza A	Influenza A
226141	PIV 1	PIV 1
226026	Influenza A	Influenza A
226032	Influenza A	Influenza A
226093	Influenza A	Influenza A
225968	Influenza B	Influenza B
225984	Neg	Neg

Fifty seven archived nasopharyngeal aspirates (NPA) samples stored at −20°C for over 3 months were tested. The samples were also tested with Binax FDA cleared fast test kits for Influenza A, Influenza B and RSV and immunofluorescence assay for Adenovirus, PIV 1 and PIV 3.

Table VII. Analytical Detection of SARS Coronavirus, Influenza A Virus, Influenza B Virus, PIV 1, PIV 3, RSV Comparing the HybriMax Test to Immunofluorescence/Rapid Antigen Test/Viral Culture

		Immunofluorescence/Rapid Antigen Test/Viral Culture		
		Positive	Negative	Total
HybriMax Test	Positive	50	1	51
	Negative	2	4	6
	Total	52	5	57

Sensitivity = No of True Positive ÷ (No of True Positive + No of False Negative) = 96.2%

Specificity = No of True Negative ÷ (No of True Negative + No of

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False Positive) = 80%

Materials:

Probe:

1. Amino modified oligonucleotide capture probes - BNIcapture (stock at (given in appendix), -20°C, 1 nmol/μl, dilute to 10 μM with 0.5 M sodium bicarbonate)

Membrane Arrays preparation reagents:

1. *Biodyne C membrane (Pall)(used for probe immobilization to capture the target labeled DNA samples used as the matrix for the low-density array)*
2. Preactivation solution: 0.1 M HCl
3. 20% EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodimmide hydrochloride) (store at 4°C) (Just before use as a 20% solution in water) (Sigma 89H0804)
5. Oligonucleotide application buffer: 0.5 M sodium bicarbonate, pH 8.4
6. Membrane quenching solution: 0.1 M NaOH
7. Hybridization and wash buffer: 2X SSC/0.1%SDS

Color Development reagents:

1. StabilGuard® - (SurModics SG01-1000) (stored at 4°C)
2. StabilZymeAP® - (SurModics SA01-1000) (4°C)
3. TBST - 50 mM Tris, 150 mM NaCl, 0.1% Tween20 (v/w), pH 7.5 (4°C)
4. Avidin-Alkaline Phosphate conjugate (from hen egg white), 1 mg/ml (4°C)
5. NBT/BCIP ready-to-use tablets (Roche 1697471) (4°C)

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Others:

1. 15 ml centrifuge tube
2. 1 ml, 200 µl, 0.5 µl pipette tips
3. pipettes (P1000, P200, P100, P2)

Instruments:

1. DNA Flow-Through Hybridization Device (DNA HybriMax)
2. Heat block or water baths
3. Thermalcycler for PCR

Methods:

Memebrane Array preparation (Covalent attachment of amino modified probes to Biodyne C membrane filter)

1. Briefly rinse the membrane with 0.1 M HCl, blot, then soak in freshly prepared 20% EDAC (1g EDAC in 5 ml Milli-Q dH_2O) for 15 min.
2. Rinse the filters in deionized water and blot to remove excess water. The carboxyl groups on the membranes are activated at this point and the filters must be processed immediately (within 0.5 hr)
3. Place filters on Parafilm, apply 0.5 µl 10 µM (5 µM/dot) of modified probes on the membrane.
4. Incubate the filters for 15 min at room temperature.
5. Quench the covalent attachment reaction, rinse the membranes thoroughly for 10 min with 0.1 M NaOH
6. Wash the membrane with four to five rinses in deionized water.

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7. The filters may be used immediately or air-dried for subsequent use. When dry, the membrane appears to be stable for indefinite periods. (Store the membrane at dark)

PCR Product- PCR amplification of the target nucleic acid(s)

1. SARS and other upper respiratory track infectious viral PCR products in the examples shown in this invention were labeled with biotin-labeled primers.
2. The PCR amplification was done using either the MJ p200 or the ABI 9700 thermalcycler (in principle, any other thermal cyclers can be used for the amplification with appropriate optimization of the cycling protocols). Single or nested PCR have been tried successfully with the one-step or the two-step processes.

Procedures for Hybridization of PCR products to filters

(Note: Cover the unused well with parafilm or alumnium foil)

1. Denature the DNA by heating at 95°C for 5 min.
2. Chill on ice for at least 2 min.
3. Place the membrane on HybriMax and set the temperature to 36°C.
4. Prehybridize the membrane with 1ml 2X SSC/0.1%SDS.
5. Add 10 µl of PCR product to 0.5 ml 2x SSC/0.1%SDS in 1.5 ml eppendorf tube.
6. Add 0.5 ml of the denatured DNAs into designated well. Incubate for 5 min for better hybridization.
7. Pump the solution out (PUMP ON).
8. (Keep PUMP ON) Wash the membrane with 2.4 ml 2X SSC/0.1%SDS.
9. PUMP OFF.

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Color Development procedure

1. Add 0.5ml of StabilGuard© to the membrane. Pump off the solution completely.
2. Pump off.
3. Block the membrane with 0.5 ml StabilGuard© for 5 min. Pump out the solution
4. Dilute avidin-AP conjugate by adding 1 µl AP stock (1 mg/ml) to 3 ml StabilZyme AP. (1:3000 dilution)
5. Add 0.5 ml diluted AP-conjugate to each well. Incubate for 3 min for better reaction.
6. Pump out the solution (PUMP ON).
7. (Keep PUMP ON) Wash the membrane with 3.2 ml TBST (**NOTE: Other developing reagents such as Avidin-colloidal gold or magnetic particles or fluorescence labels or other can be used for direct detection and save the following steps. Using these direct detection systems, the time will be less then 1 minute.)**
8. PUMP OFF. Dissolve 1 NBT/BCIP ready-to-use tablet in 10ml double distilled water.
9. Add the dissolved NBT/BCIP to each well (2.5 ml for each well).
10. Incubate until the color develops. (Around 5 minutes)
11. PUMP ON. Wash the membrane with 3ml 2XSSC/0.1%SDS followed by 1ml dH_2O after the color completely developed.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from

IDT Exhibit 1020.7321

the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

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IDT Exhibit 1020.7323

Appendix

(A) SPEICES LISTING for testing kit used in the present invention:

(i) Coronavirus (SARS) Variants used in the invention:

Known genoptypes:

Urbani;

HKU-39849;

CUHK-su10;

SIN2500;

SIN2677;

SIN2679;

SIN2748;

SIN2774;

TW1;

ZJ01;

TOR2;

CUHK-W1;

BJ01

Other variants: (once the sequences of any new type is known, the present invention can be used to develop the necessary protocols for the detection of its presence)

(ii) Multiple viral test panel (membrane array) used in this invention"

1. Respiratory syncytial virus (RSV)
2. Enterovirus (Entero)
3. Adenovirus (Adeno)

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4. Parainfluenza virus type 1 (PIV1)

5. Parainfluenza virus type 3 (PIV 3)

6. Influenza A (Inf A)

7. Influenza B (Inf B)

8. SARS coronavirus (SARS)

9. Metapneumovirus (Meta)

10. Mycoplasma pneumoniae (M. Pneu)

11. Chlamydia pneumoniae (CP)

(iii) Other viral species:

Human coronavirus 229E

Animal Viruses:

Canine coronavirus

Feline infectious peritonitis virus

Porcine transmissible gastroenteritis virus

Porcine epidemic diarrhea virus

Bovine coronavirus

Porcine hemagglutinating encephalomyelitis virus

Rat sialodacryoadenitis virus

Mouse hepatitis virus

Turkey coronavirus

Avian infectious bronchitis virus

(B) Nucleic Acid Sequences

(i) Capture probe sequences:

1. SARS - GGGTACTAACCTACCT

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1. CuBJ_1aCap - CGTTTGACAACCCTG
2. CuBJ_1aWTCap - TTTGGCAACCCTGT
3. CuBJ_1bCap - TCAAGACACTTGGGG
4. CuBJ_1bWTCap - TCAAGACATTTGGGG
5. Tor2Cap - ACCTTGCGCTTTTG
6. Tor2WTCAP - ACCTTGCTCTTTTGG
7. RSV - CCTGCATTAACACTAAATTC
8. PIV1 - TACCTTCATTATCAATTGGTAAGTCAATATATG
9. PIV3 - AAAATTCCAAAAGAGACCGGC
10. PIV3B - ACAGAACACCARAACAACAA
11. M. pneumoniae - ACTCCTACGGGAGGCAGCAGTA
12. C. pneumoniae - TCTTGCTACCTTCTGTACTAA
13. Enterovirus - GAAACACGGACACCCAAAGTA
14. Influenza A - GTCCTCATCGGAGGACTTGAATGGAATGAT
15. Influenza B - GTCAAGAGCACCGATTATCAC
16. Adenovirus - CTCGATGACGCCGCGGTGC
17. Metapneumovirus - GAAGTTTGTTCATTGAGTATGG

(ii) PCR Primer sequences

1. SARS1 - ATGAATTACCAAGTCAATGGTTAC
2. SARS2 - CATAACCAGTCGGTACAGCTAC
3. SARS3 - GAAGCTATTCGTCACGTTCG
4. SARS4 - CTGTAGAAAATCCTAGCTGGAG
5. CuBJ_1aF - TCAGGATTTATTTCTTCCATTTT
6. CuBJ_1aR - GCAGCAAAATAAATACCATCC
7. CuBJ_1bF - GAGCCATTCTTACAGCCTTT
8. CuBJ_1bR - TACCATTTTCATCATACTTGAGC

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9. SIN2696F - CAACCATTTCAACAATTTGG

10. SIN2696R - AGCATTTGTTCCAGGTCTAAT

11. RSV forward - TTAACCAGCAAAGTGTTAGA

12. RSV reverse - TGTTATAGGCATATCATTGA

13. PIV1 forward - ATTTCTGGAGATGTCCCGTAGGAGAAC

14. PIV1 reverse - CACATCCTTGAGTGATTAAGTTTGATGA

15. PIV3 forward - AGAGGTCAATACCAACAACTA

16. PIV3 reverse - TAGCAGTATTGAAGTTGGCA

17. M. Pneumoniae forward - AAGGACCTGCAAGGGTTCGT

18. M. pneumoniae reverse - CTCTAGCCATTACCTGCTAA

19. C. pneumoniae forward - TGACAACTGTAGAAATACAGC

20. C. pneumoniae reverse - CGCCTCTCTCCTATAAAT

21. Enterovirus forward - TCCTCCGGCCCCTGAATGCG

22. Enterovirus reverse - ATTGTCACCATAAGCAGCCA

23. Influenza A forward - AAGGGCTTTCACCGAAGAGG

24. Influenza A reverse - CCCATTCTCATTACTGCTTC

25. Influenza B forward - ATGGCCATCGGATCCTCAAC

26. Influenza B reverse - TGTCAGCTATTATGGAGCTG

27. Adenovirus forward - ATGACTTTTGAGGTGGATCCCATGGA

28. Adenovirus reverse - GCCGAGAAGGGCGTGCGCAGGTA

29. Metapneumovirus forward - CATGCCCACTATAAAAGGTCAG

30. Metapneumovirus reverse - CACCCCAGTCTTTCTTGAAA

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What is claimed is:

1. A method for the detection of a pathogen, comprising the steps of:

   a. selecting at least one appropriate target sequence from the pathogen;

   b. screening said sequence to obtain at least one set of appropriate primer(s) for amplification;

   c. selecting an appropriate probe from the sequence;

   d. immobilizing the allelic-specific-oligonucleotide site of each sequence onto a solid matrix suitable for capturing the target sequence;

   e. amplifying the sequence(s) wherein the steps (d) and (e) are performed separately;

   f. performing blot analyses on each sequence; and

   g. comparing the results of the blot analysis with using positive or negative controls.

2. The method of claim 1, wherein the sequence is a gene, pathogen or allelic sequence.

3. The method of claim 1, wherein the pathogen is one of the upper respiratory viruses.

4. The method of claim 1, wherein the primer and the sequence-specific-oligonucleotide site are obtained by screening data from the GenBank or by sequencing the target sequence or in combination thereof.

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5. The method of claim 1, wherein the target sequence comprises a gene, nucleic acid or DNA sequence.

6. The method of claim 1, wherein the probe is a single oligo-nucleotide.

7. The method of claim 1, wherein more than one probe is selected.

8. The method of claim 1, wherein the probe is a single nucleotide polymorphism probe.

9. The method of claim 1, wherein the solid matrix is a membrane.

10. The method of claim 1, further comprising amplifying the target sequence having a concentration less than 0.5 f moles.

11. The method of claim 1, wherein the amplification step can be performed by polymerase chain reaction amplification, isothermal amplification, nested real-time polymerase chain reaction amplification, two-step nested real-time polymerase chain reaction amplification.

12. The method of claim 11, wherein the amplification step comprises:

a. selecting an appropriate primer pair;

b. performing reverse transcription on the primer pair; and

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c. performing the first and second round of PCR in a single tube.

13. The method of claim 12, wherein the second round of PCR is performed with a thermal cycling program having the following thermal cycling profile: (1) 95°C, 5 min; (2) 95°C, 10 sec; (3) 56°C, 10sec; (4) 72°C, 30sec; (5) 39 cycle repeats on steps 2 to 4, followed by 72°C, 10 min final extension time.

14. The method of claim 12, wherein the second round PCR was done using 1ul of the 1:10 dilution of the first round product in 50ul reaction volume.

15. The method of claim 12, wherein the reverse transcription is performed with positive control RNA of about $2x10^{-5}$ copies using the Thermoscript RT-PCR system and Platinum Taq DNA polymerase.

16. The method of claim 12, wherein the second round of PCR is performed with a thermal cycling program having the following thermal cycling profile: (1) 95°C - 3min; (2) 95°C , 10sec; (3) 60°C, 10sec (drop by 1°C/cycle); (4) 72°C, 30 sec; (5) 9 cycle repeats on steps 2 to 4; (6) 95°C, 10sec; (7) 56°C, 10sec; (8) 72°C, 30sec; (9) 9 cycle repeats on steps 6 to 8; and (10) Store at 4°C.

17. The method of claim 12, wherein more than one target sequence from at least one pathogen is amplified.

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18. The method of any one of the above claims, wherein the pathogen is an upper respiratory virus.

19. The method of claim 18, wherein the virus consists of the group selected from respiratory syncytial virus (RSV), parainfluenza virus type 1 (PIV1), parainfluenza virus type 3 (PIV3), parainfluenza virus type 3B (PIV3B), *Mycoplasma pneumoniae* (MP), *Chlamydia pneumoniae* (CP), Enterovirus (EN), Influenza A (Inf A), Influenza B (Inf B); Adenovirus (Adeno), Metapneumovirus (Meta), and SARS coronavirus (SARS).

20. The method of claim 1, wherein more than one pathogen is used.

21. The method of claim 1, wherein a pair of primers are used.

22. The method of claim 1, completed within two hours.

23. The method of claim 1, complete within minutes.

24. The method of claim 1, completed within 20 minutes using enzyme link color development.

25. The method of claim 1, completed within between 2 to 3 minutes with using use colloidal gold or florescence dye.

26. The method of claim 1, used for identification of pathogens.

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27. The method of claim 26, wherein the pathogens are SARS causative pathogens.

28. The primer, not previously known, used in the method of any of claims 1-27.

29. The probe, not previously known, used in the method of any of claims 1-27.

30. A kit for detection of pathogen comprising a compartment containing the primer of claim 28 or the probe of claim 29, or a combination thereof.

31. A machine or computer capable of carrying out the method of any of claims 1-27.

32. A software dictating the complete or part of the method of any of claims 1-27.

33. The method of claim 1, wherein the solid matrix is a low-density array for detecting multiple SARS variant.

34. The method of claim 1, wherein the solid matrix is a viral detection array for detecting upper respiratory tract infection.

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35. The method of any one of claims 33 or 34, wherein the array is on a single membrane.

36. An array, not previously made, used in any of claims 33-35.

37. The method of claim 1, wherein steps (d) and (e) are performed sequentially or concurrently.

IDT Exhibit 1020.7333

Figure 1

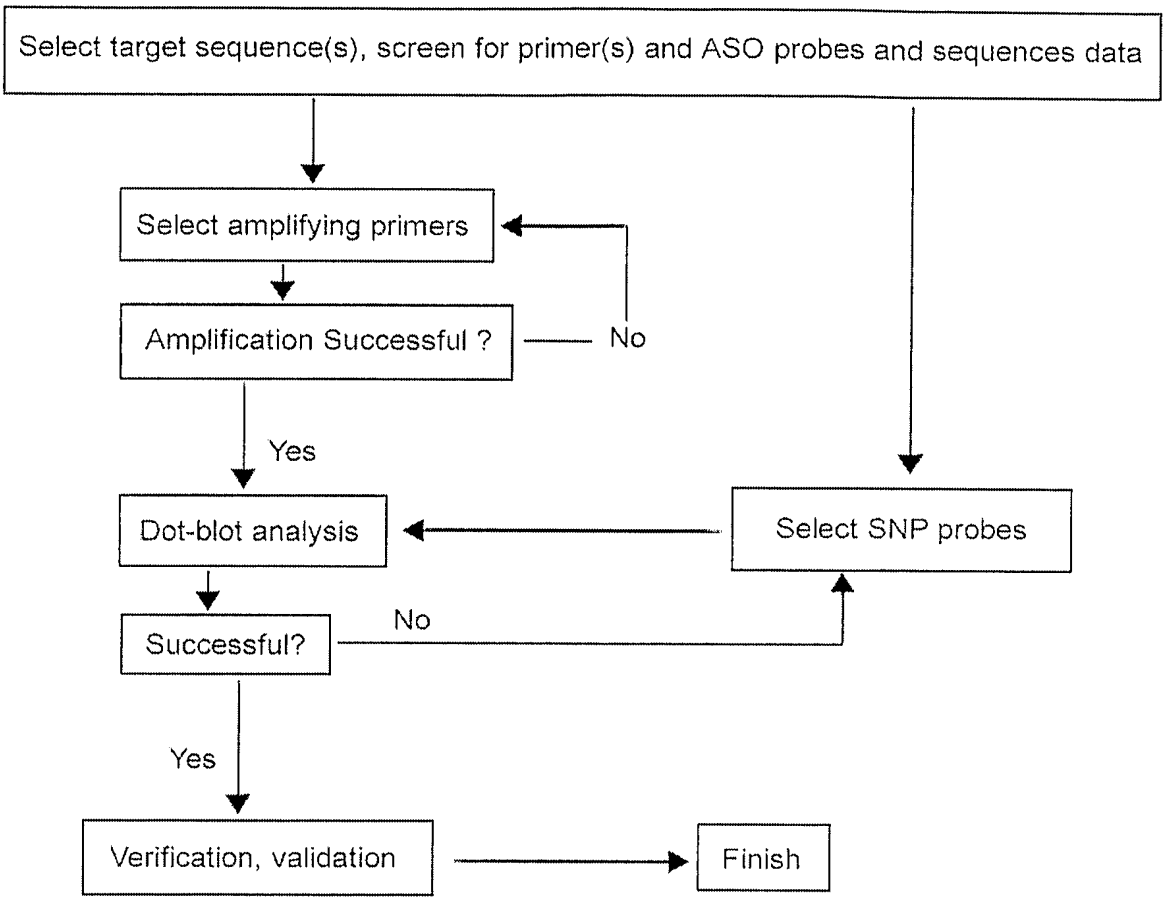

Select target sequence(s), screen for primer(s) and ASO probes and sequences data
Select amplifying primers
Amplification Successful ?
No
Yes
Dot-blot analysis
Select SNP probes
Successful?
No
Yes
Verification, validation
Finish

Figure 2

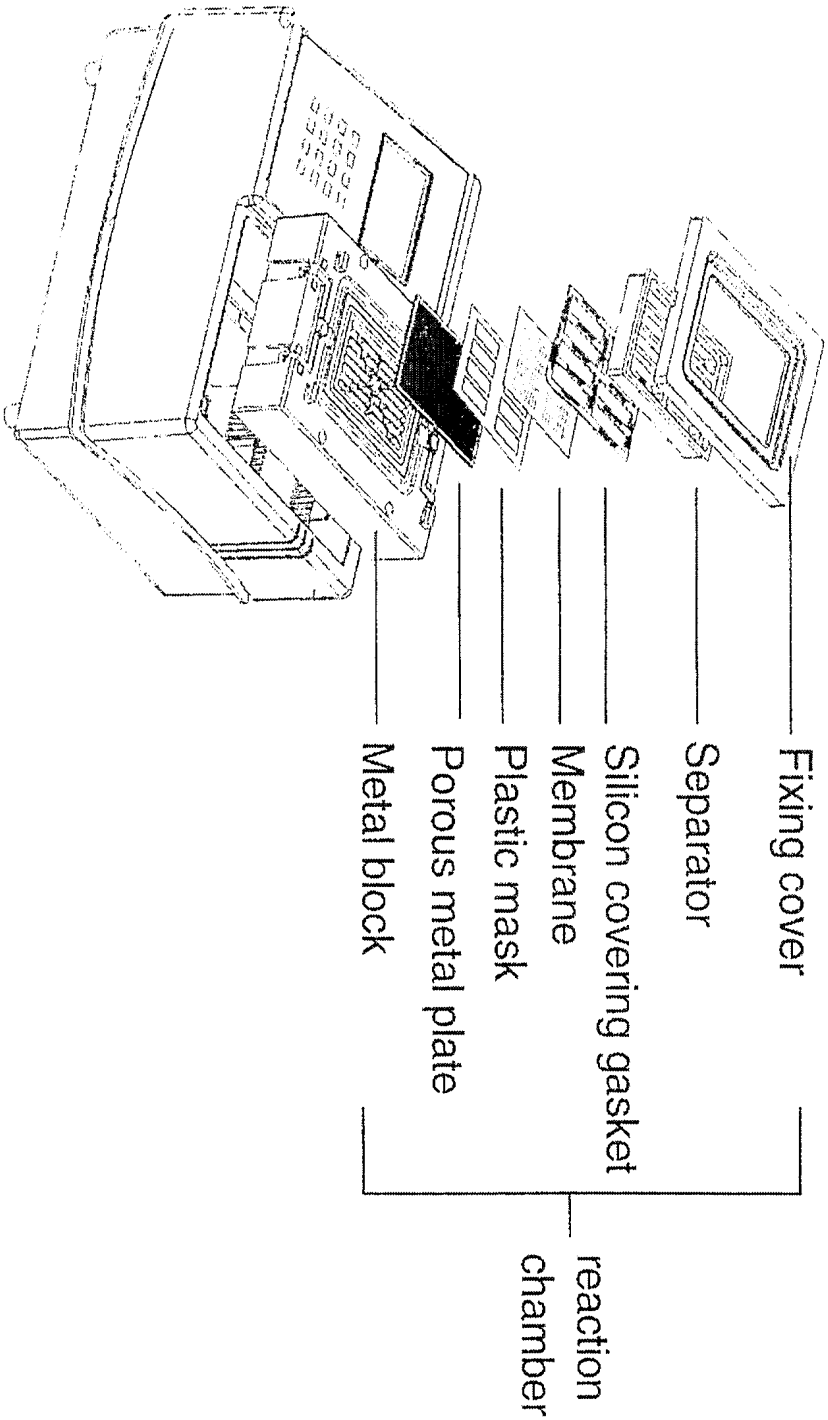

Fixing cover
Separator
Silicon covering gasket
Membrane
Plastic mask
Porous metal plate
Metal block
reaction chamber

Figure 3

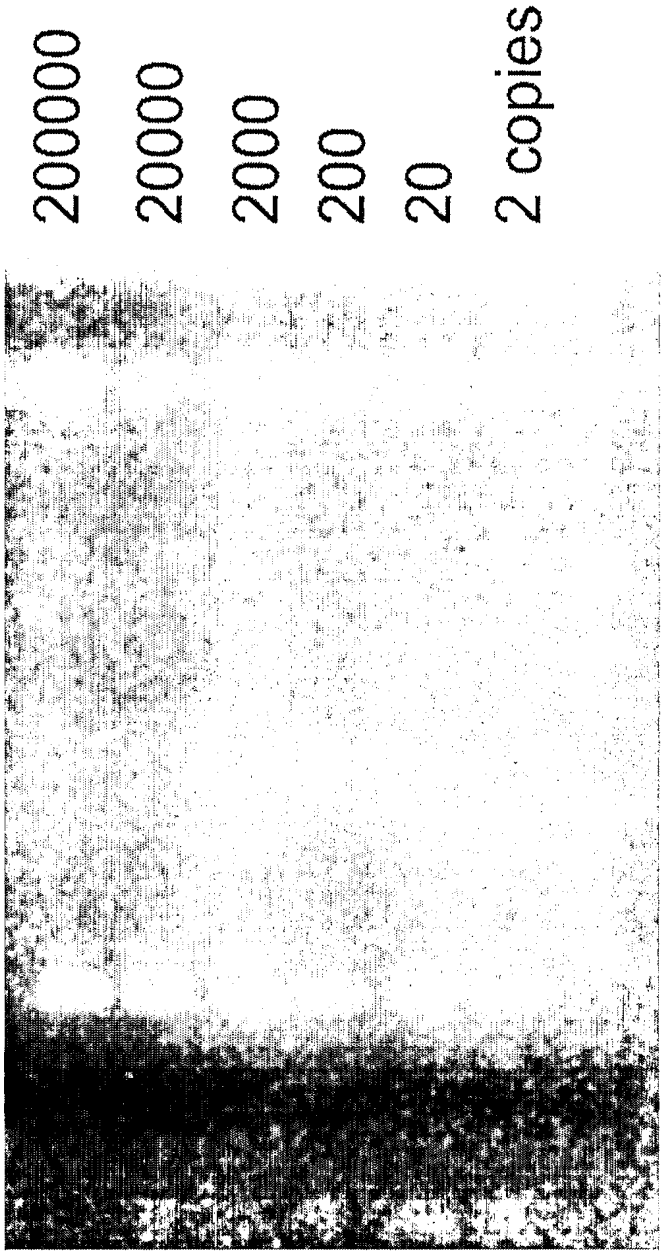


Figure 4

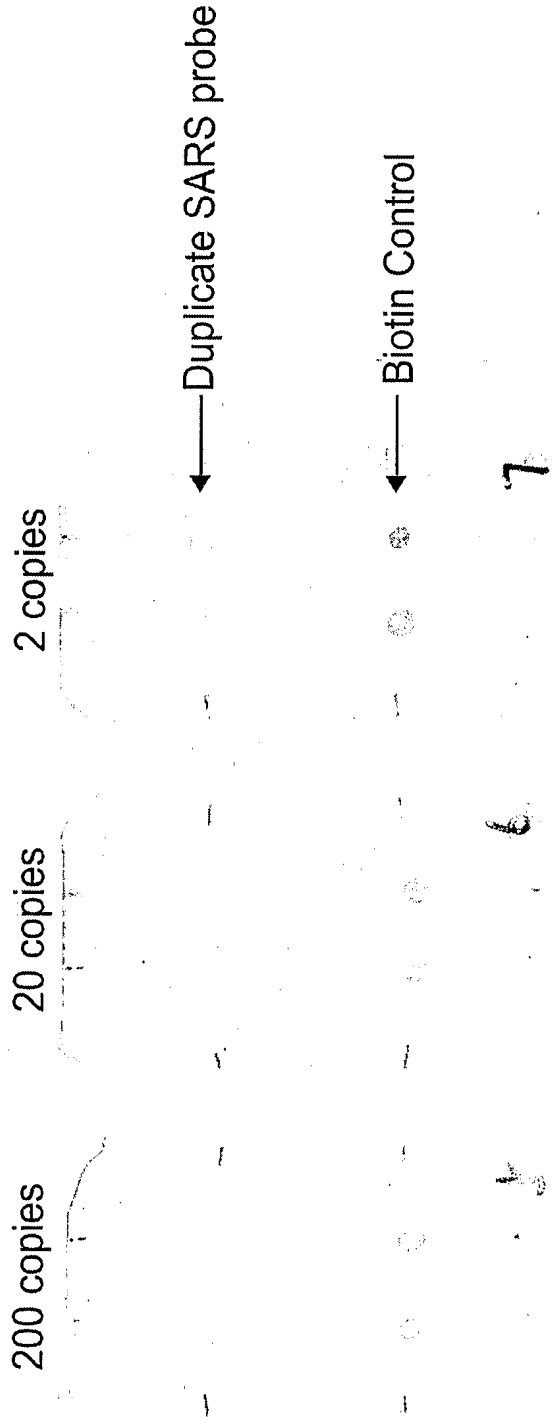

Duplicate SARS probe
Biotin Control
2 copies
20 copies
200 copies

Figure 5

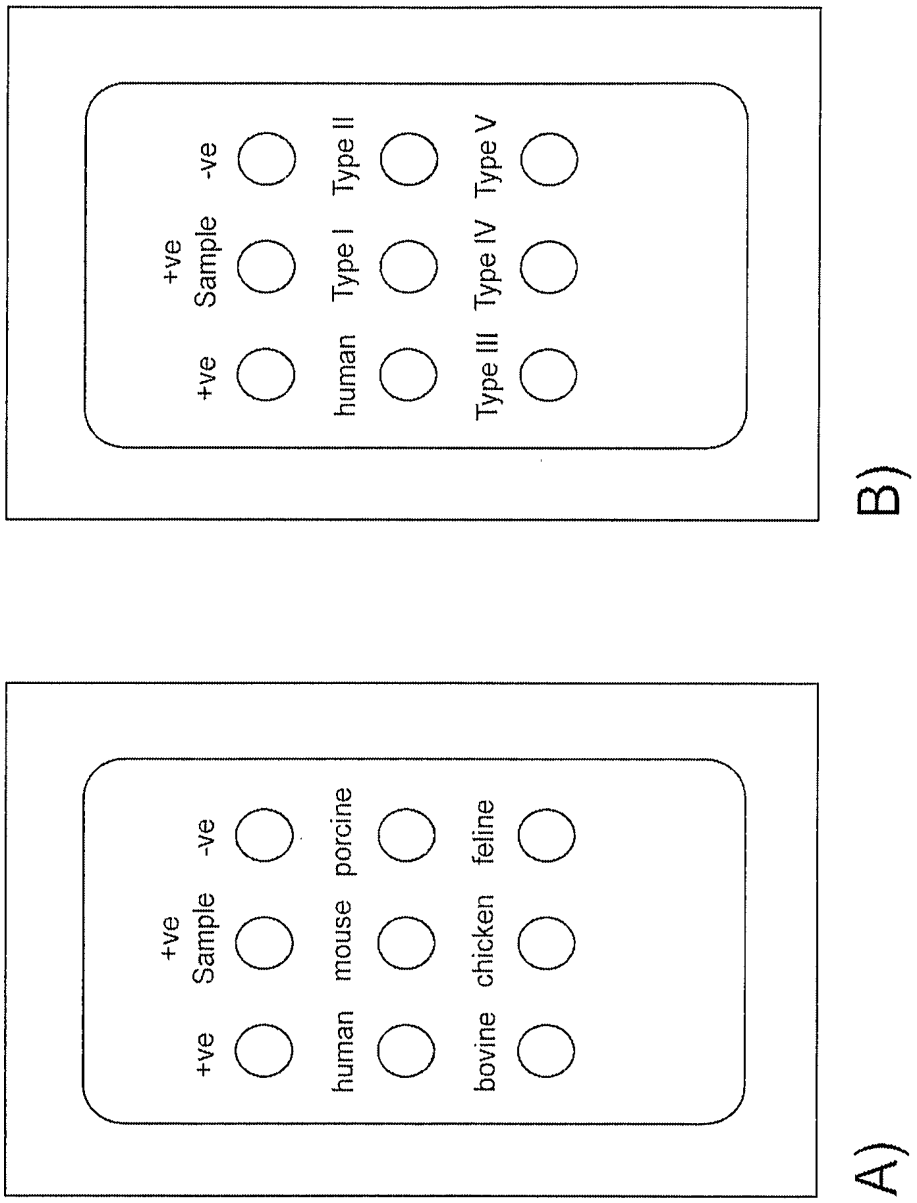
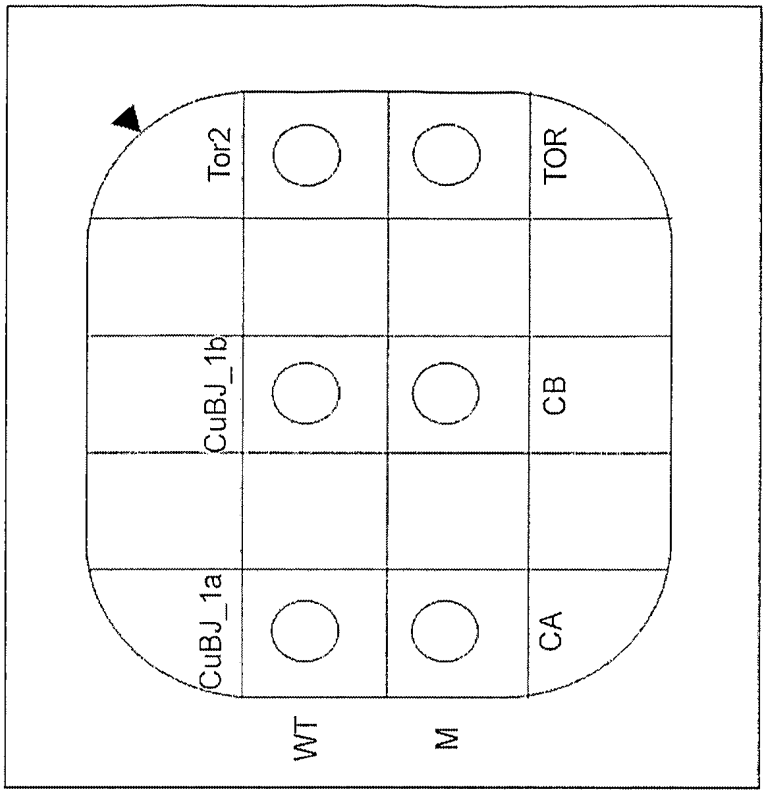

+ve
+ve
Sample
-ve
human
Type I
Type II
Type III
Type IV
Type V
B)
+ve
+ve
Sample
-ve
human
mouse
porcine
bovine
chicken
feline
A)

Figure 6



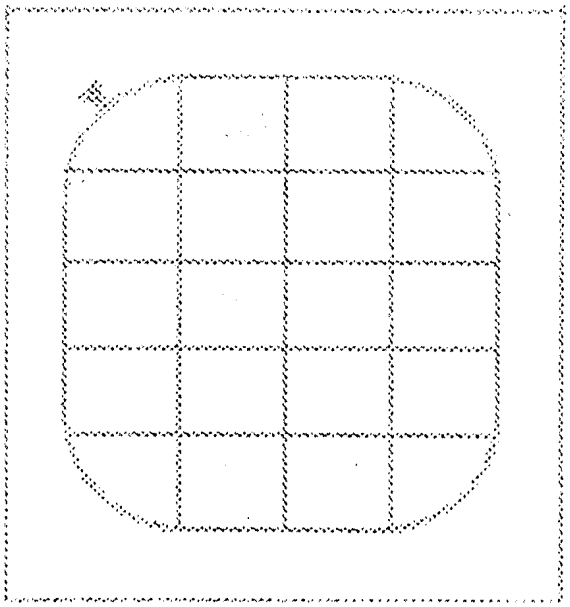


Figure 7

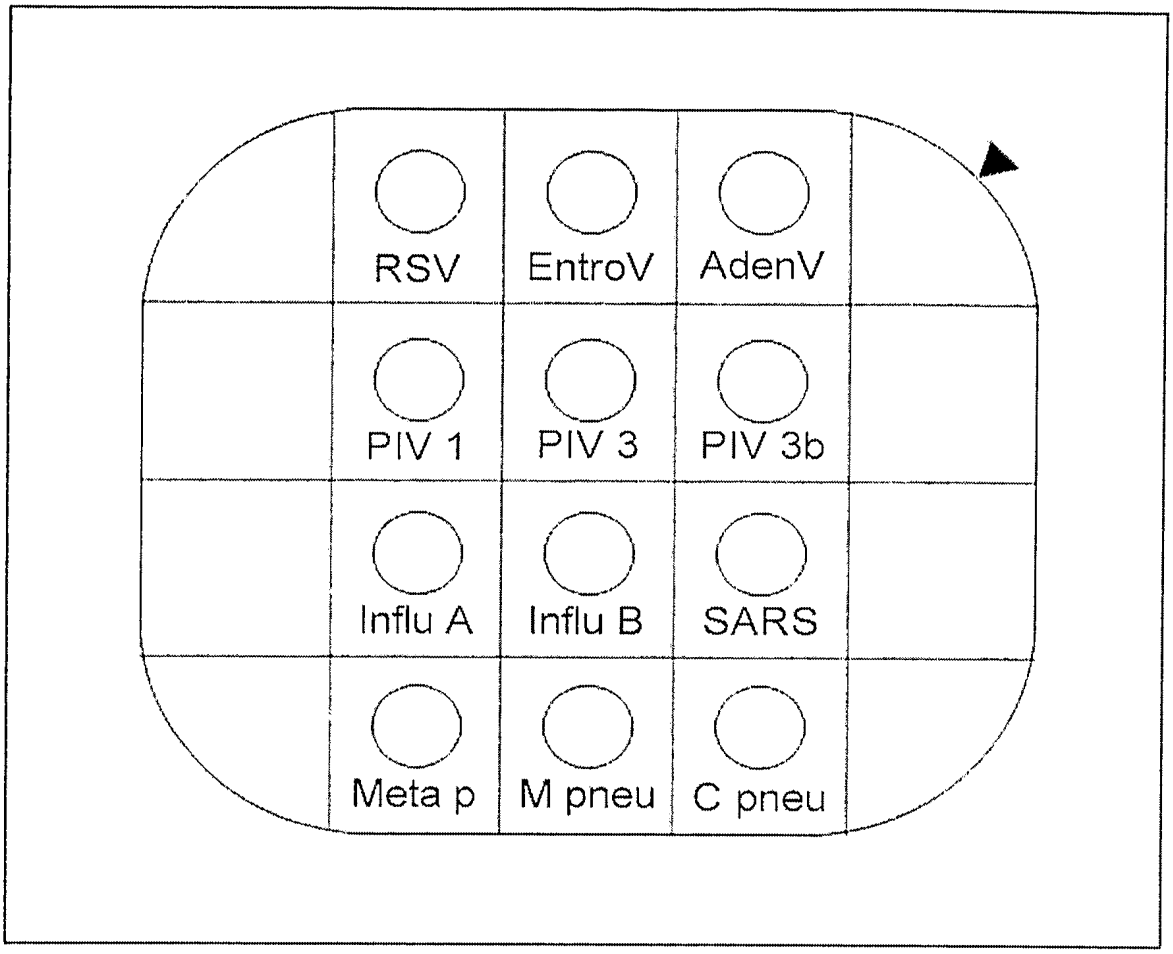
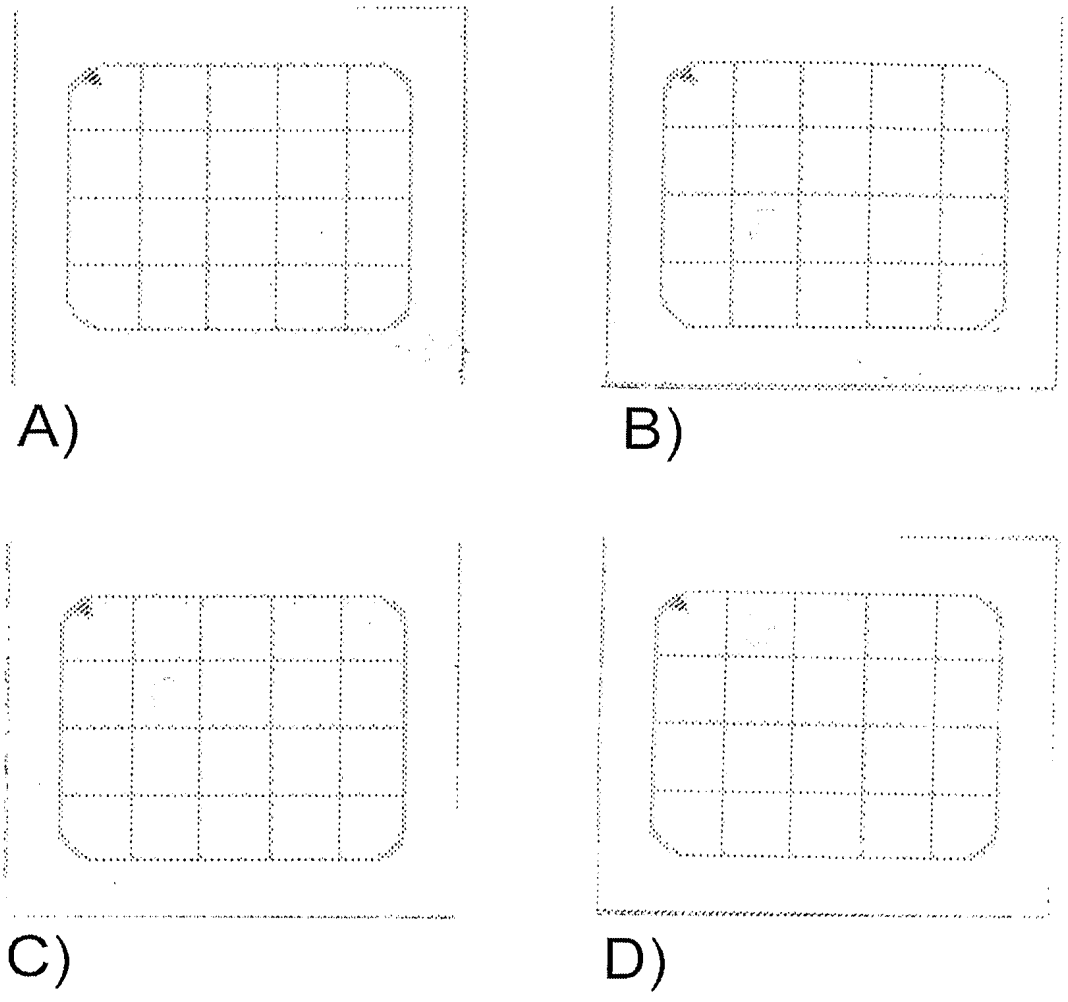

RSV
EntroV
AdenV
PIV 1
PIV 3
PIV 3b
Influ A
Influ B
SARS
Meta p
M pneu
C pneu

Figure 8



Espacenet

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METHOD FOR IDENTIFYING AND CHARACTERIZING INDIVIDUAL DNA MOLECULES

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- cooperative: **C12Q1/6883**; C12Q2600/154; C12Q2600/156

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Abstract of WO02081753 (A1)

Methods are provided that facilitate the identification and characterization of individual nucleic acid molecules.

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(54) Title: METHOD FOR IDENTIFYING AND CHARACTERIZING INDIVIDUAL DNA MOLECULES

(57) Abstract: Methods are provided that facilitate the identification and characterization of individual nucleic acid molecules.

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METHOD FOR IDENTIFYING AND CHARACTERIZING INDIVIDUAL DNA MOLECULES

This application claims priority under 35 U.S.C. §119(e) to US Provisional Application 60/281,469 filed April 4, 2001, the entire disclosure of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the fields of molecular genetics and molecular diagnostic assays. More specifically, the present invention provides methods that facilitate the identification and characterization of nucleic acid molecules present in biological samples.

BACKGROUND OF THE INVENTION

Several publications and patent documents are referenced in this application by numerals in parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

The near completion of the human genome project provides many new opportunities to more accurately study genetic alterations, mutations and DNA polymorphisms in humans (1,2). DNA sequence alterations are of particular interest because they often result in the production of defective proteins giving rise to genetic disorders. An understanding of the relationship between sequence variations and disease is highly desirable. Such knowledge facilitates the development of beneficial therapeutic agents for the treatment of genetic disorders.

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Abasic sites, or apurinic/apyrimidinic sites (AP sites), are one of the most commonly identified DNA sequence alterations. AP sites are formed when a nucleotide base, such as Adenine (A), Cytosine (C), Guanine (G), or Thymine (T) is removed from a polynucleotide strand (3, 4). Formation of AP sites occurs naturally in DNA by spontaneous depurination or deamination or by chemically-induced hydrolysis of the N-glcosylic bond, followed by the removal of the base from the polynucleotide strand by the enzymatic activity of DNA glycosylases. It has been estimated that nearly 10,000 AP sites arise per mammalian genome per day under normal physiological conditions.

AP sites are non-coding lesions on the polynucleotide strand and thus represent potentially lethal or mutagenic damages. The removal of a nucleotide base from the polynucleotide strand not only leads to an alteration in the DNA sequence, but may also lead to genetic disorders, carcinogenesis and cell death (5).

Atomic force microscopy (AFM) provides a powerful visualization tool for identifying DNA alterations, including AP sites, on individual DNA molecules (6-8). AFM is a type of scanning probe microscopy that characterizes surface properties, such as topography or friction by a spatially controlled probe. This form of microscopy is often used to capture structural images of biological molecules at nanometer resolution, including nucleic acids, proteins and cell membranes (9-11). Using AFM as opposed to electrophoretic mobility shift assays, DNase footprinting, or microfabricated DNA arrays, allows the investigator to analyze individual nucleic acid molecules of greater length.

Although several methods are presently available for quantifying the level of AP sites from a population of chromosomes or cells, no method is capable of assaying

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the physical position or the distribution of AP sites on individual DNA molecules (12-15). Such a method would be extremely beneficial for developing new technologies that are capable of rapidly comparing similarities and differences between various individual DNA molecules. This is especially true as many genetic disorders are caused by point mutations.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method for identifying and characterizing individual nucleic acid molecules is provided. An exemplary method of the invention comprises: (1) forming abasic sites specific for at least one nucleic acid base on an individual nucleic acid molecule, (2) labeling said abasic sites with a detectably labeled molecule, and (3) detecting the nature and location of the detectably labeled abasic sites. The individual nucleic acid molecule may be either DNA or cDNA, and the abasic sites are formed with a glycosylase. Suitable glycosylases for this purpose include without limitation, uracil-DNA glycosylase, 3-methyladenine-DNA glycosylase, AlkA protein, 5-methylcytosine-DNA glycosylase, Fpg protein, and Tag protein. In one embodiment, detection of the labeled abasic sites is performed by atomic force microscopy.

In an additional embodiment of the present invention, the method may be used to advantage to determine the sequence of an individual nucleic acid molecule by creating four nucleic acid sequences, wherein each of said four nucleic acid sequences contains abasic sites corresponding to one of the four particular nucleic acid bases, followed by the determination of the location of each abasic site in each of the four nucleic acid molecules.

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In yet another embodiment of the invention, the method may be used to advantage to identify and characterize individual nucleic acid molecules containing abasic sites that are naturally formed *in vivo* caused by ionizing radiation, mutagenic chemical or spontaneous mutations. The method may also be used to advantage to identify patients at risk for certain genetic disorders. Additionally, the method of the invention may be used to determine the paternity of an individual.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram outlining the method used to create AP sites on an individual DNA template. The DNA template was biotinylated at the 5'-end, and the two AP sites were formed by uracil N-glycosylase. The AP sites were then reacted with biotinylated aldehyde-reactive probe (bio-ARP) and subsequently with monomeric Avidin, approximately 16 kDa in mass.

Figures 2A-2E show atomic force microscopy height images of avidin, DNA, and Avidin-bound DNA. Height is indicated by a color code with dark (0 nm) and light (3 nm). Figure 2A shows the monomeric Avidin, 16 kDa in mass. Figure 2B shows the spin-stretched 250-bp DNA templates without any labeling. The mean and the standard deviation of the end-to-end length was measured as 77 ± 5 nm (N = 30). Figures 2C, 2D and 2E show the Avidin-DNA complexes formed after incubation with bio-ARP and monomeric Avidin.

Figure 3 shows a histogram of the distribution of bound Avidins on the stretched DNA templates. The mean and the standard deviation for the two AP sites were measured as 0.28 ± 0.02 (N = 29) and 0.35 ± 0.02 (N =

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29), which is in good agreement with the predicted values of 0.27 and 0.35, respectively.

Figures 4A-4C show atomic force microscopy height images of the end-labeled Avidin-DNA complexes. In order to illustrate three-dimensional features, the topographical images are displayed by tilting the mica surface 20 degrees. The white arrow indicates the bound Avidin monomer. Figure 4A shows the DNA templates end-labeled with Avidin. Figure 4B shows the end-labeled DNA template with one of the two AP sites labeled with Avidin. Figure 4C shows the end-labeled DNA template with both of the two AP sites labeled with Avidin.

DETAILED DESCRIPTION OF THE INVENTION

DNA is a complex biological molecule of finite chemical stability. Its sugar-phosphate backbone can be broken by oxidative stress or ionizing radiation, and its bases can be altered both enzymatically and by mutagens (16-18). Abasic or apurinic/apyrimidinic sites (AP sites) are one of the most prevalent DNA lesions. Such sites arise when a DNA base (A, C, G, or T) is removed from a polynucleotide strand (3,4). Detection of AP sites in conjunction with examination of the genetic integrity of individual DNA molecules facilitates the diagnosis of genetic disorders. Since there are different forms of DNA damage which can vary from one DNA molecule to another, a sensitive and reliable method for detecting AP sites on individual DNA molecules is highly desirable. Several methods are presently available for quantifying the level of AP sites in a population of chromosomes or cells; however, none of the current methods provide means to assess the physical position or distribution of AP sites on individual DNA molecules (13-15).

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In accordance with the present invention, a method has been developed which physically locates abasic (or AP) sites on individual nucleic acid molecules and facilitates the rapid characterization and identification of individual nucleic acid molecules using a DNA-binding agent specific for AP sites. This novel method of abasic site DNA labeling comprises the steps of: (a) creating AP sites at specific locations in an individual DNA molecule, (b) labeling the AP sites by marking them with probes specific for the AP sites, and (c) detecting the AP sites using atomic force microscopy or fluorescence microscopy.

The present invention is advantageous over existing methods for several reasons: (1) AP sites are created in a DNA sequence-dependent manner, (2) individual AP sites are labeled and detected on individual DNA molecules, and (3) single DNA molecules are characterized based on the distribution of labeled AP sites using atomic force microscopy or fluorescence microscopy. These unique features facilitate the characterization of individual DNA molecules.

Unlike conventional methods, such as color-coding with *in situ* hybridization technique or specific cuts with optical mapping techniques, the present invention places many AP sites as landmarks on individual DNA molecules in a DNA-sequence dependent manner. Therefore, the present invention provides an excellent tool for identifying similarities and differences among various DNA samples. Additionally, the present invention may be used to advantage as a tool for identifying genetic alterations which cause genetic disorders, carcinogenesis and cell death.

In an alternative embodiment of the invention, probes or PCR primers may be employed to identify AP sites on individual DNA molecules. Oligonucleotide

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probes may be used to place landmarks on individual DNA molecules by hybridizing end-labeled oligonucleotide probes to specific locations on the target molecule. Alternatively, PCR may be used to identify AP sites on individual DNA molecules by monitoring the length of PCR fragments. The presence of AP sites on a DNA molecule blocks the polymerization of new DNA fragments during PCR. Thus, shorter DNA fragments are generated from DNA molecules with AP sites than from DNA molecules without AP sites.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1997) (hereinafter "Ausubel et al.") are used.

I. Definitions

The following definitions are provided to facilitate an understanding of the present invention:

"Abasic sites" or "AP sites" are non-coding lesions on a polynucleotide strand where a single DNA base such as A, C, G or T is removed.

"Atomic Force Microscopy" is a type of scanning probe microscopy that characterizes surface properties such as topography or friction by a spatially controlled probe. AFM is a versatile tool for detecting and imaging biomolecules at nanometric i.e., single molecule, resolution. It can be applied to detect DNA, protein or DNA-binding-protein complexes.

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"Fluorescence Microscopy" is used to visualize specimens that fluoresce or emit light of one color when light of another color shines upon them. Fluorescence occurs either because of naturally occurring fluorescent substances found within a specimen such as chlorophyll or other fluorescing components (autofluorescence) or because the specimens have been coupled with a fluorescent dye.

With reference to nucleic acids used in the invention, the term "isolated nucleic acid" is sometimes employed. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. An "isolated nucleic acid molecule" may also comprise a cDNA molecule or a recombinant nucleic acid molecule.

With respect to single stranded nucleic acids, particularly oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. Appropriate conditions enabling specific hybridization of single stranded nucleic acid molecules of varying complementarity are well known in the art.

For instance, one common formula for calculating the stringency conditions required to achieve hybridization

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between nucleic acid molecules of a specified sequence homology is set forth below (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6Log [Na+] + 0.41(\% G+C) - 0.63 (\%$ formamide) - 600/#bp in duplex

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or DNA molecule, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the

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probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to a DNA oligonucleotide, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively,

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non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in US Patents 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

The term "specific binding pair" as used herein includes antigen-antibody, receptor-hormone, receptor-ligand, agonist-antagonist, lectin-carbohydrate, nucleic acid (RNA or DNA) hybridizing sequences, Fc receptor or mouse IgG-protein A, avidin-biotin, streptavidin-biotin, amine-reactive agent-amine conjugated molecule and thiol-gold interactions. Various other determinant-specific binding substance combinations are contemplated for use in practicing the methods of this invention, such as will be apparent to those skilled in the art.

The term "detectably label" is used herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or chemical means, is indicative of the presence of the target bioentity in the test sample. Representative examples of useful detectable labels, include, but are not limited to the following: molecules or ions directly or indirectly detectable based on light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or luminescence properties; molecules or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Included among the group of molecules indirectly detectable based on light absorbance or fluorescence, for example, are various enzymes which

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cause appropriate substrates to convert, e.g., from non-light absorbing to light absorbing molecules, or from non-fluorescent to fluorescent molecules.

II. Uses of Abasic DNA Labeling

In accordance with the present invention, abasic DNA labeling is a method which may be used to advantage to study individual nucleic acid molecules. Specifically, abasic DNA labeling may be used to identify AP sites that are placed as landmarks on individual nucleic acid molecules in a sequence dependent manner. By placing AP sites at known positions throughout a DNA molecule, the method may be used to confirm DNA sequence information, as well as evaluate quickly the results of DNA sequencing. Additionally, by combining four sets of data corresponding to abasic DNA molecules lacking A, C, G and T residues, abasic DNA labeling may be used for sequencing DNA molecules.

In a further embodiment of the present invention, abasic DNA labeling may be used to identify AP sites that are naturally formed by ionizing radiation, mutagenic chemicals or spontaneous mutation.

Abasic DNA labeling may also be used for restriction digest mapping on individual DNA molecules. The method may be adapted for mapping protein-DNA interaction sites, and more specifically, abasic DNA labeling may be used for mapping other forms of DNA damage, such as mismatch DNA.

In yet a further embodiment of the present invention, abasic DNA labeling may be applied to characterize methylation patterns from individual DNA molecules. Differential methylation is considered to play a role in development and progression of genetic diseases, but it is difficult to use conventional tools to detect methylation patterns with high resolution.

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Since the method described here does not require DNA amplification, the *in vivo* methylation pattern can be determined from a minute amount of DNA sample.

Abasic DNA labeling may be used as a research tool in genetic screening assays to identify those patients that may be at risk for certain genetic disorders. Such disorders include, without limitation, sickle cell anemia, cystic fibrosis, α and β thalassemias, inborn errors of metabolism, familial leukemia, fragile X syndrome, hereditary ataxias, Huntington's disease, Kennedy's disease, myotonic dystrophy, Parkinson's disease, schizophrenia, Ataxia-telangiectasia and Nijmegen breakage syndrome.

Abasic DNA labeling may also be used to advantage to identify gene loci associated with particular genetic disorders as well as for identifying alterations in particular DNA regions which give rise to genetic disorders. Abasic DNA labeling may also be adapted for purposes of identifying infectious bacteria or viruses that have been isolated from infected individuals.

Additionally, the abasic DNA labeling method of the present invention provides the basis for new and improved paternity testing as well as improved means for assessing forensic evidence.

Further details regarding the practice of this invention are set forth in the following examples, which are provided for illustrative purposes only and are in no way intended to limit the invention.

EXAMPLE I:
ABASIC DNA LABELING USING URACIL DNA GLYCOSYLASE

An atomic force microscopy-based method for detecting abasic sites (AP sites) on individual DNA molecules which is specific for the removal of thymine

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(T) residues from individual DNA molecules has been developed in accordance with the present invention. T residues were removed by uracil DNA glycosylase and replaced by uracil (19, 20). Uracil residues were subsequently removed with uracil glycosylase giving rise to AP sites on the treated DNA molecule.

To facilitate atomic force microscopy (AFM) identification of AP sites on individual DNA molecules, AP sites were labeled with biotinylated aldehyde-reactive probes (bio-ARP, 445 Da) and monomeric Avidin (approximately 16 kDa). The predominant reactive group at the AP sites was an open-chain aldehyde derived from the DNA's ribose molecules, and bio-ARP served as an initial marker of the aldehyde group on the AP sites (21, 22). The location of bio-ARPs on the individual DNA molecules was then visualized by AFM via monomeric Avidin bound to bio-ARP.

The above-described procedure was used to incorporate two uracil residues into a 250-bp DNA template. In order to determine the location of AP sites without directional ambiguity, one 5'-end of a single DNA strand was biotinylated and marked with Avidin. The DNA template was gently uncoiled and immobilized on a flat mica surface, and the location of the two Avidin-bound AP sites were identified by AFM at nanometer resolution.

The described AFM-based method facilitates analysis of biochemical reactivity at specific AP sites and can further be used as a means to determine the efficiency of AP-site labeling. AP sites exist in one of three tautomeric forms, such as an open-chain aldehyde, an open-chain hydrate, and hemiacetals, and they can be opposed to any of the four different bases on its complementary strand. In this example, the open-chain aldehyde group was targeted and labeled with bio-ARP and the two AP sites were both opposed to an Adenine residue.

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Because of tautomerization, any AP site takes the aldehyde form for approximately 1% of time, and therefore, may be labeled by aldehyde-reactive probes (22). Alternatively, there is an enzyme that specifically cleaves the AP site and generates a 5'-end base-free deoxyribose phosphate (23, 24).

I. Materials and Methods

The following protocols are provided to facilitate the practice of the present invention.

Preparation of DNA Templates

A double-stranded DNA template, 250 bp in length, was constructed which included two uracils located 163 bp and 183 bp away from the biotinylated 5'-end on the upper DNA strand (Figure 1). The two uracil residues were subsequently removed from the DNA templates using thermolabile uracil N-glycosylase (HU59100, Epicenter Technology). One hundred ng of DNA molecules were incubated for 30 minutes at room temperature in 10 µl of buffer containing 50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, and 1 U of uracil N-glycosylase.

The glycosylated DNA templates containing the two AP sites were then labeled with 5 mM N-aminooxyacetyl-N'-D-biotynoyl hydrazine (bio-ARP, Dojindo Laboratories) in a 10 µl volume of buffer consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl for 30 minutes at room temperature. The DNA templates were separated from the uracil N-glycosylase and the unreacted bio-ARP using phenol-chloroform extraction and ethanol precipitation. Lastly, the DNA sample was reacted with monomeric Avidin, approximately 16 kDa in mass (A2036, Sigma), in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM $MgCl_2$ for 30 minutes at room temperature. The ratio of

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Avidin monomers to DNA molecules in the reaction tube was approximately 10.

Uncoiling and Immobilizing DNA Samples

The glycosylated, end-biotinylated DNA templates labeled with bio-ARP and Avidin monomers were gently stretched and immobilized on a freshly cleaved mica surface (25 mm X 25 mm, Ted Pella Inc.) by the method previously described (25). Control DNA molecules were also used without glycosylation or end-biotinylation. The mica sheet was then mounted on the custom-made spin-stretcher, and the mica plate was spun at approximately 5000 rpm. A series of solutions were gently dispensed on the spinning center in the order of 50 µl of H_2O, 50 µl of 500 mM $MgCl_2$, 50 µl of H_2O, 10 µl of the DNA sample, and 50 µl of H_2O in 15 second intervals.

Imaging by Atomic Force Microscopy

A Nanoscope III atomic force microscope (Digital Instruments, Inc.) was used to capture topographical images of monomeric Avidin, DNA templates, and Avidin-DNA complexes immobilized on the mica surface. AFM was operated in the ambient air at 15-20% humidity. The tapping mode was used to reduce any damage to biological samples caused by physical contact with the tip, and the tapping frequency was set to ~290 kHz. The scanning field of view was 2 µm X 2 µm (coarse scanning) or 500 nm X 500 nm (fine scanning) with the scanning rate of 0.5-1 Hz and 512 scanning lines. The silicon tips had an estimated curvature of 10 to 20 nm. Height images in the range of 0-5 nm were flattened to remove the background curvature of the mica surface, and the images were analyzed using NIH Image 1.60 image analysis software. The normalized position corresponding to AP sites was defined as $d_1/(d_1 + d_2)$, where d_1 and d_2 were the length of DNA segments bisected by a bound Avidin monomer.

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II. Results

Labeling AP Sites with Monomeric Avidin

Prior to locating biotin-labeled AP sites on individual DNA molecules, a geometric size of the 250-bp DNA template, as well as monomeric Avidin, was determined, and proper sample preparations were evaluated. In a scanning field of 2 μm X 2 μm, approximately 30 DNA molecules were detected, and the mean and the standard deviation of end-to-end DNA length was measured as 77 ± 5 nm (N = 45, sample number; Figure 2). The predicted length in B-form was 82 nm. Monomeric Avidin was elliptical-shaped with the mean and the standard deviation of 16 ± 2 nm (major axis, N = 30) and 9 ± 1 nm (minor axis, N = 30; Figure 2). The dimension of a natural form of Avidin consisting of four monomer units was measured as 23 ± 3 (major axis, N = 30) and 16 ± 2 (minor axis, N = 30).

The monomer form of Avidin was used in this study in order to enhance resolution in determining Avidin's binding sites. When the DNA template was not biotinylated at the 5'-end, approximately 20% of the molecules were detected as a DNA-protein complex (Figure 2). The binding positions of avidin were in the vicinity of one of the two AP sites at 163 bp and 183 bp from the 5'-end. Approximately 1% of the DNA molecules exhibited two Avidin monomers closely spaced next to each other. For the DNA templates end-labeled with biotin, over 90% of the molecules were end-marked with Avidin when the ratio of DNA molecules to Avidin monomers was set to 10.

Determination of AP Sites

The location of AP sites was first determined using the DNA template lacking the biotinylated 5'-terminus. When straightened, the DNA template had an end-to-end length in the range of 77-84 nm. Since the DNA template

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was not end-marked, the DNA-bound Avidins could have been on either the segment close to the 5'-terminus or the 3'-terminus. Thus, the binding sites were assigned between 0 and 0.5 in a normalized scale where the end-to-end length of an individual DNA template was set to 1. A histogram of the stretched DNA template was then prepared which exhibited two peaks located at 0.28 ± 0.02 (mean ± standard deviation) (N = 29) and 0.35 ± 0.02 (N = 29; Figure 3). These observed peaks were in good agreement with the predicted values of 0.27 and 0.35.

Simultaneous Labeling of AP Sites and DNA Ends

In order to remove directional ambiguity and to distinguish one AP site located on a DNA segment close to the 5'-terminus from the other on the 3'-terminus, biotin was conjugated to the 5'-end of one DNA single strand. Since biotin was incorporated at the 5'-end as well as the two AP sites, all three sites could be simultaneously labeled with Avidin monomers. AFM was able to detect Avidin monomer bound to the DNA end (Figure 4). The efficiency of DNA end-labeling was approximately 90%, and approximately 25% of the end-labeled DNA molecules exhibited bound Avidin at sites close to the two AP sites. The AFM images clearly showed that the AP sites were located away from the 5'-end marked by Avidin (Figures 1 and 4). The observed distance between the two AP sites illustrated in Figure 4C was 8 nm which was in good agreement with the predicted distance of 7 nm.

III. Discussion

Two AP sites were created and then visualized on a 250-bp DNA template using the rapid and sensitive ARP-mediated method of identifying the position of DNA abasic sites by AFM. The location of the two AP sites were determined from the site of bound Avidins on the uncoiled

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DNA. The binding of Avidin to the two AP sites, as well as the biotinylated DNA end, was specific in the AFM-based assay. The average difference between the observed AP site and the predicted AP site was within a few nanometers, and the standard deviation of the distribution was approximately 6 bp (~2 nm) in the observed population of 58 bound Avidins. A pair of Avidins, apparently bound tandemly at the two AP sites separated by 20 bp, was clearly identifiable from a shape resembling a cluster of two globular structures.

Unlike conventional molecular tools, such as electrophoretic mobility shift assays or DNase footprinting, the AFM-based method described herein allows for the analysis of large DNA templates from a minute amount of sample. DNA molecules over 100 kbp in length can be straightened easily by the stretching apparatus previously developed, and determining sites of bound proteins such as Avidin along uncoiled DNA molecules is straightforward. Additionally, by end-labeling the 5'-terminus or the 3'-terminus of a single DNA strand, binding sites of interest can be identified without directional ambiguity.

In conclusion, the abasic DNA labeling method described herein provides a rapid and sensitive tool for identifying AP sites on individual DNA molecules. This method is an improvement over currently available colorimetric or fluorescence-based assays. The mean number of AP sites from a population of DNA molecules are not only quantified, but localized at nanometer resolution.

EXAMPLE II: METHYLATION-MEDIATED DNA LABELING

AFM may also be implemented for the detection of AP sites on individual DNA molecules which are created by

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the removal of methylated Cytosine residues (5-methylcytosines). Using this method, individual DNA molecules will be characterized based on the distribution of 5-methylcytosines. Removal of the 5-methylcytosines from the DNA molecule will create AP sites which can be labeled and identified by AFM.

Cytosine (C) residues, recognized at specific recognition sites by methylases, are hemi-methylated. Such recognition sites and methylases include: C in 5'-AGCT-3' by Alu I methylase, the first C in 5'-GGCC-3' by Hae III methylase, the C in 5'-GCGC-3' by HhaI methylase, the second C in 5'-CCGG-3' by HpaII methylase, the first C in 5'-CCGG-3' by MspI methylase, and C in 5'-CG-3' by SssI methylase.

AP sites will be formed by the removal of 5-methylcytosines using 5-methylcytosine-DNA glycosylase. The AP sites will be labeled by biotinylated aldehyde-reactive probes (N-aminooxylacetyl-N'-D-biotynoyl hydrazine) as described in Example 1. Monomeric Avidin molecules may then be reacted with biotin to facilitate detection of AP sites on individual DNA molecules by AFM.

EXAMPLE III:
METHODS FOR CREATING AP SITES USING VARIOUS DNA GLYCOSYLASES

A family of DNA glycosylases has been identified, and they are known to create AP sites corresponding to specific nucleotides. For instance, 3-methyladenine-DNA glycosylase, detected in humans, is able to remove 3-methyladenine which creates AP sites specific to adenine residues. The same enzyme can remove 3-methylguanine and 7-methylguanine to form AP sites specific to guanine residues. Therefore, it is possible to create AP sites corresponding to any one of the four DNA nucleotides using specific glycosylases. These glycosylases include,

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but not limited to, uracil-DNA glycosylase, 3-methyladenine-DNA glycosylase, AlkA protein, 5-methylcytosine-DNA glycosylase, Fpg protein, and Tag protein.

Thymine (T) residues may be removed using at least two glycosylases. First, uracil may be incorporated into a target DNA molecule, as described in Example 1, followed by removal of the uracil by uracil-DNA glycosylase. T residues may also be removed by incorporation of O_2-methylthymine into a target DNA molecule, followed by removal of O_2-methylthymine using AlkA protein.

Cytosine (C) residues may be removed using at least three different glycosylases. C residues may be removed by methylating C residues of a target DNA molecule, as described above in Example 2, followed by removal of 5-methylcytosine using 5-methylcytosine-DNA glycosylase. C residues may also be removed by incorporating O_2-methylcytosine into a target DNA molecule, followed by the removal of O_2-methylcytosine using AlkA protein. Lastly, C residues may be removed by incorporating 5-hydroxycytosine into a target DNA molecule, followed by the removal of 5-hydroxycytosine using Fpg protein.

Adenine (A) residues may be removed by incorporating 3-methyladenine into a target DNA molecule, followed by the removal of 3-methyladenine using 3-methyladenine-DNA glycosylase, AlkA protein, or Tag protein.

Guanine (G) residues may be removed using at least three different glycosylases. G residues may be removed by incorporating 3-methylguanine into a target DNA molecule, followed by removal of 3-methylguanine using 3-methyladenine-DNA glycosylase, AlkA protein, or Tag protein. G residues may also be removed by incorporating 7-methylguanine into a target DNA molecule, followed by the removal of 7-methylguanine using 3-methyladenine-DNA

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glycosylase, or AlkA protein. Lastly, G residues may be removed by incorporating 8-oxoguanine or 7-alkylguanine into a target DNA molecule, followed by removal of 8-oxoguanine or 7-alkylguanine using Fpg protein.

Once the specific nucleotides have been removed using any of the above-described glycosylases, the AP sites may be labeled using biotinylated aldehyde-reactive probes (N-aminooxylacetyl-N'-D-biotynoyl hydrazine) as described in Example 1. Monomeric Avidin molecules may then be reacted with the biotin to facilitate detection of the AP sites on the individual DNA molecules by AFM.

EXAMPLE IV:
FLUORESCENCE-BASED MICROSCOPY DETECTION OF AP SITES

The above described Abasic DNA labeling methods may be adapted to allow for fluorescence-based microscopy detection of AP sites on individual DNA molecules instead of AFM. The conversion may be accomplished by conjugating fluorescent dye to the biotinylated probes which will facilitate the detection and characterization of DNA molecules using fluorescence microscopy.

The fluorescent labeled probes have additional applications, including the creation of digital DNA images unique to individual DNA molecules. Digital images may then be used to identify genetic disorders or heterogeneity among populations.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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What is claimed is:

1. A method for characterizing individual nucleic acid molecules, comprising the steps of:

a) forming abasic sites specific for at least one nucleic acid base on individual nucleic acid molecules,

b) labeling said abasic sites with detectably labeled molecules, and

c) detecting the location of said detectably labeled molecules on said individual nucleic acid molecules, thereby determining the nature and location of said abasic sites.

2. The method of claim 1, wherein said nucleic acid molecules are selected from the group consisting of cDNA and DNA.

3. The method of claim 1, wherein said abasic sites are created using a glycosylase selected from the group consisting of uracil-DNA glycosylase, 3-methyladenine-DNA glycosylase, AlkA protein, 5-methylcytosine-DNA glycosylase, Fpg protein, and Tag protein.

4. The method of claim 1, wherein atomic force microscopy is performed for said detection.

5. The method of claim 1, wherein fluorescence-based microscopy is performed for said detection.

6. A method for characterizing individual nucleic acid molecules as claimed in claim 1, wherein said abasic sites formed in step a) results from removal of A residues, said method further comprising:

d) providing three additional identical nucleic

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acid molecules, where each of said three nucleic acid molecules are treated to remove C, G and T residues, respectively, thereby generating four nucleic acid sequences, each of said four nucleic acid sequences containing abasic sites corresponding to a particular nucleic acid base, and

e) determining the location of each abasic site in said four nucleic acid molecules, thereby determining the sequence of said nucleic acid molecule.

7. The method of claim 1, wherein said method is used to identify abasic sites that are naturally formed *in vivo* by a method selected from the group consisting of ionizing radiation, mutagenic chemicals and spontaneous mutations.

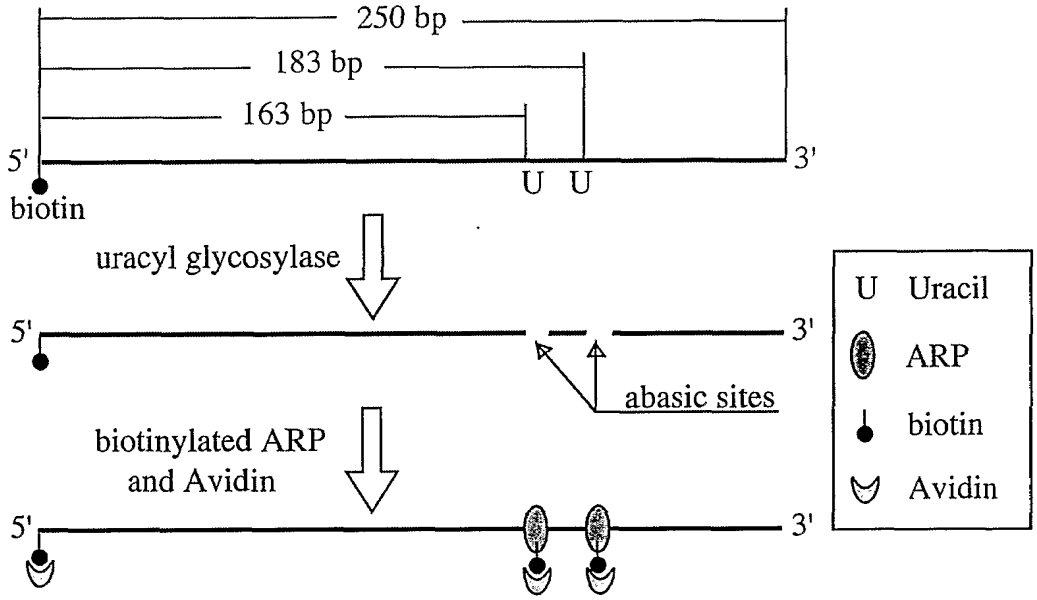
8. The method of claim 1, wherein said method is used to identify patients in need thereof who are at risk for certain genetic disorders including those genetic disorders selected from the group consisting of sickle cell anemia, cystic fibrosis, α and β thalassemias, inborn errors of metabolism, familial leukemia, fragile X syndrome, hereditary ataxias, Huntington's disease, Kennedy's disease, myotonic dystrophy, Parkinson's disease, schizophrenia, Ataxia-telangiectasia and Nijmegen breakage syndrome.

9. The method of claim 1, wherein said method is used to identify infectious bacteria or viruses present in a biological sample.

10. The method of claim 1, wherein said method is used to determine paternity of an individual.

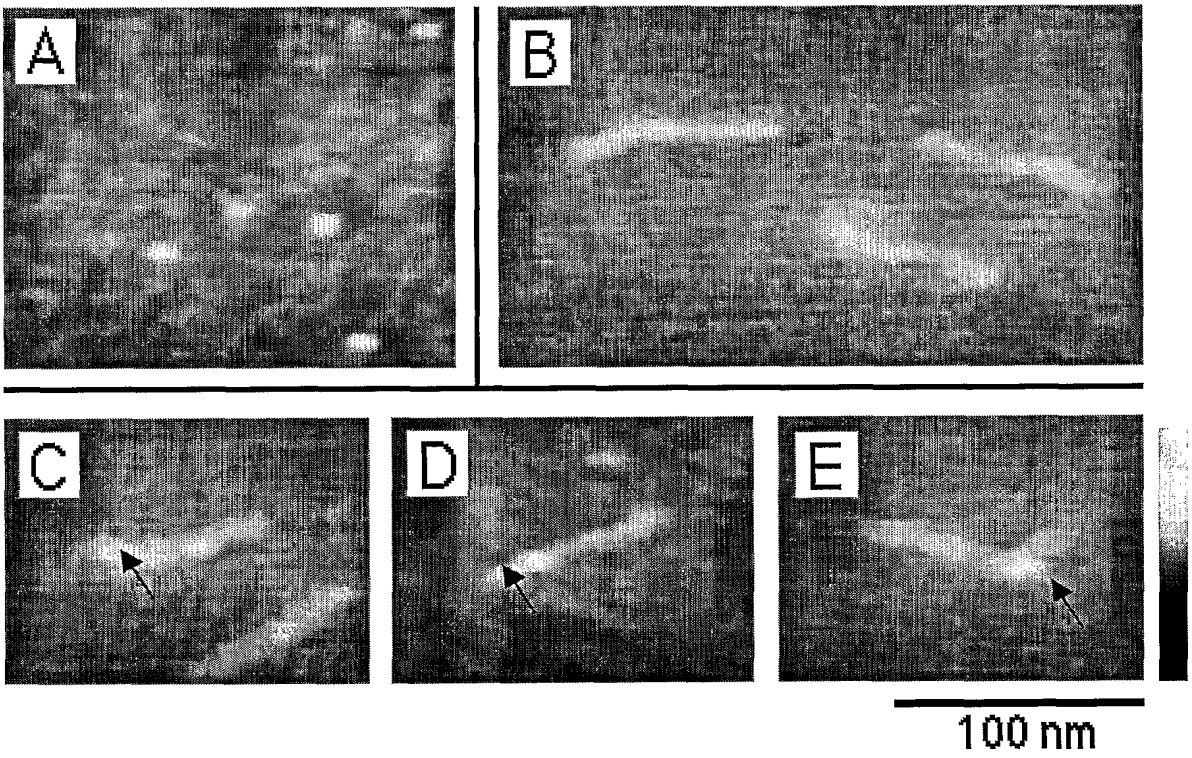
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Figure 1



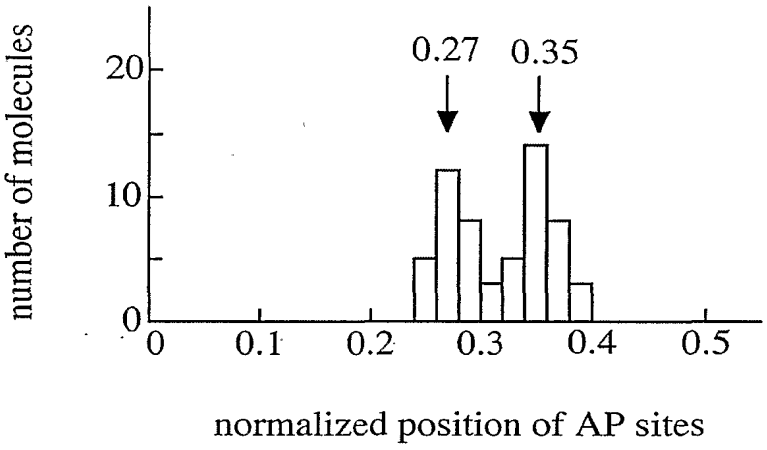
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Figure 2



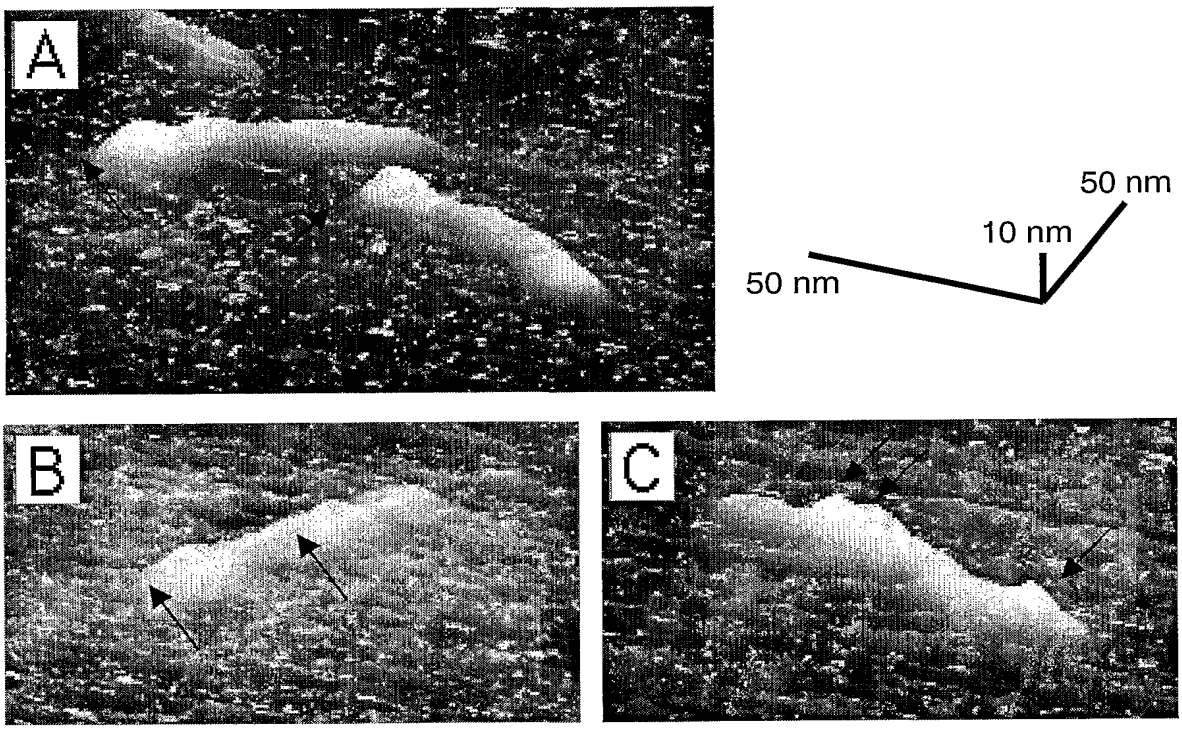
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Figure 3



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Figure 4



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/10696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q, 1/68; C70H 21/00, 21/02; C70K 17/00
US CL : 435/6; 536/22.1, 23.1; 530/350

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)
U.S. : 435/6

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)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,470,707 A (SASAKI et al) 28 November 1995 (28.11.1995), column 2, lines 48-64	1-10
Y	US 5,194,300 A (CHEUNG et al) 16 March 1993 (16.03.1993), column 7, line 54 - column 8, line 2	1-10
Y	WO 97/03210 A1 (FORFAS) 30 January 1997 (30.01.1997), page 12, line 28 - page 13, line 3 .	1-10
Y	JOST, J.P. et al. Mechanisms of DNA demethylation in chicken embryos: Purification and properties of a 5-methylcytosine-DNA glycosylase. The Journal of Biological Chemistry. April 1995, Vol. 270. No. 17, pages 9734-9739, espcially pages 9736-9737.	3
Y	BJELLAND, S. et ak, Different efficiencies of the Tag and AlkA DNA glycosylases from Escherichia coli in the removal of 3-methyladenine from single-stranded DNA. FEBS Letters. 1996, Vol.397, pages 127-129, especially page 128.	3

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

* Special categories of cited documents:

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"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

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Date of the actual completion of the international search
19 June 2002 (19.06.2002)

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/10696

Continuation of B. FIELDS SEARCHED Item 3:
Medline, CaPlus, Biosis, SciSearch; USPTO, EPO, and JPO patent databases

search terms: "atomic force microscopy," "fluorescence microscopy," "scanning electron microscopy," "tunneling electron microscopy," DNA sequence or sequencing, label, abasic, apurinic, apyrimidinic, DNA repair, DNA glycosylase, AlkA, Fpg, Tag

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(54) PROCEDE DE MARQUAGE ET DE FRAGMENTATION D'ADN
(54) METHOD FOR LABELLING AND FRAGMENTING DNA

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(54) Titre : PROCEDE DE MARQUAGE ET DE FRAGMENTATION D'ADN
(54) Title: METHOD FOR LABELLING AND FRAGMENTING DNA

(57) Abrégé/Abstract:
La présente invention concerne un procédé de marquage et de fragmentation d'un acide désoxyribonucléique (ADN) simple ou double brin comprenant les étapes suivantes : fragmenter chimiquement l'ADN en créant au moins un site abasique sur ledit ADN, attacher un marqueur sur au moins un des fragments par l'intermédiaire d'un réactif de marquage, ledit réactif se couplant de manière covalente et majoritaire sur au moins un phosphate dudit fragment. L'invention trouve une application préférentielle dans le domaine du diagnostic.

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(54) Title: METHOD FOR LABELLING AND FRAGMENTING DNA

(54) Titre : PROCÉDÉ DE MARQUAGE ET DE FRAGMENTATION D'ADN

(57) Abstract: The invention concerns a method for labelling and fragmenting a single-stranded or double-stranded deoxyribonucleic acid (DNA) comprising the following steps: chemically fragmenting the DNA by generating an abasic site on said DNA, attaching a marker on at least one of the fragments by means of a labelling reagent, said reagent being covalently and majority bonded on at least one phosphate of said fragment. The invention is applicable in the field of diagnosis.

(57) Abrégé : La présente invention concerne un procédé de marquage et de fragmentation d'un acide désoxyribonucléique (ADN) simple ou double brin comprenant les étapes suivantes : fragmenter chimiquement l'ADN en créant au moins un site abasique sur ledit ADN, attacher un marqueur sur au moins un des fragments par l'intermédiaire d'un réactif de marquage, ledit réactif se couplant de manière covalente et majoritaire sur au moins un phosphate dudit fragment. L'invention trouve une application préférentielle dans le domaine du diagnostic.

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PROCEDE DE MARQUAGE ET DE FRAGMENTATION D'ADN

La présente invention concerne un procédé de fragmentation et de marquage d'ADN ainsi que les applications de ce procédé en particulier dans le domaine du diagnostic.

L'état de la technique montre que de nombreuses méthodes existent pour marquer des acides nucléiques.

Une première méthode consiste à fixer le marqueur sur la base, que celle-ci soit naturelle ou modifiée. Une deuxième méthode propose de fixer le marqueur sur le sucre, là encore qu'il soit naturel ou modifié. Une troisième méthode a pour objet la fixation du marqueur sur le phosphate.

Le marquage sur la base a été notamment utilisé dans l'approche de marquage des acides nucléiques par incorporation de nucléotides directement marqués.

Le marquage sur le sucre est souvent utilisé dans le cas des oligonucléotides préparées par synthèse chimique.

Le marquage sur le phosphate a été aussi utilisé pour introduire des bras fonctionnalisés et des marqueurs lors de la synthèse chimique des oligonucléotides.

En fait l'homme du métier, qui doit effectuer le marquage d'un nucléotide, ou d'un analogue de nucléotide ou d'un acide nucléique, est enclin à effectuer cette fixation sur la base ou sur le sucre qui lui offre plus de commodité et d'alternatives. C'est d'ailleurs ce qui ressort de l'étude de nombreux documents, tels que EP-A-0.329.198, EP-A-0.302.175, EP-A-0.097.373, EP-A-0.063.879, US-A-5,449,767, US-A-5,328,824, WO-A-93/16O94, DE-A-39 10 151, EP-A-0.567.841 pour la base ou EP-A-0.286.898 pour le sucre.

La fixation du marqueur sur le phosphate est une technique plus complexe que la technique consistant à fonctionnaliser la base ou le sucre et a été bien moins utilisée notamment à cause de la faible réactivité du phosphate (voir par exemple Jencks W.P. et al J. Amer. Chem. Soc., 82, 1778-1785, 1960). De même dans la revue de O'Donnel et Mc Laughlin (« Reporter groups for the analysis of nucleic acid structure », p 216-243, dans « Bioorganic Chemistry : Nucleic Acids », Ed Hecht S.M., Oxford University Press, 1996) portant sur les méthodes d'introduction de sondes dans les fragments d'oligonucléotides,

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l'alkylation efficace du phosphodiester internucléotidique est considérée comme étant impossible.

Un deuxième problème se pose pour le marquage d'acides nucléiques spécialement pour les acides nucléiques de grande taille, c'est-à-dire de plus de cent (100) nucléotides, qui doivent s'hybrider avec des sondes nucléiques. Ce problème est lié à la gêne stérique ou au manque de spécificité entre l'acide nucléique et la sonde nucléique. Cela se traduit pas une perte de sensibilité en détection.

La gêne stérique peut être le fait, non seulement, de la longueur de l'acide nucléique, mais également de l'existence ou de la conservation de structures secondaires. La fragmentation permet de détruire ces structures et ainsi d'optimiser l'hybridation. Cette gêne stérique joue un rôle particulièrement important dans le cas de l'hybridation sur des surfaces contenant des sondes de capture à forte densité, par exemple les puces à ADN mises au point par la société Affymetrix ("Accessing Genetic Information with High-Density DNA arrays", M. Shee et al., Science, 274, 610-614. "Light-generated oligonucleotide arrays for rapide DNA sequence analysis", A. Caviani Pease et al., Proc. Natl. Acad. Sci. USA, 1994, 91, 5022-5026).

En ce qui concerne la fragmentation des acides nucléiques, de nombreuses méthodes sont décrites dans l'état de la technique.

Premièrement, la fragmentation peut être enzymatique, c'est-à-dire que la fragmentation des acides nucléiques peut être réalisée par des nucléases (DNases).

Deuxièmement, la fragmentation peut être chimique. Par exemple dans le cas de l'ADN, on peut effectuer la dépurination ou la dépyrimidination de l'ADN, qui est alors fragmenté en présence d'une base par un mécanisme dit de « β-élimination ». La fragmentation de l'ADN peut être réalisé par des mécanismes d'oxydation, d'alkylation, d'addition de radicaux libres entre autres.

Enfin la fragmentation peut être physique, par exemple par sonication ou par voie photochimique.

La difficulté consiste en fait à associer ces deux étapes de fragmentation et de marquage.

La demande de brevet WO-A-99/65926 décrit un procédé de marquage d'un acide ribonucléique (ARN) de synthèse ou naturel qui consiste à fragmenter l'ARN et à marquer au niveau du phosphate terminal. Ce document décrit un certain nombre de fonctions réactives pouvant être utilisées pour le marquage en liaison avec la fragmentation. Ces fonctions permettent de marquer les ARN mais il faut associer une étape de fragmentation pour avoir un marquage efficace car ce marquage se produit sur le phosphate libéré lors de la fragmentation. De plus, il faut ajouter un excès important de réactif de marquage par rapport à l'ARN pour obtenir un marquage efficace, ce qui induit des problèmes de bruit de fond généré par le marqueur en excès. Enfin cette méthode n'est pas applicable à l'ADN double brin.

Il existe donc un besoin pour une technique de marquage de l'ADN simple et efficace qui permette un bon rendement de marquage et donc une bonne sensibilité, qui soit spécifique au niveau de la position de marquage, et en particulier qui n'affecte pas les propriétés d'hybridation des bases impliquées dans la formation de la double hélice par l'intermédiaire des liaisons hydrogènes, et enfin qui permette la fragmentation de l'ADN dans le but d'hybrider ces fragments d'ADN marqués sur des sondes nucléiques, et en particulier sur des sondes nucléiques fixés sur un support solide.

La présente invention décrit un procédé de marquage et de fragmentation d'un acide désoxyribonucléique (ADN) simple ou double brin comprenant les étapes suivantes :
- fragmenter l'ADN en créant au moins un site abasique sur ledit ADN,
- attacher un marqueur sur au moins un des fragments par l'intermédiaire d'un réactif de marquage, ledit réactif se couplant de manière covalente et majoritaire sur au moins un phosphate dudit fragment.

La fragmentation et le marquage s'effectue en une étape ou en deux étapes et le marquage peut s'effectuer indifféremment avant, après ou simultanément avec la fragmentation.

De préférence, la marquage et/ou la fragmentation s'effectue(nt) en solution homogène sensiblement aqueuse. De préférence, le marquage et la fragmentation s'effectuent simultanément, c'est-à-dire que les réactifs nécessaires à ces deux étapes sont mis ensemble dans une solution sensiblement aqueuse avec l'acide nucléique par exemple. C'est notamment le cas pour la fragmentation chimique ou enzymatique. Dans le cas de la fragmentation mécanique par un moyen physique, marquage et fragmentation s'effectuant simultanément signifie que le moyen physique est appliqué à une solution contenant au moins les acides nucléiques et le réactif de marquage.

Par solution sensiblement aqueuse, on entend une solution contenant au moins 50% d'eau. Cette solution contient de préférence des sels comme une solution tampon. Par solution homogène, on entend une solution monophasique telle qu'une solution eau/DMSO, par opposition à une solution biphasique telle qu'une solution eau/chloroforme.

La fragmentation de l'ADN par l'intermédiaire de la création d'un site abasique s'effectue par voie enzymatique, chimique ou physique.

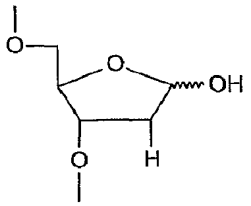
La fragmentation par voie chimique de l'ADN est réalisée en mettant en présence l'acide nucléique avec un moyen chimique de création de site abasique.

Des exemples de conditions de fragmentation chimique par l'intermédiaire d'un site abasique de l'ADN sont donnés dans G. Pratviel et al., Angew. Chem. Int. Ed. Engl., 34, p746-769, 1995; G. Pratviel et al., Adv. Inorg. Chem.,45, p251-312, 1998; D. S. Sigman et al. Chem. Rev., 93, p2295-2316, 1993 ; J. Lhomme et al., Biopolymers (Nucleic Acid Sciences), 52, p65-83, 1999.

Un site abasique est créé quand la liaison N-glycosidique reliant la base modifiée au désoxyribose est hydrolysée, laissant la liaison phosphodiester intacte. Ce phénomène est appelé dépurination (dans le cas des purines) ou dépyrimidination (dans le cas des pyrimidines). La dépurination est beaucoup plus fréquente que la dépyrimidination.

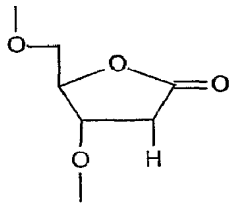
Des agents chimiques, tels que le pH acide, les agents oxydants ou les agents alkylants, peuvent induire le phénomène de dépurination et donc la formation de sites abasiques. Ces agents alkylants sont constitués d'espèces électrophiles qui réagissent avec les sites nucléophiles d'un fragment d'ADN. L'atome d'azote en position 7 de la guanine est le

site principal d'alkylation de l'ADN. En plus de la protonation des purines, l'alkylation est l'une des modifications chimiques susceptibles de rendre la liaison N-glycosidique plus labile.



Site abasique

L'oxydation de l'ADN peut également générer des lésions abasiques dites « alcalilabiles », qui conduisent à la fragmentation de l'ADN en présence d'une base. Par exemple, le résidu ribonolactone peut être généré par réaction du radical hydroxyle (OH) avec le carbone C1'.



Ribonolactone

Le site abasique est très instable. Sous sa forme aldéhydique, il peut subir facilement une β-élimination du phosphodiester fixé en position 3', ce qui conduit à la fragmentation du brin de l'ADN.

La dépurination est spontanée dans des conditions physiologiques (pH 7,4 à 37°C) mais la vitesse de la réaction est très faible de l'ordre de 3.10^{-11} dépurination par seconde, c'est-à-dire inutilisable pour une fragmentation efficace. Pour augmenter la vitesse de réaction, on utilise des agents alkylants qui fragilisent la liaison N-glycosidique ou des enzymes, comme des ADN glycosylases.

Un mode préféré de réalisation de la fragmentation est obtenu par l'utilisation d'un pH acide, c'est-à-dire un pH inférieur à 5, de préférence inférieur à 4. Avantageusement le pH est d'environ 3.

Un tampon formiate de sodium à pH 3 permet de fragmenter de manière efficace les acides nucléiques selon la présente invention. Ce tampon est compatible avec les conditions de marquage en une étape comme cela sera démontré dans les exemples. Encore plus avantageusement, un milieu acide (HCl, carbonate, H_2SO_4) est utilisé.

Dans un mode particulier de la présente invention et dans le but d'augmenter encore la fragmentation, l'acide désoxyribonucléique contient au moins une base modifiée susceptible de générer un site abasique plus facilement.

Diverses bases modifiées sont utilisables comme les N7-alkylpurines, les N3-alkylpurines, les O6-alkylpurines, les 8-bromopurines, les 8-thiopurines, les 8-alkylthiopurines, les 8 azidopurines ou les 8-alkylsulfonylpurines.

Dans le cas où l'acide nucléique à marquer est généré par une technique d'amplification enzymatique comme la PCR, l'utilisation d'une 8-bromopurine permet d'avoir une incorporation efficace dudit nucléotide pendant l'amplification ce qui facilite d'autant le procédé de fragmentation et de marquage selon l'invention, tout en conservant une sensibilité excellente pour l'étape d'amplification enzymatique. Une 8-méthylthiopurine est également incorporée sans gêner l'efficacité d'amplification et d'incorporation par PCR. Une fois incorporée, la base thioéther est oxydée par un peracide ou un dérivé de monopersulfate comme le peroxymonosulfate de potassium par exemple, en sulphonyle correspondant qui est très labile. Il a été démontré que le temps de demi-vie correspondant à l'hydrolyse d'une 8-méthylsulfonylguanosine est inférieur à 2 minutes alors que le temps de demi-vie pour son homologue naturel est de 1000 heures.

Par marqueur, on entend au moins un marqueur capable de générer directement ou indirectement un signal détectable. Une liste non limitative de ces marqueurs suit :

- les enzymes qui produisent un signal détectable par exemple par colorimétrie, fluorescence, luminescence, comme la péroxydase de raifort, la phosphatase alcaline, la β-galactosidase, la glucose-6-phosphate déshydrogénase,

- les chromophores comme les composés fluorescents, luminescents, colorants,
- les groupements à densité électronique détectable par microscopie électronique ou par leur propriété électrique comme la conductivité, l'ampérométrie, la voltamétrie, l'impédance,
- les groupements détectables, par exemple dont les molécules sont de tailles suffisantes pour induire des modifications détectables de leurs caractéristiques physiques et/ou chimiques, cette détection peut être réalisée par des méthodes optiques comme la diffraction, la résonance plasmon de surface, la variation de surface, la variation d'angle de contact ou des méthodes physiques comme la spectroscopie de force atomique, l'effet tunnel, et
- les molécules radioactives comme le ^{32}P, le ^{35}S ou le ^{125}I.

De préférence, le marqueur n'est pas un marqueur radioactif pour éviter les problèmes de sécurité liés à ces marqueurs.

Dans un mode de réalisation particulier de la présente invention, le marqueur est détectable électrochimiquement et en particulier le marqueur est un dérivé d'un complexe de Fer comme un ferrocène.

Des systèmes indirects peuvent être aussi utilisés, comme par exemple des ligands capables de réagir avec un anti-ligand. Les couples ligand/anti-ligand sont bien connus de l'homme du métier, ce qui est le cas par exemple des couples suivants : biotine/streptavidine, haptène/anticorps, antigène/anticorps, peptide/anticorps, sucre/lectine, polynucléotide/complémentaire du polynucléotide. Dans ce cas, c'est le ligand qui porte la fonction réactive. L'anti-ligand peut être détectable directement par les marqueurs décrits au paragraphe précédent ou être lui-même détectable par un autre couple ligand/anti-ligand.

Un autre exemple de systèmes indirects utilise une liaison covalente spécifique entre le ligand et l'anti-ligand, par exemple méthylcétone et alcoxyamine. Des exemples de ce système sont décrits dans les demandes de brevet WO-A-00/40590 et WO-A-98/05766.

Dans le procédé de la présente invention, la liaison entre le réactif de marquage et l'acide nucléique est covalente, mais il est décrit ci-dessus que des interactions non covalentes peuvent être utilisées notamment dans les systèmes d'empilement ou dans le cas où le marqueur est détectable indirectement. Le terme « attacher » couvre donc ces différentes possibilités.

Ces systèmes de détection indirects peuvent conduire, dans certaines conditions, à une amplification du signal et l'on pourra se reporter aux demandes de brevet antérieures WO-A-00/07982, WO-A-01/92361 et WO-A-95/08000 de la demanderesse pour des exemples d'amplification chimique en utilisant des polymères ou à la demande WO-A-01/44506, toujours de la demanderesse, pour les systèmes d'amplification chimique par empilement.

Dans un mode particulier d'amplification de signal, au moins deux marqueurs sont présents sur le réactif de marquage.

Dans un mode préféré de l'invention, le traceur est un composé fluorescent de faible encombrement stérique comme la fluorescéine, le dansyl, les chromophores du type IR (Li-COR Inc, Lincoln NE, USA), les dérivés cyanines comme le Cy5 et Cy3 (Randolph J.B. and al, Nucleic Acids Res., 25(14), p2923-2929, 1997) et en particulier les dérivés du Cy5 ou bien le traceur est un haptène de faible encombrement stérique comme la biotine ou un dérivé de l'abiétane (voir la demande WO-A-00/07982). Par faible encombrement stérique, on entend un poids moléculaire inférieur à 1000 g/mole.

Dans le cas d'un fluorophore, il est préférable de travailler avec des fluorophores dont la longueur d'onde d'excitation est supérieure à 450 nm, de préférence supérieure à 600 nm.

Dans le cas où le traceur est un haptène qui ne produit pas de signal par lui-même, comme par exemple la biotine, la détection est réalisée par la réaction d'un anti-ligand comme décrit plus haut. Dans le cas de la biotine, on utilise de préférence de la streptavidine ou un anticorps anti-biotine couplé à un composé fluorescent comme la fluorescéine, Cy5 ou la Phycoérythrine. Dans le cas de l'abiétane, on utilise un anticorps monoclonal comme décrit dans WO-A-00/07982.

Le terme « acide désoxyribonucléique» ou ADN signifie un enchaînement d'au moins deux désoxyribonucléotides comprenant éventuellement au moins un nucléotide modifié, par exemple au moins un nucléotide comportant une base modifiée tel que l'inosine, la méthyl-5-désoxycytidine, la diméthylamino-5-désoxyuridine, la désoxyuridine, la diamino-2,6-purine, la bromo-5-désoxyuridine ou toute autre base modifiée permettant l'hybridation.

Cet ADN peut aussi être modifié au niveau de la liaison internucléotidique comme par exemple les phosphorothioates, les H-phosphonates, les alkyl-phosphonates, au niveau du squelette comme par exemple les alpha-oligonucléotides (FR-A-2.607.507) ou les PNA (M. Egholm et al., J. Am. Chem. Soc., 114, 1895-1897, 1992). L'ADN est naturel ou synthétique, et/ ou sous la forme de fragment. L'ADN est simple ou double brin.

En particulier l'ADN est obtenu par une technique d'amplification enzymatique telle que :

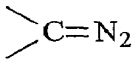
- la PCR (Polymerase Chain Reaction), décrite dans les brevets US-A-4,683,195, US-A-4,683,202 et US-A-4,800,159, et sa dérivée RT-PCR (Reverse Transcription-PCR), notamment dans un format en une étape tel que décrit dans la demande de brevet EP-A-0.569.272,
- la LCR (Ligase Chain Reaction), exposée par exemple dans la demande de brevet EP-A-0.201.184,
- la RCR (Repair Chain Reaction), décrite dans la demande de brevet WO-A-90/01069.

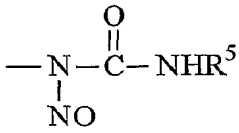
On parle alors d'amplicons pour désigner l'ADN généré par une technique d'amplification enzymatique. L'ADN peut aussi comprendre des ribonucléotides en faible proportion, par exemple inférieur à 10%.

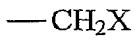
Chacune de ces modifications peut être prise en combinaison pour peu qu'au moins un phosphate soit présent dans l'ADN.

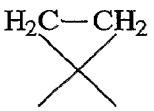
Le réactif de marquage comprend, comme fonction réactive, un motif choisi parmi les composés suivants : diazométhyle ; halogénure d'alkyle ; nitrosourée ; spirocyclopropane ; aziridine ; époxyde ; trifluorosulfonates.

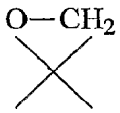
Les fonctions réactives sont décrites ci-dessous :

diazométhyle 

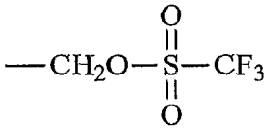
nitrosourée 

halogénure d'alkyle 

spirocyclopropane 

époxyde 

aziridine 

trifluorosulfonate 

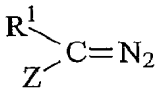
Pour la fonction réactive halogénure d'alkyle, X signifie Br, Cl ou I. Avantageusement le réactif de marquage est la 5-(bromométhyl) fluorescéine.

Pour la fonction réactive nitrosourée, R^5 est un alkyle ou H.

La fonction diazométhyle a déjà été utilisée pour l'alkylation des groupements phosphates, mais un certain nombre de problèmes se pose. D'une part, les dérivés diazo en général sont instables eux-mêmes, ce qui génère des problèmes pour l'utilisation de ces réactifs de marquage dans un kit de marquage, et d'autre part, le produit de couplage est instable ce qui est rédhibitoire si le produit marqué a pour fonction de mettre en évidence la présence d'une molécule cible biologique dans un échantillon quelconque, ou si c'est la cible marquée que l'on souhaite détecter.

Enfin les dérivés portant la fonction diazométhyle sont insolubles dans l'eau ce qui conduit à utiliser des conditions biphasiques pour le couplage avec des acides nucléiques, qui ne sont solubles et stables que dans l'eau ou des tampons aqueux, mais ces conditions ralentissent la vitesse de réaction et donc nuisent à l'efficacité du couplage.

Dans un mode préféré du procédé selon l'invention, le réactif de marquage est choisi parmi les composés de formule (1) :

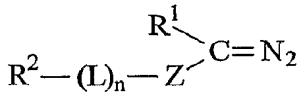


dans laquelle :

- R^1 représente H ou un groupe alkyle, alkyle substitué, aryle ou aryle substitué,
- Z comprend un marqueur détectable.

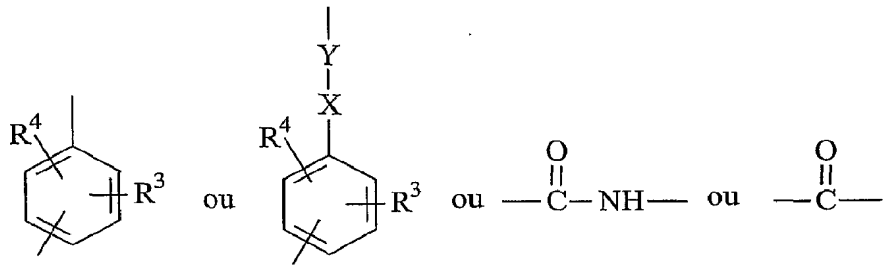
Z et/ou R^1 sont choisis pour stabiliser la fonction diazométhyle, c'est-à-dire au moins un des deux groupes Z ou R^1 a un noyau phényle.

De préférence, le réactif de marquage est choisi parmi les composés de formule (2) :



dans laquelle :

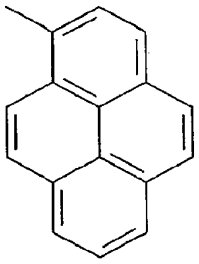
- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 est un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n est égal à 0 ou 1,et
- Z est choisi parmi :



dans lequel :

- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle,
- -Y-X- représente -CONH-, -NHCO-, $-CH_2O-$, $-CH_2S-$.

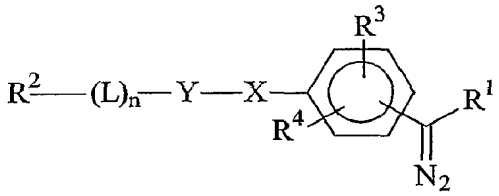
Dans un mode particulier selon la formule (1), Z a la structure suivante :



Dans ce cas et si R^1 est égal à H, le réactif de marquage est le 1-Pyrényldiazométhane (PDAM).

Bien que ce marqueur soit fluorescent, la longueur d'onde d'excitation est trop proche de celles des acides nucléiques. Un détection indirecte par l'utilisation d'un anticorps monoclonal dirigé contre le motif pyrène est préférée. Le mode d'obtention de cet anticorps est bien connu de l'homme du métier (voir par exemple la demande de brevet WO-A-00/07982).

Dans un mode préféré de réalisation du procédé, le réactif de marquage est de formule (3) :

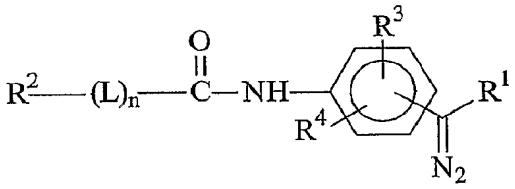


dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,
- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle,
- -Y-X- représente -CONH-, -NHCO-, $-CH_2O-$, $-CH_2S-$.

Avantageusement le réactif est de formule (4) :

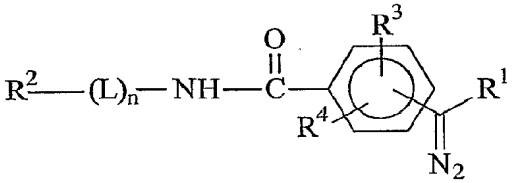
IDT Exhibit 1020.7391



dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,
- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle.

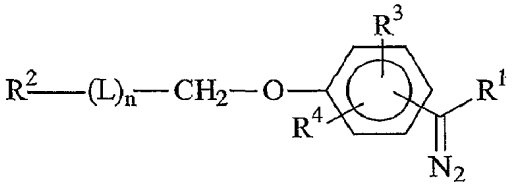
Avantageusement le réactif est de formule (5) :



dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,
- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle.

Avantageusement le réactif est de formule (6) :

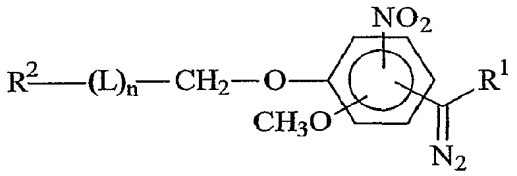


dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,
- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle.

Dans les formules ci-dessus (3) à (6), avantageusement R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, OCH_3.

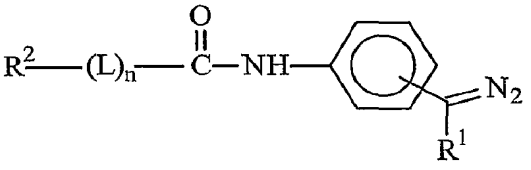
Ainsi, un composé préféré selon la formule (6) est de formule (6') :



dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1.

Un composé préféré selon la formule (4) est de formule (4') :



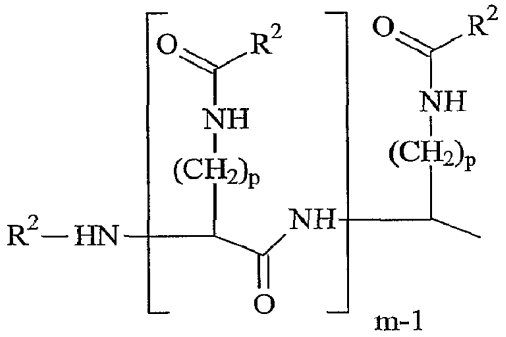
dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,

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- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1.

Dans un mode particulier du procédé où l'on veut amplifier le signal, au moins deux marqueurs sont présents sur le réactif de marquage. En particulier un réactif qui permet de mettre en oeuvre l'amplification de signal selon la présente invention possède une structure R^2-$(L)_n$- de formule (7) ci dessous :



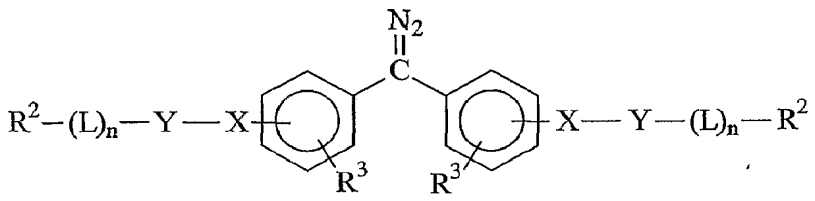
m-1

dans laquelle :

- R^2 représente un marqueur détectable,
- m est un nombre entier compris entre 1 et 100, de préférence compris entre 1 et 20,
- p est un nombre entier compris entre 1 et 10, avantageusement 2 à 6, de préférence 4.

Cette structure de R^2-$(L)_n$ s'applique indifféremment aux formules (2) à (6) précédentes.

Un autre réactif de marquage préféré pour l'amplification de signal est le réactif de formule (8) :

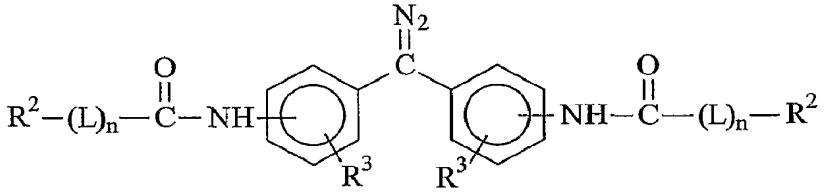


dans laquelle :

- R^2 représente un marqueur détectable,

- R^3 représente H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,
- -Y-X- représente -CONH-, -NHCO-, $-CH_2O-$, $-CH_2S-$.

Avantageusement le réactif pour l'amplification de signal a la formule (9)

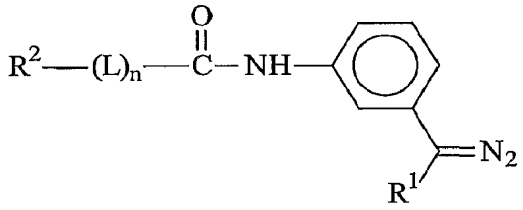


dans laquelle :

- R^2 représente un marqueur détectable,
- R^3 représente H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle, de préférence R^3 représente H, NO_2 ou OCH_3.,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1.

Certains réactifs avantageux de l'invention sont :

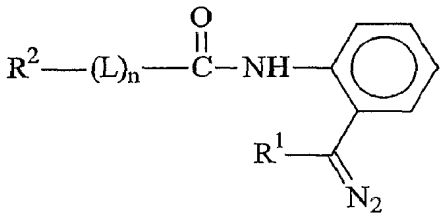
a) de formule (10) :



dans laquelle :

- R^1 représente H ou un groupe alkyle ou aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,

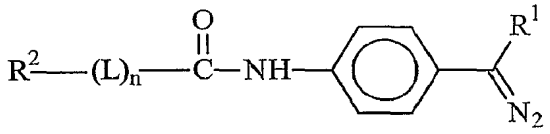
b) de formule (11) :



dans laquelle :

- R^1 représente H ou un groupe alkyle ou aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1,

c) de formule (12) :

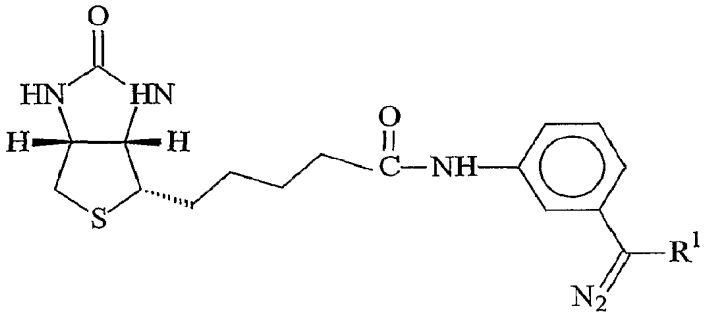


dans laquelle :

- R^1 représente H ou un groupe alkyle ou aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1.

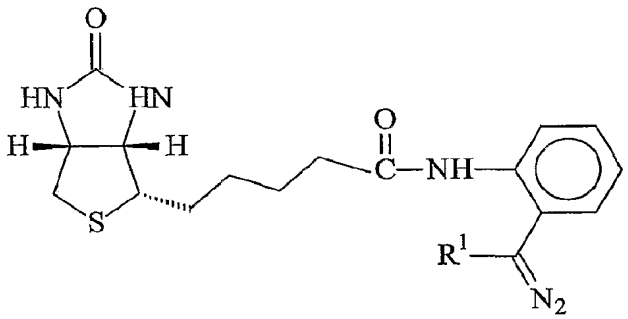
De préférence, le réactif de marquage a :

a) la structure



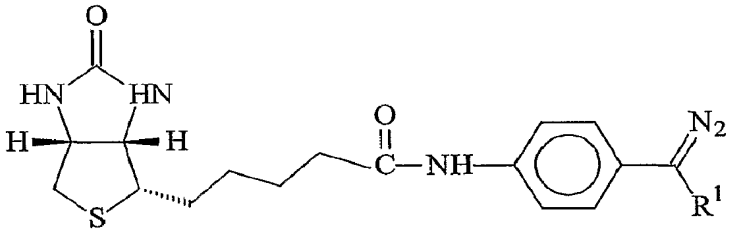
dans laquelle R^1 représente un groupe méthyle ou un phényle, ou

b) la structure :



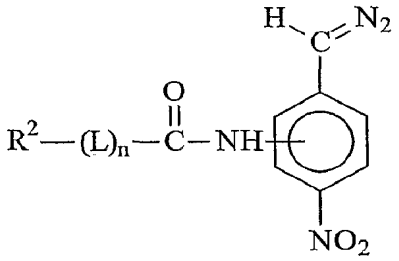
dans laquelle R^1 représente un groupe méthyle ou phényle, ou

c) la structure :



dans laquelle R^1 représente un groupe méthyle ou phényle.

D'autres réactifs préférés selon l'invention ont la formule (13) :



dans laquelle :

- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n un nombre entier égal à 0 ou 1.

En particulier les réactifs de marquage selon le procédé de l'invention sont solubles dans des solvants polaires miscibles à l'eau comme le DMF, le DMSO, CH_3CN, THF, DMA (diméthylacétamide), NMP (N-méthylpyrrolidone), DME (diméthoxyéthane).

De préférence, les réactifs de marquage sont solubles dans le DMSO ou l'eau.

Par solvant miscible à l'eau, on entend un solvant qui est miscible dans une proportion d'au moins 5% en volume avec de l'eau ou un tampon aqueux contenant des sels.

Avantageusement dans les formules précédentes, le bras L comprend un motif éthylène glycol ou polyéthylène glycol pour augmenter la solubilité du réactif dans l'eau.

Ces réactifs peuvent ainsi se fixer en phase homogène sur les acides nucléiques, la phase homogène étant constituée d'une solution sensiblement aqueuse, c'est-à-dire contenant au moins 50% d'eau.

Un objet de la présente invention est de décrire un procédé de détection d'un acide désoxyribonucléique cible (ADN) simple ou double brin comportant les étapes suivantes :

a) fragmenter et marquer lesdits ADN selon l'un quelconque des procédés décrits précédemment,

b) hybrider les fragments marqués sur au moins une sonde nucléique suffisamment spécifique de l'acide nucléique cible,

c) détecter l'hybride formé par l'intermédiaire du marqueur.

Comme cela sera démontré dans les exemples, la dénaturation de l'ADN avant hybridation permet d'augmenter la sensibilité. Cette étape de dénaturation a lieu après la fragmentation et le marquage.

Dans un mode particulier du procédé, une étape d'amplification enzymatique a lieu avant la fragmentation et le marquage. L'étape d'amplification enzymatique génère des amplicons ADN à partir d'un acide nucléique cible ADN, mais aussi à partir d'un acide nucléique cible ARN comme une ARN messager, un ARN de transfert ou un ARN ribosomal. La technique de RT-PCR par exemple est un moyen connu pour amplifier l'ARN.

De préférence, les étapes de fragmentation, marquage et dénaturation ont lieu en même temps, aussi bien dans le cas d'un acide nucléique cible naturel que dans le cas d'un acide nucléique cible obtenu par une technique d'amplification enzymatique.

La fragmentation par création d'un site abasique implique la perte d'une base. Dans les procédés de détection d'acide nucléique cible et en particulier dans les procédés de génotypage c'est-à-dire dans les procédés où l'acide nucléique cible présente un

polymorphisme réparti sur sa séquence, il est nécessaire d'identifier une pluralité de modifications de séquences dont certaines représentent la modification d'une seule base entre la cible et les sondes nucléiques dont la fonction est d'identifier cette modification (sondes de capture). La spécificité est donc essentielle dans ce contexte. De manière surprenante, la présente invention démontre que cette perte d'une base n'affecte en rien la spécificité de l'hybridation pour un acide nucléique marqué et fragmenté selon l'invention.

La présente invention a aussi pour objet un procédé de mise en évidence d'un polymorphisme, réparti en des positions prédéterminées ou non d'un acide nucléique cible par la présence d'une pluralité de délétions et/ou insertions et/ou mutations dans la séquence dudit acide nucléique cible par rapport à une séquence, dite de référence, comportant les étapes suivantes :

a) disposer d'un ADN cible comportant l'ensemble du polymorphisme à étudier, ledit ADN étant éventuellement généré par une technique d'amplification enzymatique,
b) fragmenter et marquer ledit ADN par un des procédés décrits précédemment,
c) hybrider lesdits fragments sur une pluralité de sondes nucléiques dites sondes de capture, la pluralité de sondes de capture étant fixé sur un support solide et la pluralité de sondes de capture couvrant dans son ensemble au moins le polymorphisme à étudier,
d) détecter les hybrides formés entre les fragments marqués et au moins une partie des sondes nucléiques par l'intermédiaire du marqueur et en déduire le polymorphisme de l'ADN cible.

Comme indiqué précédemment une étape de dénaturation après l'étape de fragmentation et marquage permet d'améliorer la sensibilité du procédé, et cette étape est réalisée de préférence simultanément avec l'étape de marquage et de fragmentation.

Le procédé de fragmentation et de marquage selon l'invention est particulièrement utile dans le cas où l'ADN marqué et fragmenté doit s'hybrider avec une multitude d'acides nucléiques, notamment oligonucléotides, fixés sur le support solide à une position prédéterminée pour former une puce à ADN. Par "puce à ADN", on entend un support solide de dimension réduite où sont fixés une multitude de sondes de capture à des positions

prédéterminées. Par multitude, on entend un support solide comportant au moins dix (10) sondes nucléiques de séquences différentes, avantageusement au moins quatre cents (400), de préférence au moins mille (1000).

En effet, la densité des acides nucléiques fixés sur le support solide impose des contraintes stériques importantes lors de l'hybridation et la fragmentation permet d'améliorer cette étape d'hybridation. Des exemples de ces puces à ADN sont donnés par exemple dans les publications de G. Ramsay, Nature Biotechnology, 16, p40-44, 1998; F. Ginot, Human Mutation, 10, p1-10, 1997 ; J. Cheng et al, Molecular diagnosis, 1(3), p183-200, 1996 ; T. Livache et al, Nucleic Acids Research, 22(15), p2915-2921, 1994 ; J. Cheng et al, Nature Biotechnology, 16, p541-546, 1998. Le terme "support solide" tel qu'utilisé ici inclut tous les matériaux sur lesquels peut être fixé un acide nucléique. Le support solide peut être sous la forme d'une plaque de microtitration, d'une membrane, d'une particule ou d'une plaque sensiblement plane.

L'invention concerne l'utilisation d'un ADN marqué, tel que défini ci-dessus, comme sonde de détection d'une acide nucléique cible.

Pour permettre la détection et/ou la quantification et/ou la purification de l'acide nucléique cible, l'ADN marqué est capable de former un complexe d'hybridation avec une sonde nucléique. A titre d'exemple, l'acide nucléique marqué est suffisamment complémentaire de la cible pour s'hybrider spécifiquement en fonction des conditions de réaction et notamment de la température ou de la salinité du milieu réactionnel.

Le procédé de détection est applicable pour le séquençage, le profil d'expression des ARN messagers ou le criblage de mutations à des fins de recherche ainsi que le criblage de drogues dans l'industrie pharmaceutique, le diagnostic de maladies infectieuses (aussi bien en bactériologie, virologie, parasitologie) ou génétiques, le contrôle alimentaire ou industriel.

De nombreuses publications décrivent ce type d'applications : Antoine de Saizieu et al, Nature Biotechnology, 16, p44-48, 1998 ;Thomas R. Gingeras et al Genome Research, 8, p435-448, 1998 ; David J. Lockhart et al, Nature Biotechnology, 14, p1675-1680, 1996 ; Daniel D. Shoemaker et al, Nature Genetics, vol 14, p450-456, 1996 ; R.J. Lipshutz et al;

BioTechniques, 19(3), p442-447, 1995; David G. Wang et al, Science, 280, p1077-1082, 1998 ; Kevin L. Gunderson et al; Genome Research, 8, p1142-1152, 1998 ; Joseph G. Hacia et al; Nature Genetics, vol 14, p441-447, 1996.

La tendance en matière de diagnostic et notamment pour les maladies infectieuses (SIDA ou Tuberculose par exemple) est de baisser le niveau de sensibilité jusqu'à la détection d'une molécule unique dans un échantillon, qui peut représenter plusieurs millilitres dans le cas d'un prélèvement liquide type sang ou urine ou liquide céphalo-rachidien. Ce niveau de sensibilité ne peut être obtenu que si toutes les étapes depuis le prélèvement de l'échantillon jusqu'au rendu de résultat sont optimisées. En particulier dans le cas où une étape d'amplification enzymatique est nécessaire pour obtenir la sensibilité nécessaire (infection virale ou bactérienne comme VIH, VHC ou Tuberculose), un procédé de marquage et/ou fragmentation comme décrit dans la présente invention permet de ne pas affecter la sensibilité de la technique d'amplification, soit parce qu'il n'est pas nécessaire de remplacer les désoxyribonucléotides utilisés dans la technique d'amplification enzymatique, soit parce ce que les désoxyribonucléotides incorporés n'altèrent pas la sensibilité.

Des informations complémentaires peuvent être trouvées dans une autre demande de brevet de la Demanderesse, déposée le 4 mai 2001 sous le numéro d'enregistrement FR01/06040, ainsi que de son extension internationale déposée le même jour que la présente invention.

Les figures et exemples ci-joints représentent des modes particuliers de réalisation et ne peuvent pas être considérés comme limitant la portée de la présente invention.

La figure 1 représente les formules développées de différents réactifs utilisés dans la présente invention ainsi que l'abréviation les désignant (o- signifie *ortho*, m- *méta* et p- *para*).

Les figures 2A à 2I représentent les profils, en fonction du temps, analysés par électrophorèse capillaire du couplage covalent de différents réactifs portant une fonction

diazométhyle sur l'uridine 3' monophosphate (3'-UMP) selon l'exemple 6.1. Les molécules sont les suivantes :

- PDAM en figure 2A,
- DPDAM à 2 mM (millimole(s) par litre) en figure 2B,
- DPDAM à 20 mM en figure 2C,
- PMDAM en figure 2D,
- NPDAM en figure 2E,
- BioDPDAM en figure 2F,
- *méta*-BioPMDAM en figure 2G,
- *para*-BioPMDAM en figure 2H, et
- *ortho*-BioPMDAM en figure 2I.

Les figure 3A à 3D représentent les profils en fonction du temps analysée par électrophorèse capillaire de la réaction du *méta*-BioPMDAM sur quatre (4) nucléotides 3'-monophosphate selon l'exemple 6.2. Les molécules sont les suivantes :

- 3'-CMP en série ribonucléotide selon la figure 3A,
- 3'-AMP en série ribonucléotide selon la figure 3B,
- 3'-GMP en série ribonucléotide selon la figure 3C, et
- 3'-TMP en série désoxyribonucléotide selon la figure 3D.

La figure 4 représente les profils en fonction du temps analysée par électrophorèse capillaire de la réaction du *méta*-BioPMDAM sur un dinucléotide 5'-ApUp selon l'exemple 6.3.

Les figures 5A à 5D représentent le spectre RMN du proton dans D_2O des différents conjugués entre le réactif *méta*-BioPMDAM et quatre (4) ribonucléotides 3'-monophosphate selon l'exemple 6.4. Les molécules sont les suivantes :

- 3'-GMP en figure 5A,
- 3'-AMP en figure 5B,
- 3' CMP en figure 5C, et

- 3' UMP en figure 5D.

La figure 6 représente un schéma de synthèse d'un réactif de marquage portant deux biotines pour l'amplification chimique du signal.

La figure 7 représente le mécanisme de fragmentation en milieu acide par la formation de site abasique.

La figure 8 montre, selon l'exemple 8.1, la cinétique de dégradation à pH acide pour différents nucléosides modifiés (8-bromo-2'-désoxyadénosine (8-BrdA) et la 5-bromo-2'-désoxycytidine (5-BrdC)) ainsi que les quatre nucléosides naturels (dA, dC, dG et dT). Les résultats sont représentés sous forme de pourcentage d'hydrolyse du nucléoside de départ (en ordonnée) par rapport au temps de réaction en minutes (en abscisse).

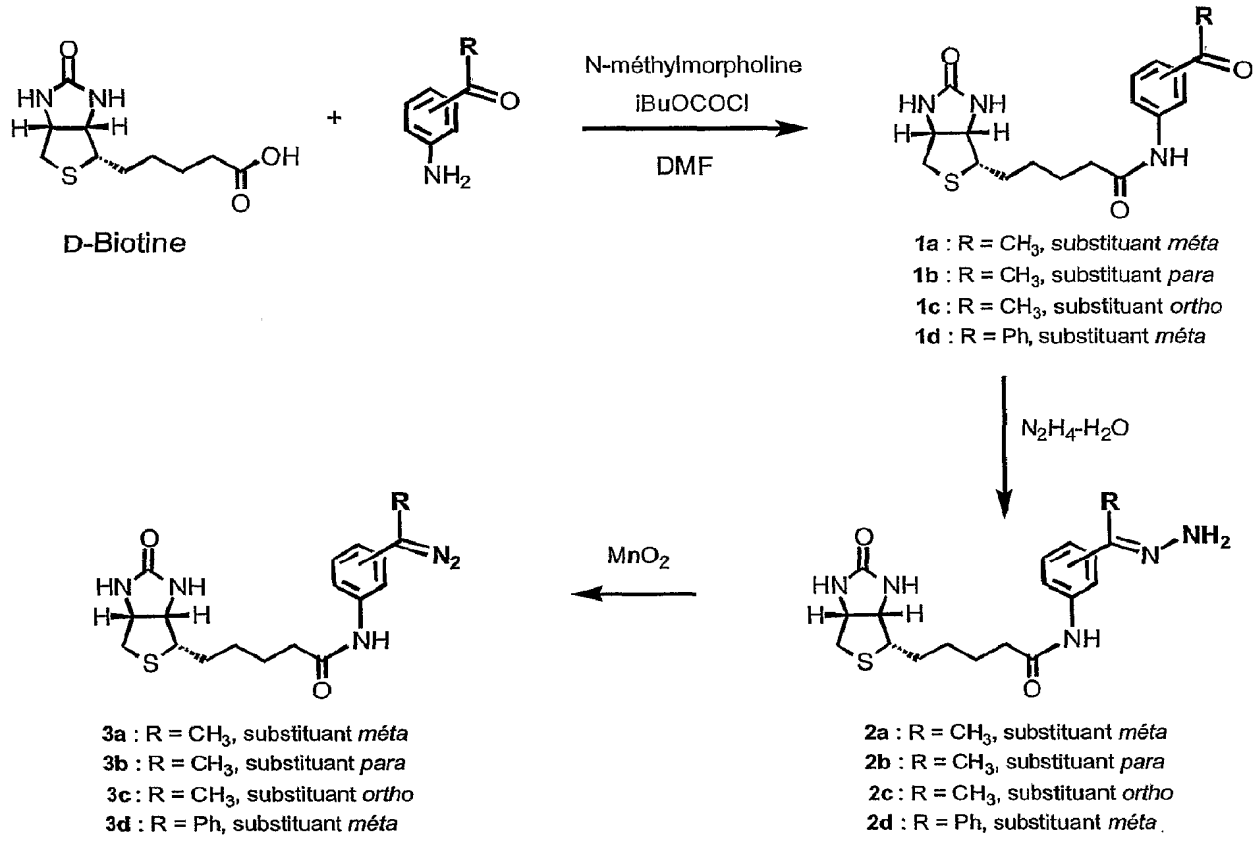
La figure 9 représente, selon l'exemple 11.2, la cinétique de marquage en fonction du temps à une température de 60°C avec le réactif PDAM sur un ODN synthétique 5'-phosphate. Les résultats sont représentés sous forme de pourcentage de marquage par rapport au temps de réaction exprimé en minutes (en abscisse).

La figure 10 représente, selon l'exemple 11.3, le pourcentage de marquage en fonction de la température de réaction. Les résultats sont présentés sur la figure 10 avec en ordonnée le pourcentage de marquage et en abscisse la température de réaction en °C.

La figure 11 représente une voie de synthèse pour un réactif selon la formule 4', en utilisant le réactif commercial 5-Nitrovanilline. La fonction aldéhyde est le précurseur de la fonction diazométhyle.

Exemple 1 : Synthèse de réactifs avec la biotine :

Schéma général de synthèse :



Exemple 1.1 : Synthèse du *méta*-BioPMDAM :

• Composé biotine *méta*-acétophénone **1a** :

On solubilise la D-biotine (1,0 gramme (g), 4,1 millimoles (mmol)) dans 45 millilitres (mL) de DMF anhydre à chaud. On refroidit à 0°C sous argon, puis on ajoute successivement la *N*-méthylmorpholine (590 microlitres (µL), 5,33 mmol) et le chloroformiate d'isobutyle (840 µL, 6,60 mmol). On laisse sous agitation pendant 30 minutes (mn), puis on ajoute la 3-aminoacétophénone (824 mg, 6,10 mmol) et la *N*-méthylmorpholine (480 µL, 4,35 mmol) dans 10 mL de DMF. La solution est maintenue sous agitation à 0°C pendant 2 heures (h), puis on évapore à sec. On reprend le résidu dans 3 mL de MeOH, puis on ajoute 50 mL d'eau. Le précipité obtenu est filtré, lavé avec de l'eau, du CH_2Cl_2 et de l'éther pour donner 1,2 g (80 %) de produit **1a** brut. Une recristallisation dans le couple MeOH-H_2O donne **1a** (1,01 g, 70 %) sous forme d'une poudre blanche.

F 145°C. - IR (KBr) : 3280, 2931, 2857, 1691, 1590,1540, 1487, 1434, 1298, 1266 cm^{-1}. - RMN ^1H (300 MHz, DMSO-d_6) δ = 1,3-1,7 (m, 6 H) ; 2,33 (t, J = 8 Hz, 2 H) ; 2,55 (s, 3 H) ; 2,58 ; (d, J = 12 Hz, 1 H) ; 2,83 (dd, J = 12 et 5 Hz, 1 H) ; 3,13 (m, 1 H) ; 4,15 (m, 1 H) ; 4,31 (m, 1 H) ; 6,34 (s, 1 H) ; 6,41 (s, 1 H) ; 7,44 (t, J = 8 Hz, 1 H) ; 7,64 (d, J = 8 Hz, 1 H) ; 7,85 (d, J = 8 Hz, 1 H) ; 8,17 (s, 1 H) ; 10,05 (s, 1 H). - MS (FAB/glycérol), *m/z*: 362 $[M+H]^+$.

• Composé *méta*-hydrazone **2a :**

Une solution de **1a** (500 mg, 1,38 mmol) et d'hydrazine monohydrate (200 µL, 4,15 mmol) dans de l'éthanol absolu (8 mL) est chauffée à reflux pendant 2 h. Après refroidissement à température ambiante, le précipité blanc est filtré, lavé avec de l'eau, puis avec de l'éther et séché. On obtient ainsi 385 mg (74 %) de produit **2a** sous forme d'une poudre blanche.

F 185°C. - IR (KBr) : 3298, 2931, 2857, 1698, 1665, 1626, 1541, 1494, 1470, 1446, 1330, 1265 cm^{-1}. - RMN ^1H (300 MHz, DMSO-d_6) δ = 1,3-1,7 (m, 6 H) ; 1,98 (s, 3 H) ; 2,26 (t, J = 8 Hz, 2 H) ; 2,56 ; (d, J = 12 Hz, 1 H) ; 2,81 (dd, J = 12 et 5 Hz, 1 H) ; 3,11 (m, 1 H) ; 4,13 (m, 1 H) ; 4,29 (m, 1 H) ; 6,39 (s, 3 H) ; 6,42 (s, 1 H) ; 7,22 (m, 2 H) ; 7,50 (d, J = 8 Hz, 1 H) ; 7,84 (s, 1 H) ; 9,82 (s, 1 H). - MS (FAB/glycérol), *m/z*: 376 $[M+H]^+$.

• Composé *méta*-diazométhane **3a** :

On solubilise **2a** (180 mg, 0,48 mmol) dans 2 mL de DMF. On ajoute alors MnO_2 (340 mg, 3,9 mmol). Après 30 minutes d'agitation à température ambiante, le mélange est filtré à travers un entonnoir fritté contenant de la célite (épaisseur : 0,5 cm) et des tamis moléculaires en poudre 3 Å (0,5cm). Le mélange réactionnel est concentré jusqu'à un volume d'environ 0,5 mL, puis 5 mL d'éther sont ajoutés. Le précipité résultant est filtré, lavé à l'éther puis séché. Le composé **3a** (170 mg, 95 %) est obtenu sous forme d'une poudre rose.

F 160°C. - IR (KBr) : 3278, 2935, 2859, 2038, 1704, 1666, 1605, 1577, 1536, 1458, 1430, 1263 cm^{-1}. - RMN ^1H (300 MHz) δ = 1,3-1,7 (m, 6 H) ; 2,11 (s, 3 H); 2,28 (t, J = 8 Hz, 2 H); 2,57 ; (d, J = 12 Hz, 1 H) ; 2,81 (dd, J = 12 et 5 Hz, 1 H) ; 3,11 (m, 1 H) ; 4,13 (m, 1 H) ; 4,29 (m, 1 H) ; 6,33 (s, 1 H) ; 6,41 (s, 1 H) ; 6,60 (m, 1 H) ; 7,25 (m, 3 H) ; 9,84 (s, 1 H).).

Exemple 1.2 : Synthèse du *para*-BioPMDAM :

• Composé biotine *para*-acétophénone **1b** :

On solubilise la D-biotine (1 g, 4,1 mmol) dans 45 mL de DMF anhydre à chaud. On refroidit à 0°C sous argon puis on ajoute successivement la *N*-méthylmorpholine (590 µL, 5,33 mmol) et chloroformiate d'isobutyle (840 µL, 6,60 mmol). On laisse sous agitation pendant 30 min, puis on ajoute la 4-aminoacétophénone (824 mg, 6,10 mmol). La solution est maintenue sous agitation à 0°C pendant 2 h puis on évapore à sec. On reprend le résidu dans 50 mL d'eau. Le précipité obtenu est filtré, lavé avec de l'eau puis avec 50mL de MeOH à chaud. Le précipité blanc est dissous dans du DMF en chauffant puis la solution obtenue est filtrée et lavée au MeOH. Le filtrat est récupéré et évaporé à sec pour donner 888 mg de **1b** (2,46 mmol, 60 %).

F 260°C. - IR (KBr) : 3260, 2930, 2358, 1706, 1673, 1610, 1526, 1401, 1380, 1322, 1257, 1150 cm^{-1}. - RMN ^1H (200 MHz, DMSO-d_6) δ = 8,82 (s, 1H, NH-CO) ; 7,57 (d, 2H, J = 9 Hz, Ar-H) ; 6,83 (d, 2H, J = 9 Hz, Ar-H) ; 6,40 (s large, 1H, NH-CO-NH) ; 6,32 (s large, 1H, NH-CO-NH) ; 4,28 (m, 1H, CH_2-CH-NH) ; 4,12 (m, 1H, CH-CH-NH) ; 3,11 (m, 1H, CH-S) ; 2,80 et 2,55 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,35 (t, 2H, J = 8 Hz, CH_2-CO) ; 2,10 (s, 3H, CH_3) ; 1,60-1,34 (m, 6H, $(CH_2)_3$).

• Composé *para*-hydrazone **2b** :

Le composé **1b** (870 mg, 2,4 mmol) est dissous à chaud dans l'éthanol (99 %, 8 mL) puis l'hydrazine monohydrate (995 µL, 19,5 mmol) est ajoutée. La solution est chauffée à reflux pendant 3 h. Le précipité blanc obtenu est filtré, lavé avec de l'eau glacée. On obtient ainsi 820 mg (90 %) de produit **2b** sous forme d'une poudre blanche.

F 305°C. – IR (KBr) : 3281, 3183, 2930, 2857, 1698, 1658, 1593, 1521, 1459, 1401, 1325, 1263, 1187 cm^{-1}. - RMN ^1H (200 MHz, DMSO-d_6) δ = 9,68 (s, 1H, NH-CO) ; 7,52 (s, 4H, J = 9 Hz, Ar-H) ; 6,43 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 6,21 (s, 2H, NH_2) 4,29 (m, 1H, CH_2-CH-NH) ; 4,12 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,81 et 2,56 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,32 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,97 (s, 3H, CH_3) ; 1,63-1,36 (m, 6H, $(CH_2)_3$).

• Composé *para*-diazométhane **3b** :

On solubilise **2b** (200 mg, 0,53 mmol) dans 10 mL de DMF. On ajoute alors 800 mg de MnO_2. Après 10 minutes d'agitation, le mélange est filtré à travers une couche mixte Célite (0,5 cm) - tamis moléculaire (0,5 cm en poudre). Le mélange réactionnel est évaporé à sec puis lavé à l'éther et séché. Le composé **3b** (190 mg, 96 %) est obtenu sous forme d'une poudre rose.

F 180°C (dec). - IR (KBr) : 3257, 2930, 2857, 2032, 1698, 1597, 1524, 1510, 1455, 1404, 1307, 1259, 1180 cm^{-1}. - RMN ^1H (200 MHz, DMSO-d_6) δ = 10,18 (s, 1H, NH-CO) ; 7,88 (d, 2H, J = 6 Hz, Ar-H) ; 7,7 (d, 2H, J = 6 Hz, Ar-H) ; 6,41 (s large, 1H, NH-CO-NH) ; 6,34 (s large, 1H, NH-CO-NH) ; 4,28 (m, 1H, CH_2-CH-NH) ; 4,12 (m, 1H, CH-CH-NH) ; 3,11 (m, 1H, CH-S) ; 2,80 et 2,55 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,35 (t, 2H, J = 8 Hz, CH_2-CO) ; 2,10 (s, 3H, CH_3) ; 1,60-1,34 (m, 6H, $(CH_2)_3$).

Exemple 1.3 : Synthèse de l'*ortho*-BioPMDAM :

• Composé biotine *ortho*-acétophénone **1c** :

On solubilise la D-biotine (1 g, 4,1 mmol) dans 45 mL de DMF anhydre à chaud. On refroidit à 0°C sous argon puis on ajoute successivement la *N*-méthylmorpholine (590 µL, 5,33 mmol) et chloroformiate d'isobutyle (840 µL, 6,60 mmol). On laisse sous agitation pendant 30 min, puis on ajoute la 2-aminoacétophénone (824 mg, 6,10 mmol). La solution est maintenue sous

agitation à température ambiante pendant 3 h 30 puis on évapore à sec. On reprend le résidu dans 50 mL d'eau. Le précipité obtenu est filtré, lavé avec de l'eau puis avec 50mL de MeOH à chaud. Le précipité obtenu est filtré, lavé avec de l'eau. Une recristallisation est faite en dissolvant le produit dans du MeOH à chaud et en reprécipitant par addition d'eau. Le précipité est filtré, lavé à l'eau, puis à l'éther pour donner 1,1 g (2,95 mmol, 72 %) de produit **1c** brut.

F 150°C. - IR (KBr) : 3248, 2930, 2857, 2359, 1691, 1669, 1651, 1582, 1528, 1448, 1354, 1310, 1245, 1161 cm^{-1}. - RMN ^1H (200 MHz, DMSO-d_6) δ = 11,24 (s, 1H, NH-CO) ; 8,33 (d, 1H, J = 8,5 Hz, Ar-H) ; 7,97 (d, 2H, J = 8 Hz, Ar-H) ; 7,57 (t, 1H, J = 7 Hz, Ar-H) ; 7,18 (t, 1H, J = 7 Hz, Ar-H) ; 6,44 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 4,30 (m, 1H, CH_2-CH-NH) ; 4,14 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,80 et 2,55 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,61 (s, 3H, CH_3) ; 2,37 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,62-1,38 (m, 6H, $(CH_2)_3$).

• Composé *ortho*-hydrazone **2c** :

Le composé **1c** (500 mg, 1,38 mmol) est dissous à chaud dans l'éthanol (99 %, 8 mL) puis l'hydrazine monohydrate (572 µL, 11,1 mmol) est ajoutée. La solution est chauffée à reflux pendant 50 minutes. La solution est évaporée à sec. Le précipité blanc obtenu est filtré, lavé avec de l'eau puis séché à l'éther. On obtient ainsi 416 mg (11,1 mmol, 80 %) de produit **2c** sous forme d'une poudre blanche.

F 161°C. – IR (KBr) : 3412, 3240, 2930, 2857, 2351, 1706, 1677, 1604, 1590, 1531, 1463, 1444, 1372, 1303, 1270, 1169 cm^{-1}.- RMN ^1H (200 MHz, DMSO-d_6) δ =11,97 (s, 1H, NH-CO) ; 8,35 (d, 1H, J = 8 Hz, Ar-H) ; 7,45 (d, 1H, J = 7 Hz, Ar-H) ; 7,19 (t, 1H, J = 7,5 Hz, Ar-H) ; 7,04 (t, 1H, J = 7 Hz, Ar-H) ; 6,61 (s, 2H, NH_2) ; 6,42 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 4,32 (m, 1H, CH_2-CH-NH) ; 4,14 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,81 et 2,56 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,31 (t, 2H, J = 8 Hz, CH_2-CO) ; 2,09 (s, 3H, CH_3) ; 1,63-1,36 (m, 6H, $(CH_2)_3$).

• Composé *ortho*-diazométhane **3c** :

On solubilise **2c** (200 mg, 0,53 mmol) dans 10 mL de DMF. On ajoute alors 800 mg de MnO_2. Après 15 minutes d'agitation, le mélange est filtré à travers une couche mixte Célite(0,5cm)-tamis moléculaire(0,5cm en poudre). Le mélange réactionnel est évaporé à sec puis lavé à l'éther et séché. Le composé **3c** (130 mg, 65 %) est obtenu sous forme d'une poudre rose.

F 110°C. - IR (KBr) : 3248, 2930, 2857, 2367, 2342, 2038, 1699, 1521,1456 cm^{-1}. - RMN ^1H (200 MHz, DMSO-d_6) δ = 9,37 (s, 1H, NH-CO) ; 7,26-7,00 (m, 4H, Ar-H) ; 6,43 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 4,30 (m, 1H, CH_2-CH-NH) ; 4,15 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,82 et 2,54 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,24 (t, 2H, J = 8 Hz, CH_2-CO) ; 2,12 (s, 3H, CH_3) ; 1,63-1,37 (m, 6H, $(CH_2)_3$).

Exemple 1.4 : Synthèse du *méta*-BioDPDAM :

• Composé *méta*-benzophénone **1d** :

On solubilise la D-biotine (500 mg, 2,05 mmol) dans 23 mL de DMF anhydre à chaud. On refroidit à 0°C sous argon, puis on ajoute successivement la *N*-méthylmorpholine (295 µL, 2,67 mmol) et le chloroformiate d'isobutyle (420 µL, 3,28 mmol). On laisse sous agitation pendant 30 min, puis on ajoute la 3-aminobenzophénone (605 mg, 3,07 mmol) et la *N*-méthylmorpholine (240 µL, 2,17 mmol) dans 7 mL de DMF. La solution est maintenue sous agitation à 0°C pendant 2 h, puis on évapore à sec. On reprend le résidu dans 1 mL de MeOH, puis on ajoute 25 mL d'eau. Le précipité obtenu est filtré, lavé avec de l'eau, puis avec de l'éther pour donner 810 mg (93 %) de produit **1d** brut. Une recristallisation dans le couple MeOH-H_2O donne **1d** (630 mg, 72 %) sous forme d'une poudre blanche.

IDT Exhibit 1020.7409

RMN ^{1}H (200MHz, DMSO-d_6) δ =10,10 (s, 1H, NH-CO) ; 8-7,39 (m, 9H, Ar-H) ; 6,43 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 4,27 (m, 1H, CH_2-CH-NH) ; 4,13 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,84 et 2,55 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2, 31 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,59-1,36 (m, 6H, $(CH_2)_3$).

• Composé *méta*-hydrazone **2d** :

On solubilise **1d** (350 mg, 0,83 mmol) dans 5,5 mL d'éthanol absolu puis on ajoute l'hydrazine monohydrate (140 µL, 2,48 mmol). La solution est chauffée à reflux pendant une nuit. Après évaporation, le produit est repris dans 1 mL d'éthanol et de l'eau. Le précipité blanc est recristallisé : on le dissout dans un minimum d'éthanol à chaud et l'on ajoute de l'eau jusqu'à apparition d'un léger trouble. Après refroidissement, le précipité obtenu est lavé à l'eau puis séché à l'éther. On obtient ainsi 264 mg (73 %) de produit **2d** sous forme d'une poudre blanche.

RMN ^{1}H (200 MHz, DMSO-d_6) δ = 9,99 (s, 1H, NH-CO) ; 9,80 (s, 2H, NH_2) ; 7,54-6,88 (m, 9H, Ar-H) ; 6,26 (s large, 1H, NH-CO-NH) ; 6,21 (s large, 1H, NH-CO-NH) ; 4,28 (m, 1H, CH_2-CH-NH) ; 4,13 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,78 et 2,59 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,27 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,57-1,36 (m, 6H, $(CH_2)_3$).

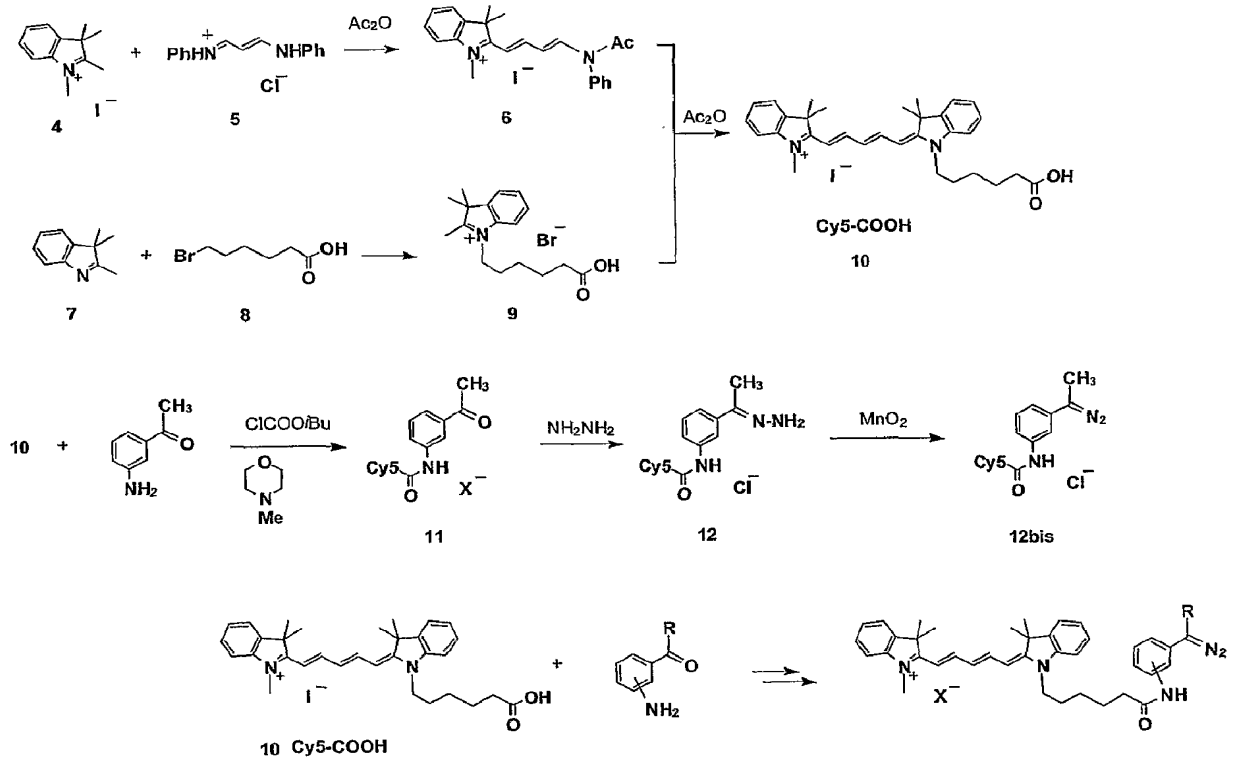
• Composé *méta*-diazodiphényl **3d** :

On solubilise **3d** (500 mg, 0,53 mmol) dans 1 mL de THF. On ajoute alors 80 mg de MnO_2 activé. Après 5 minutes d'agitation à température ambiante, le mélange est filtré à travers une couche mixte Célite (0,5 cm) -tamis moléculaire (0,5 cm en poudre). Le mélange réactionnel est évaporé à sec. Le composé **3d** (47 mg, 100 %) est obtenu sous forme d'une huile violette.

RMN ^{1}H (200 MHz, DMSO-d_6) δ = 9,95 (s, 1H, NH-CO) ; 7,60-6,9 (m, 9H, Ar-H) ; 6,42 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; 4,28 (m, 1H, CH_2-CH-NH) ; 4,14

(m, 1H, CH-C<u>H</u>-NH) ; 3,12 (m, 1H, CH-S) ; 2,83 et 2,59 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,27 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,58-1,35 (m, 6H, $(CH_2)_3$).

<u>Exemple 2 : Réactif de marquage avec le Cy5 : Cy5-PMDAM :</u>



• <u>Composé Iodure de 2-[4-(*N*-acétyl-*N*-phénylamino)buta-1,3-diényl]-1,2,3,3-tétraméthyl[3H] indolium **6** :</u>

Le mélange du monochlorhydrate de malonaldehyde-bis(phénylimine) **5** (18,3 g ; 70,0 mmole), de NaOAc (9,0 g ; 110 mmole) et de l'iodure de 1,2,3,3- tétraméthyl[3H]indolium **4** (4,25 g ; 14,1 mmole) dans de l'anhydride acétique (75 mL) est chauffé à 110°C pendant 20 min. Après refroidissement, on ajoute de l'éther (350 mL) et le solide brun précipité est filtré, lavé avec de l'éther (3 × 100 mL). Le solide est redissout dans 150 mL de CH_2Cl_2, filtré (élimination des sels minéraux) puis évaporé pour donner un solide brun (6,0 g, 90 %).

RMN ^1H ($CDCl_3$) : δ = 8,64 (d ; 1H ; J = 12 Hz ; 1-H) ; 8,14 (t ; 1H ; J = 16 ; 12 Hz ; 3-H) ; 7,63-7,19 (m ; 9H) ; 6,90 (d ; 1H ; J = 15 Hz ; 4-H) ; 5,82 (t ; 1H ; J = 12 ; 13 Hz ; 2-H) ; 4,06 (s ; 3H ; NCH_3) ; (2,16 (s ; 3H ; -$COCH_3$) ; 1,74 (s ; 6H ; CH_3).

• Composé Bromure de 1-(5-carboxypentyl)-2,3,3-triméthyl[3H]indolium **9** :

On mélange le 2,3,3-triméthylindole **7** (10,0 g ; 62,8 mmole) et l'acide 6-bromohexanoïque **8** (12,3 g ; 62,8 mmole) sans solvant et chauffe à 110°C pendant 12 h sous argon. Le mélange réactionnel pâteux rouge-violet est lavé avec de l'acétate d'éthyle (2 × 60 mL, on triture la pâte avec la spatule et on décante le surnageant), puis avec de l'acétone (50 mL, la pâte solidifie). Le solide rose est filtré puis séché sous vide (16,0 g ; 73 %).

• Composé Cy5COOH **10** :

Le mélange de l'iodure **6** (6,0 g ; 12,7 mmole), du bromure **9** (4,5 g ; 12,7 mmole) et de NaOAc (2,6 g ; 32 mmole) dans de l'anhydride acétique (35 mL) est chauffé à 110 °C pendant 20 min. Après refroidissement, on ajoute de l'éther (150 mL) et le précipité est filtré et lavé avec de l'éther (3 × 50 mL). Le solide est dissous dans 100 mL de CH_2Cl_2, filtré et purifié par chromatographie sur colonne de SiO_2 (éluant : MeOH 5-10 %/CH_2Cl_2). On obtient 3,4 g (44 %).

RMN ^1H ($CDCl_3$) : δ = 8,03 (t ; 2H ; J = 10 ; 11 Hz, 2-H, 4-H) ; 7,38-6,91 (m ; 9H ; Ar-H, 3-H) ; 6,41 (d ; 1H ; J = 14 Hz ; 1-H) ; 6,31 (d ; 1H ; J = 13 Hz ; 5-H) ; 4,07 (t ; 2H, J = 7 ; 7 Hz ; α-CH_2) ; 3,68 (s ; 3H ; NCH_3) ; 2,47 (t ; 2H, J = 7 ; 7 Hz ; ε-CH_2) ; 1,71 (m ; 18H ; CH_3, β,γ et δ-CH_2).

• Composé de couplage de la 3-aminoacétophénone avec le Cy5COOH **10** (produit **11**) :

À une solution de Cy5COOH **10** (1,19 g ; 1,9 mmole) dans 12 mL de CH_2Cl_2, on ajoute *N*-méthylmorpholine (360 µL ; 3,2 mmole). La solution est refroidie avec un bain de glace, puis on ajoute le chloroformiate d'isobutyle (480 µL ; 3,7 mmole). Après 5 minutes d'agitation, on additionne la 3-aminoacétophénone (488 mg ; 3,6 mmole). On agite le mélange à température

ambiante pendant 3 h. En ajoutant 250 mL d'éther, on obtient un solide pâteux. Après agitation, on laisse reposer le solide au fond de ballon et on décante le surnageant. On ajoute de nouveau 50 mL d'éther et on triture avec une spatule pour obtenir un solide. Celui-ci est filtré, lavé avec de l'eau, de l'éther, puis séché sous vide. Le produit (iodure) est ensuite dissous dans de l'éthanol, passé sur colonne d'amberlite IRA900 (Cl^- ; 15 g). La solution éthanolique recueillie est évaporée à sec puis passée sur colonne de SiO_2. On obtient 0,93 g (77 %) de solide bleu.

• Composé Cy5-hydrazone **12** :

On ajoute à une solution de l'acétophénone **11** (0,93 g ; 1,46 mmole) dans 5 mL d'éthanol absolu l'hydrazine monohydraté (180 µL ; 3,1 mmole) que l'on agite à température ambiante pendant 7 h. On ajoute 50 mL d'éther et le précipité est filtré et lavé avec de l'éther. On dissout le produit brut dans 50 mL de CH_2Cl_2, la solution est filtrée puis concentrée à 10 mL. Le produit est précipité par ajout de 100 mL d'éther, filtré, lavé à l'éther et séché sous vide. On obtient 540 mg de produit **12** (57 %).

• Composé Cy5PMDAM **12bis** :

A une solution de 100 mg d'hydrazone **12** dans 2 mL de DMF, on ajoute 300 mg de MnO_2 et l'on agite vigoureusement pendant 10 min. On filtre la suspension à travers une couche de célite et lavée avec DMF (3×500 µL). On ajoute 50 mL d'éther et l'huile déposée est triturée avec une spatule et le surnageant est décanté. On répète l'opération de lavage trois fois avec 25 mL d'éther et le solide ainsi obtenu est filtré, séché. On obtient 65 mg (65 %) de produit **12bis**. La pureté du produit est d'environ 80-85 % (RMN ^1H).

Exemple 3 : Autres réactifs synthétisés :

Exemple 3.1 : Synthèse de *para*-nitrophényldiazométhane (NPDAM) :

Le 4-nitrobenzaldéhyde hydrazone est commercialement disponible (référence 28,118-2, Aldrich, France).

On travaille donc sur 600 mg (3,64 mmole) de ce produit que l'on dissout dans 9 mL de THF. La solution est laissée sous agitation pendant 5 minutes puis 1,26 g (4 équivalent, 14,56 mmole) de MnO_2 sont additionnés avec précaution. Le mélange est laissé sous agitation pendant 10 minutes, puis filtré. Le filtrat récupéré est évaporé à sec. Après lavage au pentane, le composé *para*-nitrophényldiazométhane est obtenu sous forme d'une poudre orange vif avec un rendement de 79 % (468 mg, 2,87 mmole).

F 80-82°C. - RMN ^1H (300 MHz, DMSO-d_6) δ = 8,11 (d, 2H, J = 9 Hz, Ar-$\underline{H}_3$) ; 7,18 (d, 2H, J = 9 Hz, Ar-$\underline{H}_2$); 6,06 (s, 1H, C$\underline{H}_1$-N_2).

Exemple 3.2 : Synthèse du phénylméthyldiazométhane (PMDAM) :

Hydrazone de l'acétophénone : On dilue l'acétophénone (2,0 g, 16 mmole) dans 16 mL d'éthanol absolu puis on ajoute l'hydrazine (2,3 mL, 48 mmole). On porte au reflux. Après 2 h, on évapore le solvant et on reprend le résidu dans de l'éther (150 mL). On lave avec de l'eau (100mL). Après séchage sur Na_2SO_4, on évapore l'éther. On obtient l'huile jaune pâle (1,5 g, 11 mmole, 69 %).

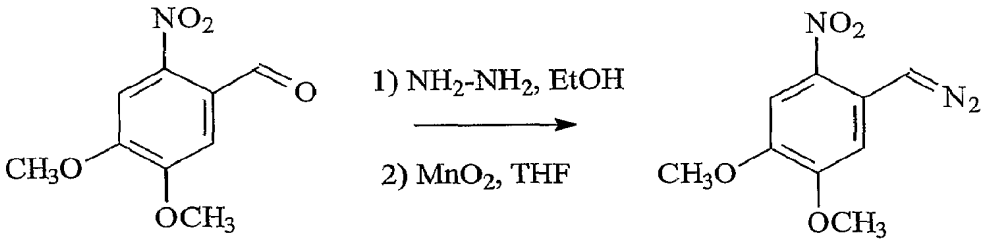
Phénylméthyldiazométhane (PMDAM) : On dissout l'hydrazone (150 mg, 1,1 mmole) dans 3 mL de THF. On ajoute le MnO_2 (480 mg, 5,5 mmole). On agite pendant 30 min à température ambiante. Le milieu se colore en rouge. On filtre et on évapore le solvant. On obtient l'huile rouge (145 mg, 100 %). Ce réactif est utilisé sans purification.

Exemple 3.3 : Synthèse du diphényldiazométhane (DPDAM) :

La Benzophénone hydrazone est commerciale (référence B 960-2 Aldrich, France).

On travaille donc sur 196 mg (1,0 mmole) que l'on dissout dans 5 mL de THF. 435 mg (5 éq, 5,0 mmole) de MnO_2 sont additionnés, le mélange est laissé sous agitation pendant 10 minutes, puis filtré. Le filtrat récupéré est évaporé à sec. On obtient 193 mg (0,99 mmole). Ce réactif est utilisé sans purification.

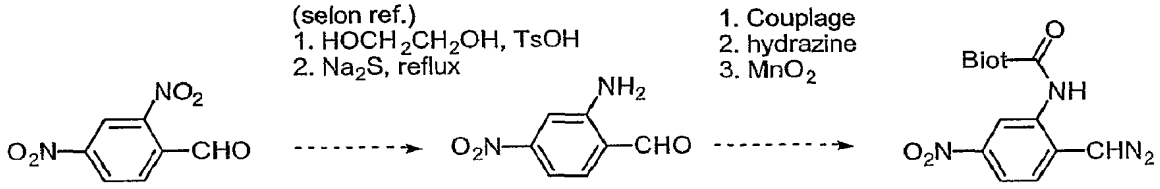
Exemple 3.4 : Synthèse du NVDAM :



NVDAM

La synthèse est réalisée selon le protocole décrit précédemment à partir de 6-nitrovératraldéhyde (Aldrich, référence 27,960-9).

Exemple 4 : Synthèse du dérivé biotinylé à partir du NPDAM :



M. E. Wall et al., *J. Med. Chem.*, **1993**, 36, 2689.

Le dérivé 2-amino-4-nitro benzaldéhyde est préparé selon la méthode de ME Wall et al référencé ci-dessus.

La préparation du diazométhane NPDAM est identique à celle décrite dans l'exemple 3.1 ci-dessus.

Exemple 5 : Préparation des acides nucléiques ADN :

Les amplicons ADN sont générés par PCR à partir de cibles d'ADN génomique *Mycobacterium tuberculosis* 16S (10^{+4} copies comme cibles de départ) en utilisant le kit Fast

Start de Roche, 0,2 mM de chaque désoxyribonucléotide (d-ATP, d-CTP, d-GTP, d-TTP), 0.3 µM d'amorces et 0,4 µL d'enzyme.

Les paramètres de la PCR sont les suivants :

- 95°C : 4 min puis 35 cycles (95°C : 30 sec ; 55°C : 30 sec ; 72°C : 30 sec) puis 4°C .

Les amplicons sont analysés qualitativement par électrophorèse sur gel d'agarose (1,5%, TBE 0,5X). Le volume déposé est de 5 µL et la migration s'effectue durant 20 min à 100 Volts (V). La visualisation des produits PCR est réalisée sous lampe UV après coloration au bromure d'éthidium.

Les conditions pour la culture, l'extraction des Mycobactéries ainsi que les amorces d'amplification sont données dans la demande de brevet WO-A-99/65926.

Exemple 6 : Réactivité des réactifs de marquage sur des nucléotides modèles

La synthèse des réactifs de marquage est décrite dans les exemples 1 à 4 ci-dessus. Le PDAM décrit dans la présente invention est commercialement disponible. (référence P1405, Molecular Probes, Eugene, OR)

Exemple 6.1 : Marquage des monomères UMP 3'-phosphate :

La réactivité des réactifs de marquage portant une fonction diazométhyle a été étudiée pour contrôler la spécificité de la réaction.

Un protocole a été mis au point qui consiste à étudier cette réactivité par électrophorèse capillaire sur un composé modèle le 3'-UMP (Uridine 3'monophosphate, référence U1126 Sigma) dans les conditions standards suivantes :

3'-UMP 0,04 mM ; H_3BO_3 2,0 mM ; Marqueur 2,0 mM [ajouté avec un solvant organique approprié (THF, AcOEt ou DMSO)] ; Solvants H_2O - CH_3CN -Solvant organique (rapport : 1/3/1).

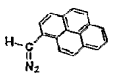
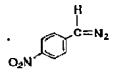
On répartit cette solution en dix fractions de 250 µL que l'on chauffe à 60 °C. Après une durée définie, chaque fraction est traitée avec 250 µL de dichlorométhane. Après agitation, on élimine la phase organique (phase inférieure). On répète cette opération encore deux fois. Après centrifugation (5 min, 5000 tours par minutes (rpm)), la phase aqueuse est analysée par électrophorèse capillaire. Les conditions de l'électrophorèse capillaire (EC) sont les suivantes : l'analyse EC a été effectuée par l'appareil Beckman P/ACE 5000. Un capillaire de silice fondue non traité (75 µm × 50 cm) a été utilisé. Le voltage appliqué est de 30 kV (polarité normale) et la température du capillaire a été maintenue à 23°C. Les électrophorégrammes ont été enregistrés à 254 nm. La solution tampon borate (0,1 M, pH 8,3) a été préparée à partir de l'acide borique en ajustant le pH avec une solution NaOH et filtrée à travers un filtre 0,2 µm. Les échantillons ont été injectés sous pression (0,5 psi, 5 secondes). Chaque analyse est précédée de la régénération du capillaire par passages successifs d'une solution de NaOH (0,1 M, 2 min), eau (2 min) et de tampon borate (2 min) sous pression (20 psi).

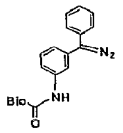
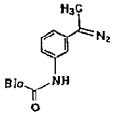
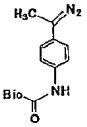
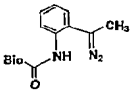
La réaction est effectuée en faisant varier le temps de réaction entre 0 et 4 heures, comme indiqué sur chaque figure, et les résultats sont présentés pour chaque réactif testé sur les figures 2A à 2I avec la concentration en réactif utilisé.

Le temps de réaction est indiqué sur chaque électrophorégramme.

Avec tous les réactifs testés, on obtient la formation exclusive d'un produit monoalkylé, ce qui prouve la spécificité de la réaction.

La réactivité, c'est-à-dire le temps de demi réaction du réactif sur le 3'-UMP, peut être ainsi calculée, par comparaison de la hauteur des pics, et les résultats sont présentés dans le tableau 1 ci-dessous (conditions standards décrites ci-dessus) :

Formules chimiques et noms	 PDAM	 DPDAM	 PMDAM	 NPDAM
Réactivité	5 min	20 h	30 min	4 h

Formules chimiques et noms	 BioDPDAM	 *m*-BioPMDAM	 *p*-BioPMDAM	 *o*-BioPMDAM *
Réactivité	10 h	15 min	5 min	1 min

* Avec le *o*-BioPMDAM, la réactivité est estimée car dans les conditions de concentration de 2 mM en réactif de marquage, la réaction est complète en moins de 5 min. La figure 2I utilise donc une concentration de 0,2 mM.

Tableau 1 : Réactivité (temps de demi réaction) de réactifs

On peut néanmoins noter que, la réaction étant très spécifique et ne conduisant pas à des sous produits pour tous les réactifs testés, il est possible d'augmenter la concentration du réactif de marquage sans conséquence du point de vue de la sélectivité sur le marquage.
Ainsi pour le réactif DPDAM, si l'on augmente la concentration à 20 mM (voir figure 2C), la réactivité (temps de demi réaction) est de 2 heures. Le même résultat est obtenu avec le BioDPDAM (figure 2F) où la réactivité est de 45 min à une concentration de 30 mM.

Exemple 6.2 : Test de différents nucléotides 3'-monophosphate :

De manière à éviter toute erreur d'interprétation, une étude complémentaire sur le marqueur *méta*-BioPMDAM, pris à titre d'exemple significatif, a été effectuée avec les autres nucléotides 3'-monophosphate.

Les nucléotides testés sont les suivants : 3'-AMP (référence 85,194-9, Aldrich), 3'-GMP (référence 151214, ICN), 3'-CMP (référence C1133, Sigma), 3'-TMP (série désoxyribo) (référence T1008, Sigma). Les électrophorégrammes obtenus avec les différents nucléotides sont représentés sur les figures 3A à 3D. Les temps de réaction indiqués sur la figure 3A sont identiques pour les figures 3B à 3D.

Quel que soit le nucléotide de départ (série ribo ou désoxyribo), nous observons la formation exclusive et complète du produit alkylé en 130 min à 60°C. Il est important de noter que dans

le cas de la guanine (base la plus réactive avec les réactifs alkylants usuels), seul le produit alkylé au phosphate est observé prouvant la très grande sélectivité de la réaction.
Cette étude permet également de vérifier que la vitesse de la réaction ne dépend pas de la nature de nucléotide en tant que substrat.

Exemple 6.3 : Etude d'un dinucléotide :

L'alkylation du dinucléotide ApUp (référence A4298, Sigma) a été effectuée avec le *méta*-BioPMDAM afin de vérifier la sélectivité de la réaction vis-à-vis du phosphate terminal par rapport au phosphate internucléotidique. Le suivi de la réaction par électrophorèse est représenté sur la figure 4. Les conditions sont les conditions standards de l'exemple 6.1 déjà décrit.

La formation exclusive d'un seul produit est observée montrant une bonne sélectivité du réactif *méta*-BioPMDAM vis-à-vis du phosphate terminal par rapport au phosphate internucléotidique.

Exmple 6.4 : Caractérisation des adduits avec les quatre (4) nucléotides 3'-monophosphates :

Pour s'assurer que les produits obtenus provenaient bien d'une alkylation au phosphate, la synthèse des adduits des monophosphates 3'-UMP, 3'-CMP, 3'-GMP et 3'-AMP avec le réactif *méta*-BioPMDAM a été effectuée. La réaction d'alkylation est réalisée à l'échelle préparative comme indiqué ci-dessous. Les adduits, obtenus avec des rendements de l'ordre de 70 % sont purifiés puis étudiés par RMN du proton et du phosphore.

Protocole de préparation :

On dissout le 3'-UMP (sous forme de sel disodique ; 9,3 mg ; 21,1 µmol) dans 2 mL d'une solution aqueuse 0,1 M de H_3BO_3, puis on ajoute successivement 2 mL de CH_3CN, 6 mL de MeOH puis le réactif *méta*-BioPMDAM (75 mg ; 0,20 mmol). La réaction est réalisée pendant

2,5 h à température ambiante. Elle est suivie par électrophorèse capillaire. On ajoute 3 mL d'eau, puis l'excès du réactif est éliminé par extraction avec CH_2Cl_2. La phase aqueuse est évaporée. Le résidu est dissous dans une petite quantité d'eau et purifié par passage sur une colonne de gel de silice en phase inverse (Lichroprep RP-18, Merck ; élution MeOH/H_2O (20/80)). On obtient 10 mg (69 %) de l'adduit du 3'-UMP.

Les spectres de RMN du proton obtenus pour les adduits du 3'-NMP (N= G, U, C, A) sont présentés sur les figures 5A à 5D. L'identification des adduits a été réalisé par des expériences de RMN à deux dimensions $^1H/^1H$ (COSY). Deux diastéréoisomères pour chacun de ces adduits dans un rapport 1/1 sont présents.
Un seul pic est présent pour la RMN du phosphore vers 0 ppm (300 MHz, D_2O).
Ces expériences démontrent que la réaction est bien spécifique, qu'un seul adduit est observé et que le marquage a bien lieu sur le phosphore. Il n'y a pas de réaction secondaire d'alkylation sur les bases. Les produits de marquage sont donc particulièrement adaptés pour une étape d'hybridation.

Exemple 7 : Etude de stabilité à la température :

Tous les dérivés du diazométhane, décrit dans le tableau 1 de l'exemple 6.1 ci-dessus, sont conservé à l'état solide dans le congélateur à -20 °C pendant au moins trois (3) mois et aucune perte de réactivité n'est observée.
La stabilité à température ambiante sur la paillasse a été déterminée par RMN ^1H pour les deux réactifs NPDAM et *méta*-BioPMDAM. Nous n'avons observé aucune décomposition en laissant le NPDAM pendant un mois sur la paillasse sans précaution particulière. Nous observons environ 50% de décomposition en laissant le *méta*-BioPMDAM sur la paillasse pendant vingt-cinq (25) jours.
La stabilité à la température du réactif de marquage est une caractéristique essentielle. En effet, la destination finale d'un tel réactif pour une application industrielle est un kit de marquage. Un réactif qui n'est pas stable au moins pendant quinze (15) jours à -20°C, de préférence un (1) mois est invendable. Même s'il existe des moyens de stockage et d'envoi

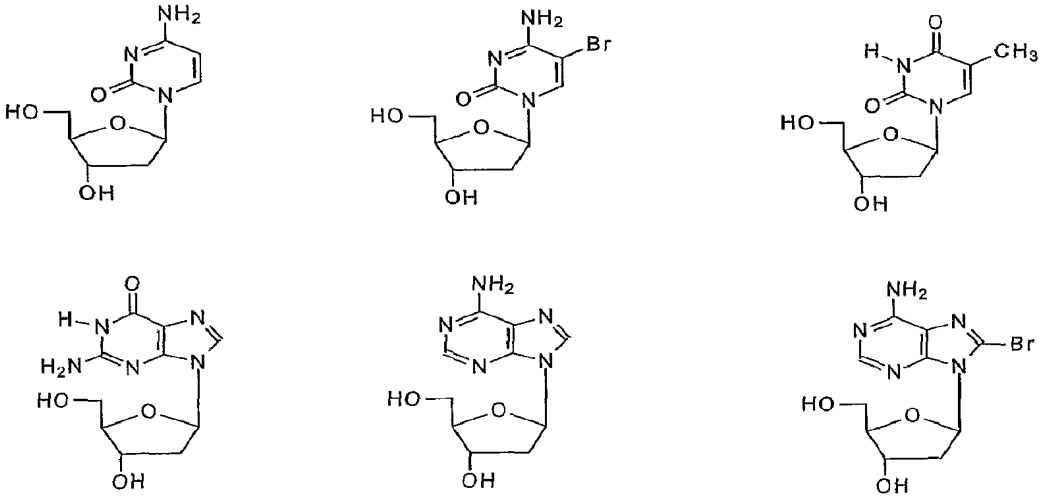
jusqu'à -110°C, il existe une relation entre la stabilité à -110°C et à -20°C et c'est pourquoi la valeur de quinze (15) jours à -20°C, de préférence un (1) mois à -20°C est un minimum industriel. Au delà de -110°C, les laboratoires ne disposent pas des équipements nécessaires (congélateur) pour stocker ces réactifs du point de vue de l'utilisateur et du fabricant, et il n'y a pas de moyen simple type carboglace pour les expédier du point de vue du fabricant.

En ce qui concerne la stabilité à température ambiante, une stabilité de quelques heures, de préférence de un (1) jour, est suffisante pour permettre à l'utilisateur d'effectuer le marquage.

Exemple 8 : Etude de la fragmentation de l'ADN :

Exemple 8.1 : Hydrolyse de différents nucléosides en milieu acide :

Le but de cette étude est de démontrer la différence en terme de stabilité à pH acide entre les nucléosides naturels, les nucléosides modifiés ainsi que les nucléosides de type puriniques et pyrimidiniques. Cette étude permet également de mieux contrôler la fragmentation de l'ADN en tenant compte de sa composition en bases.



Deux nucléosides modifiés, la 8-bromo-2'-désoxyadénosine (8-BrdA) et la 5-bromo-2'-désoxycytidine (5-BrdC) ainsi que les quatre (4) nucléosides naturels (dA, dC, dG et dT) ont été utilisés dans cette étude.

50 nanomoles (nmol) de chaque nucléoside sont incubées dans 50 mM de formiate de sodium pH 3 à 95°C. Les temps d'incubation varient de 0 à 45 min. Après séchage sous vide et reprise par 20 µl d'eau pure, les échantillons (10 nmol) sont alors analysés par HPLC en phase inverse. Les résultats sont donnés sous forme de pourcentage d'hydrolyse du nucléoside de départ (en ordonnée) par rapport au temps d'incubation en minutes (en abscisse) voir figure 8.

Les courbes de la figure 8 montrent que la modification de l'adénine en position 8 par un atome de brome rend ce nucléoside moins stable que le nucléoside naturel. D'autre part, les résultats montrent que, dans les conditions utilisées, la dépurination est beaucoup plus importante que la dépyrimidination.

Cette étude montre que la fragmentation de l'ADN par la dépurination ou la dépyrimidination peut être contrôlée en optimisant les conditions d'hydrolyse, ou en incorporant soit des bases modifiées moins stables que les bases naturelles soit des bases pouvant être modifiées et hydrolysées après leur incorporation.

Exemple 8.2 : Fragmentation de l'ADN double brin incorporant ou non un nucléotide modifié :

Trois amplifications PCR ont été réalisées en parallèle à partir de cible ADN génomique *Mycobacterium tuberculosis* 16S (10^{+4} copies de départ) en utilisant le kit *Fast Start* de Roche, 0,2 mM de chaque désoxyribonucléotide (d-ATP, d-CTP, d-GTP, d-TTP), 0,3 µM d'amorces et 0,4 µL d'enzyme.

Les paramètres de la PCR sont ceux de l'exemple 5.

Dans le premier cas, le protocole est utilisé tel quel : cas de la PCR dite naturelle.

Dans le deuxième cas, le protocole est modifié pour obtenir une PCR à 30 % de 8Br-dATP. Ceci est réalisé en introduisant 0,2 mM de d-CTP, d-GTP et d-TTP. On introduit également 0,14 mM de d-ATP et 0,06 mM de 8-BrdATP. (La 8-BrdATP est commerciale (référence N-2005-1, TriLink Biotechnologies, San Diego CA).

Dans le troisième cas, le protocole est modifié pour obtenir une PCR à 50 % de 8-BrdATP. Ceci est réalisé en introduisant 0,2 mM de d-CTP, d-GTP et d-TTP. On introduit également 0,1 mM de d-ATP et 0,1 mM de 8-BrdATP.

L'étude de la fragmentation seule de ces amplicons a été réalisée dans les conditions décrites ci-dessus : 50 mM de formiate de sodium pH 3 à 95°C.
L'analyse a été effectuée sur gel dénaturant de polyacrylamide (8 % polyacrylamide, 7 M urée, 1 X TBE) utilisant coloration au bromure d'éthidium.

Après 15 min d'incubation à 95 °C dans 50 mM de formiate de sodium pH 3, aucune différence n'est visible entre les trois (3) cibles. Dans tous les cas, nous avons observé une fragmentation totale des amplicons PCR.

La dépurination des amplicons PCR a été également réalisée à différents pH et à différentes températures et temps d'incubation. L'analyse sur gel, dans les conditions ci-dessus, montre qu'à pH 3, la fragmentation est totale après seulement 10 min d'incubation à 95°C. A ce pH, la fragmentation est aussi totale à 60°C après 30 min d'incubation.
A pH 4, 30 min d'incubation sont nécessaires pour l'obtention d'une fragmentation totale des amplicons ADN même à 95°C. Ce résultat est très intéressant et montre que le site abasique généré à pH acide est instable et donc conduit à la fragmentation de la chaîne ADN sans aucun autre traitement particulier.

Exemple 9 : Marquage et fragmentation de l'ADN par le dérivé *méta*-bioPMDAM (3a) en deux étapes :

Le dérivé *méta*-bioPMDAM (3a) a été obtenu selon le schéma réactionnel décrit dans l'exemple 1.1.
Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5.

Marquage

A 10 µl de PCR, 38 µL d'eau pure (sigma), 50 µL de formiate de sodium à pH 3 (100 mM dans l'eau pure) sont ajoutés et le mélange est incubé pendant 30 minutes à 60°C. Puis 2 µL de *méta*-bioPMDAM (100 mM dans DMSO) sont ajoutés ensuite. La solution a été mélangée par vortex, puis incubée 15 minutes supplémentaires à 60°C.

Les essais sont réalisés en duplicata afin de pouvoir analyser la fragmentation de l'ADN sur gel et l'efficacité de marquage par hybridation et lecture de la puce à ADN.

Purification

La purification est réalisée sur colonnes QIAQUICK™ (Nucleotide Removal kit, Qiagen, Hilden, Allemagne). Le protocole de purification utilisé est celui recommandé par le fournisseur.

Après purification, l'éluat est transféré dans un tube propre contenant 400 µL de tampon d'hybridation (1,75 mL 20X SSPE ; 2,9 mL Bétaine 5M ; 290 µL DTAB 0,1M ; 10 µL Anti-mousse 30%) sont ajoutés. Les références de ces substances sont, pour :

- la bétaine référence B-2754 Sigma, et
- la DTAB référence D-5047 Sigma.

La solution est mélangée par vortex et incubée 10 min à 95°C, afin de séparer les brins d'ADN qui ne sont pas séparés lors de l'étape de marquage pendant la fragmentation (étape de dénaturation). Le tube est ensuite plongé dans un mélange eau-glace à 0°C avant hybridation sur puce à ADN.

Hybridation sur puce à ADN

Après l'étape de marquage pendant la fragmentation, les fragments obtenus sont hybridés sur les puces à ADN conçues pour l'analyse de la région 213-415 de la séquence M20940 « Genbank » de l'ARN 16S de *Mycobacterium tuberculosis*. Cette puce à ADN est décrite dans A. Troesch et al, J. Clin Microbiol., 37(1), p49-55, 1999.

Les étapes d'hybridation ont été réalisées sur les stations fluidiques (Affymetrix, Santa Clara, CA) en utilisant le protocole d'hybridation et les tampons décrits dans A. Troesch et al, J. Clin

Microbiol., 37(1), p49-55, 1999. Une étape supplémentaire est nécessaire pour révéler la biotine (détection indirecte).

L'hybridation est révélée par le couplage de la streptavidine marquée à la phycoérythrine (PE) qui interagit avec la biotine de la *méta*-BioPMDAM dans les conditions suivantes : 300 µL d'eau pure ; 300 µL de tampon Tris 100 mM pH 7 / NaCl 1M / tween 0,05% / Antimousse 0,005% ; 6 µL de BSA (50 mg/mL) ; 6 µL de streptavidine-PE (300 µg / mL). Les références de ces substances sont, pour :

- la Streptavidine-Phycoérythrine : référence R0438, Dako, Danemark,
- la Streptavidine-CY5 : référence C0050, Dako, Danemark,
- l'Antimousse référence M5-575, Ultra Additives Inc., et
- le Tween référence P-7949, Sigma.

Lecture de la puce à ADN :

La lecture de la fluorescence émise à la surface de la puce à ADN après marquage et hybridation ainsi que la génération des données en termes d'intensité du signal et du pourcentage d'homologie sont réalisés par les systèmes de lecture et le logiciel fournis par Affymetrix (GeneChip® Instrument System et GeneChip® Information System, Santa Clara CA).

Le système de lecture fournit des intensités en signal et bruit de fond exprimées en rfu (« relative fluorescence unit »). Le pourcentage d'homologie est donné par rapport à une séquence référence qui dans ce cas est la séquence de *Mycobacterium tuberculosis*.

Les résultats en termes d'intensité moyenne du signal (I), du bruit de fond (B) et du pourcentage d'homologie (%) sont donnés dans le tableau 2 ci-dessous :

De manière générale, on considère qu'un pourcentage d'homologie supérieur à 90% est un résultat satisfaisant bien que l'on cherche en général un résultat supérieur à 95%. Au dessus de 95%, les valeurs ne sont plus indiquées car elles ne sont pas significatives dans le cas de la puce à ADN Mycobactéries. Une intensité élevée avec un bruit de fond faible est le deuxième résultat recherché dans les exemples qui suivent. Dans tous les résultats, le bruit de fond B est déduit de l'intensité moyenne I.

Analyse sur gel de polyacrylamide

Les échantillons destinés à être analysés sur gel sont séchés sous vide, repris par 10 µL d'eau pure et 10 µL de bleu formamide 2X.

La migration est effectuée sur gel d'acrylamide 8%, dans du TBE 1X, une (1) heure à 150 V.

Le pH acide a été utilisé pour la fragmentation de l'ADN. En effet à ce pH, le phénomène de dépurination engendre des sites abasiques très instables conduisant à une fragmentation presque immédiate des séquences ADN à température élevée. Ce type de fragmentation produit des fragments ADN-5' phosphate.

L'analyse sur gel montre que l'incubation des amplicons PCR à 60°C pendant 30 min en solution dans le tampons formiate (50 mM, pH 3) conduit à une fragmentation totale de ces amplicons. Ceci nous a permis d'évaluer le marquage pendant la fragmentation des amplicons ADN en présence du marqueur *méta*-bioPMDAM.

Les résultats de marquage pendant la fragmentation des amplicons ADN en termes de pourcentage d'homologie, d'intensité des signaux et du bruit de fond sont donnés dans le tableau 2 ci-dessous.

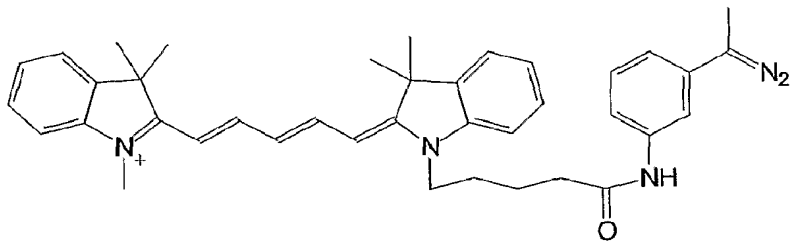
Conditions de marquage des amplicons PCR	Homologie (%)	I (rfu)	B (rfu)	I/B
Tampon : formiate pH3, 50 mM Marqueur : 2 mM *méta*-bioPMDAM Incubation : 30 min à 60°C	> 95	4456	593	7,5

Tableau 2 : Marquage et fragmentation des amplicons ADN en termes d'homologie, d'intensité des signaux (I) et du bruit de fond (B)

Cet exemple montre que les dérivés de l'invention peuvent être utilisés pour le marquage des fragments ADN produits par amplification enzymatique dans un protocole en deux étapes. Ils peuvent être également utilisés pour le marquage de l'ADN naturel non amplifié.

Exemple 10 : Marquage de l'ADN par le dérivé Cy5-PMDAM (12bis) :

Le marquage de l'ADN par ce nouveau marqueur portant le fonction diazométhyle a été évalué en utilisant un fragment ADN synthétique.



Cy5-PMDAM (12bis)

Le réactif de marquage Cy5-PMDAM (12bis) est préparé selon le protocole décrit dans l'exemple 2.

Un oligodésoxyribonucléotide (ODN) vingt (20) mers est préparé selon la méthode dite au phosphoramidite. On introduit un phosphate à l'extrémité 5' par un réactif de phosphorylation standard compatible avec la chimie au phosphoramidite. La séquence de cet ODN est constituée de toutes les bases naturelles de l'ADN (séquence de l'ODN : 5'-CTGAACGGTAGCATCTTGAC-3'). Cette séquence est complémentaire de séquences de capture d'une puce à ADN dite « modèle », synthétisée selon la technologie Affymetrix. Cette puce à ADN contient des sondes de capture identiques en séquence et qui sont réparties en damier sur sa surface. La lecture de cette puce à ADN donne des informations quant à la performance du marquage en terme d'intensité mais pas de résultat d'homologie.

Marquage : A 50 picomoles (pmoles) de cet ODN, 10 µL de Cy5-PMDAM (100 mM dans le DMSO) sont ajoutés. Le volume final est de 100 µL. Après homogénéisation, l'incubation est réalisée à 60°C pendant 30 minutes.

Purification et lecture : La purification pour éliminer l'excès de réactif de marquage est réalisée selon l'exemple 9. La lecture sur puce à ADN est effectuée selon l'exemple 17.

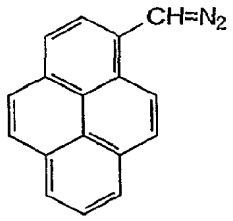
Résultats :

La moyenne des intensités de marquage (I) lue sur la puce à ADN est de 16 644 rfu pour un bruit de fond (B) de 450 rfu.

Ce niveau d'intensité est très élevé et montre que le réactif de marquage Cy5-PMDAM (12bis) est tout à fait compatible avec le marquage des fragments d'ADN sur le groupement phosphate.

Exemple 11 : Marquage et fragmentation de l'ADN par le réactif PDAM :

Exemple 11.1 : Marquage par le 1-Pyrényldiazométhane (PDAM) :



1-Pyrényldiazométhane

Le PDAM est obtenu chez Molecular Probes (Eugene, OR) et solubilisé dans du DMSO anhydre.

Deux ODN de vingt (20) mers ont été utilisés comme modèles ADN : un ODN de 20mer 5'-hydroxyle et le même ODN de 20mer portant un phosphate à l'extrémité 5'. La séquence de l'ODN est décrite dans l'exemple 10. La réaction de marquage est réalisée dans un mélange à 50 % en DMSO et 1,5 mM en 1-Pyrényldiazométhane (PDAM) à 60°C pendant 30 minutes ou une heure.

L'efficacité du marquage a été évaluée par chromatographie sur couche mince (en phase normale) dans un éluant isopropanol/ammoniaque/eau 60/10/30. Après 30 minutes, le couplage est total sur l'ODN 5'-phosphate. Il faut une heure pour obtenir un couplage partiel sur l'ODN 5'-hydroxyle c'est à dire environ 50%.

Les résultats de l'exemple 6 sont confirmés sur une séquence modèle de 20 bases en ce qui concerne le marquage préférentiel des réactifs portant une fonction diazométhyle sur le phosphate terminal. Le marquage sur un phosphate intranucléotidique n'est pas un inconvénient rédhibitoire puisque cela peut conduire à une augmentation de la sensibilité par introduction de plus d'un marqueur sur le fragment d'acide nucléique. Cela permet à l'acide nucléique de s'hybrider avec une bonne sensibilité sur la cible complémentaire tout en conservant une bonne spécificité d'hybridation.

L'homme du métier par des réactions d'optimisation peut ainsi contrôler la spécificité du marquage, en jouant par exemple sur le réactif de marquage, le temps de réaction et la température, pour avoir un marquage exclusif sur le phosphate terminal.

Exemple 11.2 : Etude cinétique de la réaction de marquage par le PDAM :

Cette étude a été réalisée en utilisant l'ODN 20mer 5'-phosphate dans les conditions précédentes en faisant varier le temps de réaction. Les rendements de marquage ont été évalués par analyse chromatographie liquide haute pression (CLHP) en phase inverse dans les conditions suivantes :

Phase inverse colonne Spheri-5 RP-18 5 µm, 220x 4,6 mm (Perkin Elmer). Les tampons et le gradient utilisés sont :

- Tampon A : 0,1 M TEAA ; Tampon B = 50% Tampon A + 50 % CH_3CN, et
- Gradient de 10 à 50 % de B en 30 min à 1 ml/min à température ambiante.

Les résultats sont représentés sur la figure 9 avec en abscisse le temps de réaction, exprimé en minutes, et en ordonnée le pourcentage de marquage.

Le rendement est proche de 90% après seulement 10 min d'incubation à 60°C.

Exemple 11.3 : Effet de la température sur le marquage au PDAM :

Le marquage a été réalisée en utilisant l'ODN 20mer 5'-phosphate dans les conditions précédentes en faisant varier la température d'incubation et avec un temps d'incubation de 10 minutes dans chaque cas.

Les rendements de marquage ont été évalués par analyse CLHP en phase inverse.
Les résultats sont présentés sur la figure 10 avec en ordonnée le pourcentage de marquage et en abscisse la température de réaction en °C
Il est très important de noter que même à température ambiante (25°C), nous observons un marquage de l'ODN. Après 10 minutes d'incubation à 25 °C, le rendement de marquage est environ de 25%. A des températures supérieures à 50°C, des rendements supérieurs à 80% sont obtenus.
Ceci montre l'efficacité et la flexibilité de cette chimie de marquage de l'ADN par les réactifs portant une fonction diazométhyle.

Exemple 12 : Marquage et fragmentation des amplicons ADN obtenus par amplification PCR avec le réactif de marquage *méta*-bioPMDAM (3a) :

Le dérivé *méta*-bioPMDAM (3a) a été obtenu selon le schéma réactionnel décrit dans l'exemple 1.1.
Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5.

Exemple 12.1 : Comparaison du marquage avec et sans fragmentation :

a. Marquage dans les conditions de fragmentation :
A 10 µL de PCR sont ajoutés 50 µL de formiate de sodium pH 3 (50 mM) et 2 µL de *méta*-BioPMDAM 100 mM dans du DMSO). Le volume est ajusté à 100 µL. La solution est incubée 30 min à 60°C.

b. Marquage sans fragmentation :
A 10 µL de PCR sont ajoutés 2 µL de *méta*-BioPMDAM (100 mM dans du DMSO). Le volume est ajusté à 100 µL. La solution est incubée 30 min à 60°C.

Le reste du protocole est identique à celui de l'exemple 9.

Résultats

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage dans les conditions de fragmentation	>95	3995	569	7,0
b. Marquage sans fragmentation	94,1	500	542	0,9

Tableau 3 : Comparaison du marquage avec et sans fragmentation

Les résultats dans le tableau 3 ci-dessus montre que sans fragmentation, la moyenne des intensités obtenues est au même niveau que le bruit de fond (500 rfu). Le marquage pendant la fragmentation donne un niveau d'intensité bien supérieur (environ 4000 rfu) et un très bon pourcentage d'homologie. La combinaison des deux étapes représente donc bien une amélioration significative pour la détection d'un acide nucléique de longueur supérieure à cent nucléotides.

Exemple 12.2 : Effet de la dénaturation avant hybridation sur puce à ADN :

Deux réactions de marquage ont été réalisées en parallèle dans deux tubes séparés selon le protocole suivant : A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH 3 (50 mM) et 2 µL de *méta-bio*PMDAM (100mM dans du DMSO). Le volume total est ajusté à 100 µL et incubé 30 min à 60°C.

Après purification sur colonne (exemple 9), la solution issue du premier tube est incubée 10 min à 95°C (afin de désapparier le double brin d'ADN), puis le tube est plongé dans un mélange eau-glace à 0°C jusqu'à l'hybridation sur la puce ADN.

La solution issue du deuxième tube est hybridée sur la puce à ADN sans dénaturation préalable.

Les fragments biotinylés hybridés sur les sondes de capture à la surface de la puce à ADN sont révélés par l'introduction d'une streptavidine marquée par la phycoérythrine en utilisant les conditions décrites dans l'exemple 9.

Résultats

Conditions utilisées	Homologie (%)	I (rfu)	B (rfu)	I/B
Avec dénaturation	>95	22812	570	40,1
Sans dénaturation	93,5	4795	681	7,0

Tableau 4 : Effet de la dénaturation avant hybridation sur puce à ADN

Les résultats obtenus, présentés dans le tableau 4, avec la dénaturation pré-hybridation sont supérieurs à ceux obtenus sans l'étape de dénaturation. Ceci montre que la dénaturation de l'ADN est nécessaire pour obtenir un bon niveau d'intensité. La fragmentation via les sites abasiques est un moyen pour faciliter la dénaturation d'un ADN double brin et renforcer l'hybridation sur les sondes de captures.

Pour tester d'autres réactifs de marquage et compte tenu des résultats obtenus avec les différentes conditions ci-dessus, nous avons défini un protocole de référence utilisant la fragmentation au tampon formiate de sodium (50 mM, pH 3) et un étape de dénaturation pré-hybridation.

Exemple 13 : Marquage et fragmentation des amplicons PCR par les réactifs biotinylés dans un protocole en une étape :

Les dérivés *méta-*, *ortho-*, et *para*-bioPMDAM ont été préparés selon le protocole décrit dans les exemples 1.1, 1.2 et 1.3. Ils ont été solubilisés dans du DMSO anhydre à une concentration de 100 mM.

Le protocole est identique à celui de l'exemple 12.2 ci-dessus (marquage et fragmentation en une étape puis étape de dénaturation pré-hybridation).

Résultats

Marqueur	Homologie (%)	I (rfu)	B (rfu)	I/B
ortho-bioPMDAM	>95	25951	820	31,6
méta-bioPMDAM	>95	22960	581	39,5
para-bioPMDAM	94,1	43785	1205	36,3

Tableau 5 : Marquage et fragmentation des amplicons PCR par les réactifs biotinylés dans un protocole en une étape

Le protocole optimisé avec fragmentation et marquage en une étape donne d'excellents résultats avec différents réactifs de marquage avec la fonction réactive diazométhyle, comme le montrent les résultats exposés dans le tableau 5.

Exemple 14 : Marquage et fragmentation de l'ADN par le BioDPDAM :

Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5.

La synthèse du réactif de marquage est décrite dans l'exemple 1.

Le protocole est identique à celui de l'exemple 12.2, y compris la dénaturation à 95 °C avant l'étape d'hybridation.

Résultats

Marqueur utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
BioDPDAM	93,0	32359	3610	9,1

Tableau 6 : Marquage et fragmentation de l'ADN par le BioDPDAM

Ce résultat, décrit dans le tableau 6, montre que des substitutions aussi importantes que le groupement phényle peuvent être utilisées pour optimiser la réactivité des réactifs de marquage portant une fonction diazométhyle.

Exemple 15 : Marquage et fragmentation de l'ADN par la 5-(bromométhyl)fluorescéine :

Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5.

A 10 µL de PCR sont ajoutés 50 µL de formiate de sodium pH3 (100 mM) et 2 µL de 5-(bromométhyl)fluorescéine (Molecular probes, Eugen, OR) (100 mM dans du DMSO). Le volume est ajusté à 100 µL. La solution est incubée 30 min à 60°C.

Les conditions de purification sont conformes à celles de l'exemple 9. Une étape de dénaturation est effectuée comme décrit dans l'exemple 12.2.

Les autres conditions d'hybridation et de lecture sont identiques à celles décrites dans l'article de A. Troesch et al. J. Clin. Microbiol., 37(1), p 49-55 1999. La fluorescéine est directement détectable sur le lecteur.

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
Marquage et fragmentation des amplicons PCR avec la 5-(bromo-méthyl)fluorescéine	>95	855	183	4,7

Tableau 7 : Marquage et fragmentation de l'ADN par la 5-(bromométhyl)fluorescéine

Ce résultat du tableau 7 montre que la fragmentation de l'ADN par la création de sites abasiques est tout à fait compatible avec un réactif de marquage portant une fonction réactive halogénure d'alkyle. Ce protocole est réalisé en une étape (fragmentation et marquage) mais avec des intensités plus faibles qu'avec les marqueurs portant une fonction diazométhyle.

Exemple 16 : Marquage et fragmentation des amplicons ADN en présence d'un autre agent chimique de fragmentation dérivé de la phénanthroline :

Des amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Deux types de conditions sont utilisées :

Conditions a :

A 10 µL de PCR sont ajoutés 20 µL de phénanthroline-$FeSO_4$ (25 mM) et 2 µL de *méta*-BioPMDAM (100 mM dans DMSO). Le volume total est ajusté à 100 µL. Le mélange est incubé 60 min à 95°C.

Conditions b :

A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH3 (100 mM dans eau pure) et 2 µL de de *méta*-BioPMDAM (100 mM dans DMSO). Le volume total est ajusté à 100 µL. Le mélange est incubé 60 min à 95°C.

Les autres conditions du protocole sont identiques à celles de l'exemple 9.

Conditions	Homologie (%)	I (rfu)	B (rfu)	I/B
Phénanthroline FeSO4(5 mM) *méta*-bioPMDAM (2 mM) 60 min à 95°C	>95	2236	500	4,5
Tampon formiate (50mM) pH3 *méta*-bioPMDAM (2 mM) 60 min à 95°C	>95	6786	565	12,0

Tableau 8 : Marquage et fragmentation des amplicons ADN en présence de la phénanthroline

Les deux conditions de fragmentation permettent un résultat satisfaisant comme démontré dans le tableau 8.

Le meilleur résultat est obtenu avec les conditions (b) utilisant la fragmentation à pH acide.

Exemple 17 : Fragmentation des amplicons PCR marqués par incorporation de d-UTP-fluorescéine :

Incorporation du nucléotide marqué

Une amplification PCR a été réalisée selon le protocole suivant afin générer des amplicons PCR marqués par la fluorescéine (marquage sur les bases).

A partir de cible ADN génomique *Mycobacterium tuberculosis* 16S (10^{+4} copies) en utilisant le kit *Fast Start* de Roche, 0,2 mM des désoxyribonucléotides d-ATP, d-CTP et d-GTP ainsi que 0,14 mM de d-TTP et 0,06 mM de d-UTP-12-fluoresceine, 0,3 µM d'amorces et 0,4 µL d'enzyme. Le pourcentage du nucléotide marqué par rapport à son homologue naturel d-UTP est de 30%. C'est ce rapport qui est généralement utilisé dans les réactions de marquage des amplicons par incorporation de nucléotides marqués.

La d-UTP-12-fluoresceine est disponible commercialement chez Roche Diagnostics référence 1373242, Mannheim, Allemagne).

Les paramètres de la PCR sont ceux de l'exemple 5.

a. Fragmentation des amplicons PCR marqués à 30% par d-UTP-fluorescéine :

A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH 3 (50 mM). Le volume est ajusté à 100 µL. La solution est ensuite incubée 30 min à 60°C.

b. Marquage pendant la fragmentation des amplicons PCR contenant 30% par d-UTP-fluorescéine :

A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH 3 (50 mM) et 2 µL de *méta*-BioPMDAM (100 mM dans du DMSO). Le volume est ajusté à 100 µL. La solution est ensuite incubée 30 min à 60°C. Cet essai correspond à un protocole de référence qui permet de comparer les différentes stratégies de marquage en s'affranchissant de la variabilité due à l'étape d'amplification.

Une étape de purification sur colonne et une étape de dénaturation à 95°C sont effectuées dans tous les cas comme dans l'exemple 9.

Protocole (a1) :

Les acides nucléiques obtenus par fragmentation des amplicons marqués à la d-UTP-fluorescéine (conditions *a*)) sont hybridés sur puce à ADN et détectés dans un premier temps par lecture directe des signaux fluorescents émis par la fluorescéine comme décrit dans l'exemple 15.

Protocole (a2) :

Une étape d'amplification du signal a été utilisée pour améliorer la sensibilité de marquage. L'amplification du signal a été réalisée par l'introduction, lors de l'étape d'hybridation, d'un anticorps anti-fluorescéine biotinylé (référence 216-065-084, Jackson ImmunoResearch) puis d'une streptavidine marquée par la phycoérythrine en utilisant les conditions successives suivantes :

- 300 µL d'eau pure,

- 300 µL de tampon Tris 100 mM pH 7 / NaCl 1M / tween 0,05% / Antimousse 0,005% , 2,4 µL de BSA (50 mg/mL), 1,2 µL d'anticorps anti-fluorescéine biotinylé. (1 mg / mL),
- 300 µL d'eau pure, et
- 300 µL de tampon Tris 100 mM pH 7 / NaCl 1M / tween 0,05% / Antimousse 0,005% ; 6 µL de BSA (50 mg/mL) ; 6 µL de streptavidine-PE. (300 µg / mL).

Dans ce protocole, la fluorescéine agit comme un haptène (traceur détectable indirectement par un anticorps marqué) et non pas comme un fluorophore.

Protocole (b) :

Les fragments biotinylés (condition *b)* hybridés sur puce à ADN sont révélés par l'introduction d'une streptavidine marquée par la phycoérythrine en utilisant les conditions suivantes :

- 300 µL d'eau pure, et
- 300 µL de tampon Tris 100 mM pH 7 / NaCl 1M / tween 0,05% / Antimousse 0,005% ; 6 µL de BSA (50 mg/mL) ; 6 µL de streptavidine marquée. (300 µg / mL).

La lecture de la fluorescence émise à la surface de la puce à ADN après marquage et hybridation ainsi que la génération des données en termes d'intensité du signal et du pourcentage d'homologie sont réalisés par les systèmes de lecture et le logiciel fournis par Affymetrix. A ce propos, il est important de noter que le système de lecture utilisé contient deux filtres permettant de détecter directement :

- la fluorescéine dans le cas où les amplicons sont marqués au d-UTP-fluorescéine uniquement, selon le protocole *a1*, ou bien
- la phycoérythrine dans le cas où les amplicons sont marqués :
  - au d-UTP-fluorescéine avec amplification du signal, selon le protocole *a2*, ou
  - par le *méta*-bioPMDAM pendant leur fragmentation, selon le protocole *b*.

Dans les deux cas où la révélation s'effectue par la phycoérythrine, l'utilisation d'un filtre permet de s'affranchir du signal généré par la fluorescéine et c'est bien le signal de la phycoérythrine qui est détecté.

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Résultats

Protocole utilisé	Marqueur détecté	Homologie (%)	I (rfu)	B (rfu)	I/B
a1. Fragmentation des amplicons PCR marqués par la d-UTP-fluorescéine	Flu*	81,6	595	342	1,7
a2. Fragmentation des amplicons PCR marqués par la d-UTP-fluorescéine avec amplification du signal	PE*	>95	22107	3461	6,4
b. Fragmentation et marquage sur les mêmes amplicons PCR modifiés par la d-UTP-fluorescéine et marqués par la méta-BioPMDAM	PE*	>95	21700	1503	14,4

* Flu =Fluorescéine et PE = Phycoérythrine

Tableau 9 : Fragmentation des amplicons PCR marqués par incorporation de d-UTP-fluorescéine

Les résultats, du tableau 9 ci-dessus, montrent que la fragmentation chimique utilisant la création de site abasique est compatible avec le marquage enzymatique des amplicons ADN et que le marquage peut avoir lieu avant la fragmentation.

Les niveaux d'intensité ainsi que le pourcentage d'homologie obtenus avec ce protocole d'incorporation enzymatique du fluorophore sont faibles en comparaison de ceux obtenus avec le marquage pendant la fragmentation utilisant le réactif de marquage avec une fonction diazométhyle comme le *méta*-bioPMDAM (conditions (b)).

Pour atteindre le niveau d'intensité obtenue avec le dérivé *méta*-bioPMDAM, une étape d'amplification du signal est nécessaire (conditions a2). Ceci montre bien l'efficacité de la fonction réactive diazométhyle par rapport à l'incorporation traditionnelle de base modifiée comme le d-UTP fluorescéine (protocole de référence (b)).

Exemple 18 : Fragmentation de l'ADN double brin par sonication :

Des amplicons ADN ont été obtenus en utilisant le protocole décrit dans l'exemple 5. Ces amplicons ont été fragmentés par sonication en présence et en absence du marqueur.

a. Marquage des amplicons PCR pendant la sonication :

A 10 µL de réaction PCR sont ajoutés 2 µL de *méta*-BioPMDAM (100 mM dans DMSO). Le volume est ajusté à 100 µL par de l'eau pure le pH est ajusté à 6,5. Le mélange est incubé 30 min à 60°C dans un bain d'une cuve à ultrasons (fréquence 35kHz, modèle T460-H, Bioblock, France).

b. Marquage pendant la fragmentation chimique des amplicons PCR (protocole de référence en une étape) :

A 10 µL de réaction PCR sont ajoutés 50 µL de formiate de sodium pH 3 (50 mM) et 2 µL de *méta*-BioPMDAM (100 mM dans du DMSO). Le volume est ajusté à 100 µL. La solution est incubée 30 min à 60°C.

Les essais sont réalisés en duplicata afin de pouvoir analyser la fragmentation de l'ADN sur gel et l'efficacité de marquage par hybridation et lecture de la puce à ADN comme décrit précédemment (exemple 9 sur la détection de la phycoérythrine).

Analyse sur gel

L'analyse a été effectuée sur gel dénaturant de polyacrylamide (8 % polyacrylamide, 7 M urée, 1 X TBE) utilisant coloration au bromure d'éthidium.

L'analyse sur gel montre que les amplicons ADN sont fragmentés par sonication à 60°C.

Résultats

Conditions	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage pendant la fragmentation par sonication	93,8	2271	631	3,6
b. Marquage pendant la fragmentation chimique (conditions de référence)	>95	19639	1459	13,5

Tableau 10 : Fragmentation de l'ADN double brin par sonication

Les résultats de marquage du tableau 10 pendant la sonication (conditions *a*) sont satisfaisants. Ceci montre que la fragmentation physique par sonication des cibles ADN est compatible avec la chimie de marquage par des réactifs de marquage portant une fonction diazométhyle.

Les faibles résultats de marquage dans ce cas sont certainement dus au fait que le marqueur se dégrade sous l'effet des ultrasons. Les résultats avec la fragmentation par création de site abasique par action du pH acide sont meilleurs.

Exemple 19 : Marquage, fragmentation et dénaturation de l'ADN en une étape :

Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5.

Deux réactions de marquage ont été réalisées :

a. Marquage, fragmentation et dénaturation à 95°C en une seule étape :

A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH3 (50 mM) et 2 µL du marqueur *méta*-BioPMDAM (100mM dans du DMSO anhydre). Le volume final est ajusté à 100 µL. La solution est ensuite incubée 30 min à 95°C. Dans ce cas, le mélange réactionnel a été hybridé sur puce à ADN sans aucune purification préalable.

b. Marquage et fragmentation à 60°C :

A 10 µL de PCR sont ajoutés 50 µL de tampon formiate de sodium pH3 (50 mM) et 2 µL du marqueur *méta*-BioDPDAM (100mM dans du DMSO anhydre). Le volume final est ajusté à 100 µL. La solution est ensuite incubée 30 min à 60°C. Le mélange réactionnel a été ensuite purifié selon le protocole décrit auparavant. Dans ce protocole et avant hybridation sur puce à ADN, les fragments ont été dénaturés selon le protocole décrit dans l'exemple 12.2.

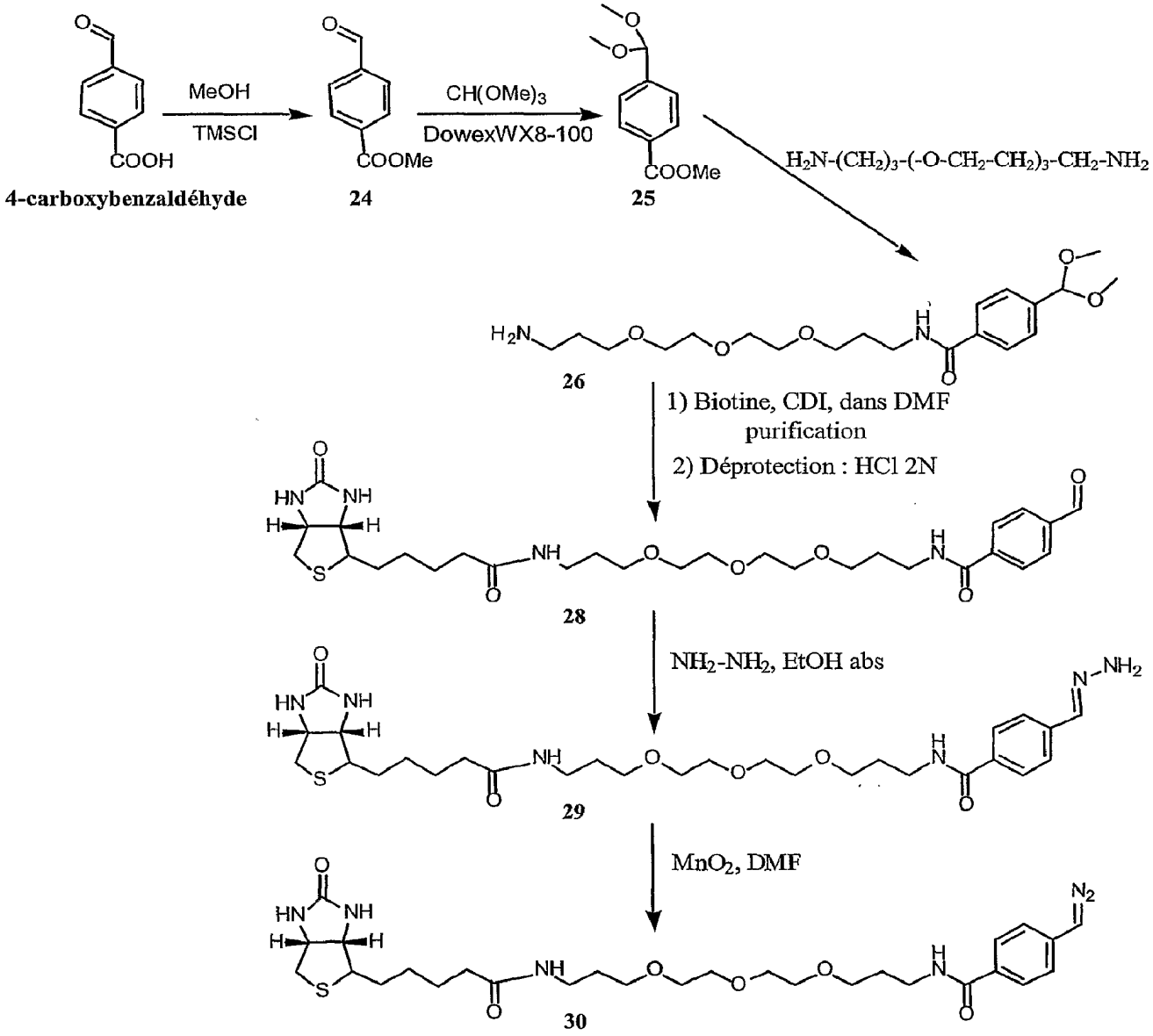
Résultats

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage, fragmentation et dénaturation à 95°C	>95	5426	506	10,7
b. Marquage et fragmentation à 60°C puis dénaturation à 95°C	>95	7015	818	6,8

Tableau 11 : Marquage, fragmentation et dénaturation de l'ADN en une étape

L'exemple 12.2 a démontré l'importance de la dénaturation de l'ADN double brin pour la sensibilité de détection. Ces résultats du tableau 11 montrent qu'avec l'approche fragmentation par création de site abasique, le marquage, la fragmentation et la dénaturation de l'ADN peuvent être réalisées en une seule étape, ce qui représente une amélioration notable du point de vue de la simplicité et du temps pour l'utilisateur et cela sans affecter la sensibilité de détection.

Exemple 20 : Synthèse du *para*-Bio-EG3-PDAM :



- Protection du 4-carboxylbenzaldéhyde :

Le 4-carboxybenzaldéhyde est commercial. On le dissout (3 g ; 20 mmol) dans une solution de chlorure de triméthylsilyle (10 g ; 92 mmol) dans 100 mL de MeOH. Le mélange est laissé sous agitation pendant 40 h à température ambiante. Après évaporation, un solide blanc

correspondant au 4-méthoxycarbonylbenzaldéhyde **24** est isolé, caractérisé par RMN et utilisé tel quel pour la réaction suivante.

RMN ^1H (200MHz, $CDCl_3$) δ =10,07 (s, 1H, -CHO) ; 8,17 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,92 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 3,93 (s, 3H, CO-O-CH_3).

- Protection du 4'-méthoxycarbonylbenzaldéhyde :

Le 4-méthoxycarbonylbenzaldéhyde (3,35 g ; 20 mmol) est mis en solution avec de l'orthoformiate de triméthyle (4,8 g ; 40 mmol) en présence de Dowex 50WX8-100 (1 g). Le mélange est chauffé à reflux pendant 2 h, puis filtré et évaporé. Après un essai de recristallisation, une analyse par RMN montre que la réaction n'est pas complète et celle-ci est relancée dans 30 mL de MeOH, 30 mL de $CH(OMe)_3$ et 1 g de Dowex 50WX8-100 à température ambiante. On réalise une filtration puis une évaporation, afin d'obtenir 3,55 g (16,89 mmol, 84 %) de produit **25**.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,01 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,50 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 5,41 (s, 1H, CH) ; 3,93 (s, 3H, -CO-O-CH_3) ; 3,29 (s, 6H, -O-CH_3).

- Composé **26** :

On solubilise le composé **25** (3,1 g ; 14,8 mmol) dans 16 mL (73 mmol) de 4,7,10-Trioxa-1,13-tridécanediamine. La solution obtenue est chauffée à 140-150°C pendant 2 h. Le mélange est ensuite dissous dans 100 mL de DCM (dichlorométhane ou CH_2Cl_2) et lavé 6 fois avec 10 mL d'eau. La phase organique est séchée avec Mg SO_4 puis évaporée jusqu'à l'obtention d'une huile. Cette huile est lavée avec du pentane 3 fois de suite par décantation, puis une nouvelle extraction avec DCM et H_2O est réalisée. Après séchage sur $MgSO_4$ et évaporation, le produit **26** est isolé avec un rendement de 63 % (9,27 mmol).

RMN ^1H (200MHz, $CDCl_3$) δ = 7,78 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,46 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 5,39 (s, 1H, CH) ; 3,62-3,47 (m, 14H, $H_{7',8',10',11'}$ et $H_{5'}$, $_{13'}$ et $H_{3'}$) ; 3,29 (s, 6H, -O-CH_3) ; 2,72 (m, 2H, $H_{15'}$) 1,87 (m, 2H, $H_{4'}$) ; 1,64 (m, 2H, $H_{14'}$) ; 1,30 (s large, 2H, NH_2).

● Composé biotinylé **27** :

La biotine (500 mg ; 2,05 mmol) est mise en suspension dans 10 mL de DMF puis 365 mg (2,25 mmol) de CDI sont ajoutés. Cette solution est laissée sous agitation pendant 30 min à température ambiante. Le composé **26** (900 mg ; 2,26 mmol) est dissous dans 1 mL de DMF, puis ajouté petit à petit à la solution précédente. Le mélange ainsi obtenu est laissé sous agitation pendant 1 h à température ambiante. Après évaporation, une purification sur colonne par chromatographie-flash (colonne de 20 mm de diamètre) est réalisée avec 250 mL de MeOH-DCM 6 %, puis avec 200 mL de MeOH-DCM 7 % et enfin 200 mL de MeOH-DCM 8 %. Les fractions correspondant au produit **27** sont rassemblées puis évaporées à sec pour donner 1,00 g d'huile avec un rendement estimé à 50 %.

RMN ^{1}H (200MHz, $CDCl_3$) δ = 9,50 (s large, 1H, $NH_{imidazole}$) ; 7,80 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,64 (s, 1H, $H_{imidazole}$) ; 7,46 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$ et 1H, $NH_{2'}$) ; 7,05 (s, 2H, $H_{imidazole}$) ; 6,76 (t, 1H, $NH_{16'}$) , 6,20 (s large, 1H, $N\underline{H}_{B1}$) ; 5,44 (s large, 1H, $N\underline{H}_{B3}$) ; 5,37 (s, 1H, CH) ; 4,42 (m, 1H, H_{B6a}) ; 4,24 (m, 1H, H_{B3a}) ; 3,59-3,44 (m, 14H, $H_{7',8',10',11'}$ et $H_{5',13'}$ et $H_{3'}$) ; 3,29 (m, 8H, $H_{15'}$ et 2-O-CH_3) ;3,07 (m, 1H, H_{B4}) ; 2,84 et 2,66 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,13 (t, 2H, J = 8 Hz, H_{B10}) ;1,85 (m, 2H, $H_{4'}$) ; 1,66 (m, 2H, $H_{14'}$) ; 1,40-1,37 (m, 6H, $H_{B7, B8, B9}$).

● Composé aldéhydique **28** :

L'acétal **27** est dissous dans 50 mL de chloroforme, puis 20 mL de HCl 2N sont ajoutés . Le mélange diphasique est vigoureusement agité pendant 15 min. On récupère la phase organique qu'on sèche sur $NaHCO_3$ anhydre. On filtre, on évapore et le composé **28** est obtenu sous forme d'une pâte (495 mg ; 0,855 mmol) avec un rendement global de 42 % à partir de la biotine.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 10,05 (s, 1H, CHO) ; 7,98 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,92 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 7,58 (t, 1H, $NH_{2'}$) ; 6,46 (t, 1H, $NH_{16'}$) , 6,02 (s large, 1H, $N\underline{H}_{B1}$) ;

5,19 (s large, 1H, N$\underline{H}_{B3}$) ; 4,46 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 3,66-3,56 (m, 10H, $H_{7'}$, $_{8',10',11'}$ et $H_{5'}$) ; 3,50-3,29 (m, 4H, $H_{3',13'}$) ; 3,28 (m, 2H, $H_{15'}$) ; 2,95 (m, 1H, H_{B4}) ; 2,84 et 2,71 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,15 (t, 2H, J = 8 Hz, H_{B10}) ;1,89 (m, 2H, $H_{4'}$) ; 1,72-1,63 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 23 (m, 2H, H_{B8}).

- Composé hydrazone **29** :

L'aldéhyde **28** (495 mg ; 0,855 mmol) est dissous dans 10 mL d'éthanol absolu. L'hydrazine (350 µL ; 7,20 mmol) est ajoutée, puis le mélange réactionnel est chauffé à reflux pendant 1 h. L'huile obtenue après évaporation est dissoute dans EtOH abs, pour être de nouveau évaporée. On obtient alors une mousse qu'on triture au pentane. La pâte correspondant au produit **29** (511 mg ; 0,862 mmol) est obtenue avec un rendement de 100 %.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 7,76 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,72 (s, 1H, CH) ; 7,56 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 7,34 (t, 1H, $NH_{2'}$) ; 6,45 (t, 1H, $NH_{16'}$) , 5,98 (s large, 1H, N$\underline{H}_{B1}$) ; 5,78 (s large, 2H, NH_2) ; 5,18 (s large, 1H, N$\underline{H}_{B3}$) ; 4,44 (m, 1H, H_{B6a}) ; 4,26 (m, 1H, H_{B3a}) ; 3,62-3,56 (m, 10H, $H_{7'}$, $_{8',10',11'}$ et $H_{5'}$) ; 3,48-3,45 (m, 4H, $H_{3',13'}$) ; 3,27 (m, 2H, $H_{15'}$) ; 3,07 (m, 1H, H_{B4}) ; 2,84 et 2,68 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,11 (t, 2H, J = 8 Hz, H_{B10}) ;1,86 (m, 2H, $H_{4'}$) ; 1,72-1,59 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 21 (m, 2H, H_{B8}).

- Composé diazo **30** :

On solubilise l'hydrazone **29** (357 mg ; 0,602 mmol) dans 17,5 mL de DMF. On ajoute alors MnO_2 (700 mg ; 7,7 mmol). Après 12 min d'agitation à température ambiante, le mélange est filtré sur millipore contenant de la célite (épaisseur : 2 cm) et du tamis moléculaire en poudre 3 Å (0,5 cm). Le mélange réactionnel est évaporé à sec. L'huile résiduelle obtenue est lavée à l'éther, trois fois de suite. Le composé **30** (290 mg, 0,491 mmol) est obtenu sous forme d'un solide légèrement rose avec un rendement de 82 %.

RMN ^{1}H (300MHz, DMSO-d_6) δ = 8,28 (t, 1H, $NH_{2'}$) ; 7,77 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,74 (t, 1H, $NH_{16'}$) ; 7,00 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 6,38 (s large, 1H, N$\underline{H}_{B1}$) ; 6,32 (s large, 1H, N$\underline{H}_{B3}$) ; 5,80 (s, 1H, C$\underline{H}$-N_2) ; 4,27 (m, 1H, H_{B6a}) ; 4,11 (m, 1H, H_{B3a}) ; 3,51-3,44 (m, 10H, $H_{7', 8', 10', 11'}$ et $H_{5'}$) ; 3,37 (m, 2H, $H_{15'}$) ; 3,32 (m, 4H, $H_{3',13'}$) ; 3,05 (m, 1H, H_{B4}) ; 2,79 et 2,58 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,02 (t, 2H, J = 8 Hz, H_{B10}) ;1,69 (m, 2H, $H_{4'}$) ; 1,59-1,48 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 25 (m, 2H, H_{B8}).

La réactivité du composé **30** a été testée sur 3'-Uridine-monophosphate et suivie par électrophorèse capillaire. Les conditions d'analyse sont celles de l'exemple 6.1. Les résultats montrent un temps de demi vie de 45 minutes.

Le réactif est stable à -20°C pendant au moins 1 mois.

Exemple 21 : Marquage et fragmentation des amplicons d'ADN avec le réactif de marquage *para*-Bio-EG3-PDAM :

Les intérêts principaux de ce genre de molécules, c'est-à-dire de dérivés de PDAM portant un bras de liaison à base de polyéthylène glycol, sont de permettre l'éloignement de la fonction diazo par rapport à la biotine, et d'augmenter la solubilité et, au final, la réactivité de ces molécules.

Le dérivé *para*-Bio-EG3-PDAM **30** a été obtenu selon le schéma réactionnel décrit dans l'exemple 20. Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Deux réactions de marquage ont été réalisées.

a. Marquage par le réactif *para*-Bio-EG3-PDAM :

A 10 µL de PCR sont ajoutés 10 µL de *para*-Bio-EG3-PDAM (100 mM dans du DMSO) et 77 µL d'eau Dnase/Rnase free. La solution est homogène et ne présente pas de précipités. Cette solution est incubée 10 min à 95°C, puis 3 µL d'HCl à 0,1 M sont ajoutés et la solution est incubée 10 min à 95°C.

Le reste du protocole est identique à celui de l'exemple 8.

b. Marquage par le réactif *méta*-BioPMDAM :

A 10 µL de PCR sont ajoutés 10 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 77 µL d'eau Dnase/Rnase free. La synthèse de ce produit est évoqué dans l'exemple 1.1. La solution présente un léger précipité. Cette solution est incubée 10 min à 95°C. Puis 3 µL d'HCl 0,1 M sont ajoutés et la solution est incubée 10 min à 95°C.
Le reste du protocole est identique à celui de l'exemple 8.

Résultats :

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage par le réactif *para*-Bio-EG3-PDAM	> 95%	15151	621	24,4
b. Marquage par le réactif *méta*-BioPMDAM	> 95%	11226	515	21,8

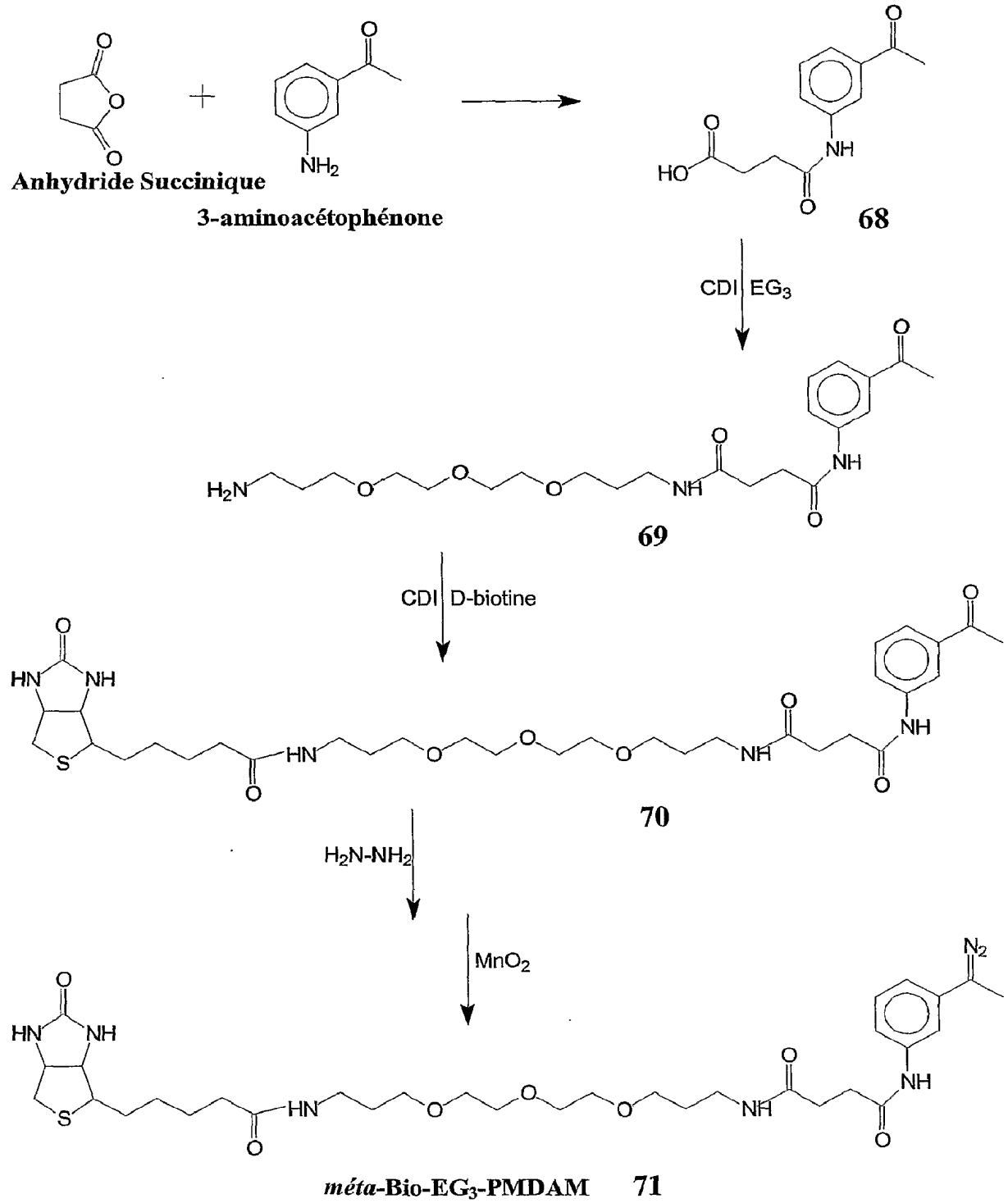
Tableau 12 : Etude comparative du marquage et de la fragmentation d'amplicons d'ADN avec le *para*-Bio-EG3-PDAM et le *méta*-BioPMDAM

Les intensités de signal obtenues dans ce tableau 12 sont très satisfaisantes et le pourcentage d'homologie est élevé. Ce résultat montre que l'introduction d'un bras polyéthylène glycol sur la molécule de marquage diazo permet d'augmenter la solubilité en phase aqueuse du réactif. Le test est donc homogène. De plus, l'augmentation de la solubilité permet d'augmenter la réactivité du marqueur.

Exemple 22 : Synthèse d'autres dérivés PDAM comportant un bras de liaison à base de polyéthylène glycol :

Exemple 22.1 : Synthèse du *méta*-Bio-EG3-PMDAM :

Schéma de synthèse



Composé **68** :

On solubilise la 3-aminoacetophenone (14,5 g, 107 mmol) dans 50 ml de DMF anhydre. On ajoute l'anhydride succinique (10,7 g, 107 mmol) et on laisse sous agitation, sous argon et a température ambiante. Après 6 h, la solution est concentrée sous vide et 50 ml de méthanol sont ajoutés. Le précipité obtenu est filtré et lavé avec méthanol et éther. On obtient ainsi 19,4 g (81%) de produit **68** sous forme d'une poudre de couleur blanc cassé.
RMN ^1H (200 MHz, DMSO-d_6): d = 2,5-2,6 (m, 7H); 7,45 (t, 1H); 7,64 (d, 1H); 7,83 (d, 1H); 8,19 (s, 1H); 10,16 (s, 1H); 12,12 (s, 1H).

Composé **69** :

On solubilise 5,07 g (22 mmol) du composé **68** dans 10 ml de DMF anhydre, sous argon. On met sur glace et on ajoute 5,00 g (32 mmol) de carbonyldiimidazole. Apres 20 min, on ajoute lentement 20 ml (94,6 mmol) du 4, 7, 10-trioxatridecanediamine (EG_3). Après 3h de réaction à température ambiante, on évapore le DMF et reprend le résidu dans 100 ml de CH_2CL_2. On fait des extractions avec du $NaHCO_3$ saturé et H_2O, après quoi la phase organique est séchée avec du Na_2SO_4 anhydre et le solvant évaporé. On obtient ainsi 4,34 g (46%) du produit **69**.
RMN ^1H (200 MHz, DMSO-d_6): d = 1,59 (m, 2H); 1,87 (m, 2H); 2,16 (s, 3H); 2,40 (m, 2H); 2,55 (m, 2H); 3,08 (m, 2H); 3,45 (m, 16H); 7,30 (t, 1H); 7,42 (d, 1H); 7,70 (d, 1H); 7,83 (t, 1H); 7,97 (s, 1H); 10,00 (s, 1H).

Composé biotinylé **70** :

On solubilise la D-biotine (1,0 g, 4,1 mmol) dans 10 ml de DMF anhydre, sous argon. On refroidit sur glace et on ajoute le carbonyldiimidazole (CDI) (0,665 g, 4,1 mmol) dans 10 ml de DMF anhydre. Après 15 min, on ajoute le composé **69** (1,8 g, 4,1 mmol) dans 2 ml de DMF anhydre. On laisse réagir 3 h à 35°C, puis on évapore le DMF et on reprend dans 100ml de CH_2CL_2. On fait des extractions avec du $NaHCO_3$ saturé et H_2O, après quoi la phase organique est séchée avec du Na_2SO_4 anhydre et le solvant évaporé. La caractérisation par

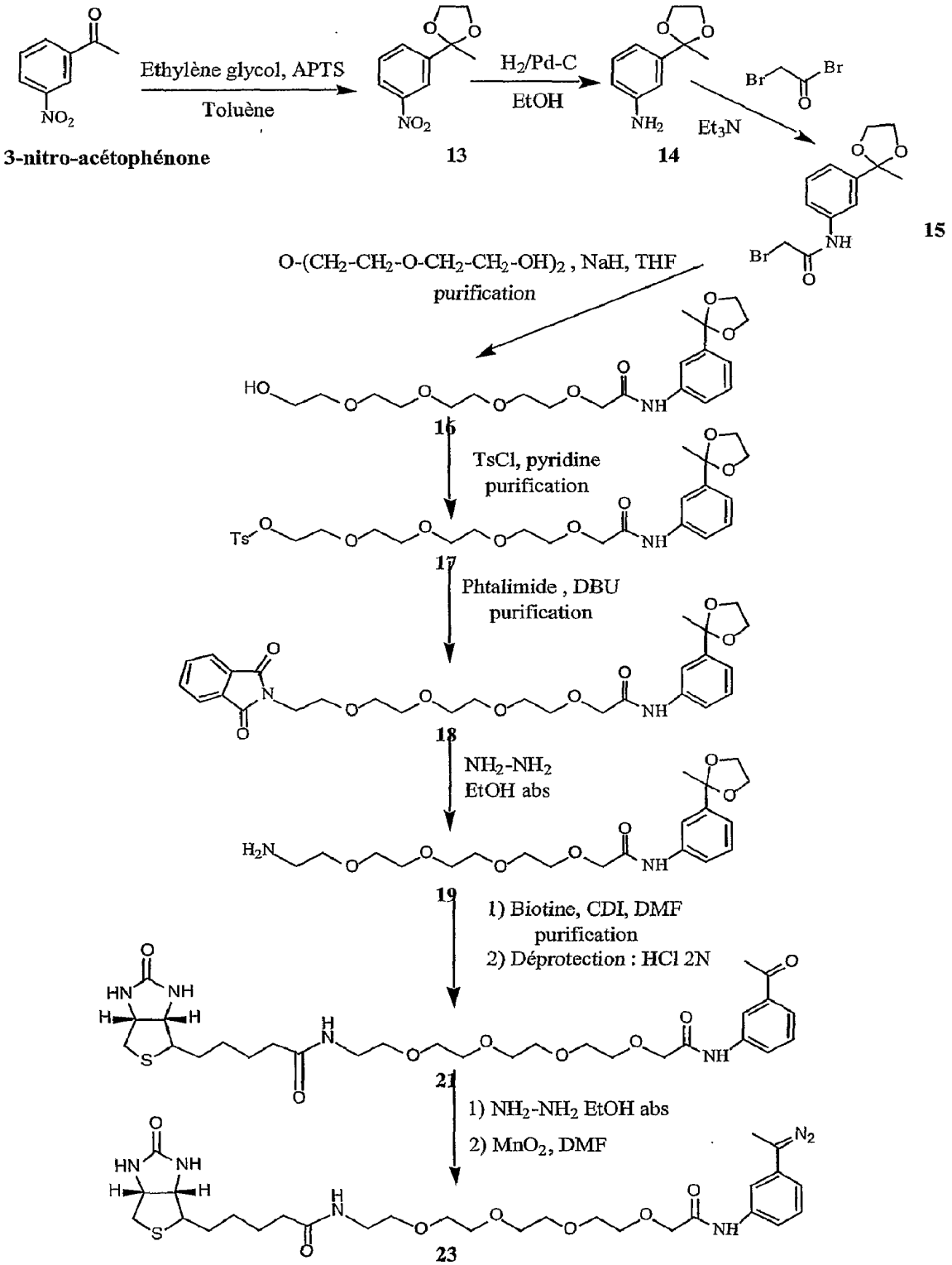
RMN du produit ainsi obtenu montre qu'on obtient un mélange du produit **70** et du EG_3 libre. Une autre étape de purification ets effectuée avant de continuer la synthèse.
Le composé final, *méta*-Bio-EG_3-PMDAM, est obtenu après deux étapes de synthèse selon le schéma décrit dans l'exemple 1.

L'intérêt de cette synthèse est double. D'une part, on obtient le produit **69** en seulement deux étapes; ce produit peut être utilisé comme précurseur du diazo avec la possibilité d'y accrocher des molécules détectables de nature différente, grâce au groupement amine terminal. Ce groupement permet aussi de greffer le composé **69** sur des supports solides, avec l'objectif d'immobiliser des acides nucléiques. D'une autre part, le composé **71** possède le même centre réactif que la *méta*-Bio-PMDAM (notre molécule de référence), ce qui facilite l'analyse des avantages liés à l'inclusion du bras éthylèneglycol (EG_3).

Le réactif est stable à -20°C pendant au moins 1 mois.

Exemple 22.2 : Synthèse du *méta*-Bio-EG4-PMDAM :

Schéma de synthèse :


Ethylène glycol, APTS
Toluène
H_2/Pd-C
EtOH
Et_3N
3-nitro-acétophénone
13
14
15
O-(CH2-CH2-O-CH2-CH2-OH)2 , NaH, THF
purification
16
TsCl, pyridine
purification
17
Phtalimide , DBU
purification
18
NH2-NH2
EtOH abs
19
1) Biotine, CDI, DMF
purification
2) Déprotection : HCl 2N
21
1) NH2-NH2 EtOH abs
2) MnO2, DMF
23

● Protection de la 3-nitro-acétophenone **13** :

On dissout 33 g (0,20 mol) de 3-nitro-acétophénone dans 400 mL de toluène, puis on ajoute 40 mL (0,717 mol) d'éthylène glycol et 600 mg (3,15 mmol) d'acide *para*-toluène sulfonique (APTS). On monte un système Dean Stark. On chauffe la solution pendant 3 h à 130°C. Après avoir laissé la solution revenir à température ambiante, on ajoute 400 mL d'acétate d'éthyle, puis on lave celle-ci avec 8 mL d'une solution de $NaHCO_3$ saturée. On sèche la phase organique sur $MgSO_4$. Après évaporation, on obtient un solide jaune pâle **13** (39,72 g ; 0,190 mol) avec un rendement de 95 %.

RMN ^1H (200MHz, $CDCl_3$) δ =7,11 (t, 1H, J = 8 Hz, Ar-H) ; 6,87-6,78 (m, 2H, Ar-H) ; 6,59 (dd, 1H, J = 6,5 Hz, Ar-H) ; 4,00 (m, 2H, $\underline{H_2}C_{acétale}$) ; 3,79 (m, 2H, $\underline{H_2}C_{acétale}$) ; 1,61 (s, 3H, CH_3).

● Préparation de l'amine **14** :

Le composé **13** (39,7 g ; 0,190 mol) est dissous dans 500 mL d'EtOH, puis 1 g de palladium sur charbon 10 % est ajouté. On chauffe pour tout dissoudre, puis on laisse la solution revenir à température ambiante. Après avoir fait le vide et mis la solution sous H_2, on laisse sous forte agitation pendant 5 h. La solution est ensuite filtrée à chaud puis évaporée. Le produit **14** est lavé au pentane, et isolé sous forme d'un solide (34 g ; 0,189 mol) avec un rendement de 99 %.

RMN ^1H (200MHz, $CDCl_3$) δ =7,14 (t, 1H, J = 8 Hz, Ar-H) ; 6,85 (m, 2H, J = 7,5 Hz, Ar-H) ; 6,79 (s, 1H, Ar-H) ; 6,59 (dd, 1H, J = 6,5 Hz, Ar-H) ; 4,00 (m, 2H, $\underline{H_2}C_{acétale}$) ; 3,77 (m, 2H, $\underline{H_2}C_{acétale}$) ; 1,61 (s, 3H, CH_3).

● Composé bromé **15** :

L'amine **14** (12,3 g ; 68,7 mmol) et la triéthylamine (7 g ; 69 mmol) sont mises en solution dans 150 mL de DCM sous argon. On ajoute à –5 °C au goutte-à-goutte une solution de 13,8 g (60 mmol) de bromure de bromoacétyle dissous dans 150 mL de DCM. À la fin de l'addition,

on ajoute 100 mL de $NaHCO_3$ aqueux 1 N. On lave la phase organique deux fois de suite avec $NaHCO_3$ aqueux et on sèche sur $MgSO_4$. Après évaporation à sec, 22,6 g d'huile marron est obtenue correspondant au composé **15** et utilisée telle quelle pour la réaction suivante.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,29 (s large, 1H, NH) ; 7,62 (dt, 1H_4, J = 5 Hz, Ar-H) ; 7,47 (s, 1H, Ar-H_2) ; 7,38-7,19 (m, 2H, Ar-$H_{5,6}$) ; 4,00 (m, 2H, Br-CH_2) ; 3,75 (m, 4H, H_2C-$H_2C_{acétale}$) ; 1,61 (s, 3H, CH_3).

- Composé alcool **16** :

L'hydrure de sodium (3,3 g ; 82,5 mmol) est lavé trois fois avec du pentane puis mis en suspension dans 150 mL de THF, le tétra éthylène glycol (50 mL ; 0,29 mol) est alors ajouté à température ambiante. On laisse la réaction sous agitation pendant 15 min, puis on refroidit la solution à –5°C.

L'ajout du composé **15** préalablement dilué dans 25 mL de THF se fait au goutte-à-goutte. On laisse sous agitation pendant 30 min pour laisser revenir à température ambiante. On concentre la solution à 100 mL, puis on dilue celle-ci avec 500 mL de $CHCl_3$. On lave trois fois de suite cette phase organique avec 250 mL de $NaHCO_3$ aqueux 1 N, puis on la sèche sur $MgSO_4$ avant de l'évaporer. On purifie le produit sur colonne de silice par chromatographie-flash (colonne de 65 mm de diamètre) avec 1,5 L de MeOH-DCM 5 %, puis avec 500 mL de MeOH-DCM 7 %, et enfin 500 mL de MeOH-DCM 10 %. Les fractions correspondant au composé **16** sont rassemblées, puis évaporées à sec pour donner 17,4 g (42,1 mmol) de produit, avec un rendement de 61 %.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,86 (s large, 1H, NH) ; 7,71 (d, 1H, J = 7,5 Hz, Ar-H_4) ; 7,51 (s, 1H, Ar-H_2) ; 7,29-7,24 (m, 2H, Ar-$H_{5,6}$) ; 4,09 (m, 2H, CO-CH_2-O) ; 3,99 (m, 2H, $H_2C_{acétale}$) ; 3,72-3,53 (m, 20H, O-CH_2-CH_2-O, $H_2C_{acétale}$ et HO-CH_2) ; 1,61 (s, 3H, CH_3).

- Composé tosylate **17** :

L'alcool **16** (4,13 g ;10,0 mmol) est mis en solution dans 5 mL de pyridine. On ajoute ensuite 2,0 g (10,5 mmol) de chlorure de tosyle à température ambiante. On met sous agitation sous

argon pendant 10 h. On dilue avec 100 mL de DCM, on lave trois fois la phase organique avec 20 mL de $NaHCO_3$ aqueux 1 N, puis on la sèche sur $MgSO_4$ avant de la co-évaporer avec du toluène. Une purification sur colonne par chromatographie-flash (colonne de 50 mm de diamètre) est réalisée avec 500 mL de MeOH-DCM 2 %, puis 500 mL de MeOH-DCM 3 %, et enfin 500 mL de MeOH-DCM 4 %. Les fractions correspondant au produit **17** sont rassemblées puis évaporées à sec pour donner 3,68 g (6,48 mmol) d'huile avec un rendement estimé à 65 %.

RMN ^1H (300MHz, $CDCl_3$) δ = 8,86 (s large, 1H, NH) ; 7,76 (d, 4H, J = 5,5 Hz, Ar-H_{tosyl}) ; 7,60 (d, 1H, J = 7,5 Hz, Ar-H_4) ; 7,50 (s, 1H, Ar-H_2) ; 7,32-7,22 (m, 2H, Ar-$H_{5,6}$) ; 4,10 (m, 2H, CO-CH_2-O) ; 4,00 (m, 2H, $H_2C_{acétale}$) ; 3,73-3,54 (m, 20H, O-CH_2-CH_2-O, $H_2C_{acétale}$ et HO-CH_2) ; 2,42 (s, 3H, Ar-CH_3) ; 1,61 (s, 3H, CH_3).

- Composé phtalimide **18** :

On met le tosylate **17** (3,68 g ; 6,48 mmol) en solution avec 1,52 g (10,0 mmol) de DBU (1,8-diazobicyclo-[5.4.0]-undecène) puis on ajoute le phtalimide (1,47 g ; 10 mmol). La solution ainsi obtenue est chauffée à 85-90°C pendant 17 h puis évaporée. On purifie le produit sur colonne de silice par chromatographie-flash (colonne de 50 mm de diamètre) avec 1 L d'acétone-DCM 15 %, puis avec 1 L d'acétone-DCM 20 %. Les fractions correspondant au composé **18** sont rassemblées, puis évaporées à sec pour donner 3,15 g (5,8 mmol) de produit, avec un rendement de 90 %.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,73 (s large, 1H, NH) ; 7,79 (m, 2H, Ar-H_{phta}) ; 7,99 (m, 2H, Ar-H_{phta} et Ar-H_4) ; 7,49 (s, 1H, Ar-H_2) ; 7,27-7,18 (m, 2H, Ar-$H_{5,6}$) ; 4,10 (m, 2H, CO-CH_2-O) ; 4,00 (m, 2H, $H_2C_{acétale}$) ; 3,69-3,56 (m, 20H, O-CH_2-CH_2-O, $H_2C_{acétale}$ et N_{phta}-CH_2) ; 1,61 (s, 3H, CH_3).

● Composé amine **19** :

Le produit **18** est dissous dans 20 mL d'EtOH absolu en chauffant à reflux à 75-80°C. L'hydrazine (1,07 mL ; 22,1 mmol) est ensuite ajoutée, et la réaction est laissée sous agitation pendant 1 h 15. Le précipité obtenu est filtré sur fritté et la phase éthanolique évaporée. Le précipité blanc est ensuite lavé au DCM, et la phase DCM est évaporée. L'huile jaune obtenue (2,3 g ; 5,57 mmol) est directement utilisée pour la réaction suivante, même si elle contient de l'imidazole qui pourra être éliminé par la suite, lors de l'étape de déprotection de l'acétal.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 8,83 (s large, 1H, NH) ; 7,69 (d, 1H, J = 7,5 Hz, Ar-H_4) ; 7,51 (s, 1H, Ar-H_2) ; 7,30-7,19 (m, 2H, Ar-$H_{5,6}$) ; 4,10 (m, 2H, CO-C$\underline{H_2}$-O) ; 4,00 (m, 2H, $\underline{H_2}C_{acétale}$) ; 3,69-3,56 (m, 20H, O-C$\underline{H_2}$-C$\underline{H_2}$-O, $\underline{H_2}C_{acétale}$ et H_2N-C$\underline{H_2}$) ; 1,61 (s, 3H, CH_3).

● Composé biotinylé **20** :

On solubilise la D-Biotine (1,05 g ; 4,32 mmol) dans 10 mL de DMF anhydre. On ajoute sous argon 790 mg (4,87 mmol) de carbonyle diimidazole (CDI). Après 10 min d'agitation, on ajoute l'amine **19** diluée dans 5 mL de DMF. La solution est laissée 40 min sous agitation, puis évaporée avant d'être purifiée sur colonne par chromatographie-flash.
Pour ceci on utilise une colonne de 50 mm de diamètre avec comme éluant 500 mL de MeOH-DCM 5 %, puis 500 mL de MeOH-DCM 10 %, et enfin 500 mL de MeOH-DCM 15 %. Les fractions correspondant au produit **20** sont rassemblées puis évaporées à sec pour donner une huile jaune (1,66 g ; 2,6 mmol).
L'huile jaune obtenue (2,4 g) contenant d'après le spectre RMN environ 30 % en poids d'imidazole. On en déduit donc que le rendement de la réaction aboutissant au produit **20** est d'environ 60 % par rapport à la biotine de départ.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 8,80 (s large, 1H, NH) ; 7,66 (m, 3H, Ar-H_4 et $H_{imidazole}$) ; 7,54 (s, 1H, Ar-H_2) ; 7,28-7,24 (m, 2H, Ar-$H_{5,6}$) ; 7,07 (s, 2H, $H_{imidazole}$), 6,59 (t, 1H, $NH_{15'}$) ; 6,06 (s large, 1H, N$\underline{H}_{B1}$) ; 5,19 (s large, 1H, N$\underline{H}_{B3}$) ; 4,45 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 4,10 (s, 2H, $H_{3'}$) ; 4,00 (m, 2H, $\underline{H_2}C_{acétale}$) ; 3,75-3,49 (m, 18H, O-C$\underline{H_2}$-C$\underline{H_2}$-O et $\underline{H_2}C_{acétale}$,) ;

3,36 (m, 2H, $H_{14'}$) ; 3,09 (m, 1H, H_{B4}) ; 2,85 et 2,66 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,16 (t, 2H, J = 8 Hz, H_{B10}) ; 1,61 (s, 3H, CH_3) ; 1,59-1,3 (m, 6H, $H_{B9, B8, B7}$).

● Composé cétone **21** :

L'acétal **20** est dissous dans 80 mL de chloroforme, puis 30 mL de HCl 2 N sont ajoutés. On laisse sous forte agitation pendant 45 min. La phase organique est récupérée puis séchée sur $NaHCO_3$ anhydre. Après filtration, la solution est évaporée et l'huile obtenue est lavée au pentane pour donner le produit **21** (1,48 g ; 2,48 mmol) avec un rendement de 99 %.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 8,99 (s large, 1H, NH) ; 8,07 (s, 1H, Ar-H_2) ; 7,98 (d, 2H, J = 8 Hz, Ar-H_4) ; 7,66 (d, 2H, J = 8 Hz, Ar-H_6) ; 7,42 (t, 2H, J = 8 Hz, Ar-H_5) ; 6,38 (t, 1H, $NH_{15'}$) ; 5,78 (s large, 1H, N$\underline{H}_{B1}$) ; 4,96 (s large, 1H, N$\underline{H}_{B3}$) ; 4,47 (m, 1H, H_{B6a}) ; 4,29 (m, 1H, H_{B3a}) ; 4,13 (s, 2H, $H_{3'}$) ; 3,76-3,37 (m, 16H, O-C$\underline{H}_2$-C$\underline{H}_2$-O) ; 3,32 (m, 2H, $H_{14'}$) ; 3,11 (m, 1H, H_{B4}) ; 2,89 et 2,75 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,59 (s, 3H, CH_3) ; 2,16 (t, 2H, J = 8 Hz, H_{B10}) ; 1,64-1,40 (m, 6H, $H_{B9, B8, B7}$).

● Composé hydrazone **22** :

La cétone **21** est dissoute dans 20 mL d'EtOH absolu. On chauffe à reflux à 75 –80°C. On ajoute ensuite l'hydrazine (816 µL ; 16,81 mmol) et on laisse sous agitation pendant 3 h. Après filtration, on évapore à sec, on redissout dans l'éthanol jusqu'à l'obtention d'une mousse blanche collante. Dans un deuxième temps, on dissout cette mousse dans 50 mL de chloroforme puis on ajoute 20 mL d'une solution de $NaHCO_3$ saturée. On lave bien puis on récupère la phase organique. On la sèche sur Na_2CO_3 anhydre et après filtration, on évapore à sec afin d'obtenir une nouvelle mousse collante. Celle-ci correspond au produit **22** (842 mg g ; 1,38 mmol) et est obtenue avec un rendement de 66 %.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 8,81 (s large, 1H, NH) ; 8,82 (s, 1H, Ar-H_2) ; 7,64 (d, 2H, J = 8 Hz, Ar-H_4) ; 7,32 (m, 4H, Ar-$H_{5,6}$) ; 6,43 (t, 1H, $NH_{15'}$) ; 5,89 (s large, 1H, N$\underline{H}_{B1}$) ; 5,46 (s

large, 2H, NH_2) ; 4,99 (s large, 1H, $N\underline{H}_{B3}$) ; 4,44 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 4,11 (s, 2H, $H_{3'}$) ; 3,70-3,37 (m, 16H, O-$C\underline{H}_2$-$C\underline{H}_2$-O) ; 3,32 (m, 2H, $H_{14'}$) ; 3,08 (m, 1H, H_{B4}) ; 2,87 et 2,67 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,11 (m, 5H, CH_3 et H_{B10}) ; 1,64-1,40 (m, 6H, $H_{B9, B8, B7}$).

- Composé diazo **23** :

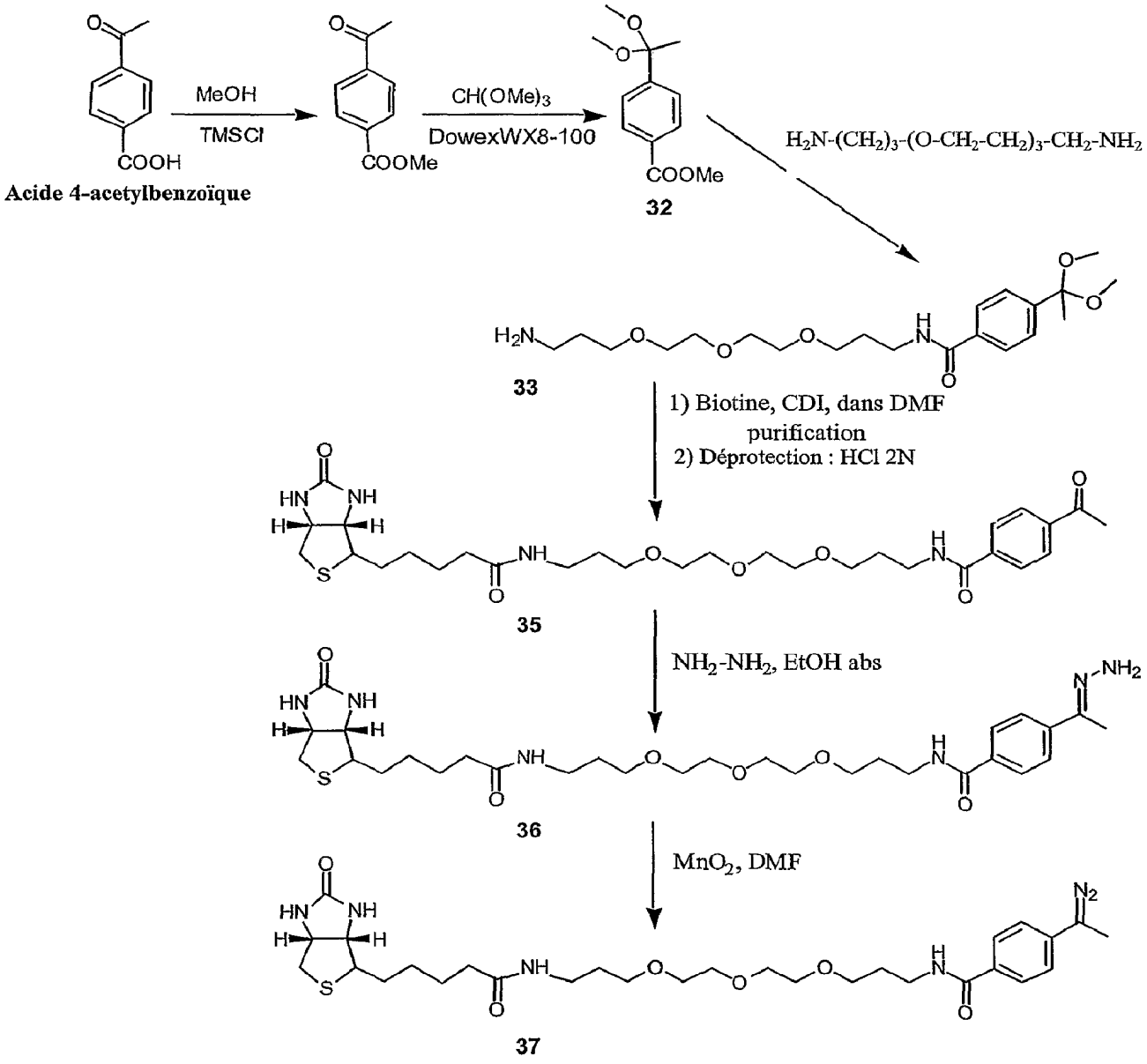
L'hydrazone **22** (100mg ; 0,164 mmol) est dissoute dans 1 mL de DMF sous argon. On ajoute 80 mg de MnO_2 activé et on laisse sous forte agitation pendant 30 min. Le mélange est filtré à travers une couche mixte Célite (3 cm) - tamis moléculaire en poudre (1 cm). La solution est ensuite évaporée à sec. L'huile obtenue en fin d'évaporation est triturée jusqu'à l'obtention d'un poudre rose correspondant au composé **23** (78 mg ; 0,128 mmol ; 78 %).

RMN ^1H (300MHz, DMSO-d_6) δ = 9,60 (s large, 1H, NH) ; 7,89 (s, 1H, Ar-H_2) ; 7,76 (t, 1H, $NH_{15'}$) ; 7,35-7,25 (m, 4H, Ar-$H_{5,6}$) ; 6,64 (d, 2H, J = 8 Hz, Ar-H_4) ; 6,36 (s large, 1H, $N\underline{H}_{B1}$) ; 6,32 (s large, 1H, $N\underline{H}_{B3}$) ; 4,28 (m, 1H, H_{B6a}) ; 4,08 (m, 1H, H_{B3a}) ; 4,06 (s, 2H, $H_{3'}$) ; 3,55-3,31 (m, 16H, O-$C\underline{H}_2$-$C\underline{H}_2$-O) ; 3,17 (m, 2H, $H_{14'}$) ; 3,08 (m, 1H, H_{B4}) ; 2,80 et 2,59 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,13 (m, 5H, CH_3) ; 2,13 (t, 2H, J = 8 Hz, H_{B10}) ; 1,99-1,30 (m, 6H, $H_{B9, B8, B7}$).

La réactivité du composé **23** a été testée sur 3'-Uridine-monophosphate et suivie par électrophorèse capillaire. Les conditions d'analyse sont celles de l'exemple 6.1. Les résultats montrent un temps de demi vie de 30 minutes.
La stabilité du réactif est supérieure à 1 mois à -20°C.

Exemple 22.3 : Synthèse du *para*-Bio-EG3-PMDAM :

Schéma de synthèse :



● Protection de l'acide 4-acétylbenzoïque :

On dissout l'acide 4-acétylbenzoïque (1 g ; 6,1 mmol) dans une solution de chlorure de triméthylsilyle (TMSCl, 10 g ; 92 mmol) dans 5 mL de MeOH. Le mélange est chauffé à 90°C pendant une nuit. Après évaporation, un solide blanc correspondant au composé **31** (1,21 g ; 5,75 mmol) est isolé, caractérisé par RMN et utilisé tel quel pour la réaction suivante.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,08 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,59 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 3,18 (s, 6H, -O-CH_3) ; 1,53 (s, 3H, CH_3).

- Composé **32** :

On dissout le composé **31** (1,21 g ; 5,75 mmol) dans 5 ml d'orthoformiate de triméthyle en présence de Dowex 50WX8-100 (0,3 g). Le mélange est chauffé à 60°C pendant une nuit, puis filtré et évaporé pour donner le composé **32** (1,19 g ; 5,3 mmol) avec un rendement de 87 %.
RMN ^1H (200MHz, $CDCl_3$) δ = 8,00 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,54 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 3,89 (s, 1H, CO-O- CH_3) ; 3,16 (s, 6H, -O-CH_3) ; 1,51 (s, 3H, CH_3).

- Composé **33** :

On solubilise le composé **32** (1,17 g ; 5,22 mmol) dans 5 mL (22,7 mmol) de 4,7,10-trioxa-1,13-tridécanediamine. La solution obtenue est chauffée à 140°C pendant 4 h. Le mélange est ensuite dissous dans 30 mL de DCM et lavé 3 fois avec 10 mL d'eau. La phase organique est séchée avec Mg SO_4, puis évaporée jusqu'à l'obtention d'une huile correspondant au produit **33** (1,44 g ; 3,49 mmol) avec un rendement de 67 %.

RMN ^1H (200MHz, $CDCl_3$) δ = 7,76 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,51 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 3,62-3,47 (m, 14H, $H_{7',8',10',11'}$ et $H_{5',13'}$ et $H_{3'}$) ; 3,15 (s, 6H, -O-CH_3) ; 2,73 (m, 2H, $H_{15'}$) 1,88 (m, 2H, $H_{4'}$) ; 1,65 (m, 2H, $H_{14'}$) ; 1,38 (s large, 2H, NH_2).

- Composé biotinylé **34** :

La biotine (780 mg ; 3,19 mmol) est mise en suspension dans 13 mL de DMF. On ajoute ensuite 590 mg (3,60 mmol) de CDI. Cette solution est laissée sous agitation pendant 30 min à température ambiante. Le composé **33** est dissous dans 1 mL de DMF, puis ajouté petit à petit à la solution précédente. Le mélange ainsi obtenu est laissé sous agitation pendant 1 h à température ambiante. Après évaporation du DMF, une purification sur colonne par chromatographie-flash (colonne de 35 mm de diamètre) est réalisée avec 500 mL de MeOH-

DCM 6 %, puis avec 250 mL de MeOH-DCM 8 %, et enfin 250 mL de MeOH-DCM 8 %. Les fractions correspondant au produit **34** sont rassemblées, puis évaporées à sec pour donner 1,05 g d'huile avec un rendement estimé à 30 %.

RMN ^1H (300MHz, $CDCl_3$) δ = 8,49 (s large, 1H, $NH_{imidazole}$) ; 7,79 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,66 (s, 1H, $H_{imidazole}$) ; 7,50 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 7,38 (t, 1H, $NH_{2'}$) ; 7,11 (s, 2H, $H_{imidazole}$) ; 6,67 (t, 1H, $NH_{16'}$) , 5,99 (s large, 1H, N<u>H</u>$_{B1}$) ; 5,15 (s large, 1H, N<u>H</u>$_{B3}$) ; 4,46 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 3,61-3,45 (m, 14H, $H_{7',8',10',11'}$ et $H_{5',13'}$ et $H_{3'}$) ; 3,28 (m, 2H, $H_{15'}$) ; 3,15 (s, 6H, -OCH_3) ; 2,85 (m, 1H, H_{B4}) ; 2,85 et 2,69 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,14 (t, 2H, J = 8 Hz, H_{B10}) ;1,86 (m, 2H, $H_{4'}$) ; 1,69 (m, 2H, $H_{14'}$) ; 1,49 (s, 3H, CH_3) ; 1,42-1,39 (m, 6H, $H_{B7, B8, B9}$).

- <u>Composé **35** :</u>

L'acétal **34** est dissous dans 45 mL de chloroforme, puis 10 mL de HCl 2N sont ajoutés . Le mélange diphasique est vigoureusement agité pendant 5 min. On récupère la phase organique qu'on sèche sur $NaHCO_3$ anhydre. On filtre, on évapore, et le composé **35** est obtenu sous forme d'un solide jaune clair (504 mg ; 0,87 mmol) avec un rendement global de 27 % à partir de la biotine.

RMN ^1H (300MHz, $CDCl_3$) δ = 7,97 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,91 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 7,51 (t, 1H, $NH_{2'}$) ; 6,50 (t, 1H, $NH_{16'}$) , 6,05 (s large, 1H, N<u>H</u>$_{B1}$) ; 5,23 (s large, 1H, N<u>H</u>$_{B3}$) ; 4,45 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 3,62-3,56 (m, 10H, $H_{7',8',10',11'}$ et $H_{5'}$) ; 3,48-3,46 (m, 4H, $H_{3',13'}$) ; 3,27 (m, 2H, $H_{15'}$) ; 3,10 (m, 1H, H_{B4}) ; 2,85 et 2,71 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,60 (s, 3H, CH_3) ; 2,14 (t, 2H, J = 8 Hz, H_{B10}) ;1,89 (m, 2H, $H_{4'}$) ; 1,72-1,61 (m, 6H, $H_{14'}$, $H_{B7\ B9}$) ; 1, 40 (m, 2H, H_{B8}).

- <u>Composé hydrazone **36** :</u>

La cétone **35** (500 mg ; 0,864 mmol) est dissoute dans 11 mL de EtOH absolu. L'hydrazine (335 µL ; 6,911 mmol) est ajoutée, puis le mélange réactionnel est chauffé à reflux pendant 1

h. L'huile obtenue après évaporation, est dissoute dans EtOH abs, pour être de nouveau évaporée. On obtient alors une mousse collante correspondant au produit **36** (488 mg ; 0,823 mmol) avec un rendement de 95 %.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 7,76 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,67 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 7,29 (t, 1H, $NH_{2'}$) ; 6,46 (t, 1H, $NH_{16'}$) , 5,98 (s large, 1H, N$\underline{H}_{B1}$) ; 5,55 (s large, 2H, NH_2) ; 5,14 (s large, 1H, N$\underline{H}_{B3}$) ; 4,45 (m, 1H, H_{B6a}) ; 4,24 (m, 1H, H_{B3a}) ; 3,62-3,51 (m, 10H, $H_{7', 8',10',11'}$ et $H_{5'}$) ; 3,47-3,45 (m, 4H, $H_{3',13'}$) ; 3,27 (m, 2H, $H_{15'}$) ; 3,07 (m, 1H, H_{B4}) ; 2,84 et 2,69 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,11 (t, 2H, J = 8 Hz, H_{B10} et s, 3H, CH_3) ; 1,86 (m, 2H, $H_{4'}$) ; 1,72-1,59 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 21 (m, 2H, H_{B8}).

- Composé diazo **37** :

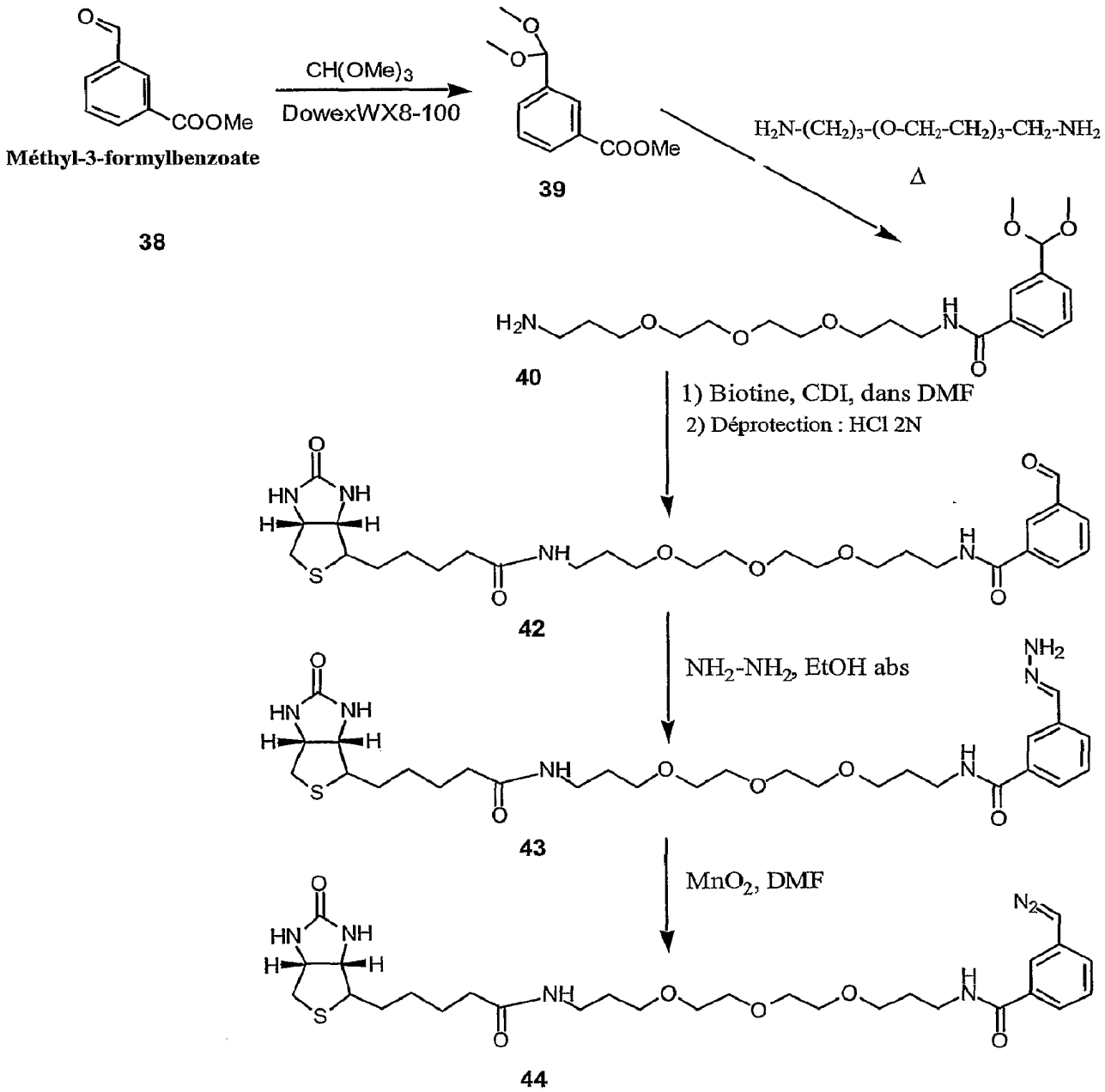
On solubilise l'hydrazone **36** (200 mg ; 0,337 mmol) dans 5 mL de DMF. On ajoute alors MnO_2 (450 mg ; 5,17 mmol). Après 15 min d'agitation à température ordinaire, le mélange est filtré sur millipore contenant de la célite (épaisseur : 2 cm) et du tamis moléculaire en poudre 3 Å (0,5 cm). Le mélange réactionnel est évaporé à sec. L'huile résiduelle obtenue est lavée à l'éther trois fois de suite, jusqu'à l'obtention d'une poudre. Le composé **37** (290 mg, 0,491 mmol) est obtenu sous forme d'un solide rose avec un rendement de 93 %.

RMN ^{1}H (300MHz, DMSO-d_6) δ = 8,33 (t, 1H, $NH_{2'}$) ; 7,83 (d, 2H, J = 8 Hz, Ar-$H_{2,6}$) ; 7,73 (t, 1H, $NH_{16'}$) ; 6,98 (d, 2H, J = 8 Hz, Ar-$H_{3,5}$) ; 6,39 (s large, 1H, N$\underline{H}_{B1}$) ; 6,33 (s large, 1H, N$\underline{H}_{B3}$) ; 4,30 (m, 1H, H_{B6a}) ; 4,12 (m, 1H, H_{B3a}) ; 3,51-3,45 (m, 16H, $H_{7', 8', 10', 11'}$ et $H_{5'}$ et $H_{15'}$ et $H_{3',13'}$) ; 3,07 (m, 1H, H_{B4}) ; 2,79 et 2,58 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,14 (s, 3H, CH_3) ; 2,04 (t, 2H, J = 8 Hz, H_{B10}) ;1,77 (m, 2H, $H_{4'}$) ; 1,62-1,48 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 31 (m, 2H, H_{B8}).

La réactivité du composé **37** a été testée sur 3'-Uridine-monophosphate et suivie par électrophorèse capillaire. Les conditions d'analyse sont celles de l'exemple 6.1. Les résultats montrent un temps de demi vie de 60 minutes.
Le réactif est stable à -20°C pendant au moins 1 mois.

Exemple 22.4 : Synthèse du *méta*-Bio-EG3-PDAM :

Schéma de synthèse :



- Protection du méthyl-3-formylbenzoate **38** :

La résine Dowex 50WX8-100 (2 g) est mise en solution dans 25 mL de MeOH et 25 mL d'orthoformiate de triméthyle puis laissé sous agitation pendant 15 min. Après décantation, on lave la résine deux fois de suite avec 20 mL de MeOH. On met ensuite cette résine dans 100 mL de MeOH, 50 mL de $CH(OMe)_3$ et on ajoute 7,12 g (43,4 mmol) de méthyl-3-formylbenzoate. La solution est laissée 15 min sous agitation, puis filtrée sur papier plissé avant évaporation. Le produit **39** (9 g ; 43,1 mmol) est isolé sous forme d'un liquide jaune clair avec un rendement de 99 %.

RMN ^1H (200MHz, $CDCl_3$) δ = 8,10 (s, H, Ar-H_2) ;7,9 (d, H, J = 8 Hz, Ar-H_4) ; 7,63 (d, H, J = 8 Hz, Ar-H_6) ; 7,42 (t, H, J = 8 Hz, Ar-H_5) ; 5,40 (s, 1H, CH) ; 3,90 (s, 3H, -CO-O-CH_3) ; 3,31 (s, 6H, -O-CH_3).

- Composé **40** :

On solubilise le composé **39** (2 g ; 9,5 mmol) dans 10,4 mL (47,6 mmol) de 4,7,10-Trioxa-1,13-tridécanediamine. La solution obtenue est chauffée à 165°C pendant 2 h. Le mélange est ensuite dissous dans 80 mL de DCM et lavé 4 fois avec 20 mL d'eau. Après séchage sur $MgSO_4$ et évaporation, le produit **40** est isolé avec un rendement de 60 % (2,27 g ; 5,69 mmol).

RMN ^1H (200MHz, $CDCl_3$) δ = 7,84 (s, H, Ar-H_2) ;7,75 (d, H, J = 8 Hz, Ar-H_4) ; 7,53 (d, H, J = 8 Hz, Ar-H_6) ; 7,39 (t, H, J = 8 Hz, Ar-H_5) ; 5,38 (s, 1H, CH) ; 3,64-3,43 (m, 14H, $H_{7',8',10',11'}$ et $H_{5',13'}$ et $H_{3'}$) ; 3,29 (s, 6H, -O-CH_3) ; 2,72 (m, 2H, $H_{15'}$) 1,87 (m, 2H, $H_{4'}$) ; 1,64 (m, 2H, $H_{14'}$) ; 1,30 (s large, 2H, NH_2).

- Composé biotinylé **41** :

La D-biotine (344 mg ; 1,40 mmol) est mise en suspension dans 4 mL de DMF puis 250 mg (1,54 mmol) de CDI sont ajoutés. Cette solution est laissée sous agitation pendant 30 min à température ambiante. Le composé **40** (616 mg ; 1,54 mmol) est dissous dans 2 mL de DMF, puis ajouté petit à petit à la solution précédente. Le mélange ainsi obtenu est laissé sous

agitation pendant 50 min à température ambiante. Après évaporation, une purification sur colonne par chromatographie-flash (colonne de 30 mm de diamètre) est réalisée avec 750 mL de MeOH-DCM 10 %, puis avec 250 mL de MeOH-DCM 15 %. Les fractions correspondant au produit **41** sont rassemblées puis évaporées à sec pour donner 740 mg d'huile avec un rendement estimé à 50 %.

RMN ^{1}H (200MHz, $CDCl_3$) δ = 7,87 (s, H, Ar-H_2) ;7,78 (d, H, J = 8 Hz, Ar-H_4) ; 7,65 (s, 1H, $H_{imidazole}$) ; 7,53 (d, H, J = 8 Hz, Ar-H_6) ; 7,39 (t, H, J = 8 Hz, Ar-H_5) ; 7,07 (s, 2H, $H_{imidazole}$) ; 6,65 (t, 1H, $NH_{16'}$) , 5,95 (s large, 1H, N$\underline{H}_{B1}$) ; 5,38 (s, 1H, CH) ; 5,15 (s large, 1H, N$\underline{H}_{B3}$) ; 4,43 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 3,59-3,44 (m, 14H, $H_{7',8',10',11'}$ et $H_{5',13'}$ et $H_{3'}$) ; 3,29 (m, 8H, $H_{15'}$ et 2-O-CH_3) ;3,07 (m, 1H, H_{B4}) ; 2,84 et 2,66 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,13 (t, 2H, J = 8 Hz, H_{B10}) ;1,85 (m, 2H, $H_{4'}$) ; 1,66 (m, 2H, $H_{14'}$) ; 1,40-1,37 (m, 6H, $H_{B7, B8, B9}$).

- Composé aldéhydique **42** :

L'acétal **41** est dissous dans 20 mL de chloroforme, puis 5 mL de HCl 2N sont ajoutés . Le mélange diphasique est vigoureusement agité pendant 15 min. On récupère la phase organique qu'on sèche sur $NaHCO_3$ anhydre. On filtre, on évapore et le composé **42** est obtenu sous forme d'une huile jaune (593 mg ; 1,02 mmol) avec un rendement global de 87 % à partir de la biotine.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 10,04 (s, 1H, CHO) ;8,34 (s, H, Ar-H_2) ; 8,16 (d, H, J = 8 Hz, Ar-H_4) ; 7,96 (d, H, J = 8 Hz, Ar-H_6) ; 7,72 (t, 1H, $NH_{2'}$) ; 7,39 (t, H, J = 8 Hz, Ar-H_5) ; 6,51 (t, 1H, $NH_{16'}$) ; 6,00 (s large, 1H, N$\underline{H}_{B1}$) ; 5,30 (s large, 1H, N$\underline{H}_{B3}$) ; 4,46 (m, 1H, H_{B6a}) ; 4,27 (m, 1H, H_{B3a}) ; 3,66-3,56 (m, 10H, $H_{7', 8',10',11'}$ et $H_{5'}$) ; 3,50-3,29 (m, 4H, $H_{3',13'}$) ; 3,28 (m, 2H, $H_{15'}$) ; 2,95 (m, 1H, H_{B4}) ; 2,84 et 2,71 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,15 (t, 2H, J = 8 Hz, H_{B10}) ;1,89 (m, 2H, $H_{4'}$) ; 1,72-1,63 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 23 (m, 2H, H_{B8}).

- Composé hydrazone **43** :

L'aldéhyde **42** (593 mg ; 1,02 mmol) est dissous dans 10 mL d'éthanol absolu. L'hydrazine (400 µL ; 8,19 mmol) est ajoutée, puis le mélange réactionnel est chauffé à reflux pendant 50 min. L'huile jaune obtenue après évaporation, est triturée avec de l'éther jusqu'à l'obtention d'une poudre beige correspondant au produit **43** (404 mg ; 0,68 mmol) avec un rendement de 66 %. Une purification sur colonne par chromatographie-flash (colonne de 15 mm de diamètre) est réalisée ensuite sur un échantillon de 150 mg (0,253 mmol) avec 200 mL de MeOH-DCM 20% . Les fractions sont rassemblées puis évaporées à sec pour donner 144 mg de produit **43** avec un rendement de 76 %.

RMN ^{1}H (300MHz, $CDCl_3$) δ = 7,95 (s, H, Ar-H_2) ; 8,16 (d, H, J = 8 Hz, Ar-H_4) ; 7,76 (s, 1H, CH) ; 7,96 (d, H, J = 8 Hz, Ar-H_6) ; 7,38 (t, H, J = 8 Hz, Ar-H_5) ; 6,45 (t, 1H, $NH_{16'}$) ; 5,98 (s large, 1H, $N\underline{H}_{B1}$) ; 5,72 (s large, 2H, NH_2) ; 5,18 (s large, 1H, $N\underline{H}_{B3}$) ; 4,44 (m, 1H, H_{B6a}) ; 4,26 (m, 1H, H_{B3a}) ; 3,62-3,56 (m, 10H, $H_{7', 8',10',11'}$ et $H_{5'}$) ; 3,48-3,45 (m, 4H, $H_{3',13'}$) ; 3,27 (m, 2H, $H_{15'}$) ; 3,07 (m, 1H, H_{B4}) ; 2,84 et 2,68 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,11 (t, 2H, J = 8 Hz, H_{B10}) ;1,86 (m, 2H, $H_{4'}$) ; 1,72-1,59 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 21 (m, 2H, H_{B8}).

- <u>Composé diazo 44 :</u>

On solubilise l'hydrazone **43** (100 mg ; 0,187 mmol) dans 4 mL de DMF. On ajoute alors MnO_2 (200 mg ; 2,3 mmol). Après 13 min d'agitation à température ordinaire, le mélange est filtré sur millipore contenant de la célite (épaisseur : 2 cm) et du tamis moléculaire en poudre 3 Å (0,5 cm). Le mélange réactionnel est évaporé à sec. L'huile résiduelle obtenue est lavée à l'éther, trois fois de suite. Le composé **44** (290 mg, 0,491 mmol) est obtenu sous forme d'un solide orange avec un rendement de 83 %.

RMN ^{1}H (300MHz, DMSO-d_6) δ = 8,39 (t, 1H, $NH_{2'}$) ; 7,78 (t, 1H, $NH_{16'}$) ; 7,39-7,34 (m, Ar-H) ; 7,09 (d, Ar-H) ; 6,38 (s large, 1H, $N\underline{H}_{B1}$) ; 6,32 (s large, 1H, $N\underline{H}_{B3}$) ; 5,78 (s, 1H, C<u>H</u>-N_2) ; 4,27 (m, 1H, H_{B6a}) ; 4,11 (m, 1H, H_{B3a}) ; 3,51-3,44 (m, 10H, $H_{7', 8', 10', 11'}$ et $H_{5'}$) ; 3,37 (m, 2H, $H_{15'}$) ; 3,32 (m, 4H, $H_{3',13'}$) ; 3,05 (m, 1H, H_{B4}) ; 2,79 et 2,58 (système ABX, 2H,

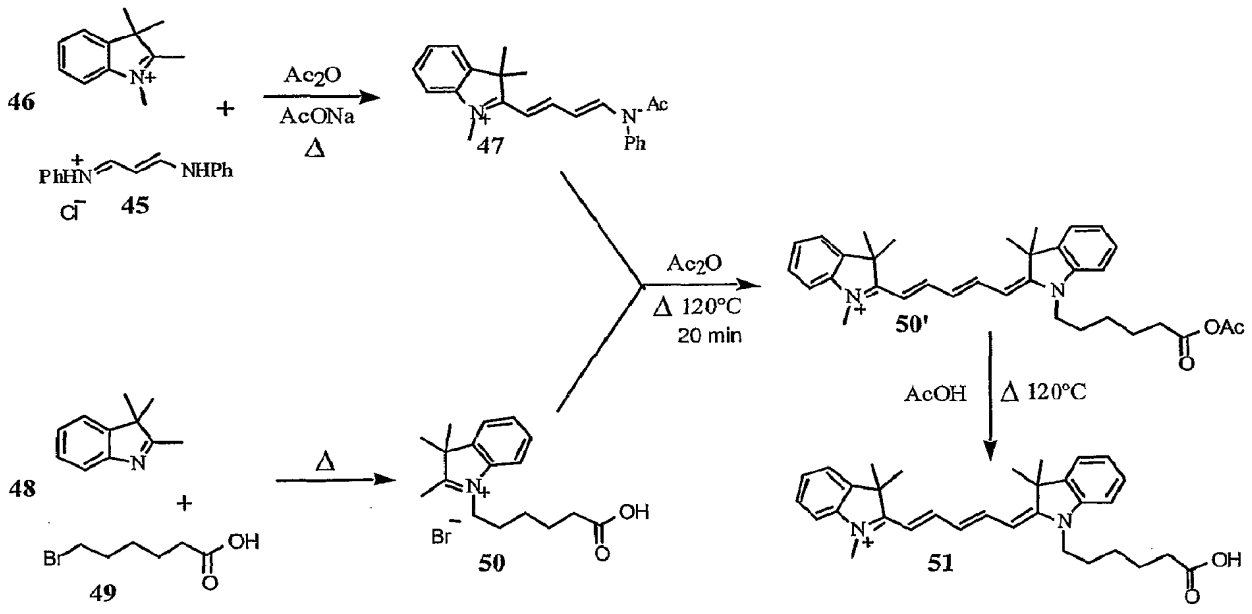
$^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, H_{B6}) ; 2,02 (t, 2H, J = 8 Hz, H_{B10}) ;1,69 (m, 2H, $H_{4'}$) ; 1,59-1,48 (m, 6H, $H_{14'}$, $H_{B7, B9}$) ; 1, 25 (m, 2H, H_{B8}).

La stabilité du produit est supérieure à 1 mois à -20°C.

Exemple 23 : Synthèse du *para*-Cy5-EG3-PDAM :

Comme cela a déjà été évoqué dans l'exemple 2, la biotine peut être remplacée par un autre marqueur tel que le Cy5. Cet exemple montre que l'on peut également relier la fonction diazo, portée par le PDAM, à ce marqueur Cy5 par l'intermédiaire d'un bras de liaison polyéthylène glycol.

Schéma de synthèse : Le contre ion I^- n'est pas représenté sur les formules 46, 47, 50' et 51.



- Iodure de 2-[4-(*N*-acétyl-*N*-phénylamino)buta-1,3-diényl]-1,2,3,3-tétraméthyl[3H]indolium 47 :

Le mélange du monochlorhydrate de malonaldehyde-bis(phénylimine) **45** (13 g ; 50,2 mmol), de NaOAc (6,0 g ; 69,7 mmol) et de l'iodure de 1,2,3,3- tétraméthyl[3H]indolium **46** (3,01 g ; 10 mmol) dans de l'anhydride acétique (50 mL) est chauffé à 100 °C pendant 20 min précisément. Après refroidissement, on ajoute de l'éther (350 mL) et le solide brun précipité est filtré, lavé avec de l'éther (3 × 100 mL). Le solide est redissout dans 150 mL de CH_2Cl_2, filtré (élimination des sels minéraux) puis précipité avec 350 mL d'éther pour donner un solide brun (3,54 g, 54 %).

RMN ^1H ($CDCl_3$) : δ = 8,64 (d ; 1H ; J = 12 Hz ; 1-H) ; 8,14 (t ; 1H ; J = 16 ; 12 Hz ; 3-H) ; 7,63-7,19 (m ; 9H) ; 6,90 (d ; 1H ; J = 15 Hz ; 4-H) ; 5,82 (t ; 1H ; J = 12 ; 13 Hz ; 2-H) ; 4,06 (s ; 3H ; NCH_3) ; (2,16 (s ; 3H ; -$COCH_3$) ; 1,74 (s ; 6H ; CH_3).

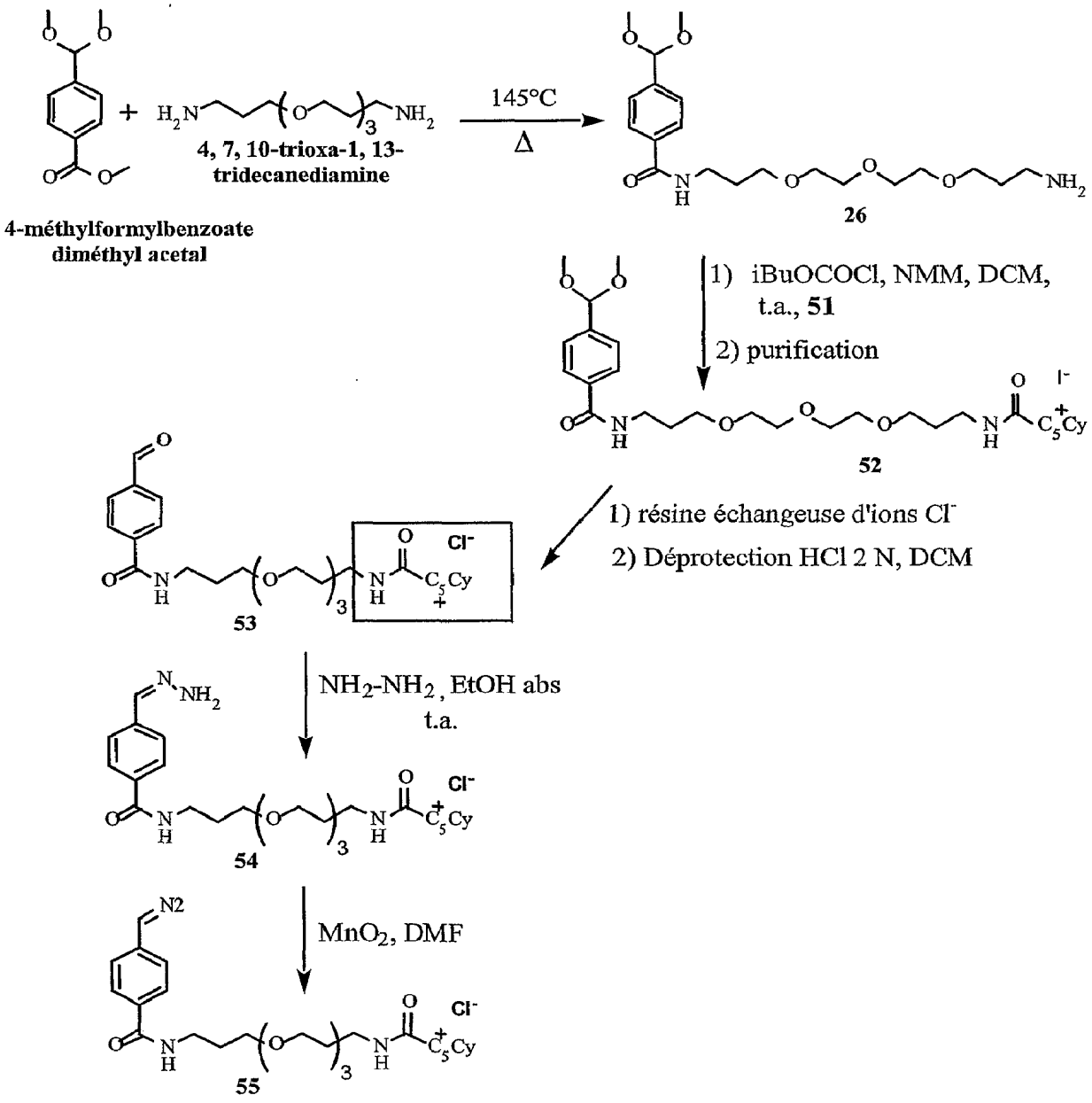
- Bromure de 1-(5-carboxypentyl)-2,3,3-triméthyl[3H]indolium **50** :

On mélange le 2,3,3-triméthylindole **48** (10,0 g ; 62,8 mmole) et l'acide 6-bromohexanoïque **49** (12,3 g ; 62,8 mmole) sans solvant et chauffe à 110 °C pendant 12 h sous argon. Le mélange réactionnel pâteux rouge violet est lavé avec de l'acétate d'éthyle (2 × 60 mL, on triture la pâte avec la spatule et on décante le surnageant), puis avec de l'acétone (50 mL, la pâte solidifie). Le solide rose est filtré puis séché sous vide (16,0 g ; 73 %).

- Composé Cy5COOH **51** :

Le mélange de l'iodure **47** (2,5 g ; 5,3 mmole), du bromure **50** (1,87 g ; 5,3 mmole) et de NaOAc (1,08 g ; 12,1 mmole) dans de l'anhydride acétique (11 mL) est chauffé à 120 °C pendant 25 min. Après refroidissement, on ajoute de l'éther (200 mL) et le précipité est filtré et lavé avec de l'éther (3 × 50 mL). Le solide correspondant au produit **50'** est dissous dans 100 mL de CH_2Cl_2 puis évaporé. On le dissout ensuite dans 15 mL d'acide acétique et l'on agite pendant 30 min à 120 °C. Le précipité correspondant au produit **51** est ainsi obtenu après ajout de 200 mL d'éther et filtration sur fritté, avec un rendement de 84 % (2,71 g ; 4,44 mmol).

RMN ^1H ($CDCl_3$) : δ = 8,03 (t ; 2H ; J = 10 ; 11 Hz, 2-H, 4-H) ; 7,38-6,91 (m ; 9H ; Ar-H, 3-H) ; 6,41 (d ; 1H ; J = 14 Hz ; 1-H) ; 6,31 (d ; 1H ; J = 13 Hz ; 5-H) ; 4,07 (t ; 2H, J = 7 ; 7 Hz ; α-CH_2) ; 3,68 (s ; 3H ; NCH_3) ; 2,47 (t ; 2H, J = 7 ; 7 Hz ; ε-CH_2) ; 1,71 (m ; 18H ; CH_3, β,γ et δ-CH_2).



- Couplage du composé **26** avec le Cy5COOH **51** (produit **52**) :

À une solution de Cy5COOH **51** (1,5 g ; 2,46 mmole) dans 15 mL de CH_2Cl_2, on ajoute *N*-méthylmorpholine (NMM, 405 µL ; 3,68 mmole). La solution est refroidie avec un bain de glace et mise sous argon, puis on ajoute le chloroformiate d'isobutyle (494 µL ; 3,81 mmole). Après 10 min d'agitation, on additionne l'amine **26** (1,86 mg ; 4,67 mmole) diluée dans 8 mL de CH_2Cl_2. On laisse le mélange sous agitation à température ambiante pendant 1 h 30. On ajoute 20 mL de CH_2Cl_2 et on lave avec 25 mL de $NaHCO_3$ (1 N) trois fois de suite. Après séchage sur Na_2CO_3, on filtre la solution pour récupérer la phase dichlorométhane que l'on évapore.

Une purification sur colonne par chromatographie-flash (colonne de 45 mm de diamètre, fractions de 20 mL)) est réalisée avec comme éluant du MeOH-DCM 10% . Les fractions correspondant au produit **52** sont rassemblées puis évaporées à sec pour donner un solide bleu qui est dissous dans CH_2Cl_2. Le produit **52** est alors précipité et lavé à l'éther pour donner un produit bleu avec un rendement de 72 % (1,45 g ; 1,77 mmol).

Le produit **52** (iodure) est ensuite dissous dans 54 mL de méthanol puis passé sur colonne d'amberlite IRA900 (Cl^- ; 15 g). La solution méthanolique recueillie est évaporée à sec pour donner une huile collante que l'on redissout dans CH_2Cl_2 . L'évaporation permet l'obtention du produit **52'** avec un rendement de 87 %.

● Aldéhyde **53** :

L'acétal **52'** est dissous dans 10 mL de DCM, puis 10 mL de HCL 2N sont ajoutés. La solution est laissée sous forte agitation pendant 3h 30 min. Après ajout de 20 mL de DCM, la phase dichlorométhane est récupérée puis séchée sur $NaHCO_3$. Le produit obtenu après évaporation est lavé avec de l'éther pour donner l'aldéhyde **53** avec un rendement de 90 % (1,18g ; 1,46 mmol).

● Hydrazone **54** :

On dissout l'aldéhyde **53** (200 mg ; 0,247 mmole) dans 1 mL d'éthanol absolu et on ajoute l'hydrazine monohydratée (15,6 µL ; 0,321 mmole). La solution est agitée à température

ambiante pendant 30 min. On ajoute 8 mL d'éther ; on lave à l'éther par décantation, trois fois de suite puis on sèche sous vide. On obtient 172 mg d'hydrazine **54** (0,209 mmol ; 85 % de rendement) que l'on conserve au congélateur.

● Diazo **55** :

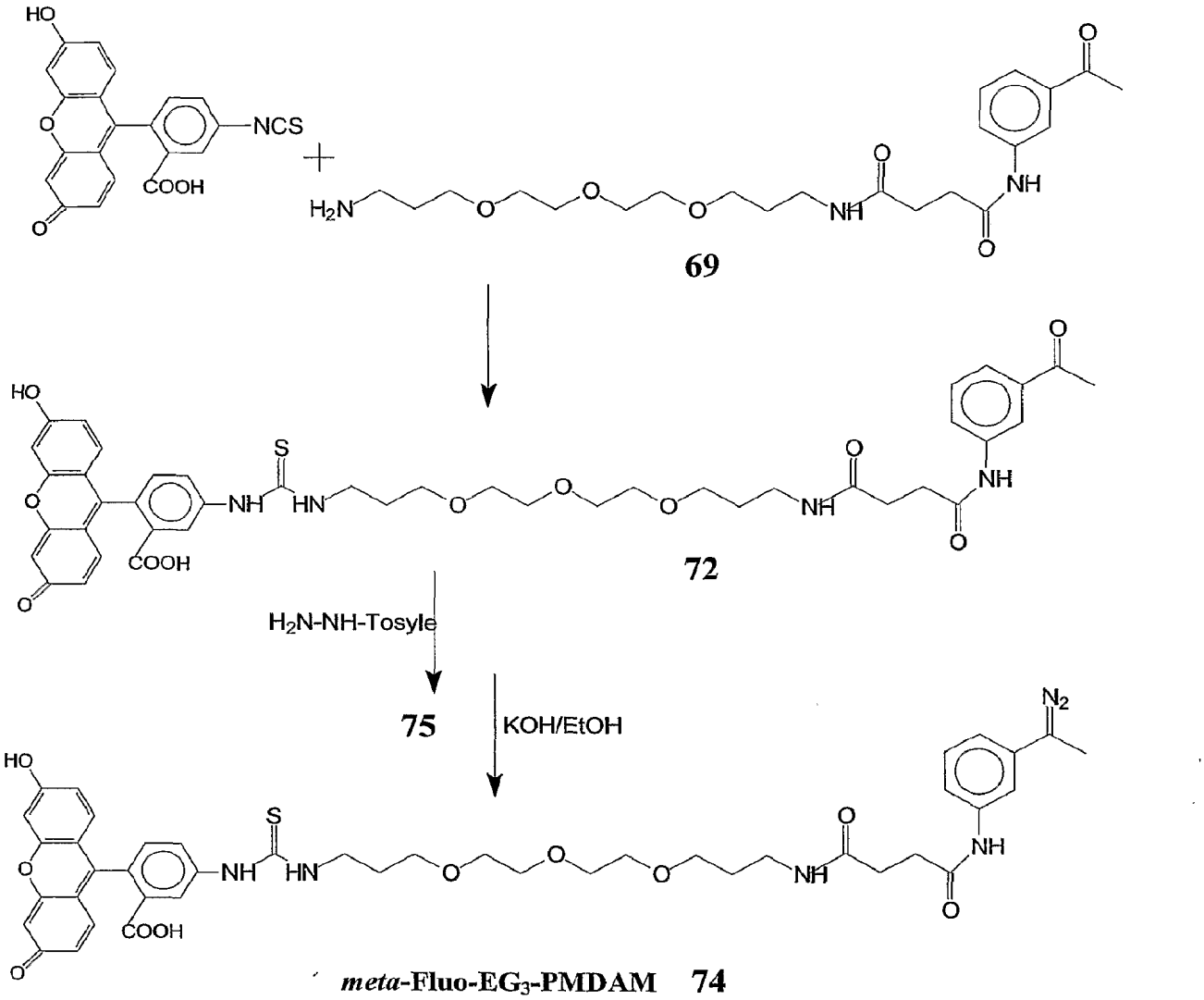
À une solution de 20 mg (0,243 mmol) d'hydrazone **54** dans 2 mL de DMF, on ajoute 100 mg de MnO_2 et agite vigoureusement pendant 5 min sous argon à température ambiante. On filtre la suspension à travers une couche du célite (épaisseur : 2 cm) et du tamis moléculaire en poudre 3 Å (0,5 cm) et lave avec DMF. On évapore la solution puis on triture à l'éther. Le solide ainsi obtenu est séché. On obtient 18 mg (0,185 mmol ; 76%) de diazo **55.**

La stabilité du réactif est supérieure à 1 mois à -20°C.

Exemple 24 : Synthèse du *méta*-Fluo-EG3-PMDAM :

Comme cela a déjà été évoqué dans l'exemple 23, la biotine peut être remplacée par un autre marqueur. Cet exemple montre que l'on peut également relier la fonction diazo, portée par le PMDAM, à ce marqueur fluorescéine par l'intermédiaire d'un bras de liaison polyéthylène glycol.

Schéma de synthèse :



Composé **72** :

On solubilise l'isothiocyanate de fluorescéine- (250 mg, 0,64 mmol) dans 1,6 ml de DMF anhydre, avec 2% de pyridine, sous argon. On ajoute le produit **69** (0,356 g, 0,81 mmol) dissout dans 1,6 ml de DMF anhydre. On laisse réagir 3,5 h à température ambiante, puis on évapore le DMF et on reprend dans 25 ml de H_2O. On fait trois extractions avec 50 ml de CH_2Cl_2, et la phase aqueuse est évaporée. On obtient 255 mg (48%) du produit **72**.

Composé *méta*-Fluo-EG_3-Hydrazone-Tosyle **75** :

On dissout le composé **72** (255 mg, 0,31 mmol) dans 1,5 ml d'éthanol à reflux. On ajoute la *p*-toluen-sulfonyl-hydrazine (69,2 mg, 0,37 mmol) dans 1,5 ml d'éthanol et on laisse réagir pendant 6 h. On évapore a sec, et on lave le solide avec CH_2Cl_2, H_2O et éther. On obtient 18,5 mg (74%) du produit **75** sous forme d'une poudre orange.
RMN ^1H (200 MHz, DMSO-d_6): d = 1,6-1,8 (m, 4H); 2,13 (s, 1H); 2,28 (s, 1H); 2,36 (s, 1H); 2,80 (m, 1H); 3,07 (m, 2H); 3,46 (m, 12H); 6,5-6,7 (m, 6H); 7,1-8,3 (m, 9H).

Composé *méta*-Fluo-EG_3-PMDAM **74** :

On solubilise l'hydrazone **75** (176 mg, 0,18 mmol) dans 720 µl d'une solution de KOH à 10% dans du méthanol anhydre. La solution est laissée a reflux pendant 3 h. On laisse refroidir la solution et un précipité apparaît. La solution est filtrée et évaporée a sec. Le résidu est lavé a l'éther et séché.
L'analyse par RMN montre la disparition des signaux a 2,36 et 2,13 ppm (correspondant aux Méthyles du tosyle et de l'hydrazone) et l'apparition d'un pic à 1,96 ppm (correspondant au méthyle du diazo).

Exemple 25 : Intermédiaire diazométhyle permettant un marquage ultérieur :

Il peut être intéressant d'avoir non pas un marquage direct par le réactif de marquage diazométhyle portant aussi le marqueur R^2 mais de procéder en deux étapes avec un marquage indirect. Dans ce cas le réactif de marquage comprenant la fonction diazométhyle est dit préfonctionnalisé c'est à dire qu'il comprend aussi une fonction chimique apte à réagir ultérieurement avec des marqueurs directs ou indirects. La pré-fonctionnalisation peut intervenir par introduction d'une fonction réactive de covalence au sein du réactif de marquage qui peut réagir avec une fonction anti-réactive de covalence du marqueur direct ou indirect. Ces fonctions peuvent être constituées par une fonction chimique organique électrophile et une fonction chimique organique nucléophile, ou inversement.

Un exemple d'une telle stratégie de marquage est illustrée par le schéma ci-dessous



Dans lequel le réactif de marquage comprend, outre une fonction diazométhyle, une fonction W^1 électrophile ou nucléophile susceptible de réagir avec un marqueur R^2 comprenant une fonction W^2 complémentaire de W^1.

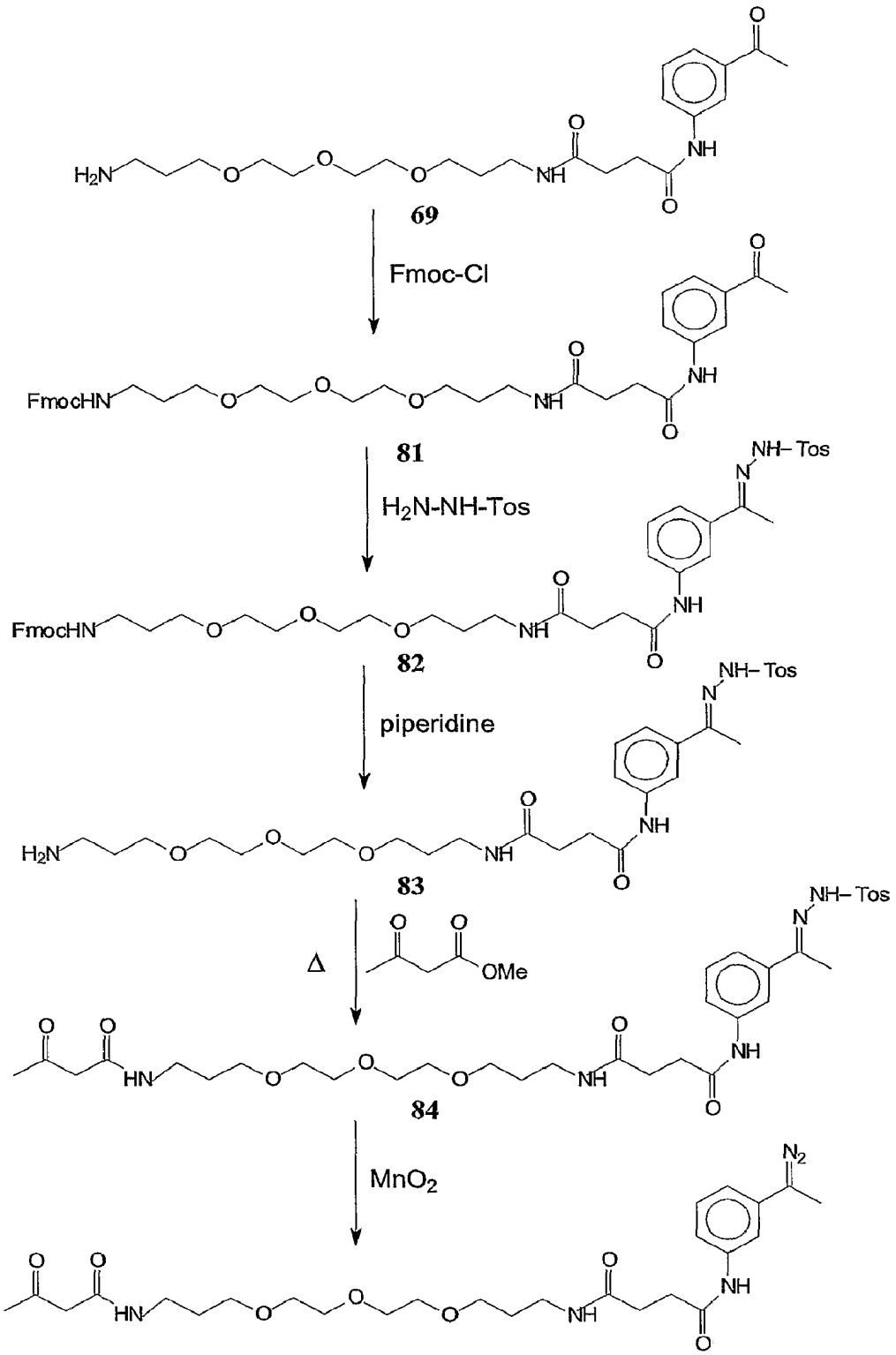
Par exemple si W^1 est une fonction méthylcétone ou aldéhyde, W^2 est une fonction alcoxyamine.

Dans un procédé de marquage d'une molécule biologique comme un acide nucléique, l'acide nucléique est mis en contact avec le réactif de marquage coprenant la fonction diazométhyle et dans une étape ultérieure, le marqueur W^2- R^2 réagit avec l'acide nucléique par l'intermédiaire de la fonction W^1.

L'une des utilisations est par exemple constituée par un procédé d'amplification d'une séquence d'un acide nucléique ou par un procédé d'amplification du signal. Des informations complémentaires sur ce type de marquage peuvent être trouvées dans la demande de brevet WO-A-98/05766, sous priorité du 2 août 1996. et dans la demande de brevet WO-A-00/40590 sous priorité du 05 Janvier 1999.

Exemple 25.1 : Synthèse du MeCO-PMDAM :

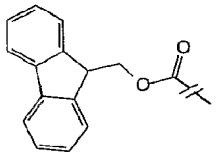
Schéma de synthèse :



MeCO-PMDAM **85**

Le produit **85**, dont la synthèse est décrite dans cet exemple, permet de réaliser le marquage des acides nucléiques naturels, grâce à la réactivité de la fonction diazométhyl avec les groupements phosphate, et d'introduire ainsi une fonction méthylcétone, qui peut être utilisée par la suite pour introduire une molécule détectable (fluorescente, biotine) possédant un groupement alcoxyamine.

Cette synthèse est basée sur des méthodes connues et utilisées en routine en chimie. Le produit de départ est le même que pour la synthèse des marqueurs **71** et **74**. La première étape consiste en la protection de l'amine terminale par le Fluorenylmethyl-formate (Fmoc, **99**). Le choix de ce groupement protecteur est basé sur sa stabilité et conditions de clivage.

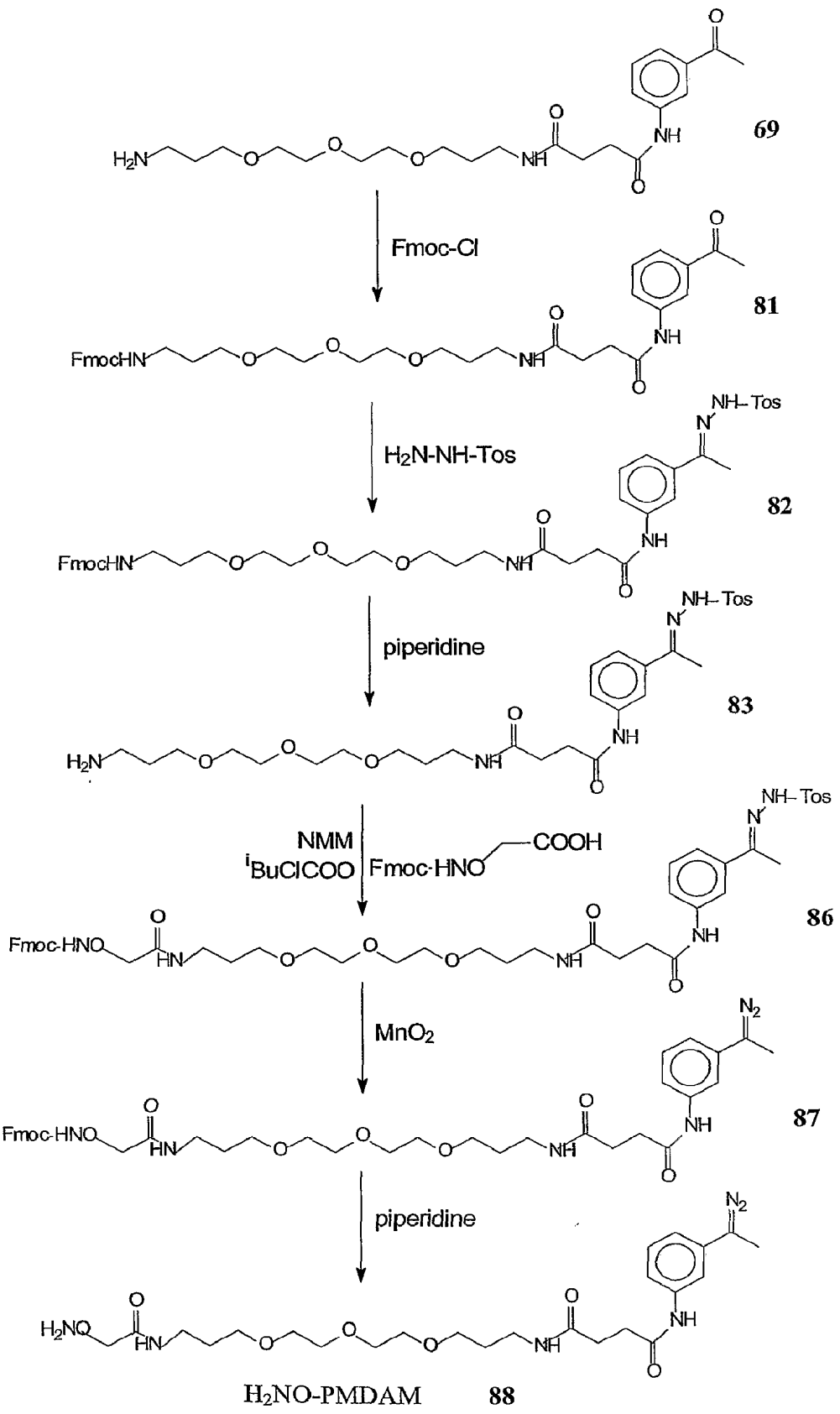


99

Après formation de l'hydrazone protégée **82** par la méthode utilisée précédemment (exemple *méta*-Fluo-EG_3-PMDAM,), l'amine terminale est déprotégée dans des conditions basiques douces qui garantissent la stabilité de l'hydrazone. L'acétoacétate de méthyle est utilisé pour créer la fonction méthylcetone par une réaction d'acylation de l'amine terminale (voir formation des composés **26** et **36**). La formation du diazométhyl est ensuite réalisée par l'une des méthodes décrites précédemment.

Exemple 25.2 : Synthèse du H_2NO-PMDAM :

Schéma de synthèse :


69
Fmoc-Cl
81
H2N-NH-Tos
82
piperidine
83
NMM
iBuClCOO
Fmoc-HNO
COOH
86
MnO2
87
piperidine
H2NO-PMDAM
88

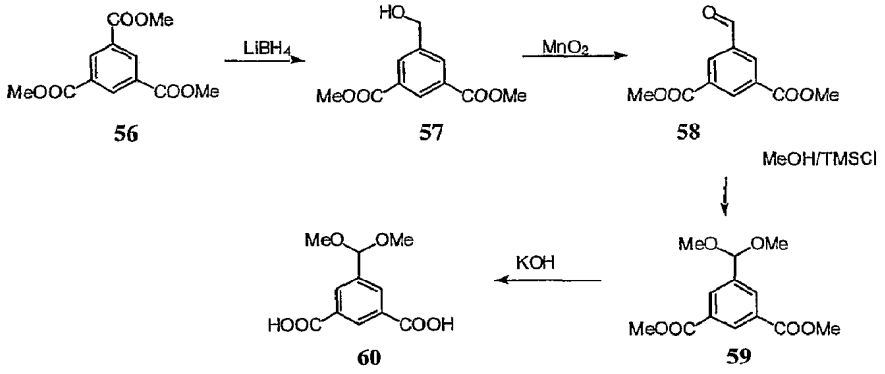
Le produit **88**, dont la synthèse est décrite dans cet exemple, permet de réaliser le marquage des acides nucléiques naturels, grâce a la réactivité de la fonction diazométhyl avec les groupements phosphate, et d'introduire ainsi une fonction alcoxyamine, qui peut être utilisée par la suite pour introduire une molécule détectable (fluorescente, biotine) possédant un groupement méthylcétone.

Cette synthèse est basée sur le modèle de la précédente, c'est-à-dire l'utilisation du précurseur **69**, du Fmoc pour la protection de l'amine et du tosyle pour la protection de l'hydrazone. L'introduction de la fonction alcoxyamine (composé **86**) se fait par l'usage du carboxyméthoxylamine (commercial) protégé par la fonction Fmoc (Thèse E. Trévisiol, LEDSS Grenoble, 1999). Etant donné les conditions douces de la déprotection finale (composé **88**), celle ci est réalisée de suite après la formation du diazométhyl.

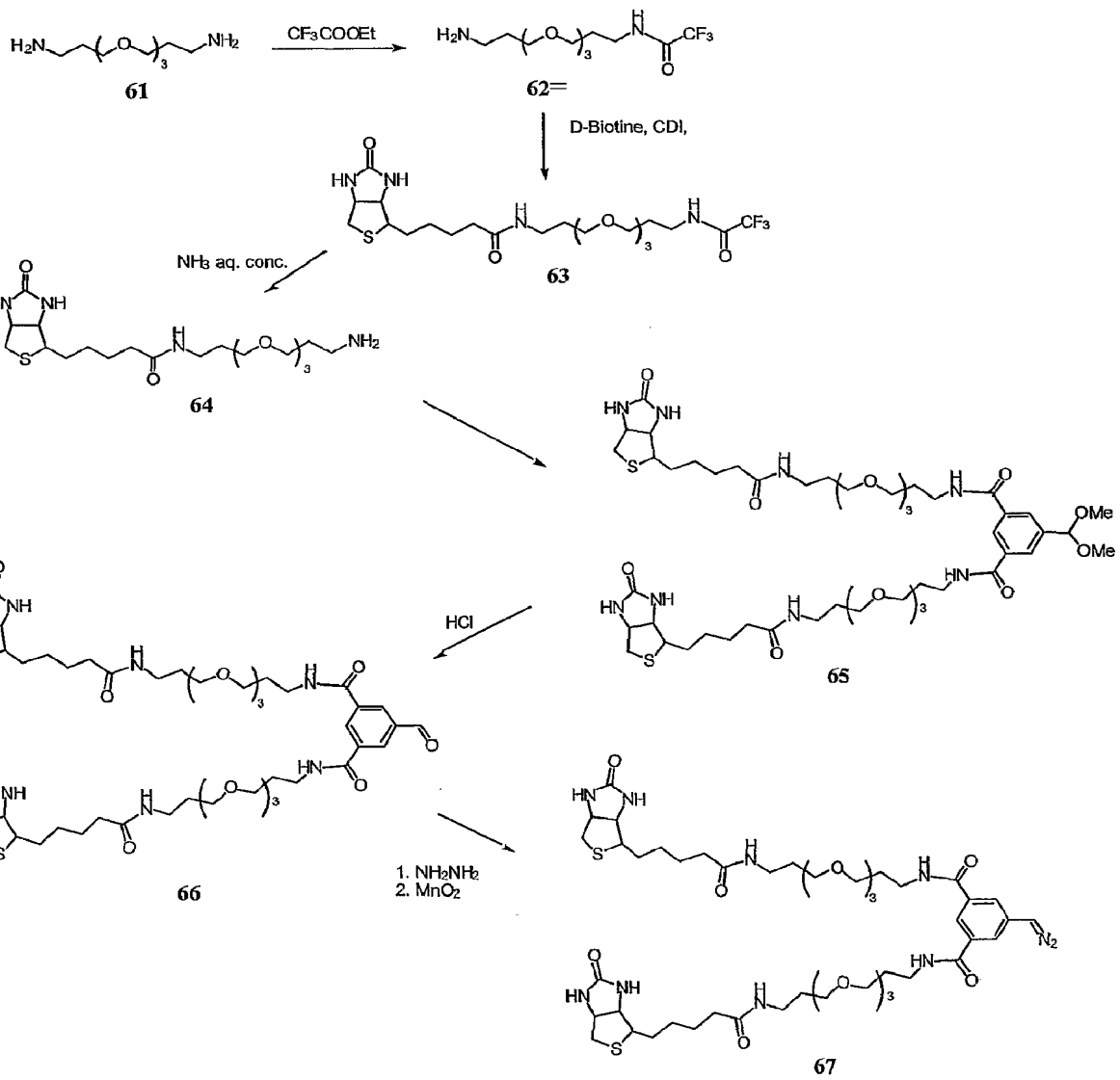
Exemple 26 : Préparation de dérivés PDAM permettant l'amplification du signal :

Exemple 26.1 : Synthèse de marqueurs bis-biotinylés tels que le [Bio-EG3]2-PDAM :

Schéma de synthèse :



56 57 58 59 60



61 62= 63 64 65 66 67

● Réduction du benzène-1,3,5-tricarboxylate de triméthyle **56** en alcool **57** :

On dissout le triester **56** (12,6 g ; 50,0 mmol) dans 100 mL de THF puis ajoute 1,1 g (50,5 mmol) de $LiBH_4$ à température ambiante. La solution rouge est chauffée à 40-45°C sous agitation sous argon pendant 1 h. Après refroidissement (glace), on détruit l'hydrure en excès avec précaution (dégagement de H_2) par l'addition d'eau (200 mL) puis de HCl 2N (30 mL). On observe le virage de couleur en jaune claire. On extrait cette solution avec CH_2Cl_2 (100mL puis 3 fois 50 mL), la phase organique est lavée avec $NaHCO_3$ anhydre, séchée sur $MgSO_4$ puis le solvant est évaporé jusqu'à l'obtention d'une huile (11,1 g). Par chromatographie flash sur colonne de silice (diamètre = 40 mm, éluant : acétate d'éthyle/cyclohéxane = 1/1), on obtient l'alcool **57** (6,38 g, 57 %).

RMN ^1H (200 MHz, $CDCl_3$) : δ = 8,53(t, 1H, J = 2 Hz) ; 8,18 (d, 2H, J = 2 Hz) ; 4,76 (s, 2H) ; 3,91 (s, 6H) ; 2,30 (s, 1H).

● Oxydation de l'alcool **57** en aldéhyde **58** :

On dissout l'alcool **57** (5,86 g ; 26,1 mmol) dans 100 mL de THF puis ajoute 40,0 g de MnO_2 petit à petit en 5 min à température ambiante. La solution est laissée une nuit sous agitation sous argon. On filtre la solution à travers un entonnoir Büchner muni d'une couche de Célite 545, on lave avec CH_2Cl_2 puis évapore les solvants. Le solide brut (4,4 g) est purifié par chromatographie flash sur une colonne de silice (diamètre = 50 mm, éluant : acétate d'éthyle/cyclohéxane 3/7). On obtient 3,44 g (59 %) de l'aldéhyde **58**.

RMN ^1H (200 MHz, $CDCl_3$) : δ = 10,11 (s, 1H) ; 8,89(t, 1H, J = 1 Hz) ; 8,69 (d, 2H, J = 1 Hz) ; 3,98 (s, 6H).

● Formation de l'acétal **59** :

On dissout l'aldéhyde **58** (3,21 g ; 14,4 mmol) dans 30 mL de méthanol puis ajoute 6,0 mL de TMSCl. La solution est laissée à température ambiante sous agitation sous argon pendant 1 h.

On dilue la solution avec 200 mL de CH_2Cl_2, et agite avec 1 M $NaHCO_3$ (100 mL) (attention dégagement de CO_2). On sépare les deux phases, extrait la phase aqueuse trois fois avec CH_2Cl_2 (25 mL), on rassemble les phases organiques, on sèche sur $MgSO_4$ puis évapore le solvant. On obtient 3,55 g (92 %) de l'acétal **59**.

RMN ^1H (200 MHz, $CDCl_3$) : δ = 8,63(t, 1H, J = 2 Hz) ; 8,29 (d, 2H, J = 2 Hz) ; 5,45 (s, 2H) ; 3,93 (s, 6H) ; 3,32 (s, 6H).

- Hydrolyse du diester **59** en diacide **60** :

On dissout le diester **59** (3,18 g ; 11,9 mmol) dans 10 mL de THF puis ajoute une solution de KOH (2,0 g, pastille 85 %) dans 10 mL de méthanol. Après 15 min à température ambiante, on évapore les solvants. Le résidu est dissous dans H_2O (50 mL). On ajoute H_3PO_4 (environ 2,5 mL, 85 %) jusqu'à pH 3 et le précipité blanc est filtré sur le fritté (#3), lavé avec de l'eau et séché sous vide. On obtient 2, 59 g (91 %) du diacide **60**.

RMN ^1H (200 MHz, DMSO-d_6) : δ = 8,43 (t, 1H, J = 1 Hz) ; 8,15 (d, 2H, J = 1 Hz) ; 5,53 (s, 1H) ; 3,27 (s, 6H).

- Trifluoracétamide **62** :

On dissout la diamine **61** (66 g ; 0,30 mol) dans 250 mL de CH_2Cl_2 puis ajoute le trifluoracétate d'éthyle (11,8 mL, 0,10 mol) goutte à goutte en 5 min à 10 °C sous agitation sous argon. Après 15 min à température ambiante, on transfère la solution dans une ampoule à décanter, on lave avec H_2O (3 × 100 mL), on sèche sur $MgSO_4$ et évapore le solvant. On obtient 22,4 g (71 %) du monoamide **62** de pureté d'environ 85 % (déterminée par F^{19} RMN). Ce composé est conservé à –20 °C et utilisé sans purification.

RMN ^1H (200 MHz, $CDCl_3$) : δ = 3,5-3,6 (m, 12H) ; 3,42 (t, 2H, J = 6 Hz) ; 2,75 (t, 2H, J = 6 Hz) ; 1,81 (quantiplet, 2H, J = 6 Hz) ; 1,67 (quantiplet, 2H, J = 6 Hz) ; 1,30 (s. large, 2H).

RMN ^{19}F (190 MHz, $CDCl_3$) : δ = –76,3.

● Composé **63** :

A une suspension de D-biotine (6,39 g ; 26,2 mmol) dans 50 mL de DMF, on ajoute le carbonyldiimidazole (CDI, 6,32 g , 80 %, 31,2 mmol). Le mélange est chauffé à 55-60 °C sous agitation sous argon pendant 30 min. On observe initialement une dissolution complète de matériel puis une prise en masse avec une précipitation de solide blanc (CO_2 évolution). On ajoute l'amine (huile) à l'aide de 5 mL de CH_2Cl_2 pour rincer et le mélange est chauffé à 55-60 °C pendant 3 h. On évapore le DMF sous vide (< 1 mmHg) et le résidu est agité avec CH_2Cl_2 (700 mL) et 2N HCl (100 mL). Après une filtration des deux phases à travers une couche de Célite 545, on sépare les phases, la phase aqueuse est extraite avec CH_2Cl_2 (15 × 100 mL), les phases organiques sont rassemblées, séchées sur $NaHCO_3$ anhydre et $MgSO_4$, puis le solvant est évaporé. Le résidu huileux est trituré avec 150 mL d'éther pour obtenir une suspension. Le solide pâteux est difficile à filtrer. On décante le surnageant et on répète le lavage à l'éther. Après séchage sous vide, on obtient 9,13 g (64 %) de composé **63**.
RMN ^1H (200 MHz, $CDCl_3$) : δ = 3,5-3,6 (m, 12H) ; 3,42 (t, 2H, J = 6 Hz) ; 2,75 (t, 2H, J = 6 Hz) ; 1,81 (quantiplet, 2H, J = 6 Hz) ; 1,67 (quantiplet, 2H, J = 6 Hz) ; 1,30 (s. large, 2H).

● Composé **64** :

On chauffe une solution du composé **63** dans l'ammoniaque (100 mL, 22 % aqueuse) dans un ballon de 250 mL bouché avec un septum à 55-60 °C pendant 2 h. Après refroidissement, on évapore le solvant. Le résidu est dissous dans le méthanol (20 mL) et passé sur une colonne résine échangeuse d'anion Dowex 21K [hauteur 12 cm × diamètre 35 mm, forme OH^- obtenu par lavage préalable avec NaOH 1N (1,5 L) puis H_2O (1,5 L) puis méthanol (1,5 L)]. Le composé **64** libre de l'ion trifluoracétate passe dans les premières fractions avec 200 mL du méthanol. Après évaporation, on triture le résidu avec 50 mL d'éther puis on le décante. Le lavage à l'éther est répété cinq fois de suite. Après séchage, on obtient le composé **64** (6,43 g, 86 %).

RMN ^1H (300 MHz, $CDCl_3$) : δ = 6,77 (t, 1H, J = 4 Hz) ; 6,32 (s, 1H) ; 5,52 (s, 1H) : 4,45 (m, 1H) ; 4,28 (m, 1H), 3,50-3,68 (m, 12H) ; 3,30 (m, 2H), 3,11 (m, 1H) ; 2,86 (dd, 1H, J = 13 et 5 Hz), 2,75 (t, 2H, J = 13 Hz), 2,68 (d, 1H, J = 13 Hz) ; 2,16 (t, 2H, J = 7 Hz) ; 1,60-1,85 (m, 8H) ; 1,41 (m, 2H).

- [Bio-EG_3]$_2$- acétal **65** :

A une suspension du diacide **60** (120 mg ; 0,500 mmol) dans le dichloroéthane (5 mL), on ajoute le carbonyldiimidazole (225 mg, 90 %, 1,25 mmol) et on chauffe à 55-60 °C pendant 30 min sous agitation sous argon. On ajoute l'amine **64** (550mg ; 1,23 mmol) et la solution est chauffée à 55-60 °C pendant 6 h. Après évaporation, on passe sur une colonne de silice (diamètre : 25 mm, éluant : méthanol 15-30 % dans CH_2Cl_2). On obtient 413 mg (75 %) de composé **65**.

RMN ^1H (300 MHz, $CDCl_3$) : δ = 8,34 (s, 1H) ; 8,06 (s, 2H) ; 7,87 (m, 2H) ; 6,85 (m, 2H) ; 6,60 (s, 2H) ; 5,93 (s, 2H) : 5,40 (s, 1H) ; 4,45 (m, 2H) ; 4,27 (m, 2H), 3,43-3,68 (m, 24H) ; 3,31 (s, 6H) ; 3,25 (m, 4H), 3,08 (m, 2H) ; 2,83 (dd, 2H, J = 13 et 5 Hz), 2,70 (t, 2H, J = 13 Hz) ; 2,13 (t, 4H, J = 7 Hz) ; 1,89 (quintuplet, 4H, J = 7 Hz) ; 1,55-1,70 (m, 12H) ; 1,37 (m, 4H).

- [Bio-EG_3]$_2$- aldéhyde **66** :

L'acétal **65** (413 mg ; 0,376 mmol) en solution dans le méthanol est traité avec HCl 2N (0,5 mL). Après évaporation et lavage avec l'éther, on obtient l'aldéhyde **66** (0,37 g, 90 %).

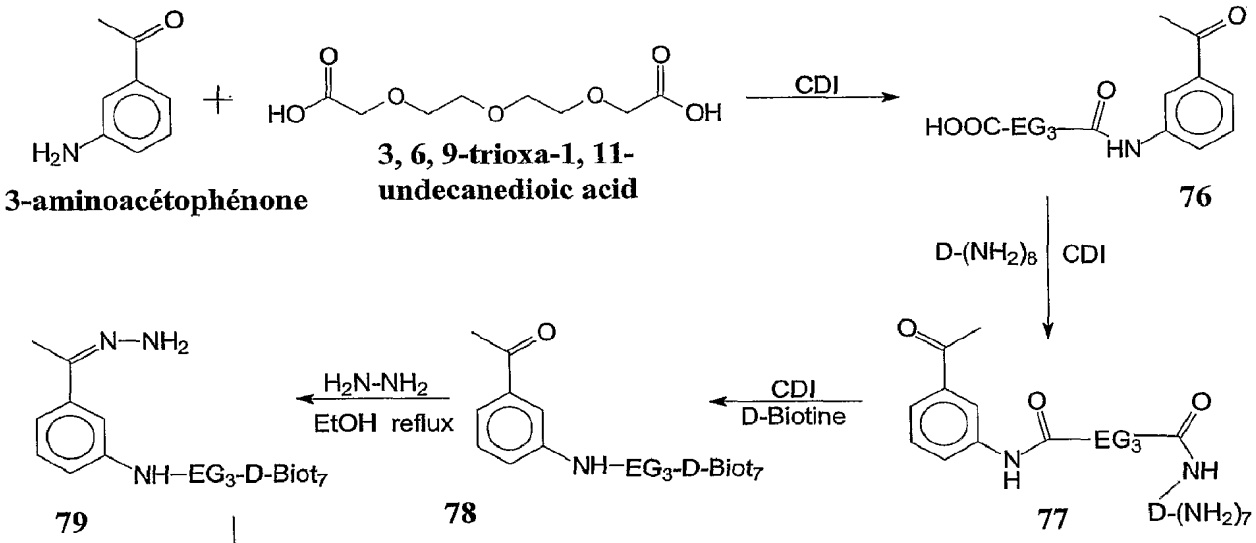
RMN ^1H (300 MHz, DMSO-d_6) : δ = 10,11 (s,1H) ; 8,82 (t, 2H, J = 6 Hz) ; 8,62 (s, 1H) ; 8,47 (s, 2H) ; 7,73 (t, 2H, J = 5 Hz) ; 4,30 (m, 2H) ; 4,11 (m, 2H), 3,30-3,60 (m, 24H) ; 3,06 (m, 6H) ; 2,80 (dd, 2H, J = 12 et 5 Hz), 2,56 (t, 2H, J = 12 Hz) ; 2,03 (t, 4H, J = 7 Hz) ; 1,78 (quintuplet, 4H, J = 7 Hz) ; 1,35-1,60 (m, 12H) ; 1,28 (m, 4H).

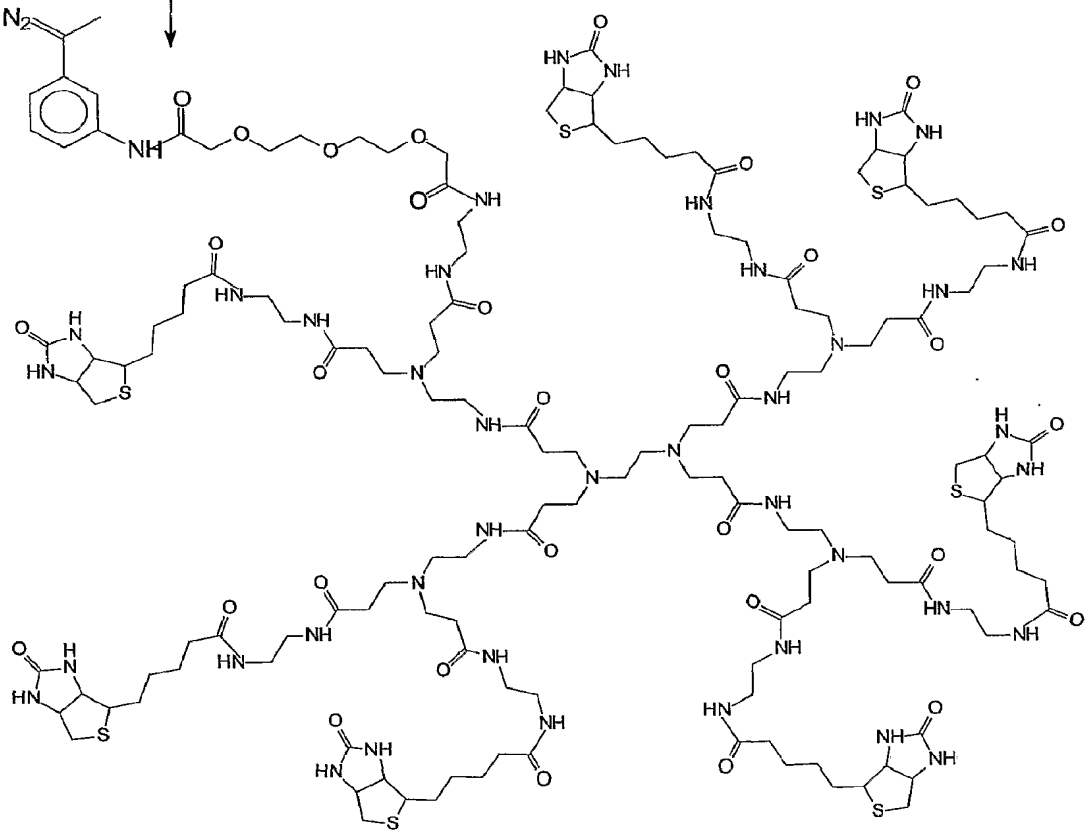
- [Bio-EG_3]$_2$- PDAM **67** :

L'aldéhyde **66** est transformé en diazométhane **67** selon la méthode utilisée pour la préparation des diazométhanes (Exemple 1).
La stabilité du réactif est supérieure à 1 mois à -20°C.

Exemple 26.2 : Synthèse du *méta*-Bio7-EG3-PMDAM :

Schéma de synthèse :





méta-Bio$_7$-EG$_3$-PMDAM **80**

Composé EG_3-acétophénone **76** :

Le 3, 6, 9-trioxa-1, 11-undecanedioic acid (EG_3, 12,64 ml, 74 mmol) est dissout dans 80 ml de DMF anhydre sous argon et refroidit dans un bain de glace. Le dicyclohexylcarbodiimide (DCC, 11,45 g, 55,5 mmol)) est ensuite dissout dans 20 ml de DMF anhydre et ajouté lentement. Après 30 min, on ajoute le 3-aminoacétophénone (5,0 g, 37 mmol) et on laisse réagir 1 h à température ambiante sous argon. Le DMF est ensuite évaporé sous vide et 70 ml de CH_2CL_2 sont ajoutés. La solution est filtrée et extraite avec 3x25 ml d'acide acétique 1%. Les phases aqueuses sont réunies et lavées avec 25 ml de CH_2CL_2. Les phases organiques sont mélangées, séchées avec du sulfate de sodium anhydre et évaporées à sec. Le produit est recristallisé dans le couple MeOH:H_2O. On obtient ainsi 8,74 g (70%) du produit **76**.

RMN ^1H (200 MHz, DMSO-d_6): d = 2,55 (s, 3H); 3,5-3,7 (m, 8H); 4,0 (s, 2H); 4,1 (s, 2H); 7,45 (t, 1H); 7,65 (d, 1H); 7,90 (d, 2H); 8,2 (s, 1H); 9,8 (s, 1H).

Composé $(NH_2)_7$-EG_3-acétophénone **77** :

Le produit **76** (120mg, 0,35 mmol) est dissout dans 15 ml de DMF anhydre sous argon, refroidit sur glace, et le DCC (110 mg, 0,53 mmol) est ensuite ajouté. Après 30 min, on ajoute cette solution sur une solution du dendrimère commercial « Starburst PAMAM Dendrimer, Generation 1 » (Aldrich, St Quentin Fallavier) (1 g, 0,71 mmol, dans 5 ml de méthanol), lentement et sous forte agitation. On laisse réagir 1 h à température ambiante et on évapore. Le résidu est repris dans 10 ml de CH_2CL_2 et extrait deux fois avec 30 ml d'acide acétique 1%.

Composé $Biot_7$-EG_3- acétophenone **78** :

La D-Biotine (1,73 g, 7,08 mmol) est solubilisée dans 80 ml de DMF anhydre sous argon, et la solution est refroidie sur glace. On ajoute successivement la N-méthylmorpholine (NMM, 856 µl, 7,7 mmol) et le chloroformiate d'isobutyle (1022 µl, 7,7 mmol). Après 30 min, on ajoute le produit **77** (1,13 g, 0,7 mmol, dans 5 ml de méthanol) et on laisse réagir 3 h sur glace et sous argon. On concentre sous vide jusqu'à 50 ml et on ajoute 100 ml de CH_2Cl_2 . Un précipité se

forme, qui est filtré, lavé à l'éther, et séché sous vide. On obtient 1,3 g de **78** sous forme d'une poudre blanche.

Composé $Biot_7$-EG_3-Hydrazone **79** :

Le composé **78** (300 mg, 0,09 mmol) est dissout dans 10 ml d'éthanol absolu à reflux. On ajoute l'hydrazine monohydrate (20 ml, 0,40 mmol) et on laisse réagir 3 h à reflux. Après refroidissement, un précipité se forme, qui est filtré, lavé a l'éther et séché sous vide. On obtient ainsi 109 mg (36%) du produit **79**, sous forme d'une poudre blanche.

Composé $Biot_7$-EG_3-PMDAM **80** :

On solubilise l'hydrazone **79** (100 mg, 0,03 mmol) dans 5 ml de DMF anhydre à 70°C. On laisse revenir à température ambiante et on ajoute le MnO_2 (31 mg, 0, 36 mmol). On laisse réagir 10 min et on élimine l'oxyde de manganèse par filtration sur un fritté avec de la célite (0,5 cm et du tamis moléculaire en poudre (0,5 cm). Le filtrat est évaporé a sec, lavé a l'éther e seché sous vide. On obtient ainsi 78 mg (78%) du produit **80**.

Les dendrimères sont des molécules arborescentes possédant aux extrémités plusieurs groupements réactifs tels que des amines, carboxyles, hydroxyles ou autres (pour revue, voir Newcome et al, (1996) *Dendritic Molecules: Concept, Syntheses, Perspectives*. VCH Ed., Weinheim, Germany). La synthèse de ces molécules est aujourd'hui bien maîtrisée, et des nombreux dendrimères sont commercialisés par l'industrie chimique. Le choix du PAMAM (Sigma-Aldrich) a été fait sur la base de sa stabilité, solubilité et souplesse, puisque plusieurs versions de cette molécule, avec un nombre et type de terminaisons différentes, sont disponibles. Le "PAMAM Generation 1" permet d'ajouter sept molécules du marqueur (en une seule étape de synthèse) pour chaque groupement diazométhyl.

Exemple 27 : Marquage et fragmentation en deux étapes d'amplicons ADN avec le *méta*-BioPMDAM :

Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Deux réactions de marquage ont été réalisées.

a. Marquage et fragmentation en deux étapes :

A 10 µL de PCR sont ajoutés 10 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 77 µL d'eau Dnase/Rnase free. La solution est incubée 10 min à 95°C. Puis 3 µL d'HCl à 0,1M sont ajoutés et la solution est incubée 10 min à 95°C.

b. Marquage et fragmentation en une étape :

A 10 µL de PCR sont ajoutés 10 µL de *méta*-BioPMDAM (100 mM dans du DMSO), 5 µL d'HCl à 0,1 M et 75 µL d'eau Dnase/Rnase free. La solution est incubée 30 min à 60°C.
Le reste du protocole est identique à celui de l'exemple 9.

Résultats :

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage et fragmentation en deux étapes	99,5	14129	624	22,7
b. Marquage et fragmentation en une étape	98,9	4431	667	6,6

Tableau 13 : Etude comparative du marquage et de la fragmentation en deux étapes distinctes et en une seule étape

Comme le démontre le tableau 13, les résultats obtenus avec le protocole en une étape sont satisfaisants. Ceux obtenus avec un marquage et une fragmentation en deux étapes sont encore meilleurs. Cet exemple montre que les étapes de marquage et clivage peuvent être dissociées pour améliorer le marquage en fonction de la cible utilisée.

Exemple 28 : Marquage et fragmentation d'amplicons ADN dans différents formats de réaction :

Les amplicons d'ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Trois réactions de marquage ont été réalisées.

a. Marquage et fragmentation dans un format de 250 µL :

A 50 µL de PCR sont ajoutés 75 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 102,5 µL d'eau Dnase/Rnase free. La solution est incubée 25 min à 95°C. Puis 22,5 µL d'HCl à 0,1 M sont ajoutés et la solution est incubée 5 min à 95°C.

b. Marquage et fragmentation dans un format de 200 µL :

A 50 µL de PCR sont ajoutés 75 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 52,5 µL d'eau Dnase/Rnase free. La solution est incubée 25 min à 95°C. Puis 22,5 µL d'HCl à 0,1 M sont ajoutés et la solution est incubée 5 min à 95°C.

c. Marquage et fragmentation dans un format de 150 µL :

A 50 µL de PCR sont ajoutés 75 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 2,5 µL d'eau Dnase/Rnase free. La solution est incubée 25 min à 95°C.
Puis 22,5 µL d'HCl 0,1M sont ajoutés et la solution est incubée 5 min à 95°C.

Le reste du protocole est identique à celui de l'exemple 9.

Résultats :

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Format de 250 µL	100,0	5606	549	10,2
b. Format de 200 µL	99,4	5886	557	10,6
c. Format de 150 µL	99,4	6800	537	12,7

Tableau 14 : Marquage et fragmentation selon différents formats

Les résultats obtenus en terme de signal et de pourcentage d'homologie sont très satisfaisants dans tous les cas de figures. De plus, bien que le format de la réaction varie de 150 à 250 µL, les résultats ont des valeurs similaires.

Cet exemple montre une flexibilité du format réactionnel du protocole de marquage pouvant accepter différents volumes et notamment différents volumes de produits d'amplification.

Exemple 29 : Comparaison entre un protocole utilisant une étape de purification avant fragmentation et un protocole utilisant une étape de purification après fragmentation :

Les amplicons d'ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Deux réactions de marquage ont été réalisées.

a. Marquage purification puis fragmentation des amplicons ADN :

A 10 µL de PCR sont ajoutés 10 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 80 µL d'eau Dnase/Rnase free. La solution est incubée 10 min à 95°C. Puis la purification est réalisée selon le protocole décrit dans l'exemple 9. A la solution d'amplicons marqués purifiés 6 µL d'HCl à 0,1 M sont ajoutés. La solution est incubée 10 min à 95°C. Quatre cents (400) µL de tampon d'hybridation préchauffé à 95°C durant 10 min sont ajoutés.

La composition du tampon d'hybridation ainsi que le reste du protocole sont identiques à celui de l'exemple 9.

b. Marquage fragmentation puis purification des amplicons ADN :

A 10 µL de PCR sont ajoutés 10 µL de *méta*-BioPMDAM (100 mM dans du DMSO) et 77 µL d'eau Dnase/Rnase free. La solution est incubée 10 min à 95°C.
Puis 3 µL d'HCl à 0,1 M sont ajoutés et la solution est incubée de nouveau 10 min à 95°C. Le reste du protocole est identique à celui de l'exemple 9.

Résultats :

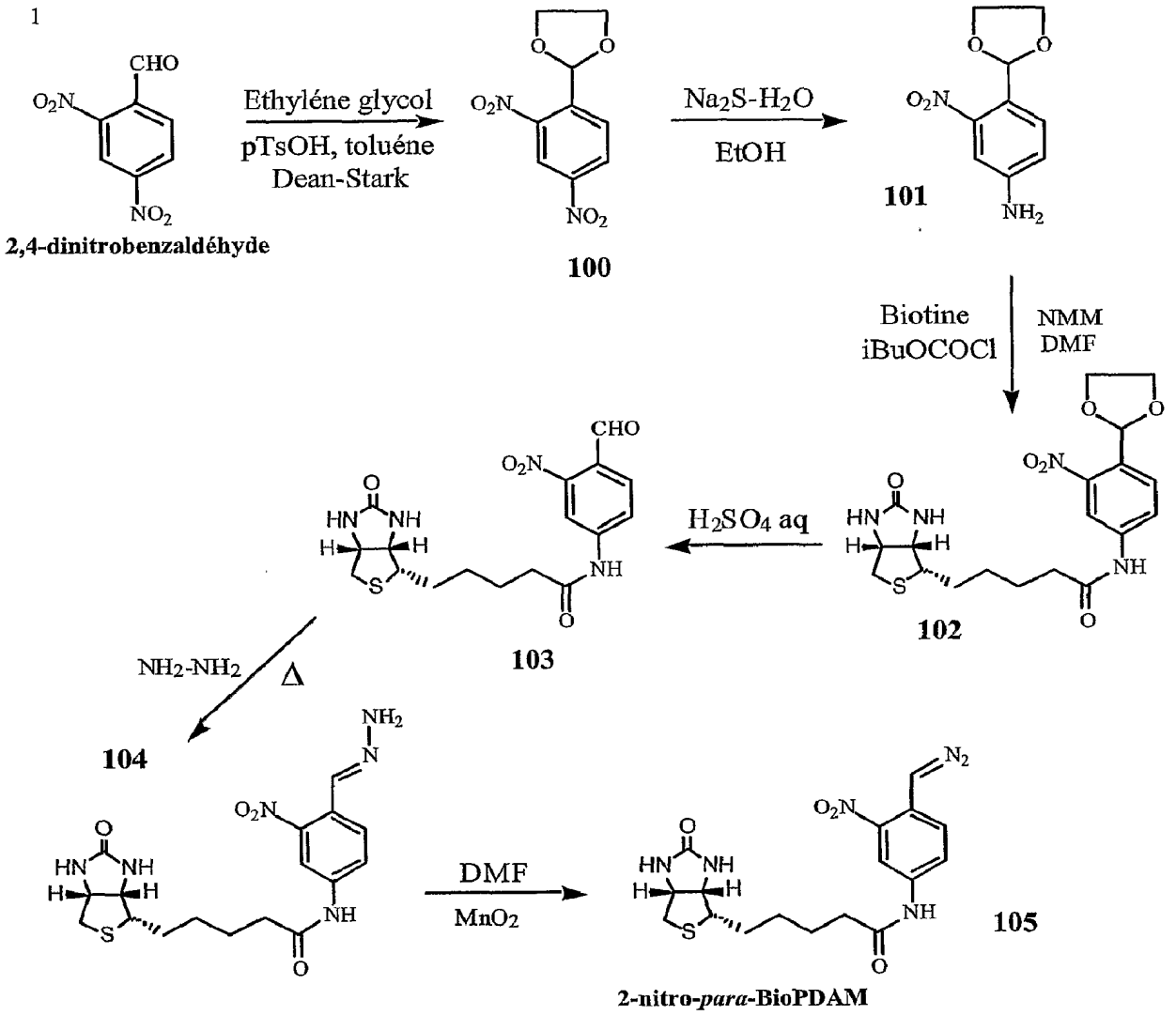
Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Purification avant fragmentation	98,9	6256	473	13
b. Fragmentation avant purification	96,1	6066	556	11

Tableau 15 : Comparaison entre protocole utilisant une étape de purification avant fragmentation et protocole utilisant une étape de purification après fragmentation

Ce résultat, présenté dans le tableau 15, montre que l'étape de purification peut être introduite entre les étapes de marquage et de fragmentation. De plus l'introduction de la purification entre les étapes de marquage et de fragmentation permet de réaliser la dénaturation pendant le clivage et d'hybrider sur la puce la totalité des fragments d'amplicons marqués.

Exemple 30 : Synthèse du 2-nitro-*para*-BioPDAM :

Schéma de synthèse :



● Protection de l'aldéhyde :

On dissout 5 g (25,5 mmol) de 2,4-dinitrobenzaldéhyde dans 250 mL de toluène, et l'on ajoute 20 mL d'éthylène glycol et 150 mg d'acide *para*-toluènesulfonique. On chauffe à reflux en récupérant l'eau dans un système Dean-Stark pendant 6 h. On traite avec 150 mL d'EtOAc et 100 mL d'H_2O. On extrait deux fois la solution à l'acétate d'éthyle, on sèche la phase organique avec $MgSO_4$ puis on l'évapore. L'huile obtenue correspondant au produit **100**, est utilisée pour la réaction suivante.

RMN ^{1}H (200 MHz, $CDCl_3$) : δ = 8,70 (d, $1H_{aro}$, J = 2 Hz, H_3) ; 8,44 (dd, $1H_{aro}$, J = 2 Hz, J = 6 Hz, H_5) ; 8,02 (d, $1H_{aro}$, J = 8 Hz, H_6) ; 6,49 (s, 1H, CH) ; 4,12-4,06 (m, 4H, CH_2-CH_2).

● Réduction du dérivé dinitro **100** :

Le 2-4-dinitrobenzaldéhyde protégé (6,4 g ; 25,5 mmol) est dissous dans un mélange éthanol-eau (6/1), puis 2 équivalents de Na_2S nonahydraté (12,3 g ; 51,1 mmol) sont ajoutés. Le mélange réactionnel est ensuite chauffé pendant 30 min. Une évaporation puis une extraction au dichlorométhane sont réalisées. Après séchage et filtration, le milieu réactionnel est évaporé afin d'obtenir une huile, qui est directement purifiée sur colonne de silice (cyclohexane-acétate d'éthyle 60/40). Le composé **101** est isolé avec un rendement de 45 %.

Composé **101** : F 58-60°C. - RMN ^{1}H (200 MHz, $CDCl_3$) : 7,49 (d, $1H_{aro}$, J = 2 Hz, H_3) ; 7,09 (d, $1H_{aro}$, J = 2 Hz, H_6) ; 6,80 (dd, $1H_{aro}$, J = 2 Hz, J = 6 Hz, H_5) ; 6,27 (s, 1H, CH) ; 3,99-3,97 (m, 4H, CH_2-CH_2).

● Couplage avec la biotine :

On solubilise la D-biotine (1,0 g ; 4,1 mmol) dans 20 mL de DMF anhydre et 600 µL de N-méthylmorpholine. On ajoute sous argon le chloroformiate d'isobutyle (700 µL ; 5,5 mmol) en refroidissant dans un bain glacé. On laisse sous agitation pendant 5 min, puis on ajoute 1 g (4,75 mmol) du composé **101** et 500 µL de N-méthylmorpholine. La solution est maintenue sous agitation à température ambiante pendant 4 h, puis évaporée à sec. L'huile obtenue est passée directement sur colonne de silice avec comme solvant d'élution MeOH-DCM 7 % puis 10 %. Le produit **102** (1,1 g ; 2,52 mmol) est obtenu avec un rendement de 62 %.
RMN ^{1}H (200MHz, DMSO-d_6) δ =10,40 (s, 1H, NH-CO) ; 8,31 (d, $1H_{aro}$, J = 2 Hz, H_3) ; 7,77 (dd, $1H_{aro}$, J = 2 Hz, J = 6 Hz, H_5) ; 7,68 (d, $1H_{aro}$, J = 2 Hz, H_6) ; 6,43 (s large, 1H, NH-CO-NH) ; 6,36 (s large, 1H, NH-CO-NH) ; 6,23 (s, 1H, CH) ; 4,28 (m, 1H, CH_2-CH-NH) ; 4,14 (m, 1H, CH-CH-NH) ; 3,92 (s, 4H, CH_2-CH_2) ; 3,12 (m, 1H, CH-S) ; 2,85 et 2,76 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,29 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,61-1,39 (m, 6H, $(CH_2)_3$).

● Déprotection de l'acétal :

On met le produit **102** (768 mg ; 1,76 mmol) en suspension dans 25 mL de THF. Tout se dissout après ajout de 4 mL de H_2SO_4 2N. On laisse sous agitation pendant 2 h. On évapore puis on rince et lave à l'eau sur fritté. Le composé **103** (694 mg) est obtenu sous forme d'une poudre jaune avec un rendement de 90 %.

F 165°C. - RMN ^{1}H (200MHz, DMSO-d_6) δ =10,69 (s, 1H, NH-CO) ;10,09 (s, H, CHO) ; 8,43 (d, 1H_{aro}, J = 2 Hz, H_3) ; 7,91 (s, 2H_{aro}, H_5 et H_6) ; 6,42 (s large, 1H, NH-CO-NH) ; 6,35 (s large, 1H, NH-CO-NH) ; δ = 6,23 (s, 1H, CH) ; 4,29 (m, 1H, CH_2-CH-NH) ; 4,13 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,84 et 2,78 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,29 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,61-1,39 (m, 6H, $(CH_2)_3$).

● Formation de l'hydrazone **104** :

On met l'aldéhyde **103** en suspension dans l'éthanol et l'on chauffe à 80°C. Quand on ajoute l'hydrazine, tout se dissout et la solution se colore immédiatement en orange. Un précipité se forme après 5 min. On chauffe sous agitation pendant 1 h. On filtre sur fritté puis on sèche le précipité. Le produit **104** (700 mg ; 690 mmol) est obtenu avec un rendement de 98 %.

F 169°C. - RMN ^{1}H (200MHz, DMSO-d_6) δ =10,31 (s, 1H, NH-CO) ; 8,31 (d, 1H_{aro}, J = 2 Hz, H_3) ; 7,96 (s, H, CHO) ; 7,87 (d, 1H_{aro}, J = 2 Hz, H_6) ; 7,68 (dd, 1H_{aro}, J = 2 Hz, J = 6 Hz, H_5) ; 7,31 (s, 2H, NH_2) ; 6,42 (s large, 1H, NH-CO-NH) ; 6,34 (s large, 1H, NH-CO-NH) ; 4,29 (m, 1H, CH_2-CH-NH) ; 4,13 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,84 et 2,78 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,29 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,61-1,39 (m, 6H, $(CH_2)_3$).

● Formation du diazo **105** :

On dissout le composé **104** (200 mg ; 0,492 mmol) dans 8 mL de DMF. On ajoute 400 mg de MnO_2. On agite vigoureusement pendant 10 min. On filtre sur millipore contenant de la célite (épaisseur : 2 cm) et du tamis moléculaire en poudre 3 Å (0,5 cm). On évapore à sec puis on lave à l'éther. On filtre de nouveau sur millipore. Le composé **105** (180 mg ; 0,445 mmol) est obtenu sous forme d'une poudre orange avec un rendement de 98 %.

F 155°C. - RMN ^{1}H (200MHz, DMSO-d_6) δ =10,21 (s, 1H, NH-CO) ; 8,60 (d, 1H$_{aro}$, J = 2 Hz, H_3) ; 7,77 (d, 1H$_{aro}$, J = 6 Hz, H_5) ; 7,22 (d, 1H$_{aro}$, J = 6 Hz, H_6) ; 6,60 (s, H, CH-N) ; 6,41 (s large, 1H, NH-CO-NH) ; 6,33 (s large, 1H, NH-CO-NH) ; 4,29 (m, 1H, CH_2-CH-NH) ; 4,13 (m, 1H, CH-CH-NH) ; 3,12 (m, 1H, CH-S) ; 2,84 et 2,78 (système ABX, 2H, $^2J_{AB}$= 5 Hz, $^3J_{AX}$ = 12 Hz, $^3J_{BX}$ = 0 Hz, CH_2-S) ; 2,29 (t, 2H, J = 8 Hz, CH_2-CO) ; 1,61-1,39 (m, 6H, $(CH_2)_3$).

La réactivité du composé **105** a été testée sur 3'-Uridine-monophosphate suivie par électrophorèse capillaire. Les conditions d'analyse sont celles de l'exemple 6.1. Les résultats montrent un temps de demi réaction de 45 minutes.
La stabilité du réactif est supérieure à 1 mois à -20°C

Exemple 31 : Marquage et fragmentation des amplicons d'ADN avec le réactif de marquage 2-nitro-*para*-BioPDAM :

Le dérivé 2-nitro-*para*-BioPDAM a été obtenu selon le schéma réactionnel décrit dans l'exemple 30. Les amplicons ADN ont été préparés par amplification PCR selon le protocole décrit dans l'exemple 5. Deux réactions de marquage ont été réalisées.

a. Marquage par le réactif 2-nitro-*para*-BioPDAM :

A 10 µL de PCR sont ajoutés 2 µL de 2-nitro-*para*-BioPDAM (100 mM dans du DMSO), 5µL d'HCl à 0,1 M et 83 µL d'eau Dnase/Rnase free. Cette solution est incubée 30 min à 60°C.

b. Marquage par le réactif *méta*-bioPMDAM :

A 10 µL de PCR sont ajoutés 2 µL de *méta*-BioPMDAM (100 mM dans du DMSO), 5µL d'HCl à 0,1 M et 83 µL d'eau Dnase/Rnase free. Cette solution est incubée 30 min à 60°C.

Le reste du protocole est identique à celui de l'exemple 9.

Résultat :

Protocole utilisé	Homologie (%)	I (rfu)	B (rfu)	I/B
a. Marquage par le réactif 2-nitro-*para*-BioPDAM	100,0	24392	899	27,1
b. Marquage par le réactif méta-BioPMDAM	98,9	21883	774	28,3

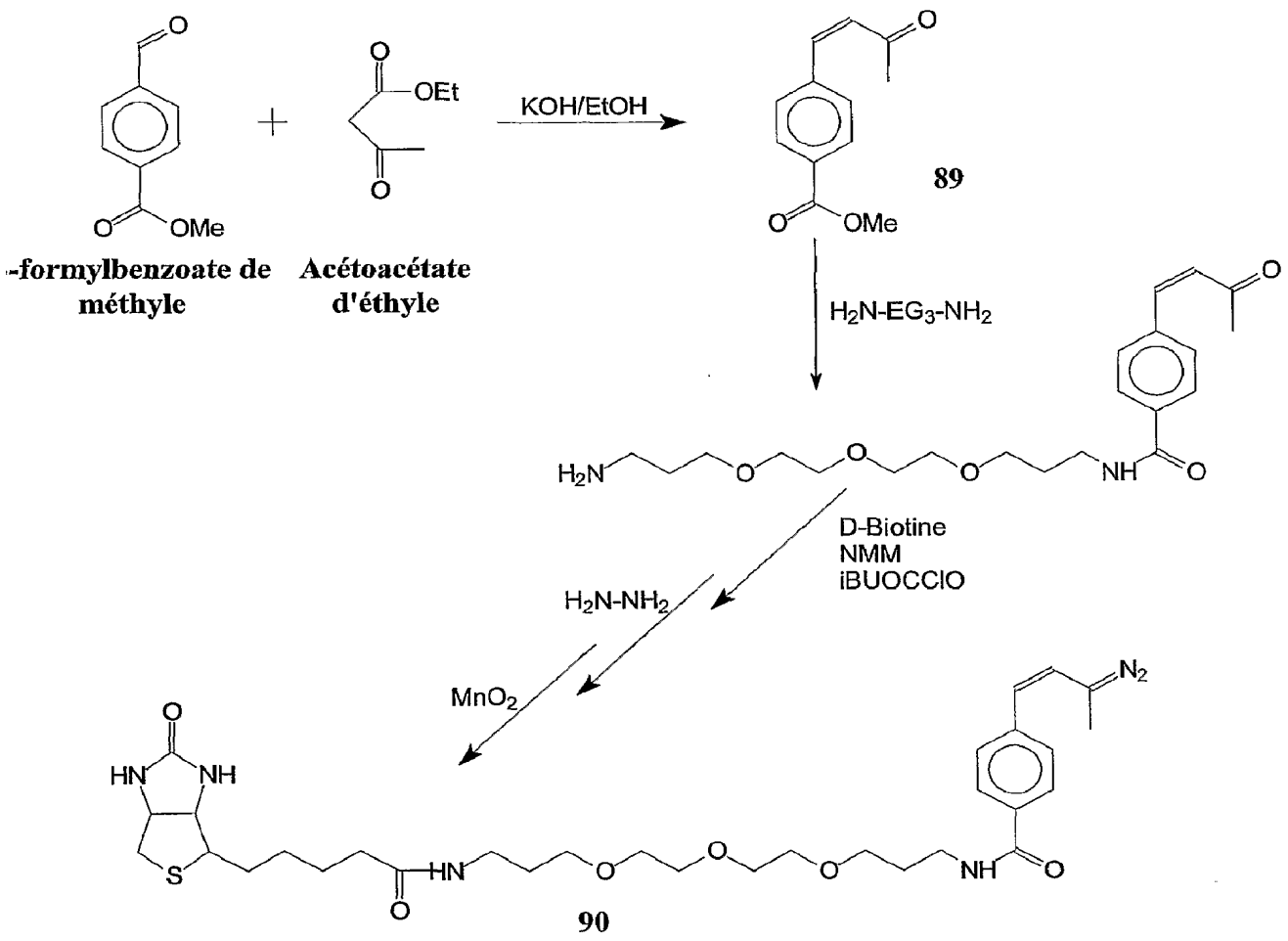
Tableau 16 : Etude comparative du marquage d'ADN par le dérivé 2-nitro-*para*-BioPDAM vis-à-vis du dérivé *méta*-BioPMDAM

Le réactif 2-nitro-*para*-BioPDAM utilisé pour le marquage de l'ADN donne des résultats intéressants en terme d'intensité de marquage et de pourcentage d'homologie.

Exemple 32 : Insertion d'une double liaison entre la fonction diazométhyle et le noyau phenyle, éloignement du DAM et synthèse d'une molécule particulièrement adaptée à cet éloignement :

L'objectif visé est l'éloignement de la fonction diazométhyle (DAM) de la structure aromatique pour minimiser l'effet de l'encombrement stérique lors de l'alkyaltion des phosphates et aussi lors de l'hybridation de l'acide nucléique marqué avec sa séquence complémentaire.

Schéma de synthèse :



-formylbenzoate de méthyle

Acétoacétate d'éthyle

Pour la réaction aldolique de formation du (*para*-méthoxycarbonyl)-styrylméthylcétone **89**, on utilise l'acétoacétate d'éthyle pour la forte acidité des protons du méthylène, ce qui facilite l'attaque du groupement formyl, avec élimination postérieure de H_2O (favorisée par la conjugaison de la double liaison avec l'anneau aromatique) et décarboxylation par hydrolyse due au milieu basique. Le reste de la synthèse est similaire a ce qui a été montré dans les autres exemples.

Le produit final *para*-Bio-EG_3-SMDAM **90**, possède deux carbones de plus entre le diazométhyle et l'anneau aromatique, ce qui limite les possibles problèmes stériques, tout en conservant la stabilisation du diazométhyle par le système aromatique par conjugaison.

Exemple 33 : Capture et détection d'un acide nucléique sur un support solide portant des groupements diazométhyles :

La réactivité d'une résine portant des groupements diazométhyles a été étudiée pour déterminer sa capacité à lier des acides nucléiques.

La 4-(Diazomethyl)phenoxymethyl-polystyrene (référence 17338, Fluka) est une résine décrite pour son pouvoir à lier les groupements carboxyliques, en particuliers ceux présents dans les protéines (G. Bhalay, A.R. Dunstan, Tetrahedron Lett. *39*, 7803 – 1998), mais elle n'est pas décrite pour sa capacité à lier les molécules d'ADN. Nous avons testé la possibilité de capturer des acides nucléiques avec ce réactif, et de les révéler par un test colorimétrique.

L'expérience est réalisée avec une partie des réactifs présents dans le kit HLA-DR oligo-détection (référence 33 202, bioMérieux, France principe de base décrit dans le brevet EP 549 776-B1), permettant la détection d'acides nucléiques amplifiés par PCR dans des microplaques, par lecture colorimétrique. Dans le cadre de l'expérience décrite, on fait réagir simultanément des acides nucléiques produits par PCR avec la résine testée, ainsi qu'avec une molécule de *para*-Bio-EG3-PDAM, dont la synthèse est évoquée dans l'exemple 20. Si l'ADN réagit avec les fonctions diazométhyle présentes sur les deux composés, il sera possible de le révéler, après lavage et élimination des molécules fixées de manière non covalente, grâce à une réaction colorimétrique faisant intervenir une enzyme couplée à la streptavidine. La streptavidine est associée à une péroxydase de raifort, cet enzyme pouvant décomposer une molécule d'OrthoPhénylène Diamine (réactif Color 1 du kit) en un composé détectable à une longueur d'onde de 492 nm.

Exemple 33.1 : Capture et détection de l'ADN :

On incube 10 mg de résine pendant 30 minutes à 60°C avec 50 µL de PCR réalisées comme décrit dans l'exemple 5, dans 400 µL d'eau pure (Sigma) additionnée de 5 µL de *para*-Bio-EG3-PDAM. On lave ensuite cette résine par 500 µL de tampon PBS tween (Réactif Color 0

HLA du kit, PBS pH 7,0 ; Tween 1 %, BND 0,2 g/L ; Ciproflaxacine 0,01 g/L). On remet en suspension alors la résine dans 100 µL de PBS Tween, et 250 µL de tampon d'hybridation streptavidine (BPS pH 7,0 TWEEN 0,5 %) additionné de streptavidine HRP (S-911, MOLECULAR PROBES, EUGENE, OR, USA) dilué au 1/10000. On incube le mélange réactionnel 30 min à température ambiante. On lave ensuite la résine trois (3) fois par 500 µL de tampon PBS tween, et on l'incube à température ambiante en présence de réactif chromogénique (1 Comprimé Color 1, Chlorhydrate d'orthophénylène diamine, dilué dans 5 mL de tampon Color 2, Phosphate de sodium 100 mM, acide citrique 50 mM, H_2O_2 0,03%). Après une incubation de 20 min à l'obscurité, la réaction est ensuite bloquée par 50 µL de H_2SO_4 (1, 8 N réactif Color 3). Le surnageant est alors pipeté et placé dans une microplaque pour lire l'absorbance du milieu réactionnel à 492 nm.

Exemple 33.2 : Témoin sans acide nucléique :

On incube 10 mg de résine pendant 30 minutes à 60°C dans 425 µL d'eau pure (Sigma) additionnée de 5 µL de *para*-Bio-EG3-PDAM. On lave ensuite cette résine par 500 µL de tampon PBS. On traite alors l'échantillon de façon identique à processus décrit à l'exemple 33.1.

Exemple 33.3 : Témoin avec PCR réalisée sans cibles :

On incube 10 mg de résine dans 400 µL d'eau pure additionnée de 5 µL de *para*-Bio-EG3-PDAM pendant 30 minutes à 60°C, avec 50 µL de PCR réalisée avec un volume de 25 µL d'eau pure à la place du volume l'ADN génomique décrit. On lave ensuite cette résine par 500 µL de tampon PBS. On traite alors l'échantillon de façon identique au processus décrit à l'exemple 33.1.

Exemple 33.4 : Témoin avec PCR sans molécule de révélation :

On incube 10 mg de résine avec 50 µL de PCR dans 400 µL d'eau pure pendant 30 minutes à 60°C. On lave ensuite cette résine par 500 µL de tampon PBS. On traite alors l'échantillon de façon identique au processus décrit à l'exemple 33.1.

Exemple 33.5 : Témoin avec acide nucléique non capturé :

On incube 10 mg de résine pendant 30 minutes à 60°C avec 400 µL d'eau pure additionnée de 5 µL de *para*-Bio-EG3-PDAM. On lave ensuite cette résine par 500 µL de tampon PBS tween (Réactif Color 0 HLA du kit, PBS pH 7,0 ; Tween 1 %, BND 0,2 g/L ; Ciproflaxacine 0,01 g/L). On resuspend alors la résine dans 100 µL de PBS Tween, et 250 µL de tampon d'hybridation streptavidine additionné de streptavidine HRP dilué au 1/10000 ème. A cette préparation est rajoutée 50µL d'une préparation d'ADN préparée comme suit :

On rajoute 5 µL de *para*-Bio-EG3-PDAM et 70 µL d'eau pure à 25 µL d'ADN provenant d'une PCR préparé comme décrit dans l'exemple 5. Ce mélange est incubé 30 min à 60°C, puis l'excès de marqueur est éliminé en soumettant la préparation à une purification sur colonne QIAquick (Nucleotide Removal Kit, Qiagen, Hilden, Allemagne) suivant le protocole recommandé par le fournisseur, en effectuant une élution finale dans un volume de 50 µL.

Le mélange réactionnel est incubé 30 min à température ambiante, puis il est traité suivant le processus décrit à l'exemple 33.1.

Résultats

Conditions	Absorbance à 492 nm
Ex. 33.1 : ADN capturé sur Résine	527
Ex. 33.2 : Sans acide nucléique	249
Ex. 33.3 : PCR sans cible	261
Ex. 33.4 : Témoin sans marqueur	264
Ex. 33.5 : Acide nucléique non capturé	249

Tableau 17 : Etude de la réactivité d'une résine portant des groupements diazométhyle

Dans le tableau 17, une forte valeur colorimétrique indique une forte concentration d'enzymes dans le milieu réactionnel, correspondant à une présence importante d'acide nucléique portant des dérivés biotines. Les témoins indiquent que le signal n'est pas dû à une adsorption non spécifique de l'ADN sur la bille, à une réaction du *para*-Bio-EG3-PDAM sur la résine, ou à une adsorption de la streptavidine HRP sur la résine, mais bien à la présence d'ADN capturé de façon covalente et marqué par le *para*-Bio-EG3-PDAM.

Exemple 34 : Marquage d'un produit PCR permettant sa capture et sa détection dans une microplaque :

Il est montré dans cet exemple la possibilité de marquer une molécule d'ADN avec un seul type de molécule portant une fonction diazométhyle, de façon à capturer et à détecter cet acide nucléique en une seule étape, sur une microplaque.

L'expérience est réalisée avec une partie des réactifs présent dans le kit HLA-DR oligo-détection (référence 33 202, bioMérieux), permettant la détection d'acides nucléiques amplifiés par PCR dans des microplaques, par lecture colorimétrique. Dans le cadre de l'expérience décrite, on fait réagir du *para*-Bio-EG3-PDAM, dont la synthèse est décrite dans l'exemple 20, sur des acides nucléiques produits par PCR. L'ADN réagit avec les groupements diazométhyle de la molécule, et se trouve ainsi doté de biotines greffé sur ses phosphate. Il sera alors possible de capturer l'acide nucléique par incubation sur une microplaque où des molécule de streptavidine sont adsorbées, et de le révéler, grâce à une réaction colorimétrique.On utilise un réactif de détection qui est aussi une molécule de streptavidine, associée à une peroxydase de raifort (Horse Radish Peroxidase, HRP). Dans les condition utilisées, la péroxydase peut décomposer une molécule d'OrthoPhénylène Diamine (réactif Color 1 du kit) en un composé détectable à une longueur d'onde de 492 nm.

Exemple 34.1 : Capture et détection de l'ADN issu de PCR, sur microplaque :

On marque 10 µL d'ADN obtenu par amplification PCR comme décrit dans l'exemple 5 en l'incubant 30 min à 60°C dans 80 µL d'eau pure (Sigma) additionnée de 10 µL de *para*-Bio-EG3-PDAM. Après marquage, l'ADN est purifié sur colonne QIAquick (Nucleotide Removal Kit, Qiagen, Hilden, Allemagne) suivant le protocole recommandé par le fournisseur, et l'éluat final est recueilli dans 50 µL de tampon EB (Tris EDTA 10 mM, pH 8,5). Vingt (20) µL de cet éluat est dilué dans 180 µL de tampon PEG (0,1 M Na PO3 ; 0,5 M NaCl ; 0,65% Tween 20 ; 0,14 mg/mL ADN de sperme de Saumon (Gibco) ; 2 % PEG 4000) additionné de streptavidine HRP (S-911, Molecular Probes, Eugene, OR, USA) diluée au 1/10 000. Cent (100) µL de cette préparation sont ensuite incubés 1 heure à 37°C, soit dans un puits de Combiplate 8, streptavidine coated, (référence 95029263, Labsystem, Helsinki, Finlande) soit dans un puits témoin, provenant d'une barrette Maxisorb. (Nunc, Danemark).

Exemple 34.2 : Réalisation de témoins :

Des témoins sont simultanément réalisés de la manière suivante :

A - Témoin de marquage sans ADN :
Quatre-vingt-dix (90) µL d'eau pure additionnés de 10 µL de *para*-Bio-EG3-PDAM sont incubés 30 min à 60°C. Le mélange réactionnel est ensuite traité de façon similaire au processus précédemment décrit à l'exemple 34.1.

B - Témoin de marquage sans marqueurs :
Dix (10) µL d'ADN obtenus par amplification PCR, comme décrit dans l'exemple 5, sont incubés 30 min à 60°C dans 90 µL d'eau pure. Le mélange réactionnel est ensuite traité de façon similaire au processus précédemment décrit à l'exemple 34.1.

Toutes les barrettes sont ensuite lavées par trois fois 100 µl de tampon PBS Tween (Réactif Color 0 HLA du kit, PBS pH 7,0 ; Tween 1 %, BND 0,2 g/L ; Ciproflaxacine 0,01

g/L) puis la présence de streptavidine HRP est révélée par addition de 100 µL du réactif chromogénique (1 Comprimé Color 1, Chlorydrate d'orthophénylène diamine, dilué dans 5 mL de tampon Color 2, Phosphate de sodium 100 mM, acide citrique 50 mM, H_2O_2 0,03%), incubé 20 min dans l'obscurité, la réaction étant ensuite bloquée par 50 µL de H_2SO_4 (1,8 N Réactif Color3). L'absorbance du milieu réactionnel est alors mesurée à 492 nm.

Résultats :

Conditions	Combiplate	Témoin maxisorp
A - ADN marqué	1382	152
B - ADN non marqué	178	136
C - Sans ADN	140	192

Tableau 18 : Détection d'ADN capturé et marqué par du *para*-Bio-EG3-PDAM

L'expérience, exposée dans le tableau 18, montre donc que l'ADN marqué par du *para*-Bio-EG3-PDAM peut être capturé et détecté en une seule étape dans un puits de microplaque. Comme l'indique les témoins de réaction, le signal généré est uniquement dû à l'ADN et ne résulte pas d'une adsorption non spécifique de l'acide nucléique sur la paroi de la microplaque, ou sur la streptavidine, ou bien à une réaction non spécifique de la streptavidine HRP sur l'ADN non marqué, ou sur le plastique de la microplaque.

Exemple 35 : Double marquage d'un produit PCR permettant sa capture et sa détection sur un support solide de type microplaque :

Il est montré dans cet exemple la possibilité de marquer avec deux molécules, porteuses de fonctions diazométhyles, et en une seule étape, une molécule d'ADN, de façon la capturer et à la détecter sur une microplaque.

L'expérience est réalisée avec une partie des réactifs présent dans le kit HLA-DR oligo-détection, permettant la détection d'acides nucléiques amplifié par PCR dans des microplaques, par simple lecture colorimétrique. Dans le cadre de l'expérience décrite, on fait réagir simultanément sur des acides nucléiques produits par PCR du :

- 1-Pyrényldiazométhane (PDAM), et
- *para*-Bio-EG3-PDAM.

Si l'ADN réagit avec les fonctions diazométhyle présentes sur les deux composés, il pourra se fixer sur un support portant des anticorps anti-pyrène, et il sera possible de le révéler avec une molécule de streptavidine associée à une péroxydase de raifort. Cette enzyme peut décomposer une molécule d'OrthoPhénylène Diamine, faisant office de réactif de révélation, en un composé détectable à une longueur d'onde de 492 nm.

Exemple 35.1 : Double marquage de l'ADN et détection sur microplaque :

On a adsorbé sur des barrettes Maxisorp huit (8) puits, des anticorps anti-pyrène, en incubant une nuit à température ambiante 100 µL d'une solution de 1,1 µL d'anticorps anti-pyrène dilués dans 100 µL de tampon bicarbonate (0,05M pH 9,6). De tels anticorps, appelés Rabbit anti-pyrène (Réf. : YSRT-AHP236) sont disponibles chez Accurate Chemical & Scientific (Westbury, New York, Etats-Unis d'Amérique). Bien entendu, ceci aurait pu être réalisé avec d'autres anticorps commercialement disponibles, sans qu'il y ait de résultats divergents par rapport à ceux que nous avons obtenus dans cet exemple.

On a ensuite marqué 10 µL d'ADN obtenu par amplification PCR, comme décrit dans l'exemple 5, en l'incubant 30 min à 60°C dans 40 µL d'eau pure (Sigma), 10 µL de *para*-Bio-EG3-PDAM, 2 µL de PDAM (P-1405, 1-pyrenyldiazomethane, Molecular Probes, Eugene, OR, USA) et 38 µL de DMSO.

Après marquage, l'ADN a été purifié par utilisation d'un kit QIAquick (QIAGEN) et l'éluat final à été recueilli dans 50 µL de tampon EB (Tris EDTA 10 mM, pH 8,5). Vingt (20) µL de cet éluat a été dilué dans 180 µL de tampon PEG (0,1 M Na PO3 ; 0,5 M NaCl ; 0,65 % Tween 20 ; 0,14 mg/mL ADN de sperme de Saumon (Gibco) ; 2 % PEG 4000) additionné de streptavidine HRP (S-911, MOLECULAR PROBES, EUGENE, OR,

USA) diluée au 1/10 000. Cent (100) µL de cette préparation ont été ensuite incubés 1 heure à 37°C, soit dans un puits de barrette Maxisorp adsorbé, soit dans un puits témoin, non adsorbé.

Exemple 35.2 : Réalisation de témoins :

Des témoins ont été simultanément réalisés de la manière suivante :

A - Témoin de marquage avec *para*-Bio-EG3-PDAM seul :

Dix (10) µL d'ADN obtenus par amplification PCR, comme décrit dans l'exemple 5, sont marqués par incubation de 30 min à 60°C dans 90 µL d'eau pure additionnée de 10 µL de *para*-Bio-EG3-PDAM. Après marquage, l'ADN est purifié par utilisation d'un kit QIAquick et l'éluat final est recueilli dans 50 µL de tampon EB. Vingt (20) µL de cet éluat est dilué dans 180 µL de tampon PEG additionné de streptavidine HRP diluée au 1/10 000. Cent (100) µL de cette préparation sont ensuite incubés 1 heure à 37°C, soit dans un puits de barrette Maxisorp adsorbé, soit dans un puits non adsorbé.

B - Témoin de marquage avec PDAM seul :

Dix (10) µL d'ADN obtenus par amplification PCR, comme décrit dans l'exemple 5, sont marqués par incubation de 30 min à 60°C dans 90 µL d'eau pure additionnée de 2 µL de PDAM et 38 µL de DMSO. Après marquage, l'ADN est purifié par utilisation d'un kit QIAquick et l'éluat final est recueilli dans 50 µL de tampon EB. Vingt (20) µL de cet éluat est dilué dans 180 µL de tampon PEG additionné de streptavidine HRP diluée au 1/10 000. Cent (100) µL de cette préparation sont ensuite incubés 1 heure à 37°C, soit dans un puits de barrette Maxisorp adsorbé, soit dans un puits non adsorbé.

C - Témoin sans marquage :

Dix (10) µL d'ADN obtenus par amplification PCR, comme décrit dans l'exemple 5, sont incubés 30 min à 60°C dans 100 µL d'eau pure. Après marquage, l'ADN est purifié par utilisation d'un kit QIAquick et l'éluat final est recueilli dans 50 µL de tampon EB. Vingt (20) µL de cet éluat est dilué dans 180 µL de tampon PEG additionné de streptavidine

HRP diluée au 1/10 000. Cent (100) µL de cette préparation sont ensuite incubés 1 heure à 37°C, soit dans un puits de barrette Maxisorp adsorbé, soit dans un puits non adsorbé.

Les barrettes sont ensuite lavées par trois fois 100 µL de tampon PBS Tween (color 0) puis la présence de streptavidine HRP est révélée par addition de 100 µL du réactif chromogénique (Color 2), incubé 20 min dans l'obscurité, la réaction étant ensuite bloquée par 50 µL de H_2SO_4 (Color3). L'absorbance du milieu réactionnel est alors mesurée à 492 nm.

Résultats :

Conditions	Anticorps anti-pyrène adsorbés sur plaque (rfu)	Témoin plaque non adsorbée (rfu)
Ex. 35.1 : ADN marqué PDAM + *para*-Bio-EG3-PDAM	348	16
Ex. 35.2.A : ADN marqué *para*-Bio-EG3-PDAM	44	19
Ex. 35.2.B : ADN marqué PDAM	68	12
Ex. 35.2.C : ADN non marqué	75	19

Tableau 19 : Double marquage de l'ADN par le PDAM et le *para*-Bio-EG3-PDAM

Le résultat du tableau 19 montre clairement un signal important résultant de la capture de l'ADN dans les puits par les anticorps anti-pyrène, ainsi que le marquage simultané de celui-ci par la streptavidine HRP, qui s'est fixée. Comme le montre l'absence de signal au niveau des témoins, cette détection est spécifique de l'ADN marqué, et n'est pas dû à l'adsorption non spécifique de l'ADN ou de la streptavidine HRP sur le plastique, ou à une liaison non spécifique de l'enzyme sur l'ADN capturé. Cet exemple montre donc qu'il est possible de

réaliser un double marquage de l'ADN en une seule étape, ce double marquage pouvant être utilisé pour le capturer et le détecter simultanément.

Exemple 36 : Marquage d'un produit PCR permettant simultanément sa capture et sa détection par sondes nucléiques complémentaires : :

Cette expérience permet de démontrer qu'il est possible de détecter spécifiquement un ADN, ledit ADN étant capturé sur une surface solide en utilisant la réactivité de la fonction diazométhyle vis-à-vis d'un groupement phosphate de l'ADN.

L'expérience est réalisée avec une partie des réactifs présents dans le kit HLA-DR oligo-détection (référence 33 202, bioMérieux, France), permettant la détection d'acides nucléiques amplifié par PCR dans des microplaques, par lecture colorimétrique. Dans le cadre de l'expérience décrite, on fait réagir du *para*-Bio-EG3-PDAM sur des acides nucléiques produit par PCR. L'ADN réagit avec les fonctions diazométhyle de la molécule, et se trouve ainsi doté de biotines greffées sur ses phosphates. Il sera alors possible de capturer l'acide nucléique par incubation sur une microplaque où des molécule de streptavidine sont adsorbées, et de le révéler, grâce à une sonde constitué par un oligonucléotide complémentaire de la séquence capturée, associée à une péroxydase de raifort, cette enzyme pouvant décomposer des molécules incolores d'OrthoPhénylène Diamine (réactif Color 1 du kit) en un composé détectable à une longueur d'onde de 492 nm.

Exemple 36.1 : Capture et détection spécifique de l'ADN sur microplaque :

On réalise en double le marquage de 10 µL d'ADN obtenu par amplification PCR, en l'incubant 30 min à 60°C avec 20 µL de *para*-Bio-EG3-PDAM. Après marquage, l'ADN est purifié sur colonne QIAquick (Nucleotide Removal Kit, Qiagen, Hilden, Allemagne) suivant le protocole recommandé par le fournisseur et l'éluat final est recueuilli dans 50 µL de tampon EB (Tris-HCl 10 mM, pH 8,5). Quatre-vingt-cinq (85) µL du mélange de ces

éluats sont dénaturés par 8,5 µL de réactif R4 (NaOH 2N) durant 5 min à température ambiante, puis la solution est ensuite neutralisée par 8,5 µL de réactif R5 (acide acétique 2 N). On rajoute au mélange 850 µL de tampon d'hybridation (R6 – Tris-HCL 10 mM, pH 7,0 BND 0,2 g/L, Ciproflaxacine 0,01 g/L.) et 85 µL d'oligonucléotide de détection (R7 – Phosphate de sodium 4 mM, Phosphate de potassium 1 mM, pH 7,0, Sérum albumine bovine 0,1 %, Phénol 0,5%). Cent (100) µL de cette préparation sont déposés soit sur le contrôle positif d'une barrette R1 fournie avec le kit, (capture par une séquence consensus du gène amplifié), soit sur une plaque Combiplate 8 streptavidine coated (référence 95029263, Labsystem, Helsinki, Finlande), soit sur une plaque témoin Maxisorp (Nunc, Danemark).

Parallèlement, la même réaction d'hybridation est réalisée sur une dilution, dans du tampon EB, au dixième et au centième, de la préparation d'ADN, afin de tester la sensibilité de la technique.

Exemple 36.2 : Réalisation des témoins :

Des témoins ont été simultanément réalisés de la manière suivante :

A - Comparaison au kit HLA-DR :

Dix (10) µL d'ADN obtenus par amplification PCR, comme décrit à l'exemple 5, sont incubés 30 min à 60°C avec 20 µL de *para*-Bio-EG3-PDAM. Après marquage, l'ADN est purifié sur colonne QIAquick, et l'éluat final est recueilli dans un volume de 50 µL de tampon EB. Les 45 µL d'éluat sont dénaturés par 4,5 µL de réactif R4 durant 5 min à température ambiante, puis la solution est neutralisée par 4,5 µL de réactif R5. On rajoute au mélange 450 µL de tampon d'hybridation R6 et 45 µL d'oligonucléotide de détection. Cent (100) µL de cette préparation sont déposés soit sur le contrôle positif d'une barrette R1 fournie avec le kit (hybridation avec une séquence consensus du gène amplifié), soit sur une plaque Combiplate 8 streptavidine, soit sur une plaque témoin Maxisorp.

B - Hybridation réalisée sur un ADN ne s'hybridant pas à la sonde spécifique :

Dix (10) µL d'ADN obtenu par amplification PCR, comme décrit dans l'exemple 5, sont incubés 30 min à 60°C avec 20 µL de *para*-Bio-EG3-PDAM. On traite alors l'échantillon de façon identique à processus décrit précédemment dans l'exemple A.

C - Témoin sans ADN :

Dix (10) µL de réactif R6 (tampon d'hybridation) et 100 µL de réactif R7 (oligonucléotide de détection) sont déposés soit sur le contrôle positif d'une barrette R1 fournie avec le kit, sur une plaque streptavidine, soit sur une plaque témoin de type Maxisorp.

Toutes les barrettes des protocoles ci-dessus sont incubées une heure et demie à 37°C, puis lavées par trois fois 100 µL de tampon PBS Tween (réactif color 0 HLA) puis la présence de la sonde de détection spécifique est révélée par addition de 100 µL du réactif chromogénique (Réactif Color 2, , PBS pH 7,0 ; Tween 1 %, BND 0,2 g/L ; Ciproflaxacine 0,01 g/L), incubé 20 min dans l'obscurité, la réaction étant ensuite bloquée par 50 µL de H_2SO_4 (1,8 N réactif Color 3). L'absorbance du milieu réactionnel est alors mesurée à 492 nm.

Résultats :

Conditions	Barette R1 Kit HLA DR (Capture spécifique)	Combiplate (Streptavidine)	Maxisorp (Témoin)
A - ADN marqué - Ex 36.1	**1215**	**2160**	**16**
A - ADN marqué (dilué au 1/10ème)	NA	**900**	NA
A - ADN marqué (dilué au 1/100ème)	NA	**53**	NA
B - Témoin ADN non marqué - Ex 36.2 A	**1153**	**40**	**17**
C- Témoin ADN bactérien - Ex 36.2 B	**24**	**15**	NA
D - Témoin sans ADN - Ex 36.2 C	**13**	**12**	**17**

Tableau 20 : Détection spécifique sur microplaque d'un ADN issus de PCR

Les résultats du tableau 20 indiquent une excellente amplification de la cible permettant d'envisager une utilisation dans un cadre diagnostic. Cet exemple montre que le marquage au niveau des groupements phosphate permet la capture de l'ADN et n'empêche par l'hybridation spécifique sur celui-ci.

Exemple 37 : Capture et amplification d'ADN issu d'un lysat bactérien et marqué par *para*-Bio-EG3-PDAM :

Cet exemple montre qu'il est possible de capturer et d'amplifier un ADN bactérien en utilisant une capture basée sur la réactivité de la fonction diazométhyle sur le phosphate de l'acide nucléique.

Dans le cas présent, les acides nucléiques contenus dans un lysat bactérien et marqués par du *para*-Bio-EG3-PDAM, sont capturés sur des billes magnétiques recouvertes de streptavidine. L'utilisation de billes magnétiques permet d'immobiliser celles-ci par aimantation lors des lavages successifs visant à éliminer les résidus cellulaires, présents dans le milieu réactionnel, ces résidus devant être éliminés car ils peuvent inhiber l'amplification par PCR qui est pratiqué par la suite.
Les produits d'amplification ont pu être analysés par passage sur puces à ADN.

L'ADN bactérien est obtenu par lyse des cellules contenues dans une culture de *Mycobacterium tuberculosis*. La lyse s'effectue par lyse mécanique. Plus précisément, elle est réalisée par sonication, l'échantillon liquide traité contenant des billes de verre. Un tel procédé est déjà bien décrit par la Demanderesse dans sa demande de brevet WO-A-99/15621, en ce qui concerne les billes, et dans sa demande de brevet WO-A-00/60049, en ce qui concerne la sonication. La sonication peut également être réalisée à l'aide d'un bain liquide.
Toutefois d'autres techniques, connues de l'homme du métier, peuvent être utilisées comme celles décrites dans le brevet US-A-5,902,746 et les demandes de brevet WO-A-98/54306 et WO-A-00/05338. Tous ces titres de Propriété Industrielle appartiennent à la Demanderesse.

L'ADN bactérien a été quantifié par Picogreen (P-7589 ; Molecular Probes, Eugene, OR, USA)suivant le protocole décrit par le fournisseur, à une concentration de 10^7 copies par µL.

Dix (10) µL de lysat sont incubés en présence de 20 µL de *para*-Bio-EG3-PDAM durant 30 minutes à 60°C. Parallèlement, 10 µL de lysat sont incubés dans 20 µL d'eau pure (Sigma) dans les mêmes conditions.

Le milieu réactionnel est ensuite purifié sur colonne QIAquick (Nucleotide Removal Kit, Qiagen, Hilden, Allemagne). Le protocole de purification utilisé est celui recommandé par le fournisseur. Le volume final d'élution est de 50 µL.

Les fragments d'ADN marqués sont ensuite capturés sur des billes magnétiques Dynal (Dynabeads M-280 streptavidin ; référence 112.05 ; Dynal Biotech ASA, Oslo, Norway), qui sont préparées selon le protocole suivant :
Quatre-vingt-dix (90) µL de billes Dynal sont lavés deux fois par 200 µL d'eau pure Free (Sigma), puis sont repris par 200 µL de tampon PEG (0,1 M Na PO_4, pH 7, 0,5 M NaCl ; 0,65 % Tween 20 ; 0,14 mL ADN de sperme de Hareng (Référence 15634-017, GibcoBRL) ; 2 % PEG 4000) et incubés 30 min à 37°C. Elles sont ensuite lavées deux fois par 200 µL de tampon PBS 1X Tween 20 0,5%, puis finalement reprises par 90 µL du même tampon.

Dix (10) µL des éluats d'ADN marqués ou non marqués sont incubés 5 min à température ambiante avec 40 µL de tampon PEG et 2,5 µL de la préparation de billes magnétiques décrite précédemment.

Les billes sont ensuite lavées trois fois par 200 µL de tampon PBS 1 X tween 0,5%, reprises dans 200 µL d'eau et incubées 20 min à 60°C, puis lavées de nouveau quatre fois par 200 µL de PBS tween. Les billes sont finalement reprises par 25 µL d'eau et une PCR est réalisée en suivant le protocole décrit à l'exemple 5. Deux contrôles réactionnels sont effectués l'un avec 25 µL d'eau pure et l'autre avec 2,5 µL de billes préparées et lavées dans les mêmes conditions que les échantillons biologiques, et reprises dans 25 µL d'eau.

Résultat :

Les produits de PCR sont ensuite quantifiés par Picogreen (P-7589 ; Molecular Probes, Eugene, OR, USA) suivant le protocole décrit par le fournisseur. Cette méthode repose sur l'utilisation d'une molécule (Picogreen) ayant la particularité de devenir fluorescente uniquement lorsqu'elle se positionne à l'intérieure d'une molécule d'ADN (en s'intercalant entre les bases). En raison du caractère très spécifique de cette intercalation, et du fait que le signal fluorescent produit est directement proportionnel à la quantité d'ADN présent dans le milieu, il est possible de doser de cette manière, de façon très précise, la concentration d'acide nucléique présente dans un échantillon. Le signal est alors exprimé en rfu (relative fluorescent unit).

L'analyse des résultats de PCR sur gel montre la présence d'une seule bande spécifique à la taille attendue dans les échantillons réalisé à partir d'ADN génomique marqué par *para*-Bio-EG3-PDAM. Les bandes ne sont pas détectées lorsque la PCR a été effectuée à partir d'ADN génomique non marqué. Une quantification de l'ADN par Picogreen permet de confirmer la production d'ADN à partir d'ADN génomique capturé sur billes.

Conditions	rfu
Témoins non Marqués	51
ADN marqués	170
Bruit de fond	20

Tableau 21 : Quantification de l'ADN produit par PCR, à partir d'un lysat bactérien, capturé et purifié par le *para*-Bio-EG3-PDAM

Une analyse sur puce à ADN, suivant le protocole décrit à l'exemple 8 permet de confirmer la spécificité de l'amplification comme le montre le taleau 34 ci-dessous.

	Homologie %	I (rfu)	B(rfu)	I/B
Echantillon	98	11531	723	16

Tableau 22 : Détection spécifique de la cible capturée et purifiée par le *para*-Bio-EG3-PDAM

Cet exemple montre qu'il est possible de préparer un échantillon biologique de façon à amplifier l'acide nucléique qu'il contient, en utilisant une technique de capture basée sur la réactivité de la fonction diazométhyle sur les groupements phosphate de celui-ci.

Exemple 38 : Amplification successive de deux gènes à partir d'un ADN bactérien capturé sur un support solide :

Cet exemple montre qu'il est possible d'amplifier à plusieurs reprises et sur des cibles différentes un ADN capturé grâce à la réactivité de la fonction diazométhyle sur les groupements phosphates de celui-ci .

Dans le cas présent, les acides nucléiques, contenus dans un lysat bactérien et marqués par du *para*-Bio-EG3-PDAM, sont capturés sur des billes magnétiques recouvertes de streptavidine. L'utilisation de billes magnétiques permet de conserver celles-ci par aimantation lors des lavages successifs visant à éliminer les résidus cellulaires présents dans le milieu réactionnel, ces résidus devant être éliminés car ils peuvent inhiber les amplifications par PCR, qui seront pratiquées par la suite. Ces amplifications vont avoir lieu sur deux gènes différents présents dans l'ADN génomiques, respectivement désignés sous les noms de 16S et rpoB. Ces deux gênes sont ensuite analysés par utilisation de puces à ADN.

L'ADN bactérien est obtenu par lyse des cellules contenues dans une culture de *Mycobacterium tuberculosis*, suivant le protocole déjà décrit dans l'exemple 37. L'ADN bactérien a été quantifié par Picogreen suivant le protocole décrit par le fournisseur, à une concentration de 10^7 copies par µL.

Dix (10) µL de lysat sont incubés en présence de 20 µL de *para*-Bio-EG3-PDAM durant 30 minutes à 60°C. Parallèlement, 10 µL de lysat sont incubés dans 20 µL d'eau pure (Sigma) dans les mêmes conditions.

Le milieu réactionnel est ensuite purifié sur colonne QIAquick (Nucleotide Removal Kit, Qiagen, Hilden, Allemagne). Le protocole de purification utilisé est celui recommandé par le fournisseur. Le volume final d'élution est de 50 µL.

Les fragments d'ADN marqués sont ensuite capturés sur des billes magnétiques Dynal, qui sont préparées suivant le protocole suivant :
Quatre-vingt-dix (90) µL de billes Dynal sont lavées deux fois par 200 µL d'eau pure Free (Sigma), puis sont reprises par 200 µL de tampon PEG (0,1 M Na PO_4, pH 7, 0,5 M NaCl ; 0,65 % Tween 20 ; 0,14 mL ADN de sperme de saumon (Gibco) ; 2 % PEG 4000) et incubées 30 min à 37°C. Elles sont ensuite lavées deux fois par 200 µL de tampon PBS 1X Tween 20 0.5%, puis finalement reprises par 90 µL du même tampon.

Dix (10) µL des éluats d'ADN marqués ou non marqués sont incubés 5 min à température ambiante avec 40 µL de tampon PEG et 2,5 µL de la préparation de billes magnétiques décrite précédemment.

Les billes sont ensuite lavées trois fois par 200 µL de tampon PBS 1 X tween 0,5 %, reprise dans 200 µL d'eau et incubées 20 min à 60°C, puis lavées de nouveau quatre fois par 200 µL de PBS tween. Les billes sont finalement reprises par 25 µL d'eau et une PCR est réalisée en suivant le protocole décrit dans l'exemple 5. Deux contrôles réactionnels sont effectués l'un avec 25 µL d'eau pure (Sigma) et l'autre avec 2,5 µL de billes préparées et lavées dans les même, conditions que les échantillons biologiques, et reprise, dans 25 µL d'eau.

Après amplification, le milieu réactionnel est recueilli, et les billes sont séparées et lavées par 150 µL de PBS 1 X Tween 0,5%, puis resuspendues dans 25 µL d'eau pure (Sigma). Une nouvelle amplification est réalisée sur les billes, mais en présence d'amorces destinées à amplifier le gène rpoB.

Des amplifications témoins réalisés à partir d'ADN génomique non capturé sont réalisées en parrallèle sur les deux systèmes d'amplification (rpoB et 16S).

Résultat :

Les produits de PCR obtenus sont ensuite analysés par puces à ADN suivant le protocole décrit à l'exemple 8.

Conditions		Homologie (%)	I (rfu)	B (rfu)	I/B
PCR témoin 16S	16S	96	4662	387	12
Résultats sur séquence rpoB	RpoB	23	237	358	1
PCR témoin rpoB	RpoB	98	5438	397	14
Résultats sur séquence 16S	16S	17	183	391	1
PCR 16S sur billes	16S	98	2726	534	5
Résultats sur séquence rpoB	RpoB	8	161	480	<1
PCR rpoB sur billes lavées	RpoB	97	3205	349	9
Résultats sur séquence 16S	16S	14	84	358	<1

Tableau 23 : Analyse sur puces à ADN des amplicons ADN issus des PCR amplifiées successivement ou non

L'analyse des résultats de PCR sur gel montre la présence d'une seule bande spécifique à la taille attendue dans les échantillons réalisés à partir d'ADN génomique marqué par *para*-Bio-EG3-PDAM. Les bandes ne sont pas détectées lorsque la PCR a été effectuée à partir d'ADN génomique non marqué.

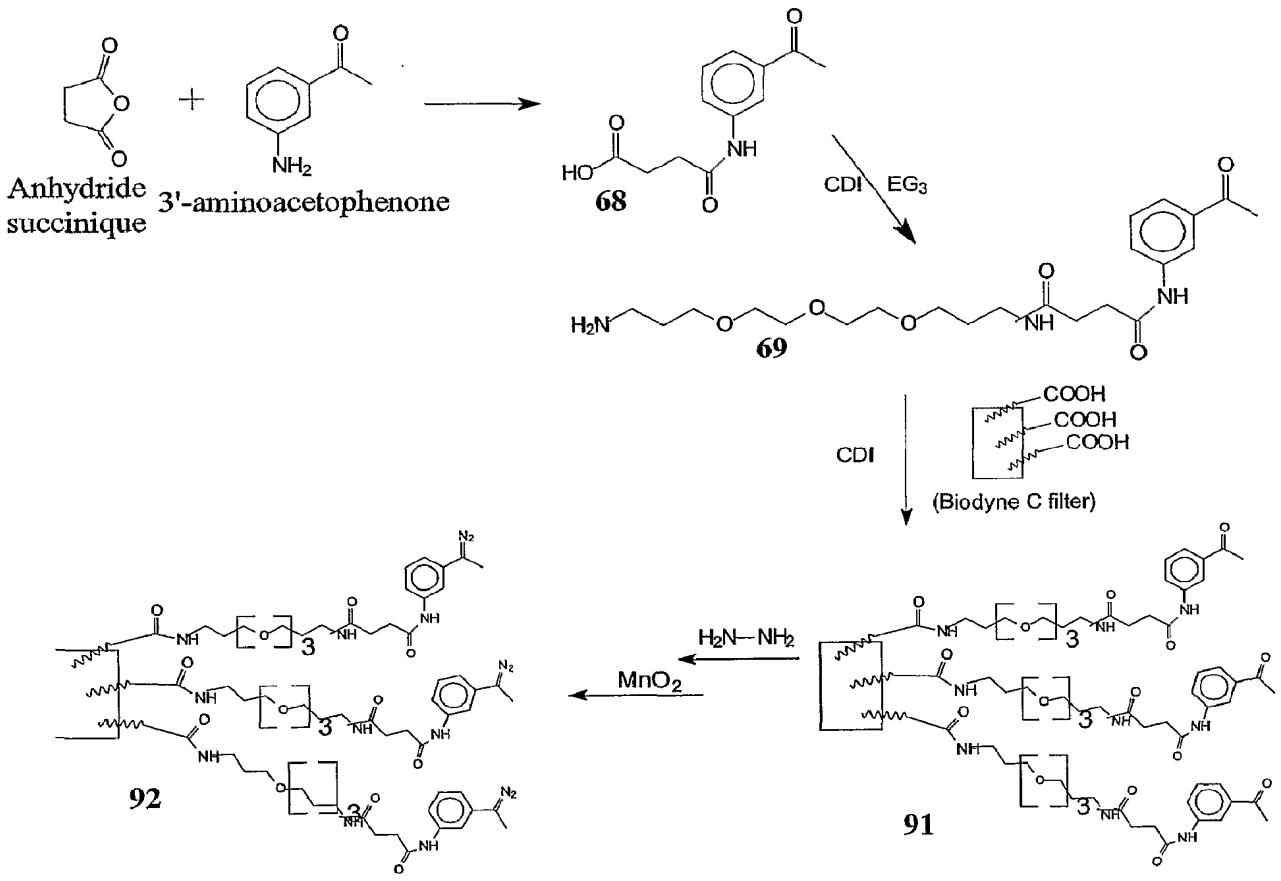
Une analyse par puce à ADN, telle que présentée sur le tableau 23 ci-dessus, permet de confirmer la spécificité des deux amplifications successives, et donc la capacité de réaliser une amplification successive de plusieurs gènes à partir d'ADN immobilisé sur un support solide, ceci permettant d'éviter la mise au point de systèmes multiplex, qui réduisent souvent de façon importante la sensibilité et l'efficacité des amplifications d'acides nucléiques.

Exemple 39 : Capture et Amplification d'ADN sur une membrane de Nylon portant des groupements diazométhyles :

Une membrane de nylon activée de façon à porter des groupements diazométhyles a été utilisée afin de capturer de l'ADN bactérien, dans le but de l'amplifier par PCR.

Exemple 39.1 : Modification du filtre Biodyne C :

Schéma de synthèse :



Composé **68** :

On solubilise la 3'-aminoacetophenone (14,5 g, 107 mmol) dans 50 ml de DMF anhydre. On ajoute l'anhydride succinique (10,7 g, 107 mmol) et on laisse sous agitation, sous argon et à température ambiante. Après 6 h, la solution est concentrée sous vide et 50 ml de méthanol sont ajoutés. Le précipité obtenu est filtré et lavé avec méthanol et éther. On obtient ainsi 19,4 g (81 %) de produit **68** sous forme d'une poudre de couleur blanc cassé.

RMN ^{1}H (200 MHz, DMSO-d_6): d= 2,5-2,6 (m, 7H); 7,45 (t, 1H); 7,64 (d, 1H); 7,83 (d, 1H); 8,19 (s, 1H); 10,16 (s, 1H); 12,12 (s, 1H).

Composé **69** :

On solubilise 5,07 g (22 mmol) du composé **68** dans 10 ml de DMF anhydre, sous argon. On met sur glace et on ajoute 5,00 g (32 mmol) de carbonyldiimidazole. Apres 20 min, on ajoute lentement 20 ml (94,6 mmol) du 4,7,10-trioxatridecanediamine (EG_3). Après 3h de réaction à température ambiante, on évapore le DMF et on reprend le résidu dans 100 ml de CH_2Cl_2. On fait des extractions avec du $NaHCO_3$ saturé et H_2O, après quoi la phase organique est séchée avec du Na_2SO_4 anhydre et le solvant évaporé. On obtient ainsi 4,34 g (46 %) du produit **69**.

RMN ^{1}H (200 MHz, DMSO-d_6): d= 1,59 (m, 2H); 1,87 (m, 2H); 2,16 (s, 3H); 2,40 (m, 2H); 2,55 (m, 2H); 3,08 (m, 2H); 3,45 (m, 16H); 7,30 (t, 1H); 7,42 (d, 1H); 7,70 (d, 1H); 7,83 (t, 1H); 7,97 (s, 1H); 10,00 (s, 1H).

Composé **91** :

Un rectangle de 4 cm^2 est découpé sur une feuille de filtre Biodyne C (référence 60314 ; Pall Gelman Laboratory ; Ann Arbor ; Michigan ; USA), introduit dans un flacon et mis en contact avec 0,97 g (6 mmol) de carbonyldiimidazole (CDI) dans 3 ml de DMF anhydre, sur glace, sous argon et sous forte agitation. Après 20 min, la solution est éliminée et le filtre lavé avec du DMF. Une quantité de 0,53 g du produit **68** (1 mmol) dans 3 ml de DMF anhydre est ensuite ajoutée, et la réaction se réalise pendant la nuit à température ambiante. La solution est ensuite enlevée et le filtre est rincé avec de l'éthanol, séché sous vide et gardé sous argon.

Composé **92** :

Le filtre modifié **91** est mis dans une solution de 97 ml d'hydrazine hydrate (2 mmol) dans 4 ml d'éthanol absolu. La solution est mise à reflux pendant 5 h. Après avoir laissé refroidir, le filtre est lavé avec H_2O, éthanol et éther, séché sous vide et mis sous argon. Ensuite, on ajoute 4 ml de DMF anhydre et 86 mg de MnO_2 (1 mmol), et on laisse réagir sous forte agitation. Après 20 min, la solution est rejetée, et le filtre est rincé avec du DMF et de l'éther. Le filtre modifié-diazométhyl **92** est conservé sous argon, à une température de -19 à -31°C.

Exemple 39.2 : Essais biologiques :

La membrane activée est découpée en petits fragments de 2 mm^2 qui vont être incubés pendant 30 minutes à température ambiante dans 25 µl de lysat bactérien de *Mycobacterium tuberculosis*, préparé par lyse mécanique, suivant la même technique et la même concentration finale que dans l'exemple 37, et 375 µl d'eau pure (Sigma).

La membrane est ensuite placée à 65°C pendant 60 min dans 100 mL de tampon de lavage (5 % Formamide (Sigma), 1X SSPE (Perbio), 0.01 % triton X-100) afin d'éliminer les acides nucléiques non spécifiques adsorbés sur la membrane, puis celle-ci est stockée dans 1 mL d'eau pure avant amplification.

La PCR est pratiquée comme décrite dans le paragraphe 5.1, le volume réactionnel étant complété par une quantité suffisante d'eau pure.

Parallèlement, des contrôles sont effectués suivant le même processus avec des membranes ne pouvant pas lier de façon covalente les acides nucléiques :

- Membrane Biodyne C non modifiée (membrane A),
- Membrane Biodyne modifiée chimiquement suivant le processus décrit, mais non traitée par du DMF anhydre et du MnO_2 ; ce contrôle permet de vérifier le comportement de la membrane en l'absence de groupements diazométhyles (membrane B), et

- Membrane Biodyne C non modifiée, mais traitée 20 min sous forte agitation par du DMF anhydre et du MnO_2, ce contrôle permettant de vérifier que cette dernière étape ne modifie pas l'adsorption de l'ADN sur la membrane (membrane C).

Afin de contrôler l'absence d'inhibition de la PCR provoquée par le traitement des membranes, un autre fragment des membranes A, B, C est amplifié simultanément avec 25 µl de lysat bactériens.(tubes A', B', C')

Les produits de PCR sont ensuite quantifiés par Picogreen suivant le protocole décrit par le fournisseur.

Résultat :

Tests effectués	Signal (rfu)
Membrane modifiée	111
Membrane non modifiée (A)	18
Membrane non modifiée, co-amplifiée avec 25 µL de lysat bactérien (A')	260
Membrane modifiée non activée (B)	26
Membrane modifiée non activée, co-amplifiée avec 25 µL de lysat bactérien (B')	264
Membrane non modifiée ayant subit une activation (C)	21
Membrane non modifiée ayant subit une activation, co-amplifiée avec 25 µL de lysat bactérien (C')	268

Tableau 24 : Quantification de l'ADN obtenu par PCR à partir d'ADN de lysat bactérien capturé sur support solide

Ces résultats du tableau 24 indiquent qu'il est possible de capturer de façon covalente sur un support solide, des acides nucléiques, issus de lysat, grâce à la chimie diazométhyle. L'amplification observée n'est pas due à une adsorption non spécifique de l'ADN sur la membrane. D'autre part, on observe avec les contrôles effectués avec les PCR que les membranes ne provoquent pas d'inhibition de la réaction d'amplification.

Afin de contrôler la nature du produit amplifié sur la membrane, le produit d'amplification a été analysé par passage sur puce à ADN, suivant le protocole décrit auparavant.

5

IDT Exhibit 1020.7520

REVENDICATIONS

1. Procédé de marquage et de fragmentation d'un acide désoxyribonucléique (ADN) simple ou double brin comprenant les étapes suivantes :
- fragmenter l'ADN en créant au moins un site abasique sur ledit ADN,
- attacher un marqueur sur au moins un des fragments par l'intermédiaire d'un réactif de marquage, ledit réactif se couplant de manière covalente et majoritaire sur au moins un phosphate dudit fragment.

2. Procédé, selon la revendication 1, caractérisé par le fait que la fragmentation et le marquage sont effectués en deux étapes.

3. Procédé, selon la revendication 1, caractérisé par le fait que la fragmentation et le marquage sont réalisés en une étape.

4. Procédé, selon l'une quelconque des revendications 1 à 3, caractérisé par le fait que le site abasique est généré par action d'un milieu aqueux acide.

5. Procédé, selon la revendication 4, caractérisé par le fait que le pH du milieu acide est inférieur à 5, de préférence inférieur à 4.

6. Procédé, selon la revendication 5, caractérisé par le fait que le milieu aqueux acide est un tampon formiate de sodium à pH d'environ 3.

7. Procédé, selon l'une quelconque des revendications 1 à 6, caractérisé par le fait que l'ADN contient au moins une base modifiée susceptible de générer un site abasique.

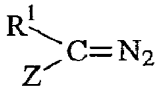
8. Procédé, selon la revendication 7, caractérisé par le fait que la base modifiée susceptible de générer un site abasique est choisi parmi les dérivés de la 8-bromo purine.

9. Procédé, selon l'une quelconque des revendications 1 à 3, caractérisé par le fait que le site abasique est généré par un agent alkylant ou un agent oxydant.

10. Procédé de marquage et de fragmentation, selon l'une quelconque des revendications 1 à 9, caractérisé par le fait que le réactif de marquage comprend, comme fonction réactive, un motif choisi parmi les composés suivants : diazométhyle ; halogénure d'alkyle ; nitrosourée ; spirocyclopropane ; aziridine ; époxyde ; trifuorosulfonates.

11. Procédé, selon la revendication 10, caractérisé par le fait que ledit réactif de marquage est la 5-(bromométhyl)fluorescéine.

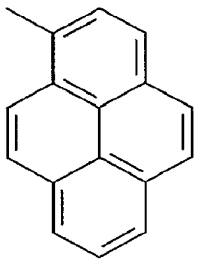
12. Procédé, selon la revendication 10, caractérisé par le fait que le réactif de marquage est choisi parmi les composés de formule (1):



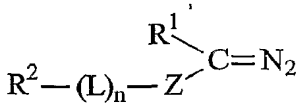
dans laquelle :

- R^1 représente H ou un groupe alkyle, alkyle substitué, aryle ou aryle substitué, et
- Z comprend un marqueur détectable.

13. Procédé, selon la revendication 12, caractérisé par le fait que Z est :

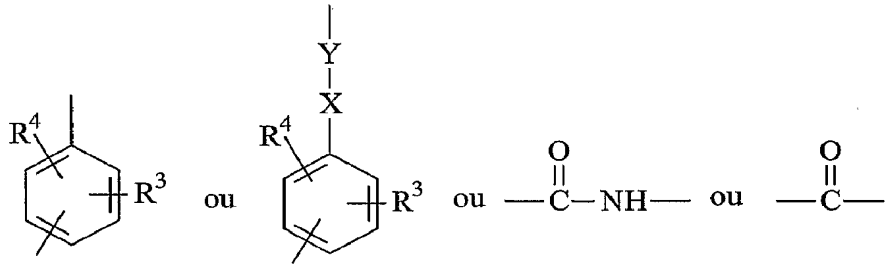


14. Procédé, selon la revendication 12, caractérisé par le fait que le réactif de marquage est choisi parmi les composés de formule (2) :



dans laquelle

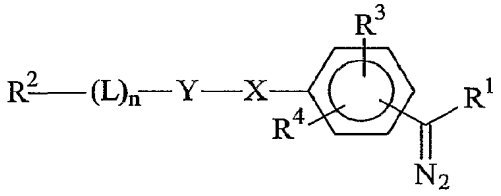
- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 est un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins deux liaisons covalentes et n est égal à 0 ou 1, et
- Z est choisi parmi :



dans lequel :

- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle, et
- -Y-X- représente -CONH-, -NHCO-, $-CH_2O-$, $-CH_2S-$.

15. Procédé, selon la revendication 14, caractérisé par le fait que le réactif de marquage a la formule (3) :



dans laquelle :

- R^1 représente H ou un groupe alkyle, aryle ou aryle substitué,
- R^2 représente un marqueur détectable,
- L est un bras de liaison comportant un enchaînement linéaire d'au moins 2 liaisons covalentes et n un nombre entier égal à 0 ou 1,
- R^3 et R^4 représentent indépendamment l'un de l'autre : H, NO_2, Cl, Br, F, I, OR, SR, NR_2, R, NHCOR, CONHR, COOR avec R = alkyle ou aryle, et
- -Y-X- représente -CONH-, -NHCO-, $-CH_2O-$, $-CH_2S-$.

16. Procédé, selon l'une quelconque des revendications 14 ou 15, caractérisé par le fait que R^2 est un composé fluorescent ou un haptène.

17. Procédé, selon l'une quelconque des revendications 10 à 16, caractérisé par le fait que ledit réactif de marquage est soluble dans un solvant miscible à l'eau.

18. Procédé de détection d'un acide désoxyribonucléique cible (ADN) simple ou double brin comportant les étapes suivantes :

- fragmenter et marquer lesdits ADN, selon l'un quelconque des procédés selon les revendications 1 à 17,
- hybrider les fragments marqués sur au moins une sonde nucléique suffisamment spécifique de l'acide nucléique cible, et
- détecter l'hybride formé par l'intermédiaire du marqueur.

19. Procédé de détection d'un acide désoxyribonucléique cible (ADN) double brin, selon la revendication 18, comportant en outre une étape de dénaturation après la fragmentation et le marquage.

20. Procédé, selon la revendication 19, caractérisé par le fait que la fragmentation, le marquage et la dénaturation sont réalisés en une seule étape.

21. Procédé de détection d'un acide nucléique cible comportant les étapes suivantes :

- amplifier enzymatiquement l'acide nucléique cible pour générer une multitude d'amplicons ADN double brin,
- fragmenter et marquer lesdits amplicons ADN, selon l'un quelconque des procédés selon les revendications 1 à 17,
- hybrider les fragments marqués sur au moins une sonde nucléique suffisamment spécifique de l'acide nucléique cible, et
- détecter l'hybride formé par l'intermédiaire du marqueur.

22. Procédé, selon la revendication 21, comportant en outre une étape de dénaturation après l'étape de fragmentation et marquage.

23. Procédé, selon la revendication 22, caractérisé par le fait que la fragmentation, le marquage et la dénaturation sont réalisés en une seule étape.

24. Procédé de mise en évidence d'un polymorphisme réparti en des positions prédéterminées ou non d'un acide nucléique cible par la présence d'une pluralité de délétions et/ou insertions et/ou mutations dans la séquence dudit acide nucléique cible par rapport à une séquence dite de référence comportant les étapes suivantes :

- disposer d'un ADN cible comportant l'ensemble du polymorphisme à étudier, ledit ADN étant éventuellement généré par une technique d'amplification enzymatique.
- fragmenter et marquer ledit ADN par un procédé, selon l'une quelconque des revendications 1 à 17.
- hybrider lesdits fragments sur une pluralité de sondes nucléiques dites sondes de capture, la pluralité de sondes de capture étant fixé sur un support solide et la pluralité de sondes de capture couvrant dans son ensemble au moins le polymorphisme à étudier.
- détecter les hybrides formés entre les fragments marqués et au moins une partie des sondes nucléiques par l'intermédiaire du marqueur et en déduire le polymorphisme de l'ADN cible.

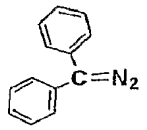
25. Procédé, selon la revendication 24, comportant en outre une étape de dénaturation après l'étape de fragmentation et marquage.

26. Procédé, selon la revendication 25, caractérisé par le fait que la fragmentation, le marquage et la dénaturation sont réalisés en une seule étape.

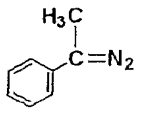
27. Procédé, selon l'une quelconque des revendications 24 à 26, caractérisé par le fait que le support solide comporte au moins dix (10) sondes nucléiques de séquences différentes, avantageusement au moins quatre cents (400), de préférence au moins mille (1000).

28. Utilisation d'un fragment d'acide nucléique marqué, selon le procédé de l'une quelconque des revendication 1 à 17, comme sonde de détection d'un acide nucléique cible.

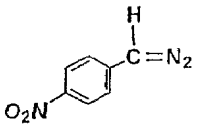
Fig. 1



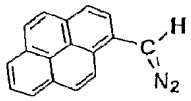
DPDAM
(DiPhénylDAM)



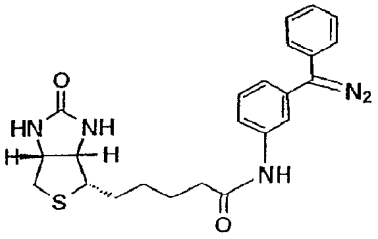
PMDAM
(PhénylMéthylDAM)



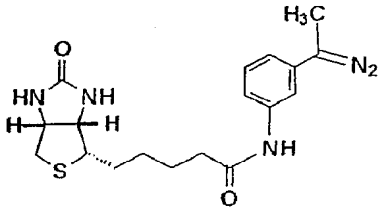
NPDAM
(NitroPhénylDAM)



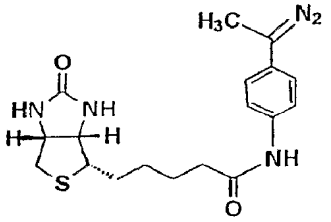
PDAM
(PyrénylDiAzoMéthane)



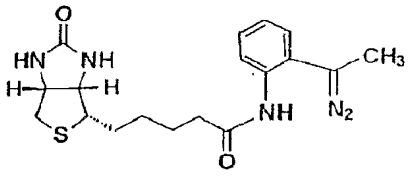
BioDPDAM
(BiotinylDPDAM)



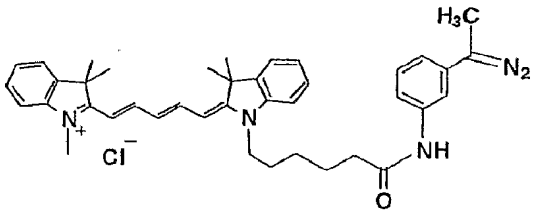
m-BioPMDAM
(*meta*-BiotinylPMDAM)



p-BioPMDAM
(*para*-BiotinylPMDAM)



o-BioPMDAM
(*ortho*-BiotinylPMDAM)



Cy5PMDAM

Fig. 2A

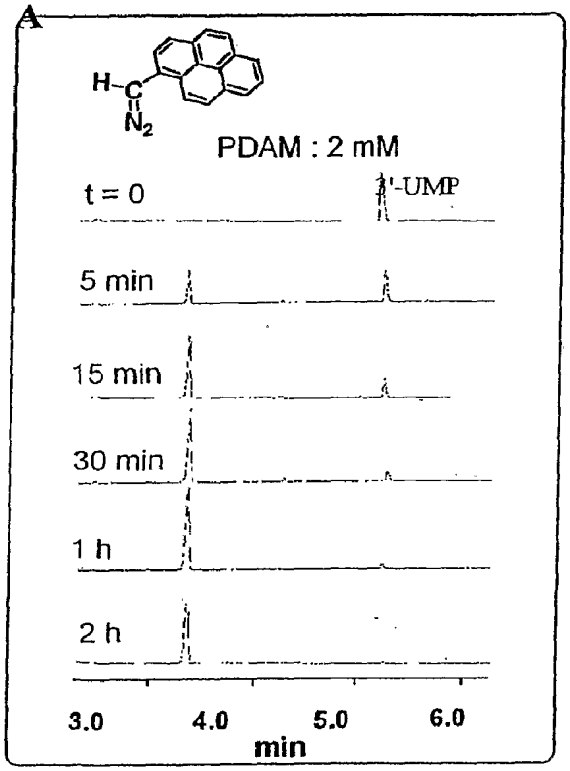


Fig. 2B

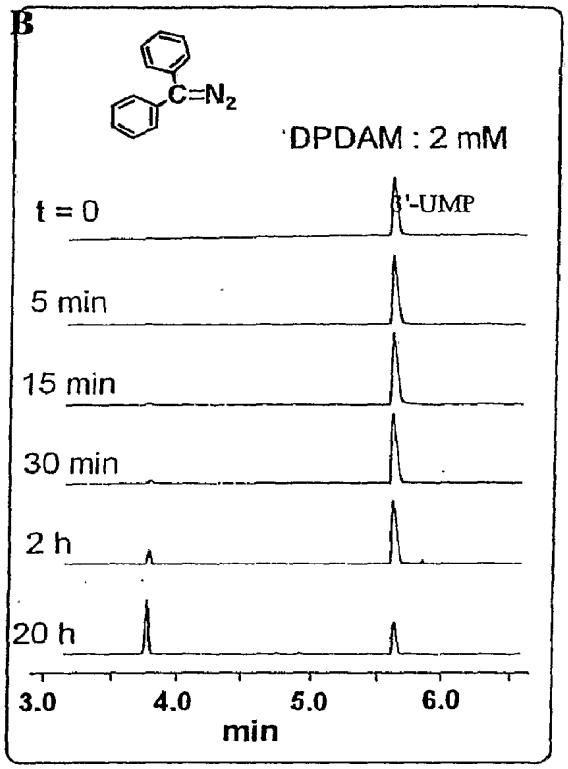


Fig. 2C

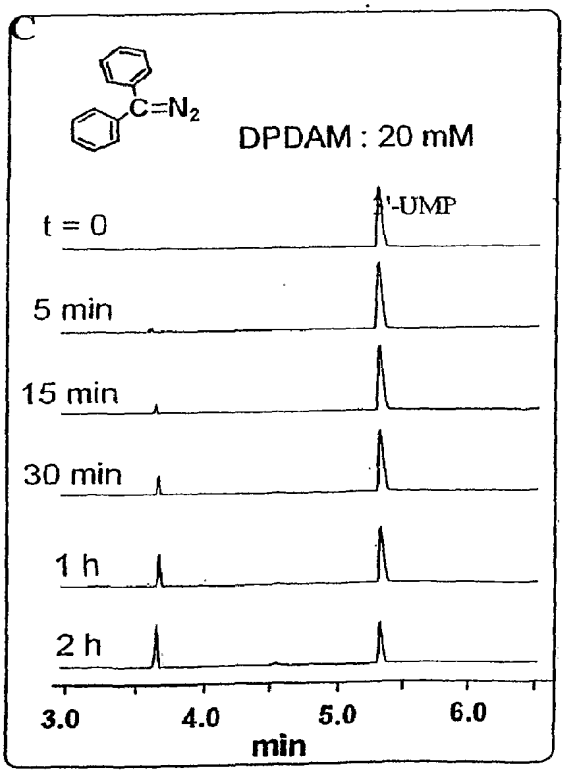


Fig. 2D

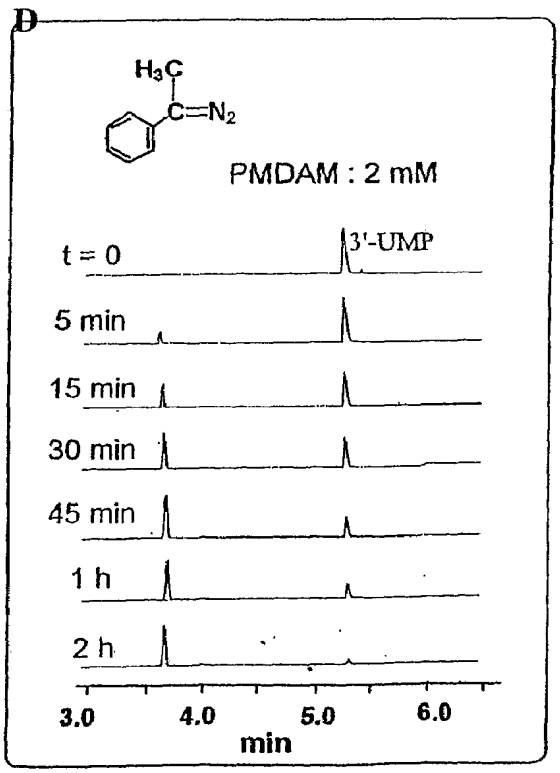


Fig. 2E

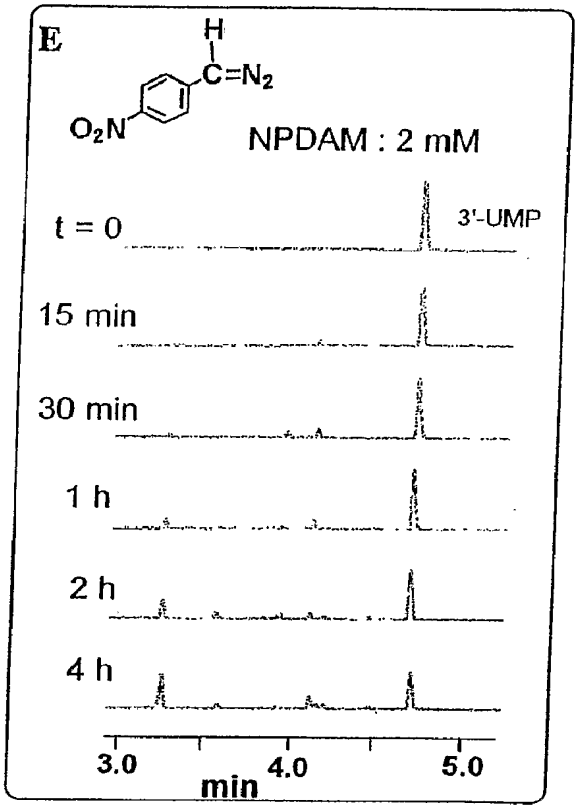


Fig. 2F

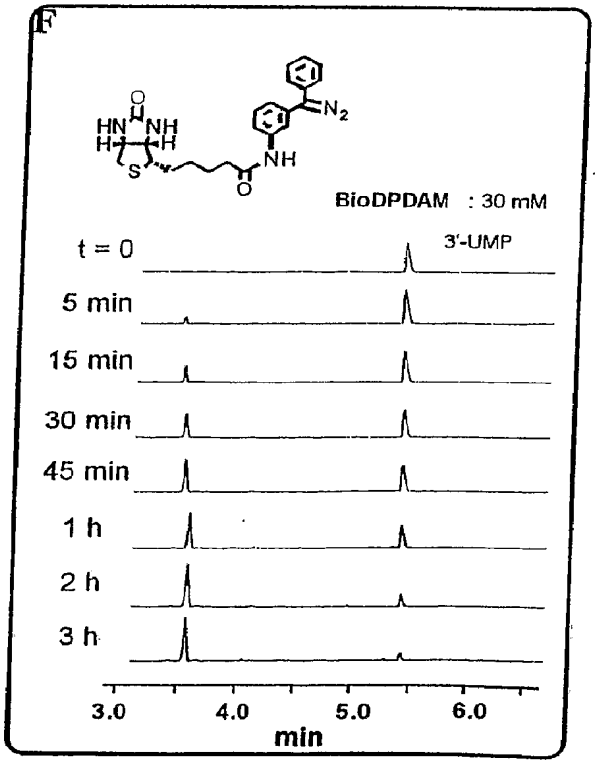


Fig. 2G

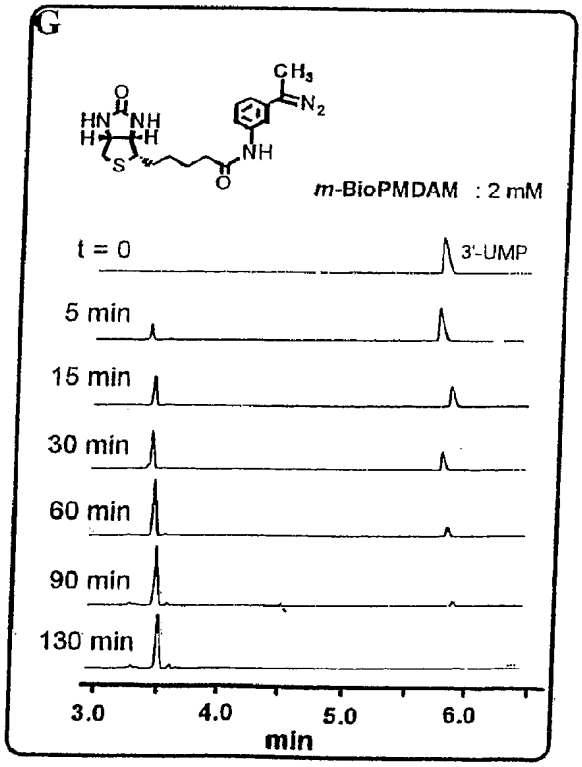


Fig. 2H

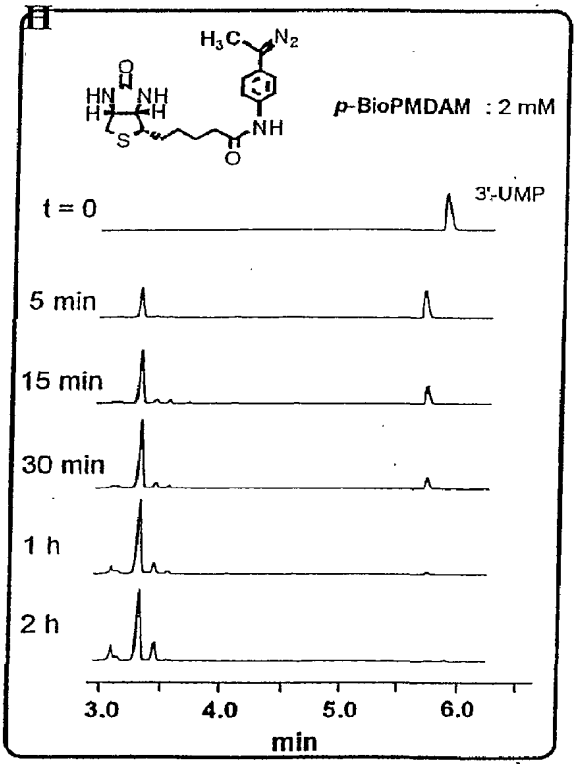

H
p-BioPMDAM : 2 mM
3'-UMP
t = 0
5 min
15 min
30 min
1 h
2 h
3.0
4.0
5.0
6.0
min

Fig. 2I

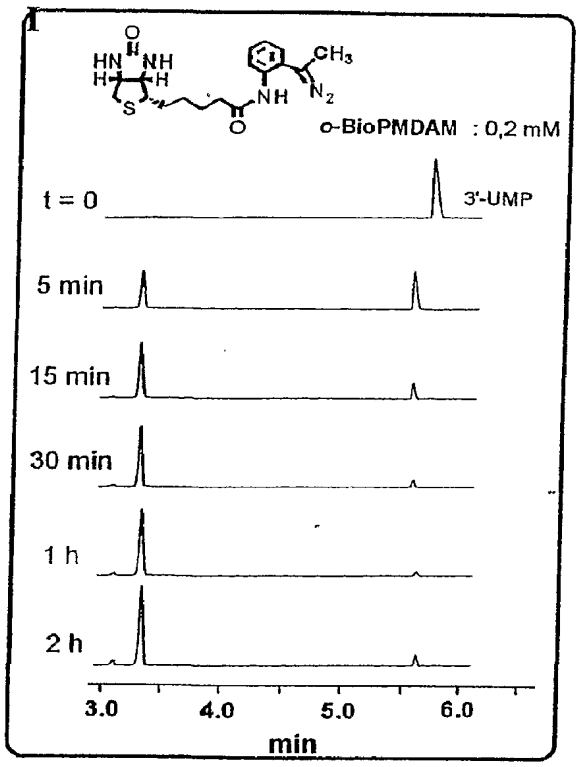

I
o-BioPMDAM : 0,2 mM
3'-UMP
t = 0
5 min
15 min
30 min
1 h
2 h
3.0
4.0
5.0
6.0
min

Fig. 3A

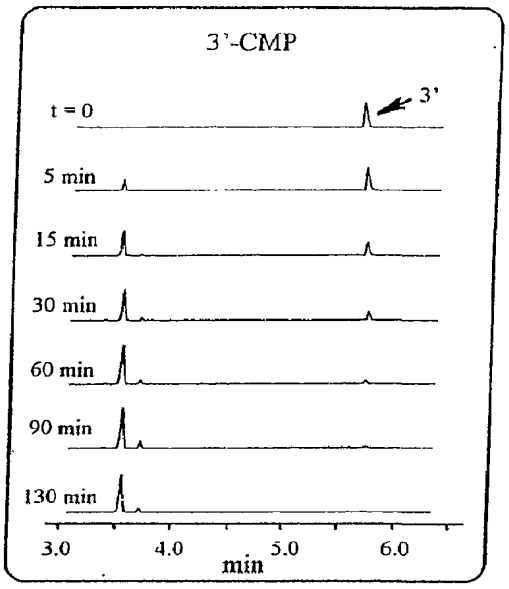


Fig. 3B

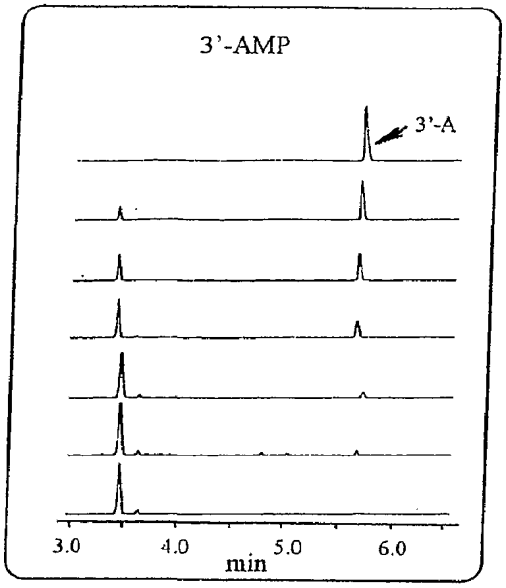


Fig. 3C

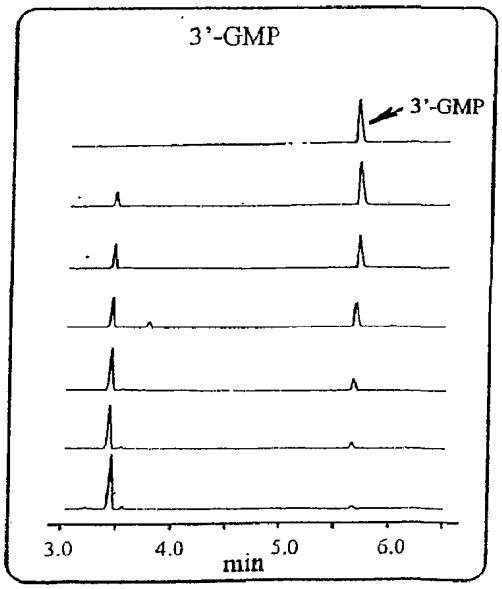


Fig. 3D

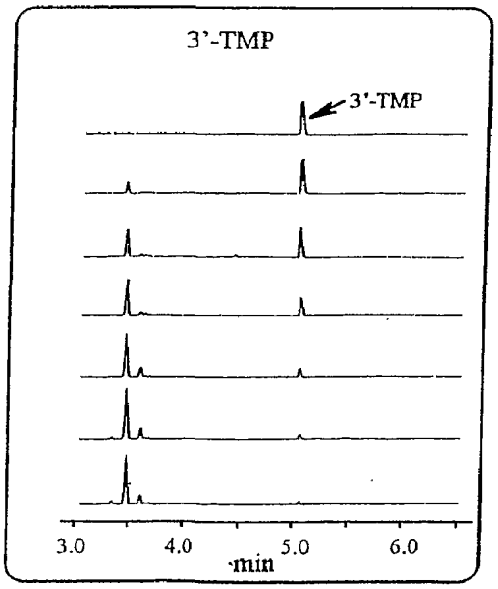
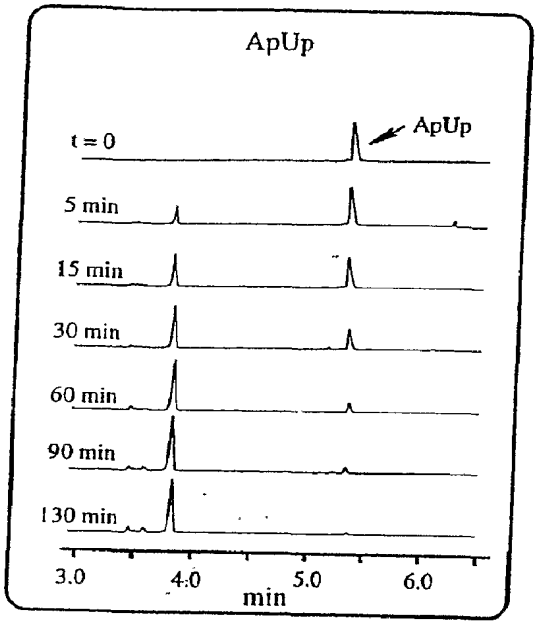


Fig. 4


ApUp
t = 0
ApUp
5 min
15 min
30 min
60 min
90 min
130 min
3.0
4.0
5.0
6.0
min

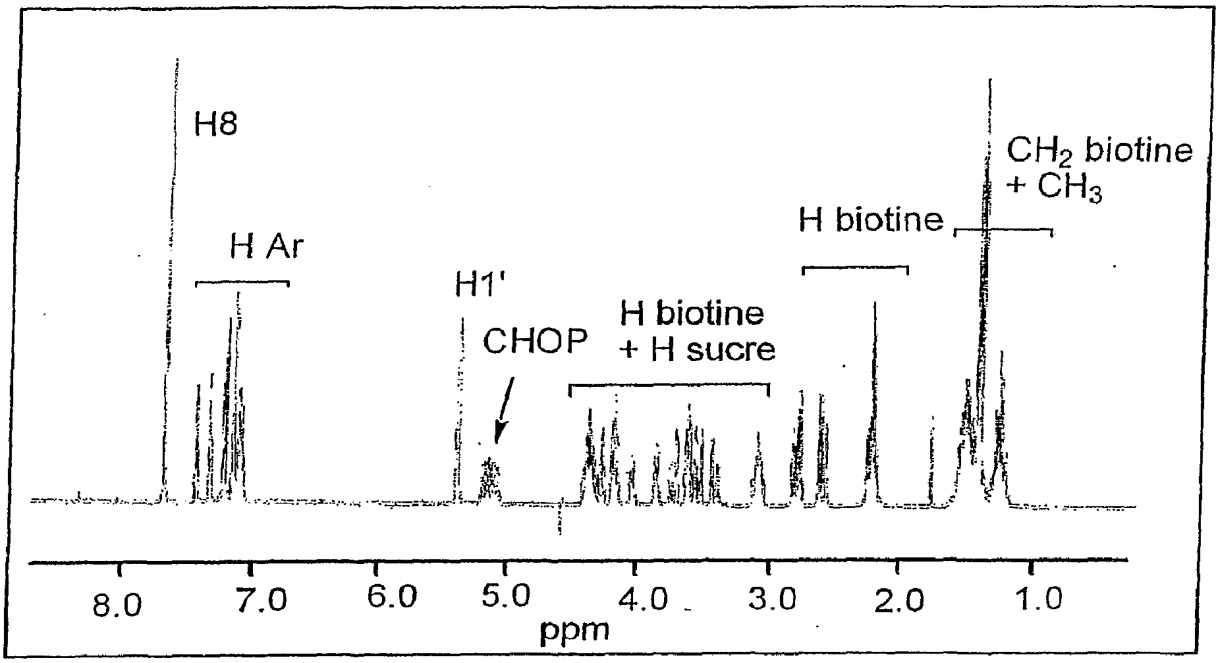

H8
H Ar
H1'
CHOP
H biotine
+ H sucre
H biotine
CH2 biotine
+ CH3
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm

Fig. 5B

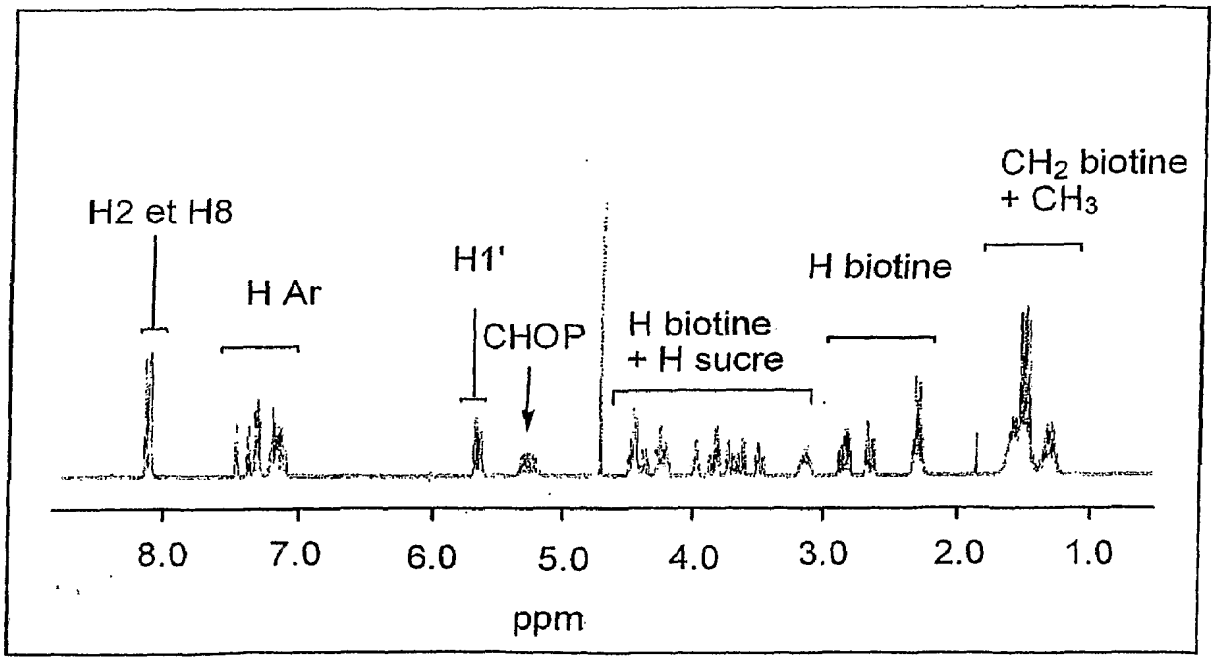

H2 et H8
H Ar
H1'
CHOP
H biotine
+ H sucre
H biotine
CH2 biotine
+ CH3
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm

Fig. 5C

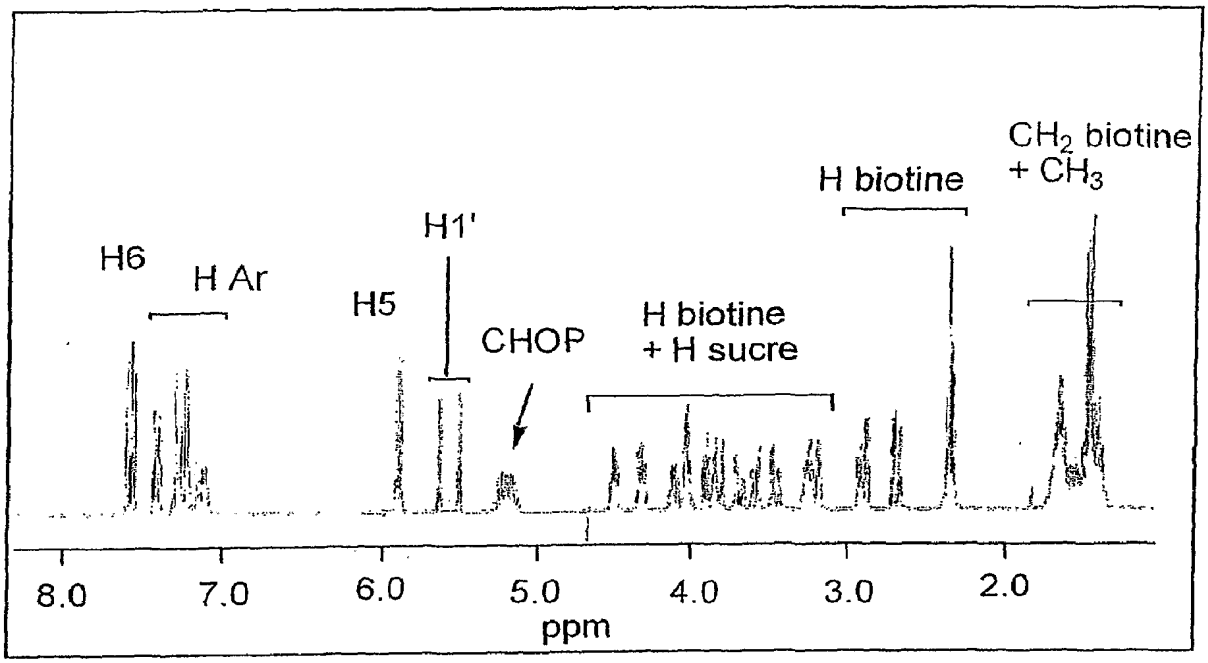
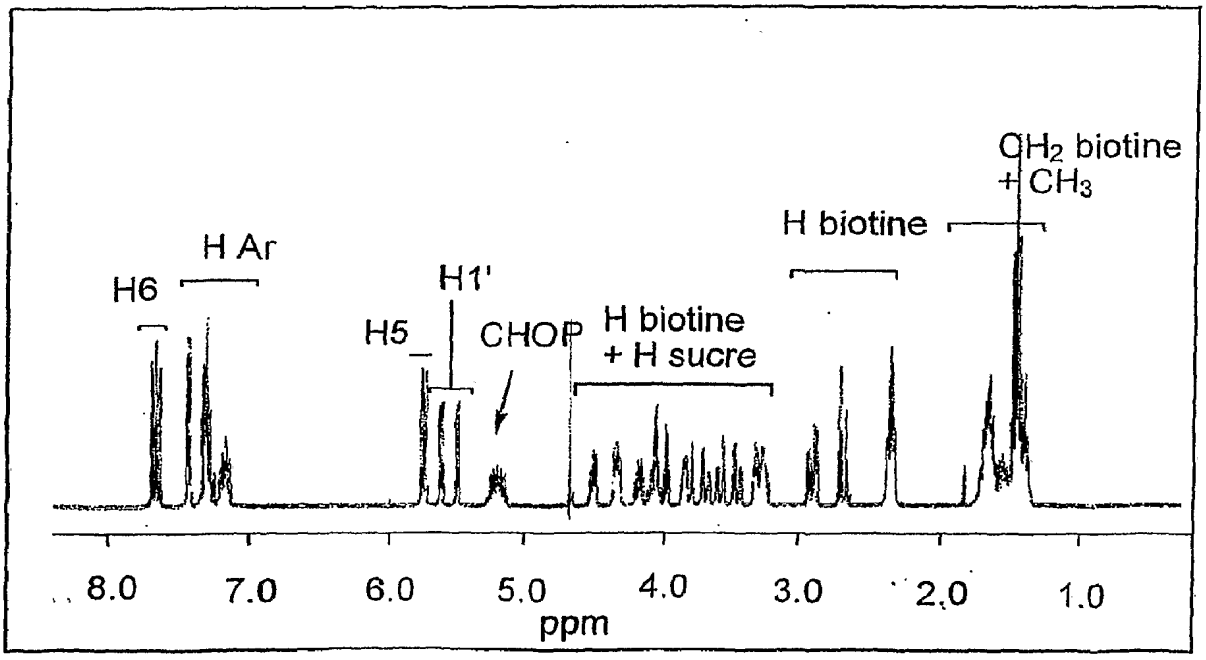

H6
H Ar
H5
H1'
CHOP
H biotine
+ H sucre
H biotine
CH2 biotine
+ CH3
8.0
7.0
6.0
5.0
4.0
3.0
2.0
ppm

Fig. 5D


H6
H Ar
H5
H1'
CHOP
H biotine
+ H sucre
H biotine
CH2 biotine
+ CH3
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ppm

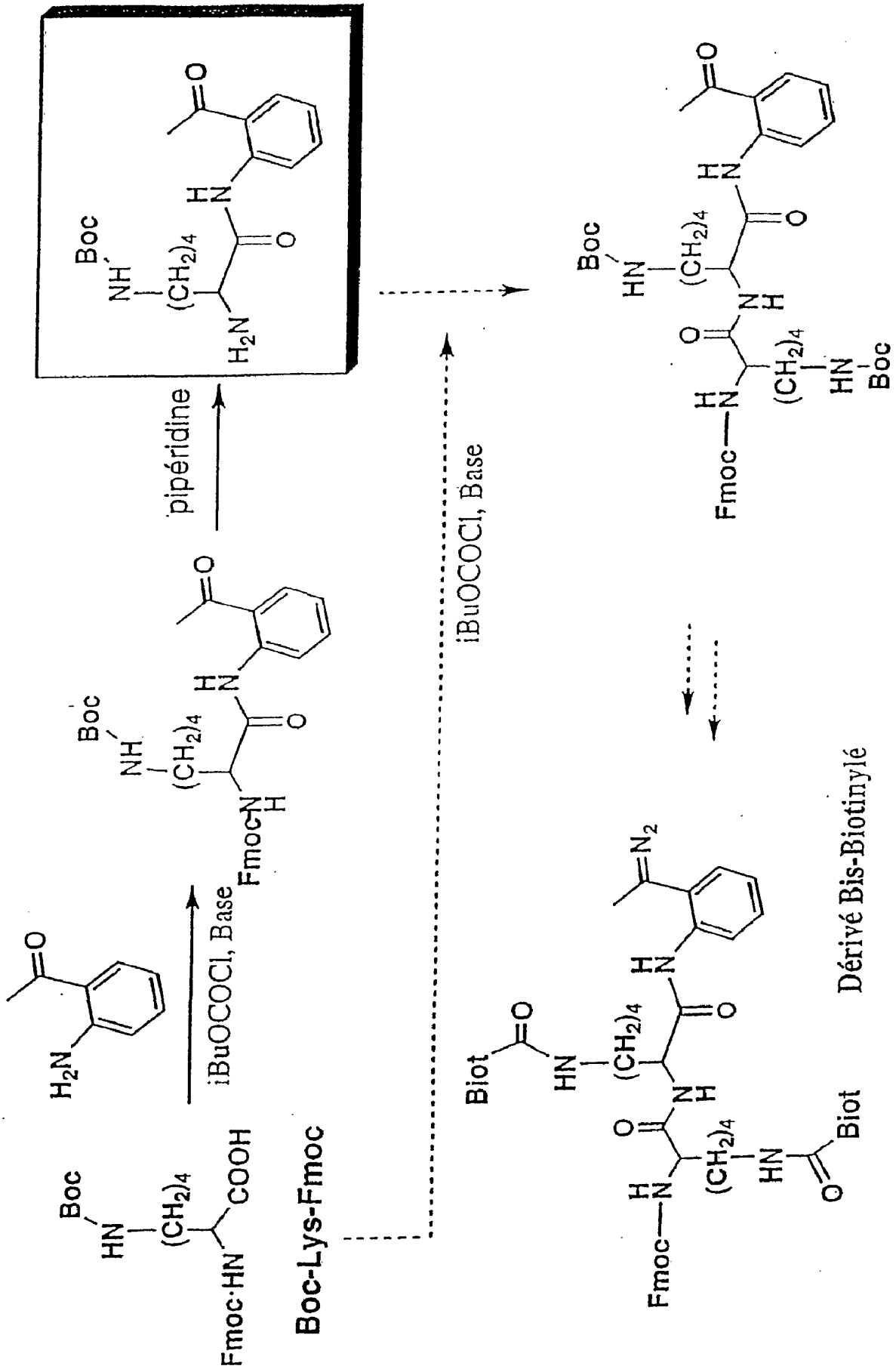

Boc-Lys-Fmoc
iBuOCOCl, Base
pipéridine
iBuOCOCl, Base
Dérivé Bis-Biotinylé

Fig. 6

Fig 7

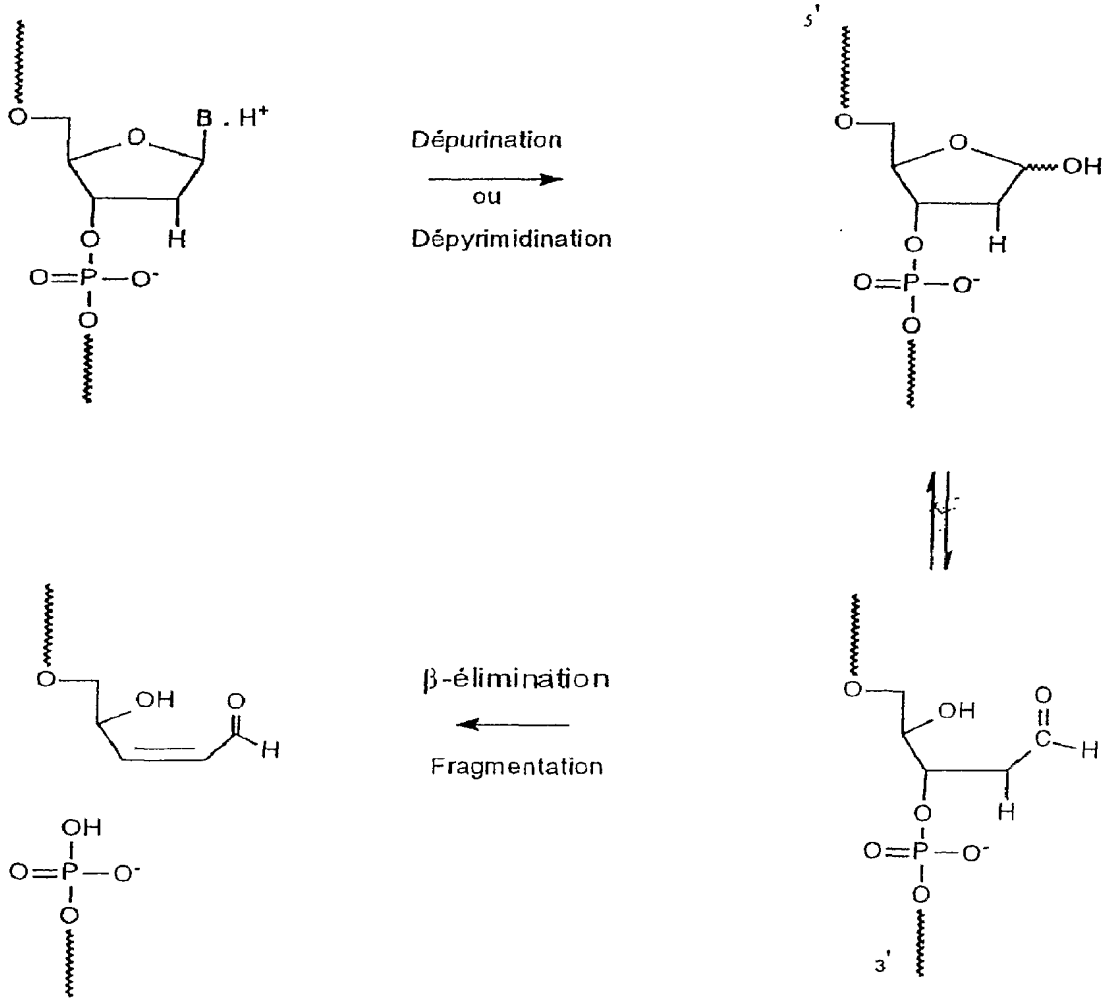

Dépurination
ou
Dépyrimidination
β-élimination
Fragmentation
5'
3'

Fig 8

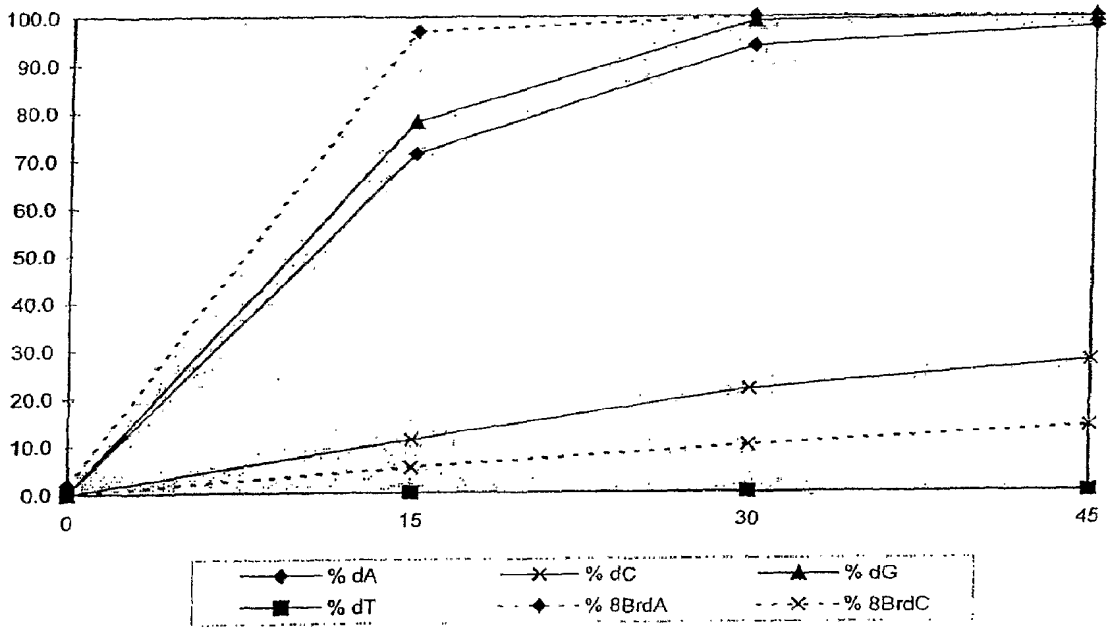

100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
15
30
45
% dA
% dC
% dG
% dT
% 8BrdA
% 8BrdC

Fig 9

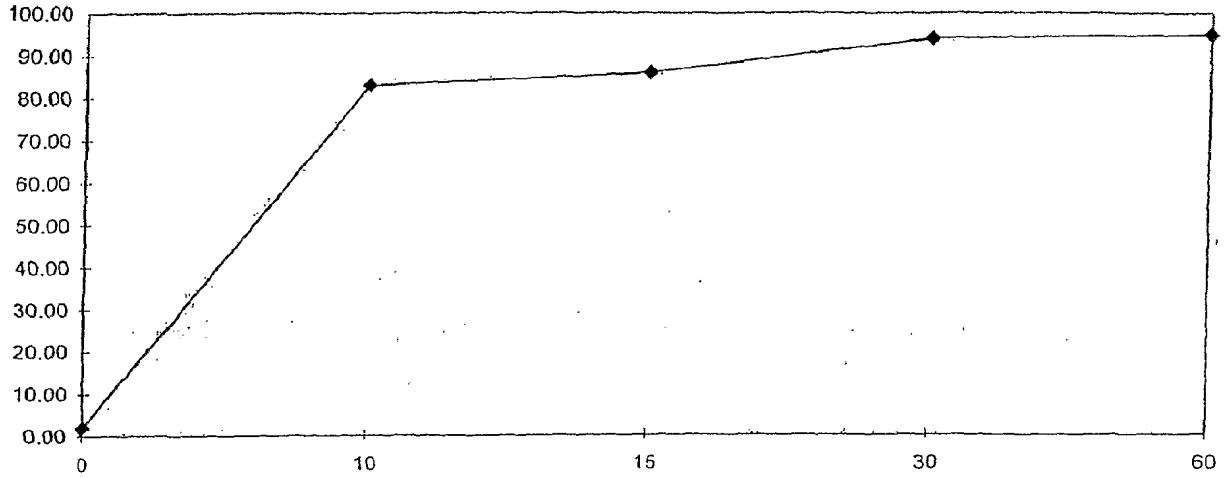
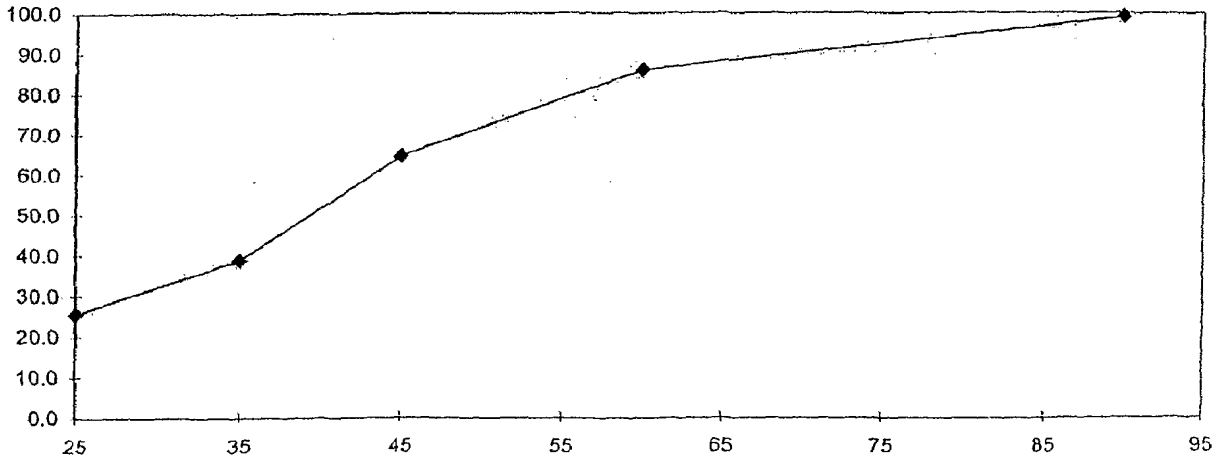

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
10
15
30
60

Fig 10


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
25
35
45
55
65
75
85
95

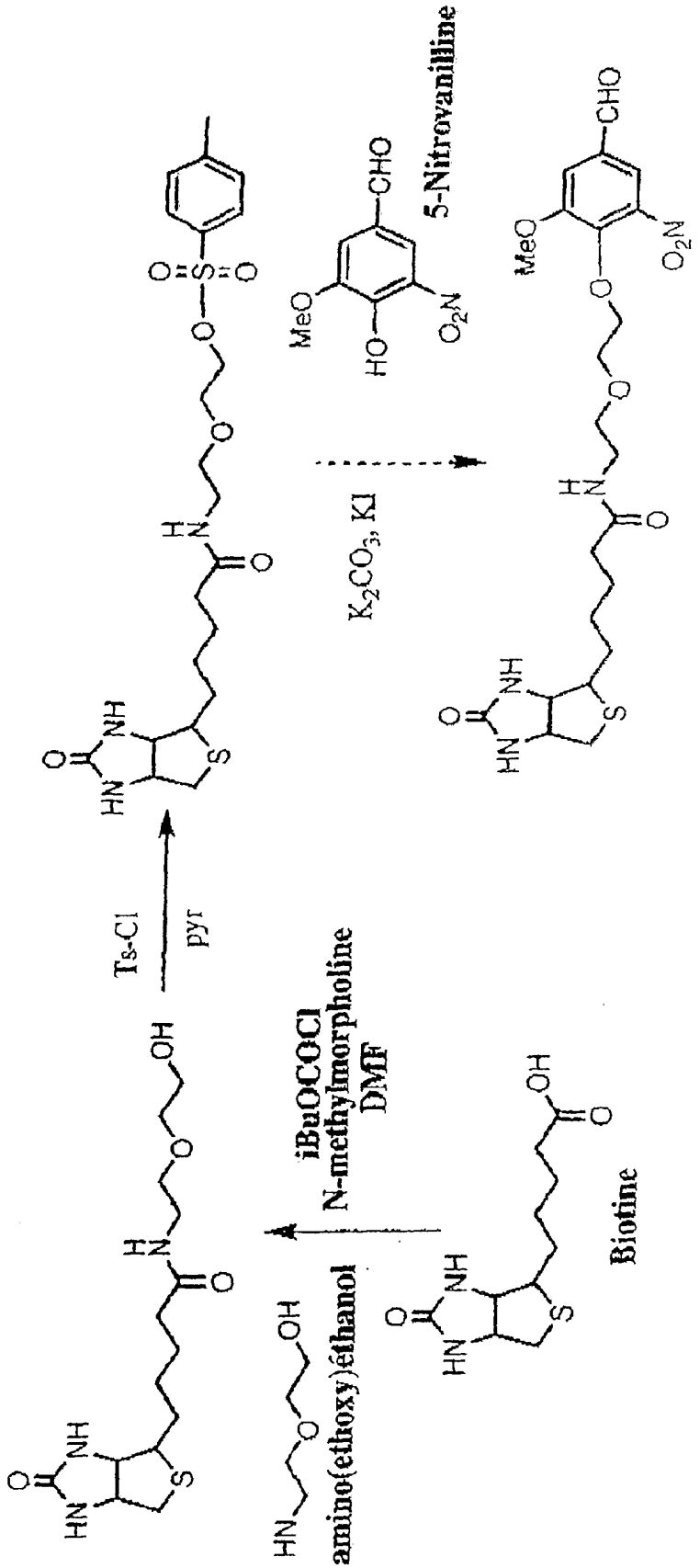

Biotine
iBuOCOCl
N-methylmorpholine
DMF
amino(ethoxy)éthanol
Ts-Cl
pyr
5-Nitrovanilline
K_2CO_3, KI

Fig 11

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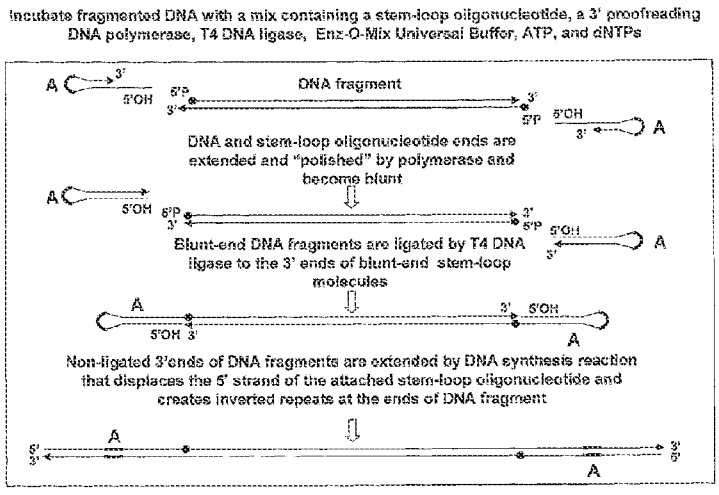
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *with international search report*

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR PROCESSING AND AMPLIFICATION OF DNA, INCLUDING USING MULTIPLE ENZYMES IN A SINGLE REACTION



(57) Abstract: The present invention concerns preparation of DNA molecules, such as a library, using a stem-loop oligonucleotide. In particular embodiments, the invention employs a single reaction mixture and conditions. In particular, at least part of the inverted palindrome is removed during the preparation of the molecules to facilitate amplification of the molecules. Thus, in specific embodiments, the DNA molecules are suitable for amplification and are not hindered by the presence of the palindrome.

WO 2007/018601 A1

IDT Exhibit 1020.7540

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

COMPOSITIONS AND METHODS FOR PROCESSING AND AMPLIFICATION OF DNA, INCLUDING USING MULTIPLE ENZYMES IN A SINGLE REACTION

[0001] The present invention claims priority to U.S. Provisional Patent Application Serial No. 60/704,932, filed August 2, 2005, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention generally concerns the fields of molecular biology and cellular biology. In particular, the present invention regards preparation and amplification of molecules, optionally utilizing a novel single-step reaction.

BACKGROUND OF THE INVENTION

[0003] Enzymatic reactions that involve DNA and RNA are numerous, and they play a central role in maintenance and propagation of living cells. Since the discovery of the DNA double helix structure in 1953, researchers have found, isolated and introduced into practice a multitude of different enzymes that can, for example, cut, nick, trim, join, unwind, phosphorylate, de-phosphorylate, methylate, de-methylate, recombine, replicate, transcribe, repair, and perform many other reactions with nucleic acid molecules. These enzymes are now actively used in many areas of biology, biotechnology and medicine to clone, amplify, sequence, identify mutations, quantify gene copy number, establish expression patterns, determine DNA methylation status, *etc.*

[0004] Frequently the process of DNA analysis involves multiple enzymatic reactions that are performed in a sequential manner, with intermediate purification steps between the reactions. Sometimes, the reactions are multiplexed to combine in one reaction the analysis of several DNA or RNA targets, and the nucleic acid processing or analysis may involve mutiplexing of two or three enzymatic processes in one reaction. Furthermore, DNA and RNA enzymatic reactions frequently utilize synthetic nucleic acid components, such as single stranded or double stranded oligonucleotides that function as PCR or sequencing primers, ligation adaptors, or fluorescent probes, for example.

Adaptors and Their Use for DNA Processing

[0005] Supplementing DNA ends with additional short polynucleotide sequences, referred to as an adaptor or linker, is used in many areas of molecular biology. The usefulness of

adapted DNA molecules is illustrated by but not limited to several examples, such as ligation-mediated locus-specific PCR, ligation-mediated whole genome amplification, adaptor-mediated DNA cloning, DNA affinity tagging, DNA labeling, *etc.*

Ligation-mediated amplification of unknown regions flanking known DNA sequence

[0006] Libraries generated by DNA fragmentation and addition of a universal adaptor to one or both DNA ends were used to amplify (by PCR) and sequence DNA regions adjacent to a previously established DNA sequence (see U.S. Patent No. 6,777,187 and references therein, for example, all of which are incorporated by reference herein in their entirety). The adaptor can be ligated to the 5' end, the 3' end, or both strands of DNA. The adaptor can have a 3' or 5' overhang, depending on the structure of the overhang generated by restriction enzyme digestion of DNA. It can also a have blunt end, especially in the cases when DNA ends are "polished" after enzymatic, mechanical, or chemical DNA fragmentation. Ligation-mediated PCR amplification is achieved by using a locus-specific primer (or several nested primers) and a universal primer complementary to the adaptor sequence.

Ligation-mediated whole genome amplification

[0007] Libraries generated by DNA fragmentation and subsequent attachment of a universal adaptor to both DNA ends were used to amplify whole genomic DNA (whole genome amplification, or WGA) (see U.S. Patent Applications Nos. 10/797,333 and 10/795,667 and references therein, for example, all of which are incorporated by reference herein in their entirety). The adaptor can be ligated to both strands of DNA or only to the 3' end followed by extension. The adaptor can have a 3' or 5' overhang, depending on the structure of the DNA end generated by restriction enzyme or other enzyme used to digest DNA. It can also have a blunt end, such as in the cases where DNA ends after enzymatic DNA cleavage are blunt or when the ends are repaired and "polished" after enzymatic, mechanical, or chemical DNA fragmentation. Whole genome PCR amplification is achieved by using one or two universal primers complementary to the adaptor sequence(s), in specific embodiments.

Adaptor-mediated DNA cloning

[0008] Adaptors (or linkers) are frequently used for DNA cloning (see Sambrook *et al.*, 1989, for example). Ligation of double stranded adaptors to DNA fragments produced by sonication, nebulization, or hydro-shearing process followed by restriction digestion within the adaptors allows production of DNA fragments with 3' or 5' protruding ends that can be efficiently introduced into a vector sequence and cloned.

IDT Exhibit 1020.7543

Use of Stem-Loop (Hairpin) DNA Oligonucleotides for Nucleic Acid Analysis

[0009] Stem-loop (also referred to as hairpin) oligonucleotides have been used in several applications for analysis and detection of nucleic acids. These applications include molecular beacons, stem-loop PCR primers, and stem-loop DNA probes, immobilized on microarrays (Broude, 2002).

Molecular beacons

[0010] A molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by self-complementary termini, and carries a fluorophore and a quencher at the 3' and 5' ends, respectively (Tyagi and Kramer, 1996). In the absence of target the fluorophore and the quencher are in a close proximity, which quenches fluorescence. Upon hybridization with the target, the beacon changes its conformation so that the fluorophore and the quencher become separated, and fluorescence increases up to 100-200 times. Molecular beacons have found many applications for real-time monitoring of PCR (Tyagi *et al.*, 1998) and isothermal amplification (de Baar *et al.*, 2001), as microarray-immobilized probes (Liu *et al.*, 2000), as antisense probes for RNA detection *in vivo* (Sokol *et al.*, 1998), and as a probe to measure DNA polymerase activity (Summerer and Marx, 2002) and monitor conformational changes of DNA targets (Goddard *et al.*, 2000).

Stem-loop (Hairpin) PCR primers

[0011] PCR primers containing hairpin structures on their 5' ends with donor and acceptor moieties located in close proximity on the stem-loop stem have been proposed for homogeneous (a closed tube) format for amplification, real-time quantification and mismatch detection by PCR (Broude, 2002). A stem-loop primer with a fluorophore at the 5' end and a "scorpion" probe is simultaneously a molecular beacon and a PCR primer (Whitcombe *et al.*, 1999). It has a tail attached to its 5' end by a linker that prevents copying of the 5' extension. The probe element is designed so that it hybridizes to its target only when the target site has been incorporated into the same molecule by extension of the tailed primer. It was also shown that stem-loop probes can be used as primers for PCR to reduce primer-dimer formation and mispriming, thereby increasing its specificity (Kaboev *et al.*, 2000).

Stem-loop microarray probes

[0012] Stem-loop probes can also be used as capture devices if the loop is immobilized on a surface and dangling ends are used for stacking-hybridization, thus providing both faster kinetics and higher hybrid stability (Riccelli *et al.*, 2001). Immobilized molecular

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beacon probes can be used for direct detection of non-amplified target DNA and RNA molecules (Hamad-Schifferli *et al.*, 2002).

Multiplexed Reactions that Involve DNA and RNA Molecules

[0013] Several types of multiplexed reactions that involve DNA or RNA are described to date. The multiplexed reactions can be divided into two major categories: reactions where two or more DNA/RNA sequences are amplified or detected simultaneously in one enzymatic process, and reactions where several enzymatic processes occur simultaneously with one DNA or RNA template.

Several targets-one enzyme

[0014] Multiplex PCR and RT-PCR are examples of the first category of multiplexed reactions (Mackay *et al.*, 2000). In this case, several genomic or cDNA regions are amplified in one polymerase chain reaction carried out by one thermostable DNA polymerase. Whole genome or whole transcriptome amplification is another example of highly multiplexed DNA/RNA amplification reactions carried out by methophilic (Phi 29) or thermophylic (*Taq*) DNA polymerase (Sambrook *et al.*, 1989).

One target-several enzymes

[0015] "Long distance" PCR, nucleic acid sequence-based amplification (NASBA), its analog, transcription-mediated amplification (TMA), and DNA strand-displacement amplification (SDA) are examples of the second category of multiplexed DNA/RNA amplification reactions. In the "long distance" PCR method there is a mixture of *Taq* DNA polymerase and another thermo-stable DNA polymerase with 3'proofreading activity (Barns , 1994). TMA and NASBA methods utilize transcription-mediated amplification that involves three enzymes: T7 RNA polymerase, reverse transcriptase, and RNase H (Deiman *et al.*, 2001). In the SDA method, two enzymes (a DNA polymerase and a restriction endonuclease) are combined in one enzymatic step to amplify DNA (Hellyer and Naolean, 2004).

[0016] DNA nick-translation method is an example of a DNA labeling reaction that involves two enzymes. The method is based on simultaneous incubation of DNA with DNase I and DNA polymerase I. DNase I generates nicks in the DNA molecule, while DNA polymerase I incorporates labeled nucleotides by initiating DNA synthesis from the nicked sites (Sambrook *et al.*, 1989).

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[0017] dU-glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG) and endonuclease VIII can be combined to produce the enzymatic mix, or the USER enzyme for fragmentation of DNA containing dU bases (New England Biolabs; Beverly, MA) may be employed. The fragmentation process occurs through enzymatic generation and cleavage of abasic sites at positions of dU bases.

Several targets-several enzymes

[0018] There is description of multiplexed amplification and detection of several nucleic acid sequences using three-enzyme TMA and NASBA methods (van Deursen *et al.*, 1999).

SUMMARY OF THE INVENTION

[0019] The present invention overcomes deficiencies in the art by providing novel methods and compositions for amplification of a plurality of double stranded DNA molecules by incorporating at least one sequence onto the ends of the DNA molecules. However, the invention avoids the disadvantages of other methods, such as the generation of primer dimers in polymerase chain reaction, for example.

[0020] In particular, the present invention allows for the amplification of molecules having at least one double stranded region by using adaptors that avoid the limitations of some adaptor molecules, such as those having the propensity to form adaptor dimers. In certain aspects, the present invention provides an inert oligonucleotide for attachment to a double stranded molecule such that it renders the oligonucleotide-ligated molecule capable of being modified, such as amplified, for example by polymerase chain reaction. Upon attachment of the inert adaptor to the molecule, the attached oligonucleotide becomes active and suitable for providing at least in part one or more sequences employable for amplification, while the non-attached, free adaptor remains inactive. For example, during polymerase chain reaction the free, non-attached inert adaptor can neither be primed nor used as a PCR primer (until it is intentionally and specifically fragmented and converted into a PCR primer); during amplification by transcription the promoter sequence of the non-attached, free adaptor does not initiate RNA synthesis; during amplification by strand displacement, the nicking enzyme recognition sequence of the non-attached adaptor remains uncut.

[0021] In particular aspects, the inert adaptor comprises a stem-loop oligonucleotide, wherein the inertness of the stem-loop oligonucleotide is at least in part a result

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of the dormant nature of its unique structure, biochemical propereties (one enzymatically active end), and/or physico-chemical characteristics (extremely high thermostability, for example). The oligonucleotide may comprise RNA, DNA, or both. The oligonucleotide may have one or more hairpins, and it may be further described as comprising any structure with multiple secondary structure and only one end. In specific embodiments, the oligonucleotide can change its functionality upon attachment to a double stranded nucleic acid molecule (DNA, RNA, or both). In other words, because of a conformational change of the oligonucleotide upon (such as following, for example) attachment to a double stranded nucleic acid molecule, one or more functional properties of the oligonucleotide become altered, such as one or more functional properties hidden in the oligonucleotide prior to attachment manifesting upon attachment.

[0022] In addition to the advantages provided by the adaptors of the invention, the invention further provides novel conditions for modification of DNA molecules with the adaptors, and subsequent amplification. In most cases in the art, the process of DNA analysis involves multiple enzymatic reactions that are performed in a sequential manner, frequently with intermediate purification steps between at least some of the reactions. For example, preparation of DNA libraries for subsequent amplification and analysis involves ligation of adaptor sequences to DNA ends to introduce a priming site for the PCR-mediated whole genome amplification and ligation-mediated PCR, a promoter element for the transcription-mediated DNA amplification, and/or cohesive ends to facilitate DNA integration into a vector molecule for cloning. Preparation of library from genomic or viral DNA by current adaptor attachment procedures typically occur in 6 steps (FIG. 1A):

[0023] Step 1 – fragmentation of high molecular weight (HMW) DNA to a size amenable to amplification or cloning;

[0024] Step 2 – DNA purification to remove nucleases and other reagents;

[0025] Step 3 – "polishing" of DNA ends by a 3' proofreading DNA polymerase(s) to generate blunt-ended DNA fragments with the 3' hydroxyl and 5' phosphate groups at the termini;

[0026] Step 4 – DNA purification to remove the polymerase and replace the buffer, or, alternatively, heat inactivation of the DNA polymerase;

[0027] Step 5 – adaptor ligation; and

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[0028] Step 6 –3' end extension (in the case when an adaptor is ligated only to one strand of DNA, specifically to the 5' phosphate).

[0029] However, such a multi-step process takes a considerable amount of time (1-2 days) and can be a major obstacle for high throughput and diagnostic-type applications, for example. The process becomes more complicated when library preparation involves additional enzymatic steps, such as DNA or library digestion with methylation-sensitive or methylation-specific endonucleases (for example, in the preparation of Methylome libraries, such as are described in U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety), or restriction nuclease cleavage within the adaptor sequence to produce sticky ends for DNA cloning.

[0030] The present invention satisfies a long-felt need in the art for obviating the requirement for multiple manipulations for nucleic acid processing. The current invention introduces and demonstrates new methods and compositions that allow reduction of several important multi-step enzymatic DNA processes to a single-step-reaction that is performed in one reaction volume (FIGS. 1B and 1C). Such simplification has been achieved by developing a highly multiplexed enzymatic method (Enz-O-Mix) that combines a substantial number (from 2 to 10) of enzymatic processes into one complex reaction mixture that occurs under universal buffer conditions.

[0031] Specifically, the current invention introduces novel processes of DNA library preparation and/or DNA amplification (FIG. 1C) that reduce the whole multi-step process to a single-step multiplexed enzymatic reaction involving as a minimum two **Enz**ymes, a stem-loop (hairpin) **O**ligonucleotide with a special base/bonds composition, and a universal buffer (**Mix**), which is herein referred to as **Enz-O-Mix**, that supports efficient functioning of all enzymatic activities present in the mixture. The invention demonstrates that the Enz-O-Mix approach can be used to prepare a DNA library for whole genome amplification (WGA Library), a Methylome library for amplification and analysis of methylated DNA regions, and/or even directly amplify DNA in a single multi-enzyme reaction, for example.

[0032] The Enz-O-Mix library/amplification has no limitations on the size and nature of DNA in the reaction. The method can be applied to high molecular weight DNA, such as is isolated from tissues or cell culture, for example, as well as highly degraded DNA, such as

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cell-free DNA from blood and urine and/or DNA extracted from formalin-fixed, paraffin-embedded tissues, for example.

[0033] The applications of the Enz-O-Mix method are numerous. The Enz-O-Mix method is easy to automate and use in clinical diagnostic and point-of-care applications, for example. In specific embodiments, different enzyme combinations are utilized for many novel kits and assays and utilized in such areas as biotechnology, the pharmaceutical industry, molecular diagnostics, forensics, pathology, bio-defense, and/or bio-computing, for example. The Enz-O-Mix approach is a simple and cost-effective alternative to the "lab-on-a chip" microfluidic approach that is currently attempting to solve the same problem (multi-step DNA processing) by reduction of reaction volumes and integration of multiple reactions into a small format (FIG. 2). The method can be used for *in vitro* as well as for *in vivo* nucleic acid amplification and/or detection.

[0034] In specific aspects, the present invention introduces a concept of multiplexing two or more enzymatic processes in one reaction, teaches how to optimize a highly multiplexed enzymatic process, and demonstrates in multiple specific examples the efficacy of this approach. In particular embodiments, the present invention is directed to compositions and methods for simultaneous processing of DNA molecules with a combination of enzymes in a one-step-one-tube reaction and producing either a collection of molecules suitable for amplification, or amplified DNA molecules.

[0035] In particular, the present invention greatly reduces the number of steps for library preparation by consolidating a series of steps into one step. For known whole genome amplification or whole methylome methods, these protocols greatly reduce the number of steps for library syntheses that utilize adaptors. That is, in the case of WGA library preparation from high molecular weight DNA, for example, the number of steps is reduced from about 6 to 1 (FIG. 1A and 1B). In the case of WMA library preparation from high molecular weight DNA, for example, the number of steps is reduced from about 7 to 1. In the case of WGA library preparation from fragmented DNA (plasma, serum, or urine, for example), the number of steps is reduced from about 4 to 1 (FIG. 1B). In the case of Methylome library preparation from fragmented DNA, for example, the number of steps is reduced from about 5 to 1 (FIG. 1B).

[0036] In particular embodiments, the methods of the invention can be easily applied to any type of fragmented double stranded DNA including but not limited to, for

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example, free DNA isolated from plasma, serum, and/or urine; apoptotic DNA from cells and/or tissues; DNA fragmented enzymatically *in vitro* (for example, by DNase I and/or restriction endonuclease); and/or DNA fragmented by mechanical forces (hydro-shear, sonication, nebulization, *etc.*).

[0037] In other embodiments, the invention can be easily applied to any high molecular weight double stranded DNA including, for example, DNA isolated from tissues, cell culture, bodily fluids, animal tissue, plant, bacteria, fungi, viruses, *etc.*

[0038] In one embodiment of the invention, there is incubation of DNA with an enzymatic mixture (Enz-O-Mix) comprising from about 2 to about 18 different enzymes in one buffer and a stem-loop (hairpin) oligonucleotide with a specific base/bonds composition.

[0039] In particular embodiments, there is a one-step enzymatic process including one or more of the following: a) "polishing" of DNA and stem-loop oligonucleotide ends by Klenow fragment of DNA polymerase I (or other proofreading DNA polymerase); b) ligation of the 3' end of stem-loop oligonucleotide to the 5' end of DNA, such as by a ligase, for example, by T4 DNA ligase; c) "fill-in" DNA synthesis that is initiated at the 3' end of DNA, propagates towards the end of the stem-loop oligonucleotide, and stops at a replication block or at the end of the oligonucleotide; and d) cleavage in the middle of a generated inverted repeat by a restriction nuclease; and e) restriction cleavage with a mixture of methylation-sensitive restriction enzymes or methylation-specific nuclease(s) (such as in embodiments concerning whole methylome amplification library), for example.

[0040] In particular aspects of the invention, an inverted repeat (palindrome) at the end of DNA molecules is not generated (or is generated and removed), because it may inhibit the amplification step (PCR), priming of the cDNA strand synthesis (amplification by transcription), or priming of the second DNA strand synthesis (amplification by strand displacement DNA synthesis). Thus, in specific embodiments at least part of the oligonucleotide sequence is eliminated, such as, by termination of replication in the middle of a stem-loop oligonucleotide or by cutting the generated inverted repeat internally, for example.

[0041] In one specific embodiment of this invention, a chemical modification, such as hexaethylene glycol (HEG) linker, a bulky base analog, or one or several abasic sites within the loop or adjacent to it, can be introduced during oligonucleotide synthesis. Such modifications will terminate the replication within the stem-loop oligonucleotide.

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[0042] In another specific embodiment, the replication block is generated during the reaction by incorporating one or more dU bases into the stem-loop oligonucleotide design and including dU-glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG) in the reaction mix.

[0043] In some embodiments, a specific site for a cleavage enzyme is generated during "fill-in" DNA synthesis that is initiated at the 3' end of the DNA, propagates towards the end of the stem-loop oligonucleotide, and stops at the 5' end of the oligonucleotide. Such a site resides originally either completely or partially within the single-stranded loop of an oligonucleotide and becomes functional (cleavable) only after conversion of this loop into double-stranded form.

[0044] In one specific embodiment a cleavage enzyme is represented by a restriction endonuclease, in another by a homing endonuclease.

[0045] In some embodiments, the methods further comprise simultaneous digestion with an endonuclease or combination of endonucleases, such as restriction endonucleases, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonucleases, *etc.*, to generate in one step Whole Genome or Whole Methylome library even from high molecular weight (HMW) DNA.

[0046] In particular embodiments, a library generated by a one-step process is further amplified by PCR using universal primer complementary to the sequence introduced by attachment of a stem-loop oligonucleotide.

[0047] In particular embodiments, library generation and PCR amplification are combined into one integrated, closed-tube process in a thermocycler.

[0048] In one particular embodiment, an integrated, closed-tube library preparation/amplification process is supported by a universal PCR primer included in the original Enz-O-Mix.

[0049] In another particular embodiment, an integrated, closed-tube library preparation/amplification process is supported by a stem-loop oligonucleotide converted into a functional universal primer as a result of enzymatic and chemical reactions that occur before PCR amplification.

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[0050] In another embodiment, degradable stem-loop oligonucleotides are used as locus-specific primers for a hot start PCR process.

[0051] In another particular embodiment, a library generated by one-step process is further amplified by transcription utilizing a promoter sequence (T7, T3, SP6, *etc.*) introduced by attachment of a stem-loop oligonucleotide.

[0052] In particular embodiments, a stem-loop oligonucleotide design is employed for the one-step library synthesis reaction, although in alternative embodiments other adaptors may be utilized, such as a standard linear two-oligonucleotide adaptor, for example.

[0053] In particular embodiments, a stem-loop oligonucleotide provides a synthesized library with a new function that was present neither in the DNA molecule nor in the stem-loop oligonucleotide prior to its attachment. Examples include a functionally inactive T7 promoter sequence, a non-cleavable restriction site, and/or inactive nicking site residing at least partially within the loop or stem region of the stem-loop oligonucleotide and activated only by the attachment process and conversion of the single stranded loop into a double stranded molecule.

[0054] In particular embodiments, a stem-loop oligonucleotide provides a synthesized library with a 5' overhang that was not present either in the DNA molecule or in the stem-loop oligonucleotide prior to its attachment, and that can be used for covalent or non-covalent immobilization of a synthesized library on a solid support *via* hybridization or hybridization and ligation to a covalently attached oligonucleotide.

[0055] In a specific embodiment, the library synthesis and its immobilization within the tube (well) occurs simultaneously in a one Enz-O-Mix process. In this case, combinations of enzymes and stem-loop oliginucleotides are mixed together in a special tube with a covalently attached capture oligonucleotide to produce Enz-O-Mix-Immobilization kits for high throughput DNA processing and diagnostic applications in solid phase format.

[0056] In particular embodiments, combinations of enzymes and stem-loop oligonucleotides are mixed together to produce Enz-O-Mix kits for high throughput DNA processing and diagnostic applications. In particular embodiments, there is a Universal Enz-O-Mix Buffer that provides simultaneous activity of all enzymes used in the reaction.

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[0057] In particular embodiments, combinations of enzymes and stem-loop oligonucleotides are mixed together to produce integrated, closed-tube Enz-O-Mix kits for high throughput DNA fragmentation, amplification and labeling. In particular embodiments, there is a Universal Enz-O-Mix Buffer that supports simultaneous activities of all enzymes used in the reaction.

[0058] In a specific embodiment, there is a process for optimization of the Universal Buffer and other components of the multiplexed enzymatic reaction.

[0059] In particular aspects, the present invention is directed to a system and method for preparing a collection of molecules, particularly molecules suitable for amplification, such as amplification utilizing known sequences on the molecules. In specific embodiments, the oligonucleotide comprises a known sequence.

[0060] In an additional embodiment, there is a kit housed in a suitable container that comprises one or more compositions of the invention and/or comprises one or more compositions suitable for at least one method of the invention.

[0061] In one embodiment of the invention, there is a method of preparing a nucleic acid molecule, comprising: providing a double stranded nucleic acid molecule; and attaching one strand of a stem-loop oligonucleotide comprising an inverted repeat and a loop to the double stranded nucleic acid molecule to produce an oligonucleotide-attached nucleic acid molecule. The double stranded nucleic acid molecule may be a double stranded DNA molecule, in some embodiments. In specific embodiments, the attaching is further defined as attaching the oligonucleotide to the double stranded nucleic acid molecule under conditions to produce a non-covalent junction, such as a nick, a gap, or a 5' flap structure, in the oligonucleotide-attached nucleic acid molecule. In particular aspects of the invention, the attaching is further defined as ligating. The method may further comprise displacing one strand of the oligonucleotide from the oligonucleotide-attached nucleic acid molecule by strand displacement or by nick translation polymerization. In a specific embodiment, at least part of the oligonucleotide-attached nucleic acid molecule is amplified, such as by polymerase chain reaction, RNA transcription, or strand displacement, for example. Methods of the invention may further comprise amplifying an oligonucleotide-attached nucleic acid molecule, wherein at least part of the inverted repeat is excluded from the amplified oligonucleotide-attached nucleic acid molecule.

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