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(54) Title: NUCLEIC ACID AMPLIFICATION METHODS

Figure 1

1. Prepare Target DNA = A Tailed cfDNA

5' XXXXXXXXXXXXXXXXXXXX 3'
3' AZZZZZZZZZZZZZZZZZZZ 5'

T7RNA P

UIP

MBC

SBC

U2

T7 RNA Polymerase promoter sequence

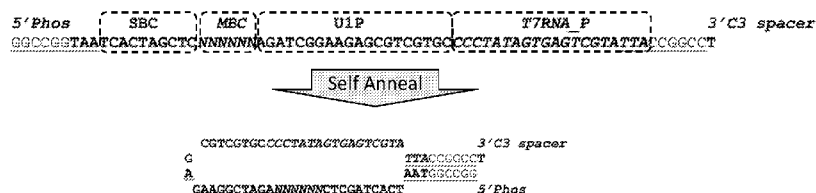
Partial Unique NGS Sequence 1

Molecular Bar Code

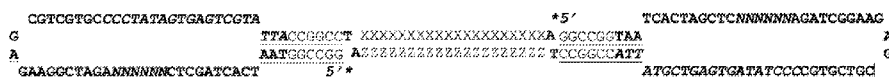
Sample Bar Code

Unique NGS Sequence 2

2. Anneal Oligo to form Circular Adapter



3. Ligate Circular Adapter to Target DNA



* Ligation

(57) Abstract: The present invention relates to a method for amplifying template DNA, the method comprising: i) providing template DNA and an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA; ii) ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule; iii) providing a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide; iv) annealing the secondary oligonucleotide to the adapter oligonucleotide-template DNA molecule; and v) transcribing the template DNA by introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule, to generate an aRNA product. The method of the present invention may further comprise vi)



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NUCLEIC ACID AMPLIFICATION METHODS

Field of the Invention

- 5 The present invention relates to improved, reliable and highly specific methods of amplifying DNA to produce an aRNA or DNA product, utilising novel adapter oligonucleotides, which can be used to identify genetic variations in biological samples.

Background to the Invention

10

The progress of Next Generation Sequencing (NGS) now enables the sequencing of entire genomes in a greatly reduced time frame.

15

The discovery of circulating cell-free DNA (cfDNA) has provided opportunities to analyse genetic materials from blood samples, without the risks typically associated with more invasive sampling methods.

20

Furthermore, small quantities of circulating tumour DNA (ctDNA) originating from tumours, especially malignant tumours (or cancers), can be found among the cfDNA in the blood of cancer patients. The ctDNA may be analysed by sequencing cfDNA to detect sequence variants that are known to be specifically associated with various types of tumours or cfDNA copy numbers to detect the presence of tumours and their progression. In addition to ctDNA the blood of cancer patients can also contain circulating tumour cells (CTCs) which may also be analysed by sequencing CTC DNA to detect sequence variants that are known to be specifically associated with various types of tumours or harbour copy number patterns linked to the presence of tumours and their progression.

25

30

However, there are a number of limitations of current NGS approaches for use in a clinical setting, especially for the early detection of cancer. For example, many current NGS protocols do not provide sufficient sensitivity needed for the limited levels of ctDNA generally available. Additionally, lack of specificity can occur due to inherent issues with the DNA polymerase chain reaction currently employed in combination with NGS as errors can be introduced during amplification. These limitations underlie the continuing need for methods that would improve specificity, sensitivity, and applicability, to reliably analyse and identify genetic cancer markers which can be used in a clinical setting.

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-2-

It is an object of the present invention to obviate or mitigate one or more of the abovementioned problems. It is a further object of the present invention to provide improved methods and reagents for the identification and analysis of cfDNA. It is also desirable if such improved methods and reagents are compatible with a number of NGS platforms and equipment.

Summary of the Invention

In accordance with a first aspect of the present invention, there is provided a method for producing an aRNA product from template DNA, the method comprising:

- i) providing template DNA and an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA;
- ii) ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule;
- iii) providing a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide;
- iv) annealing the secondary oligonucleotide to the adapter oligonucleotide-template DNA molecule; and
- v) transcribing the template DNA by introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule, to generate an aRNA product.

The method of the present invention provides a number of advantages. Compared to other methods used to amplify template DNA the approach is simpler, more flexible and can provide increased sensitivity and specificity making it more suitable where input DNA is limited (for example in the analysis of cfDNA and single cells).

The term aRNA as used herein is used to refer to amplified RNA produced by an RNA polymerase from the template DNA of the present invention.

RNA polymerase/RNA polymerase promoter sequence

The method of the present invention comprises providing an adapter oligonucleotide comprising a RNA polymerase promoter sequence and transcribing template DNA by introducing a RNA polymerase. As will be appreciated by the skilled person, there are many

-3-

RNA polymerases which could be utilised in the method of the present invention, such as T3 RNA polymerase, SP6 RNA polymerase and T7 RNA polymerase. However, in a preferred embodiment, the RNA polymerase may comprise a T7 RNA polymerase and the RNA polymerase promoter sequence may comprise a T7 RNA polymerase promoter sequence.

- 5 The present inventors have shown that utilising a T7 RNA polymerase and associated promoter sequence results in a highly efficient, sensitive and specific method.

In embodiments of the present invention, the RNA polymerase promoter sequence in the adapter oligonucleotide may comprise SEQ ID NO.1 or a fragment or variant thereof. The T7
10 RNA polymerase promoter sequence is: ATTATGCTGAGTGATATCCC (SEQ ID NO. 1).

In embodiments, the RNA polymerase promoter sequence in the adapter oligonucleotide consists of SEQ ID NO.1.

- 15 In embodiments, the RNA polymerase promoter sequence in the secondary oligonucleotide may comprise SEQ ID NO.2 or a fragment or variant thereof. The Reverse T7 RNA polymerase promoter sequence is: TAATACGACTCACTATAGGG (SEQ ID NO. 2).

In embodiments, the RNA polymerase promoter sequence in the secondary oligonucleotide
20 consists of SEQ ID NO.2.

The phrase "fragment or variant" as used herein is used in its broadest sense to refer to a fragment or variant of the sequence disclosed which retains its function, i.e. in this case, the ability to act as an RNA polymerase promoter sequence thereby allowing an RNA
25 polymerase to bind.

Preferably, the fragment or variant comprises at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity with SEQ ID NO.1 or 2.

- 30 In embodiments of the present invention, the fragment or variant has 100% sequence identity at positions 7, 8, 9, 10 and 11 of SEQ ID NO.1, as shown underlined below:

ATTATGCTGAGTGATATCCC

- 35 In embodiments of the present invention, the fragment or variant has 100% sequence identity at positions 7, 8, 9, 10 and 11 of SEQ ID NO:2, as shown underlined below:

TAATACGACTCACTATAGGG

In the method of the present invention, the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide. This sequence complementarity may be by way of the RNA polymerase promoter sequences present in the adapter oligonucleotide and secondary oligonucleotide respectively. The RNA polymerase promoter sequences in the adapter oligonucleotide and secondary oligonucleotide may be complementary or at least partially complementary in sequence. For example, in embodiments, the RNA polymerase promoter sequence in the adapter oligonucleotide comprises SEQ ID NO:1 or a fragment or variant thereof and the RNA polymerase promoter sequence in the secondary oligonucleotide comprises SEQ ID NO:2 or a fragment or variant thereof. These sequences are complementary in that they can anneal to each other in step iv) of the method of the present invention.

The secondary and adapter oligonucleotides may comprise further complementary sequences, in addition to the RNA polymerase promoter sequences.

Step ii) - Ligation

Step ii) of the method of the present invention comprises ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule. In the present invention, the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA. The skilled person will appreciate that there are numerous ways in which the adapter oligonucleotide may be configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA. For example, in embodiments, the adapter oligonucleotide may comprise a T-tail. In such embodiments, the template DNA may comprise an A-tail, facilitating adapter oligonucleotide and template DNA annealing (due to the corresponding A- and T-tails) such that they are in proximity for subsequent ligation. Alternatively, the adapter oligonucleotide may comprise a 5' sequence which is complementary to a 3' sequence of the corresponding DNA template. For example, the DNA template may have been treated with a restriction enzyme resulting in an overhanging sequence. In such embodiments, the 5' end of the adapter oligonucleotide may have a sequence corresponding to the overhanging sequence of the DNA template such that the

adapter oligonucleotide and template DNA can anneal such that they are in proximity for subsequent ligation.

In embodiments, step ii) may comprise ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA using a DNA ligase. The skilled person will appreciate that any DNA ligase suitable of ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA may be used. The DNA ligase may comprise a bacterial, bacteriophage (e.g. T4 DNA ligase) or mammalian DNA ligase (e.g. DNA ligase I, II, III or IV), for example.

In embodiments of the present invention, the DNA ligase may comprise a T4 DNA ligase, for example KAPA-hyper DNA ligase, NEB Quick Ligation Kit, NEB T4 DNA Ligase, NEB Blunt/TA Ligase Master Mix or NEBNext® Ultra™ II Ligation Module.

In preferred embodiments of the present invention, the DNA ligase may comprise KAPA-hyper DNA ligase. The present inventors found that the use of KAPA-hyper DNA ligase is particularly effective in the method of the present invention (see for example, Figure 4).

As discussed above, the design of the adapter oligonucleotide allows ligation of the 5' end of the adapter oligonucleotide to the 3' end of the template DNA. This results in a contiguous molecule incorporating the RNA polymerase promoter at the 3' end and the template DNA at the 5' end. Due to the orientation of the adapter oligonucleotide-template DNA molecule there is no need to convert to a dsDNA molecule for the RNA polymerisation step since the ligated adapter oligonucleotide-template DNA molecule can be utilised as a template directly by the RNA polymerase.

Furthermore, unlike PCR based methods which require ligation to both 3' and 5' ends of template DNA, there are amplified products from each ligated end of the DNA template using the present method, regardless of whether ligation has also occurred at the opposite end. This is particularly advantageous when dealing with sub-optimal ligation brought about by poor input DNA, enzyme deterioration or the presence of inhibitors.

In embodiments of the present invention, ligation of the 5' end of the adapter oligonucleotide to the 3' end of the template DNA takes place between around 14 and 22°C, preferably between around 16 and 22°C, preferably at 20°C. The inventors have shown that particularly favourable results are achieved when the ligation step takes place at this temperature.

-6-

In embodiments of the present invention, the method may further comprise dephosphorylating the template DNA, preferably prior to the ligation of step ii) of the present invention. In embodiments, a 5' phosphate of the template DNA is removed by treatment with a phosphatase enzyme.

5

As will be appreciated by the skilled person, any suitable phosphatase enzyme could be utilised, for example Shrimp Alkaline Phosphatase, Antarctic Phosphatase, Calf Intestinal Alkaline Phosphatase or FastAP Thermosensitive Alkaline Phosphatase. However, preferably the phosphatase comprises a heat labile phosphatase such as FastAP

10 Thermosensitive Alkaline Phosphatase.

The present inventors have found that the removal of the 5' phosphate from the template DNA reduces DNA self ligation and also reduces or blocks ligation of the 3' end of the adapter to the 5' of the template DNA. It is highly advantageous to reduce the ligation of the

15 3' end of the adapter to the 5' of the template DNA since this can result in circular ligation products which are not compatible with subsequent steps leading to NGS library generation. The use of a dephosphorylation step is in contrast to current NGS PCR amplification approaches which incorporate phosphorylation of template DNA. Instead, the present invention preferably makes use of dephosphorylated template DNA which results in aRNA

20 production.

This is in contrast to current NGS PCR amplification approaches which incorporate phosphorylation of input target DNA. Instead, the present invention preferably makes use of dephosphorylated target DNA which results in improved aRNA production.

25

In certain aspects of the present invention, the method comprises a multiplex method. Such a multiplex method may utilise sample bar codes allowing for the multiplexing of tens to thousands of samples following the ligation of the adapter oligonucleotide and the template

30 DNA.

The method may further comprises a restriction enzyme digestion of the template DNA prior to the ligation of step ii). Various restriction enzymes are suitable to perform the restriction enzyme digestion which are known in the art. The restriction enzyme may produce "blunt

35 ends" or "sticky ends". Restriction enzymes suitable to produce blunt ends may be selected from MLyl, BsuRI, HaeIII, EcoRV, SmaI. Restriction enzymes suitable to produce sticky

-7-

ends may be selected from NIAIII, Hsp92II, Hin1II, MspI. Wherein sticky ends are produced by the restriction enzymes the target DNA may then undergo end repair and/or addition of an A tail. In a preferred embodiment a restriction enzyme which produces blunt ends may be used, more preferably MLyl or BsuRI may be used.

5

Transcribing the DNA

Step v) of the method of the present invention comprises transcribing the template DNA by introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule, to
10 generate an aRNA product. As described above, there are many RNA polymerases which could be utilised in the method of the present invention, such as T3 RNA polymerase, SP6 RNA polymerase and T7 RNA polymerase. However, in a preferred embodiment, the RNA polymerase may comprise a T7 RNA polymerase.

15 By introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule to transcribe the template DNA, linear amplification of each original DNA strand is achieved. Unlike PCR approaches, which require an initially potentially error prone polymerisation step prior to amplification, around 1000 aRNA copies are made directly from each input strand, providing a means to reduce sequencing errors and increase specificity and sensitivity when
20 detecting rare mutations in DNA mixtures.

Further steps - amplification of DNA

In embodiments of the present invention, the method further comprises the step of:

25 vi) converting the aRNA product to cDNA.

In embodiments of the present invention, the method may further comprise the steps of:

vi) converting the aRNA product to cDNA; and

vii) amplifying the cDNA.

30

In such embodiments the method of the present invention may be a method for amplifying template DNA.

The present invention therefore also provides a method for amplifying template DNA, the
35 method comprising:

-8-

i) providing template DNA and an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA;

ii) ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule;

iii) providing a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide;

iv) annealing the secondary oligonucleotide to the adapter oligonucleotide-template DNA molecule; and

v) transcribing the template DNA by introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule, to generate an aRNA product;

vi) converting the aRNA product to cDNA; and

vii) amplifying the cDNA.

As will be appreciated by the skilled person, the term cDNA refers to complementary DNA and is used to refer to DNA synthesised from single-stranded RNA, for example in a reaction catalysed by the enzyme reverse transcriptase.

Step vi) may first comprise ligating a priming site to the aRNA followed by reverse transcription using the priming site and a reverse transcriptase. Alternatively, step vi) may comprise utilising reverse transcriptase and random oligonucleotide primers or utilising reverse transcriptase and target specific oligonucleotide primers. As will be appreciated by the skilled person, any suitable reverse transcriptase enzyme could be utilised, for example the bacterial *ret* reverse transcriptase, the retroviral reverse transcriptases including RSV reverse transcriptase from Rous sarcoma virus, HIV-1 reverse transcriptase from human immunodeficiency virus type 1, MMLV reverse transcriptase from Moloney murine leukemia virus and AMV reverse transcriptase from avian myeloblastosis virus. However, preferably the reverse transcriptase comprises of an optimised commercial cloned reverse transcriptase such as the mutant MMLV Superscript IV.

The method to convert the aRNA to cDNA may be performed in a number of ways. Conversion of aRNA to cDNA may be performed via ligation of a priming site to the aRNA. The ligation may be followed by reverse transcription. Conversion of aRNA to cDNA may be performed via single stranded ligation of a priming site to the aRNA. The single stranded ligation may ligate a unique NGS primer to the target aRNA. Conversion of aRNA to cDNA

may be performed via splinted double stranded ligation to aRNA. The splinted double stranded ligation may ligate a unique NGS primer to the target aRNA. Conversion of aRNA to cDNA may be performed using random NGS primer. Conversion of aRNA to cDNA may be performed using target specific NGS primer.

5

Step vii) may comprise amplification in a next generation sequencing (NGS) protocol. Such a NGS protocol will be largely dependent upon the NGS platform chosen and advantageously, the method of the present invention can be used or adapted for use in all of the current mainstream NGS platforms and machines.

10

The present invention as defined above is compatible with all of the current NGS platforms including systems which rely on clonal amplification of adapter-ligated libraries such as Illumina and Ion Torrent as well as single molecule sequencing platforms such as Pacific Bioscience and Oxford Nanopore. Both optics-based sequencers which use visible light, collecting photons from arrays to form a visual image of bases incorporated during polymerization (e.g. Illumina) and semiconductor sequencing which rely on the detection of hydrogen ions released during the polymerisation of DNA (e.g. Ion Torrent) rely on the inclusion of specific adapter sequences which can be readily incorporated into the method of the present invention. Similarly, single molecule sequencing platforms such as Pacific Bioscience and Oxford Nanopore rely on addition of sequences that facilitate sequencing and these can also be readily incorporated into the method of the present invention.

15

20

In addition to the suitability of the method of the present invention for the current range of commercial NGS platforms, as described above, the method can be used with both capture based NGS, where specific sequences are enriched following generation of a genome wide library, and direct PCR amplicon NGS.

25

Adapter oligonucleotide

30

The method of the present invention includes providing an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA. The 5' end of the adapter oligonucleotide is ligated to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule and a secondary oligonucleotide is then annealed to the adapter oligonucleotide-template DNA molecule.

35

Preferably, the adapter oligonucleotide comprises complementary regions which are capable of annealing to one another to form a secondary structure, preferably a circular or hairpin loop structure. The skilled person will appreciate how such an adapter could be designed.

5 The use of a circular or hairpin loop structure simplifies the method of the present invention in that by using an adapter with such a structure, it is not necessary to determine the optimum molar balance which becomes necessary when multi molecule adapters are utilised.

10 In embodiments in which the adapter forms a secondary structure, step iv) of the method may further comprise the step of denaturing the adapter oligonucleotide prior to annealing the secondary oligonucleotide to the adapter oligonucleotide.

The adapter oligonucleotide may comprise a number of additional sequence features which
15 may be useful in further analysing the target DNA which has been amplified, such as molecular bar code, sample bar codes, a NGS sequence and/or a T tail.

The adapter oligonucleotide may further comprise a single region which is complementary, or partially complementary, to the stick ends produced by the restriction enzyme digestion.

20

In embodiments of the present invention the adapter oligonucleotide may further comprise a molecular barcode sequence. A molecular bar code sequence may comprise, for example, a sequence of random bases, for example, 5, 6, 7, 8, 9 or 10 bases. In embodiments, the molecular barcode sequence may include 6-8 bases. The molecular barcode provides a
25 unique bar code for each adapter oligonucleotide that in turn will be incorporated into each adapter oligonucleotide-template DNA molecule. RNA polymerisation of each adapter oligonucleotide-template DNA molecule will produce aRNA molecules each harbouring the same molecular bar code sequence as well as the same input DNA sequence enabling bioinformatics identification of all aRNA molecules originating from each single initial ligation
30 event. By identifying cohorts of aRNA molecules originating from the same starting ligation product a consensus sequence can be built up that avoids identifying errors present in single aRNA molecules which may arise due to polymerase error. This will therefore increase the specificity and sensitivity which is particularly important for detecting rare mutations in DNA mixtures. By utilising molecular barcodes along with a linear amplification step the error
35 obtained using NGS is expected to be reduced by one to three orders of magnitude.

In embodiments of the present invention the adapter oligonucleotide may further comprise a sample barcode. A sample barcode may comprise, for example, a sequence of random bases, for example, 5, 6, 7, 8, 9 or 10 bases. The incorporation of a sample bar code allows multiplexing of tens to thousands of samples following the ligation of the adapter oligonucleotide and the template DNA. For example, each adapter may comprise a unique sample bar code which is ligated to a sample from a particular source. Following ligation of the adapter oligonucleotide and the template DNA, samples could be combined for subsequent steps, thereby improving efficiency. Post-sequencing information, for example, could be identified for each sample bioinformatically, through identifying the sample barcode.

In embodiments of the present invention the adapter oligonucleotide may further comprise a NGS sequence.

In embodiments of the present invention the adapter oligonucleotide may further comprise a T-tail. As described above, in such embodiments, the template DNA may comprise an A-tail, encouraging the adapter oligonucleotide and template DNA to anneal (due to the corresponding A- and T-tails) such that they are in proximity for subsequent ligation.

In embodiments of the present invention, the template DNA is double-stranded. In embodiments of the present invention, the adapter oligonucleotide may only anneal to a single strand of said double-stranded DNA.

Unlike PCR based methods which require ligation to both the 3' and 5' ends of the input DNA, there are amplified products from each ligated end using the method of the present invention, regardless of whether ligation has occurred at both ends.

Wherein the adapter oligonucleotide forms secondary structure the adapter oligonucleotide may further comprise an overhang of oligonucleotides. This may be given the designation NNNNNNNN. The overhang may be positioned after one of the complementary regions which are capable of annealing to one another. The overhang may comprise a random sequence of oligonucleotides, or the sequence may be selected to be complementary to the target DNA. The overhang may be 5' or 3'. In an embodiment the adapter oligonucleotide forms a circular or hairpin secondary structure and comprises an overhang. The oligonucleotide overhang may comprise about 6 to about 12 oligonucleotides, preferably about 8 to about 10 oligonucleotides, more preferably about 8 oligonucleotides. This format of the adapter oligonucleotide may be particular suitable for use in single strand ligation.

Template DNA

The skilled person will appreciate that the method of the present invention may have a
5 multitude of applications and is especially applicable in the clinical or medical research fields.

The template DNA may be derived from a biological sample. The biological sample may
comprise blood. The template DNA may comprise cfDNA, gDNA and/or single cell DNA.

10 The skilled person will appreciate how such template DNA can be obtained. For example,
suitable commercial kits are available for obtaining template DNA, including cfDNA, gDNA or
single cell DNA. Suitable kits include for example QIAamp DNA Blood Mini Kit (Qiagen),
QIAamp circulating nucleic acid kit (Qiagen) and MagMAX Cell-free DNA Isolation Kit
(Thermo Fisher Scientific).

15 In embodiments of the present invention, the template DNA is double-stranded DNA or
single-stranded DNA. Ligation to single-stranded DNA can incorporate improved ligation
conditions including use of optimised ligase (Gansauge and Meyer, 2013) or primer design
incorporating ligation guides or splinter oligonucleotides (Gansauge *et al*, 2017; Kwok *et al*,
20 2013).

Other aspects of the invention

In accordance with a further aspect of the present invention, there is provided an adapter
25 oligonucleotide for producing an aRNA product from a DNA template, the adapter
oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter
oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to
a 3' end of a template DNA.

30 There is also provided an adapter oligonucleotide for amplifying a DNA template, the adapter
oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter
oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to
a 3' end of a template DNA.

35 The adapter of the present invention may be for use in the method of the present invention,
as defined above.

The RNA polymerase promoter sequence may comprise a T7 RNA polymerase promoter sequence, and such a sequence may comprise SEQ ID NO.1 or a fragment or variant thereof. In embodiments, the RNA polymerase promoter sequence may consist of SEQ ID
5 NO.1.

The adapter may comprise complementary regions which are capable of annealing to one another to form circular or hairpin loop structure.

- 10 The adapter may comprise a number of additional sequence features which may be useful in further analysing the target DNA which has been amplified, such as molecular bar code, sample bar codes, a NGS sequence and/or a T tail.

The adapter may be in a buffered (or other) solution so as to enable storage and
15 transportation of the adapter. The skilled person will appreciate suitable solutions in which the adapter may be present. Furthermore, the adapter may be in vial or other vessel suitable for storage, transportation and mixing with other reagents if desired.

Preferably, the adapter is for use as a research reagent/tool.

20

In accordance with a further aspect of the present invention, there is provided a kit for producing an aRNA product from a DNA template, the kit comprising:

- i) an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter
25 oligonucleotide can be ligated to a 3' end of a template DNA; and
- ii) a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide.

- 30 There is also provided a kit for amplifying a DNA template, the kit comprising:

- i) an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of a template DNA; and

-14-

ii) a secondary oligonucleotide comprising a RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide.

- 5 Preferably, the RNA polymerase promoter sequence in the adapter oligonucleotide comprises a T7 RNA polymerase promoter sequence with a sequence comprising SEQ ID NO.1 or a fragment or variant thereof. In embodiments, the RNA polymerase promoter sequence in the adapter oligonucleotide consists of SEQ ID NO.1.
- 10 Preferably, the RNA polymerase promoter sequence in the secondary oligonucleotide comprises a T7 RNA polymerase promoter sequence with a sequence comprising SEQ ID NO.2 or a fragment or variant thereof. In embodiments, the RNA polymerase promoter sequence in the adapter oligonucleotide consists of SEQ ID NO.2.
- 15 The adapter oligonucleotide of the kit may comprise complementary regions which are capable of annealing to one another to form circular or hairpin loop structure as described above in relation to the method of the present invention.

The adapter oligonucleotide of the kit may comprise a number of additional sequence
20 features which may be useful in further analysing the target DNA which has been amplified, such as molecular bar code, sample bar codes, a NGS sequence and/or a T tail as described above in relation to the method of the present invention.

The adapter oligonucleotide and/or secondary oligonucleotide of the kit may be in a buffered
25 solution. The skilled person will appreciate suitable solutions in which the adapter may be present.

In accordance with a further aspect of the present invention, there is provided a method for detecting a genetic variant or genetic signature in template DNA, comprising amplifying
30 template DNA in the method as herein above described and analysing the amplified cDNA for the genetic variation or signature.

The method of the present invention can be used to identify a wide range of genetic signatures including the presence of specific point mutations, mutational signature and
35 structural alterations including copy number gains and losses. The information generated can be used to identify mutations linked to specific therapy, monitor disease, predict clinical

outcomes and response to therapy. It is also envisaged that the invention will be particularly suited to identifying differences in DNA methylation that are related to the detection of tumour DNA in patient blood samples. Differences in DNA methylation may comprise differences in total methylation or DNA methylation patterns or signatures (such as described in Shen, S.Y., *et. al.*, (2018) and Shina. A. A. I. *et. al.*, (2018)).

The method of the present invention can be used to identify the presence, variations, or signatures, of ctDNA in the blood of cancer patients (or patients who are at a high risk of developing cancer or having a reoccurrence of cancer) and can also be used to identify the presence, variation or signature of circulating tumour cells (CTCs). The method can be used to detect sequence variants that are known to be specifically associated with various types of tumours or harbour copy number patterns linked to the presence of tumours and their progression.

In accordance with a further aspect of the present invention, there is provided a method of preparing template DNA for amplification, wherein the method comprises dephosphorylating the template DNA. Preferably, dephosphorylating the template DNA is achieved using a phosphatase enzyme. The method preferably removes the 5' phosphate of the template DNA. The phosphatase enzyme may comprise one or more selected from the following: Shrimp Alkaline Phosphatase; Antarctic Phosphatase; Calf Intestinal Alkaline Phosphatase; and/or or FastAP Thermosensitive Alkaline Phosphatase. Preferably the phosphatase enzyme comprises a heat labile phosphatase such as FastAP Thermosensitive Alkaline Phosphatase.

In accordance with a related further aspect of the present invention, there is provided use of a phosphatase enzyme for dephosphorylating template DNA prior to amplification or modification prior to amplification. The use will preferably be to remove the 5' phosphate of the template DNA. The phosphatase enzyme may comprise one or more selected from the following: Shrimp Alkaline Phosphatase; Antarctic Phosphatase; Calf Intestinal Alkaline Phosphatase; and/or or FastAP Thermosensitive Alkaline Phosphatase. Preferably the phosphatase comprises a heat labile phosphatase such as FastAP Thermosensitive Alkaline Phosphatase.

In accordance with a yet further related aspect of the present invention, there is provided a method for amplifying template DNA, the method comprising:

-16-

i) providing template DNA and an adapter oligonucleotide, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA;

ii) dephosphorylating the template DNA;

5 iii) ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule; and

iv) amplifying the adapter oligonucleotide-template DNA molecule.

Prior to step iv), the method may further comprise transcribing the template DNA by
10 providing a RNA polymerase promoter sequence in the adapter oligonucleotide sequence and introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule under conditions so to generate an aRNA product, and to converting the aRNA product to cDNA and subsequently amplifying the cDNA.

15 Preferably, dephosphorylating the template aRNA or DNA is achieved using a phosphatase enzyme. The method preferably removes the 5' phosphate of the template aRNA or DNA. The phosphatase enzyme may comprise one or more selected from the following: Shrimp Alkaline Phosphatase; Antarctic Phosphatase; Calf Intestinal Alkaline Phosphatase; and/or or FastAP Thermosensitive Alkaline Phosphatase. Preferably the phosphatase enzyme
20 comprises a heat labile phosphatase such as FastAP Thermosensitive Alkaline Phosphatase.

The described and illustrated embodiments are to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been
25 shown and described and that all changes and modifications that come within the scope of the inventions as defined in the claims are desired to be protected.

The optional features set out herein may be used either individually or in combination with each other where appropriate and particularly in the combinations as set out in the
30 accompanying claims. The optional features for each aspect or exemplary embodiment of the invention as set out herein are also to be read as applicable to any other aspect or exemplary embodiments of the invention, where appropriate. In other words, the skilled person reading this specification should consider the optional features for each exemplary embodiment of the invention as interchangeable and combinable between different
35 exemplary embodiments.

It should be understood that while the use of words such as “preferable”, “preferably”, “preferred” or “more preferred” in the description suggest that a feature so described may be desirable, it may nevertheless not be necessary and embodiments lacking such a feature may be contemplated as within the scope of the invention as defined in the appended claims.

5 In relation to the claims, it is intended that when words such as “a,” “an,” or “at least one,” are used to preface a feature there is no intention to limit the claim to only one such feature unless specifically stated to the contrary in the claim.

Detailed Description of the Invention

10

The present invention will now be further described with reference to the following figures which show:

Figure 1 is a schematic diagram for amplifying cfDNA as described in Example 1;

15

Figure 2 is a schematic diagram for amplifying genomic and circulating cell-free DNA as described in Example 2 (where steps 4-6 are carried out as depicted in Figure 1);

Figure 3 is a schematic diagram for amplifying genomic and circulating cell-free DNA as
20 described in Example 3, where steps 4-6 are carried out as depicted in Figure 1;

Figure 4 shows the result of the cfDNA ligation kit selection experiments as described in Example 4 for a dsDNA cfDNA T7 library preparation comparing NEB quick, NEB ultra and KAPA ligases, where Figure 3A shows a photograph of a electrophoretic gel and Figure 4B is
25 a graph;

Figure 5 shows the result of the cfDNA ligation optimisation experiments as described in Example 5, where the experiments were conducted between 4 - 16°C and where Figure 5A shows a photograph of a electrophoretic gel and Figure 5B is a graph;

30

Figure 6 shows the result of the cfDNA ligation optimisation experiments as described in Example 6, where the experiments were conducted between 12 - 20°C and where Figure 6A shows a photograph of a electrophoretic gel and Figure 6B is a graph;

Figure 7 shows the result of the cfDNA ligation optimisation experiments as described in Example 7, where Figure 7A shows a photograph of a electrophoretic gel illustrating the results of the KAPA ligase T7 library preparation and Figure 7B is a graph;

- 5 **Figure 8** shows the result of NGS analysis of HNV and cancer patient cfDNA as described in Example 8, where Figure 8A shows a photograph of a electrophoretic gel and Figure 8B shows plots of genome wide copy number changes;

10 **Figure 9** shows that the present DNA amplification method is compatible with DNA methylation analysis. Figure 9A shows a photograph of an electrophoretic gel showing index PCR products from the present method, lanes 2 and 3 show input (no enrichment control) pool 1 and 2 (In1 and In2) and lanes 3 and 4 show methylation-enriched pool 1 and 2 (Me1 and Me2). Figure 9B shows the results of sample bar code demultiplexing;

- 15 **Figure 10** shows that the present DNA amplification method is compatible with DNA methylation analysis and delivers improved results compared to a non-UMI-based NGS library preparation method. Figure 10A shows Methylation Enrichment QC which demonstrates greater enrichment using the present method (T7 pool and individual) compared to non-UMI based method (current method). Figure 10B shows Methylation
20 background QC and demonstrates a reduction in background noise with the present method (T7 pool and individual) compared to non-UMI based method (current method). More than double the number of reads in the non-UMI based method (current method) do not contain a CpG. Figure 10C shows PCA plot separates tumour and normal samples. Figure 10D shows HOXA9 tumour vs. normal samples and demonstrates that the present method can identify
25 tumour-specific methylation;

Figure 11 shows comparative data of the present DNA amplification method (T7) vs a commercial amplification method using UMI NGS adapters (IDT). Figure 11A shows analysis of the methylation enrichment, the present method shows improvement in enrichment over
30 the commercial method. Figure 11B shows analysis of the methylation background, the present method shows a significant reduction in the amount of background compared to the commercial method. Figure 11C shows that the present method has enhanced sensitivity over the commercial method;

- 35 **Figure 12** shows amplification performed on DNA digested using three restriction enzymes, MLyl, BsuRI and NIAIII. Both MLyl and BsuRI produce blunt ends and NIAIII produces a 3'

overhang. Figure 12A shows the restriction sites recognised by MLyl, BsuRI and NIAIII and a photograph of an electrophoretic gel of the DNA post digestion with each of the restriction enzymes individually. Figure 12B shows a photograph of an electrophoretic gel of the digested DNA after performing amplification using the present method. Figure 12C shows a schematic of the method for amplifying genomic and single cell gDNA comprising the steps of: Step 1: prepare gDNA by utilising a MLyl or BsuRI restriction enzyme digest, resulting in blunt end DNA fragments, which are then A-tailed. Step 2: anneal the oligonucleotide so as to form a circular adapter. Step 3: ligates the circular adapter to target DNA;

Figure 13 shows the results of the in-silico folding of the adapter using Quickfold software. This demonstrates the complementary regions of the adapter are capable of self-annealing and forming circular secondary structure;

Figure 14 demonstrates the suitability of pooling multiple samples after ligation. Pooled NGS library samples were sequenced and then demultiplexed;

Figure 15 shows conversion of aRNA to cDNA. Figure 15A demonstrates the conversion of aRNA to cDNA using the ligation approach or a random primer approach. Figure 15B shows optimisation of the conversion of aRNA to cDNA using the ligation approach or a random primer approach. For the ligation approach one sample has been treated with phosphatase and one sample has not been treated with phosphatase prior to the ligation step. For the random primer approach one sample has used 100 μ M primer and one sample has used 50 μ M primer. Figure 15 C and D show schematics of the conversion of aRNA to cDNA using the ligation approach (15C) or a random primer approach (15D); and

Figure 16 shows amplification of cfDNA. Figure 16A shows amplification of cfDNA using the present method with different input amounts of cfDNA. Figures 16B and C show RNaseP qPCR quantification and RNaseP qPCR fold increase in aRNA relative to input DNA respectively.

Example 1 - Method for amplifying cfDNA

The method for amplifying cfDNA, in accordance with the present invention, is schematically shown in Figure 1 and has the following steps:

Step 1: prepare target cfDNA to be A-tailed cfDNA and dephosphorylated.

-20-

Step 2: utilise a unique oligonucleotide sequence to form self-annealing circular adapter.

Step 3: ligates the circular adapter to target DNA. Ligation only occurs to one strand so as to enable separate independent sequencing of both upper and lower strands of the DNA.

5

Step 4: denatures and anneals a T7 RNA polymerase complementary oligonucleotide. This step does not require a 2nd polymerisation step to produce dsDNA and is therefore simpler and more efficient than prior art DNA polymerase amplification.

10 Step 5: employs a T7 RNA polymerase reaction on the complementary oligonucleotide and amplify the strand. In this step, all amplified aRNA products are generated by producing up to 1,000 aRNA copies of each original unaltered strand.

15 Step 6: converts the aRNA into cDNA in preparation for sequencing. It will be apparent to the skilled person that this step will be dependent upon which sequencing platform will be utilised and would typically be selected from one of the following outlined below:

20 Step 6a: converts aRNA to cDNA via ligation to aRNA. This has the benefit of preserving the original fragment length, but does involve an extra possible inefficient step in the process.

Step 6b: converts aRNA to cDNA using random NGS primer. This has the benefit of being simple and efficient, but the original fragment length is lost.

25 Step 6c: converts aRNA to cDNA using target specific NGS primer. This has the benefit of being highly specific, but is limited to the target.

Example 2 - Method for amplifying genomic and circulating cell-free DNA

30 A method for amplifying genomic and circulating cell-free DNA, according to the present invention, is schematically shown in Figure 2 and has the following steps 1 - 3:

Step 1: prepare gDNA by utilising a Hsp92II restriction enzyme digest.

Step 2: anneal the oligonucleotide so as to form a circular adapter.

35

-21-

Step 3: ligates the circular adapter to target DNA. Since the adapter T7_U1_ Hsp92II3c3 contains only 3 of the 4 bases of the overhang, this reduces primer dimer. Furthermore, adapter T7_U1_ Hsp92II2c3 contains only 2 of the 4 bases of the overhang – further reducing primer dimer.

5

The method may additionally comprise steps 4 – 6 (not shown):

Step 4: denature and anneal the T7 RNA polymerase complementary oligonucleotide.

10 Step 5: employs a T7 RNA polymerase reaction on the complementary oligonucleotide and amplify the strand.

Step 6: converts the aRNA into cDNA in preparation for sequencing. It will be apparent to the skilled person that this step will be dependent upon which sequencing platform will be
15 utilised and would typically be selected from one of the following outlined below:

Step 6a: converts aRNA to cDNA via ligation to aRNA. This has the benefit of preserving the original fragment length, but does involve an extra possible inefficient step in the process.

20

Step 6b: converts aRNA to cDNA using random NGS primer. This has the benefit of being simple and efficient, but the original fragment length is lost.

Step 6c: converts aRNA to cDNA using target specific NGS primer. This has the
25 benefit of being highly specific, but is limited to the target.

Further methods for converting aRNA into cDNA in preparation for sequencing are shown in Figures 15C and 15D.

30 Figure 15C shows step 6a(i) involves adding a priming site to aRNA via ligation to U2 oligonucleotide to ligate U2 ligation adapter to aRNA. The next steps would be the conversion of aRNA to full length cDNA using a primer complementary to U2 oligo and the formation of a NGS library with full length cDNA.

35 Figure 15C shows step 6a(ii) involves converting aRNA to cDNA via improved ligation to aRNA. A U2 ligation adapter is ligated to aRNA. The rationale is that the NN adapter

-22-

promotes annealing to aRNA thereby improving efficiency of ligation compared to U2 oligonucleotide alone (as set out in step 6a above). The next steps would be the conversion of aRNA to full length cDNA using primer complementary to U2 oligonucleotide and the formation of a NGS Library with full length cDNA.

5

Figure 15D shows step 6b(i) involves preparing cDNA from aRNA using a U2 random primer. aRNA is converted to cDNA in a reverse transcriptase reaction. The next steps would be the formation of a NGS Library direct with cDNA from the reverse transcriptase reaction.

- 10 Figure 15D shows step 6b(ii) involves promoting full length reverse transcriptase products using a random primer adapter. aRNA is converted to cDNA in a reverse transcriptase reaction. The rationale is that the U2 Random Primer adapter promotes priming from the end of each aRNA by sterically blocking/reducing internal priming and this will provide more full length cDNA than U2 Random Primer alone (as set out in step 6c above). The next steps
- 15 would be the formation of a NGS library direct with cDNA from the reverse transcriptase reaction.

Example 3 - Method for amplifying single stranded genomic and circulating cell-free DNA

- 20 A method for amplifying single stranded genomic and circulating cell-free DNA, according to the present invention, is schematically shown in Figure 3 and has the following steps:

Step 1: prepare single stranded DNA by denaturation (heat or chemical).

- 25 Step 2: anneal the oligonucleotide so as to form a circular adapter.

Step 3: anneal and ligate the circular adapter to target DNA.

- Step 4: denature and anneal a T7 RNA polymerase complementary oligonucleotide. This
- 30 step does not require a 2nd polymerisation step to produce dsDNA and is therefore simpler and more efficient than prior art DNA polymerase amplification.

- Step 5: employs a T7 RNA polymerase reaction on the complementary oligonucleotide and amplify the strand. In this step, all amplified aRNA products are generated by producing up
- 35 to 1,000 aRNA copies of each original unaltered strand.

-23-

Step 6: converts the aRNA into cDNA in preparation for sequencing. It will be apparent to the skilled person that this step will be dependent upon which sequencing platform will be utilised and would typically be selected from one of the following outlined below:

5 Step 6a: converts aRNA to cDNA via ligation to aRNA. This has the benefit of preserving the original fragment length, but does involve an extra possible inefficient step in the process.

10 Step 6b: converts aRNA to cDNA using random NGS primer. This has the benefit of being simple and efficient, but the original fragment length is lost.

Step 6c: converts aRNA to cDNA using target specific NGS primer. This has the benefit of being highly specific, but is limited to the target.

15 **Example 4 - cfDNA ligation kit selection**

Experiments were conducted to assess the suitability and efficiency of NEB Quick Ligation Kit (New England BioLabs Inc.), NEB Ultra Ligation Kit (New England BioLabs, Inc.) and KAPA Ligation Kit (Kapa Biosystems) for the amplification of cfDNA.

20 In the experiments, 10 ng cfDNA were used in each reaction with 0.03 ng max carried through to qPCR. 0.03 ng was loaded as input cfDNA. As shown in Figure 3A and 3B, the approximate amplification produced by the kits were as follows: Quick 40x; Ultra 10x; and Kapa 80x. Clear aRNA products from cfDNA with qPCR indicating 25-80x amplification was seen (not shown) and NTC shows primer aRNA products overlapping in size with cfDNA
25 aRNA products.

Example 5 - cfDNA ligation optimisation conducted between 4 - 16°C

Experiments were conducted to optimise cfDNA ligation conducted between 4 - 16°C assessed using the KAPA Ligation Kit.

30

Figure 5A and 5B show the results of the experiments shows the fold change relative to input of: 4°C overnight ligation = 4.9; 10°C overnight ligation = 12.1; and 16°C overnight ligation = 26.5. The experiments also showed clear aRNA products from cfDNA with qPCR indicating 25-80x amplification (not shown) and NTC shows primer aRNA products overlapping in size
35 with cfDNA aRNA products.

Example 6 - cfDNA ligation optimisation conducted between 12 - 20°C

Experiments were conducted to optimise cfDNA ligation conducted between 12 - 20°C. As shown in Figures 6A and 6B, 20°C gave best results and low NTC.

5 Example 7 - cfDNA ligation optimisation inclusion of phosphatase treatment of cfDNA

Experiments were conducted to optimise cfDNA ligation by examining the effect of phosphatase treatment of the input cfDNA. For 2 of the 4 samples, phosphatase (5 µl FastAP Thermosensitive Alkaline Phosphatase (1 U/µl)) was added to 42 µl polished and A-tailed cfDNA (Example 1, step 1) and all 4 samples were subjected to cfDNA ligation conducted at
 10 20°C using the KAPA Ligation Kit. As shown in Figures 7A and 7B phosphatase treatment of the input cfDNA resulted in improved aRNA production with >300x amplification seen in the phosphatase treated samples.

Example 8 – T7 cfDNA Library Preparation Method

15 Experiments were conducted to produce a T7 cfDNA library.

Step 1 was to polish and add a dA tail to the DNA. In PCR tubes, all samples were adjusted to 35 µl with nuclease-free water. To each 35 µl of sample the following products were added (NOTE: defrost and place the reagents on ice, then prepare the tailing reaction on the bench
 20 at room temp):

	Vol (µl)
NEBNext Ultra II End Prep Enzyme Mix	2.1
NEBNext Ultra II End Prep Reaction Buffer	4.9

Table 1

Following mixing, the samples were placed into PCR block and the following applied:

Step	Temp	Time	Heated Lid
1	20°C	30 min	75°C
2	65°C	30 min	75°C
3	4°C	Hold	75°C

Table 2

25

No clean up between tailing and ligation was necessary.

Step 2 was to ligate the adapter to tailed DNA under the following conditions (NOTE: When Step 1 is running prepare T7dT_U1_Lcf1 oligo):

-25-

	T7dT_U1_Lcf1 oligo	TE	Final conc (μM)
Dilution 1	4.4 μL of 100uM Stock	35.6 μL	11
Dilution 2	10 μl of dilution 1	40 μL	2.2

Table 3

Followed by annealing the entire 50 μl Dilution 2:

Step	Temp	Time	Heated Lid
1	95°C	1 min	105°C
2	Ramp from 95°C - 4°C	4°C/sec	105°C
3	4°C	Hold	105°C
4	Wet ice	Transfer	NA

5 **Table 3**

(NOTE: Annealed T7 oligo, and subsequently master mix, maintained at 4°C or on ice until start of ligation)

A ligation master mix was then prepared on ice as followed:

Kapa Ligation module	Vol (μl)	master mix (x7)
KAPA-Hyper Ligation Buffer	21	147
KAPA-Hyper DNA ligase	7	49
Water	3.5	24.5
Annealed T7 Adapter (2.2uM)	3.5	24.5

10 **Table 4**

35 μl of the ligation master mix was then added to the 42 μl of tailed DNA and incubated at 20°C overnight.

15 Step 3 was to clean-up post ligation:

Sample Volume (μl)	Bead ratio	Bead vol (μl)	Elution vol (μl)
77	0.8	61.6	11

Table 5

8.5 μL of eluate was transferred to fresh a PCR tube and either processed immediately or stored at -20°C.

-26-

Step 4 was to anneal to produce dsDNA T7 promoter. The T7_prim1 oligo was prepared as follows:

	T7_prim1	Diluent	Final conc μM
Dilution 1	2 μ l of 100uM stock	18 μ l of TE	10
Dilution 2	2 μ l of dilution 1	23 μ l of nuclease free water	0.8

Table 6

- 5 1.2 μ l of Dilution 2 (0.8 μ M) was added to the 8.5 μ l of purified ligation product, placed into a thermocycler and run using the following programme:

Step	Temp	Time	Heated Lid
1	95°C	1 min	105°C
2	Ramp from 95°C - 37°C	4°C/sec	105°C
3	37°C	Hold	105°C

Table 7

Step 5 was the T7 aRNA production. On wet ice a T7 master mix was prepared as follows:

Reaction component	Vol (μl)	x8
10X Reaction Buffer	2.5	20
ATP (100 mM)	2.5	20
GTP (100 mM)	2.5	20
UTP (100 mM)	2.5	20
CTP (100 mM)	2.5	20

10 **Table 8**

- 12.5 μ l of the T7 master mix was then dispensed into six PCR tubes and the tubes were placed into the thermocycler on hold at 37°C. The master mix was left in the block for 1 min to reach 37°C. Then 12.1 μ l of the pre-warmed T7 master mix was transferred to each tube containing the annealed T7_Prim1/ligation product, which is already on hold at 37°C. 2.4 μ l of T7 polymerase was then added to each tube and mixed by pipetting 10 times.
- 15

A T7 run off positive control reaction was prepared as follows:

Reaction component	Vol (μl)
FLuc Control Template (10ng/ μ l)	1
Pol	2.4
Master mix	12.1

-27-

Nuclease-free water	8.7
---------------------	-----

Table 9

The positive control tubes were then placed on the block on hold at 37°C and left to run overnight with the other reactions.

5

Step 6 involved the clean-up post aRNA production. An Ampure bead clean was performed as follows (exclude the positive control):

Sample Volume (µl)	Bead ratio	Bead vol (µl)	Elution vol (µl)
24.25	1	24.25	11.5

Table 10

- 10 10 µl of eluate was transferred to a fresh PCR tube and placed on ice. In a QC step, 1 µl of purified aRNA was run on a gel to assess output (along with 5 µl of 1:100 diluted positive control reaction).

Step 7 was to convert aRNA to cDNA. The following oligonucleotide was prepared:

- 15 U2P_NNv1 100 µM GACGTGTGCTCTTCCGATCTNNNNNNNNN

The oligonucleotide was then mixed with the aRNA on ice at the following concentrations/volumes:

Component	Concentration	Volume (µl)
U2P_NNv1 oligo	100 µM	1.4
aRNA	N/A	8.6

Table 11

20

The denaturing and annealing were performed on a thermocycler using the following conditions:

Step	Temp	Time	Heated Lid
1	72°C	3 min	105°C
2	4°C	Hold	105°C

Table 12

- 25 The RT master mix was then prepared and put on ice:

Component	Concentration	Volume (30 µl reaction)	Final conc.	x7
dNTPs	10 mM	1.0	0.33 mM	7

-28-

buffer	5X	6.0	5x	42
DTT	0.1M	1.0	3.3 mM	7
Superscript IV	200 U/μl	1.0	200 U	
H ₂ O		11.0		77

Table 13

19 μl of RT master mix was added to the 10 μl of RNA/U2P_NNv1 mix. Then 1 μl of Superscript IV was added to each reaction and mixed by pipetting 10 times.

5

The mixture was then placed on a thermocycler and run using the following programme:

Step	Temp	Time	Heated Lid
1	50°C	10 min	105°C
2	80°C	10 min	105°C
3	4°C	Hold	105°C

Table 14

Step 8 involved the clean-up post aRNA RT. An Ampure bead clean-up was performed as follows:

10

Sample Volume (μl)	Bead ratio	Bead vol (μl)	Elution vol (μl)
30	1.5	45	23

Table 15

20 μl of eluate was transferred to fresh a PCR tube and placed on ice.

15 Step 9 involves the NGS PCR. A NGS PCR master mix was prepared as follows:

Reagent	Vol (μl) X1	Vol (μl) X7
Kapa Polymerase*	1	7
Kapa Buffer*	10	70
dNTPs*	1.5	10.5
NEB Universal**	2.5	17.5
Water	12.5	87.5

Table 16 (*From Kapa HiFi Hot Start PCR Kit with dNTPs, **From NEBNext Multiplex Oligos for Illumina index primers)

-29-

27.5 µl of NGS PCR mastermix and 2.5 µl of appropriate indexing oligo was added to 20 µl of purified RT reaction and the protocol below followed:

Step	Temp	Time	Heated Lid
1	98°C	30 sec	105°C
2	98°C	10 sec	105°C
3	65°C	30 sec	105°C
4	72°C	30 sec	105°C
Repeat Steps 2-4 a total of 8 times			
5	72°C	5 min	105°C
6	4°C	Hold	105°C

Table 17

- 5 Step 10 involved the clean-up post NGS PCR. Ampure bead clean was performed as follows:

Sample Volume (µl)	Bead ratio	Bead vol (µl)	Elution vol (µl)
50	1.8	90	33

Table 18

- 10 30 µl of eluate containing each NGS library was transferred into a PCR tube and stored at -20°C.

- Step 11 involved NGS. Libraries were quantified using Kapa qPCR according to FRM-94. Libraries were pooled in equimolar or amounts to generate a composite library sample. Composite library was quantified using KAPA qPCR according to FRM-94. The composite library was denatured and diluted to 12.5 pM and supplemented with 10% PhiX (2 pM final conc.). The NGS run was performed on the Miseq using Miseq Kit (300 cycles) and paired end sequencing.

- 20 Table 19 below and Figure 6 shows the results of a T7 cfDNA Library which was run on an electrophoretic gel and shows very high banding with a library prep loading of 25 ng, but good banding too at 5.845 ng.

Sample details	Sample ID	Index
HNV-377	1	1
HNV-378	2	2
HNV-380	3	3

-30-

NSCLC-12275_cfDNA	4	4
NSCLC-12136_cfDNA	5	6
NSCLC-12210_cfDNA	6	12

Table 19

It will be appreciated that numerous modifications to the above described method may be made without departing from the scope of the invention as defined in the appended claims.

5

Example 9 – production of methylation enriched T7 gDNA library

Experiments were conducted to produce a methylation enriched T7 gDNA library. The agarose gel in Figure 9A shows the NGS library product for a methylation enriched library and a no enrichment control for the same set of samples. Six samples were pooled to generate the methylation enriched T7 gDNA library. The results of demultiplexing of the NGS reads based on the sample barcode present in the circular adapter are shown in Figure 9B. The protocol for generating the methylation enriched T7 gDNA library is as follows:

15

Step 1 end repair and dA-tailing

End repair and dA-tailing was performed followed by ligations performed in duplicate to create two pools. 'Sample set' is a dilution series of sheared gDNA from SCLC cell line H446 diluted in sheared gDNA from HNV PBMCs as shown below in Table 20.

Sample Number	Sample details	Sample ID	cfDNA volume (μL)	Water volume (μL)	cfDNA amount (ng)	SBC
1	100 % H446	AC-MBDSeq-040-1a&b	10.0	25.0	10	T7dT_U1_Lcf1
2	5 % H446	AC-MBDSeq-040-2a&b	10.0	25.0	10	T7dT_U1_Lcf2
3	2.5 % H446	AC-MBDSeq-040-3a&b	10.0	25.0	10	T7dT_U1_Lcf4
4	1 % H446	AC-MBDSeq-040-4a&b	10.0	25.0	10	T7dT_U1_Lcf6
5	0.1 % H446	AC-MBDSeq-040-5a&b	10.0	25.0	10	T7dT_U1_Lcf14
6	0.01 % H446	AC-MBDSeq-040-6a&b	10.0	25.0	10	T7dT_U1_Lcf15
7	0.001 % H446	AC-MBDSeq-040-7a&b	10.0	25.0	10	T7dT_U1_Lcf21
8	100 % PBMC	AC-MBDSeq-040-8a&b	10.0	25.0	10	T7dT_U1_Lcf25

20

Table 20

To each sample the following products were added as detailed in Table 21 below:

	Vol (μL)
NEBNext Ultra II End Prep Enzyme Mix	2.1
NEBNext Ultra II End Prep Reaction Buffer	4.9

Table 21

Following mixing, the samples were placed into PCR block and the following conditions applied (as detailed in Table 22 below):

Step	Temp	Time	Heated Lid
1	20°C	30 min	85°C
2	65°C	30 min	
3	4°C	Hold	

Table 22

Step 2 dephosphorylation

- 10 Dephosphorylation of the DNA samples was performed using Thermo AP fast phosphatase. To each sample 5µL of AP fast phosphatase (1U/µL) was added. Following mixing the samples were placed into PCR block and the following conditions applied (as detailed below in Table 23):

Step	Temp	Time	Heated Lid
1	37°C	30 min	85°C
2	80°C	5 min	85°C
3	4°C	Hold	85°C

Table 23

Step 3 preparation of the T7dT_U1_LcfX oligo hairpin for ligation

The preparation of the T7dT_U1_LcfX oligo hairpin for ligation was in accordance with values detailed below in Table 24.

	T7dT_U1_Lcf oligo	Volume of TE (µL)	Final conc (µM)
Dilution 1	4.4 µL of Stock	35.6	11
Dilution 2	2 µL of dilution 1	8	2.2

Table 24

All of dilution 2 was used for annealing using the following conditions (as detailed in Table 25 below):

-32-

Step	Temp	Time	Heated Lid
1	95 °C	1 min	105 °C
2	Ramp	0.4 °C/sec	
3	4 °C	Hold	
4	Transfer to ice		

Table 25

The annealed T7 oligo and subsequently the master mix were held on ice until the start of ligation.

Step 4 ligation

The ligation master mix was prepared as follows (as detailed in Table 26):

Kapa Ligation module	Vol for 1 rxn (μL)	Vol for master mix (μL)
KAPA-Hyper Ligation B	21	357
KAPA-Hyper DNA ligase	7	119
Water	3.5	59.5
T7 Adaptor (2.2uM)	3.5	

Table 26

On ice, 3.5 μL of appropriate T7dT_U1_Lcf oligo (2.2 μM) was added to the dephosphorylated DNA and mixed thoroughly. Then 31.5 μL of master mix was added to dephosphorylated DNA, and mixed. Ligations were placed into the PCR machine running at 20 °C overnight, with heated lid off.

Step 5 preparation of the sample pool is shown below in Table 27.

Total volume of ligated DNA (μL)	82	
Pool all of each reaction mix	Volume per sample (μL)	82
Prepare two pools poolA and poolB	Total samples in pool	8
	Total volume of pool (μL)	656

Table 27

Clean-up of the pooled samples was performed post ligation. Bead clean-up was performed, and samples were eluted in low EDTA buffer as detailed below in Table 28:

Sample	Sample Volume (μL)	Bead ratio	Bead Vol (μL)	Elution vol (μL)
Pooled sample*	656	0.8	524.8	53

Table 28

45 µL of eluate was used for methylation pull down and 5 µL of the eluate was used as a no-methylation enrichment control.

Step 6 methylated DNA enrichment

5

Methylated DNA enrichment was performed as per the manufacturer's protocol.

MBD2-Fc was prebound to Protein A magnetic beads. For each sample a mix of protein and beads was prepared containing the following ingredients (as detailed in Table 29 below):

	Per tube
Protein A magnetic beads	10 uL
MBD2-Fc	1 uL
1 X Bind/Wash buffer	11 uL

10 **Table 29**

- Resuspend the beads by pipetting up and down
- Incubate at room temperature for 15 minutes with occasional gentle shaking
- Add 950 µL 1X Bind/Wash Buffer

- 15
- Incubate at room temperature for 3 minutes with occasional gentle shaking
 - Place on magnetic rack for 2 minutes
 - Remove supernatant

- Add 950 µL 1X Bind/Wash Buffer
- Incubate at room temperature for 3 minutes with occasional gentle shaking

- 20
- Place on magnetic rack for 2 minutes
 - Remove supernatant, spin for 5 s and place back on the magnet to remove the residual supernatant
 - Remove the tube from the rack and add 11 uL of 1X Bind/Wash Buffer

- 25
- Capture of the methylated CpG DNA was performed using the components as detailed in Table 30.

	Sample per tube
5X Bind/Wash Buffer (uL)	20
Fragmented sample DNA (uL)	45
MBD2-Fc/Protein A Magnetic Bead mix (uL)	10
DNase-free water (uL)	25
Total (uL)	100

Table 30

-34-

Wash off unbound DNA;

- Place on magnetic rack for 3 minutes

- Remove supernatant and save as non-captured DNA fraction

5 - Add 950 μ L 1X Bind/Wash Buffer

- Incubate at room temperature for 3 minutes with occasional gentle shaking

- Place on magnetic rack for 2 minutes

- Remove supernatant

- Add 950 μ L 1X Bind/Wash Buffer

10 - Incubate at room temperature for 3 minutes with occasional gentle shaking

- Place on magnetic rack for 2 minutes

- Remove supernatant

- Add 950 μ L 1X Bind/Wash Buffer

- Incubate at room temperature for 3 minutes with occasional gentle shaking

15 - Place on magnetic rack for 2 minutes

- Remove supernatant, spin for 5 s and place back on the magnet to remove the residual supernatant

Elute captured methylated CpG DNA

20 - Add 37 μ L DNase-free water to washed beads and mix by pipetting up and down 10 times

- Incubate in heat block at 65 °C for 15 minutes, with frequent mixing

- Spin down for 5 seconds in microcentrifuge

- Place on magnetic rack for 3 minutes

25 - Remove 34 μ L supernatant, place in PCR tubes and save as enriched methyl CpG containing DNA fraction

- Use all 34 μ L in the T7 amplification

Step 7 T7 amplification of methylation-enriched tumour DNA to aRNA was performed according to Table 31 below.

Tube Number	Sample details	Sample ID	Sample description	Volume into reaction (μ L)
1	Postive control	-	-	4.0
2	H446 dilution series	AC-MBDSeq-040-PoolA-M	Me-Cap - pool	34.0
3	H446 dilution series	AC-MBDSeq-040-PoolB-M	Me-Cap - pool	34.0
4	H446 dilution series	AC-MBDSeq-040-PoolA-I	Input - pool	5 + 3.5 μ L water
5	H446 dilution series	AC-MBDSeq-040-PoolB-I	Input - pool	5 + 3.5 μ L water
6	Postive control	-	-	1.0

Table 31

-35-

Step 8 Annealing

For Me-cap samples the conditions detailed in Table 32 were used.

	Stock conc.	Final conc.	Vol (μL)
Purified Me-enriched sample			34
T7_prim1	0.8μM	100nM	4.8
Total			38.8

Table 32

5

For input samples the conditions detailed in Table 33 were used.

	Stock conc.	Final conc.	Vol (μL)
Purified Ligation reaction			8.5
T7_prim1	0.8μM	100nM	1.2
Total			9.7

Table 33

- 10 For T7_prim1 dilution to 0.8 μM from 100μM stock. Dilute 1:10 in TE to make 10 μM dilution, then 2μl of the 1:10 dilution (10 μM) and 23μL H2O.

Step 9 T7 run off

For Me-cap samples the components detailed in Table 34 were used.

	Vol for 1 rxn (μL)	Vol for master mix (μL)
10X Reaction Buffer	10	40
ATP (100 mM)	10	40
GTP (100 mM)	10	40
UTP (100 mM)	10	40
CTP (100 mM)	10	40

15

Table 34

For each reaction, add 50 μl of mastermix to a PCR tube and place on the thermocycler at 37 °C for 1 min. Transfer 48.5 μL of warm master mix to 38.8 μL of Prim1/ligation product that is already on hold at 37 °C. Add 9.7 μL T7 Polymerase to each tube. Include a positive control as indicated below in Table 35.

20

Template	Template (μL)	T7 polymerase (μL)	Mastermix (μL)	Water (μL)
Ligations	38.8	9.7	48.5	0
FLuc Control Template (1	4	9.7	48.5	34.8

Table 35

- 25 Bead clean-up was then performed, and samples were eluted in low EDTA TE buffer as detailed below in Table 36:

-36-

Sample Volume (μL)	Bead ratio	Bead Vol (μL)	Elution vol (μL)
97	1	97	33

Table 36

For input samples the components in Table 37 were utilised.

	Vol for 1 rxn (μL)	Vol for master mix (μL)
10X Reaction Buffer	2.5	10
ATP (100 mM)	2.5	10
GTP (100 mM)	2.5	10
UTP (100 mM)	2.5	10
CTP (100 mM)	2.5	10

Table 37

For each reaction, add 12.5 μl of master mix to a PCR tube and place on the thermocycler at 37 °C for 1 min. Transfer 12.1 μL of warm master mix to 9.7 μL of Prim1/ligation product that is already on hold at 37 °C. Add 2.4 μL T7 Polymerase to each tube. Include a positive control as indicated below in Table 38.

Template	Template (μL)	T7 polymerase (μL)	Mastermix (μL)	Water (μL)
Ligations	9.7	2.4	12.1	0
FLuc Control Templ	1	2.4	12.1	8.7

Table 38

Bead clean-up was then performed, and samples were eluted in low EDTA TE buffer in the quantities detailed in Table 39.

Sample Volume (μL)	Bead ratio	Bead Vol (μL)	Elution vol (μL)
24.2	1	24.2	33

Table 39

Step 10 ssLigation, RT and Index PCR

Sample Summary is detailed in Table 40 below.

Tube Number	Sample details	Sample ID	Adapter concentration	Ligase	NEB Index
1	MeCap - aRNA - PoolA	AC-MBDseq-042A-Me	10 μM	mut T4 RNA ligase 2	2
2	MeCap - aRNA - PoolB	AC-MBDseq-042B-Me	10 μM	mut T4 RNA ligase 2	4
3	Input - aRNA - PoolA	AC-MBDseq-042A_In	10 μM	mut T4 RNA ligase 2	7
4	Input - aRNA - PoolB	AC-MBDseq-042B_In	10 μM	mut T4 RNA ligase 2	16

Table 40

Step 11 dephosphorylation of aRNA

-37-

8 μ L of each aRNA sample was combined with 1 μ L of AP fast phosphatase and placed into PCR block and the conditions detailed in Table 41 below applied.

Temp (°C)	Time (min)	Cycles
37 °C	30	1
80 °C	5	1
4 °C	Hold	1

Table 41

5

Step 12 ssLigation

20 μ L of a 10 μ M solution of Adapter 5'Adenyl-U2-C3sp was prepared. The following ligation master mix was also prepared (as detailed in Table 42):

Component	Volume (μ L)	Volume for mastermix (μ L)	Final concentration
10X ligation buffer	2	10	1X
50 % PEG 8000	5	25	12.50%
Water	2	10	1X
mut T4 RNA ligase 2	1	5	1X

10 **Table 42**

1 μ L of adapter was added to each aRNA sample followed by 10 μ L of ligation master mix. Ligation was then performed at 16°C overnight.

15 **Step 13 ligation reaction clean up**

Clean up of the ligation products was performed using Ampure bead clean up according to the quantities detailed in Table 43:

Sample Volume (μ L)	Bead ratio	Bead Vol (μ L)	Elution vol (μ L)	Ethanol
20	1	20	22.5	80%

Table 43

20

Step 14 RT reaction

The RT oligo was prepared as follows. RT_U2P_primer (100 μ M) (GACGTGTGCTCTTCCGATCT) was used.

1 μ L of RT_U2P_primer (100 μ M) was added to each adapter-ligated aRNA sample. The samples were then denatured and annealed using the following conditions (as detailed in Table 44).

Temp °C	Time (min)	Cycles
72	3	1
4	∞	1

Table 44

The RT master mix was prepared as follows (as detailed in Table 45):

Component	Concentration	Volume (30 μ L reaction)	Final conc.	Volume for MM (μ L)
dNTPs	10mM	1.0	0.33mM	5
buffer	5X	6.0	1X	30
DTT	0.1M	1.0	3.3mM	5
Superscript IV	200U/ μ L	1.0	200U	
H2O		0.0		0

Table 45

5

1 μ L of Superscript IV was added to the samples and placed in the PCR block under the following conditions (as detailed in Table 46):

Temp °C	Time (min)	Cycles
50	10	1
80	10	1
4	∞	1

Table 46

10

Clean up of the samples was then performed using Ampure bead clean up. Products were eluted in low EDTA TE buffer in quantities detailed in Table 47 below.

Sample Volume (μ L)	Bead ratio	Bead Vol (μ L)	Elution vol (μ L)	Ethanol
30	1.5	45	32.5	70%

Table 47

15

Step 15 indexing PCR

The master mix for the indexing PCR was prepared as follows (as detailed in Table 48 below).

Reagent	Vol (μ L) X1	Vol (μ L)
Kapa Polymerase*	1	5
Kapa Buffer*	10	50
dNTPs*	1.5	7.5
NEB Universal**	2.5	12.5
NEB Index**	2.5	
Water	2.5	12.5
*From Kapa HiFi Hot Start PCR Kit with dNTPs		
**From NEBNext Multiplex Oligos for Illumina inc		

20

Table 48

17.5 μ L of the PCR master mix was added to 30 μ L of purified RT reactions. 2.5 μ L of the indexing oligo was added. Then the following PCR conditions were applied (as detailed in Table 49):

Temperature	Time	Cycles
98°C	30 Seconds	1
98°C	10 Seconds	7 cycles
65°C	30 Seconds	
72°C	30 Seconds	
72°C	5 Minutes	1
4°C	∞	

5 **Table 49**

Clean up of the product from the index PCR reaction was then performed using Ampure bead clean up. Products were eluted in low EDTA TE buffer in quantities detailed in Table 50 below.

Sample Volume (μ L)	Bead ratio	Bead Vol (μ L)	Elution vol (μ L)
25	1.8	45	32.5

10

Table 50

Samples were stored for KAPA qPCR quantification and NGS.

15 **Example 10: Comparison of methylation enriched libraries prepared by the present method and a commercially available method**

Experiments were conducted to compare the methylation enriched libraries generated by the present method (T7 method) with a commercial non-UMI based NGS library preparation method (current method). The commercial method comprises end repair and A tailing of the DNA, followed by ligation of the circular adapter from the NEBNext Multiplex Oligos for Illumina Index Primers kit (New England BioLabs Inc.) using KAPA-Hyper DNA ligase and KAPA-Hyper ligation buffer. The circular adapter is opened using USER enzyme digestion from the NEBNext Multiplex Oligos for Illumina Index Primers kit (New England BioLabs Inc.). The adapter ligated DNA was enriched for methylated DNA using the EpiMark Methylated DNA Enrichment Kit (New England BioLabs Inc.). Methylation enriched DNA was amplified in an NGS PCR, which added NGS adapters and a sample index. Samples were pooled and quantified by KAPA qPCR, and then run on an Illumina NextSeq instrument. Individual methylation enriched NGS libraries were prepared for each sample using the T7 method described in the present invention. In addition, samples were also pooled after the first ligation of the T7 circular adapter and this pool of samples was enriched for methylated

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DNA and NGS libraries generated using the T7 method described in the present invention. Figure 10 A and B show that the T7 method results in a much higher enrichment of methylated DNA as well as reducing the background of non-methylated DNA (reads that do not contain a CpG). Comparison of the T7 method run on a pool of samples compared to individual samples shows that pooling the samples results in more uniform data. Figure 10C shows that tumour and matched normal samples can be separated based on the methylation patterns determined by the T7 method. Figure 10D shows that the method is picking out known tumour-specific methylation, that is not detected in the matched normal tissue sample.

Example 11 - Further comparison of methylation enriched libraries prepared by the present method and a commercially available method

Experiments were conducted to compare the methylation enriched libraries generated by the T7 method described in the present invention with a commercial UMI based NGS library preparation method. The samples used were a dilution series of sheared H446 cell line DNA diluted in sheared gDNA from healthy normal volunteer PBMCs. The dilution series was 100% cell line, 5% cell line, 2.5% cell line, 1.0% cell line, 0.1% cell line, 0.01% cell line, 0.001% cell line and 0% cell line (100% HNV PBMC). Briefly the commercial UMI based NGS library preparation method started with end repair and A tailing of the DNA, followed by ligation of the xGen Dual Index UMI Adapters from IDT (Integrated DNA Technologies) using KAPA-Hyper DNA ligase and KAPA-Hyper ligation buffer. The adapter ligated DNA was pooled and enriched for methylated DNA using the EpiMark Methylated DNA Enrichment Kit (New England BioLabs Inc.). Methylation enriched DNA was amplified in an NGS PCR. Samples were quantified by KAPA qPCR, and then run on an Illumina NextSeq instrument. Figure 11 A and B show that the T7 method results in a slightly higher enrichment of methylated DNA, but greatly reduces the background of non-methylated DNA (reads that do not contain a CpG). Figure 11C shows that in windows (of 200bp) that are methylated in the cell line but not in the HNV PBMCs, there are more reads in the T7 method at lower dilutions of the cell line, indicating that the T7 method is more sensitive than the commercial UMI adapters.

Example 12 – Fragmentation of DNA by restriction enzyme digest prior to ligation with circular adapter

Experiments were performed to assess the suitability of fragmenting DNA by restriction enzyme digest prior to ligation to the circular adapter. As outlined in the table in Figure 12A, two restriction enzymes which result in blunt end DNA fragments were tested, MLyl and BsuRI. A third restriction enzyme, NlaIII, which results in an overhang of 4 nucleotides at the 3' end of the fragment (was also tested. The agarose gel in Figure 12A shows that all three

restriction enzymes result in fragmentation of the high molecular weight gDNA. The circular adapter was ligated to the digested DNA products and aRNA products generated. The agarose gel in Figure 12B shows that DNA digested by the two blunt end restriction enzymes (BsuRI and MLyI) give the most aRNA product.

5

Example 13 - In-silico folding of the adapter.

In-silico folding of the circular adapter with 50 different barcodes was performed using Quikfold software. Table 51 below demonstrates the folding of the hairpin adapter oligonucleotide is unaffected by the sequence of the barcode.

10

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 1 (SEQ ID NO. 26)	GGCCGGTAATCACTA GCTCNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	TCACTAG CTC	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -7.47$	$\Delta H = -83.00$	$\Delta S = -257.65$	T _m = 49.0°C	YES
T7dT_U1_Lcf 2 (SEQ ID NO. 27)	GGCCGGTAAAACCAA CCAGNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AACCAAC CAG	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 3 (SEQ ID NO. 28)	GGCCGGTAAAACCAA GACNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AACCAAG ACC	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 4 (SEQ ID NO. 29)	GGCCGGTAAAACCAA TGANNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AACCAAT GGA	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 5 (SEQ ID NO. 30)	GGCCGGTAAAATCCA TCCGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AATCCAT CCG	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 6 (SEQ ID NO. 31)	GGCCGGTAAAATCCT ATTANNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AATCCTA TTA	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 7 (SEQ ID NO. 32)	GGCCGGTAAAATCG GTAGANNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AATCGGT AGA	DNA at 20°C, [Na+] = 0.01 M, [Mg++] = 0 M	$\Delta G = -7.32$	$\Delta H = -96.70$	$\Delta S = -304.90$	T _m = 44.0°C	NO

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 8 (SEQ ID NO. 33)	GGCCGGTAAAGAAC GGTCGNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AGAACGG TCG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -8.18$	$\Delta H = -134.50$	$\Delta S = -430.91$	T _m = 39.0°C	NO
T7dT_U1_Lcf 9 (SEQ ID NO. 34)	GGCCGGTAAAGAACT CAGGNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AGAACTC AGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 10 (SEQ ID NO. 35)	GGCCGGTAAAGAAG ACCGCNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AGAAGAC CGC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.33$	$\Delta H = -130.30$	$\Delta S = -419.48$	T _m = 37.5°C	NO
T7dT_U1_Lcf 11 (SEQ ID NO. 36)	GGCCGGTAAATACGA ATACNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGTC GTATTACCGGCCT	ATACGAA TAC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -8.55$	$\Delta H = -111.80$	$\Delta S = -352.21$	T _m = 44.3°C	NO
T7dT_U1_Lcf 12 (SEQ ID NO. 37)	GGCCGGTAAATACTC AACGNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGTC GTATTACCGGCCT	ATACTCA ACG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.03$	$\Delta H = -114.20$	$\Delta S = -365.58$	T _m = 39.2°C	NO
T7dT_U1_Lcf 13 (SEQ ID NO. 38)	GGCCGGTAAATACTT GGCCNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	ATACTTG GCC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 14 (SEQ ID NO. 39)	GGCCGGTAACAGGA GGTTANNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CAGGAG GTTA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 15 (SEQ ID NO. 40)	GGCCGGTAACAGGC CATCCNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CAGGCCA TCC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 16 (SEQ ID NO. 41)	GGCCGGTAACAGGC GCTGNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CAGGCG CTGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -8.10$	$\Delta H = -133.70$	$\Delta S = -428.45$	T _m = 38.9°C	NO
T7dT_U1_Lcf 17 (SEQ ID NO. 42)	GGCCGGTAACCTTAC GTTCNNNNNNNAGATCGGAAGAGCGTCGT GCCCCTATAGTGAGTC GTATTACCGGCCT	CCTTACG TTC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.86$	$\Delta H = -137.80$	$\Delta S = -443.25$	T _m = 37.7°C	NO

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 18 (SEQ ID NO. 43)	GGCCGGTAACCTTAG TTGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CCTTAGT TGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 19 (SEQ ID NO. 44)	GGCCGGTAACCTTAT ACTGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CCTTATA CTG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 20 (SEQ ID NO. 45)	GGCCGGTAACCTCCA GAGGCNNNNNNNAGA TCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CTCCAGA GGC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 21 (SEQ ID NO. 46)	GGCCGGTAACCTCCTA CCAANNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CTCCTAC CAA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 22 (SEQ ID NO. 47)	GGCCGGTAACCTCGAA TCGNNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CTCGAAT CGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -8.21$	$\Delta H = -110.30$	$\Delta S = -348.25$	T _m = 43.6°C	NO
T7dT_U1_Lcf 23 (SEQ ID NO. 48)	GGCCGGTAAGCCAAAT ACGNNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	GCCAAATA CGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES
T7dT_U1_Lcf 24 (SEQ ID NO. 49)	GGCCGGTAAGCCAG CCATGNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	GCCAGC CATG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES
T7dT_U1_Lcf 25 (SEQ ID NO. 50)	GGCCGGTAAGCCAG TTCTANNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	GCCAGTT CTA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES
T7dT_U1_Lcf 26 (SEQ ID NO. 51)	GGCCGGTAAGTATAG TTGCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	GTATAGT TGC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES
T7dT_U1_Lcf 27 (SEQ ID NO. 52)	GGCCGGTAAGTATCC TACGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	GTATCCT ACG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 28 (SEQ ID NO. 53)	GGCCGGTAAGTCAA GGTAANNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	GTCAAGG TAA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.80$	$\Delta H = -75.80$	$\Delta S = -235.37$	T _m = 48.9°C	YES
T7dT_U1_Lcf 29 (SEQ ID NO. 54)	GGCCGGTAATCTGCC ATTCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	TCTGCCA TTC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.47$	$\Delta H = -83.00$	$\Delta S = -257.65$	T _m = 49.0°C	YES
T7dT_U1_Lcf 30 (SEQ ID NO. 55)	GGCCGGTAATCTGGT ATAGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	TCTGGTA TAG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.47$	$\Delta H = -83.00$	$\Delta S = -257.65$	T _m = 49.0°C	YES
T7dT_U1_Lcf 31 (SEQ ID NO. 56)	GGCCGGTAATCTGGT CGTCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	TCTGGTC GTC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.47$	$\Delta H = -83.00$	$\Delta S = -257.65$	T _m = 49.0°C	YES
T7dT_U1_Lcf 32 (SEQ ID NO. 57)	GGCCGGTAAAATGG CAAGCNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AATGGCA AGC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 33 (SEQ ID NO. 58)	GGCCGGTAAACCAA GCCTCNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	ACCAAGC CTC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 34 (SEQ ID NO. 59)	GGCCGGTAAACCAGT AATGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	ACCAGTA ATG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 35 (SEQ ID NO. 60)	GGCCGGTAACTAATG GCTCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CTAATGG CTC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 36 (SEQ ID NO. 61)	GGCCGGTAACTATCT TATCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	CTATCTT ATC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES
T7dT_U1_Lcf 37 (SEQ ID NO. 62)	GGCCGGTAACTCAAT GGCGNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CTCAATG GCG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 38 (SEQ ID NO. 63)	GGCCGGTAAAACCA GAAGGNNNNNNAGAG TCGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AACCAGA AGG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 39 (SEQ ID NO. 64)	GGCCGGTAAAACCA GGCTANNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AACCAGG CTA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 40 (SEQ ID NO. 65)	GGCCGGTAAAACCA GTTACNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AACCAGT TAC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 41 (SEQ ID NO. 66)	GGCCGGTAAAATCTC CGCCNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AATCTCC GCC	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -7.08$	$\Delta H = -127.90$	$\Delta S = -412.14$	T _m = 37.2°C	NO
T7dT_U1_Lcf 42 (SEQ ID NO. 67)	GGCCGGTAAAATGAA TCAANNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AATGAAT CAA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.94$	$\Delta H = -102.40$	$\Delta S = -325.64$	T _m = 41.3°C	NO
T7dT_U1_Lcf 43 (SEQ ID NO. 68)	GGCCGGTAAAATGAG AGTANNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AATGAGA GTA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 44 (SEQ ID NO. 69)	GGCCGGTAAAGAAG CGATGNNNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	AGAAGCG ATG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 45 (SEQ ID NO. 70)	GGCCGGTAAAGAAGT ATGANNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AGAAGTA TGA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 46 (SEQ ID NO. 71)	GGCCGGTAAAGAATA ATTGNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	AGAATAA TTG	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 47 (SEQ ID NO. 72)	GGCCGGTAAATAGAA TTGANNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	ATAGAAT TGA	DNA at 20°C, [Na+] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES

-46-

Name	Hairpin adapter oligo	Sample Barcode	Quikfold parameters	ΔG	ΔH	ΔS	T _m (°C)	Expected folding
T7dT_U1_Lcf 48 (SEQ ID NO. 73)	GGCCGGTAAATAGAC CTTGNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	ATAGACC TTG	DNA at 20°C, [Na ⁺] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 49 (SEQ ID NO. 74)	GGCCGGTAAATAGAG AGCCNNNNNNNAGATC GGAAGAGCGTCGTG CCCCTATAGTGAGTC GTATTACCGGCCT	ATAGAGA GCC	DNA at 20°C, [Na ⁺] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.92$	$\Delta H = -76.30$	$\Delta S = -236.67$	T _m = 49.2°C	YES
T7dT_U1_Lcf 50 (SEQ ID NO. 75)	GGCCGGTAACAGGT AATAANNNNNNAGAT CGGAAGAGCGTCGT GCCCCTATAGTGAGT CGTATTACCGGCCT	CAGGTAA TAA	DNA at 20°C, [Na ⁺] = 0.01 M, [Mg ⁺⁺] = 0 M	$\Delta G = -6.77$	$\Delta H = -75.30$	$\Delta S = -233.77$	T _m = 49.0°C	YES

Table 51**Example 14 – Formation of circular adapter using various buffers.**

Experiments were conducted to assess the formation of the circular adapter in various buffers. The buffers tested were TE buffer (Invitrogen, Fisher Scientific), TNE buffer (Tris 100mM, NaCl 500mM and EDTA 10mM) and JENA buffer 5 (Jena Bioscience). The TNE buffer and JENA buffer 5 were also tested with increasing amounts of PEG present in the reaction. aRNA products formed following T7 RNA transcription were analysed by agarose gel and showed that the hairpin formation works in all buffer conditions (data not shown).

Table 52 below shows the number of copies of aRNA produced under each condition as quantified by RNase P qPCR.

Annealing Buffer	Mean copies per μ l			
	Duplicate A	Duplicate B	Mean	Fold change relative to input
TE	921.76	951.96	936.86	74.48
TNE	648.64	871.07	759.86	60.40
TNE + 5% PEG	1024.58	927.22	975.90	77.58
TNE + 10% PEG	900.02	726.39	813.21	64.65
Jena	888.76	751.95	820.36	65.21
Jena + 5% PEG	846.25	882.96	864.60	68.73
Jena + 10% PEG	1181.77	956.31	1069.04	84.98
Input	12.58			

Table 52**Example 15 – Pooling of multiple samples post ligation**

Experiments were conducted to assess the suitability of pooling multiple samples after ligation of the circular adapter each containing an individual sample barcode. In total circular adapters containing 38 different sample barcodes were ligated to cfDNA samples. These

-47-

were pooled and NGS libraries were prepared and sequenced on an Illumina MiSeq instrument. Samples were demultiplexed and the plot in Figure 14 shows the distribution of sequencing reads across the 38 samples.

5 Example 16 – Optimisation of addition of second NGS adapter to aRNA

Experiments were conducted to optimise the addition of the second NGS adapter to aRNA comparing the four approaches described in Figure 15C and 15D. For the single strand ligation approach (6a(i)), a 5'-phosphorylated NGS adapter was ligated to aRNA using T4 RNA ligase (New England BioLabs Inc.). This was compared to a second T4 RNA ligase, mutant T4 RNA Ligase 2, truncated KQ (New England BioLabs Inc.), that was used to ligate a 5'-adenylated NGS adapter to aRNA. Also compared was the necessity for a bead-based clean up step after the ligation step, before converting to cDNA by RT using a primer complementary to the ligated NGS adapter (U2P_RT_1). A splint double strand ligation (Figure 15C part 6a(ii)) was tested with and without the presence of 12.5% PEG-8000. The RT with random primer method (Figure 1 step 6b and Figure 15D step 6b) was tested with (Figure 15D step 6b(ii)) and without (Figure 15D step 6b(i)) the presence of double stranded primer to guide annealing to the end of the aRNA molecule. Finally, all cDNA products from each method were amplified in an index PCR, which added the final NGS adapter sequences and a library index. The agarose gel in Figure 15A shows that the best conditions were the use of the mutant T4 RNA Ligase 2, truncated KQ (New England BioLabs Inc.) with a post-ligation clean up step.

Experiments were conducted to compare methods for the addition of the second NGS adapter (Figure 1 step 6). Single strand ligation using mutant T4 RNA Ligase 2, truncated KQ (New England BioLabs Inc.) to ligate a 5'-adenylated NGS adapter was performed with and without phosphatase treatment of the aRNA prior to ligation. The RT with random primer method (Figure 1 step 6b) was tested with two different concentrations of random primer, 100 μ M and 50 μ M. Single strand ligation with phosphatase treatment of the aRNA are the best conditions as shown in the agarose gel on Figure 15A.

The protocol for optimised single strand ligation of aRNA is as follows;

aRNA was generated as described in steps 1-6 of Example 8.

Samples were prepared as shown below in Table 53

Tube Number	Sample details	Sample ID	Ligase	NEB index
1	AC-T7-003 aRNA	AC-T7-10-1a	mut T4 RNA ligase 2	1
2	AC-T7-003 aRNA	AC-T7-10-1b	mut T4 RNA ligase 2	2

Table 53

Step 7 – dephosphorylation of aRNA

8µL of AC-T7-003_aRNA_ was combined with 1µL of AP fast phosphatase (1U/µL) and placed on the PCR block using the conditions detailed in Table 54 below;

Template Name	Temp (°C)	Time (min)	Cycles
	37 °C	30	1
	80 °C	5	1
	4 °C	Hold	1

Table 54

Step 8 – ssligation

A 1µM solution of the adapter oligonucleotide 5'Adenyl-U2-C3sp (AGATCGGAAGAGCACACGTC, 5' modification = adenylation, 3' modification = C3 spacer) was prepared. The following ligation master mix was prepared according to the conditions outlined in Table 55 below;

Component	Volume (µL)	Volume for mastermix (µL)	Final concentration
10X ligation buffer	2	10	1X
50 % PEG 8000	5	25	12.50%
Water	2	10	1X
mut T4 RNA ligase 2	1	5	1X

Table 55

1µL of adapter was added to each sample followed by 10µL of ligation mastermix. Ligation was then performed at 16°C overnight.

Clean-up of the ligation products was performed by Ampure bead clean up according to the conditions in Table 56. Products were eluted in a low EDTA TE buffer

Sample Volume (µL)	Bead ratio	Bead Vol (µL)	Elution vol (µL)	Ethanol
20	1	20	22.5	80%

Table 56

Step 9 – preparation of RT oligo

The components were combined as outlined in Table 57.

Component	Concentration	Volume (µL)
RT_U2P_primer	100 µM	1.0
adapter-ligated aRNA	From step 8	20.0

Table 57

The components outlined in Table 57 were denatured and annealed using the conditions outlined in Table 58 below.

Temp °C	Time (min)	Cycles
72	3	1
4	∞	1

Table 58

5

The RT reaction master mix was prepared as outlined in Table 59.

Component	Concentration	Volume (30 μ L reaction)	Final conc.	Volume for MM (μ L)
dNTPs	10mM	1.0	0.33mM	5
buffer	5X	6.0	1X	30
DTT	0.1M	1.0	3.3mM	5
Superscript IV	200U/ μ L	1.0	200U	
H2O		0.0		0

Table 59

- 10 8 μ L of RT reaction master mix was added to the aRNA/U2P_primer mix, followed by 1 μ L of Superscript IV. Samples were placed in the PCR block and the conditions outlined in Table 60 were applied

Temp °C	Time (min)	Cycles
50	10	1
80	10	1
4	∞	1

Table 60

15

The RT reaction products were cleaned up using Ampure bead clean up using the conditions outlined in Table 61. Products were eluted in low EDTA TE buffer.

Sample Volume (μ L)	Bead ratio	Bead Vol (μ L)	Elution vol (μ L)
30	1.5	45	32.5

Table 61

20

Step 10 – Indexing PCR

The indexing PCR master mix was prepared using the conditions outlined in Table 62.

Reagent	Vol (μ L) X1	Vol (μ L)
Kapa Polymerase*	1	5
Kapa Buffer*	10	50
dNTPs*	1.5	7.5
NEB Universal**	2.5	12.5
NEB Index**	2.5	
Water	2.5	12.5
*From Kapa HiFi Hot Start PCR Kit with dNTPs		
**From NEBNext Multiplex Oligos for Illumina index primers		

Table 62

17.5μL of the master mix was added to 30μL of the purified RT reactions. 2.5μL of the indexing oligo was added and the sample placed in the PCR block. The conditions outlined in Table 63 were applied.

Temperature	Time	Cycles
98°C	30 Seconds	1
98°C	10 Seconds	7 cycles
65°C	30 Seconds	
72°C	30 Seconds	
72°C	5 Minutes	1
4°C	∞	

Table 63

Samples were then clean-up using Ampure bead clean up and products eluted in low EDTA TE buffer. Samples were stored before quantification and NGS.

Example 17 – Amplification performed with various amounts of cfDNA.

Experiments were conducted to assess the efficiency of the method on a range of cfDNA input amounts from 2 ng to 25 ng. Figures 16A-C show that aRNA is produced at all inputs of cfDNA. The fold change of aRNA relative to the input DNA shows that the method is as efficient even to low levels of cfDNA.

The foregoing embodiments are not intended to limit the scope of the protection afforded by the claims, but rather to describe examples of how the invention may be put into practice.

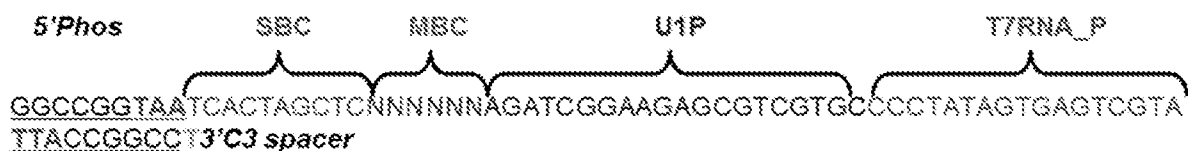
-51-

Sequences

The following sequences were used in the experiments undertaken by the present inventors:

Key

T7RNA_P	T7 RNA Polymerase promoter sequence
MBC	Molecular Bar Code
SBC	Sample Bar Code
U1P	Partial Unique NGS Sequence 1
U2	Unique NGS Sequence 2

T7dT U1 Lcf1 (SEQ ID NO.3)**T7dT U1 Lcf1 Reverse (SEQ ID NO.4)****U2P Lig1 (SEQ ID NO.5)**

modified with a phosphate group at 5' and a C3 spacer (blocker) at the 3'

U2P RT 1 (SEQ ID NO.6)

5' U2 3'
 GACGTGTGCTCTTCCGATCT

U2P RT 1 Reversed (SEQ ID NO.7)

TCTAGCCTTCTCGTGTGCAG
 3' U2 5'

T7 U1 Hsp92II02c3 (SEQ ID NO.8)

- Designed anneal to Hsp92II digested DNA
- Contains 2 of the 4 bases in the overhang – prevents primer dimer
- Can order without 3'C3 spacer

5'Phos SBC MBC U1P T7RNA_P

AGGCCGGTAATCACTAGCTCNNNNNNAGATCGGAAGAGCGTCGTGCCCTATAGTGAGTCGTA
 TTACCGGCCTCA 3'C3 spacer

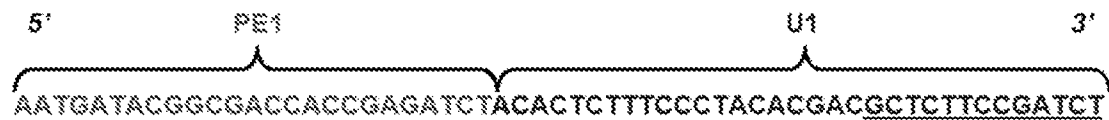
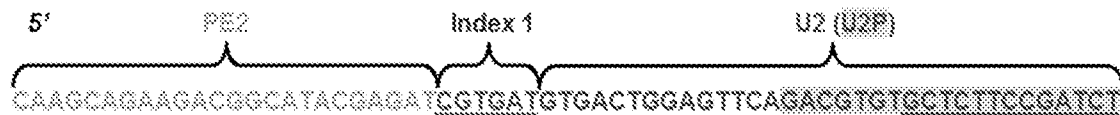
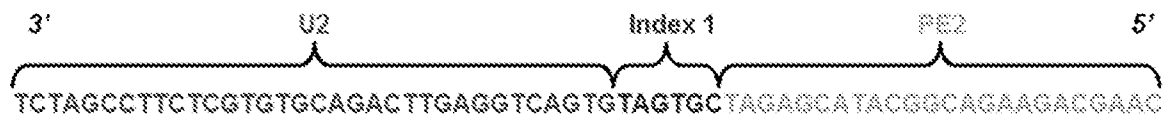
T7 U1 Hsp92II03c3 (SEQ ID NO.9)

- Designed anneal to Hsp92II digested DNA
- Contains 3 of the 4 bases in the overhang – prevents primer dimer
- Can order without 3'C3 spacer

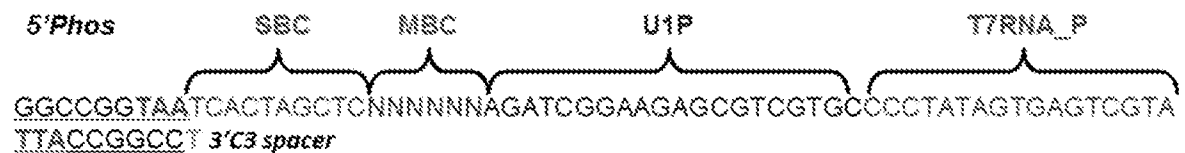
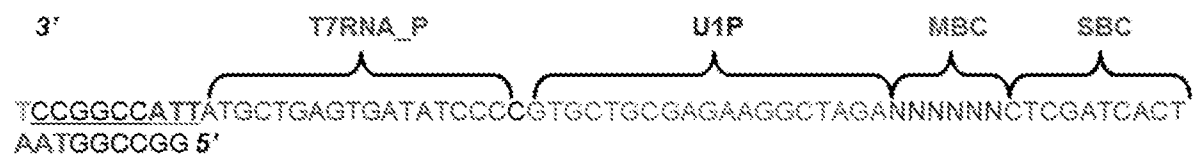
5'Phos SBC MBC U1P T7RNA_P

AGGCCGGTAATCACTAGCTCNNNNNNAGATCGGAAGAGCGTCGTGCCCTATAGTGAGTCGTA
 TTACCGGCCTCAT 3'C3 spacer

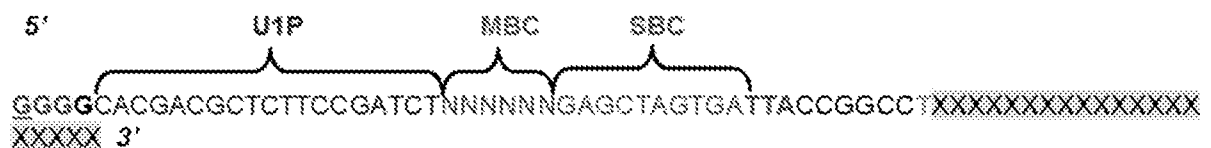
-53-

Illumina Universal (SEQ ID NO.10)Illumina Index (1) (SEQ ID NO.11)REVERSE OF Illumina Index (1) (SEQ ID NO.12)

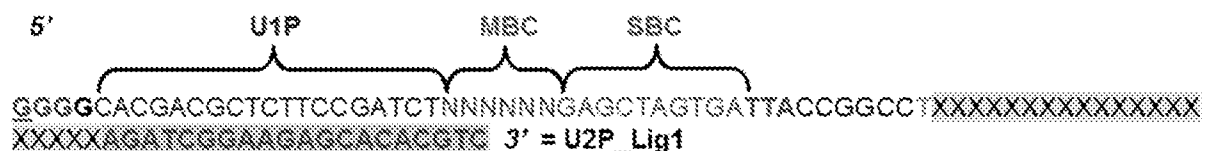
Tailed - cfDNA T7 Primers and Process

T7dT U1 Lcf1 (SEQ ID NO.13)T7dT U1 Lcf1 Reverse (SEQ ID NO.14)

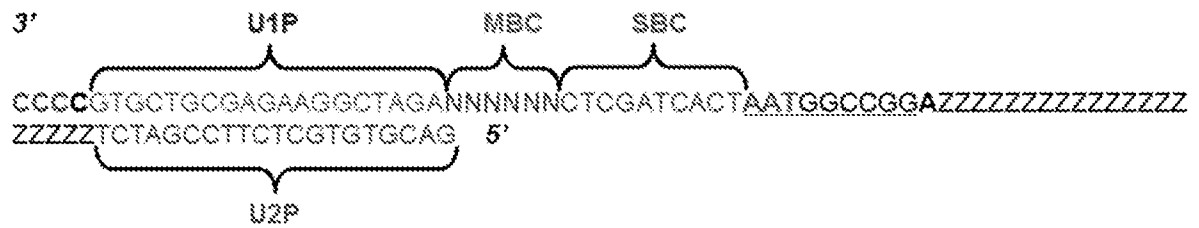
-54-

T7dT U1 Lcf1 cfDNA Ligation Product (SEQ ID NO.15)T7U1 Prim1 (SEQ ID NO.16)T7dT U1 Lcf1 cfDNA Predicted aRNA (SEQ ID NO.17)aRNA Ligation Product (SEQ ID NO.18)

Using U2P_Lig1

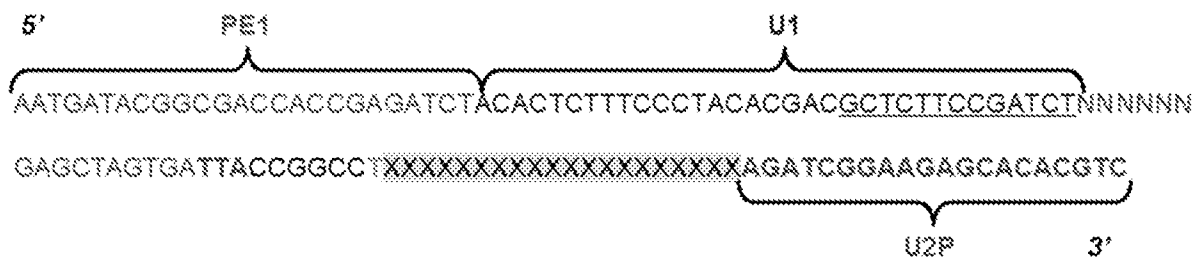
Ligated aRNA 1st RT Product (SEQ ID NO.19)

Using U2P_RT_1



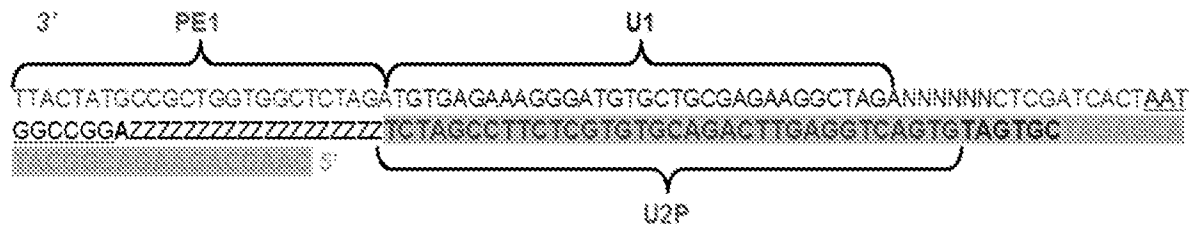
NGS PCR 1st Cycle on RT Product (SEQ ID NO.20)

Using Illumina Universal



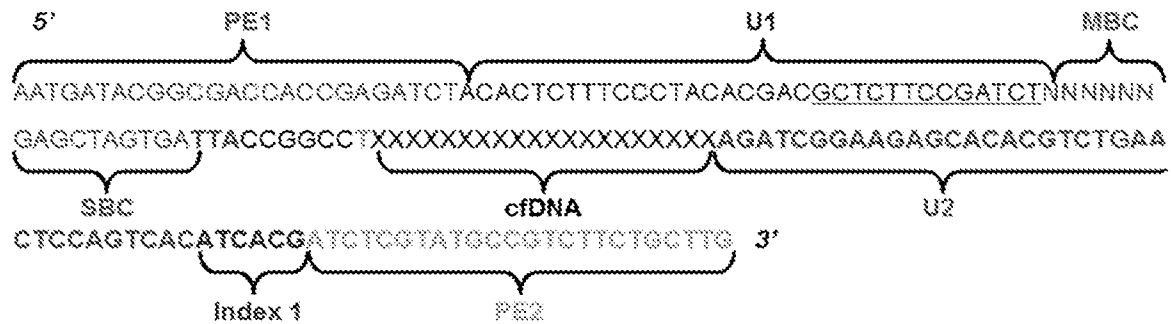
NGS PCR 2nd Cycle on 1st Cycle Product

Using Illumina Index (SEQ ID NO.21)



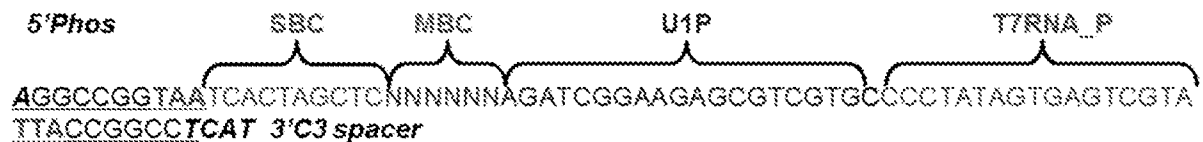
Reverse (SEQ ID NO.22)

-56-

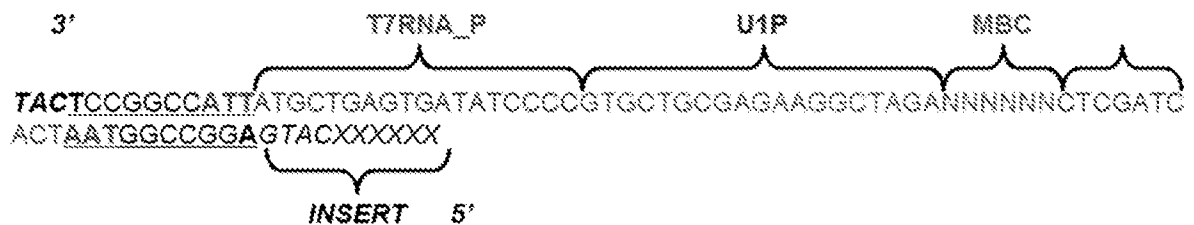


T7 U1 Hsp92II03c3 (SEQ ID NO.23)

- Designed anneal to Hsp92II digested DNA
- Contains 3 of the 4 bases in the overhang – prevents primer dimer
- Can order without 3'C3 spacer



T7 U1 Hsp92II3c3 Ligation Product Linearised (SEQ ID NO.24)



T7 U1 Hsp92II3c3 Ligation Product Predicted aRNA (SEQ ID NO.25)



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Claims

1. A method for amplifying template DNA, the method comprising:
 - i) providing template DNA and an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of the template DNA;
 - ii) ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA to produce an adapter oligonucleotide-template DNA molecule;
 - iii) providing a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide;
 - iv) annealing the secondary oligonucleotide to the adapter oligonucleotide-template DNA molecule; and
 - v) transcribing the template DNA by introducing an RNA polymerase to the adapter oligonucleotide-template DNA molecule, to generate an aRNA product.
2. The method according to claim 1 wherein the method further comprises the steps of:
 - vi) converting the aRNA product to cDNA; and
 - vii) amplifying the cDNA.
3. The method according to either claim 1 or claim 2, wherein step vi) first comprises ligating a priming site to the aRNA.
4. The method according to either claim 1 or claim 2, wherein step vi) comprises utilising reverse transcriptase and random oligonucleotide primers.
5. The method according to either claim 1 or claim 2, wherein step vi) comprises utilising reverse transcriptase and target specific oligonucleotide primers.

6. The method according to any one of claims 2-5, wherein step vii) comprises amplification in a next generation sequencing (NGS) protocol.
7. The method according to any preceding claim wherein the RNA polymerase promoter sequence comprises a T7 RNA polymerase promoter sequence.
8. The method according to any preceding claim, wherein the RNA promoter sequence in the adapter oligonucleotide comprises SEQ ID NO:1 or a fragment or variant thereof.
9. The method according to any preceding claim wherein the RNA promoter sequence in the secondary oligonucleotide comprises SEQ ID NO:2 or a fragment or variant thereof.
10. The method according to any preceding claim wherein the method further comprises dephosphorylating the template DNA prior to the ligation of step ii).
11. The method according to claim 10, wherein a 5' phosphate of the template DNA is removed by treatment with a phosphatase enzyme.
12. The method according to claims 1-9 wherein the method further comprises a restriction enzyme digestion of the template DNA prior to the ligation of step ii).
13. The method according to any preceding claim wherein step ii) comprises ligating the 5' end of the adapter oligonucleotide to the 3' end of the template DNA using a DNA ligase.
14. The method according to claim 13 wherein the DNA ligase comprises KAPA-hyper DNA ligase.
15. The method according to any preceding claim, wherein the adapter oligonucleotide comprises complementary regions which are capable of annealing to one another to form circular or hairpin loop structure.

-60-

16. The method according to claim 15, wherein prior to step iv), the method further comprises the step of denaturing the adapter oligonucleotide prior to annealing the secondary oligonucleotide to the adapter oligonucleotide.
17. The method according to any preceding claim, wherein the adapter oligonucleotide comprises a molecular bar code sequence.
18. The method according to any preceding claim, wherein the adapter oligonucleotide comprises a sample bar code.
19. The method according to any preceding claim, wherein the adapter oligonucleotide comprises a NGS sequence.
20. The method according to any preceding claim, wherein the adapter oligonucleotide comprises a T tail.
21. The method according to any one of claims 12 to 20, wherein the adapter oligonucleotide further comprises a single region which is complementary to the stick ends produced by the restriction enzyme digestion.
22. The method according to any one of claims 12 to 20, wherein the adapter oligonucleotide further comprise a single region which is partially complementary to the stick ends produced by the restriction enzyme digestion.
23. The method according to any preceding claim, wherein the template DNA is derived from a biological sample, optionally blood.
24. The method according to any preceding claim, wherein the template DNA comprises cfDNA and/or ctDNA.
25. The method according to any one of claims 1 to 19, wherein the template DNA comprises gDNA.
26. The method according to any one of claims 1 to 19, wherein the template DNA comprises single cell DNA.

-61-

27. An adapter oligonucleotide for producing an aRNA product from a DNA template, the adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to the 3' end of a template DNA.
28. The adapter according to claim 27, wherein the RNA polymerase promoter sequence comprises a T7 RNA polymerase promoter sequence.
29. The adapter according to either claim 27 or 28, wherein the RNA promoter sequence in the adapter oligonucleotide comprises SEQ ID NO:1 or a fragment or variant thereof
30. The adapter according to any of claims 27-29, wherein the adapter oligonucleotide comprises complementary regions which are capable of annealing to one another to form circular or hairpin loop structure.
31. The adapter according to any one of claims 27-30, wherein the adapter oligonucleotide further comprises a molecular bar code sequence.
32. The adapter according to any one of claims 27-31, wherein the adapter further comprises a sample bar code.
33. The adapter according to any one of claims 27-32, wherein the adapter oligonucleotide further comprises a NGS sequence.
34. The adapter according to any one of claims 27-33, wherein the adapter oligonucleotide further comprises a T tail.
35. The adapter according to any one of claims 30-34, wherein the adapter further comprises an overhang comprising 6 to 12 oligonucleotides.
36. A kit for producing an aRNA product from a DNA template, the kit comprising:
 - i) an adapter oligonucleotide comprising an RNA polymerase promoter sequence, wherein the adapter oligonucleotide is configured so that a 5' end of the adapter oligonucleotide can be ligated to a 3' end of a template DNA; and

-62-

- ii) a secondary oligonucleotide comprising an RNA polymerase promoter sequence, wherein the sequence of the secondary oligonucleotide is complementary to the sequence of the adapter oligonucleotide.
- 37. The kit according to claim 36 wherein the RNA polymerase promoter sequence comprises a T7 RNA polymerase promoter sequence.
 - 38. The kit according to either claim 36 or 37, wherein the RNA promoter sequence in the adapter oligonucleotide comprises SEQ ID NO.1 or a fragment or variant thereof.
 - 39. The kit according to any one of claims 36-38 wherein the RNA promoter sequence in the secondary oligonucleotide comprises SEQ ID NO.2 or a fragment or variant thereof.
 - 40. The kit according to any one of claims 36-39, wherein the adapter oligonucleotide comprises complementary regions which are capable of annealing to one another to form circular or hairpin loop structure.
 - 41. The kit according to any one of claims 36-40, wherein the adapter oligonucleotide further comprises a molecular bar code sequence.
 - 42. The kit according to any one of claims 36-41, wherein the adapter further comprises a sample bar code.
 - 43. The kit according to any one of claims 36-42, wherein the adapter oligonucleotide further comprises a NGS sequence.
 - 44. The kit according to any one of claims 36-43, wherein the adapter oligonucleotide further comprises a T tail.
 - 45. A method for detecting a genetic variant or genetic signature in template DNA, comprising amplifying template DNA in the method according to any one of claims 1-23 and analysing the amplified cDNA for the genetic variation or signature.

-63-

46. The method according to claim 45 wherein the genetic variation or signature comprises one or more of the following: DNA methylation, tumour-associated mutations or sequence copy numbers.
47. Use of a phosphatase enzyme for dephosphorylating template DNA prior to amplification, or modification, prior to amplification.
48. Use according to claim 47, wherein the phosphatase enzyme is for removing the 5' phosphate of the template DNA.
49. Use according to either claim 47 or 48, wherein phosphatase enzyme is selected from one or more of the following: Shrimp Alkaline Phosphatase; Antarctic Phosphatase; Calf Intestinal Alkaline Phosphatase; and/or or FastAP Thermosensitive Alkaline Phosphatase.

Figure 1

1. Prepare Target DNA = A Tailed cfDNA

5'XXXXXXXXXXXXXXXXXXXXA3'

3'AAAAAAAAAAAAAAAAAAAAZZZ5'

T7RNA_P

U1P

MBC

SBC

U2

T7 RNA Polymerase promoter sequence

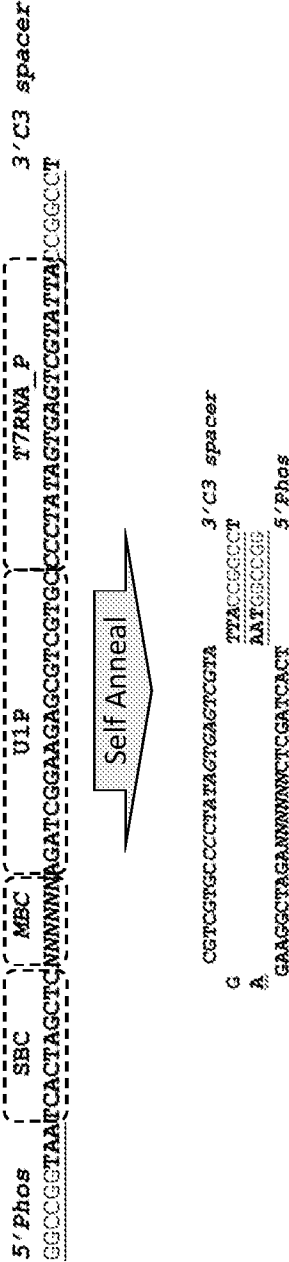
Partial Unique NGS Sequence 1

Molecular Bar Code

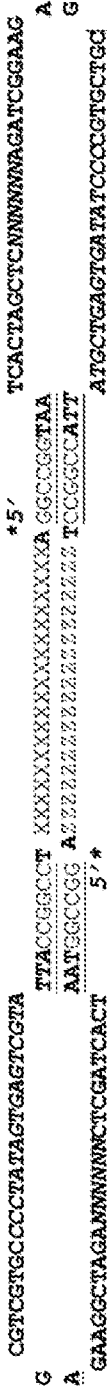
Sample Bar Code

Unique NGS Sequence 2

2. Anneal Oligo to form Circular Adapter



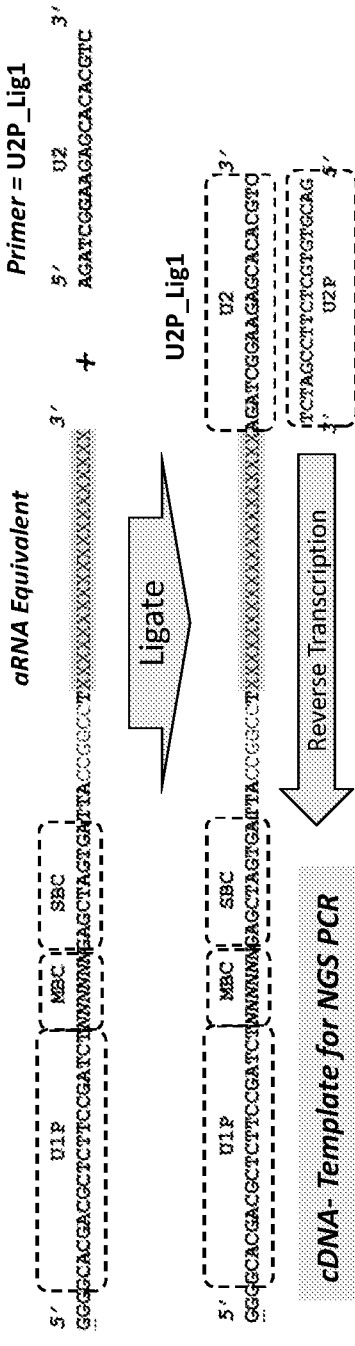
3. Ligate Circular Adapter to Target DNA



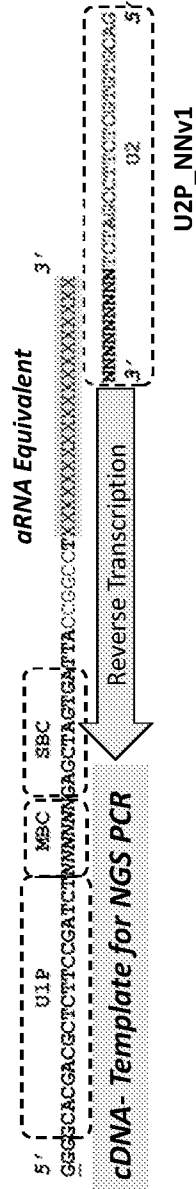
* Ligation

Figure 1 cont.

6a. Convert aRNA to cDNA via Ligation to aRNA



6b. Convert aRNA to cDNA using Random NGS primer in RT Reaction



6c. Convert aRNA to cDNA using Target Specific NGS primer

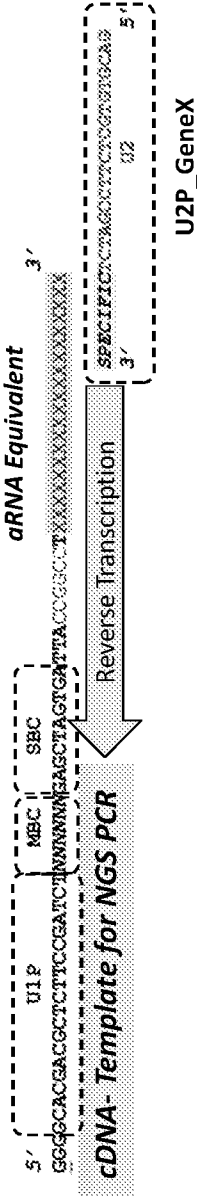


Figure 2

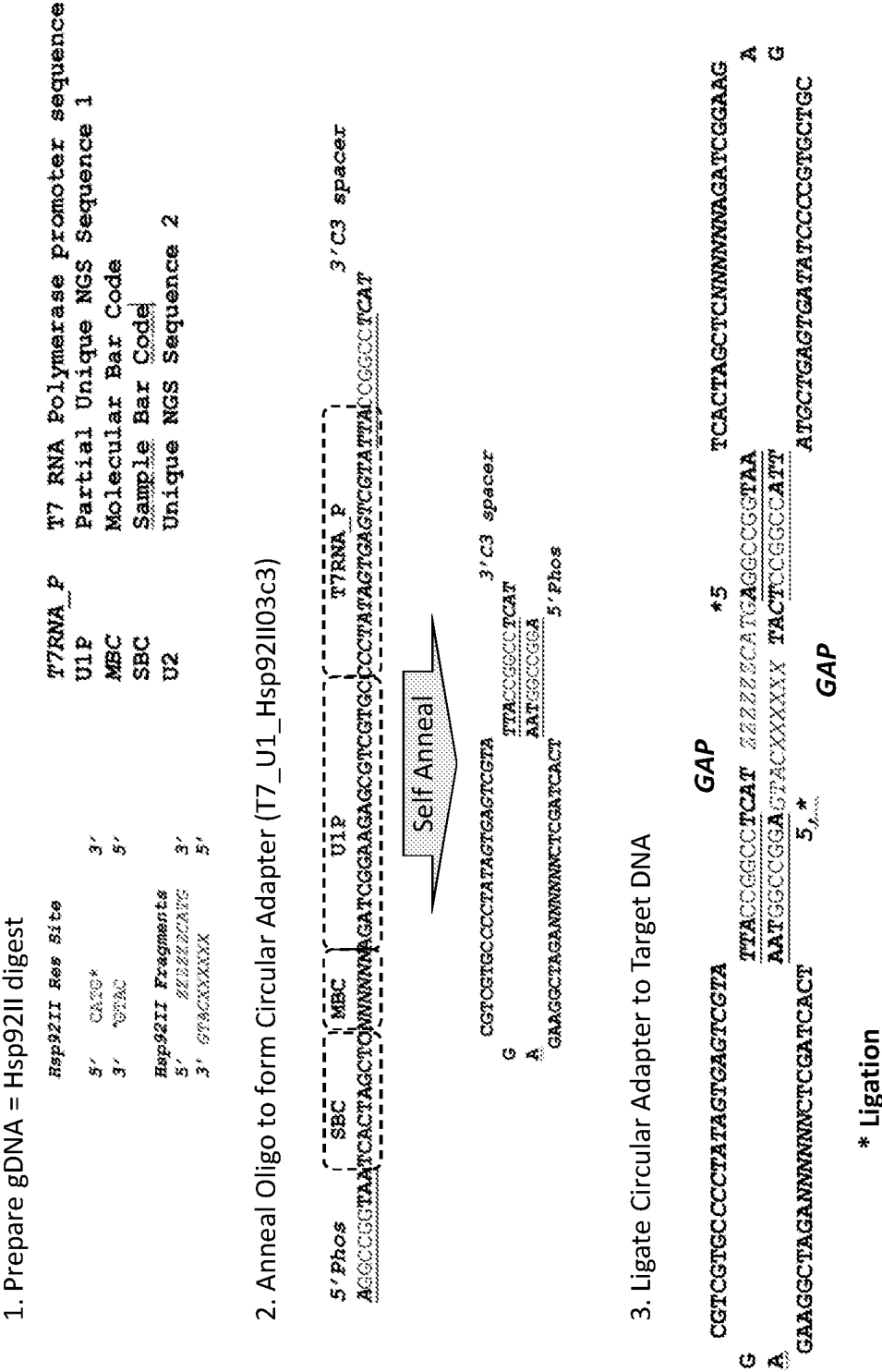
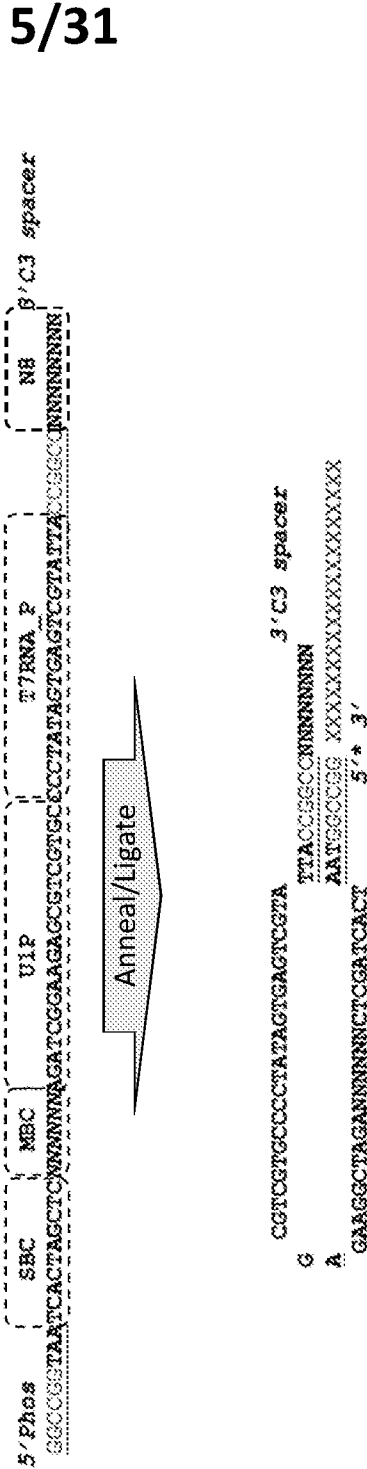


Figure 3

1. Denature input DNA to produce ssDNA

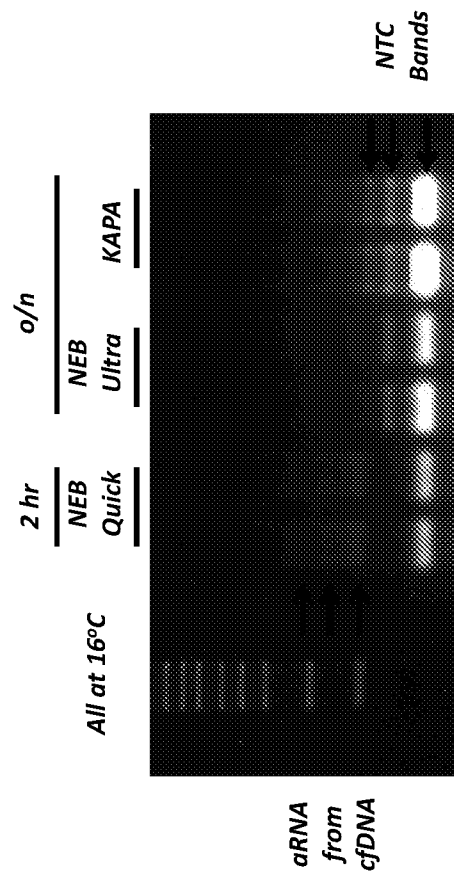


2. Anneal Oligo to form Circular Adapter and anneal/ligate to ssDNA (T7dT_U1_N8)



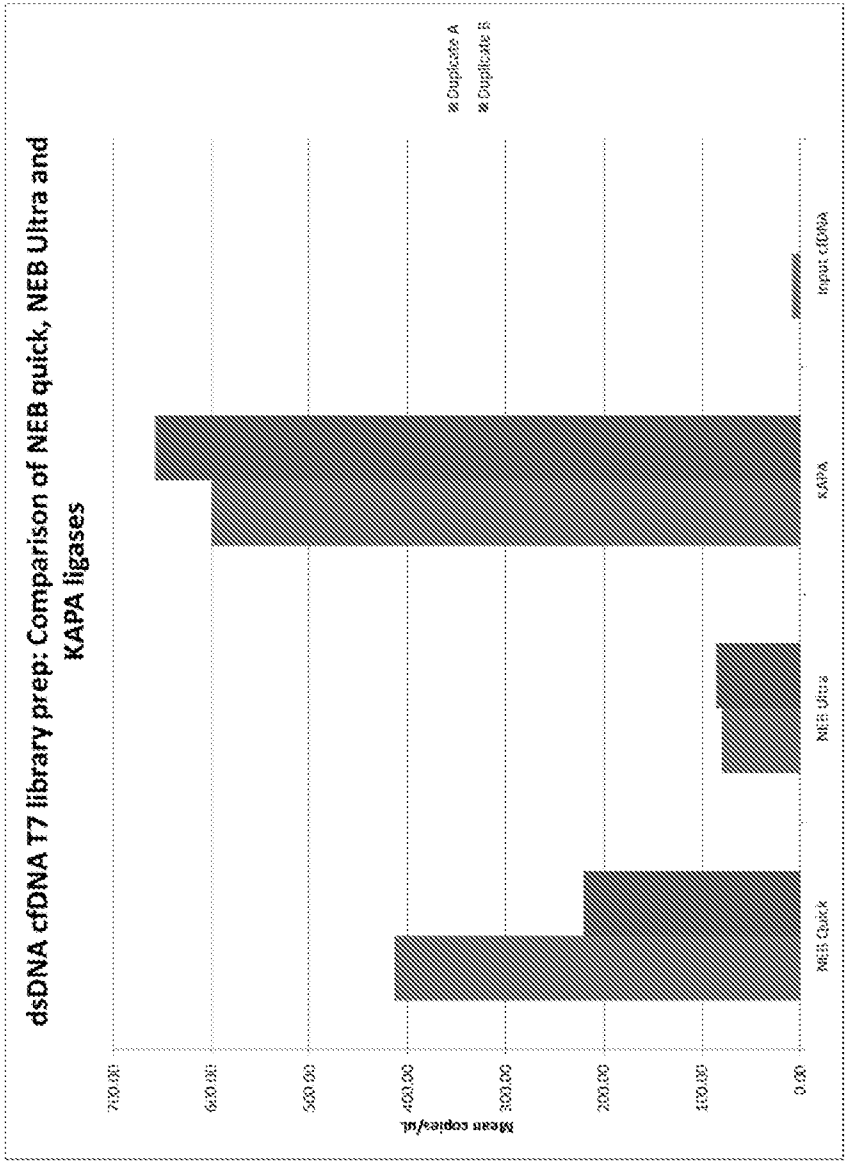
* Ligation

Figure 4A



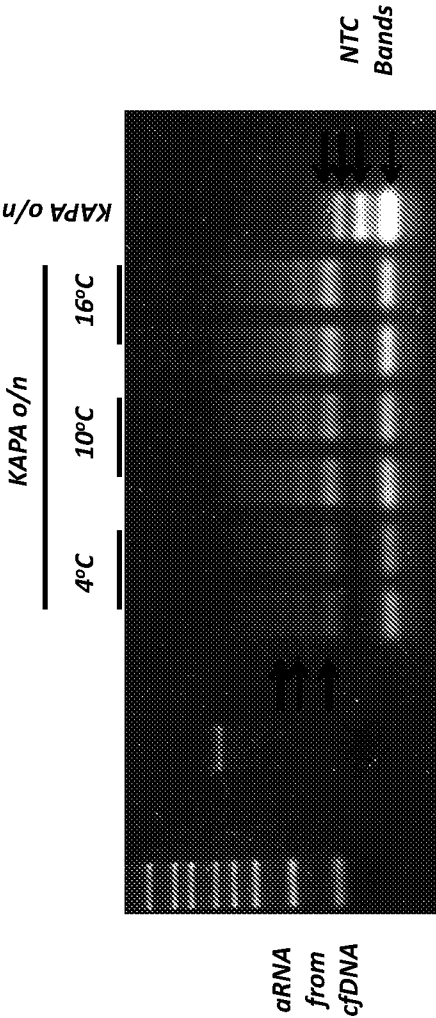
7/31

Figure 4B



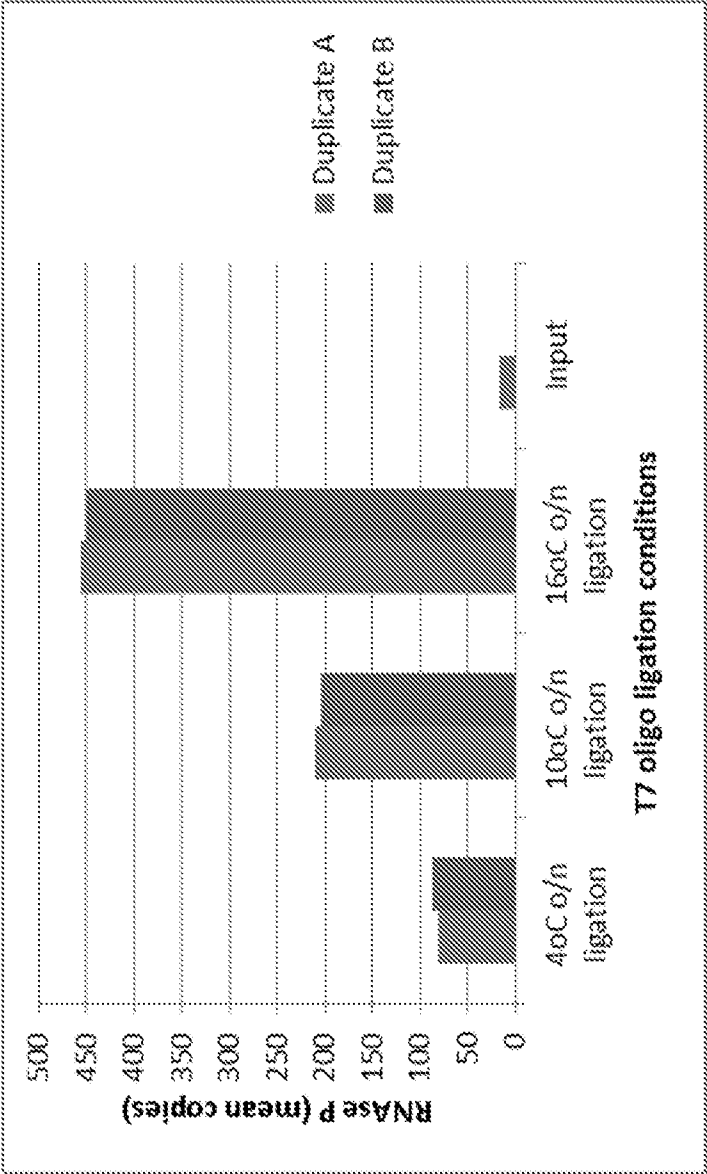
8/31

Figure 5A



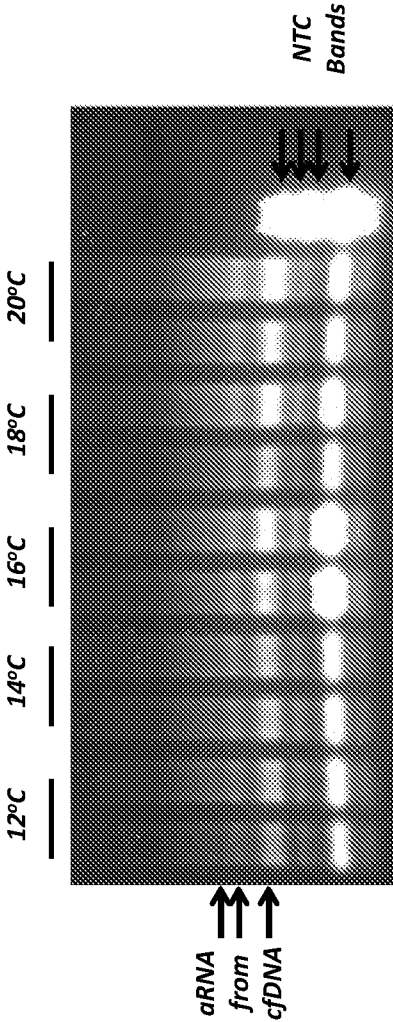
9/31

Figure 5B



10/31

Figure 6A



11/31

Figure 6B

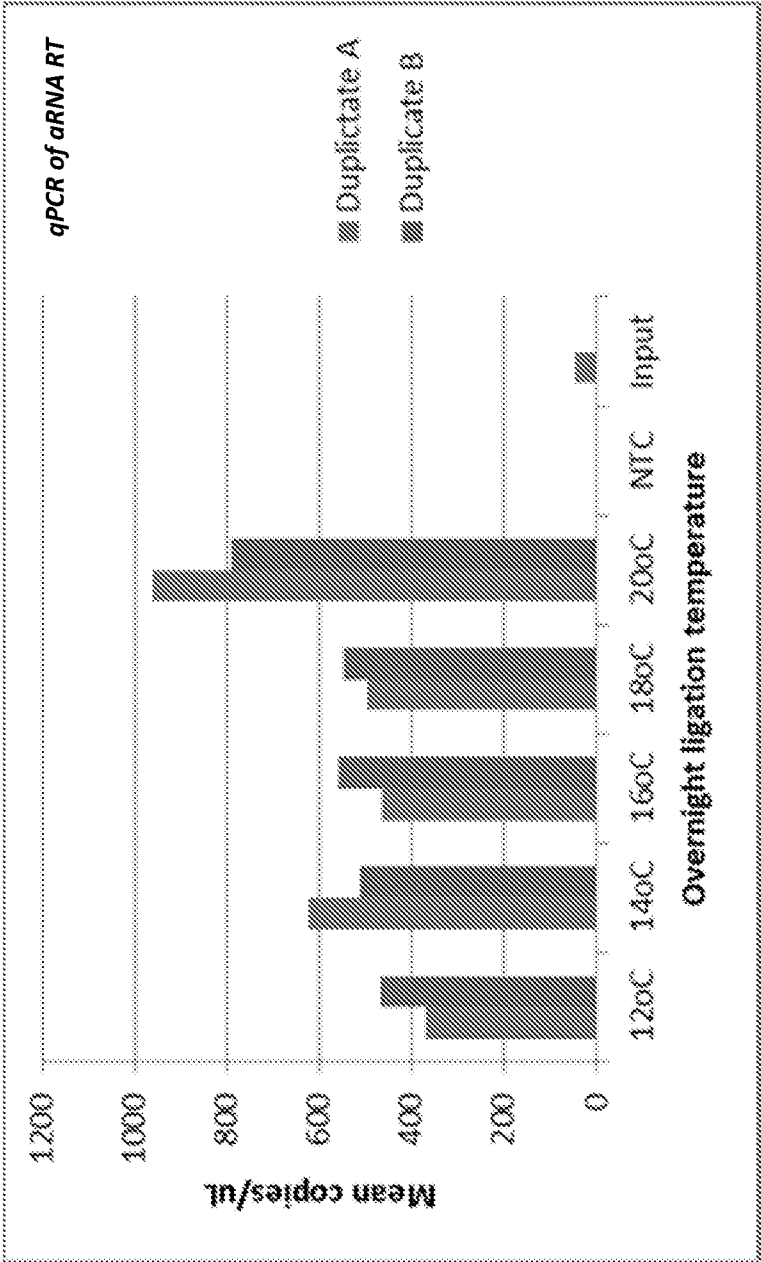


Figure 7A

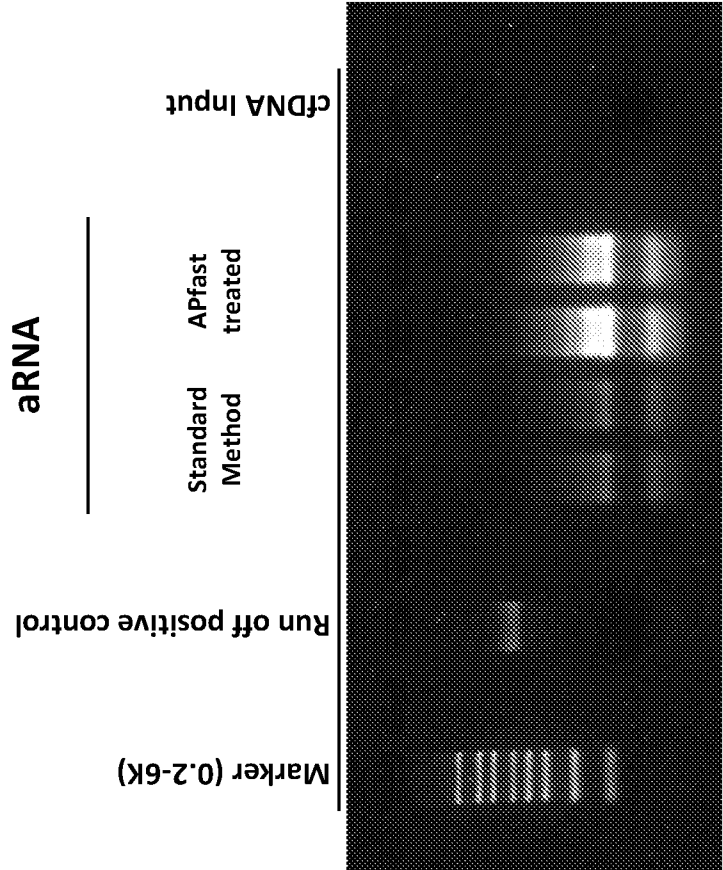
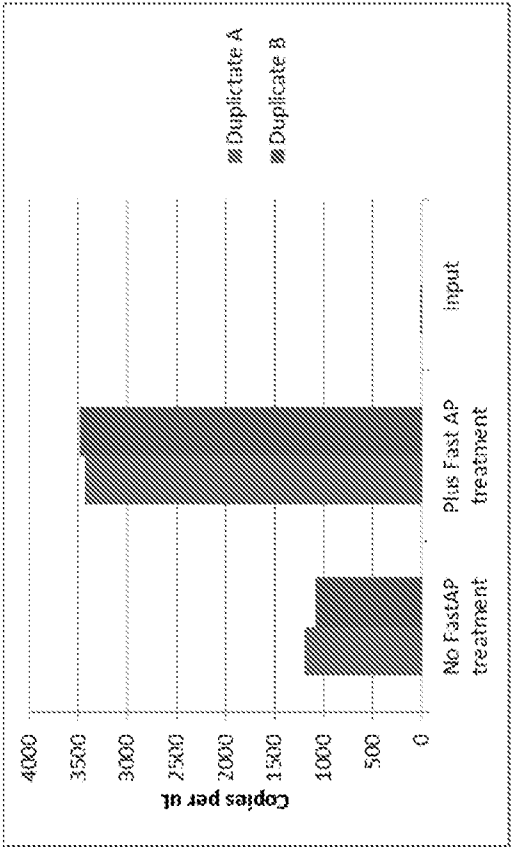


Figure 7B



	Mean copies per µl		Mean	Fold change relative to input
	Duplicate A	Duplicate B		
No Fast AP treatment	1194.28	1079.18	1136.73	119.03
Plus Fast AP treatment	3425.79	3489.07	3457.43	362.03
Input	9.55			

Figure 8A

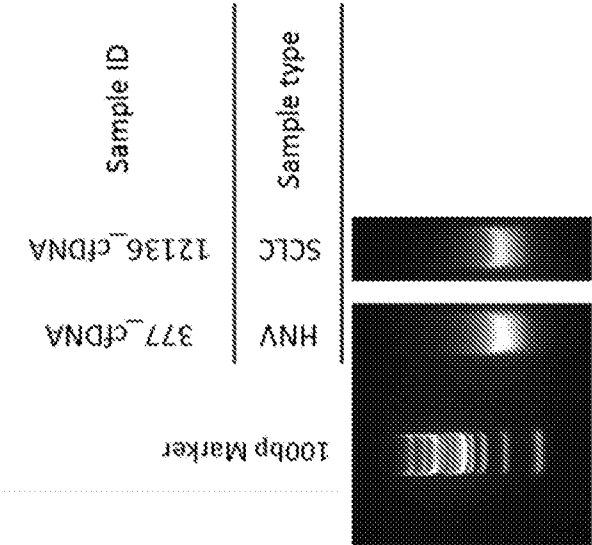


Figure 8B

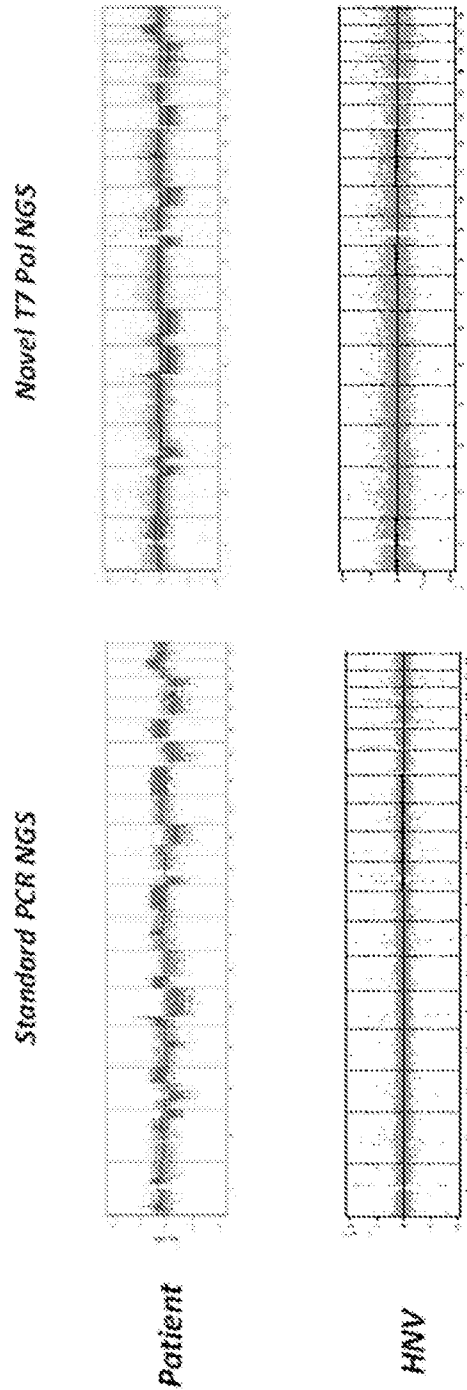


Figure 9A

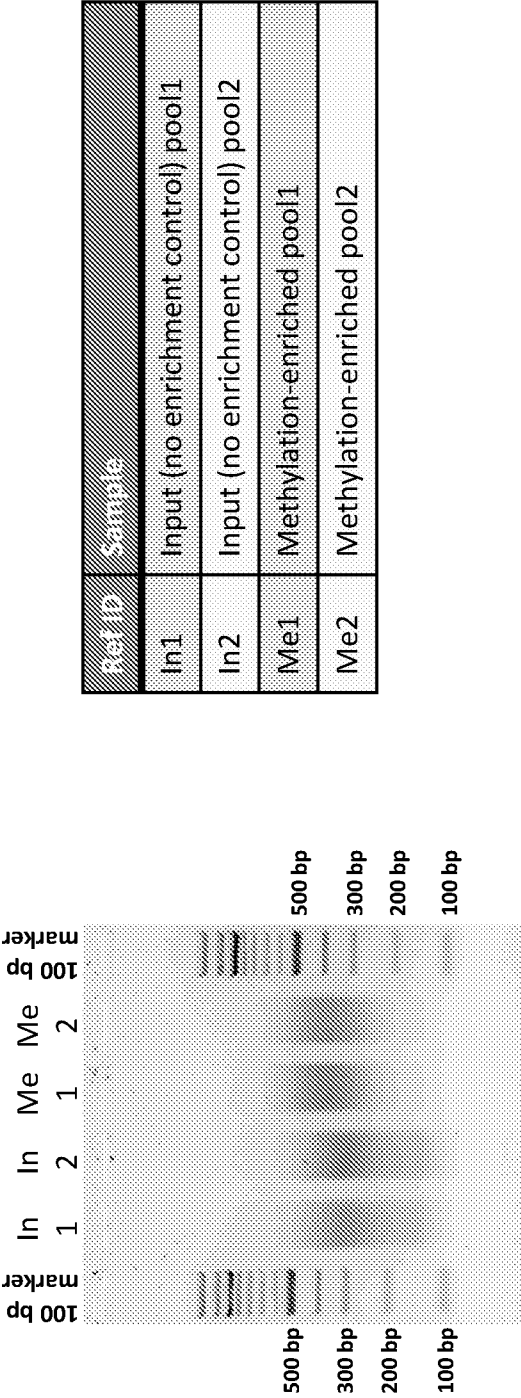


Figure 9B

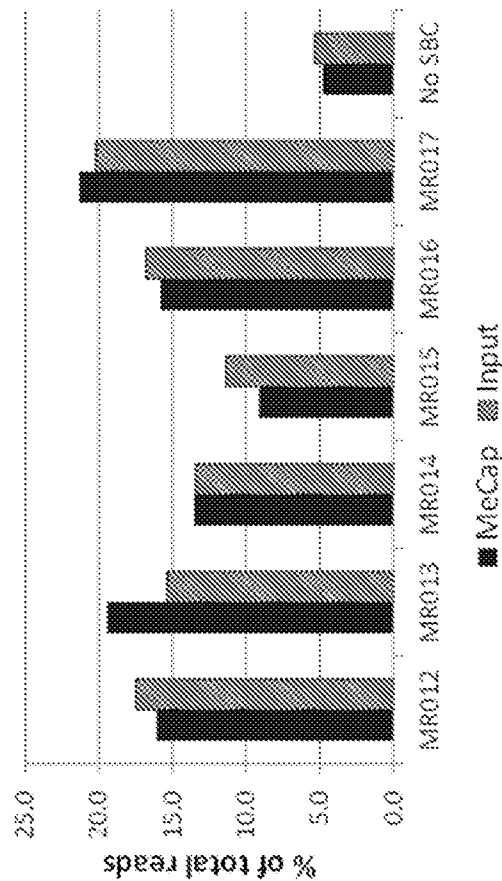


Figure 10

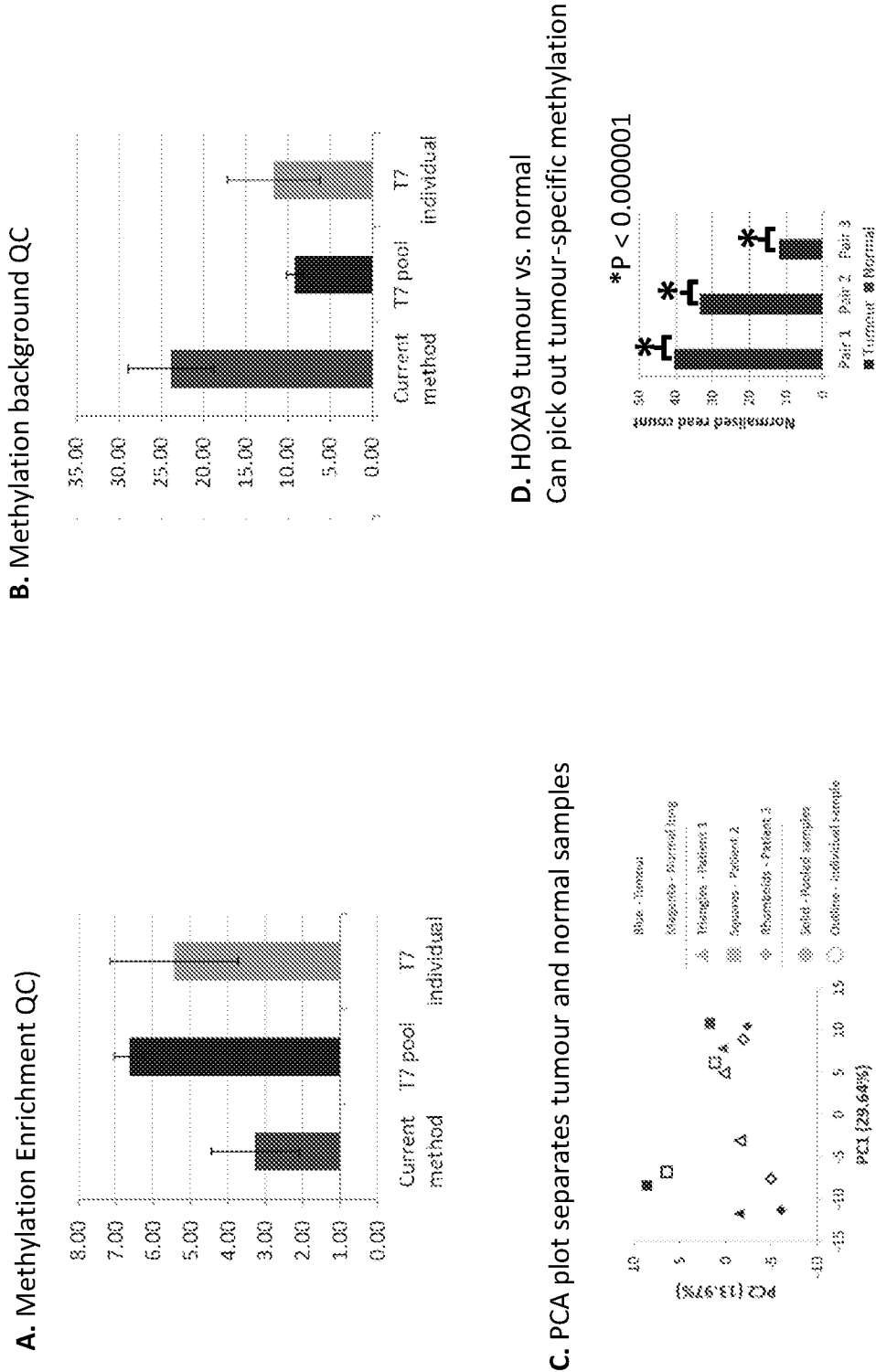
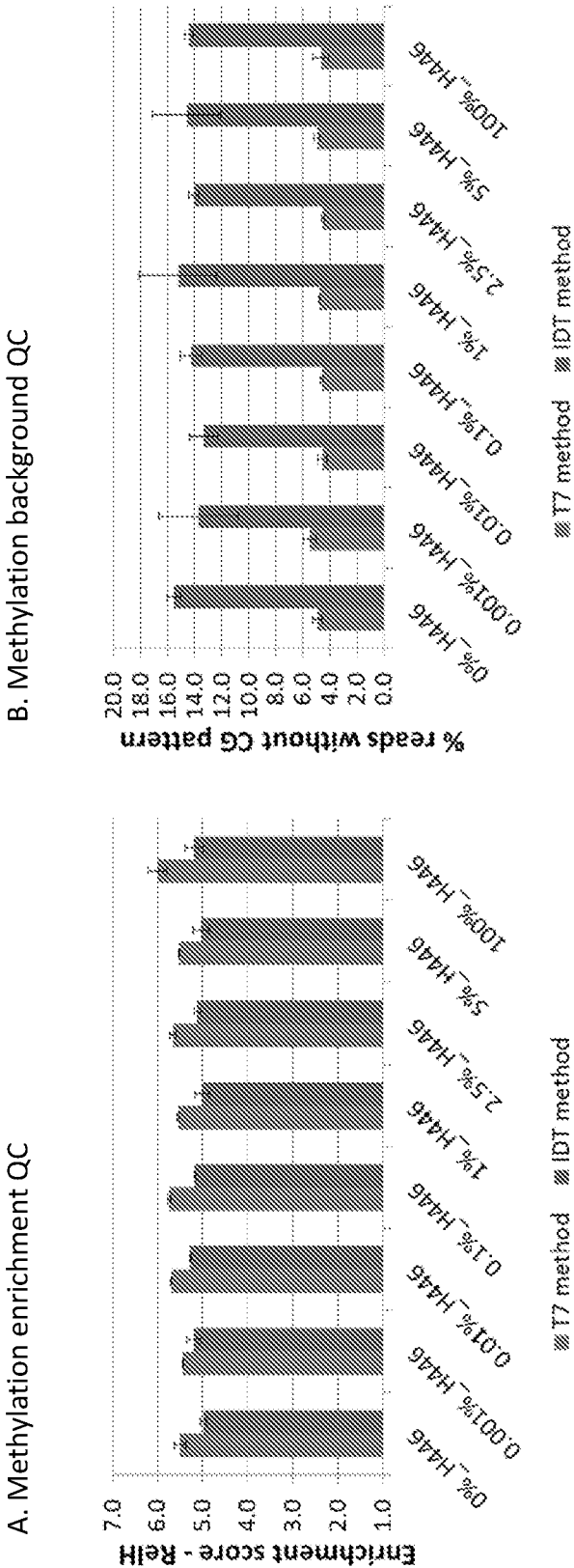


Figure 11A and B



20/31

Figure 11C

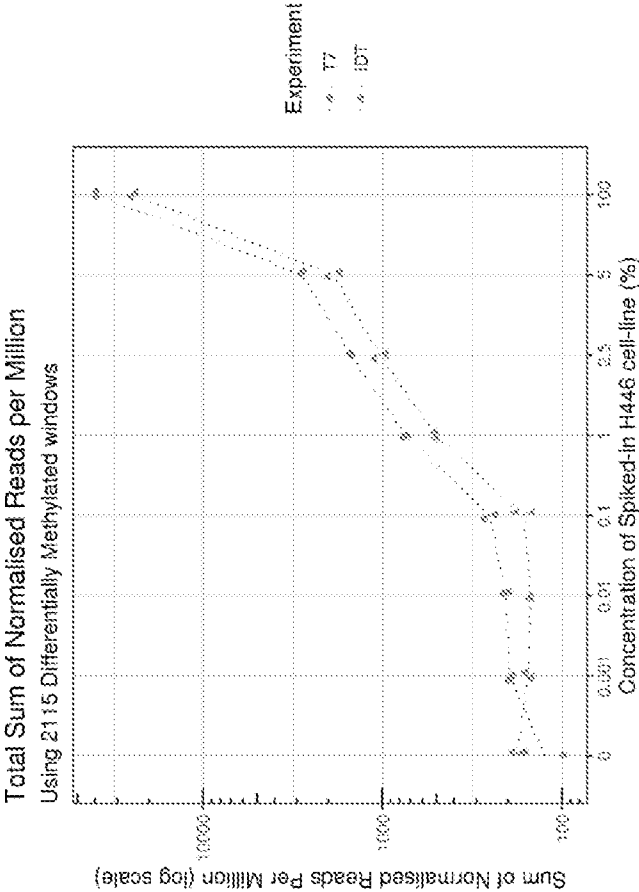


Figure 12A

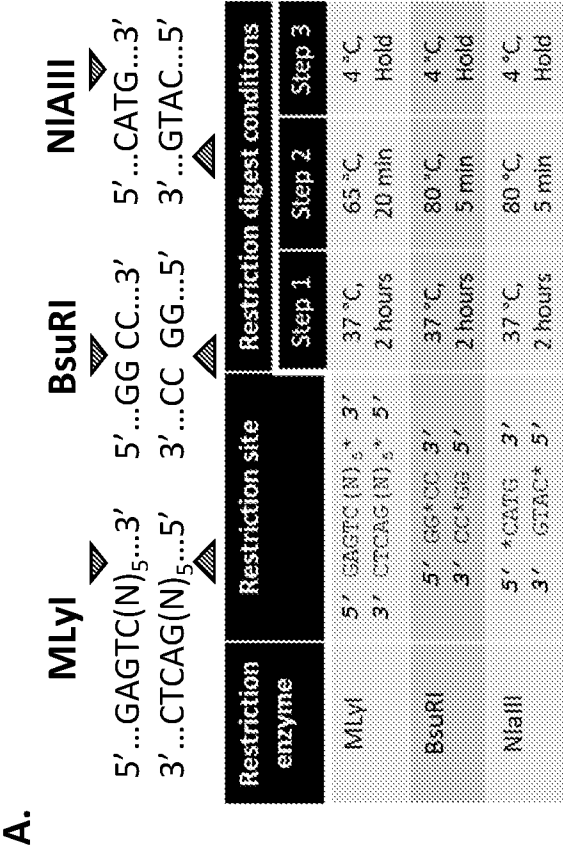
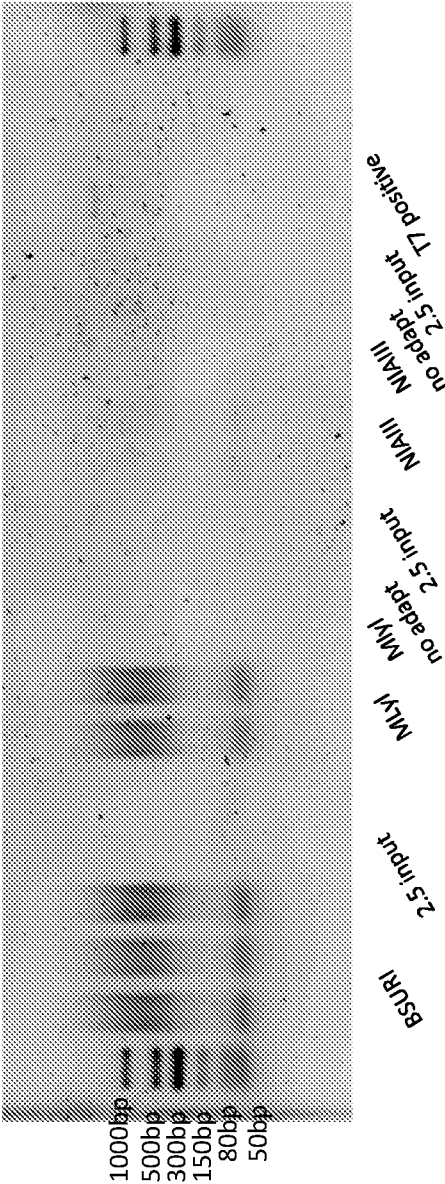


Figure 12B

B. aRNA post T7 RNA transcription amplification



24/31

Figure 13

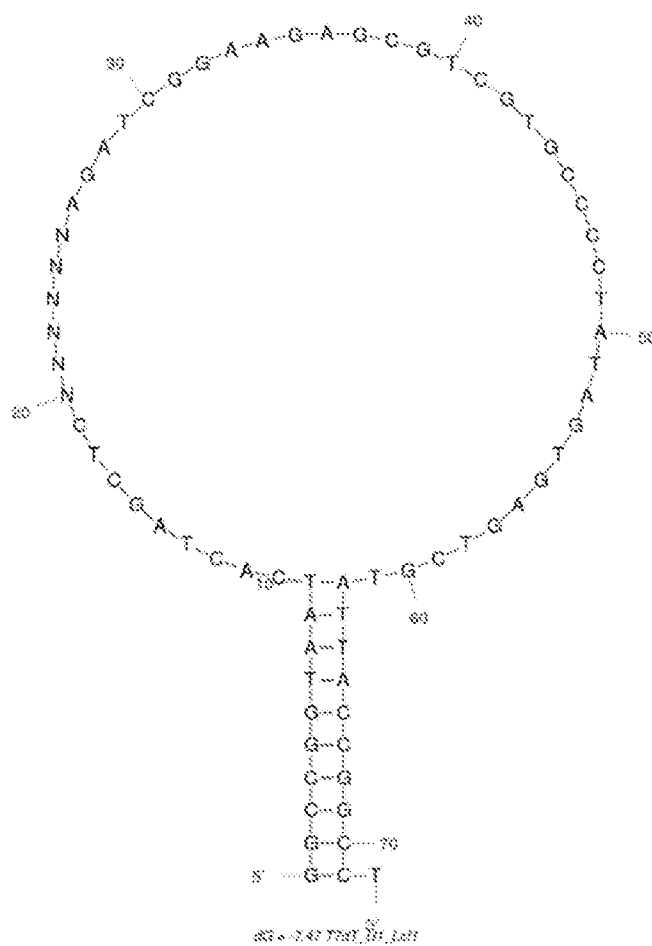


Figure 14

T7 sample barcode distribution

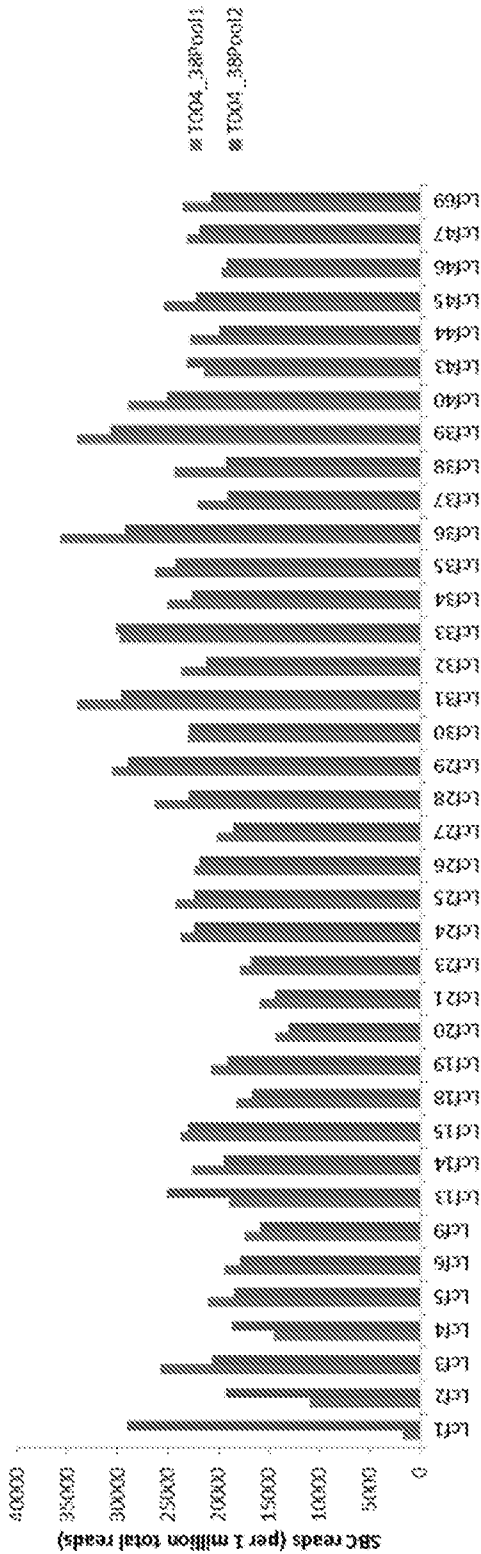


Figure 15A

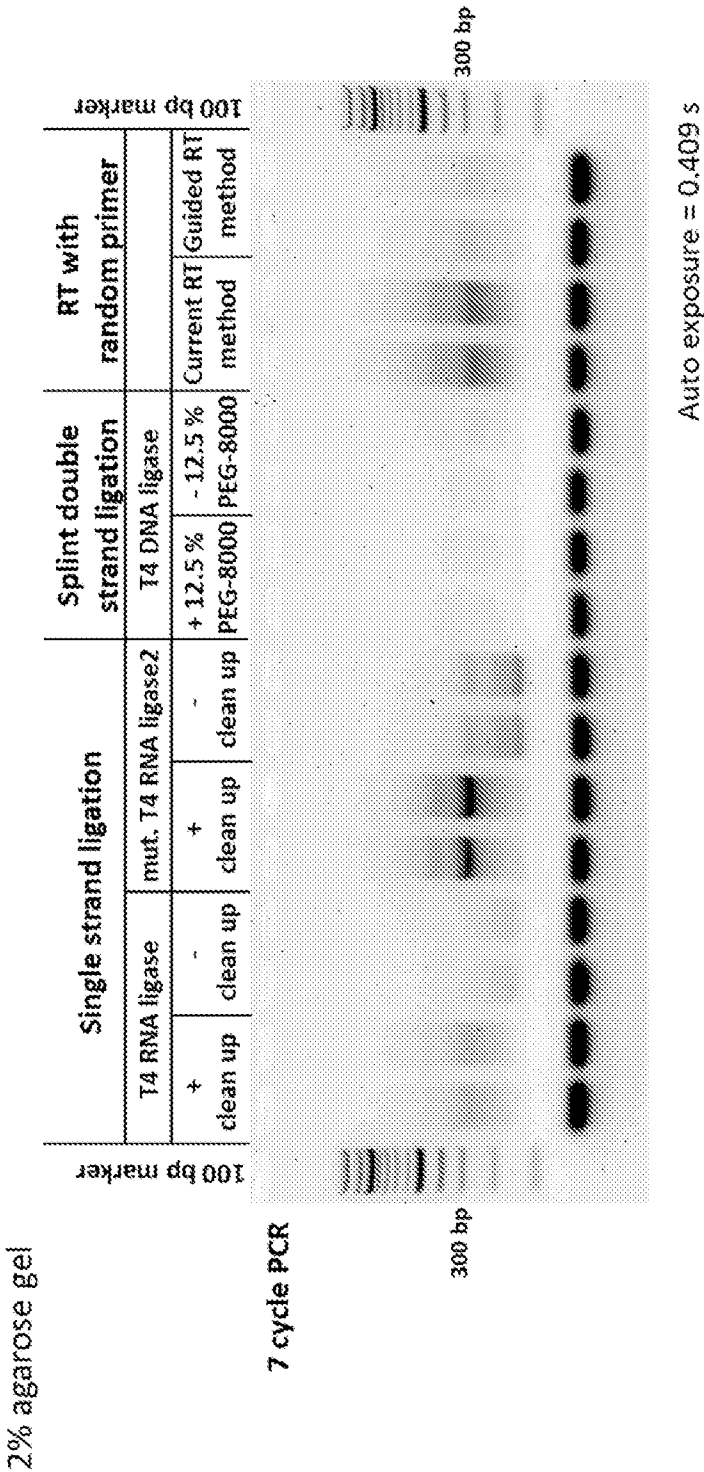


Figure 15B

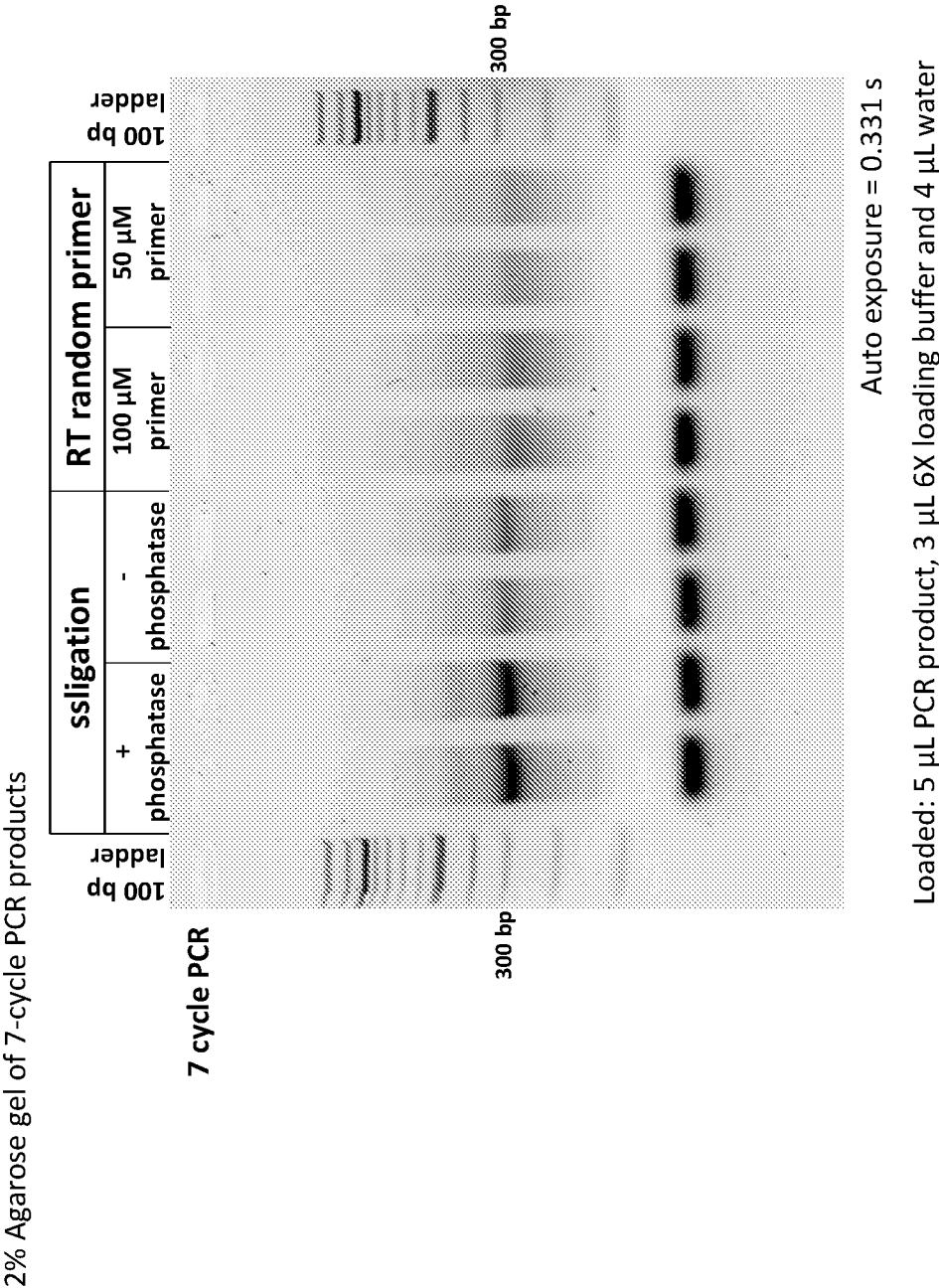
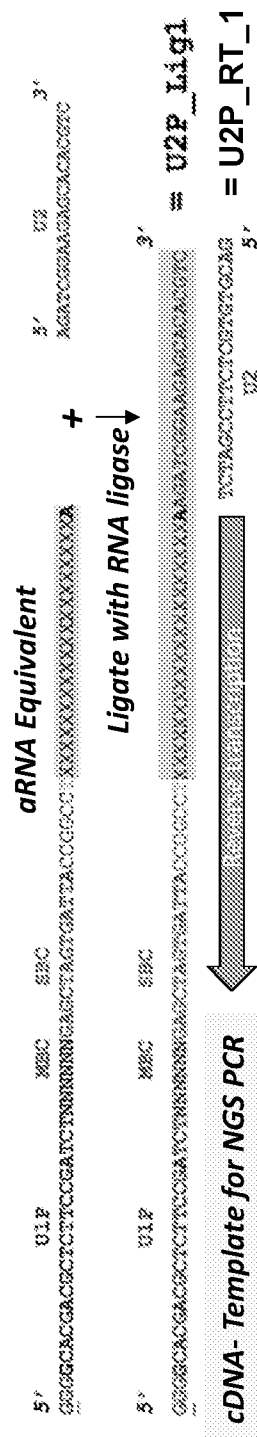


Figure 15C

6a(i). Convert α RNA to cDNA via Ligation to α RNA



6a(ii). Convert aRNA to cDNA via splinted DNA ligation to aRNA

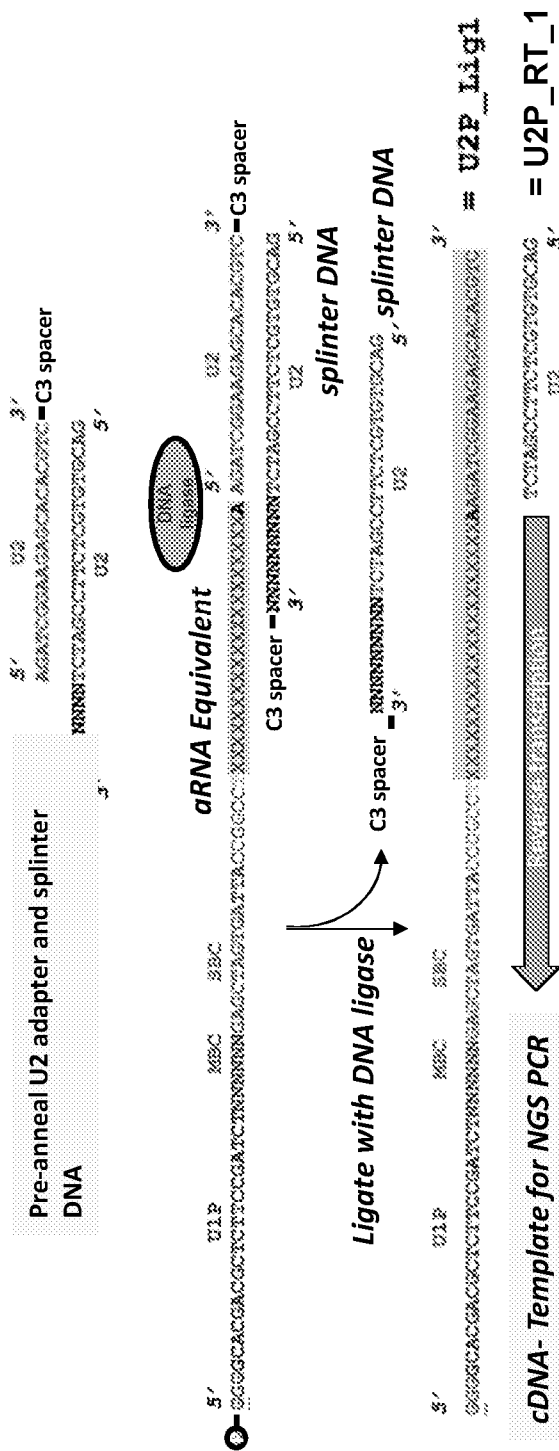


Figure 16A

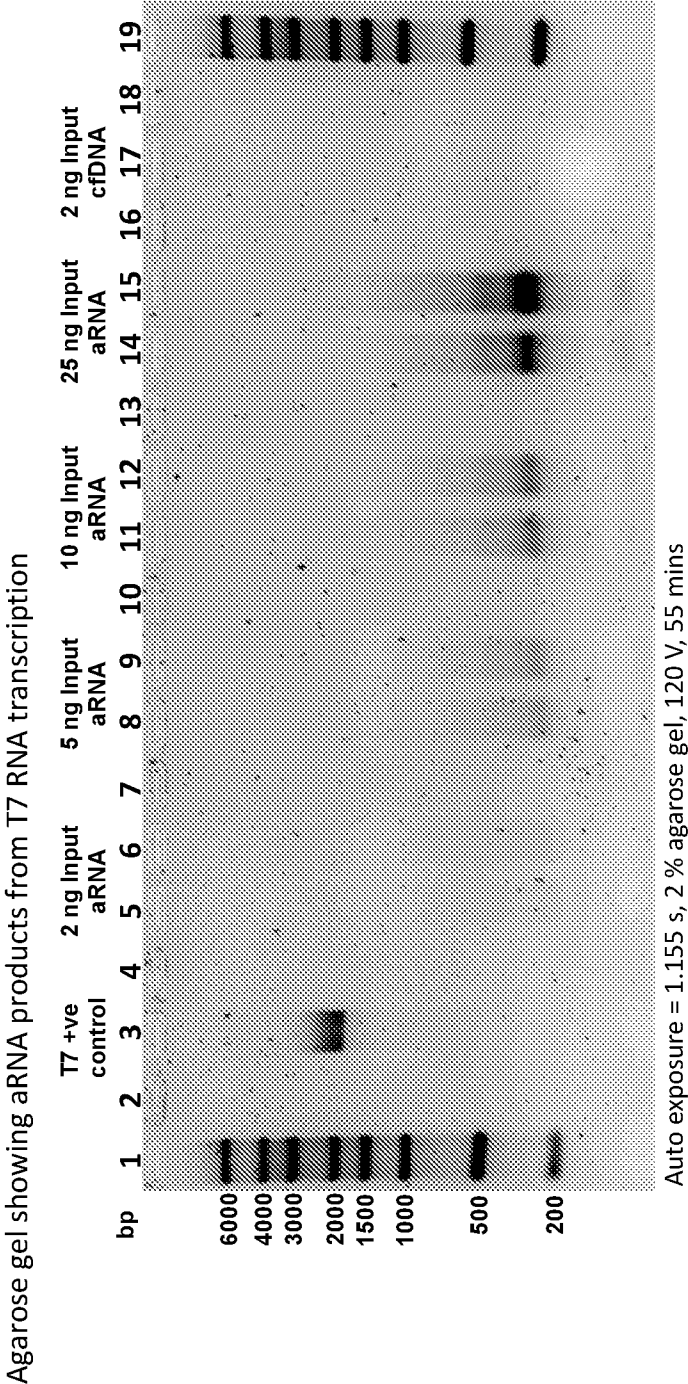
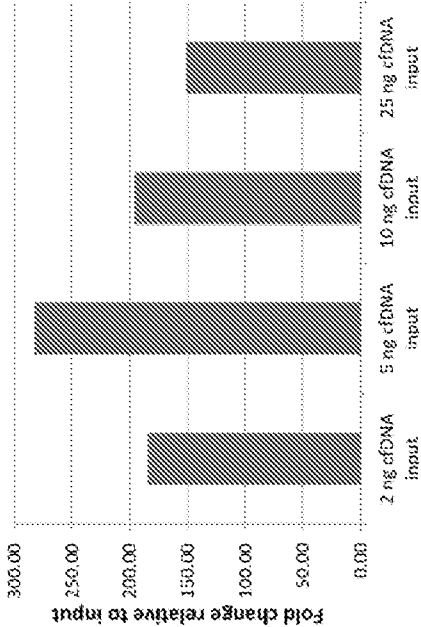
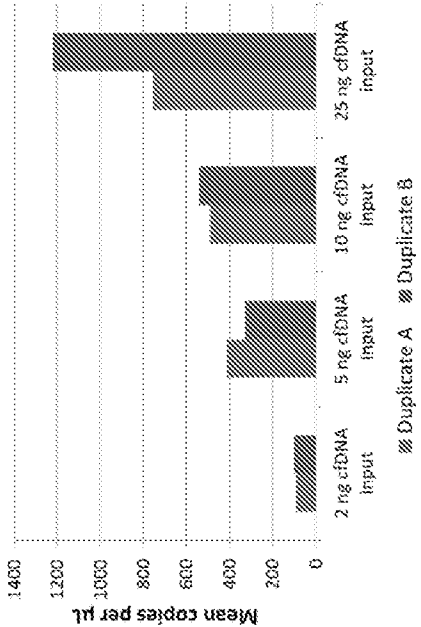


Figure 16B and C

C. RNase P qPCR fold increase of aRNA relative to input DNA



B. RNase P qPCR quantification



INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2020/050635

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/6865 C12Q1/6855
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/112949 A2 (ARCHERDX INC [US]) 30 July 2015 (2015-07-30) the whole document	1-46
X	US 2003/104432 A1 (XU ZHIDONG [US] ET AL) 5 June 2003 (2003-06-05) the whole document	1-46
X	WO 2004/101749 A2 (GENISPHERE INC [US]; GETTS ROBERT C [US]; SCHWALM JAIME [US]) 25 November 2004 (2004-11-25) the whole document	1-46
X	WO 91/18115 A1 (LIFE TECHNOLOGIES INC [US]) 28 November 1991 (1991-11-28) the whole document	1-46
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See patent family annex.

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Date of the actual completion of the international search

15 May 2020

Date of mailing of the international search report

25/05/2020

Name and mailing address of the ISA/

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Authorized officer

Bradbrook, Derek

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2020/050635

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92/18521 A1 (LIFE TECHNOLOGIES INC [US]) 29 October 1992 (1992-10-29) the whole document -----	1-46
X	WO 97/47762 A1 (SARNOFF CORP [US]) 18 December 1997 (1997-12-18) the whole document -----	1-46
X	WO 2015/117040 A1 (SWIFT BIOSCIENCES INC [US]) 6 August 2015 (2015-08-06)	47-49
A	paragraph [0013]; claims 1-55 paragraph [0151] -----	1-46
A	MORGANE BOONE ET AL: "Capturing the 'ome': the expanding molecular toolbox for RNA and DNA library construction", NUCLEIC ACIDS RESEARCH, vol. 46, no. 6, 5 March 2018 (2018-03-05), pages 2701-2721, XP055681576, ISSN: 0305-1048, DOI: 10.1093/nar/gky167 the whole document -----	1-49

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2020/050635

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WO 2015117040 A1	06-08-2015	AU 2015210705 A1 CA 2938213 A1 CN 106459968 A CN 109971826 A EP 3099796 A1 EP 3363904 A2 EP 3604544 A1 JP 6633684 B2 JP 2017504360 A JP 2018138052 A KR 20160115938 A US 2016340746 A1 US 2016362726 A1 US 2019241935 A1 WO 2015117040 A1	11-08-2016 06-08-2015 22-02-2017 05-07-2019 07-12-2016 22-08-2018 05-02-2020 22-01-2020 09-02-2017 06-09-2018 06-10-2016 24-11-2016 15-12-2016 08-08-2019 06-08-2015