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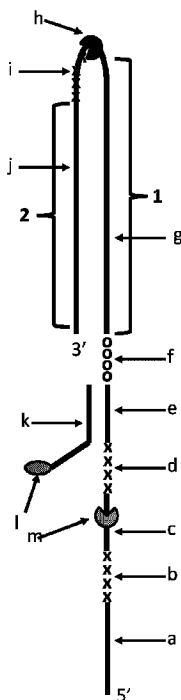
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(57) Abrégé/Abstract:

The invention relates to new methods of controlling the movement of polynucleotides through transmembrane pores. The invention also relates to new methods of characterising target polynucleotides using helicases.

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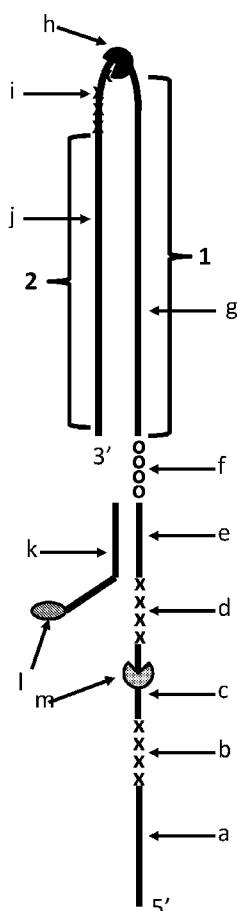
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(54) Title: METHOD FOR CONTROLLING THE MOVEMENT OF A POLYNUCLEOTIDE THROUGH A TRANSMEMBRANE PORE

(57) Abstract: The invention relates to new methods of controlling the movement of polynucleotides through transmembrane pores. The invention also relates to new methods of characterising target polynucleotides using helicases.

Figure 1



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METHOD FOR CONTROLLING THE MOVEMENT OF A POLYNUCLEOTIDE THROUGH A TRANSMEMBRANE PORE

Field of the invention

The invention relates to new methods of controlling the movement of polynucleotides through transmembrane pores. The invention also relates to new methods of characterising target polynucleotides using helicases.

Background of the invention

There is currently a need for rapid and cheap polynucleotide (e.g. DNA or RNA) sequencing and identification technologies across a wide range of applications. Existing technologies are slow and expensive mainly because they rely on amplification techniques to produce large volumes of polynucleotide and require a high quantity of specialist fluorescent chemicals for signal detection.

Transmembrane pores (nanopores) have great potential as direct, electrical biosensors for polymers and a variety of small molecules. In particular, recent focus has been given to nanopores as a potential DNA sequencing technology.

When a potential is applied across a nanopore, there is a change in the current flow when an analyte, such as a nucleotide, resides transiently in the barrel for a certain period of time. Nanopore detection of the nucleotide gives a current change of known signature and duration. In the strand sequencing method, a single polynucleotide strand is passed through the pore and the identities of the nucleotides are derived. Strand sequencing can involve the use of a polynucleotide binding protein to control the movement of the polynucleotide through the pore.

Summary of the invention

The inventors have surprisingly demonstrated that the movement of a polynucleotide through a transmembrane pore is improved if it is controlled by one or more helicase in combination with one or more molecular brakes. The one or more helicases and one or more molecular brakes typically start at different positions on the polynucleotide and are brought together as the polynucleotide moves through the pore. Accordingly, the invention provides a method for controlling the movement of a polynucleotide through a transmembrane pore, comprising:

(a) providing the polynucleotide with one or more helicases attached to the polynucleotide and one or more molecular brakes attached to the polynucleotide;

(b) contacting the polynucleotide provided in step (a) with the pore; and

(c) applying a potential across the pore such that the one or more helicases and the one or more molecular brakes are brought together and both control the movement of the polynucleotide through the pore.

The invention also provides a method of characterising a target polynucleotide,
5 comprising:

(a) carrying out the method of the invention; and

(b) taking one or more measurements as the polynucleotide moves with respect to the pore wherein the measurements are indicative of one or more characteristics of the polynucleotide and thereby characterising the target polynucleotide.

10 The invention further provides a kit for controlling the movement of a polynucleotide through a transmembrane pore, wherein the kit comprises one or more helicases and one or more molecular brakes.

The invention further provides a series of one or more helicases and one or more molecular brakes attached to a polynucleotide.

Description of the Figures

Figure 1 shows DNA construct Y which was used in Example 1. Section a of DNA construct Y corresponds to SEQ ID NO: 27. Section b corresponds to four iSpC3 spacers. Section c corresponds to SEQ ID NO: 28. Section c is one of the regions of construct Y to which
20 the helicase enzymes T4 Dda – E94C/A360C or T4 Dda – E94C/C109A/C136A/A360C (depending on the experiment) bound (labelled m). The length of section c corresponded to the footprint (binding region) of one enzyme e.g. it was long enough to allow one enzyme to bind to this region. Section d corresponds to four iSpC3 spacers. Section e corresponds to SEQ ID NO: 26. Section f corresponds to four 5'-nitroindoles. Section g corresponds to SEQ ID NO: 29 (this
25 section of the strand was referred to as region 1 of DNA construct Y). Section i corresponds to four iSpC3 spacers. The TrwC Cba-Q594A helicase (SEQ ID NO: 25 with the mutation Q594A) which bound to part of SEQ ID NO: 29 is labelled h. Section j corresponds to SEQ ID NO: 30 (this section of the strand was referred to as region 2 of DNA construct Y). Section k corresponds to SEQ ID NO: 31 which was attached at its 3' end to six iSp18 spacers which were
30 attached at the opposite end to two thymines and a 3' cholesterol TEG. It was possible to distinguish between regions 1 and 2 as they translocated through a nanopore as they produced different characteristics. Furthermore, the section i spacers (four iSpC3 spacers) produced a current spike in the current trace which aided identification of the transition from region 1 to region 2.

Figure 2 shows a number of example current traces after helicase controlled DNA movement detection (all traces have the following axes labels y-axis label = Current (pA), x-axis label = Time (seconds)). The traces in section A show single DNA strands moving through a nanopore under the control of only the T4 Dda – E94C/A360C helicase, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of DNA construct Y (see Figure 1). The traces in section B show single DNA strands moving through a nanopore under the control of both T4 Dda – E94C/A360C and TrwC Cba-Q594A helicases, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of DNA construct Y (see Figure 1). The traces in section C show single DNA strands moving through a nanopore under the control of only the TrwC Cba-Q594A helicase, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of DNA construct Y (see Figure 1). Traces A and C show that unequal regions 1 and 2 are obtained when DNA construct Y was translocated through the pore under the control of only one type of helicase either T4 Dda – E94C/A360C helicase (section A) or TrwC Cba-Q594A helicase (section B). Trace B shows improved helicase-controlled DNA movement when construct Y translocated through the pore under the control of both T4 Dda – E94C/A360C and TrwC Cba-Q594A helicase (in this trace regions 1 and 2 were approximately equal). When both enzymes were used to control the movement of region 2 of construct Y through the nanopore the translocation speed was slower and the number of observed stepwise changes in the measured current levels was higher than when a single enzyme was used, and the number of observed stepwise changes in the measured current levels was approximately the same as region 1. This meant that more information was obtained from region 2 when it translocated through the pore under the control of the two enzymes rather than one and therefore improved movement was observed.

Figure 3 shows example plots of when either the helicases T4 Dda – E94C/A360C only (Section A) or both T4 Dda – E94C/A360C and TrwC Cba-Q594A (Section B) controlled the translocation of DNA construct Y (see Figure 1 for details) through an MspA nanopore. The x-axis corresponds to the movement index and the y-axis corresponds to the current (pA). For each DNA strand which moved through the pore the current was measured as a function of time. The moving DNA resulted in stepwise changes in the measured current levels. The observed current levels were fitted to obtain a mean current for each step, and assigned an incrementing movement index point. The mean current against movement index therefore closely approximated the original current signal, and was used to characterise the translocated DNA. Plots A and B each showed single DNA strands moving through the nanopore under the control of helicases, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of

DNA construct Y (see Figure 1). Trace A shows the movement index observed when construct Y was translocated through the pore under the control of a single T4 Dda – E94C/A360C helicase only. Trace B shows the movement index observed when construct Y was translocated through the pore under the control of both T4 Dda – E94C/A360C and TrwC Cba-Q594A helicases. As region 1 and region 2 were approximately the same length, the movement index observed for each region would have been expected to have had approximately the same number of points. Plot A shows a significantly reduced number of points in the movement index for region 2 when compared to region 1, therefore, less information was derived from region 2 than region 1. However, plot B (where the movement of construct Y was controlled by both T4 Dda – E94C/A360C and TrwC Cba-Q594A helicases) showed many more points in the movement index of region 2 (and approximately the same amount as in region 1), which indicated that approximately the same amount of information was derived from region 2 as region 1. Using the combination of helicases (T4 Dda – E94C/A360C and TrwC Cba-Q594A) to control the movement of construct Y provided improved movement as more information was derived from region 2 than when a single helicase controlled the movement.

Figure 4 shows example plots of when either the helicases T4 Dda – E94C/C109A/C136A/A360C only (Section A) or both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A (Section B) controlled the translocation of DNA construct Y (see Figure 1 for details) through an MspA nanopore. The x-axis corresponds to the movement index (see Figure 3's figure legend for description of movement index) and the y-axis corresponds to the current (pA). Plots A and B each showed a single DNA strand moving through the nanopore under the control of helicases, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of DNA construct Y (see Figure 1). Trace A shows the movement index observed when construct Y was translocated through the pore under the control of a single T4 Dda – E94C/C109A/C136A/A360C helicase only. Trace B shows the movement index observed when construct Y was translocated through the pore under the control of both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A helicases. As region 1 and region 2 were approximately the same length, the movement index observed for each region would have been expected to have had approximately the same number of points. Plot A shows a significantly reduced number of points in the movement index for region 2 when compared to region 1, therefore, less information was derived from region 2 than region 1. However, plot B (where the movement of construct Y was controlled by both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A helicases) showed many more points in the movement index of region 2 (and approximately the same amount as in region 1), which indicated that approximately the same

amount of information was derived from region 2 as region 1. Using the combination of helicases (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A) to control the movement of construct Y provided improved movement as more information was derived from region 2 than when a single helicase controlled the movement.

Figure 5 shows example current traces of when either the helicase T4 Dda – E94C/C109A/C136A/A360C only (Section A) or both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C (Section B) controlled the translocation of DNA construct Y (see Figure 1 for details) through an MspA nanopore. The x-axis corresponds to the time (s) and the y-axis corresponds to the current (pA). Plots A and B each showed a single DNA strand moving through the nanopore under the control of helicases, the labelled regions 1 and 2 corresponded to the translocation of region 1 and 2 of DNA construct Y (see Figure 1). Trace A shows a current trace observed when construct Y was translocated through the pore under the control of a single T4 Dda – E94C/C109A/C136A/A360C helicase only. Trace B shows a current trace observed when construct Y was translocated through the pore under the control of both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C helicases. Plot A shows a significantly reduced number of observed stepwise changes in the measured current levels in the current trace for region 2 when compared to region 1, therefore, less information was derived from region 2 than region 1. However, plot B (where the movement of construct Y was controlled by both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C helicases) showed many more observed stepwise changes in the measured current levels in the current trace of region 2 (and approximately the same amount as in region 1), which indicated that approximately the same amount of information was derived from region 2 as region 1. Using the combination of helicases (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C) to control the movement of construct Y provided improved movement as more information was derived from region 2 than when a single helicase controlled the movement.

Figure 6 shows two histogram plots which show the base calling accuracy (as a percentage) for helicase controlled DNA movement events detected in the experiments carried out in Example 3 when either a single enzyme (T4 Dda – E94C/C109A/C136A/A360C) or two enzymes (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C) controlled the movement of region 1(trace A) or region 2 (trace B) of the DNA construct Y. The x-axis label was count and the y-axis label was % base calling accuracy for either region 1 (trace A) or region 2 (trace B) based on the known sequence of construct Y. Plot A shows the base calling accuracy of the helicase controlled translocation of region 1 (shown in Figure 1). Plot B

shows the base calling accuracy of the helicase controlled translocation of region 2 (shown in Figure 1). Each helicase controlled DNA translocation was categorised as either having more observed stepwise changes in the measured current levels in region 1 (shown as black bars which was indicative of T4 Dda – E94C/C109A/C136A/A360C only) or more observed stepwise

5 changes in the measured current levels in region 2 (shown as grey bars, which was indicative of both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C bound to construct Y). As the TrwC Cba-L376C/Q594A/K762C only affected the movement of region 2, the sequencing accuracies for region 1 of the strand have the same distribution for both class of strand (either one or two enzymes bound). However, the sequencing accuracy of the region 2 of

10 construct Y was improved as there were more observed stepwise changes in the measured current levels in region 2 when TrwC Cba-L376C/Q594A/K762C (grey bars) was bound. Therefore, the bulk accuracy of the base calling distribution was improved by approximately 5-10% when both enzymes were bound (the grey bars shown in trace B).

15 **Description of the Sequence Listing**

SEQ ID NO: 1 shows the codon optimised polynucleotide sequence encoding the MS-B1 mutant MspA monomer. This mutant lacks the signal sequence and includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K.

SEQ ID NO: 2 shows the amino acid sequence of the mature form of the MS-B1 mutant

20 of the MspA monomer. This mutant lacks the signal sequence and includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K.

SEQ ID NO: 3 shows the polynucleotide sequence encoding one monomer of α -hemolysin-E111N/K147N (α -HL-NN; Stoddart *et al.*, PNAS, 2009; 106(19): 7702-7707).

SEQ ID NO: 4 shows the amino acid sequence of one monomer of α -HL-NN.

25 SEQ ID NOs: 5 to 7 show the amino acid sequences of MspB, C and D.

SEQ ID NO: 8 shows the polynucleotide sequence encoding the Phi29 DNA polymerase.

SEQ ID NO: 9 shows the amino acid sequence of the Phi29 DNA polymerase.

SEQ ID NO: 10 shows the codon optimised polynucleotide sequence derived from the *shcB* gene from *E. coli*. It encodes the exonuclease I enzyme (EcoExo I) from *E. coli*.

30 SEQ ID NO: 11 shows the amino acid sequence of exonuclease I enzyme (EcoExo I) from *E. coli*.

SEQ ID NO: 12 shows the codon optimised polynucleotide sequence derived from the *xthA* gene from *E. coli*. It encodes the exonuclease III enzyme from *E. coli*.

SEQ ID NO: 13 shows the amino acid sequence of the exonuclease III enzyme from *E. coli*. This enzyme performs distributive digestion of 5' monophosphate nucleosides from one strand of double stranded DNA (dsDNA) in a 3' – 5' direction. Enzyme initiation on a strand requires a 5' overhang of approximately 4 nucleotides.

5 SEQ ID NO: 14 shows the codon optimised polynucleotide sequence derived from the *recJ* gene from *T. thermophilus*. It encodes the RecJ enzyme from *T. thermophilus* (*TthRecJ-cd*).

SEQ ID NO: 15 shows the amino acid sequence of the RecJ enzyme from *T. thermophilus* (*TthRecJ-cd*). This enzyme performs processive digestion of 5' monophosphate nucleosides from ssDNA in a 5' – 3' direction. Enzyme initiation on a strand requires at least 4 nucleotides.

SEQ ID NO: 16 shows the codon optimised polynucleotide sequence derived from the bacteriophage lambda *exo* (*redX*) gene. It encodes the bacteriophage lambda exonuclease.

15 SEQ ID NO: 17 shows the amino acid sequence of the bacteriophage lambda exonuclease. The sequence is one of three identical subunits that assemble into a trimer. The enzyme performs highly processive digestion of nucleotides from one strand of dsDNA, in a 5' - 3' direction (<http://www.neb.com/nebecomm/products/productM0262.asp>). Enzyme initiation on a strand preferentially requires a 5' overhang of approximately 4 nucleotides with a 5' phosphate.

20 SEQ ID NO: 18 shows the amino acid sequence of Hel308 Mbu.

SEQ ID NO: 19 shows the amino acid sequence of Hel308 Csy.

SEQ ID NO: 20 shows the amino acid sequence of Hel308 Tga.

SEQ ID NO: 21 shows the amino acid sequence of Hel308 Mhu.

SEQ ID NO: 22 shows the amino acid sequence of TraI Eco.

25 SEQ ID NO: 23 shows the amino acid sequence of XPD Mbu.

SEQ ID NO: 24 shows the amino acid sequence of Dda 1993.

SEQ ID NO: 25 shows the amino acid sequence of Trwc Cba.

SEQ ID NO: 26 shows a polynucleotide sequence used in Example 1 and 2.

SEQ ID NO: 27 shows a polynucleotide sequence used in Example 1 and 2.

30 SEQ ID NO: 28 shows a polynucleotide sequence used in Example 1 and 2.

SEQ ID NO: 29 shows a polynucleotide sequence used in Example 1 and 2.

SEQ ID NO: 30 shows a polynucleotide sequence used in Example 1 and 2.

SEQ ID NO: 31 shows a polynucleotide sequence used in Example 1 and 2.

Detailed description of the invention

It is to be understood that different applications of the disclosed products and methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

In addition as used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to “a polynucleotide” includes two or more polynucleotides, reference to “a helicase” includes two or more helicases, reference to “a molecular brake” refers to two or more molecular brakes, reference to “a transmembrane pore” includes two or more pores and the like.

Method of the invention

The invention provides a method of controlling the movement of a polynucleotide through a transmembrane pore. The polynucleotide is provided with one or more helicases and one or more molecular brakes. The polynucleotide, the one or more helicases and the one or more molecular brakes are contacted with a transmembrane pore. Once a potential is applied, the polynucleotide moves through the pore and brings the one or more helicases and the one or more molecular brakes together and they both control the movement of the polynucleotide through the pore. The combination of the one or more helicases and the one or more molecular brakes results in an improved movement of the polynucleotide through the pore.

Helicases can control the movement of polynucleotides in at least two active modes of operation (when the helicase is provided with all the necessary components to facilitate movement, e.g. ATP and Mg^{2+}) and one inactive mode of operation (when the helicase is not provided with the necessary components to facilitate movement or is modified to prevent or hinder movement). When provided with all the necessary components to facilitate movement, the helicase moves along the polynucleotide in a 5' to 3' or a 3' to 5' direction (depending on the helicase), but the orientation of the polynucleotide in the pore (which is dependent on which end of the polynucleotide is captured by the pore) means that the helicase can be used to either move the polynucleotide out of the pore against the applied field or move the polynucleotide into the pore with the applied field. When the end of the polynucleotide towards which the helicase moves is captured by the pore, the helicase works against the direction of the field resulting from the applied potential and pulls the threaded polynucleotide out of the pore and into the *cis*

chamber. However, when the end away from which the helicase moves is captured in the pore, the helicase works with the direction of the field resulting from the applied potential and pushes the threaded polynucleotide into the pore and into the *trans* chamber.

When the helicase is not provided with the necessary components to facilitate movement it can bind to the polynucleotide and act as a brake slowing the movement of the polynucleotide when it is pulled into the pore by the field resulting from the applied potential. In the inactive mode, it does not matter which end of the polynucleotide is captured, it is the applied field which pulls the polynucleotide into the pore towards the *trans* side with the helicase acting as a brake. When in the inactive mode, the movement control of the polynucleotide by the helicase can be described in a number of ways including ratcheting, sliding and braking.

In the method of the invention, the one or more helicases preferably control the movement of the target polynucleotide through the pore with the field resulting from the applied potential. In one preferred embodiment, the one or more helicases are used in the active mode and the end away from which the one or more helicases move is captured by the pore such that the one or more helicases work with the field resulting from the applied potential and push the polynucleotide through the pore. If the one or more helicases move in the 5' to 3' direction, the 5' end of the polynucleotide is preferably captured by the pore. In such embodiments, the one or more helicases move along the polynucleotide in the 5' to 3' direction. If the one or more helicases move in the 3' to 5' direction, the 3' end of the polynucleotide is preferably captured by the pore. In such embodiments, the one or more helicases move along the polynucleotide in the 3' to 5' direction.

In another preferred embodiment, the one or more helicases are used in the inactive mode such that the applied field pulls the polynucleotide through the pore and the one or more helicases act as a brake. In another preferred embodiment, the one or more helicases are modified such that they retain their polynucleotide binding ability but lack helicase activity (i.e. the ability to actively move along the polynucleotide) such that the applied field pulls the polynucleotide through the pore and the one or more helicases act as a brake. In the method of the invention, the one or more helicases preferably slow or brake the movement of the polynucleotide through the pore with the field resulting from the applied potential. In either case, the one or more helicases are typically too large to move through the pore and the pore pushes the one or more helicases along the polynucleotide as the polynucleotide moves through the pore with the field resulting from the applied potential. This brings to the one or more helicases and one or more molecular brakes together.

The method of controlling the movement of a polynucleotide through a transmembrane pore can be helpful during characterisation of the polynucleotide using the pore, for instance during strand sequencing. The invention also provides a method of characterising a target polynucleotide. Once a potential is applied, the polynucleotide moves through the pore and
5 brings the one or more helicases and the one or more molecular brakes together and they both control the movement of the polynucleotide through the pore. The method also comprises taking one or more measurements as the polynucleotide moves with respect to the pore. The measurements are indicative of one or more characteristics of the polynucleotide, such as the sequence.

10 It has been shown that double stranded polynucleotides can be effectively characterised using a transmembrane pore if they are modified to include a Y adaptor (a double stranded stem and two non-complementary arms) containing a leader sequence and a bridging moiety adaptor, such as a hairpin loop adaptor (WO 2013/014451). It is preferred that that Y adaptor containing the leader sequence is attached to one end of the polynucleotide and the bridging moiety adaptor
15 is attached to the other end. The leader sequence preferentially threads into the nanopore and the bridging moiety connecting the two strands of the polynucleotide allows both strands to be investigated as the polynucleotide unzips and both strands (connected via the bridging moiety) move through the pore. This is advantageous because it doubles the amount of information obtained from a single double stranded polynucleotide. Moreover, because the sequences in the
20 two strands are complementary, the information from the two strands can be combined informatically. This mechanism provides an orthogonal proof-reading capability that provides higher confidence observations.

One or more helicases may be attached to the Y adaptor and used to control the movement of both strands of the double stranded polynucleotide (connected via the bridging
25 moiety) through the pore. The inventors have shown that, once the one or more helicases move past the bridging moiety and control the movement of the second strand of the double stranded polynucleotide, the one or more helicases are less effective at controlling the movement of the second strand through the pore and less information is derived from the second strand. The invention overcomes this decrease in efficiency of movement control by using one or more
30 molecular brakes. When the one or more helicases and one or more molecular brakes are brought together, they effectively control the movement of the second strand through the pore. The one or more molecular brakes are preferably attached to the bridging moiety so that the one or more helicases and one or more molecular brakes are brought together at the appropriate time. The increased efficiency of movement control means that more information is derived from the

second strand. In particular, approximately the same amount of information can be derived from both strands.

Polynucleotide

5 A polynucleotide, such as a nucleic acid, is a macromolecule comprising two or more nucleotides. The polynucleotide or nucleic acid may comprise any combination of any nucleotides. The nucleotides can be naturally occurring or artificial. One or more nucleotides in the polynucleotide can be oxidized or methylated. One or more nucleotides in the polynucleotide may be damaged. For instance, the polynucleotide may comprise a pyrimidine
10 dimer. Such dimers are typically associated with damage by ultraviolet light and are the primary cause of skin melanomas. One or more nucleotides in the polynucleotide may be modified, for instance with a label or a tag. Suitable labels are described below. The polynucleotide may comprise one or more spacers.

A nucleotide typically contains a nucleobase, a sugar and at least one phosphate group.
15 The nucleobase and sugar form a nucleoside.

The nucleobase is typically heterocyclic. Nucleobases include, but are not limited to, purines and pyrimidines and more specifically adenine (A), guanine (G), thymine (T), uracil (U) and cytosine (C).

The sugar is typically a pentose sugar. Nucleotide sugars include, but are not limited to,
20 ribose and deoxyribose. The sugar is preferably a deoxyribose.

The nucleotide in the polynucleotide is typically a ribonucleotide or deoxyribonucleotide. The polynucleotide may comprise the following nucleosides: adenosine, uridine, guanosine and cytidine. The nucleotide is preferably a deoxyribonucleotide. The polynucleotide preferably comprises the following nucleosides: deoxyadenosine (dA), deoxyuridine (dU) and/or thymidine
25 (dT), deoxyguanosine (dG) and deoxycytidine (dC).

The nucleotide typically contains a monophosphate, diphosphate or triphosphate. Phosphates may be attached on the 5' or 3' side of a nucleotide.

Suitable nucleotides include, but are not limited to, adenosine monophosphate (AMP), guanosine monophosphate (GMP), thymidine monophosphate (TMP), uridine monophosphate
30 (UMP), cytidine monophosphate (CMP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP) and deoxycytidine monophosphate (dCMP). The nucleotides are preferably selected from AMP, TMP, GMP, CMP, UMP, dAMP, dTMP, dGMP, dCMP and

dUMP. The nucleotides are most preferably selected from dAMP, dTMP, dGMP, dCMP and dUMP. The polynucleotide preferably comprises the following nucleotides: dAMP, dUMP and/or dTMP, dGMP and dCMP.

The nucleotides in the polynucleotide may be attached to each other in any manner. The nucleotides are typically attached by their sugar and phosphate groups as in nucleic acids. The nucleotides may be connected via their nucleobases as in pyrimidine dimers.

The polynucleotide may be single stranded or double stranded. At least a portion of the polynucleotide is preferably double stranded.

The polynucleotide can be a nucleic acid. The polynucleotide may be any synthetic nucleic acid known in the art, such as peptide nucleic acid (PNA), glycerol nucleic acid (GNA), threose nucleic acid (TNA), locked nucleic acid (LNA) or other synthetic polymers with nucleotide side chains. The PNA backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The GNA backbone is composed of repeating glycol units linked by phosphodiester bonds. The TNA backbone is composed of repeating threose sugars linked together by phosphodiester bonds. LNA is formed from ribonucleotides as discussed above having an extra bridge connecting the 2' oxygen and 4' carbon in the ribose moiety.

The polynucleotide is most preferably ribonucleic acid (RNA) or deoxyribonucleic acid (DNA).

The polynucleotide may be any length. For example, the polynucleotide can be at least 10, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400 or at least 500 nucleotides in length. The polynucleotide can be 1000 or more nucleotides, 5000 or more nucleotides in length or 100000 or more nucleotides in length.

The helicase may move along the whole or only part of the polynucleotide in the method of the invention. The whole or only part of the target polynucleotide may be characterised using the method of the invention.

The polynucleotide may be single stranded. At least a portion of the polynucleotide is preferably double stranded. Helicases typically bind to single stranded polynucleotides. If at least a portion of the polynucleotide is double stranded, the polynucleotide preferably comprises a single stranded region or a non-hybridised region. The one or more helicases are capable of binding to the single stranded region or one strand of the non-hybridised region. The polynucleotide preferably comprises one or more single stranded regions or one or more non-hybridised regions.

The one or more spacers are preferably included in the single stranded region or the non-hybridised region of the polynucleotide. The polynucleotide may comprise more than one

single stranded region or more than one non-hybridised region. The polynucleotide may comprise a single stranded region or a non-hybridised region within its sequence and/or at one or both ends. The one or more spacers may be included in the double stranded region of the polynucleotide.

5 If the one or more helicases used in the method move in the 5' to 3' direction, the polynucleotide preferably comprises a single stranded region or a non-hybridised region at its 5' end. If the one or more helicases used in the method move in the 3' to 5' direction, the polynucleotide preferably comprises a single stranded region or a non-hybridised region at its 3' end. If the one or more helicases are used in the inactive mode (i.e. as a brake), it does not
10 matter where the single stranded region or the non-hybridised region is located.

 The single stranded region preferably comprises a leader sequence which preferentially threads into the pore. This is discussed in more detail below.

 If at least a portion of the polynucleotide is double stranded, the two strands of the double stranded portion are preferably linked using a bridging moiety, such as a hairpin or a hairpin
15 loop. This facilitates characterisation method of the invention and is discussed in more detail below.

 The polynucleotide is present in any suitable sample. The invention is typically carried out on a sample that is known to contain or suspected to contain the polynucleotide. The invention may be carried out on a sample to confirm the identity of one or more polynucleotides
20 whose presence in the sample is known or expected.

 The sample may be a biological sample. The invention may be carried out *in vitro* on a sample obtained from or extracted from any organism or microorganism. The organism or microorganism is typically archaeal, prokaryotic or eukaryotic and typically belongs to one of the five kingdoms: plantae, animalia, fungi, monera and protista. The invention may be carried
25 out *in vitro* on a sample obtained from or extracted from any virus. The sample is preferably a fluid sample. The sample typically comprises a body fluid of the patient. The sample may be urine, lymph, saliva, mucus or amniotic fluid but is preferably blood, plasma or serum. Typically, the sample is human in origin, but alternatively it may be from another mammal animal such as from commercially farmed animals such as horses, cattle, sheep, fish, chickens or
30 pigs or may alternatively be pets such as cats or dogs. Alternatively, the sample may be of plant origin, such as a sample obtained from a commercial crop, such as a cereal, legume, fruit or vegetable, for example wheat, barley, oats, canola, maize, soya, rice, rhubarb, bananas, apples, tomatoes, potatoes, grapes, tobacco, beans, lentils, sugar cane, cocoa, cotton.

The sample may be a non-biological sample. The non-biological sample is preferably a fluid sample. Examples of a non-biological sample include surgical fluids, water such as drinking water, sea water or river water, and reagents for laboratory tests.

The sample is typically processed prior to being used in the invention, for example by centrifugation or by passage through a membrane that filters out unwanted molecules or cells, such as red blood cells. The sample may be measured immediately upon being taken. The sample may also be typically stored prior to assay, preferably below -70°C.

Helicases

Any helicase may be used in the invention. The helicase may be or be derived from a Hel308 helicase, a RecD helicase, such as TraI helicase or a TrwC helicase, a XPD helicase or a Dda helicase. The helicase may be any of the helicases, modified helicases or helicase constructs disclosed in International Application Nos. PCT/GB2012/052579 (published as WO 2013/057495); PCT/GB2012/053274 (published as WO 2013/098562); PCT/GB2012/053273 (published as WO2013/098561); PCT/GB2013/051925 (published as WO 2014/013260); PCT/GB2013/051924 (published as WO 2014/013259) and PCT/GB2013/051928 (published as WO 2014/013262); and in UK Application No. 1318464.3 filed on 18 October 2013. In particular, the one or more helicases are preferably modified to reduce the size of an opening in the polynucleotide binding domain through which in at least one conformational state the polynucleotide can unbind from the helicase. This is disclosed in WO 2014/013260.

The one or more helicases may be derived from any helicase, such as Hel308 Mbu (SEQ ID NO: 18), Hel308 Csy (SEQ ID NO: 19), Hel308 Tga (SEQ ID NO: 20), Hel308 Mhu (SEQ ID NO: 21), TraI Eco (SEQ ID NO: 22), XPD Mbu (SEQ ID NO: 23) or a variant thereof.

The helicase preferably comprises the sequence shown in SEQ ID NO: 25 (Trwc Cba) or as variant thereof, the sequence shown in SEQ ID NO: 18 (Hel308 Mbu) or a variant thereof or the sequence shown in SEQ ID NO: 24 (Dda) or a variant thereof.

Variants may differ from the native sequences in any of the ways discussed below for transmembrane pores. Variants retain helicase activity. This can be assayed using known methods and the methods disclosed in the Examples. In particular, over the entire length of the amino acid sequence of SEQ ID NO: 18, 19, 20, 21, 22, 23, 24 or 25, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 18, 19, 20, 21, 22, 23, 24 or 25

15

over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 100 or more, for example 125, 150, 175 or 200 or more, contiguous amino acids ("hard homology").

A preferred variant of SEQ ID NO: 24 comprises (or only comprises) (a) E94C/A360C, 5 (b) E94C/A360C and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2), (c) E94C/A360C/C109A/C136A or (d) E94C/A360C/C109A/C136A and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2).

Other preferred variants of SEQ ID NO: 24 comprise W378A. Preferred variants of SEQ ID NO: 24 comprise (or comprise only) (a) E94C/A360C/ W378A, (b) E94C/A360C/ 10 W378A and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2), (c) E94C/A360C/C109A/C136A/ W378A or (d) E94C/A360C/C109A/C136A/ W378A and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2).

Any number of helicases may be used in accordance with the invention. For instance, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more helicases may be used

15 If two or more helicases are used, they may be attached to one another. The two or more helicases may be covalently attached to one another. The helicases may be attached in any order and using any method. Preferred helicase constructs for use in the invention are described in International Application Nos. PCT/GB2013/051925 (published as WO 2014/013260); PCT/GB2013/051924 (published as WO 2014/013259) and PCT/GB2013/051928 (published as 20 WO 2014/013262); and in UK Application No. 1318464.3 filed on 18 October 2013.

If two or more helicases are used, they are preferably not attached to one another except via the polynucleotide. The two or more helicases are more preferably not covalently attached to one another.

The one or more helicases may be any of those discussed below with reference to the 25 molecular brakes, including all variants of helicases.

Any steps in the method using one or more helicases are typically carried out in the presence of free nucleotides or free nucleotide analogues and an enzyme cofactor that facilitates the action of the one or more helicases. The free nucleotides may be one or more of any of the individual nucleotides discussed above. The free nucleotides include, but are not limited to, 30 adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), thymidine monophosphate (TMP), thymidine diphosphate (TDP), thymidine triphosphate (TTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP),

cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate (dAMP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate (dGDP), deoxyguanosine triphosphate (dGTP), deoxythymidine monophosphate (dTMP), deoxythymidine diphosphate (dTDP), deoxythymidine triphosphate (dTTP), deoxyuridine monophosphate (dUMP), deoxyuridine diphosphate (dUDP), deoxyuridine triphosphate (dUTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP) and deoxycytidine triphosphate (dCTP). The free nucleotides are preferably selected from AMP, TMP, GMP, CMP, UMP, dAMP, dTMP, dGMP or dCMP. The free nucleotides are preferably adenosine triphosphate (ATP). The enzyme cofactor is a factor that allows the construct to function. The enzyme cofactor is preferably a divalent metal cation. The divalent metal cation is preferably Mg^{2+} , Mn^{2+} , Ca^{2+} or Co^{2+} . The enzyme cofactor is most preferably Mg^{2+} .

15 *Molecular brakes*

The one or more molecular brakes may be any compound or molecule which binds to the polynucleotide and slows the movement of the polynucleotide through the pore.

The one or more molecular brakes preferably comprise one or more compounds which bind to the polynucleotide. The one or more compounds are preferably one or more macrocycles. Suitable macrocycles include, but are not limited to, cyclodextrins, calixarenes, cyclic peptides, crown ethers, cucurbiturils, pillararenes, derivatives thereof or a combination thereof. The cyclodextrin or derivative thereof may be any of those disclosed in Eliseev, A. V., and Schneider, H-J. (1994) *J. Am. Chem. Soc.* 116, 6081-6088. The agent is more preferably heptakis-6-amino- β -cyclodextrin (am_7 - β CD), 6-monodeoxy-6-monoamino- β -cyclodextrin (am_1 - β CD) or heptakis-(6-deoxy-6-guanidino)-cyclodextrin (gu_7 - β CD).

The one or more molecular brakes are preferably not one or more single stranded binding proteins (SSB). The one or more molecular brakes are more preferably not a single-stranded binding protein (SSB) comprising a carboxy-terminal (C-terminal) region which does not have a net negative charge or (ii) a modified SSB comprising one or more modifications in its C-terminal region which decreases the net negative charge of the C-terminal region. The one or more molecular brakes are most preferably not any of the SSBs disclosed in International Application No. PCT/GB2013/051924 (published as WO 2014/013259).

The one or more molecular brakes are preferably one or more polynucleotide binding proteins. The polynucleotide binding protein may be any protein that is capable of binding to the

polynucleotide and controlling its movement through the pore. It is straightforward in the art to determine whether or not a protein binds to a polynucleotide. The protein typically interacts with and modifies at least one property of the polynucleotide. The protein may modify the polynucleotide by cleaving it to form individual nucleotides or shorter chains of nucleotides, such as di- or trinucleotides. The moiety may modify the polynucleotide by orienting it or moving it to a specific position, i.e. controlling its movement.

The polynucleotide binding protein is preferably derived from a polynucleotide handling enzyme. A polynucleotide handling enzyme is a polypeptide that is capable of interacting with and modifying at least one property of a polynucleotide. The enzyme may modify the polynucleotide by cleaving it to form individual nucleotides or shorter chains of nucleotides, such as di- or trinucleotides. The enzyme may modify the polynucleotide by orienting it or moving it to a specific position. The polynucleotide handling enzyme does not need to display enzymatic activity as long as it is capable of binding the polynucleotide and controlling its movement through the pore. For instance, the enzyme may be modified to remove its enzymatic activity or may be used under conditions which prevent it from acting as an enzyme. Such conditions are discussed in more detail below.

The one or more molecular brakes are preferably derived from a nucleolytic enzyme. The enzyme is more preferably derived from a member of any of the Enzyme Classification (EC) groups 3.1.11, 3.1.13, 3.1.14, 3.1.15, 3.1.16, 3.1.21, 3.1.22, 3.1.25, 3.1.26, 3.1.27, 3.1.30 and 3.1.31. The enzyme may be any of those disclosed in International Application No. PCT/GB10/000133 (published as WO 2010/086603).

Preferred enzymes are polymerases, exonucleases, helicases and topoisomerases, such as gyrases. Suitable enzymes include, but are not limited to, exonuclease I from *E. coli* (SEQ ID NO: 11), exonuclease III enzyme from *E. coli* (SEQ ID NO: 13), RecJ from *T. thermophilus* (SEQ ID NO: 15) and bacteriophage lambda exonuclease (SEQ ID NO: 17), TatD exonuclease and variants thereof. Three subunits comprising the sequence shown in SEQ ID NO: 15 or a variant thereof interact to form a trimer exonuclease. The polymerase may be PyroPhage® 3173 DNA Polymerase (which is commercially available from Lucigen® Corporation), SD Polymerase (commercially available from Bioron®) or variants thereof. The enzyme is preferably Phi29 DNA polymerase (SEQ ID NO: 9) or a variant thereof. Modified versions of Phi29 polymerase (SEQ ID NO: 8) which act as molecular brakes are disclosed in US Patent No. 5,576,204. The topoisomerase is preferably a member of any of the Moiety Classification (EC) groups 5.99.1.2 and 5.99.1.3.

The one or more molecular brakes are most preferably derived from a helicase, such as Hel308 Mbu (SEQ ID NO: 18), Hel308 Csy (SEQ ID NO: 19), Hel308 Tga (SEQ ID NO: 20), Hel308 Mhu (SEQ ID NO: 21), TraI Eco (SEQ ID NO: 22), XPD Mbu (SEQ ID NO: 23) or a variant thereof. The one or more helicases and the one or more molecular brakes derived from
 5 helicases are different from one another. In other words, the one or more helicases are not the same as the one or more molecular brakes derived from helicases.

Any helicase may be used in the invention. The helicase may be or be derived from a Hel308 helicase, a RecD helicase, such as TraI helicase or a TrwC helicase, a XPD helicase or a Dda helicase. The helicase may be any of the helicases, modified helicases or helicase
 10 constructs disclosed in International Application Nos. PCT/GB2012/052579 (published as WO 2013/057495); PCT/GB2012/053274 (published as WO 2013/098562); PCT/GB2012/053273 (published as WO2013098561); PCT/GB2013/051925 (published as WO 2014/013260); PCT/GB2013/051924 (published as WO 2014/013259) and PCT/GB2013/051928 (published as WO 2014/013262); and in UK Application No. 1318464.3 filed on 18 October 2013.

15 The helicase preferably comprises the sequence shown in SEQ ID NO: 25 (Trwc Cba) or as variant thereof, the sequence shown in SEQ ID NO: 18 (Hel308 Mbu) or a variant thereof or the sequence shown in SEQ ID NO: 24 (Dda) or a variant thereof. Variants may differ from the native sequences in any of the ways discussed below for helicases or transmembrane pores.

Preferred molecular brake variants of SEQ ID NO: 25 comprises (or only comprises) (a)
 20 Q594A, (b) L376C/Q594A/K762C, (c) L376C/Q594A/A779C, (d) Q346C/Q594A/A779C, (e) Q346C/Q594A/A783C, (f) D411/Q594A/A783C, (g) Q594A/R353C/E722C, (h) Q594A/Q357C/T720C, (i) Q594A/R358C/T720C, (j) Q594A/H354C/T720C, (k) Q594A/F374C/E722C or (l) Q594A/S350C/E722C. Any of (a) to (l) may further comprise and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2. Other Preferred variants are
 25 discussed above.

Any number of helicases may be used as molecular brakes. For instance, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more helicases may be used as molecular brakes. If two or more helicases are be used as molecular brakes, the two or more helicases are typically the same helicases. The two or more helicases may be different helicases.

30 The two or more molecular brakes may be any combination of the helicases mentioned above. The two or more molecular brakes may be two or more Dda helicases. The two or more molecular brakes may be one or more Dda helicases and one or more TrwC helicases. The two or more molecular brakes may be different variants of the same helicase.

If two or more molecular brakes are used, they may be attached to one another. The two or more molecular brakes may be covalently attached to one another. The molecular brakes may be attached in any order and using any method.

If two or more molecular brakes are used, they are preferably not attached to one another except via the polynucleotide. The two or more molecular brakes are more preferably not covalently attached to one another.

The one or more molecular brakes derived from helicases are preferably modified to reduce the size of an opening in the polynucleotide binding domain through which in at least one conformational state the polynucleotide can unbind from the helicase. This is disclosed in WO 2014/013260.

Preferred helicase constructs for use in the invention are described in International Application Nos. PCT/GB2013/051925 (published as WO 2014/013260); PCT/GB2013/051924 (published as WO 2014/013259) and PCT/GB2013/051928 (published as WO 2014/013262); and in UK Application No. 1318464.3 filed on 18 October 2013.

A variant of SEQ ID NOs: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22, 23, 24 or 25 is an enzyme that has an amino acid sequence which varies from that of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22, 23, 24 or 25 and which retains polynucleotide binding ability. This can be measured using any method known in the art. For instance, the variant can be contacted with a polynucleotide and its ability to bind to and move along the polynucleotide can be measured. The variant may include modifications that facilitate binding of the polynucleotide and/or facilitate its activity at high salt concentrations and/or room temperature. Variants may be modified such that they bind polynucleotides (i.e. retain polynucleotide binding ability) but do not function as a helicase (i.e. do not move along polynucleotides when provided with all the necessary components to facilitate movement, e.g. ATP and Mg^{2+}). Such modifications are known in the art. For instance, modification of the Mg^{2+} binding domain in helicases typically results in variants which do not function as helicases. These types of variants may act as molecular brakes (see below).

Over the entire length of the amino acid sequence of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22, 23, 24 or 25, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant polypeptide may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22, 23, 24 or 25 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino

acid identity over a stretch of 200 or more, for example 230, 250, 270, 280, 300, 400, 500, 600, 700, 800, 900 or 1000 or more, contiguous amino acids (“hard homology”). Homology is determined as described above. The variant may differ from the wild-type sequence in any of the ways discussed above with reference to SEQ ID NO: 2 and 4 above. The enzyme may be covalently attached to the pore. Any method may be used to covalently attach the enzyme to the pore.

A preferred molecular brake is TrwC Cba-Q594A (SEQ ID NO: 25 with the mutation Q594A). This variant does not function as a helicase (i.e. binds to polynucleotides but does not move along them when provided with all the necessary components to facilitate movement, e.g. ATP and Mg^{2+}). The one or more molecular brake helicases can be used in any direction and/or mode discussed above.

One or more helicases and one or more molecular brakes

If the one or more helicases are used in the active mode (i.e. when the one or more helicases are provided with all the necessary components to facilitate movement, e.g. ATP and Mg^{2+}), the one or more molecular brakes are preferably (a) used in an inactive mode (i.e. are used in the absence of the necessary components to facilitate movement or are incapable of active movement), (b) used in an active mode where the one or more molecular brakes move in the opposite direction to the one or more helicases or (c) used in an active mode where the one or more molecular brakes move in the same direction as the one or more helicases and more slowly than the one or more helicases.

If the one or more helicases are used in the inactive mode (i.e. when the one or more helicases are not provided with all the necessary components to facilitate movement, e.g. ATP and Mg^{2+} or are incapable of active movement), the one or more molecular brakes are preferably (a) used in an inactive mode (i.e. are used in the absence of the necessary components to facilitate movement or are incapable of active movement) or (b) used in an active mode where the one or more molecular brakes move along the polynucleotide in the same direction as the polynucleotide through the pore.

The one or more helicases and one or more molecular brakes may be attached to the polynucleotide at any positions so that they are brought together and both control the movement of the polynucleotide through the pore. The one or more helicases and one or more molecular brakes are at least one nucleotide apart, such as at least 5, at least 10, at least 50, at least 100, at least 500, at least 1000, at least 5000, at least 10,000, at least 50,000 nucleotides or more apart. If the method concerns characterising a double stranded polynucleotide provided with a Y

adaptor at one end and a bridging moiety adaptor, such as a hairpin loop adaptor, at the other end, the one or more helicases are preferably attached to the Y adaptor and the one or more molecular brakes are preferably attached to the bridging moiety adaptor. In this embodiment, the one or more molecular brakes are preferably one or more helicases that are modified such that they bind the polynucleotide but do not function as a helicase. The one or more helicases attached to the Y adaptor are preferably stalled at a spacer as discussed in more detail below. The one or more molecular brakes attached to the bridging moiety adaptor are preferably not stalled at a spacer. The one or more helicases and the one or more molecular brakes are preferably brought together when the one or more helicases reach the bridging moiety. The one or more helicases may be attached to the Y adaptor before the Y adaptor is attached to the polynucleotide or after the Y adaptor is attached to the polynucleotide. The one or more molecular brakes may be attached to the bridging moiety adaptor before the bridging moiety adaptor is attached to the polynucleotide or after the bridging moiety adaptor is attached to the polynucleotide.

The one or more helicases and the one or more molecular brakes are preferably not attached to one another. The one or more helicases and the one or more molecular brakes are more preferably not covalently attached to one another. The one or more helicases and the one or more molecular brakes are preferably not attached as described in International Application Nos. PCT/GB2013/051925 (published as WO 2014/013260); PCT/GB2013/051924 (published as WO 2014/013259) and PCT/GB2013/051928 (published as WO 2014/013262); and in UK Application No. 1318464.3 filed on 18 October 2013.

Membrane

Any membrane may be used in accordance with the invention. Suitable membranes are well-known in the art. The membrane is preferably an amphiphilic layer. An amphiphilic layer is a layer formed from amphiphilic molecules, such as phospholipids, which have both hydrophilic and lipophilic properties. The amphiphilic molecules may be synthetic or naturally occurring. Non-naturally occurring amphiphiles and amphiphiles which form a monolayer are known in the art and include, for example, block copolymers (Gonzalez-Perez et al., Langmuir, 2009, 25, 10447-10450). Block copolymers are polymeric materials in which two or more monomer sub-units that are polymerized together to create a single polymer chain. Block copolymers typically have properties that are contributed by each monomer sub-unit. However, a block copolymer may have unique properties that polymers formed from the individual sub-units do not possess. Block copolymers can be engineered such that one of the monomer sub-

units is hydrophobic (i.e. lipophilic), whilst the other sub-unit(s) are hydrophilic whilst in aqueous media. In this case, the block copolymer may possess amphiphilic properties and may form a structure that mimics a biological membrane. The block copolymer may be a diblock (consisting of two monomer sub-units), but may also be constructed from more than two monomer sub-units to form more complex arrangements that behave as amphiphiles. The copolymer may be a triblock, tetrablock or pentablock copolymer. The membrane is preferably a triblock copolymer membrane.

Archaeobacterial bipolar tetraether lipids are naturally occurring lipids that are constructed such that the lipid forms a monolayer membrane. These lipids are generally found in extremophiles that survive in harsh biological environments, thermophiles, halophiles and acidophiles. Their stability is believed to derive from the fused nature of the final bilayer. It is straightforward to construct block copolymer materials that mimic these biological entities by creating a triblock polymer that has the general motif hydrophilic-hydrophobic-hydrophilic. This material may form monomeric membranes that behave similarly to lipid bilayers and encompass a range of phase behaviours from vesicles through to laminar membranes. Membranes formed from these triblock copolymers hold several advantages over biological lipid membranes. Because the triblock copolymer is synthesized, the exact construction can be carefully controlled to provide the correct chain lengths and properties required to form membranes and to interact with pores and other proteins.

Block copolymers may also be constructed from sub-units that are not classed as lipid sub-materials; for example a hydrophobic polymer may be made from siloxane or other non-hydrocarbon based monomers. The hydrophilic sub-section of block copolymer can also possess low protein binding properties, which allows the creation of a membrane that is highly resistant when exposed to raw biological samples. This head group unit may also be derived from non-classical lipid head-groups.

Triblock copolymer membranes also have increased mechanical and environmental stability compared with biological lipid membranes, for example a much higher operational temperature or pH range. The synthetic nature of the block copolymers provides a platform to customize polymer based membranes for a wide range of applications.

In a preferred embodiment, the invention provides a method for determining the presence, absence or one or more characteristics of two or more analytes in two or more samples, comprising (a) coupling a first analyte in a first sample to a membrane using one or more anchors comprising a triblock copolymer, optionally wherein the membrane is modified to facilitate the coupling; (b) allowing the first analyte to interact with a detector present in the

membrane and thereby determining the presence, absence or one or more characteristics of the first analyte; (c) uncoupling the first analyte from the membrane; (d) coupling a second analyte in a second sample to the membrane using one or more anchors; and (e) allowing the second analyte to interact with a detector in the membrane and thereby determining the presence,
5 absence or one or more characteristics of the second analyte.

The membrane is most preferably one of the membranes disclosed in International Application No. PCT/GB2013/052766 or PCT/GB2013/052767.

The amphiphilic molecules may be chemically-modified or functionalised to facilitate coupling of the analyte.

10 The amphiphilic layer may be a monolayer or a bilayer. The amphiphilic layer is typically planar. The amphiphilic layer may be curved. The amphiphilic layer may be supported.

Amphiphilic membranes are typically naturally mobile, essentially acting as two dimensional fluids with lipid diffusion rates of approximately 10^{-8} cm s⁻¹. This means that the
15 detector and coupled analyte can typically move within an amphiphilic membrane.

The membrane may be a lipid bilayer. Lipid bilayers are models of cell membranes and serve as excellent platforms for a range of experimental studies. For example, lipid bilayers can be used for *in vitro* investigation of membrane proteins by single-channel recording. Alternatively, lipid bilayers can be used as biosensors to detect the presence of a range of
20 substances. The lipid bilayer may be any lipid bilayer. Suitable lipid bilayers include, but are not limited to, a planar lipid bilayer, a supported bilayer or a liposome. The lipid bilayer is preferably a planar lipid bilayer. Suitable lipid bilayers are disclosed in International Application No. PCT/GB08/000563 (published as WO 2008/102121), International Application No. PCT/GB08/004127 (published as WO 2009/077734) and International Application No.
25 PCT/GB2006/001057 (published as WO 2006/100484).

Methods for forming lipid bilayers are known in the art. Suitable methods are disclosed in the Example. Lipid bilayers are commonly formed by the method of Montal and Mueller (Proc. Natl. Acad. Sci. USA., 1972; 69: 3561-3566), in which a lipid monolayer is carried on aqueous solution/air interface past either side of an aperture which is perpendicular to that
30 interface. The lipid is normally added to the surface of an aqueous electrolyte solution by first dissolving it in an organic solvent and then allowing a drop of the solvent to evaporate on the surface of the aqueous solution on either side of the aperture. Once the organic solvent has evaporated, the solution/air interfaces on either side of the aperture are physically moved up and down past the aperture until a bilayer is formed. Planar lipid bilayers may be formed across an

aperture in a membrane or across an opening into a recess.

The method of Montal & Mueller is popular because it is a cost-effective and relatively straightforward method of forming good quality lipid bilayers that are suitable for protein pore insertion. Other common methods of bilayer formation include tip-dipping, painting bilayers and patch-clamping of liposome bilayers.

Tip-dipping bilayer formation entails touching the aperture surface (for example, a pipette tip) onto the surface of a test solution that is carrying a monolayer of lipid. Again, the lipid monolayer is first generated at the solution/air interface by allowing a drop of lipid dissolved in organic solvent to evaporate at the solution surface. The bilayer is then formed by the Langmuir-Schaefer process and requires mechanical automation to move the aperture relative to the solution surface.

For painted bilayers, a drop of lipid dissolved in organic solvent is applied directly to the aperture, which is submerged in an aqueous test solution. The lipid solution is spread thinly over the aperture using a paintbrush or an equivalent. Thinning of the solvent results in formation of a lipid bilayer. However, complete removal of the solvent from the bilayer is difficult and consequently the bilayer formed by this method is less stable and more prone to noise during electrochemical measurement.

Patch-clamping is commonly used in the study of biological cell membranes. The cell membrane is clamped to the end of a pipette by suction and a patch of the membrane becomes attached over the aperture. The method has been adapted for producing lipid bilayers by clamping liposomes which then burst to leave a lipid bilayer sealing over the aperture of the pipette. The method requires stable, giant and unilamellar liposomes and the fabrication of small apertures in materials having a glass surface.

Liposomes can be formed by sonication, extrusion or the Mozafari method (Colas *et al.* (2007) *Micron* 38:841–847).

In a preferred embodiment, the lipid bilayer is formed as described in International Application No. PCT/GB08/004127 (published as WO 2009/077734). Advantageously in this method, the lipid bilayer is formed from dried lipids. In a most preferred embodiment, the lipid bilayer is formed across an opening as described in WO2009/077734 (PCT/GB08/004127).

A lipid bilayer is formed from two opposing layers of lipids. The two layers of lipids are arranged such that their hydrophobic tail groups face towards each other to form a hydrophobic interior. The hydrophilic head groups of the lipids face outwards towards the aqueous environment on each side of the bilayer. The bilayer may be present in a number of lipid phases including, but not limited to, the liquid disordered phase (fluid lamellar), liquid ordered phase,

solid ordered phase (lamellar gel phase, interdigitated gel phase) and planar bilayer crystals (lamellar sub-gel phase, lamellar crystalline phase).

Any lipid composition that forms a lipid bilayer may be used. The lipid composition is chosen such that a lipid bilayer having the required properties, such surface charge, ability to support membrane proteins, packing density or mechanical properties, is formed. The lipid composition can comprise one or more different lipids. For instance, the lipid composition can contain up to 100 lipids. The lipid composition preferably contains 1 to 10 lipids. The lipid composition may comprise naturally-occurring lipids and/or artificial lipids.

The lipids typically comprise a head group, an interfacial moiety and two hydrophobic tail groups which may be the same or different. Suitable head groups include, but are not limited to, neutral head groups, such as diacylglycerides (DG) and ceramides (CM); zwitterionic head groups, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM); negatively charged head groups, such as phosphatidylglycerol (PG); phosphatidylserine (PS), phosphatidylinositol (PI), phosphatic acid (PA) and cardiolipin (CA); and positively charged headgroups, such as trimethylammonium-Propane (TAP). Suitable interfacial moieties include, but are not limited to, naturally-occurring interfacial moieties, such as glycerol-based or ceramide-based moieties. Suitable hydrophobic tail groups include, but are not limited to, saturated hydrocarbon chains, such as lauric acid (*n*-Dodecanolic acid), myristic acid (*n*-Tetradecanoic acid), palmitic acid (*n*-Hexadecanoic acid), stearic acid (*n*-Octadecanoic) and arachidic (*n*-Eicosanoic); unsaturated hydrocarbon chains, such as oleic acid (*cis*-9-Octadecanoic); and branched hydrocarbon chains, such as phytanoyl. The length of the chain and the position and number of the double bonds in the unsaturated hydrocarbon chains can vary. The length of the chains and the position and number of the branches, such as methyl groups, in the branched hydrocarbon chains can vary. The hydrophobic tail groups can be linked to the interfacial moiety as an ether or an ester. The lipids may be mycolic acid.

The lipids can also be chemically-modified. The head group or the tail group of the lipids may be chemically-modified. Suitable lipids whose head groups have been chemically-modified include, but are not limited to, PEG-modified lipids, such as 1,2-Diacyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000]; functionalised PEG Lipids, such as 1,2-Distearoyl-sn-Glycero-3 Phosphoethanolamine-N-[Biotinyl(Polyethylene Glycol)2000]; and lipids modified for conjugation, such as 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(succinyl) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Biotinyl). Suitable lipids whose tail groups have been chemically-modified include, but are not limited to, polymerisable lipids, such as 1,2-bis(10,12-tricosadiynoyl)-sn-Glycero-3-

Phosphocholine; fluorinated lipids, such as 1-Palmitoyl-2-(16-Fluoropalmitoyl)-sn-Glycero-3-Phosphocholine; deuterated lipids, such as 1,2-Dipalmitoyl-D62-sn-Glycero-3-Phosphocholine; and ether linked lipids, such as 1,2-Di-O-phytanyl-sn-Glycero-3-Phosphocholine. The lipids may be chemically-modified or functionalised to facilitate coupling of the analyte.

5 The amphiphilic layer, for example the lipid composition, typically comprises one or more additives that will affect the properties of the layer. Suitable additives include, but are not limited to, fatty acids, such as palmitic acid, myristic acid and oleic acid; fatty alcohols, such as palmitic alcohol, myristic alcohol and oleic alcohol; sterols, such as cholesterol, ergosterol, lanosterol, sitosterol and stigmasterol; lysophospholipids, such as 1-Acyl-2-Hydroxy-sn-
10 Glycero-3-Phosphocholine; and ceramides.

 In another preferred embodiment, the membrane is a solid state layer. Solid state layers can be formed from both organic and inorganic materials including, but not limited to, microelectronic materials, insulating materials such as Si_3N_4 , Al_2O_3 , and SiO_2 , organic and inorganic polymers such as polyamide, plastics such as Teflon® or elastomers such as two-
15 component addition-cure silicone rubber, and glasses. The solid state layer may be formed from graphene. Suitable graphene layers are disclosed in International Application No. PCT/US2008/010637 (published as WO 2009/035647).

 The method is typically carried out using (i) an artificial amphiphilic layer comprising a pore, (ii) an isolated, naturally-occurring lipid bilayer comprising a pore, or (iii) a cell having a
20 pore inserted therein. The method is typically carried out using an artificial amphiphilic layer, such as an artificial triblock copolymer layer. The layer may comprise other transmembrane and/or intramembrane proteins as well as other molecules in addition to the pore. Suitable apparatus and conditions are discussed below. The method of the invention is typically carried out *in vitro*.

25

Coupling

 The polynucleotide is preferably coupled to the membrane using one or more anchors. The polynucleotide may be coupled to the membrane using any known method.

 Each anchor comprises a group which couples (or binds) to the polynucleotide and a
30 group which couples (or binds) to the membrane. Each anchor may covalently couple (or bind) to the polynucleotide and/or the membrane. If a Y adaptor and/or a bridging moiety adaptor is/are used, the polynucleotide is preferably coupled to the membrane using the adaptor(s).

 Each polynucleotide may be coupled to the membrane using any number of anchors, such as 2, 3, 4 or more anchors. For instance, one polynucleotide may be coupled to the membrane

using two anchors each of which separately couples (or binds) to both the polynucleotide and membrane.

The one or more anchors may comprise the one or more helicases and/or the one or more molecular brakes.

5 If the membrane is an amphiphilic layer, such as a copolymer membrane or a lipid bilayer, the one or more anchors preferably comprise a polypeptide anchor present in the membrane and/or a hydrophobic anchor present in the membrane. The hydrophobic anchor is preferably a lipid, fatty acid, sterol, carbon nanotube, polypeptide, protein or amino acid, for example cholesterol, palmitate or tocopherol. In preferred embodiments, the one or more
10 anchors are not the detector.

The components of the membrane, such as the amphiphilic molecules, copolymer or lipids, may be chemically-modified or functionalised to form the one or more anchors. Examples of suitable chemical modifications and suitable ways of functionalising the components of the membrane are discussed in more detail below. Any proportion of the
15 membrane components may be functionalized, for example at least 0.01%, at least 0.1%, at least 1%, at least 10%, at least 25%, at least 50% or 100%.

The polynucleotide may be coupled directly to the membrane. The one or more anchors used to couple the polynucleotide to the membrane preferably comprise a linker. The one or more anchors may comprise one or more, such as 2, 3, 4 or more, linkers. One linker may be
20 used couple more than one, such as 2, 3, 4 or more, polynucleotides to the membrane.

Preferred linkers include, but are not limited to, polymers, such as polynucleotides, polyethylene glycols (PEGs), polysaccharides and polypeptides. These linkers may be linear, branched or circular. For instance, the linker may be a circular polynucleotide. The polynucleotide may hybridise to a complementary sequence on the circular polynucleotide
25 linker.

The one or more anchors or one or more linkers may comprise a component that can be cut to broken down, such as a restriction site or a photolabile group.

Functionalised linkers and the ways in which they can couple molecules are known in the art. For instance, linkers functionalised with maleimide groups will react with and attach to
30 cysteine residues in proteins. In the context of this invention, the protein may be present in the membrane or may be used to couple (or bind) to the polynucleotide. This is discussed in more detail below.

Crosslinkage of polynucleotides can be avoided using a "lock and key" arrangement. Only one end of each linker may react together to form a longer linker and the other ends of the

linker each react with the polynucleotide or membrane respectively. Such linkers are described in International Application No. PCT/GB10/000132 (published as WO 2010/086602).

The use of a linker is preferred in the sequencing embodiments discussed below. If a polynucleotide is permanently coupled directly to the membrane in the sense that it does not uncouple when interacting with the detector (i.e. does not uncouple in step (b) or (e)), then some sequence data will be lost as the sequencing run cannot continue to the end of the polynucleotide due to the distance between the membrane and the detector. If a linker is used, then the polynucleotide can be processed to completion.

The coupling may be permanent or stable. In other words, the coupling may be such that the polynucleotide remains coupled to the membrane when interacting with the pore.

The coupling may be transient. In other words, the coupling may be such that the polynucleotide may decouple from the membrane when interacting with the pore.

For certain applications, such as aptamer detection, the transient nature of the coupling is preferred. If a permanent or stable linker is attached directly to either the 5' or 3' end of a polynucleotide and the linker is shorter than the distance between the membrane and the transmembrane pore's channel, then some sequence data will be lost as the sequencing run cannot continue to the end of the polynucleotide. If the coupling is transient, then when the coupled end randomly becomes free of the membrane, then the polynucleotide can be processed to completion. Chemical groups that form permanent/stable or transient links are discussed in more detail below. The polynucleotide may be transiently coupled to an amphiphilic layer or triblock copolymer membrane using cholesterol or a fatty acyl chain. Any fatty acyl chain having a length of from 6 to 30 carbon atom, such as hexadecanoic acid, may be used.

In preferred embodiments, a polynucleotide, such as a nucleic acid, is coupled to an amphiphilic layer such as a triblock copolymer membrane or lipid bilayer. Coupling of nucleic acids to synthetic lipid bilayers has been carried out previously with various different tethering strategies. These are summarised in Table 1 below.

Table 1

Anchor comprising	Type of coupling	Reference
Thiol	Stable	Yoshina-Ishii, C. and S. G. Boxer (2003). "Arrays of mobile tethered vesicles on supported lipid bilayers." <u>J Am Chem Soc</u> 125 (13): 3696-7.
Biotin	Stable	Nikolov, V., R. Lipowsky, et al. (2007). "Behavior of giant vesicles with anchored DNA molecules." <u>Biophys J</u> 92 (12): 4356-68

Cholesterol	Transient	Pfeiffer, I. and F. Hook (2004). "Bivalent cholesterol-based coupling of oligonucleotides to lipid membrane assemblies." <u>J Am Chem Soc</u> 126 (33): 10224-5
Surfactant (e.g. Lipid, Palmitate, etc)	Stable	van Lengerich, B., R. J. Rawle, et al. "Covalent attachment of lipid vesicles to a fluid-supported bilayer allows observation of DNA-mediated vesicle interactions." <u>Langmuir</u> 26 (11): 8666-72

Synthetic polynucleotides and/or linkers may be functionalised using a modified phosphoramidite in the synthesis reaction, which is easily compatible for the direct addition of suitable anchoring groups, such as cholesterol, tocopherol, palmitate, thiol, lipid and biotin groups. These different attachment chemistries give a suite of options for attachment to polynucleotides. Each different modification group couples the polynucleotide in a slightly different way and coupling is not always permanent so giving different dwell times for the polynucleotide to the membrane. The advantages of transient coupling are discussed above.

Coupling of polynucleotides to a linker or to a functionalised membrane can also be achieved by a number of other means provided that a complementary reactive group or an anchoring group can be added to the polynucleotide. The addition of reactive groups to either end of a polynucleotide has been reported previously. A thiol group can be added to the 5' of ssDNA or dsDNA using T4 polynucleotide kinase and ATP γ S (Grant, G. P. and P. Z. Qin (2007). "A facile method for attaching nitroxide spin labels at the 5' terminus of nucleic acids." Nucleic Acids Res **35**(10): e77). An azide group can be added to the 5'-phosphate of ssDNA or dsDNA using T4 polynucleotide kinase and γ -[2-Azidoethyl]-ATP or γ -[6-Azidoethyl]-ATP. Using thiol or Click chemistry a tether, containing either a thiol, iodoacetamide OPSS or maleimide group (reactive to thiols) or a DIBO (dibenzocyclooctyne) or alkyne group (reactive to azides), can be covalently attached to the polynucleotide. A more diverse selection of chemical groups, such as biotin, thiols and fluorophores, can be added using terminal transferase to incorporate modified oligonucleotides to the 3' of ssDNA (Kumar, A., P. Tchen, et al. (1988). "Nonradioactive labeling of synthetic oligonucleotide probes with terminal deoxynucleotidyl transferase." Anal Biochem **169**(2): 376-82). Streptavidin/biotin and/or streptavidin/desthiobiotin coupling may be used for any other polynucleotide. The Examples below describes how a polynucleotide can be coupled to a membrane using streptavidin/biotin and streptavidin/desthiobiotin. It may also be possible that anchors may be directly added to polynucleotides using terminal transferase with suitably modified nucleotides (e.g. cholesterol or palmitate).

The one or more anchors preferably couple the polynucleotide to the membrane via hybridisation. Hybridisation in the one or more anchors allows coupling in a transient manner as discussed above. The hybridisation may be present in any part of the one or more anchors, such as between the one or more anchors and the polynucleotide, within the one or more anchors or
 5 between the one or more anchors and the membrane. For instance, a linker may comprise two or more polynucleotides, such as 3, 4 or 5 polynucleotides, hybridised together. The one or more anchors may hybridise to the polynucleotide. The one or more anchors may hybridise directly to the polynucleotide or directly to a Y adaptor and/or leader sequence attached to the polynucleotide or directly to a bridging moiety adaptor, such as a hairpin loop adaptor, attached
 10 to the polynucleotide (as discussed below). Alternatively, the one or more anchors may be hybridised to one or more, such as 2 or 3, intermediate polynucleotides (or “splints”) which are hybridised to the polynucleotide, to a Y adaptor and/or leader sequence attached to the polynucleotide or to a bridging moiety adaptor attached to the polynucleotide (as discussed below).

15 The one or more anchors may comprise a single stranded or double stranded polynucleotide. One part of the anchor may be ligated to a single stranded or double stranded polynucleotide. Ligation of short pieces of ssDNA have been reported using T4 RNA ligase I (Troutt, A. B., M. G. McHeyzer-Williams, et al. (1992). "Ligation-anchored PCR: a simple amplification technique with single-sided specificity." Proc Natl Acad Sci U S A 89(20): 9823-
 20 5). Alternatively, either a single stranded or double stranded polynucleotide can be ligated to a double stranded polynucleotide and then the two strands separated by thermal or chemical denaturation. To a double stranded polynucleotide, it is possible to add either a piece of single stranded polynucleotide to one or both of the ends of the duplex, or a double stranded polynucleotide to one or both ends. For addition of single stranded polynucleotides to the a
 25 double stranded polynucleotide, this can be achieved using T4 RNA ligase I as for ligation to other regions of single stranded polynucleotides. For addition of double stranded polynucleotides to a double stranded polynucleotide then ligation can be “blunt-ended”, with complementary 3' dA / dT tails on the polynucleotide and added polynucleotide respectively (as is routinely done for many sample prep applications to prevent concatemer or dimer formation)
 30 or using “sticky-ends” generated by restriction digestion of the polynucleotide and ligation of compatible adapters. Then, when the duplex is melted, each single strand will have either a 5' or 3' modification if a single stranded polynucleotide was used for ligation or a modification at the 5' end, the 3' end or both if a double stranded polynucleotide was used for ligation.

If the polynucleotide is a synthetic strand, the one or more anchors can be incorporated during the chemical synthesis of the polynucleotide. For instance, the polynucleotide can be synthesised using a primer having a reactive group attached to it.

Adenylated polynucleotides are intermediates in ligation reactions, where an adenosine-monophosphate is attached to the 5'-phosphate of the polynucleotide. Various kits are available for generation of this intermediate, such as the 5' DNA Adenylation Kit from NEB. By substituting ATP in the reaction for a modified nucleotide triphosphate, then addition of reactive groups (such as thiols, amines, biotin, azides, etc) to the 5' of a polynucleotide can be possible. It may also be possible that anchors could be directly added to polynucleotides using a 5' DNA adenylation kit with suitably modified nucleotides (e.g. cholesterol or palmitate).

A common technique for the amplification of sections of genomic DNA is using polymerase chain reaction (PCR). Here, using two synthetic oligonucleotide primers, a number of copies of the same section of DNA can be generated, where for each copy the 5' of each strand in the duplex will be a synthetic polynucleotide. Single or multiple nucleotides can be added to 3' end of single or double stranded DNA by employing a polymerase. Examples of polymerases which could be used include, but are not limited to, Terminal Transferase, Klenow and *E. coli* Poly(A) polymerase). By substituting ATP in the reaction for a modified nucleotide triphosphate then anchors, such as a cholesterol, thiol, amine, azide, biotin or lipid, can be incorporated into double stranded polynucleotides. Therefore, each copy of the amplified polynucleotide will contain an anchor.

Ideally, the polynucleotide is coupled to the membrane without having to functionalise the polynucleotide. This can be achieved by coupling the one or more anchors, such as a polynucleotide binding protein or a chemical group, to the membrane and allowing the one or more anchors to interact with the polynucleotide or by functionalizing the membrane. The one or more anchors may be coupled to the membrane by any of the methods described herein. In particular, the one or more anchors may comprise one or more linkers, such as maleimide functionalised linkers.

In this embodiment, the polynucleotide is typically RNA, DNA, PNA, TNA or LNA and may be double or single stranded. This embodiment is particularly suited to genomic DNA polynucleotides.

The one or more anchors can comprise any group that couples to, binds to or interacts with single or double stranded polynucleotides, specific nucleotide sequences within the polynucleotide or patterns of modified nucleotides within the polynucleotide, or any other ligand that is present on the polynucleotide.

Suitable binding proteins for use in anchors include, but are not limited to, *E. coli* single stranded binding protein, P5 single stranded binding protein, T4 gp32 single stranded binding protein, the TOPO V dsDNA binding region, human histone proteins, *E. coli* HU DNA binding protein and other archaeal, prokaryotic or eukaryotic single stranded or double stranded
 5 polynucleotide (or nucleic acid) binding proteins, including those listed below.

The specific nucleotide sequences could be sequences recognised by transcription factors, ribosomes, endonucleases, topoisomerases or replication initiation factors. The patterns of modified nucleotides could be patterns of methylation or damage.

The one or more anchors can comprise any group which couples to, binds to, intercalates
 10 with or interacts with a polynucleotide. The group may intercalate or interact with the polynucleotide via electrostatic, hydrogen bonding or Van der Waals interactions. Such groups include a lysine monomer, poly-lysine (which will interact with ssDNA or dsDNA), ethidium bromide (which will intercalate with dsDNA), universal bases or universal nucleotides (which can hybridise with any polynucleotide) and osmium complexes (which can react to methylated
 15 bases). A polynucleotide may therefore be coupled to the membrane using one or more universal nucleotides attached to the membrane. Each universal nucleotide may be coupled to the membrane using one or more linkers. The universal nucleotide preferably comprises one of the following nucleobases: hypoxanthine, 4-nitroindole, 5-nitroindole, 6-nitroindole, formylindole, 3-nitropyrrole, nitroimidazole, 4-nitropyrazole, 4-nitrobenzimidazole, 5-
 20 nitroindazole, 4-aminobenzimidazole or phenyl (C₆-aromatic ring). The universal nucleotide more preferably comprises one of the following nucleosides: 2'-deoxyinosine, inosine, 7-deaza-2'-deoxyinosine, 7-deaza-inosine, 2-aza-deoxyinosine, 2-aza-inosine, 2-O'-methylinosine, 4-nitroindole 2'-deoxyribonucleoside, 4-nitroindole ribonucleoside, 5-nitroindole 2'-deoxyribonucleoside, 5-nitroindole ribonucleoside, 6-nitroindole 2'-deoxyribonucleoside, 6-
 25 nitroindole ribonucleoside, 3-nitropyrrole 2'-deoxyribonucleoside, 3-nitropyrrole ribonucleoside, an acyclic sugar analogue of hypoxanthine, nitroimidazole 2'-deoxyribonucleoside, nitroimidazole ribonucleoside, 4-nitropyrazole 2'-deoxyribonucleoside, 4-nitropyrazole ribonucleoside, 4-nitrobenzimidazole 2'-deoxyribonucleoside, 4-nitrobenzimidazole ribonucleoside, 5-nitroindazole 2'-deoxyribonucleoside, 5-nitroindazole ribonucleoside, 4-
 30 aminobenzimidazole 2'-deoxyribonucleoside, 4-aminobenzimidazole ribonucleoside, phenyl C-ribonucleoside, phenyl C-2'-deoxyribosyl nucleoside, 2'-deoxynebularine, 2'-deoxyisoguanosine, K-2'-deoxyribose, P-2'-deoxyribose and pyrrolidine. The universal nucleotide more preferably comprises 2'-deoxyinosine. The universal nucleotide is more preferably IMP or dIMP. The

universal nucleotide is most preferably dPMP (2'-Deoxy-P-nucleoside monophosphate) or dKMP (N6-methoxy-2, 6-diaminopurine monophosphate).

The one or more anchors may couple to (or bind to) the polynucleotide via Hoogsteen hydrogen bonds (where two nucleobases are held together by hydrogen bonds) or reversed
 5 Hoogsteen hydrogen bonds (where one nucleobase is rotated through 180° with respect to the other nucleobase). For instance, the one or more anchors may comprise one or more nucleotides, one or more oligonucleotides or one or more polynucleotides which form Hoogsteen hydrogen bonds or reversed Hoogsteen hydrogen bonds with the polynucleotide. These types of hydrogen bonds allow a third polynucleotide strand to wind around a double stranded helix and form a
 10 triplex. The one or more anchors may couple to (or bind to) a double stranded polynucleotide by forming a triplex with the double stranded duplex.

In this embodiment at least 1%, at least 10%, at least 25%, at least 50% or 100% of the membrane components may be functionalized.

Where the one or more anchors comprise a protein, they may be able to anchor directly
 15 into the membrane without further functionalisation, for example if it already has an external hydrophobic region which is compatible with the membrane. Examples of such proteins include, but are not limited to, transmembrane proteins, intramembrane proteins and membrane proteins. Alternatively the protein may be expressed with a genetically fused hydrophobic region which is compatible with the membrane. Such hydrophobic protein regions are known in the art.

20 The one or more anchors are preferably mixed with the polynucleotide before contacting with the membrane, but the one or more anchors may be contacted with the membrane and subsequently contacted with the polynucleotide.

In another aspect the polynucleotide may be functionalised, using methods described above, so that it can be recognised by a specific binding group. Specifically the polynucleotide
 25 may be functionalised with a ligand such as biotin (for binding to streptavidin), amylose (for binding to maltose binding protein or a fusion protein), Ni-NTA (for binding to poly-histidine or poly-histidine tagged proteins) or a peptides (such as an antigen).

According to a preferred embodiment, the one or more anchors may be used to couple a polynucleotide to the membrane when the polynucleotide is attached to a leader sequence which
 30 preferentially threads into the pore. Leader sequences are discussed in more detail below. Preferably, the polynucleotide is attached (such as ligated) to a leader sequence which preferentially threads into the pore. Such a leader sequence may comprise a homopolymeric polynucleotide or an abasic region. The leader sequence is typically designed to hybridise to the one or more anchors either directly or via one or more intermediate polynucleotides (or splints).

In such instances, the one or more anchors typically comprise a polynucleotide sequence which is complementary to a sequence in the leader sequence or a sequence in the one or more intermediate polynucleotides (or splints). In such instances, the one or more splints typically comprise a polynucleotide sequence which is complementary to a sequence in the leader
5 sequence.

An example of a molecule used in chemical attachment is EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride). Reactive groups can also be added to the 5' of polynucleotides using commercially available kits (Thermo Pierce, Part No. 22980). Suitable methods include, but are not limited to, transient affinity attachment using histidine residues and
10 Ni-NTA, as well as more robust covalent attachment by reactive cysteines, lysines or non natural amino acids.

Transmembrane pore

The method comprises taking one or more measurements as at least one strand of the
15 polynucleotide moves with respect to the transmembrane pore. A variety of different types of measurements may be made using the pore. This includes without limitation: electrical measurements and optical measurements. Possible electrical measurements include: current measurements, impedance measurements, tunnelling measurements (Ivanov AP et al., Nano Lett. 2011 Jan 12; 11(1):279-85), and FET measurements (International Application WO
20 2005/124888). Optical measurements may be combined with electrical measurements (Soni GV et al., Rev Sci Instrum. 2010 Jan; 81(1):014301). The measurement may be a transmembrane current measurement such as measurement of ionic current flowing through the pore.

Electrical measurements may be made using standard single channel recording equipment as describe in Stoddart D et al., Proc Natl Acad Sci, 12;106(19):7702-7, Lieberman
25 KR et al, J Am Chem Soc. 2010;132(50):17961-72, and International Application WO 2000/28312. Alternatively, electrical measurements may be made using a multi-channel system, for example as described in International Application WO 2009/077734 and International Application WO 2011/067559.

The method is preferably carried out with a potential applied across the membrane. The
30 applied potential may be a voltage potential. Alternatively, the applied potential may be a chemical potential. An example of this is using a salt gradient across a membrane, such as an amphiphilic layer. A salt gradient is disclosed in Holden *et al.*, J Am Chem Soc. 2007 Jul 11; 129(27):8650-5. In some instances, the current passing through the detector (or pore) as a

polynucleotide moves with respect to the pore is used to estimate or determine the sequence of the polynucleotide. This is strand sequencing.

The method comprises contacting the polynucleotide with a transmembrane pore. A transmembrane pore is a structure that crosses the membrane to some degree. It permits
5 hydrated ions driven by an applied potential to flow across or within the membrane. The transmembrane pore typically crosses the entire membrane so that hydrated ions may flow from one side of the membrane to the other side of the membrane. However, the transmembrane pore does not have to cross the membrane. It may be closed at one end. For instance, the pore may be a well, gap, channel, trench or slit in the membrane along which or into which hydrated ions
10 may flow.

Any transmembrane pore may be used in the invention. The pore may be biological or artificial. Suitable pores include, but are not limited to, protein pores, polynucleotide pores and solid state pores. The pore may be a DNA origami pore (Langecker *et al.*, Science, 2012; 338: 932-936).

15 The transmembrane pore is preferably a transmembrane protein pore. A transmembrane protein pore is a polypeptide or a collection of polypeptides that permits hydrated ions, such as analyte, to flow from one side of a membrane to the other side of the membrane. In the present invention, the transmembrane protein pore is capable of forming a pore that permits hydrated ions driven by an applied potential to flow from one side of the membrane to the other. The
20 transmembrane protein pore preferably permits analyte such as nucleotides to flow from one side of the membrane, such as a triblock copolymer membrane, to the other. The transmembrane protein pore allows a polynucleotide, such as DNA or RNA, to be moved through the pore.

The transmembrane protein pore may be a monomer or an oligomer. The pore is preferably made up of several repeating subunits, such as at least 6, at least 7, at least 8 or at least
25 9 subunits. The pore is preferably a hexameric, heptameric, octameric or nonameric pore. The pore may be a homo-oligomer or a hetero-oligomer.

The transmembrane protein pore typically comprises a barrel or channel through which the ions may flow. The subunits of the pore typically surround a central axis and contribute strands to a transmembrane β barrel or channel or a transmembrane α -helix bundle or channel.

30 The barrel or channel of the transmembrane protein pore typically comprises amino acids that facilitate interaction with analyte, such as nucleotides, polynucleotides or nucleic acids. These amino acids are preferably located near a constriction of the barrel or channel. The transmembrane protein pore typically comprises one or more positively charged amino acids, such as arginine, lysine or histidine, or aromatic amino acids, such as tyrosine or tryptophan.

These amino acids typically facilitate the interaction between the pore and nucleotides, polynucleotides or nucleic acids.

Transmembrane protein pores for use in accordance with the invention can be derived from β -barrel pores or α -helix bundle pores. β -barrel pores comprise a barrel or channel that is formed from β -strands. Suitable β -barrel pores include, but are not limited to, β -toxins, such as α -hemolysin, anthrax toxin and leukocidins, and outer membrane proteins/porins of bacteria, such as *Mycobacterium smegmatis* porin (Msp), for example MspA, MspB, MspC or MspD, lysenin, outer membrane porin F (OmpF), outer membrane porin G (OmpG), outer membrane phospholipase A and *Neisseria* autotransporter lipoprotein (NalP). α -helix bundle pores comprise a barrel or channel that is formed from α -helices. Suitable α -helix bundle pores include, but are not limited to, inner membrane proteins and α outer membrane proteins, such as WZA and ClyA toxin. The transmembrane pore may be derived from lysenin. Suitable pores derived from lysenin are disclosed in International Application No. PCT/GB2013/050667 (published as WO 2013/153359). The transmembrane pore may be derived from Msp or from α -hemolysin (α -HL).

The transmembrane protein pore is preferably derived from Msp, preferably from MspA. Such a pore will be oligomeric and typically comprises 7, 8, 9 or 10 monomers derived from Msp. The pore may be a homo-oligomeric pore derived from Msp comprising identical monomers. Alternatively, the pore may be a hetero-oligomeric pore derived from Msp comprising at least one monomer that differs from the others. Preferably the pore is derived from MspA or a homolog or paralog thereof.

A monomer derived from Msp typically comprises the sequence shown in SEQ ID NO: 2 or a variant thereof. SEQ ID NO: 2 is the MS-(B1)8 mutant of the MspA monomer. It includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K. A variant of SEQ ID NO: 2 is a polypeptide that has an amino acid sequence which varies from that of SEQ ID NO: 2 and which retains its ability to form a pore. The ability of a variant to form a pore can be assayed using any method known in the art. For instance, the variant may be inserted into an amphiphilic layer along with other appropriate subunits and its ability to oligomerise to form a pore may be determined. Methods are known in the art for inserting subunits into membranes, such as amphiphilic layers. For example, subunits may be suspended in a purified form in a solution containing a triblock copolymer membrane such that it diffuses to the membrane and is inserted by binding to the membrane and assembling into a functional state. Alternatively, subunits may be directly inserted into the membrane using the "pick and place" method

described in M.A. Holden, H. Bayley. J. Am. Chem. Soc. 2005, 127, 6502-6503 and International Application No. PCT/GB2006/001057 (published as WO 2006/100484).

Over the entire length of the amino acid sequence of SEQ ID NO: 2, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 2 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 100 or more, for example 125, 150, 175 or 200 or more, contiguous amino acids ("hard homology").

Standard methods in the art may be used to determine homology. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology, for example used on its default settings (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent residues or corresponding sequences (typically on their default settings)), for example as described in Altschul S. F. (1993) *J Mol Evol* 36:290-300; Altschul, S.F *et al* (1990) *J Mol Biol* 215:403-10. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

SEQ ID NO: 2 is the MS-(B1)8 mutant of the MspA monomer. The variant may comprise any of the mutations in the MspB, C or D monomers compared with MspA. The mature forms of MspB, C and D are shown in SEQ ID NOs: 5 to 7. In particular, the variant may comprise the following substitution present in MspB: A138P. The variant may comprise one or more of the following substitutions present in MspC: A96G, N102E and A138P. The variant may comprise one or more of the following mutations present in MspD: Deletion of G1, L2V, E5Q, L8V, D13G, W21A, D22E, K47T, I49H, I68V, D91G, A96Q, N102D, S103T, V104I, S136K and G141A. The variant may comprise combinations of one or more of the mutations and substitutions from Msp B, C and D. The variant preferably comprises the mutation L88N. A variant of SEQ ID NO: 2 has the mutation L88N in addition to all the mutations of MS-B1 and is called MS-(B2)8. The pore used in the invention is preferably MS-(B2)8. A variant of SEQ ID NO: 2 has the mutations G75S/G77S/L88N/Q126R in addition to all the mutations of MS-B1 and is called MS-B2C. The pore used in the invention is preferably MS-(B2)8 or MS-(B2C)8.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 2 in addition to those discussed above, for example up to 1, 2, 3, 4, 5, 10, 20 or 30 substitutions. Conservative substitutions replace amino acids with other amino acids of similar chemical structure, similar chemical properties or similar side-chain volume. The amino acids introduced may have similar polarity, hydrophilicity, hydrophobicity, basicity, acidity, neutrality or charge to the amino acids they replace. Alternatively, the conservative substitution may introduce another amino acid that is aromatic or aliphatic in the place of a pre-existing aromatic or aliphatic amino acid. Conservative amino acid changes are well-known in the art and may be selected in accordance with the properties of the 20 main amino acids as defined in Table 2 below. Where amino acids have similar polarity, this can also be determined by reference to the hydropathy scale for amino acid side chains in Table 3.

Table 2 – Chemical properties of amino acids

Ala	aliphatic, hydrophobic, neutral	Met	hydrophobic, neutral
Cys	polar, hydrophobic, neutral	Asn	polar, hydrophilic, neutral
Asp	polar, hydrophilic, charged (-)	Pro	hydrophobic, neutral
Glu	polar, hydrophilic, charged (-)	Gln	polar, hydrophilic, neutral
Phe	aromatic, hydrophobic, neutral	Arg	polar, hydrophilic, charged (+)
Gly	aliphatic, neutral	Ser	polar, hydrophilic, neutral
His	aromatic, polar, hydrophilic, charged (+)	Thr	polar, hydrophilic, neutral
Ile	aliphatic, hydrophobic, neutral	Val	aliphatic, hydrophobic, neutral
Lys	polar, hydrophilic, charged(+)	Trp	aromatic, hydrophobic, neutral
Leu	aliphatic, hydrophobic, neutral	Tyr	aromatic, polar, hydrophobic

Table 3 - Hydropathy scale

Side Chain	Hydropathy
Ile	4.5
Val	4.2
Leu	3.8
Phe	2.8
Cys	2.5
Met	1.9
Ala	1.8
Gly	-0.4
Thr	-0.7

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	Ser	-0.8
	Trp	-0.9
	Tyr	-1.3
	Pro	-1.6
5	His	-3.2
	Glu	-3.5
	Gln	-3.5
	Asp	-3.5
	Asn	-3.5
10	Lys	-3.9
	Arg	-4.5

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 2 may additionally be deleted from the polypeptides described above. Up to 1, 2, 3, 4, 5, 10, 20 or 30 residues may be deleted, or more.

Variants may include fragments of SEQ ID NO: 2. Such fragments retain pore forming activity. Fragments may be at least 50, 100, 150 or 200 amino acids in length. Such fragments may be used to produce the pores. A fragment preferably comprises the pore forming domain of SEQ ID NO: 2. Fragments must include one of residues 88, 90, 91, 105, 118 and 134 of SEQ ID NO: 2. Typically, fragments include all of residues 88, 90, 91, 105, 118 and 134 of SEQ ID NO: 2.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the amino terminal or carboxy terminal of the amino acid sequence of SEQ ID NO: 2 or polypeptide variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer, for example up to 50 or 100 amino acids. A carrier protein may be fused to an amino acid sequence according to the invention. Other fusion proteins are discussed in more detail below.

As discussed above, a variant is a polypeptide that has an amino acid sequence which varies from that of SEQ ID NO: 2 and which retains its ability to form a pore. A variant typically contains the regions of SEQ ID NO: 2 that are responsible for pore formation. The pore forming ability of Msp, which contains a β -barrel, is provided by β -sheets in each subunit. A variant of SEQ ID NO: 2 typically comprises the regions in SEQ ID NO: 2 that form β -sheets. One or more modifications can be made to the regions of SEQ ID NO: 2 that form β -sheets as long as the resulting variant retains its ability to form a pore. A variant of SEQ ID NO: 2 preferably includes one or more modifications, such as substitutions, additions or deletions, within its α -helices and/or loop regions.

The monomers derived from Msp may be modified to assist their identification or purification, for example by the addition of histidine residues (a hist tag), aspartic acid residues (an asp tag), a streptavidin tag or a flag tag, or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. An alternative to introducing a genetic tag is to chemically react a tag onto a native or engineered position on the pore. An example of this would be to react a gel-shift reagent to a cysteine engineered on the outside of the pore. This has been demonstrated as a method for separating hemolysin hetero-oligomers (Chem Biol. 1997 Jul; 4(7):497-505).

The monomer derived from Msp may be labelled with a revealing label. The revealing label may be any suitable label which allows the pore to be detected. Suitable labels are described below.

The monomer derived from Msp may also be produced using D-amino acids. For instance, the monomer derived from Msp may comprise a mixture of L-amino acids and D-amino acids. This is conventional in the art for producing such proteins or peptides.

The monomer derived from Msp contains one or more specific modifications to facilitate nucleotide discrimination. The monomer derived from Msp may also contain other non-specific modifications as long as they do not interfere with pore formation. A number of non-specific side chain modifications are known in the art and may be made to the side chains of the monomer derived from Msp. Such modifications include, for example, reductive alkylation of amino acids by reaction with an aldehyde followed by reduction with NaBH₄, amidination with methylacetimidate or acylation with acetic anhydride.

The monomer derived from Msp can be produced using standard methods known in the art. The monomer derived from Msp may be made synthetically or by recombinant means. For example, the pore may be synthesized by *in vitro* translation and transcription (IVTT). Suitable methods for producing pores are discussed in International Application Nos. PCT/GB09/001690 (published as WO 2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603). Methods for inserting pores into membranes are discussed.

The transmembrane protein pore is also preferably derived from α -hemolysin (α -HL).

The wild type α -HL pore is formed of seven identical monomers or subunits (i.e. it is heptameric). The sequence of one monomer or subunit of α -hemolysin-NN is shown in SEQ ID NO: 4. The transmembrane protein pore preferably comprises seven monomers each comprising the sequence shown in SEQ ID NO: 4 or a variant thereof. Amino acids 1, 7 to 21, 31 to 34, 45 to 51, 63 to 66, 72, 92 to 97, 104 to 111, 124 to 136, 149 to 153, 160 to 164, 173 to 206, 210 to

213, 217, 218, 223 to 228, 236 to 242, 262 to 265, 272 to 274, 287 to 290 and 294 of SEQ ID NO: 4 form loop regions. Residues 113 and 147 of SEQ ID NO: 4 form part of a constriction of the barrel or channel of α -HL.

In such embodiments, a pore comprising seven proteins or monomers each comprising the sequence shown in SEQ ID NO: 4 or a variant thereof are preferably used in the method of the invention. The seven proteins may be the same (homo-heptamer) or different (hetero-heptamer).

A variant of SEQ ID NO: 4 is a protein that has an amino acid sequence which varies from that of SEQ ID NO: 4 and which retains its pore forming ability. The ability of a variant to form a pore can be assayed using any method known in the art. For instance, the variant may be inserted into an amphiphilic layer, such as a triblock copolymer membrane, along with other appropriate subunits and its ability to oligomerise to form a pore may be determined. Methods are known in the art for inserting subunits into amphiphilic layers, such as triblock copolymer membranes. Suitable methods are discussed above.

The variant may include modifications that facilitate covalent attachment to or interaction with the construct. The variant preferably comprises one or more reactive cysteine residues that facilitate attachment to the construct. For instance, the variant may include a cysteine at one or more of positions 8, 9, 17, 18, 19, 44, 45, 50, 51, 237, 239 and 287 and/or on the amino or carboxy terminus of SEQ ID NO: 4. Preferred variants comprise a substitution of the residue at position 8, 9, 17, 237, 239 and 287 of SEQ ID NO: 4 with cysteine (A8C, T9C, N17C, K237C, S239C or E287C). The variant is preferably any one of the variants described in International Application No. PCT/GB09/001690 (published as WO 2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603).

The variant may also include modifications that facilitate any interaction with nucleotides.

The variant may be a naturally occurring variant which is expressed naturally by an organism, for instance by a *Staphylococcus* bacterium. Alternatively, the variant may be expressed *in vitro* or recombinantly by a bacterium such as *Escherichia coli*. Variants also include non-naturally occurring variants produced by recombinant technology. Over the entire length of the amino acid sequence of SEQ ID NO: 4, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant polypeptide may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 4 over the entire

sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 200 or more, for example 230, 250, 270 or 280 or more, contiguous amino acids (“hard homology”). Homology can be determined as discussed above.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 4 in addition to those discussed above, for example up to 1, 2, 3, 4, 5, 10, 20 or 30 substitutions. Conservative substitutions may be made as discussed above.

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 4 may additionally be deleted from the polypeptides described above. Up to 1, 2, 3, 4, 5, 10, 20 or 30 residues may be deleted, or more.

Variants may be fragments of SEQ ID NO: 4. Such fragments retain pore-forming activity. Fragments may be at least 50, 100, 200 or 250 amino acids in length. A fragment preferably comprises the pore-forming domain of SEQ ID NO: 4. Fragments typically include residues 119, 121, 135, 113 and 139 of SEQ ID NO: 4.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the amino terminus or carboxy terminus of the amino acid sequence of SEQ ID NO: 4 or a variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer, for example up to 50 or 100 amino acids. A carrier protein may be fused to a pore or variant.

As discussed above, a variant of SEQ ID NO: 4 is a subunit that has an amino acid sequence which varies from that of SEQ ID NO: 4 and which retains its ability to form a pore. A variant typically contains the regions of SEQ ID NO: 4 that are responsible for pore formation. The pore forming ability of α -HL, which contains a β -barrel, is provided by β -strands in each subunit. A variant of SEQ ID NO: 4 typically comprises the regions in SEQ ID NO: 4 that form β -strands. The amino acids of SEQ ID NO: 4 that form β -strands are discussed above. One or more modifications can be made to the regions of SEQ ID NO: 4 that form β -strands as long as the resulting variant retains its ability to form a pore. Specific modifications that can be made to the β -strand regions of SEQ ID NO: 4 are discussed above.

A variant of SEQ ID NO: 4 preferably includes one or more modifications, such as substitutions, additions or deletions, within its α -helices and/or loop regions. Amino acids that form α -helices and loops are discussed above.

The variant may be modified to assist its identification or purification as discussed above.

Pores derived from α -HL can be made as discussed above with reference to pores derived from Msp.

In some embodiments, the transmembrane protein pore is chemically modified. The pore can be chemically modified in any way and at any site. The transmembrane protein pore is preferably chemically modified by attachment of a molecule to one or more cysteines (cysteine linkage), attachment of a molecule to one or more lysines, attachment of a molecule to one or more non-natural amino acids, enzyme modification of an epitope or modification of a terminus. Suitable methods for carrying out such modifications are well-known in the art. The transmembrane protein pore may be chemically modified by the attachment of any molecule. For instance, the pore may be chemically modified by attachment of a dye or a fluorophore.

Any number of the monomers in the pore may be chemically modified. One or more, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10, of the monomers is preferably chemically modified as discussed above.

The reactivity of cysteine residues may be enhanced by modification of the adjacent residues. For instance, the basic groups of flanking arginine, histidine or lysine residues will change the pKa of the cysteines thiol group to that of the more reactive S⁻ group. The reactivity of cysteine residues may be protected by thiol protective groups such as dTNB. These may be reacted with one or more cysteine residues of the pore before a linker is attached.

The molecule (with which the pore is chemically modified) may be attached directly to the pore or attached via a linker as disclosed in International Application Nos. PCT/GB09/001690 (published as WO 2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603).

Any of the proteins described herein, such as the transmembrane protein pores, may be modified to assist their identification or purification, for example by the addition of histidine residues (a his tag), aspartic acid residues (an asp tag), a streptavidin tag, a flag tag, a SUMO tag, a GST tag or a MBP tag, or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. An alternative to introducing a genetic tag is to chemically react a tag onto a native or engineered position on the pore or construct. An example of this would be to react a gel-shift reagent to a cysteine engineered on the outside of the pore. This has been demonstrated as a method for separating hemolysin hetero-oligomers (Chem Biol. 1997 Jul; 4(7):497-505).

The pore may be labelled with a revealing label. The revealing label may be any suitable label which allows the pore to be detected. Suitable labels include, but are not limited to,

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fluorescent molecules, radioisotopes, e.g. ¹²⁵I, ³⁵S, enzymes, antibodies, antigens,
polynucleotides and ligands such as biotin.

Any of the proteins described herein, such as the transmembrane protein pores, may be made synthetically or by recombinant means. For example, the pore may be synthesized by *in vitro* translation and transcription (IVTT). The amino acid sequence of the pore may be modified to include non-naturally occurring amino acids or to increase the stability of the protein. When a protein is produced by synthetic means, such amino acids may be introduced during production. The pore may also be altered following either synthetic or recombinant production.

The pore may also be produced using D-amino acids. For instance, the pore or construct may comprise a mixture of L-amino acids and D-amino acids. This is conventional in the art for producing such proteins or peptides.

The pore may also contain other non-specific modifications as long as they do not interfere with pore formation or construct function. A number of non-specific side chain modifications are known in the art and may be made to the side chains of the protein(s). Such modifications include, for example, reductive alkylation of amino acids by reaction with an aldehyde followed by reduction with NaBH₄, amidination with methylacetimidate or acylation with acetic anhydride.

Any of the proteins described herein, such as the transmembrane protein pores, can be produced using standard methods known in the art. Polynucleotide sequences encoding a pore or construct may be derived and replicated using standard methods in the art. Polynucleotide sequences encoding a pore or construct may be expressed in a bacterial host cell using standard techniques in the art. The pore may be produced in a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide. These methods are described in Sambrook, J. and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

The pore may be produced in large scale following purification by any protein liquid chromatography system from protein producing organisms or after recombinant expression. Typical protein liquid chromatography systems include FPLC, AKTA systems, the Bio-Cad system, the Bio-Rad BioLogic system and the Gilson HPLC system.

Spacers

The one or more helicases may be stalled at one or more spacers as discussed in International Application No. PCT/GB2014/050175. Any configuration of one or more helicases and one or more spacers disclosed in the International Application may be used in this invention.

When a part of the polynucleotide enters the pore and moves through the pore along the field resulting from the applied potential, the one or more helicases are moved past the spacer by the pore as the polynucleotide moves through the pore. This is because the polynucleotide (including the one or more spacers) moves through the pore and the one or more helicases remain on top of the pore.

The one or more spacers are preferably part of the polynucleotide, for instance it/they interrupt(s) the polynucleotide sequence. The one or more spacers are preferably not part of one or more blocking molecules, such as speed bumps, hybridised to the polynucleotide.

There may be any number of spacers in the polynucleotide, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more spacers. There are preferably two, four or six spacers in the polynucleotide. There may be spacer in different regions of the polynucleotide, such as a spacer in the leader sequence and a spacer in the bridging moiety or the hairpin loop.

The one or more spacers each provides an energy barrier which the one or more helicases cannot overcome even in the active mode. The one or more spacers may stall the one or more helicases by reducing the traction of the helicase (for instance by removing the bases from the nucleotides in the polynucleotide) or physically blocking movement of the one or more helicases (for instance using a bulky chemical group).

The one or more spacers may comprise any molecule or combination of molecules that stalls the one or more helicases. The one or more spacers may comprise any molecule or combination of molecules that prevents the one or more helicases from moving along the polynucleotide. It is straightforward to determine whether or not the one or more helicases are stalled at one or more spacers in the absence of a transmembrane pore and an applied potential. For instance, this can be assayed as shown in the Examples, for instance the ability of a helicase to move past a spacer and displace a complementary strand of DNA can be measured by PAGE.

The one or more spacers typically comprise a linear molecule, such as a polymer. The one or more spacers typically have a different structure from the polynucleotide. For instance, if the polynucleotide is DNA, the one or more spacers are typically not DNA. In particular, if the polynucleotide is deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), the one or more spacers preferably comprise peptide nucleic acid (PNA), glycerol nucleic acid (GNA), threose nucleic acid (TNA), locked nucleic acid (LNA) or a synthetic polymer with nucleotide side chains. The one or more spacers may comprise one or more nucleotides in the opposite direction

from the polynucleotide. For instance, the one or more spacers may comprise one or more nucleotides in the 3' to 5' direction when the polynucleotide is in the 5' to 3' direction. The nucleotides may be any of those discussed above.

The one or more spacers preferably comprises one or more nitroindoles, such as one or more 5-nitroindoles, one or more inosines, one or more acridines, one or more 2-aminopurines, one or more 2-6-diaminopurines, one or more 5-bromo-deoxyuridines, one or more inverted thymidines (inverted dTs), one or more inverted dideoxy-thymidines (ddTs), one or more dideoxy-cytidines (ddCs), one or more 5-methylcytidines, one or more 5-hydroxymethylcytidines, one or more 2'-O-Methyl RNA bases, one or more Iso-deoxycytidines (Iso-dCs), one or more Iso-deoxyguanosines (Iso-dGs), one or more iSpC3 groups (i.e. nucleotides which lack sugar and a base), one or more photo-cleavable (PC) groups, one or more hexandiol groups, one or more spacer 9 (iSp9) groups, one or more spacer 18 (iSp18) groups, a polymer or one or more thiol connections. The one or more spacers may comprise any combination of these groups. Many of these groups are commercially available from IDT® (Integrated DNA Technologies®).

The one or more spacers may contain any number of these groups. For instance, for 2-aminopurines, 2-6-diaminopurines, 5-bromo-deoxyuridines, inverted dTs, ddTs, ddCs, 5-methylcytidines, 5-hydroxymethylcytidines, 2'-O-Methyl RNA bases, Iso-dCs, Iso-dGs, iSpC3 groups, PC groups, hexandiol groups and thiol connections, the one or more spacers preferably comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more. The one or more spacers preferably comprise 2, 3, 4, 5, 6, 7, 8 or more iSp9 groups. The one or more spacers preferably comprise 2, 3, 4, 5 or 6 or more iSp18 groups. The most preferred spacer is four iSp18 groups.

The polymer is preferably a polypeptide or a polyethylene glycol (PEG). The polypeptide preferably comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more amino acids. The PEG preferably comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more monomer units.

The one or more spacers preferably comprise one or more abasic nucleotides (i.e. nucleotides lacking a nucleobase), such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more abasic nucleotides. The nucleobase can be replaced by -H (idSp) or -OH in the abasic nucleotide. Abasic spacers can be inserted into polynucleotides by removing the nucleobases from one or more adjacent nucleotides. For instance, polynucleotides may be modified to include 3-methyladenine, 7-methylguanine, 1,N6-ethenoadenine inosine or hypoxanthine and the nucleobases may be removed from these nucleotides using Human Alkyladenine DNA Glycosylase (hAAG). Alternatively, polynucleotides may be modified to include uracil and the

nucleobases removed with Uracil-DNA Glycosylase (UDG). In one embodiment, the one or more spacers do not comprise any abasic nucleotides.

The one or more helicases may be stalled by (i.e. before) or on each linear molecule spacer. If linear molecule spacers are used, the polynucleotide is preferably provided with a double stranded region of polynucleotide adjacent to the end of each spacer past which the one or more helicases are to be moved. The double stranded region typically helps to stall the one or more helicases on the adjacent spacer. The presence of the double stranded region(s) is particularly preferred if the method is carried out at a salt concentration of about 100 mM or lower. Each double stranded region is typically at least 10, such as at least 12, nucleotides in length. If the polynucleotide used in the invention is single stranded, a double stranded region may be formed by hybridising a shorter polynucleotide to a region adjacent to a spacer. The shorter polynucleotide is typically formed from the same nucleotides as the polynucleotide, but may be formed from different nucleotides. For instance, the shorter polynucleotide may be formed from LNA.

If linear molecule spacers are used, the polynucleotide is preferably provided with a blocking molecule at the end of each spacer opposite to the end past which the one or more helicases are to be moved. This can help to ensure that the one or more helicases remain stalled on each spacer. It may also help retain the one or more helicases on the polynucleotide in the case that it/they diffuse(s) off in solution. The blocking molecule may be any of the chemical groups discussed below which physically cause the one or more helicases to stall. The blocking molecule may be a double stranded region of polynucleotide.

The one or more spacers preferably comprise one or more chemical groups which physically cause the one or more helicases to stall. The one or more chemical groups are preferably one or more pendant chemical groups. The one or more chemical groups may be attached to one or more nucleobases in the polynucleotide. The one or more chemical groups may be attached to the polynucleotide backbone. Any number of these chemical groups may be present, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more. Suitable groups include, but are not limited to, fluorophores, streptavidin and/or biotin, cholesterol, methylene blue, dinitrophenols (DNPs), digoxigenin and/or anti-digoxigenin and dibenzylcyclooctyne groups.

Different spacers in the polynucleotide may comprise different stalling molecules. For instance, one spacer may comprise one of the linear molecules discussed above and another spacer may comprise one or more chemical groups which physically cause the one or more helicases to stall. A spacer may comprise any of the linear molecules discussed above and one

or more chemical groups which physically cause the one or more helicases to stall, such as one or more abasics and a fluorophore.

Suitable spacers can be designed depending on the type of polynucleotide and the conditions under which the method of the invention is carried out. Most helicases bind and move along DNA and so may be stalled using anything that is not DNA. Suitable molecules are discussed above.

The method of the invention is preferably carried out in the presence of free nucleotides and/or the presence of a helicase cofactor. This is discussed in more detail below. In the absence of the transmembrane pore and an applied potential, the one or more spacers are preferably capable of stalling the one or more helicases in the presence of free nucleotides and/or the presence of a helicase cofactor.

If the method of the invention is carried out in the presence of free nucleotides and a helicase cofactor as discussed below (such that the one of more helicases are in the active mode), one or more longer spacers are typically used to ensure that the one or more helicases are stalled on the polynucleotide before they are contacted with the transmembrane pore and a potential is applied. One or more shorter spacers may be used in the absence of free nucleotides and a helicase cofactor (such that the one or more helicases are in the inactive mode).

The salt concentration also affects the ability of the one or more spacers to stall the one or more helicases. In the absence of the transmembrane pore and an applied potential, the one or more spacers are preferably capable of stalling the one or more helicases at a salt concentration of about 100 mM or lower. The higher the salt concentration used in the method of the invention, the shorter the one or more spacers that are typically used and *vice versa*.

Preferred combinations of features are shown in Table 4 below.

Polynucleotide	Spacer composition*	Spacer length (i.e. number of *)	Salt []	Free nucleotides?	Helicase cofactor?
DNA	iSpC3	4	1 M	Yes	Yes
DNA	iSp18	4	100-1000 mM	Yes	Yes
DNA	iSp18	6	<100-1000 mM	Yes	Yes
DNA	iSp18	2	1 M	Yes	Yes
DNA	iSpC3	12	<100-1000 mM	Yes	Yes
DNA	iSpC3	20	<100-1000 mM	Yes	Yes
DNA	iSp9	6	100-1000 mM	Yes	Yes

DNA	idSp	4	1 M	Yes	Yes
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The method may concern moving two or more helicases past a spacer. In such instances, the length of the spacer is typically increased to prevent the trailing helicase from pushing the leading helicase past the spacer in the absence of the pore and applied potential. If the method concerns moving two or more helicases past one or more spacers, the spacer lengths discussed above may be increased at least 1.5 fold, such 2 fold, 2.5 fold or 3 fold. For instance, if the method concerns moving two or more helicases past one or more spacers, the spacer lengths in the third column of Table 4 above may be increased 1.5 fold, 2 fold, 2.5 fold or 3 fold.

The two or more helicases may also be separated such that each has its own one or more spacers. This is discussed in more detail below.

Double stranded polynucleotide

The polynucleotide may be double stranded. If the polynucleotide is double stranded, the method preferably comprises providing the polynucleotide with a bridging moiety adaptor, such as a hairpin loop adaptor, at one end of the polynucleotide and separating the two strands of the polynucleotide to form a single stranded polynucleotide construct. The single stranded polynucleotide construct may then be moved through the pore in accordance with the invention. Linking and interrogating both strands on a double stranded construct in this way increases the efficiency and accuracy of characterisation.

The bridging moiety is capable of linking the two strands of the polynucleotide. The bridging moiety typically covalently links the two strands of the polynucleotide. The bridging moiety can be anything that is capable of linking the two strands of the polynucleotide, provided that the bridging moiety does not interfere with movement of the polynucleotide through the transmembrane pore.

The bridging moiety may be linked to the polynucleotide by any suitable means known in the art. The bridging moiety may be synthesized separately and chemically attached or enzymatically ligated to the polynucleotide. Alternatively, the bridging moiety may be generated in the processing of the polynucleotide.

The bridging moiety is linked to the polynucleotide at or near one end of the polynucleotide. The bridging moiety is preferably linked to the polynucleotide within 10 nucleotides of the end of the polynucleotide

Suitable bridging moieties include, but are not limited to a polymeric linker, a chemical linker, a polynucleotide or a polypeptide. Preferably, the bridging moiety comprises DNA,

RNA, modified DNA (such as abasic DNA), RNA, PNA, LNA or PEG. The bridging moiety is more preferably DNA or RNA.

The bridging moiety is most preferably a hairpin loop or a hairpin loop adaptor. Suitable hairpin loop adaptors can be designed using methods known in the art. The hairpin loop may be
5 any length. The hairpin loop is typically 110 or fewer nucleotides, such as 100 or fewer nucleotides, 90 or fewer nucleotides, 80 or fewer nucleotides, 70 or fewer nucleotides, 60 or fewer nucleotides, 50 or fewer nucleotides, 40 or fewer nucleotides, 30 or fewer nucleotides, 20 or fewer nucleotides or 10 or fewer nucleotides, in length. The hairpin loop is preferably from
10 of the hairpin loop, such as from 50 to 110 nucleotides, are preferred if the loop is involved in the differential selectability of the adaptor. Similarly, shorter lengths of the hairpin loop, such as from 1 to 5 nucleotides, are preferred if the loop is not involved in the selectable binding as discussed below.

The bridging moiety adaptor or hairpin loop adaptor may be ligated to either end of the
15 polynucleotide, i.e. the 5' or the 3' end. The bridging moiety adaptor or hairpin loop adaptor may be ligated to the polynucleotide using any method known in the art. The bridging moiety adaptor or hairpin adaptor may be ligated using a ligase, such as T4 DNA ligase, *E. coli* DNA ligase, Taq DNA ligase, Tma DNA ligase and 9°N DNA ligase.

The two strands of the polynucleotide may be separated using any method known in the
20 art. For instance, they may be separated by the one or more helicase and/or the one or more molecular brakes or using conditions which favour dehybridisation (examples of conditions which favour dehybridisation include, but are not limited to, high temperature, high pH and the addition of agents that can disrupt hydrogen bonding or base pairing, such as formamide and urea).

The bridging moiety adaptor, such as the hairpin loop adaptor, preferably comprises a
25 selectable binding moiety. This allows the polynucleotide to be purified or isolated. A selectable binding moiety is a moiety that can be selected on the basis of its binding properties. Hence, a selectable binding moiety is preferably a moiety that specifically binds to a surface. A selectable binding moiety specifically binds to a surface if it binds to the surface to a much
30 greater degree than any other moiety used in the invention. In preferred embodiments, the moiety binds to a surface to which no other moiety used in the invention binds.

Suitable selective binding moieties are known in the art. Preferred selective binding moieties include, but are not limited to, biotin, a polynucleotide sequence, antibodies, antibody fragments, such as Fab and ScSv, antigens, polynucleotide binding proteins, poly histidine tails

and GST tags. The most preferred selective binding moieties are biotin and a selectable polynucleotide sequence. Biotin specifically binds to a surface coated with avidins. Selectable polynucleotide sequences specifically bind (i.e. hybridise) to a surface coated with homologous sequences. Alternatively, selectable polynucleotide sequences specifically bind to a surface
5 coated with polynucleotide binding proteins.

The bridging moiety adaptor (such as the hairpin loop adaptor) and/or the selectable binding moiety may comprise a region that can be cut, nicked, cleaved or hydrolysed. Such a region can be designed to allow the first and/or second polynucleotide to be removed from the surface to which it is bound following purification or isolation. Suitable regions are known in
10 the art. Suitable regions include, but are not limited to, an RNA region, a region comprising desthiobiotin and streptavidin, a disulphide bond and a photocleavable region.

The one or more molecular brakes are preferably attached to the bridging moiety adaptor (such as the hairpin loop adaptor).

15 *Leader sequence*

The polynucleotide may be provided with a leader sequence which preferentially threads into the pore. The leader sequence facilitates the method of the invention. The leader sequence is designed to preferentially thread into the transmembrane pore and thereby facilitate the movement of polynucleotide analyte through the pore. The leader sequence can also be used to
20 link the polynucleotide to the one or more anchors as discussed above.

The leader sequence typically comprises a polymer. The polymer is preferably negatively charged. The polymer is preferably a polynucleotide, such as DNA or RNA, a modified polynucleotide (such as abasic DNA), PNA, LNA, polyethylene glycol (PEG) or a polypeptide. The leader preferably comprises a polynucleotide and more preferably comprises a
25 single stranded polynucleotide. The leader sequence can comprise any of the polynucleotides discussed above. The single stranded leader sequence most preferably comprises a single strand of DNA, such as a poly dT section. The leader sequence preferably comprises the one or more spacers.

The leader sequence can be any length, but is typically 10 to 150 nucleotides in length,
30 such as from 20 to 150 nucleotides in length. The length of the leader typically depends on the transmembrane pore used in the method.

Double coupling

The method of the invention may involve double coupling of double stranded polynucleotides. In a preferred embodiment, the invention provides a method of controlling the movement of a double stranded polynucleotide through a transmembrane pore, comprising:

- (a) providing the double stranded polynucleotide with a Y adaptor at one end and a
5 bridging moiety adaptor, such as a hairpin loop adaptor, at the other end, wherein the Y adaptor comprises the one or more helicases and one or more first anchors for coupling the polynucleotide to the membrane, wherein the bridging moiety adaptor comprises the one or more molecular brakes and one or more second anchors for coupling the polynucleotide to the membrane and wherein the strength of coupling of the bridging moiety adaptor to the membrane
10 is greater than the strength of coupling of the Y adaptor to the membrane;
- (b) contacting the polynucleotide provided in step (a) with the pore; and
- (c) applying a potential across the pore such that the one or more helicases and the one or more molecular brakes are brought together and both control the movement of the polynucleotide through the pore.

15 This type of method is discussed in detail in UK Application No. 1406147.7.

The double stranded polynucleotide is provided with a Y adaptor at one end and a bridging moiety adaptor at the other end. The Y adaptor and/or the bridging moiety adaptor are typically polynucleotide adaptors. They may be formed from any of the polynucleotides discussed above.

20 The Y adaptor typically comprises (a) a double stranded region and (b) a single stranded region or a region that is not complementary at the other end. The Y adaptor may be described as having an overhang if it comprises a single stranded region. The presence of a non-complementary region in the Y adaptor gives the adaptor its Y shape since the two strands typically do not hybridise to each other unlike the double stranded portion. The Y adaptor
25 comprises the one or more first anchors. Anchors are discussed in more detail above.

The Y adaptor preferably comprises a leader sequence which preferentially threads into the pore. This is discussed above.

The bridging moiety adaptor preferably comprises a selectable binding moiety as discussed above. The bridging moiety adaptor and/or the selectable binding moiety may
30 comprise a region that can be cut, nicked, cleaved or hydrolysed as discussed above.

The Y adaptor and/or the bridging moiety adaptor may be ligated to the polynucleotide using any method known in the art. One or both of the adaptors may be ligated using a ligase, such as T4 DNA ligase, *E. coli* DNA ligase, Taq DNA ligase, Tma DNA ligase and 9°N DNA

ligase. Alternatively, the adaptors may be added to the polynucleotide using the methods of the invention discussed below.

In a preferred embodiment, step a) of the method comprises modifying the double stranded polynucleotide so that it comprises the Y adaptor at one end and the bridging moiety adaptor at the other end. Any manner of modification can be used. The method preferably comprises modifying the double stranded polynucleotide in accordance with the invention. This is discussed in more detail below. The methods of modification and characterisation may be combined in any way.

The strength of coupling (or binding) of the bridging moiety adaptor to the membrane is greater than the strength of coupling (or binding) of the Y adaptor to the membrane. This can be measured in any way. A suitable method for measuring the strength of coupling (or binding) is disclosed in the Examples of UK Application No. 1406147.7.

The strength of coupling (or binding) of the bridging moiety adaptor is preferably at least 1.5 times the strength of coupling (or binding) of the Y adaptor, such as at least twice, at least three times, at least four times, at least five or at least ten times the strength of coupling (or binding) of the Y adaptor. The affinity constant (K_d) of the bridging moiety adaptor for the membrane is preferably at least 1.5 times the affinity constant of the Y adaptor, such as at least twice, at least three times, at least four times, at least five or at least ten times the strength of coupling of the Y adaptor.

There are several ways in which the bridging moiety adaptor couples (or binds) more strongly to the membrane than the Y adaptor. For instance, the bridging moiety adaptor may comprise more anchors than the Y adaptor. For instance, the bridging moiety adaptor may comprise 2, 3 or more second anchors whereas the Y adaptor may comprise one first anchor.

The strength of coupling (or binding) of the one or more second anchors to the membrane may be greater than the strength of coupling (or binding) of the one or more first anchors to the membrane. The strength of coupling (or binding) of the one or more second anchors to the bridging moiety adaptor may be greater than the strength of coupling (or binding) of the one or more first anchors to the Y adaptor. The one or more first anchors and the one or more second anchors may be attached to their respective adaptors via hybridisation and the strength of hybridisation is greater in the one or more second anchors than in the one or more first anchors. Any combination of these embodiments may also be used in the invention. Strength of coupling (or binding) may be measured using known techniques in the art.

The one or more second anchors preferably comprise one or more groups which couples(s) (or bind(s)) to the membrane with a greater strength than the one or more groups in

the one or more first anchors which couple(s) (or bind(s)) to the membrane. In preferred embodiments, the bridging moiety adaptor/one or more second anchors couple (or bind) to the membrane using cholesterol and the Y adaptor/one or more first anchors couple (or bind) to the membrane using palmitate. Cholesterol binds to triblock copolymer membranes and lipid
5 membranes more strongly than palmitate. In an alternative embodiment, the bridging moiety adaptor/one or more second anchors couple (or bind) to the membrane using a mono-acyl species, such as palmitate, and the Y adaptor/one or more first anchors couple (or bind) to the membrane using a diacyl species, such as dipalmitoylphosphatidylcholine.

10 *Adding bridging moieties and leader sequences*

Before provision with one or more helicases and one or more molecular brakes attached, a double stranded polynucleotide may be contacted with a MuA transposase and a population of double stranded MuA substrates, wherein a proportion of the substrates in the population are Y adaptors comprising the leader sequence and wherein a proportion of the substrates in the
15 population are bridging moiety adaptors, such as hairpin loop adaptors. The transposase fragments the double stranded polynucleotide analyte and ligates MuA substrates to one or both ends of the fragments. This produces a plurality of modified double stranded polynucleotides comprising the leader sequence at one end and the bridging moiety (or hairpin loop) at the other. The modified double stranded polynucleotides may then be investigated using the method of the
20 invention.

Each substrate in the population preferably comprises at least one overhang of universal nucleotides such that the transposase fragments the template polynucleotide and ligates a substrate to one or both ends of the double stranded fragments and thereby produces a plurality of fragment/substrate constructs and wherein the method further comprises ligating the
25 overhangs to the fragments in the constructs and thereby producing a plurality of modified double stranded polynucleotides. Suitable universal nucleotides are discussed above. The overhang is preferably five nucleotides in length.

Alternatively, each substrate in population preferably comprises (i) at least one overhang and (ii) at least one nucleotide in the same strand as the at least one overhang which comprises a
30 nucleoside that is not present in the template polynucleotide such that the transposase fragments the template polynucleotide and ligates a substrate to one or both ends of the double stranded fragments and thereby produces a plurality of fragment/substrate constructs, and wherein the method further comprises (a) removing the overhangs from the constructs by selectively removing the at least one nucleotide and thereby producing a plurality of double stranded

constructs comprising single stranded gaps and (b) repairing the single stranded gaps in the constructs and thereby producing a plurality of modified double stranded polynucleotides. The polynucleotide analyte typically comprises the nucleosides deoxyadenosine (dA), deoxyuridine (dU) and/or thymidine (dT), deoxyguanosine (dG) and deoxycytidine (dC). The nucleoside that is not present in the polynucleotide is preferably abasic, adenosine (A), uridine (U), 5-methyluridine (m^5U), cytidine (C) or guanosine (G) or comprises urea, 5, 6 dihydroxythymine, thymine glycol, 5-hydroxy-5 methylhydantoin, uracil glycol, 6-hydroxy-5, 6-dihydrothymine, methyltartronylurea, 7, 8-dihydro-8-oxoguanine (8-oxoguanine), 8-oxoadenine, fapy-guanine, methy-fapy-guanine, fapy-adenine, aflatoxin B1-fapy-guanine, 5-hydroxy-cytosine, 5-hydroxy-uracil, 3-methyladenine, 7-methylguanine, 1,N6-ethenoadenine, hypoxanthine, 5-hydroxyuracil, 5-hydroxymethyluracil, 5-formyluracil or a cis-syn-cyclobutane pyrimidine dimer. The at least one nucleotide preferably is 10 nucleotides or fewer from the overhang. The at least one nucleotide is the first nucleotide in the overhang. All of the nucleotides in the overhang preferably comprise a nucleoside that is not present in the template polynucleotide.

These MuA based methods are disclosed in UK Application No. 1314695.6. They are also discussed in detail in UK Application No. 1406147.7.

The one or more helicases may be attached to the MuA substrate Y adaptors before they are contacted with the double stranded polynucleotide and MuA transposase. Alternatively, the one or more helicases may be attached to the MuA substrate Y adaptors after they are contacted with the double stranded polynucleotide and MuA transposase.

The one or more molecular brakes may be attached to the MuA substrate bridging moiety (or hairpin loop) adaptors before they are contacted with the double stranded polynucleotide and MuA transposase. Alternatively, the one or more molecular brakes may be attached to the MuA substrate bridging moiety (or hairpin loop) adaptors after they are contacted with the double stranded polynucleotide and MuA transposase.

Polynucleotide characterisation

The invention provides a method of characterising a target polynucleotide. The target polynucleotide may also be called the template polynucleotide or the polynucleotide of interest.

The method of the invention involves measuring one or more characteristics of the polynucleotide. In particular, one of the methods above for controlling the movement of a polynucleotide through a transmembrane pore is carried out as step (a) and then in step (b) one or more measurements are taken as the polynucleotide moves with respect to the pore wherein the

measurements are indicative of one or more characteristics of the polynucleotide. Suitable measurements are discussed above.

Any number of target polynucleotides can be investigated. For instance, the method of the invention may concern characterising 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 50, 100 or more target polynucleotides. The target polynucleotide can be naturally occurring or artificial. For instance, the method may be used to verify the sequence of manufactured oligonucleotides. The methods are typically carried out *in vitro*.

The method may involve measuring one, two, three, four or five or more characteristics of the target polynucleotide. The one or more characteristics are preferably selected from (i) the length of the polynucleotide, (ii) the identity of the polynucleotide, (iii) the sequence of the polynucleotide, (iv) the secondary structure of the polynucleotide and (v) whether or not the polynucleotide is modified. Any combination of (i) to (v) may be measured in accordance with the invention, such as {i}, {ii}, {iii}, {iv}, {v}, {i,ii}, {i,iii}, {i,iv}, {i,v}, {ii,iii}, {ii,iv}, {ii,v}, {iii,iv}, {iii,v}, {iv,v}, {i,ii,iii}, {i,ii,iv}, {i,ii,v}, {i,iii,iv}, {i,iii,v}, {i,iv,v}, {ii,iii,iv}, {ii,iii,v}, {ii,iv,v}, {iii,iv,v}, {i,ii,iii,iv}, {i,ii,iii,v}, {i,ii,iv,v}, {i,iii,iv,v}, {ii,iii,iv,v} or {i,ii,iii,iv,v}.

For (i), the length of the polynucleotide may be measured for example by determining the number of interactions between the polynucleotide and the pore or the duration of interaction between the polynucleotide and the pore.

For (ii), the identity of the polynucleotide may be measured in a number of ways. The identity of the polynucleotide may be measured in conjunction with measurement of the sequence of the polynucleotide or without measurement of the sequence of the polynucleotide. The former is straightforward; the polynucleotide is sequenced and thereby identified. The latter may be done in several ways. For instance, the presence of a particular motif in the polynucleotide may be measured (without measuring the remaining sequence of the polynucleotide). Alternatively, the measurement of a particular electrical and/or optical signal in the method may identify the polynucleotide as coming from a particular source.

For (iii), the sequence of the polynucleotide can be determined as described previously. Suitable sequencing methods, particularly those using electrical measurements, are described in Stoddart D et al., Proc Natl Acad Sci, 12;106(19):7702-7, Lieberman KR et al, J Am Chem Soc. 2010;132(50):17961-72, and International Application WO 2000/28312.

For (iv), the secondary structure may be measured in a variety of ways. For instance, if the method involves an electrical measurement, the secondary structure may be measured using a change in dwell time or a change in current flowing through the pore. This allows regions of single-stranded and double-stranded polynucleotide to be distinguished.

For (v), the presence or absence of any modification may be measured. The method preferably comprises determining whether or not the polynucleotide is modified by methylation, by oxidation, by damage, with one or more proteins or with one or more labels, tags or spacers. Specific modifications will result in specific interactions with the pore which can be measured
5 using the methods described below. For instance, methylcytosine may be distinguished from cytosine on the basis of the current flowing through the pore during its interaction with each nucleotide.

The methods may be carried out using any apparatus that is suitable for investigating a membrane/pore system in which a pore is present in a membrane. The method may be carried
10 out using any apparatus that is suitable for transmembrane pore sensing. For example, the apparatus comprises a chamber comprising an aqueous solution and a barrier that separates the chamber into two sections. The barrier typically has an aperture in which the membrane containing the pore is formed. Alternatively the barrier forms the membrane in which the pore is present.

15 The methods may be carried out using the apparatus described in International Application No. PCT/GB08/000562 (WO 2008/102120).

The methods may involve measuring the current passing through the pore as the polynucleotide moves with respect to the pore. Therefore the apparatus may also comprise an electrical circuit capable of applying a potential and measuring an electrical signal across the
20 membrane and pore. The methods may be carried out using a patch clamp or a voltage clamp. The methods preferably involve the use of a voltage clamp.

The methods of the invention may involve the measuring of a current passing through the pore as the polynucleotide moves with respect to the pore. Suitable conditions for measuring ionic currents through transmembrane protein pores are known in the art and disclosed in the
25 Example. The method is typically carried out with a voltage applied across the membrane and pore. The voltage used is typically from +5 V to -5 V, such as from +4 V to -4 V, +3 V to -3 V or +2 V to -2 V. The voltage used is typically from -600 mV to +600mV or -400 mV to +400 mV. The voltage used is preferably in a range having a lower limit selected from -400 mV, -300 mV, -200 mV, -150 mV, -100 mV, -50 mV, -20mV and 0 mV and an upper limit independently
30 selected from +10 mV, + 20 mV, +50 mV, +100 mV, +150 mV, +200 mV, +300 mV and +400 mV. The voltage used is more preferably in the range 100 mV to 240 mV and most preferably in the range of 120 mV to 220 mV. It is possible to increase discrimination between different nucleotides by a pore by using an increased applied potential.

The methods are typically carried out in the presence of any charge carriers, such as metal salts, for example alkali metal salt, halide salts, for example chloride salts, such as alkali metal chloride salt. Charge carriers may include ionic liquids or organic salts, for example tetramethyl ammonium chloride, trimethylphenyl ammonium chloride, phenyltrimethyl ammonium chloride, or 1-ethyl-3-methyl imidazolium chloride. In the exemplary apparatus discussed above, the salt is present in the aqueous solution in the chamber. Potassium chloride (KCl), sodium chloride (NaCl), caesium chloride (CsCl) or a mixture of potassium ferrocyanide and potassium ferricyanide is typically used. KCl, NaCl and a mixture of potassium ferrocyanide and potassium ferricyanide are preferred. The charge carriers may be asymmetric across the membrane. For instance, the type and/or concentration of the charge carriers may be different on each side of the membrane.

The salt concentration may be at saturation. The salt concentration may be 3 M or lower and is typically from 0.1 to 2.5 M, from 0.3 to 1.9 M, from 0.5 to 1.8 M, from 0.7 to 1.7 M, from 0.9 to 1.6 M or from 1 M to 1.4 M. The salt concentration is preferably from 150 mM to 1 M. The method is preferably carried out using a salt concentration of at least 0.3 M, such as at least 0.4 M, at least 0.5 M, at least 0.6 M, at least 0.8 M, at least 1.0 M, at least 1.5 M, at least 2.0 M, at least 2.5 M or at least 3.0 M. High salt concentrations provide a high signal to noise ratio and allow for currents indicative of the presence of a nucleotide to be identified against the background of normal current fluctuations.

The methods are typically carried out in the presence of a buffer. In the exemplary apparatus discussed above, the buffer is present in the aqueous solution in the chamber. Any buffer may be used in the method of the invention. Typically, the buffer is phosphate buffer. Other suitable buffers are HEPES and Tris-HCl buffer. The methods are typically carried out at a pH of from 4.0 to 12.0, from 4.5 to 10.0, from 5.0 to 9.0, from 5.5 to 8.8, from 6.0 to 8.7 or from 7.0 to 8.8 or 7.5 to 8.5. The pH used is preferably about 7.5.

The methods may be carried out at from 0 °C to 100 °C, from 15 °C to 95 °C, from 16 °C to 90 °C, from 17 °C to 85 °C, from 18 °C to 80 °C, 19 °C to 70 °C, or from 20 °C to 60 °C. The methods are typically carried out at room temperature. The methods are optionally carried out at a temperature that supports enzyme function, such as about 37 °C.

30

RTC sequencing

In a preferred embodiment, a target double stranded polynucleotide is provided with a bridging moiety (or hairpin loop) adaptor at one end and the method comprises contacting the polynucleotide with a transmembrane pore such that both strands of the polynucleotide move

through the pore and taking one or more measurements as the both strands of the polynucleotide move with respect to the pore wherein the measurements are indicative of one or more characteristics of the strands of the polynucleotide and thereby characterising the target double stranded polynucleotide. Any of the embodiments discussed above equally apply to this

5 embodiment.

Uncoupling

The method of the invention may involve characterising multiple target polynucleotides and uncoupling of the at least the first target polynucleotide.

10 In a preferred embodiment, the invention involves characterising two or more target polynucleotides. The method comprises:

(a) providing a first polynucleotide in a first sample with one or more helicases attached to the first polynucleotide and one or more molecular brakes attached to the first polynucleotide;

15 (b) providing a second polynucleotide in a second sample with one or more helicases attached to the second polynucleotide and one or more molecular brakes attached to the second polynucleotide;

(c) coupling the first polynucleotide in the first sample to a membrane using one or more anchors;

20 (d) contacting the first polynucleotide with a transmembrane pore and applying a potential across the pore such that the one or more helicases and the one or more molecular brakes are brought together and both control the movement of the first polynucleotide through the pore;

(e) taking one or more measurements as the first polynucleotide moves with respect to the pore wherein the measurements are indicative of one or more characteristics of the first polynucleotide and thereby characterising the first polynucleotide;

25

(f) uncoupling the first polynucleotide from the membrane;

(g) coupling the second polynucleotide in the second sample to the membrane using one or more anchors;

30 (h) contacting the second polynucleotide with a transmembrane pore and applying a potential across the pore such that the one or more helicases and the one or more molecular brakes are brought together and both control the movement of the second polynucleotide through the pore; and

(i) taking one or more measurements as the second polynucleotide moves with respect to the pore wherein the measurements are indicative of one or more characteristics of the second polynucleotide and thereby characterising the second polynucleotide.

This type of method is discussed in detail in UK Application No. 1406155.0.

5 Step (f) (i.e. uncoupling of the first polynucleotide) may be performed before step (g) (i.e. before coupling the second polynucleotide to the membrane). Step (g) may be performed before step (f). If the second polynucleotide is coupled to the membrane before the first polynucleotide is uncoupled, step (f) preferably comprises selectively uncoupling the first polynucleotide from the membrane (i.e. uncoupling the first polynucleotide but not the second
10 polynucleotide from the membrane). A skilled person can design a system in which selective uncoupling is achieved. Steps (f) and (g) may be performed at the same time. This is discussed in more detail below.

In step (f), at least 10% of the first polynucleotide is preferably uncoupled from the membrane. For instance, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at
15 least 70%, at least 80%, at least 90% or at least 95% of the first polynucleotide may be uncoupled from the membrane. Preferably, all of the first polynucleotide is uncoupled from the membrane. The amount of the first polynucleotide uncoupled from the membrane can be determined using the pore.

The first polynucleotide and second polynucleotide may be different from one another.
20 Alternatively, the first and second polynucleotides may be different polynucleotides. In such instances, there may be no need to remove at least part of the first sample before adding the second polynucleotide. This is discussed in more detail below. If the method concerns investigating three or more polynucleotides, they may all be different from one another or some of them may be different from one another.

25 The first polynucleotide and the second polynucleotide may be two instances of the same polynucleotide. The first polynucleotide may be identical to the second polynucleotide. This allows proof reading. If the method concerns investigating three or more polynucleotides, they may all be three or more instances of the same polynucleotide or some of them may be separate instances of the same polynucleotide.

30 The first sample and second sample may be different from one another. For instance, the first sample may be derived from a human and the second sample may be derived from a virus. If the first and second samples are different from one another, they may contain or be suspected of containing the same first and second polynucleotides. If the method concerns investigating

three or more samples, they may all be different from one another or some of them may be different from one another.

The first sample and the second sample are preferably two instances of the same sample. The first sample is preferably identical to the second sample. This allows proof reading. If the method concerns investigating three or more samples, they may all be three or more instances of the same sample or some of them may be separate instances of the same sample.

Any number of polynucleotides can be investigated. For instance, the method of the invention may concern characterising 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 50, 100 or more polynucleotides. If three or more polynucleotides are investigated using the method of the invention, the second polynucleotide is also uncoupled from the membrane and the requisite number of steps are added for the third polynucleotide. The same is true for four or more polynucleotides.

The method of the invention involves uncoupling the first polynucleotide from the membrane. The method of the invention may involve uncoupling the second polynucleotide from the membrane if three or more polynucleotides are being investigated.

The first polynucleotide can be uncoupled from the membrane using any known method. The first polynucleotide is preferably not uncoupled from the membrane in step (f) using the transmembrane pore. The first polynucleotide is preferably not uncoupled from the membrane using a voltage or an applied potential.

Step (f) preferably comprises uncoupling the first polynucleotide from the membrane by removing the one or more anchors from the membrane. If the anchors are removed, the second polynucleotide is coupled to the membrane using other (or separate) anchors. The anchors used to couple the second polynucleotide may be the same type of anchors used to couple the first polynucleotide or different type of anchors.

Step (f) more preferably comprises contacting the one or more anchors with an agent which has a higher affinity for the one or more anchors than the anchors have for the membrane. A variety of protocols for competitive binding or immunoradiometric assays to determine the specific binding capability of molecules are well known in the art (see for example Maddox *et al*, J. Exp. Med. 158, 1211-1226, 1993). The agent removes the anchor(s) from the membrane and thereby uncouples the first polynucleotide. The agent is preferably a sugar. Any sugar which binds to the one or more anchors with a higher affinity than the one or more anchors have for the membrane may be used. The sugar may be a cyclodextrin or derivative thereof as discussed below.

If one or more anchors comprise a hydrophobic anchor, such as cholesterol, the agent is preferably a cyclodextrin or a derivative thereof or a lipid. The cyclodextrin or derivative thereof may be any of those disclosed in Eliseev, A. V., and Schneider, H-J. (1994) *J. Am. Chem. Soc.* 116, 6081-6088. The agent is more preferably heptakis-6-amino- β -cyclodextrin (am₇- β CD),
 5 6-monodeoxy-6-monoamino- β -cyclodextrin (am₁- β CD) or heptakis-(6-deoxy-6-guanidino)-cyclodextrin (gu₇- β CD). Any of the lipids disclosed herein may be used.

If an anchor comprise(s) streptavidin, biotin or desthiobiotin, the agent is preferably biotin, desthiobiotin or streptavidin. Both biotin and desthiobiotin bind to streptavidin with a higher affinity than streptavidin binds to the membrane and *vice versa*. Biotin has a stronger
 10 affinity for streptavidin than desthiobiotin. An anchor comprising streptavidin may therefore be removed from the membrane using biotin or streptavidin and *vice versa*.

If an anchor comprises a protein, the agent is preferably an antibody or fragment thereof which specifically binds to the protein. An antibody specifically binds to a protein if it binds to the protein with preferential or high affinity, but does not bind or binds with only low affinity to
 15 other or different proteins. An antibody binds with preferential or high affinity if it binds with a K_d of 1×10^{-6} M or less, more preferably 1×10^{-7} M or less, 5×10^{-8} M or less, more preferably 1×10^{-8} M or less or more preferably 5×10^{-9} M or less. An antibody binds with low affinity if it binds with a K_d of 1×10^{-6} M or more, more preferably 1×10^{-5} M or more, more preferably 1×10^{-4} M or more, more preferably 1×10^{-3} M or more, even more preferably 1×10^{-2} M or more.
 20 Any method may be used to detect binding or specific binding. Methods of quantitatively measuring the binding of an antibody to a protein are well known in the art. The antibody may be a monoclonal antibody or a polyclonal antibody. Suitable fragments of antibodies include, but are not limited to, F_v, F(ab') and F(ab')₂ fragments, as well as single chain antibodies. Furthermore, the antibody or fragment thereof may be a chimeric antibody or fragment thereof, a
 25 CDR-grafted antibody or fragment thereof or a humanised antibody or fragment thereof.

Step (f) preferably comprises contacting the one or more anchors with an agent which reduces ability if the one or more anchors to couple to the membrane. For instance, the agent could interfere with the structure and/or hydrophobicity of the one or more anchors and thereby reduce their ability to couple to the membrane. If an anchor comprises cholesterol, the agent is
 30 preferably cholesterol dehydrogenase. If an anchor comprises a lipid, the agent is preferably a phospholipase. If an anchor comprises a protein, the agent is preferably a proteinase or urea. Other combination of suitable anchors and agents will be clear to a person skilled in the art.

Step (f) preferably comprises uncoupling the first polynucleotide from the membrane by separating the first polynucleotide from the one or more anchors. This can be done in any

manner. For instance, the linker could be cut in an anchor comprising a linker. This embodiment is particularly applicable to anchors which involve linkage via hybridisation. Such anchors are discussed above.

Step (f) more preferably comprises uncoupling the first polynucleotide from the
 5 membrane by contacting the first polynucleotide and the one or more anchors with an agent which competes with the first polynucleotide for binding to one or more anchors. Methods for determining and measuring competitive binding are known in the art. The agent is preferably a polynucleotide which competes with the first polynucleotide for hybridisation to the one or more
 10 anchors. For instance, if the first polynucleotide is coupled to the membrane using one or more anchors which involve hybridisation, the polynucleotide can be uncoupled by contacting the one or more anchors with a polynucleotide which also hybridises to the site of hybridisation. The polynucleotide agent is typically added at a concentration that is higher than the concentration of the first polynucleotide and one or more anchors. Alternatively, the polynucleotide agent may hybridise more strongly to the one or more anchors than the first polynucleotide.

15 Step (f) more preferably comprises (i) contacting the first polynucleotide and the one or more anchors with urea, tris(2-carboxyethyl)phosphine (TCEP), dithiothreitol (DTT), streptavidin or biotin, UV light, an enzyme or a binding agent; (ii) heating the first polynucleotide and the one or more anchors; or (iii) altering the pH. Urea, tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) are capable of disrupting anchors and
 20 separating the first polynucleotide from the membrane. If an anchor comprises a streptavidin-biotin link, then a streptavidin agent will compete for binding to the biotin. If an anchor comprises a streptavidin-desthiobiotin link, then a biotin agent will compete for binding to the streptavidin. UV light can be used to breakdown photolabile groups. Enzymes and binding agents can be used to cut, breakdown or unravel the anchor. Preferred enzymes include, but are
 25 not limited to, an exonuclease, an endonuclease or a helicase. Preferred binding agents include, but are not limited to, an enzyme, an antibody or a fragment thereof or a single-stranded binding protein (SSB). Any of the enzymes discussed below or antibodies discussed above may be used. Heat and pH can be used to disrupt hybridisation and other linkages.

If the first polynucleotide is uncoupled from the membrane by separating the first
 30 polynucleotide from the one or more anchors, the one or more anchors will remain in the membrane. Step (g) preferably comprises coupling the second polynucleotide to the membrane using the one or more anchors that was separated from the first polynucleotide. For instance, the second polynucleotide may also be provided with one or more polynucleotides which hybridise(s) to the one or more anchors that remain in the membrane. Alternatively, step (g)

preferably comprises coupling the second polynucleotide to the membrane using one or more separate anchors from the ones separated from the first polynucleotide (i.e. one or more other anchors). The one or more separate anchors may be the same type of anchors used to couple the first polynucleotide to the membrane or may be different types of anchors. Step (g) preferably
5 comprises coupling the second polynucleotide to the membrane using one or more different anchors from the one or more anchors separated from the first polynucleotide.

In a preferred embodiment, steps (f) and (g) comprise uncoupling the first polynucleotide from the membrane by contacting the membrane with the second polynucleotide such that the second polynucleotide competes with the first polynucleotide for binding to the one or more
10 anchors and replaces the first polynucleotide. For instance, if the first polynucleotide is coupled to the membrane using one or more anchors which involve hybridisation, the first polynucleotide can be uncoupled by contacting the anchors with the second polynucleotide attached to polynucleotides which also hybridise to the sites of hybridisation in the one or more anchors. The second polynucleotide is typically added at a concentration that is higher than the
15 concentration of the first polynucleotide and the one or more anchors. Alternatively, the second polynucleotide may hybridise more strongly to the one or more anchors than the first polynucleotide.

Removal or washing

20 Although the first polynucleotide is uncoupled from the membrane in step (f), it is not necessarily removed or washed away. If the second polynucleotide can be easily distinguished from the first polynucleotide, there is no need to remove the first polynucleotide.

Between steps (f) and (g), the method preferably further comprises removing at least some of the first sample from the membrane. At least 10% of the first sample may be removed,
25 such as at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% of the first sample may be removed.

The method more preferably further comprises removing all of the first sample from the membrane. This can be done in any way. For instance, the membrane can be washed with a buffer after the first polynucleotide has been uncoupled. Suitable buffers are discussed below.

30

Modified polynucleotide analytes

Before characterisation, a target polynucleotide may be modified by contacting the polynucleotide analyte with a polymerase and a population of free nucleotides under conditions in which the polymerase forms a modified polynucleotide using the target polynucleotide as a

template, wherein the polymerase replaces one or more of the nucleotide species in the target polynucleotide with a different nucleotide species when forming the modified polynucleotide. The modified polynucleotide may then be provided with one or more helicases attached to the polynucleotide and one or more molecular brakes attached to the polynucleotide. This type of modification is described in UK Application No. 1403096.9. Any of the polymerases discussed above may be used. The polymerase is preferably Klenow or 9° North.

The template polynucleotide is contacted with the polymerase under conditions in which the polymerase forms a modified polynucleotide using the template polynucleotide as a template. Such conditions are known in the art. For instance, the polynucleotide is typically contacted with the polymerase in commercially available polymerase buffer, such as buffer from New England Biolabs®. The temperature is preferably from 20 to 37°C for Klenow or from 60 to 75°C for 9° North. A primer or a 3' hairpin is typically used as the nucleation point for polymerase extension.

Characterisation, such as sequencing, of a polynucleotide using a transmembrane pore typically involves analyzing polymer units made up of k nucleotides where k is a positive integer (i.e 'k-mers'). This is discussed in International Application No. PCT/GB2012/052343 (published as WO 2013/041878). While it is desirable to have clear separation between current measurements for different k-mers, it is common for some of these measurements to overlap. Especially with high numbers of polymer units in the k-mer, i.e. high values of k, it can become difficult to resolve the measurements produced by different k-mers, to the detriment of deriving information about the polynucleotide, for example an estimate of the underlying sequence of the polynucleotide.

By replacing one or more nucleotide species in the target polynucleotide analyte with different nucleotide species in the modified polynucleotide analyte, the modified polynucleotide analyte contains k-mers which differ from those in the target polynucleotide. The different k-mers in the modified polynucleotide are capable of producing different current measurements from the k-mers in the target polynucleotide analyte and so the modified polynucleotide provides different information from the target polynucleotide. The additional information from the modified polynucleotide can make it easier to characterise the target polynucleotide. In some instances, the modified polynucleotide itself may be easier to characterise. For instance, the modified polynucleotide may be designed to include k-mers with an increased separation or a clear separation between their current measurements or k-mers which have a decreased noise.

The polymerase preferably replaces two or more of the nucleotide species in the target polynucleotide with different nucleotide species when forming the modified polynucleotide. The

polymerase may replace each of the two or more nucleotide species in the target polynucleotide analyte with a distinct nucleotide species. The polymerase may replace each of the two or more nucleotide species in the target polynucleotide analyte with the same nucleotide species.

If the target polynucleotide analyte is DNA, the different nucleotide species in the modified analyte typically comprises a nucleobase which differs from adenine, guanine, thymine, cytosine or methylcytosine and/or comprises a nucleoside which differs from deoxyadenosine, deoxyguanosine, thymidine, deoxycytidine or deoxymethylcytidine. If the target polynucleotide is RNA, the different nucleotide species in the modified polynucleotide typically comprises a nucleobase which differs from adenine, guanine, uracil, cytosine or methylcytosine and/or comprises a nucleoside which differs from adenosine, guanosine, uridine, cytidine or methylcytidine. The different nucleotide species may be any of the universal nucleotides discussed above.

The polymerase may replace the one or more nucleotide species with a different nucleotide species which comprises a chemical group or atom absent from the one or more nucleotide species. The chemical group may be a propynyl group, a thio group, an oxo group, a methyl group, a hydroxymethyl group, a formyl group, a carboxy group, a carbonyl group, a benzyl group, a propargyl group or a propargylamine group.

The polymerase may replace the one or more nucleotide species with a different nucleotide species which lacks a chemical group or atom present in the one or more nucleotide species. The polymerase may replace the one or more of the nucleotide species with a different nucleotide species having an altered electronegativity. The different nucleotide species having an altered electronegativity preferably comprises a halogen atom.

The method preferably further comprises selectively removing the nucleobases from the one or more different nucleotides species in the modified polynucleotide.

25

Kits

The present invention also provides a kit for controlling the movement of a polynucleotide through a transmembrane pore, wherein the kit comprises one or more helicases and one or more molecular brakes. The one or more helicases and one or more molecular brakes may be any of those discussed above with reference to the method of the invention.

30

The kit is preferably for controlling the movement of a double stranded polynucleotide through a transmembrane pore and the kit preferably comprises a Y adaptor having one or more helicases attached and a bridging moiety (or hairpin loop) adaptor having one or more molecular brakes attached. The Y adaptor preferably comprises one or more first anchors for coupling the

polynucleotide to the membrane, the bridging moiety (or hairpin loop) adaptor preferably comprises one or more second anchors for coupling the polynucleotide to the membrane and the strength of coupling of the bridging moiety (or hairpin loop) adaptor to the membrane is preferably greater than the strength of coupling of the Y adaptor to the membrane.

5 The kit preferably further comprises a transmembrane pore. Any of the membrane and pores discussed above may be in the kit.

Any of the embodiments discussed above with reference to the method of the invention equally apply to the kits. The kit may further comprise the components of a membrane, such as the components of an amphiphilic layer or a triblock copolymer membrane.

10 The kit of the invention may additionally comprise one or more other reagents or instruments which enable any of the embodiments mentioned above to be carried out. Such reagents or instruments include one or more of the following: suitable buffer(s) (aqueous solutions), means to obtain a sample from a subject (such as a vessel or an instrument comprising a needle), means to amplify and/or express polynucleotides, a membrane as defined above or
15 voltage or patch clamp apparatus. Reagents may be present in the kit in a dry state such that a fluid sample resuspends the reagents. The kit may also, optionally, comprise instructions to enable the kit to be used in the method of the invention or details regarding for which organism the method may be used.

20 Series

The invention also provides a series of one or more helicases and one or more molecular brakes attached (or bound) to a polynucleotide. The series may comprise any number and combination of one or more helicases and one or more molecular brakes discussed above.

The one or more helicases preferably comprise a variant of SEQ ID NO: 8 comprising (or
25 comprising only) (i) E94C/A360C, (ii) E94C/A360C and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2), (iii) E94C/A360C/C109A/C136A or (iv) E94C/A360C/C109A/C136A and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2). The one or more helicases preferably comprise a variant of SEQ ID NO: 8 which comprises (or only comprises) (a) E94C/A360C/W378, (b) E94C/A360C/W378A and then
30 (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2), (c) E94C/A360C/C109A/C136A/W378A or (d) E94C/A360C/C109A/C136A/W378A and then (Δ M1)G1G2 (i.e. deletion of M1 and then addition G1 and G2).

The one or more helicases and one or more molecular brakes in the series may be separate from one another. The one or more helicases and one or more molecular brakes in the

series may be brought together. The one or more helicases and one or more molecular brakes in the series may contact one another.

If two or more helicases are present, they may be attached to one another, such as covalently attached to one another. A series of two or more attached helicases may be called a train. The two or more helicases are preferably not attached to one another except via the polynucleotide. The two or more helicases are preferably not covalently attached to one another.

If two or more molecule brakes are present, they may be attached to one another, such as covalently attached to one another. A series of two or more attached molecular brakes may be called a train. The two or more molecular brakes are preferably not attached to one another except via the polynucleotide. The two or more molecular brakes are preferably not covalently attached to one another.

The one or more helicases and the one or more molecular brakes are preferably not attached to one another except via the polynucleotide. The one or more helicases and the one or more molecular brakes are preferably not covalently attached to one another.

Polynucleotides to which the series of the invention may be attached/bound are discussed in more detail above.

The following Examples illustrate the invention.

Example 1

This example compared the use of a single T4 Dda – E94C/A360C to T4 Dda – E94C/A360C and TrwC Cba-Q594A (SEQ ID NO: 25 with the mutation Q594A) in tandem in order to control the movement of DNA construct Y (shown in Figure 1) through an MspA nanopore. T4 Dda – E94C/A360C is an active helicase which moved along the DNA when provided with appropriate fuel, whereas, TrwC Cba-Q594A is an inactive helicase (which has had its helicase activity knocked out) and therefore acted as a molecular brake. When two different helicases were used to control the movement of the construct through the nanopore then improved movement was observed in comparison to when the movement was controlled by a single helicase.

The Dda helicase used in this Example moves along the polynucleotide in a 5' to 3' direction. When the 5' end of the polynucleotide (the end away from which the helicase moves) is captured by the pore, the helicase works with the direction of the field resulting from the applied potential and moves the threaded polynucleotide into the pore and into the *trans* chamber. In this Example, slipping forward involves the DNA moving forwards relative to the

the pore (i.e. towards its 3' and away from its 5' end in this Example) at least 4 consecutive nucleotides and typically more than 10 consecutive nucleotides.

Slipping forward may involve movement forward of 100 consecutive nucleotides or more and this may happen more than once in each strand. This phenomenon was called skipping and slipping in UK Application Nos. 1406151.9.

Materials and Methods

Prior to setting up the experiment, DNA construct Y (see Figure 1 for diagram and sequences used in construct Y, final concentration added to the nanopore system 0.1 nM) was pre-incubated at room temperature for three hours with T4 Dda – E94C/A360C (final concentration added to nanopore system 10 nM, SEQ ID NO: 24 with mutations E94C/A360C, which was provided in buffer (253 mM KCl, 50 mM potassium phosphate, pH 8.0)). After three hours, TrwC Cba-Q594A (5 nM final concentration added to the nanopore system, SEQ ID NO: 25 with the mutation Q594A, which was provided in buffer (100 mM NaCl, 50 mM CAPS, pH 10) was added to the pre-mix and the mixture incubated overnight. Finally, MgCl₂ (10 mM final concentration added to the nanopore system), ATP (1 mM final concentration added to the nanopore system) and buffer (600 mM KCl, 25 mM potassium phosphate, 75 mM potassium ferrocyanide (II), 25 mM potassium ferricyanide (III) pH 8.0) were added to the pre-mix.

Electrical measurements were acquired from single MspA nanopores inserted in block co-polymer in buffer (25 mM potassium phosphate, 75 mM potassium ferrocyanide (II), 25 mM potassium ferricyanide (III), 600 mM KCl, pH 8.0). After achieving a single pore inserted in the block co-polymer, then buffer (2 mL, 25 mM potassium phosphate, 75 mM potassium ferrocyanide (II), 25 mM potassium ferricyanide (III), 600 mM KCl, pH 8.0) was flowed through the system to remove any excess MspA nanopores. The enzyme (T4 Dda – E94C/A360C, 10 nM final concentration, TrwC Cba-Q594A, 5 nM final concentration), DNA construct Y (0.1 nM final concentration), fuel (MgCl₂ 10mM final concentration, ATP 1 mM final concentration) pre-mix (300 µL total) was then flowed into the single nanopore experimental system and the experiment run at a holding potential of -120 mV for 6 hours (with potential flips to -180 mV for 2 seconds then to 0 mV for 2 seconds) and helicase-controlled DNA movement monitored.

Results

Helicase controlled DNA movement was observed for DNA construct Y using both T4 Dda – E94C/A360C and TrwC Cba-Q594A (SEQ ID NO: 25 with the mutation Q594A) (see Figures 2 and 3) in tandem. When either T4 Dda – E94C/A360C (current trace shown in Figure

2A) or TrwC Cba-Q594A (current trace shown in Figure 2C) or both T4 Dda – E94C/A360C and TrwC Cba-Q594A (current trace shown in Figure 2B) bound to DNA construct Y, then helicase controlled DNA movement through the nanopore was observed for regions 1 and 2 (see Figure 1). The movement of region 1 through the nanopore was either uncontrolled (Figure 2C –
 5 no T4 Dda – E94C/A360C bound) or controlled by T4 Dda – E94C/A360C (Figure 2A and B – T4 Dda – E94C/A360C bound). The movement of region 2 through the nanopore was either controlled by T4 Dda – E94C/A360C only (Figure 2A) or controlled by T4 Dda – E94C/A360C and TrwC Cba-Q594A (Figure 2B) or controlled by TrwC Cba-Q594A (Figure 2C) only.

The traces shown in Figure 2 section A show DNA controlled movement of regions 1 and
 10 2 through the nanopore by T4 Dda – E94C/A360C only. When region 1 translocated through the nanopore, observed stepwise changes in the measured current levels were plotted in Figure 2A. However, when region 2 translocated through the nanopore, fewer observed stepwise changes in the measured current levels were detected and plotted in Figure 2A, indicating that less
 15 information was obtained when this part of the strand translocated through the nanopore than when region 1 translocated through the nanopore. The movement control of region 2 provided by T4 Dda - E94C/A360C alone was not as consistent as for region 1, as a comparable number of observed stepwise changes in the measured current levels would have been expected for region 2 as for region 1 (owing to the DNA regions being similar in length). The enzyme was also observed to travel more quickly along region 2 of DNA construct Y. Furthermore, this
 20 inconsistency of movement resulted in slipping forward of the DNA region labelled 2 resulting in sections of DNA sequence having been missed. Figure 3A also shows an example movement index plot from a single DNA strand when the helicase T4 Dda – E94C/A360C controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. This figure showed that the movement index for region 2 had many less points than for region 1, which again
 25 indicated that less information was obtained for this region of DNA construct Y when it translocated through the nanopore and that the enzyme movement was less consistent.

The traces shown in Figure 2 section C show DNA controlled movement of regions 1 and 2 through the nanopore by TrwC Cba-Q594A only. When region 1 translocated through the nanopore, it was in an uncontrolled fashion as there was no enzyme bound to the DNA in front
 30 of region 1 to control the movement of this region of the construct. Therefore, this region translocated through the nanopore very quickly and it was not possible to pick out separate current levels. However, when the pore contacted the TrwC Cba-Q594A, the enzyme controlled the movement of region 2 through the nanopore, and it was possible to pick out observed stepwise changes in the measured current levels which were plotted in Figure 2C. The TrwC Cba-

Q594A helicase had been mutated so that its helicase activity had been removed. Therefore, the helicase acted like a molecular brake as it controlled DNA movement through the nanopore under an applied potential. This meant that the movement of region 1 of construct Y was not controlled through the nanopore and the movement of region 2 was controlled only by the TrwC Cba-Q594A helicase.

The traces shown in Figure 2 section B show DNA controlled movement of regions 1 and 2 through the nanopore by both T4 Dda – E94C/A360C and TrwC Cba-Q594A. When region 1 translocated through the nanopore under the control of T4 Dda – E94C/A360C, it was possible to pick out observed stepwise changes in the measured current levels which were plotted in Figure 2B. Moreover, when region 2 translocated through the nanopore, the movement was controlled by both T4 Dda – E94C/A360C and TrwC Cba-Q594A (the pore brought the T4 Dda – E94C/A360C helicase into contact with the TrwC Cba-Q594A molecular brake). When region 2 translocated through the nanopore under the control of the two enzymes (T4 Dda – E94C/A360C and TrwC Cba-Q594A) then the DNA movement was significantly different from that observed when a single T4 Dda - E94C/A360C or TrwC Cba-Q594A helicase controlled the movement. A similar number of observed stepwise changes in the measured current levels were detected and plotted for translocation of region 2 as were observed for translocation of region 1. This indicated that more information was obtained when this part of the strand translocated through the nanopore than when region 2 translocated through the nanopore under the control of only a single helicase either T4 Dda - E94C/A360C or TrwC Cba-Q594A. This illustrated that more consistent movement of the DNA was observed when T4 Dda - E94C/A360C and TrwC Cba-Q594A enzymes were used to control movement (e.g. slower movement or less slipping forward of the DNA region labelled 2).

Control experiments were also run using DNA construct Y when the construct was not pre-incubated with TrwC Cba-Q594A, therefore, only T4 Dda – E94C/A360C could control the DNA movement through the nanopore. The controls showed no improved strand movement when only the T4 Dda – E94C/A360C was used to control movement.

Figure 3B also shows an example movement index plot when T4 Dda – E94C/A360C and TrwC Cba-Q594A controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. This figure showed that the movement index for region 2, when the helicase movement was controlled using T4 Dda – E94C/A360C and TrwC Cba-Q594A, had many more points than for region 2 when the helicase movement was controlled by a single enzyme either T4 Dda – E94C/A360C or TrwC Cba-Q594A which again indicated that more information was obtained for this region of DNA construct Y when it translocated through the nanopore under the

control of two different enzymes and that the DNA movement was more consistent (e.g. slower movement or less slipping forward of the DNA region labelled 2). This meant that a combination of T4 Dda – E94C/ A360C and TrwC Cba-Q594A enzymes could be used to improve sequencing of a strand of DNA.

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Example 2

This example compared the use of a single T4 Dda – E94C/C109A/C136A/A360C to T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A (SEQ ID NO: 25 with the mutation Q594A) in tandem in order to control the movement of DNA construct Y (shown in Figure 1) through an MspA nanopore. T4 Dda – E94C/C109A/C136A/A360C is an active helicase which moved along the DNA when provided with appropriate fuel, whereas, TrwC Cba-Q594A is an inactive helicase (which has had its helicase activity knocked out) and therefore acted as a molecular brake. When two different helicases were used to control the movement of the construct through the nanopore then improved movement was observed in comparison to when the movement was controlled by a single helicase.

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Materials and Methods

Prior to setting up the experiment, the DNA construct Y pre-mix was prepared as described in Example 1 except the first helicase to be incubated with the DNA was T4 Dda – E94C/C109A/C136A/A360C instead of T4 Dda – E94C/ A360C.

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Electrical measurements were acquired from single MspA nanopores as described in Example 1 above, except the helicases used in this experiment were T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A.

Results

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Helicase controlled DNA movement was observed for DNA construct Y using both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A (see Figure 4) in tandem. Helicase controlled DNA movements corresponding to controlled translocation by T4 Dda – E94C/C109A/C136A/A360C only, or TrwC Cba-Q594A only or both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A bound to DNA were observed.

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The trace shown in Figure 4 section A showed an example movement index plot when the helicase T4 Dda – E94C/C109A/C136A/A360C controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. When region 1 translocated through the nanopore, it was possible to observe the movement index for region 1. However, this figure

shows that the movement index for region 2 had less points than for region 1 which indicated that less information was obtained for this region of DNA construct Y when it translocated through the nanopore. This resulted in DNA movement that was less consistent (e.g. more slipping forward of the DNA region labelled 2) and sections of DNA sequence were missed.

Figure 4B shows the movement index when T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. When region 1 translocated through the nanopore under the control of T4 Dda – E94C/C109A/C136A/A360C, it was possible to observe a movement index. Moreover, when region 2 translocated through the nanopore, the movement was controlled by both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A. When region 2 translocated through the nanopore under the control of the two enzymes (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A) then the DNA movement was significantly different from that observed when a single T4 Dda – E94C/C109A/C136A/A360C or TrwC Cba-Q594A helicase controlled the movement. This figure showed that the movement index for region 2, when the helicase movement was controlled using T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A, had many more points than for region 2 when the helicase movement was controlled by a single enzyme either T4 Dda – E94C/C109A/C136A/A360C or TrwC Cba-Q594A which indicated that more information was obtained for this region of DNA construct Y when it translocated through the nanopore under the control of two different enzymes and that the enzyme movement was more consistent (e.g. slower movement or less slipping forward of the DNA region labelled 2). This meant that a combination of T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-Q594A enzymes could be used to improve sequencing of a strand of DNA.

Control experiments were also run using DNA construct Y when the construct was not pre-incubated with TrwC Cba-Q594A, therefore, only T4 Dda – E94C/C109A/C136A/A360C controlled the DNA movement through the nanopore. The controls showed no improved strand movement when only the T4 Dda – E94C/C109A/C136A/A360C was used to control movement.

Example 3

This example compared the use of a single T4 Dda – E94C/C109A/C136A/A360C to T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C (SEQ ID NO: 25 with mutations L376C/Q594A/K762C) in tandem in order to control the movement of DNA construct Y (shown in Figure 1) through an MspA nanopore. T4 Dda – E94C/C109A/C136A/A360C is an active helicase which moved along the DNA when provided with appropriate fuel, whereas, TrwC Cba-L376C/Q594A/K762C is an inactive helicase (which

has had its helicase activity knocked out) and therefore acted as a molecular brake. This helicase has also been mutated in order to reduce the size of an opening in the polynucleotide binding domain through which in at least one conformational state the polynucleotide can unbind from the helicase. When two different helicases were used to control the movement of the construct
 5 through the nanopore then improved movement was observed in comparison to when the movement was controlled by a single helicase.

Materials and Methods

The DNA construct Y (final concentration added to the nanopore system 0.1nM) which
 10 either had both enzymes pre-bound (see Figure 5B data) or only T4 Dda – E94C/C109A/C136A/A360C pre-bound (control experiment, see Figure 5A data) was added to buffer (final concentrations added to the nanopore system were 500 mM KCl, 25 mM potassium phosphate pH 8.0), ATP (final concentration added to the nanopore system 2 mM) and MgCL2 (final concentration added to the nanopore system 2 mM). This was the pre-mix which was then
 15 added to the nanopore system (total volume 150 µL).

Electrical measurements were acquired from single MspA nanopores inserted in block co-polymer in buffer (25 mM potassium phosphate, 150 mM potassium ferrocyanide (II), 150 mM potassium ferricyanide (III), pH 8.0). After achieving a single pore inserted in the block co-polymer, then buffer (2 mL, 25 mM potassium phosphate, 150 mM potassium ferrocyanide
 20 (II), 150 mM potassium ferricyanide (III), pH 8.0) was flowed through the system to remove any excess MspA nanopores. The enzyme pre-bound to construct Y (either a single T4 Dda – E94C/C109A/C136A/A360C (control) or T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C), fuel (MgCl2 and ATP) pre-mix (150 µL total) was then flowed into the single nanopore experimental system and the experiment run at a holding potential of -
 25 120 mV for 6 hours (with potential flips to +60 mV for 2 seconds) and helicase-controlled DNA movement monitored.

Results

Helicase controlled DNA movement was observed for DNA construct Y using both T4
 30 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C in tandem (see Figure 5). Helicase controlled DNA movements corresponding to controlled translocation by T4 Dda – E94C/C109A/C136A/A360C only or both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C bound to DNA were observed.

The trace shown in Figure 5 section A showed an example current trace when the helicase T4 Dda – E94C/C109A/C136A/A360C controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. When region 1 translocated through the nanopore, it was possible to observe the current trace for region 1. However, this figure shows that the current trace for region 2 had less observed stepwise changes in the measured current levels than for region 1 which indicated that less information was obtained for region 2 of DNA construct Y when it translocated through the nanopore. This resulted in DNA movement that was less consistent (e.g. more slipping forward of the DNA region labelled 2) and sections of DNA sequence were missed.

Figure 5B shows the current trace when T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C controlled the translocation of DNA construct Y (see Figure 1) through an MspA nanopore. When region 1 translocated through the nanopore under the control of T4 Dda – E94C/C109A/C136A/A360C, it was possible to observe a current trace. Moreover, when region 2 translocated through the nanopore, the movement was controlled by both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C. When region 2 translocated through the nanopore under the control of the two enzymes (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C) then the DNA movement was significantly different from that observed when a single T4 Dda – E94C/C109A/C136A/A360C helicase controlled the movement. This figure showed that the current trace for region 2, when the helicase movement was controlled using T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C had many more observed stepwise changes in the measured current levels than for region 2 when the helicase movement was controlled by a single enzyme which indicated that more information was obtained for this region of DNA construct Y when it translocated through the nanopore under the control of two different enzymes and that the enzyme movement was more consistent (e.g. slower movement or less slipping forward of the DNA region labelled 2). This meant that a combination of T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C enzymes could be used to improve sequencing of a strand of DNA.

Figure 6 shows two histogram plots which show the base calling accuracy (as a percentage and based on the known sequence of construct Y) for helicase controlled DNA movement events detected when either a single enzyme (T4 Dda – E94C/C109A/C136A/A360C) or two enzymes (T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C) controlled the movement of region 1(trace A) or region 2 (trace B) of the DNA construct Y. Each helicase controlled DNA translocation was categorised as either having more observed

stepwise changes in the measured current levels in region 1 (shown as black bars which was indicative of T4 Dda – E94C/C109A/C136A/A360C only) or more observed stepwise changes in the measured current levels in region 2 (shown as grey bars, which was indicative of both T4 Dda – E94C/C109A/C136A/A360C and TrwC Cba-L376C/Q594A/K762C bound to construct Y). As the TrwC Cba-L376C/Q594A/K762C only affected the movement of region 2, the sequencing accuracies for region 1 of the strand have the same distribution for both class of strand (either one (black) or two enzymes (grey) bound). However, the sequencing accuracy of the region 2 of construct Y was improved as there were more observed stepwise changes in the measured current levels in region 2 when TrwC Cba-L376C/Q594A/K762C was bound. Therefore, the bulk accuracy of the base calling distribution was improved by approximately 5-10% when both enzymes were bound (the grey bars shown in trace B).

A similar experiment was carried out to compare the single enzyme T4 Dda – E94C/C109A/C136A/A360C with the following enzyme combination T4 Dda – E94C/C109A/C136A/A360C with TrwC Cba-D411C/Q594A/A783C. When helicase controlled DNA movement was compared between the single enzyme and the double enzyme combinations then improved movement of construct Y was observed when it translocated through the nanopore under the control of two different enzymes and the enzyme movement was more consistent (e.g. slower movement or less slipping forward of the DNA region labelled 2) than that observed when movement was controlled by a single enzyme.

WE CLAIM:

1. A method for controlling the movement of a polynucleotide through a transmembrane pore, comprising:

(a) providing the polynucleotide with one or more helicases attached to the polynucleotide and one or more molecular brakes attached to the polynucleotide, wherein the one or more molecular brakes comprise one or more inactive helicases;

(b) contacting the polynucleotide provided in step (a) with the pore; and

(c) applying a potential across the pore such that the one or more helicases and the one or more molecular brakes are brought together and both control the movement of the polynucleotide through the pore.

2. The method according to claim 1, wherein the one or more helicases and the one or more molecular brakes are not attached to one another except via the polynucleotide.

3. The method according to claim 1 or 2, wherein the polynucleotide is a double stranded polynucleotide.

4. The method according to claim 3, wherein in step (a) the one or more helicases are attached to a Y adaptor attached to one end of the double stranded polynucleotide and wherein the one or more molecular brakes are: (i) attached to a bridging moiety adaptor attached to the other end of the double stranded polynucleotide; or (ii) to a hairpin loop adaptor attached to the other end of the double stranded polynucleotide.

5. The method according to claim 4, wherein the one or more helicases and the one or more molecular brakes are brought together when the one or more helicases reach the bridging moiety or hairpin loop.

6. The method according to claim 4 or 5, wherein the Y adaptor comprises a leader sequence which preferentially threads into the pore.

7. The method according to any one of claims 1 to 6, wherein the polynucleotide is coupled to the membrane using one or more anchors.

8. The method according to any one of claims 1 to 7, wherein in step (a) the one or more helicases are stalled at one or more spacers and the one or more spacers comprise:

(i) one or more nitroindoles, one or more inosines, one or more acridines, one or more 2-aminopurines, one or more 2-6-diaminopurines, one or more 5-bromo-deoxyuridines, one or more inverted thymidines (inverted dTs), one or more inverted dideoxy-thymidines (ddTs), one or more dideoxy-cytidines (ddCs), one or more 5-methylcytidines, one or more 5-hydroxymethylcytidines, one or more 2'-O-Methyl RNA bases, one or more Iso-deoxycytidines (Iso-dCs), one or more Iso-deoxyguanosines (Iso-dGs), one or more C3 groups, one or more photo-cleavable (PC) groups, one or more hexandiol groups, one or more spacer 9 (iSp9) groups, one or more spacer 18 (iSp18) groups, a polymer or one or more thiol connections;

(ii) one or more abasic nucleotides; and/or

(iii) one or more chemical groups which cause the one or more helicases to stall.

9. The method according to any one of claims 1 to 8, wherein the one or more helicases and the one or more molecular brakes control the movement of the polynucleotide through the pore with the field resulting from the applied potential.

10. The method according to any one of claims 1 to 9, wherein the one or more helicases are (a) Hel308 helicases, RecD helicases, XPD helicases or Dda helicases; or (b) a combination of any of the helicases in (a).

11. The method according to any one of claims 1 to 10, wherein the pore is (i) a transmembrane protein pore or (ii) a solid state pore.

12. The method according to claim 11, wherein the transmembrane protein pore is hemolysin, leukocidin, *Mycobacterium smegmatis* porin A (MspA), MspB, MspC, MspD, lysenin, outer membrane porin F (OmpF), outer membrane porin G (OmpG), outer membrane phospholipase A, *Neisseria* autotransporter lipoprotein (NalP) or WZA.

13. The method according to claim 12, wherein the transmembrane protein pore is:
- (a) MspA formed of eight identical subunits as shown in SEQ ID NO: 2 or (b) a variant thereof in which one or more of the eight subunits has at least 50% amino acid identity to SEQ ID NO: 2 over the entire sequence and retains pore activity; or
 - (c) α -hemolysin formed of seven identical subunits as shown in SEQ ID NO: 4 or (d) a variant thereof in which one or more of the seven subunits has at least 50% amino acid identity to SEQ ID NO: 4 over the entire sequence and retains pore activity.
14. A method of characterising a target polynucleotide, comprising:
- (a) carrying out the method of any one of claims 1 to 13; and
 - (b) taking one or more measurements as the polynucleotide moves with respect to the pore wherein the measurements are indicative of one or more characteristics of the polynucleotide and thereby characterising the target polynucleotide.
15. The method according to claim 14, wherein the one or more characteristics are selected from (i) the length of the target polynucleotide, (ii) the identity of the target polynucleotide, (iii) the sequence of the target polynucleotide, (iv) the secondary structure of the target polynucleotide and (v) whether or not the target polynucleotide is modified.
16. The method according to claim 15, wherein the target polynucleotide is modified (i) by methylation; (ii) by oxidation; (iii) by damage; (iv) with one or more proteins; or (v) with one or more labels, tags or spacers.
17. The method according to any one of claims 14 to 16, wherein the one or more characteristics of the target polynucleotide are measured by electrical measurement and/or optical measurement.
18. The method according to claim 17, wherein the electrical measurement is a current measurement, an impedance measurement, a tunnelling measurement or a field effect transistor (FET) measurement.

19. A kit suitable for controlling the movement of a polynucleotide through a transmembrane pore, wherein the kit comprises one or more helicases and one or more molecular brakes, wherein the one or more molecular brakes comprise one or more inactive helicases.
20. The kit according to claim 19, wherein wherein the one or more helicases and the one or more molecular brakes are not attached to one another except via the polynucleotide.
21. The kit according to claim 19 or 20, wherein the kit further comprises one or more anchors for coupling the polynucleotide to the membrane.
22. The kit according to any one of claims 19 to 21, wherein the kit is for controlling the movement of a double stranded polynucleotide through a transmembrane pore and wherein the kit comprises a Y adaptor having one or more helicases attached and a bridging moiety adaptor or a hairpin loop adaptor having one or more molecular brakes attached.
23. The kit according to claim 22, wherein the Y adaptor comprises a first anchor for coupling the polynucleotide to the membrane, the bridging moiety adaptor or the hairpin loop adaptor comprises a second anchor for coupling the polynucleotide to the membrane and wherein the strength of coupling of the second anchor to the membrane is greater than the strength of coupling of the first anchor to the membrane.
24. A polynucleotide to which is attached a series of one or more helicases and one or more molecular brakes, wherein the one or more molecular brakes comprise one or more inactive helicases.

Figure 1

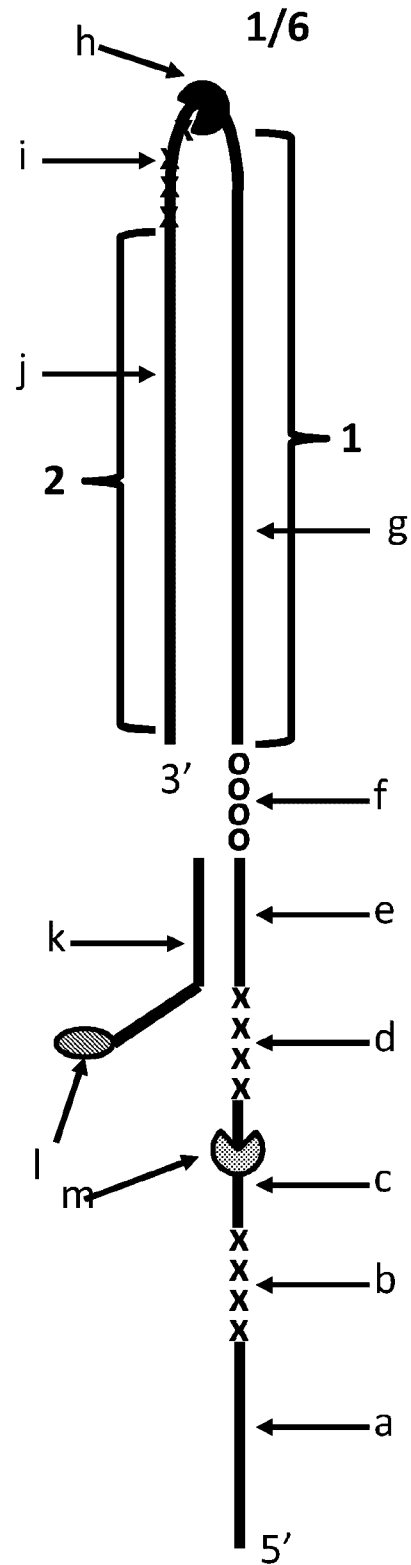


Figure 2

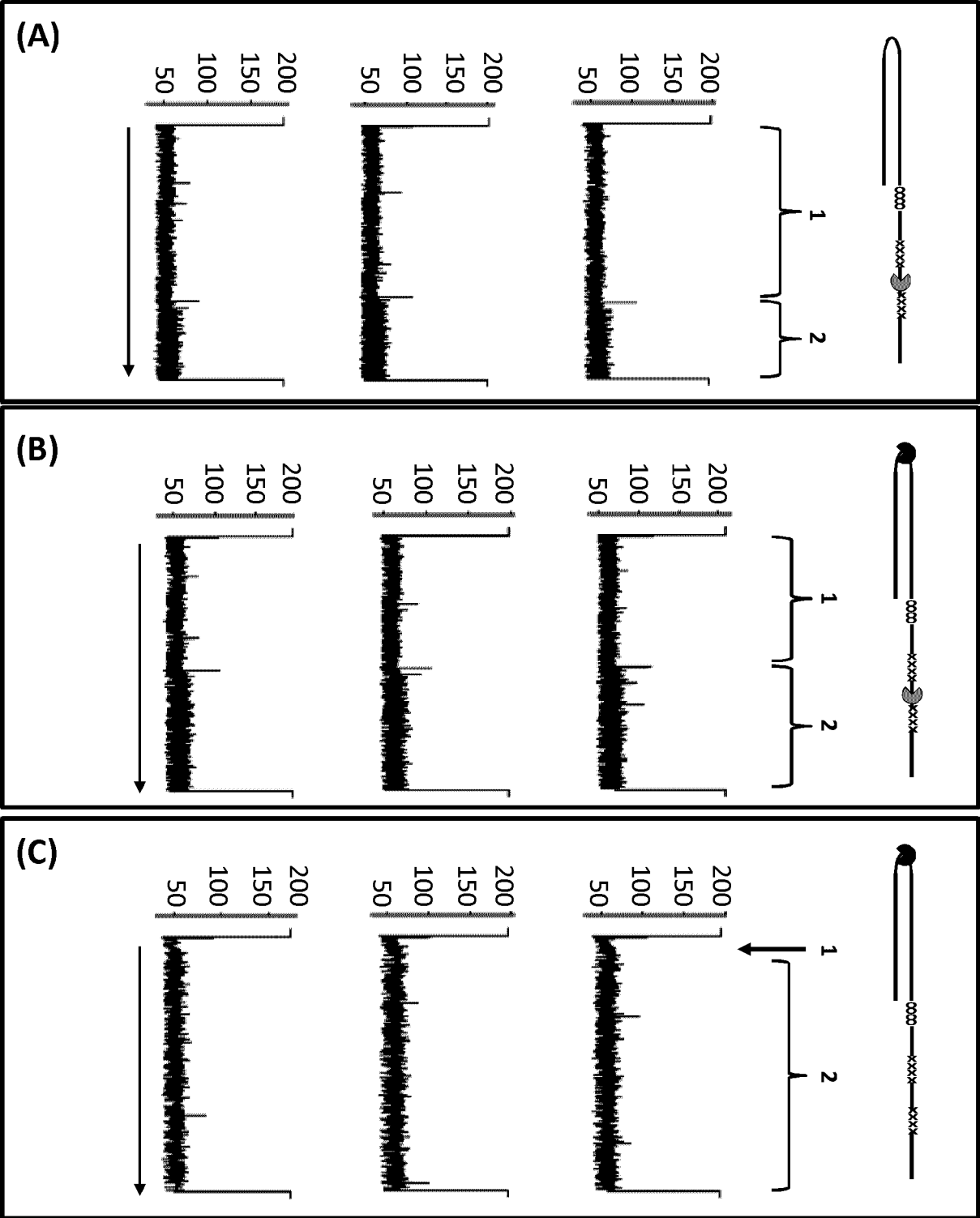
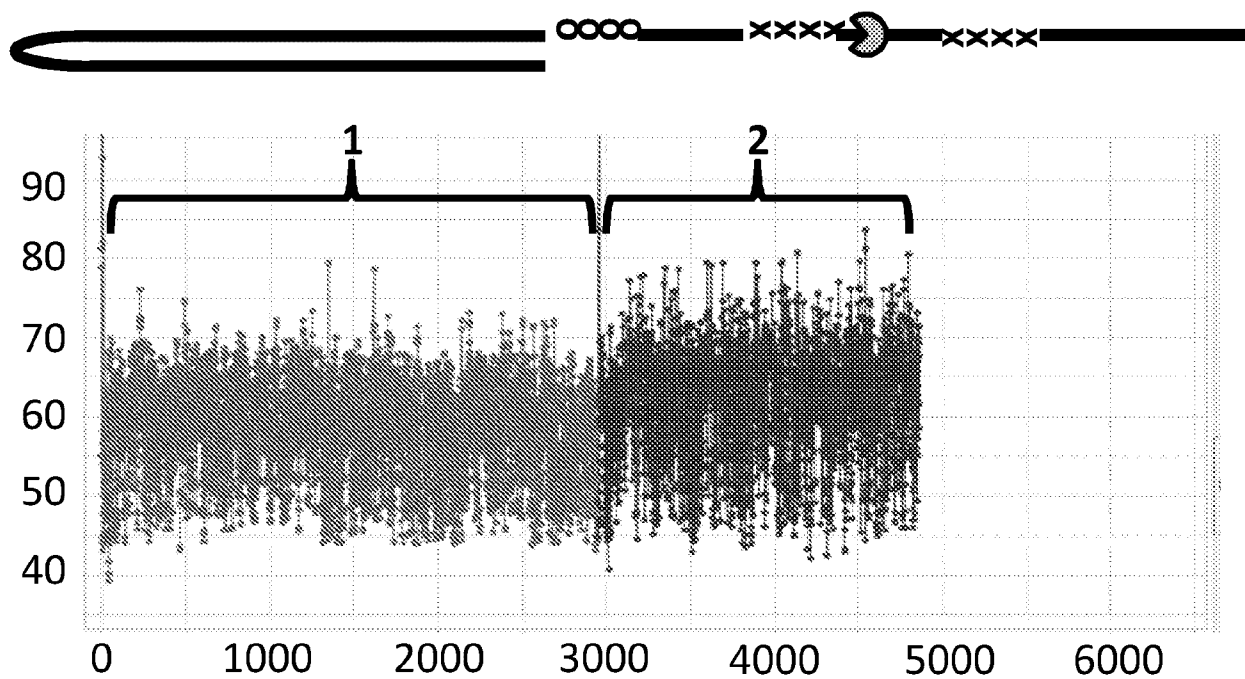


Figure 3

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(A)



(B)

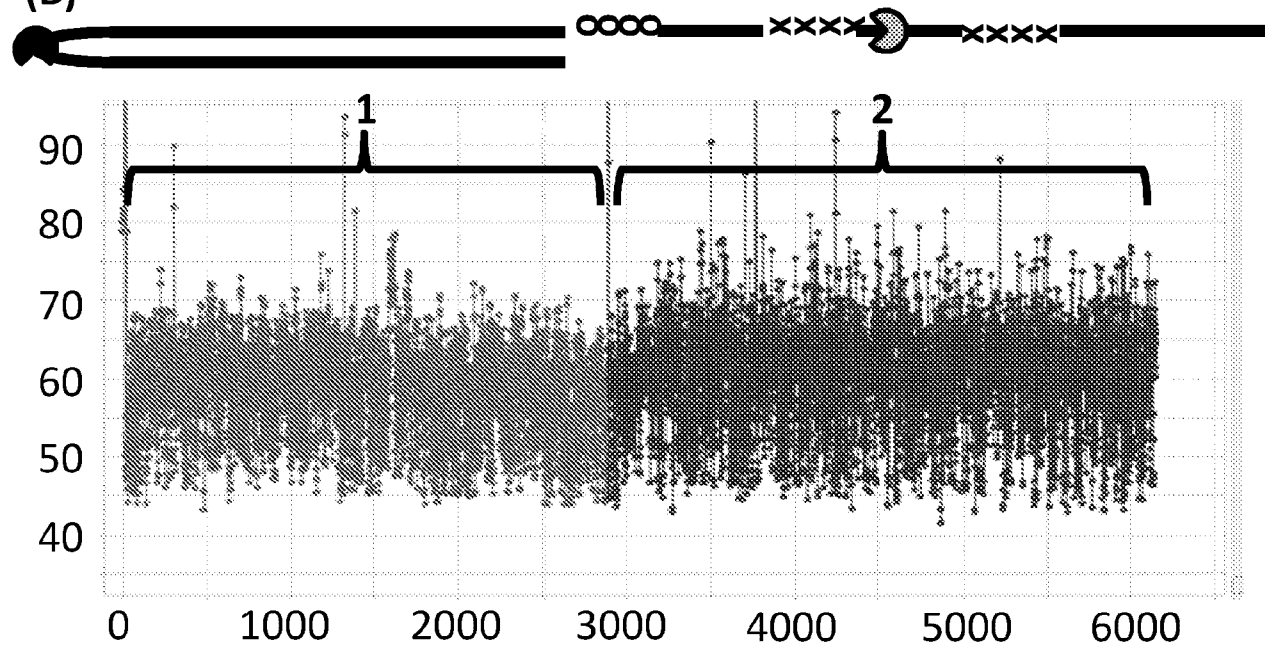
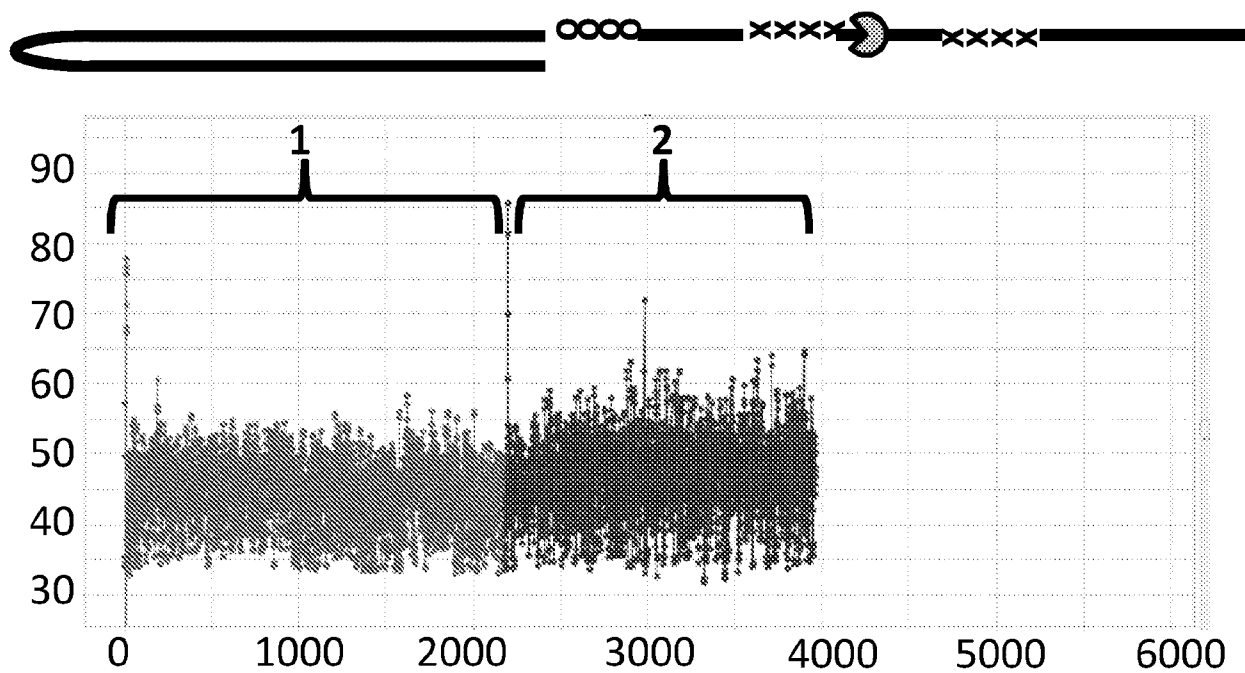


Figure 4

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(A)



(B)

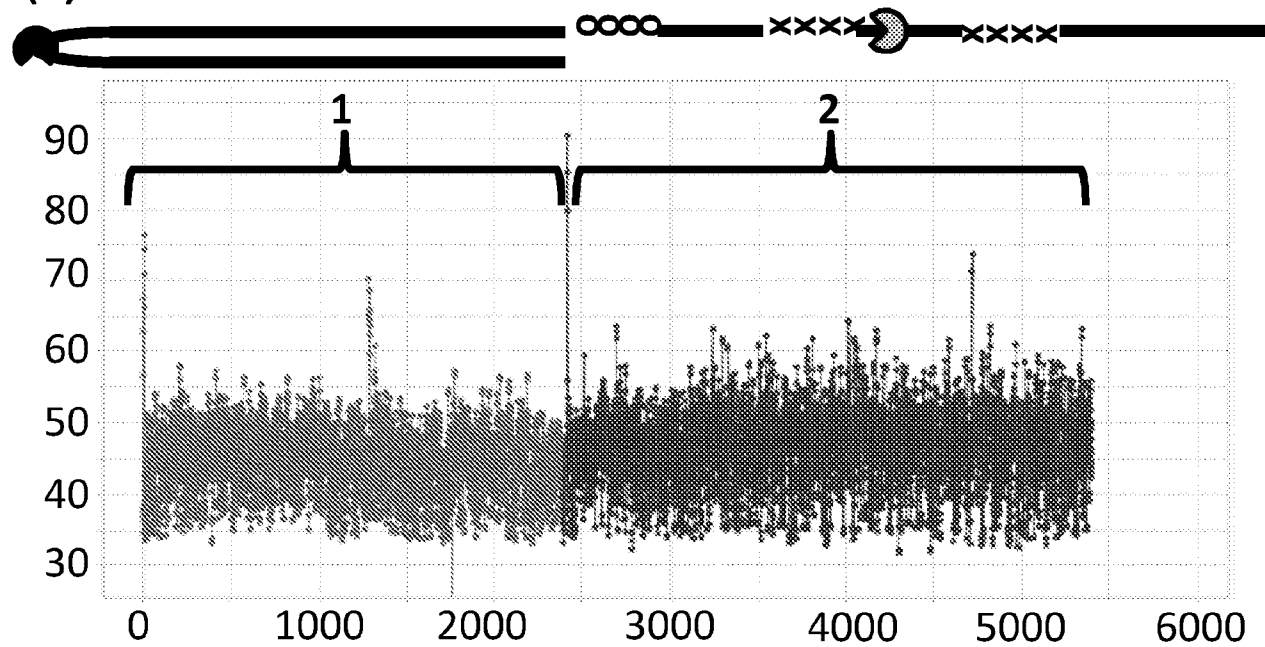
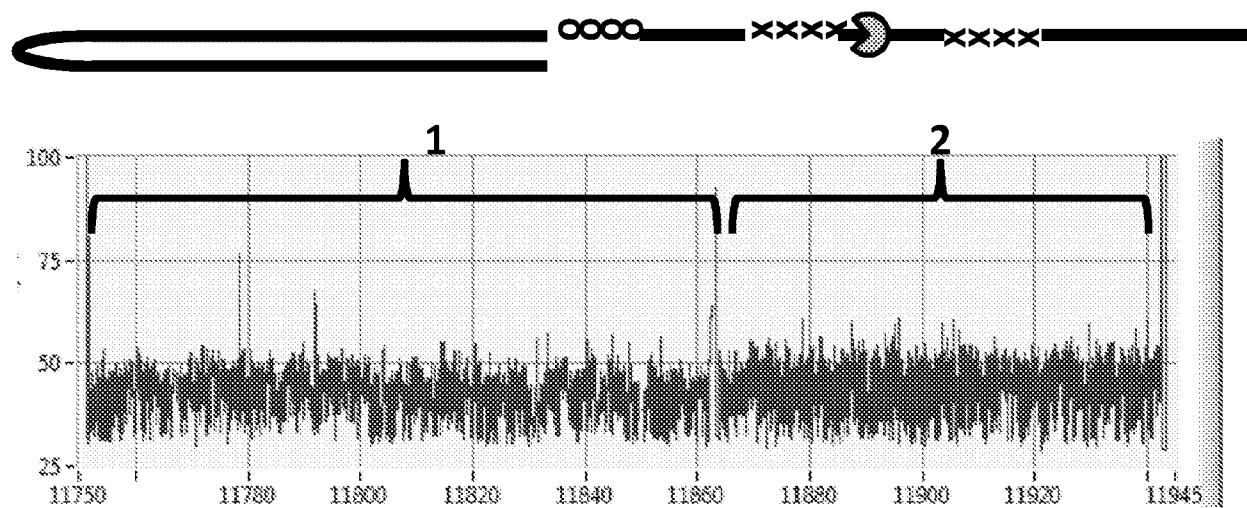


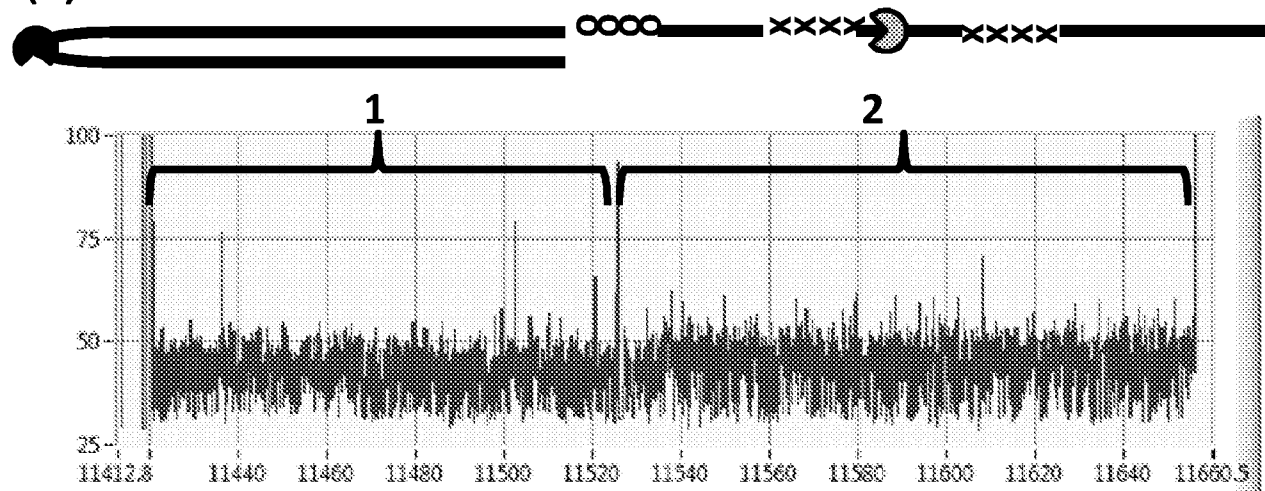
Figure 5

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(A)



(B)



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