KITS AND METHODS FOR ON-FLOW CELL LIBRARY PREPARATION AND METHYLATION DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application S.N. 63/715,069, filed November 1, 2024, and U.S. Provisional Application S.N. 63/716,148, filed November 4, 2024, and U.S. Provisional Application S.N. 63/715,958, filed November 4, 2024, and U.S. Provisional Application S.N. 63/571,297, filed March 28, 2024, the content of each of which is incorporated by reference herein in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] The Sequence Listing submitted herewith is hereby incorporated by reference in its entirety. The name of the file is ILI280BPCT_IP-2763-PCT_Sequence_Listing.xml, the size of the file is 46,700 bytes, and the date of creation of the file is March 21, 2025.

BACKGROUND

[0003] Deoxyribonucleic acid (DNA) methylation is an epigenetic mechanism in the mammalian genome that involves the transfer of a methyl group or a hydroxymethyl onto the C5 position of the cytosine to form, respectively, 5-methylcytosine or 5-hydroxymethylcytosine. DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA. DNA methylation affects the regulation of gene expression in development, in differentiation, and in diseases, such as multiple sclerosis, diabetes, schizophrenia, and cancers.

SUMMARY

[0004] The flow cell disclosed herein includes surface chemistry for on-flow cell tagmentation and extension, which enables fully adapted DNA fragments to be generated on-board the flow cell. In the examples disclosed herein, the fully adapted

DNA fragments can also be exposed to enzymatic methylation conversion on-board the flow cell. The converted single-stranded DNA fragments can then be amplified and sequenced. Thus, the examples disclosed herein provide methylated base information in addition to genome wide metrics.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] Features of examples of the present disclosure will become apparent by reference to the following detailed description and drawings, in which like reference numerals correspond to similar, though perhaps not identical, components. For the sake of brevity, reference numerals or features having a previously described function may or may not be described in connection with other drawings in which they appear.

[0006] Fig. 1 depicts an example of transposome complexes that can be immobilized in depressions of a flow cell;

[0007] Fig. 2A is a top view of an example of the flow cell that can include the transposome complexes;

[0008] Fig. 2B is an enlarged, partially cross-sectional, and perspective view of the architecture within the flow channel of the flow cell of Fig. 2A, which includes depressions separated by interstitial regions and the transposome complexes in the depressions;

[0009] Fig. 3 schematically illustrates several flow cell depressions with a DNA sample tagmented by the transposome complexes that are attached within the depressions; and

[0010] Fig. 4 illustrates an example of a method disclosed herein.

DETAILED DESCRIPTION

[0011] In the examples set forth herein, DNA sample tagmentation and extension, methylation conversion, and amplification may take place on-board the same flow cell.

[0012] ***Definitions***

[0013] Terms used herein will be understood to take on their ordinary meaning in the relevant art unless specified otherwise. Several terms used herein and their meanings are set forth below.

[0014] As used herein, the singular forms "a," "an," and "the" refer to both the singular as well as plural, unless the context clearly indicates otherwise. The term "comprising" as used herein is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps.

[0015] Reference throughout the specification to "one example," "another example," "an example," and so forth, means that a particular element (e.g., feature, structure, composition, configuration, and/or characteristic) described in connection with the example is included in at least one example described herein, and may or may not be present in other examples. In addition, it is to be understood that the described elements for any example may be combined in any suitable manner in the various examples unless the context clearly dictates otherwise.

[0016] The terms "substantially" and "about" used throughout this disclosure, including the claims, are used to describe and account for small fluctuations, such as those due to variations in processing. For example, these terms can refer to less than or equal to ±5% from a stated value, such as less than or equal to ±2% from a stated value, such as less than or equal to ±1% from a stated value, such as less than or equal to ±0.5% from a stated value, such as less than or equal to ±0.2% from a stated value, such as less than or equal to ±0.1% from a stated value, such as less than or equal to ±0.05% from a stated value.

[0017] *Adapter*: An oligonucleotide sequence that can be fused to a nucleic acid molecule, for example, by ligation or tagmentation. Suitable adapter lengths may range from about 10 nucleotides to about 100 nucleotides, or from about 12 nucleotides to about 60 nucleotides, or from about 15 nucleotides to about 50 nucleotides. The adapter may include any combination of nucleotides and/or nucleic acids. In some examples, the adapter can include an amplification domain, e.g., having a universal nucleotide sequence, such as a P5 or P7 sequence, that can serve

as a starting point for template amplification and cluster generation. In other examples, the adapter can include a sequence that is complementary to at least a portion of a flow cell surface bound primer (which includes the universal nucleotide sequence). In the latter example, the adapter sequence can hybridize to the complementary flow cell surface bound primer during amplification and cluster generation. In some examples, the adapter can also include a sequencing primer sequence (i.e., sequencing binding site) or a sequencing sample index (i.e., a barcode sequence). Combinations of different adapters may be incorporated into the nucleic acid molecule, such as the DNA fragments generated via tagmentation.

[0018] *Altered cytidine deaminase*: A cytidine deaminase including a substitution mutation at one or more residues when compared to a reference cytidine deaminase. In an example, the reference cytidine deaminase is a member of the APOBEC protein family. A substitution mutation can be at the same position or a functionally equivalent position compared to the reference cytidine deaminase. By "functionally equivalent," it is meant that the altered cytidine deaminase has the amino acid substitution at the amino acid position in a reference cytidine deaminase that has the same functional role in both the reference cytidine deaminase and the altered cytidine deaminase. The altered cytidine deaminase may be "structurally similar" to a reference cytidine deaminase if the amino acid sequence of the altered cytidine deaminase possesses a specified amount of sequence similarity and/or sequence identity compared to the reference cytidine deaminase. The skilled artisan will readily appreciate that an altered cytidine deaminase described herein is not naturally occurring.

[0019] *Amplification*: Replicating one or more nucleic acid templates, including fragments thereof, and thus creating multiple copies of the one or more nucleic acid templates. Amplification can include one or more of a bridge amplification reaction, an isothermal bridge amplification reaction, a rolling circle amplification (RCA) reaction, a modified rolling circle multiple displacement amplification, a helicase-dependent amplification reaction, a recombinase-dependent amplification reaction, a single-stranded DNA binding (SSB) protein mediated isothermal amplification, a polymerase chain reaction (PCR) reaction, a strand-displacement reaction, a ligase chain reaction,

a transcription-mediated reaction, a loop-mediated amplification reaction, other suitable reactions, and combinations thereof.

[0020] *Amplification Domain*: A portion of an adapter having a universal nucleotide sequence, such as a P5 or P7 sequence or a complement thereof, that can serve as a starting point for template amplification and cluster generation.

[0021] *Asymmetrical attachment or asymmetrically attached*: When one type of transposome complex is attached to a flow cell surface through its 5' end and the other type of transposome complex is attached to the flow cell through its 3' end.

[0022] *Attachment / Attached / Affixed / Immobilized:* These terms are used interchangeably herein. The terms refer to the state of two things being joined, fastened, adhered, connected or bound to each other, either directly or indirectly and either physically or chemically. As an example of chemical attachment, a nucleic acid can be attached to a polymeric hydrogel by a covalent or non-covalent bond. A covalent bond is characterized by the sharing of pairs of electrons between atoms. As an example, a covalent attachment includes a bond resulting from the use of click chemistry techniques. A non-covalent bond is a physical bond that does not involve the sharing of pairs of electrons and can include, for example, non-specific interactions (e.g., hydrogen bonds, ionic bonds, van der Waals forces) or specific interactions (e.g. affinity interactions (e.g., hydrophilic interactions and hydrophobic interactions), receptor-ligand interactions, antibody-epitope interactions, avidin-biotin interactions, streptavidin-biotin interactions, lectin-carbohydrate interactions, etc.). Exemplary attachments are set forth in U.S. Pat. Nos. 6,737,236 B1; 7,259,258 B2; 7,375,234 B2 and 7,427,678 B2; and U.S. Pat. Pub. 2011/0059865 A1, each of which is incorporated herein by reference in its entirety.

[0023] In certain examples, the molecules (e.g., nucleic acids, enzymes) remain immobilized or attached to the solid support under the conditions in which it is intended to use the solid support, for example in applications requiring nucleic acid amplification and/or sequencing. In other examples, the molecules are reversibly immobilized or attached and can be removed from the solid support through the use of cleavable sites, linkers, and the like.

[0024] *Cluster / Cluster of oligonucleotides / Oligonucleotide cluster / Colony:* A localized group or collection of DNA or RNA molecules on a nucleotide-sample support, such as a flow cell, particle, polymer scaffold, or other solid surface. In particular, a cluster includes tens, hundreds, thousands, or more copies of a cloned or the same DNA or RNA segment. For example, in one or more examples, a cluster includes a grouping of oligonucleotides immobilized in a section of a flow cell or other nucleotide-sample slide. In some examples, the cluster can comprise one or more concatemers, such as, for example, a polony or a nanoball. In some examples, clusters are evenly spaced or organized in a systematic structure within a patterned flow cell. By contrast, in some cases, clusters are randomly organized within a non-patterned flow cell. In typical examples, a cluster is the product of an amplification reaction. A cluster of oligonucleotides can be imaged utilizing one or more light signals, changes in pH, changes in conductance, and other signals. For instance, an oligonucleotide-cluster image may be captured by a camera during a sequencing cycle. The image captures light emitted by irradiated fluorescent labeled nucleotides incorporated into oligonucleotides, fluorescent labeled nucleotides bound but not incorporated into oligonucleotides, and other fluorescent labeled complexes associated with incorporated or bound nucleotides from one or more clusters on a flow cell. Examples of other sequencing procedures are set forth herein. In some examples, a cluster can be monoclonal or polyclonal.

[0025] *Corresponds with:* When one primer "corresponds with" an amplification domain, the primer and amplification domain may have the same sequence, so that a copy of the amplification domain generates a sequence complementary to the primer; or they may have complementary sequences when the amplification domain is introduced as part of an adapter.

[0026] *Depositing*: Any suitable application technique, which may be manual or automated, and, in some instances, results in modification of the surface properties. Generally, depositing may be performed using vapor deposition techniques, coating techniques, grafting techniques, or the like. Some specific examples include chemical vapor deposition (CVD), spray coating (e.g., ultrasonic spray coating), spin coating,

dunk or dip coating, doctor blade coating, puddle dispensing, flow through coating, aerosol printing, screen printing, microcontact printing, inkjet printing, or the like.

[0027] *Depression*: A discrete concave or recessed feature in a substrate or a layer of a substrate (e.g., a patterned resin) having a surface opening that is at least partially surrounded by interstitial region(s) of the substrate or the layer. Depressions can have any of a variety of shapes at their opening in a surface including, as examples, round, elliptical, square, polygonal, star shaped (with any number of vertices), etc. The cross-section of a depression taken orthogonally with the surface can be curved, square, polygonal, hyperbolic, conical, angular, etc. The depression may also have more complex architectures, such as ridges, step features, etc.

[0028] *DNA Sample:* Genetic material extracted from a cell, where the genetic material includes a DNA molecule. The DNA molecule is a polymeric form of nucleotides of any length that includes deoxyribonucleotides, deoxyribonucleotide analogs, or complementary deoxyribonucleotides derived from an RNA (ribonucleic acid) sample. The DNA sample is double stranded. The DNA sample may include naturally occurring DNA, which includes a nitrogen containing heterocyclic base (a nucleobase such as adenine, thymine, cytosine and/or guanine), a sugar (specifically deoxyribose, i.e., a sugar lacking a hydroxyl group that is present at the 2' position in ribose), and a backbone containing phosphodiester bonds. An analog structure can have an alternate backbone linkage including any of a variety known in the art.

[0029] The DNA sample may be genomic DNA (gDNA) that can be isolated from one or more cells, bodily fluids (e.g., whole blood, blood spots, saliva) or tissues. gDNA can be prepared by lysing a cell that contains the DNA. The cell may be lysed under conditions that substantially preserve the integrity of the cell's gDNA. In one particular example, thermal lysis may be used to lyse a cell. In another particular example, exposure of a cell to alkaline pH can be used to lyse a cell while causing relatively little damage to gDNA. Any of a variety of basic compounds can be used for lysis including, for example, potassium hydroxide, sodium hydroxide, and the like. Additionally, relatively undamaged gDNA can be obtained from a cell lysed by an enzyme that degrades the cell wall. Cells lacking a cell wall either naturally or due to enzymatic removal can also be lysed by exposure to osmotic stress. Other conditions

that can be used to lyse a cell include exposure to detergents, mechanical disruption, sonication heat, pressure differential such as in a French press device, or Dounce homogenization. Agents that stabilize gDNA can be included in a cell lysate or isolated gDNA sample including, for example, nuclease inhibitors, chelating agents, salts, buffers and the like. A crude cell lysate containing gDNA may be used without further isolation of the gDNA. In one example, a whole blood sample may be lysed using an inorganic salt free lysis buffer (containing a chaotropic detergent), and the crude lysate may be exposed to specific processing steps to generate a complexed crude lysate (e.g., as described in International Pub. No. WO 2023/122755 A2, incorporated herein by reference in its entirety). This complexed crude lysate can also be used as the DNA sample without further isolation or purification.

[0030] A DNA sample is one example of a nucleic acid sample. A nucleic acid sample is a sample, containing DNA and/or RNA, derived from any organism, including, for example, animals, plants, fungi, and microbes. Such samples may be derived from one or more biological fluids, cells, tissues, organs, or organisms, comprising a nucleic acid or a mixture of nucleic acids comprising at least one nucleic acid sequence. Such samples may include, but are not limited to, sputum/oral fluid, amniotic fluid, blood, a blood fraction, fine needle biopsy samples (such as surgical biopsy, fine needle biopsy, etc.), urine, peritoneal fluid, pleural fluid, and the like. Although the sample is often taken from a human subject (such as a patient), the sample may be from any mammal, including, but not limited to dogs, cats, horses, goats, sheep, cattle, pigs, etc. Alternatively, the sample may be microbial such as bacteria, viral, or fungal. The sample may be used directly as obtained from the biological source or following a pretreatment to modify the character of the sample. For example, such pretreatment may include preparing plasma from blood, diluting viscous fluids and so forth. Methods of pretreatment may also involve, but are not limited to, filtration, precipitation, dilution, distillation, mixing, centrifugation, freezing, lyophilization, concentration, amplification, nucleic acid fragmentation, inactivation of interfering components, the addition of reagents, lysing, etc. If such methods of pretreatment are employed with respect to the sample, such pretreatment methods are typically such that the nucleic acid(s) of interest remain in the test sample, sometimes

at a concentration proportional to that in an untreated test sample (such as namely, a sample that is not subjected to any such pretreatment method(s)). Such "treated" or "processed" samples are still considered to be biological "test" samples with respect to the methods described herein. A "nucleic acid sample" may also include nucleic acid sequence information stored in a memory, and which was originally obtained from a source such as one or more biological fluids, cells, tissues, organs, or organisms.

[0031] *Each*: When used in reference to a collection of items, each identifies an individual item in the collection, but does not necessarily refer to every item in the collection. Exceptions can occur if explicit disclosure or context clearly dictates otherwise.

[0032] *Flow Cell*: A vessel having an enclosed flow channel where a reaction can be carried out, or a vessel having a channel that is open to a surrounding environment and in which a reaction can be carried out. The vessel with an open flow channel may be referred to herein as an open wafer flow cell. Any example of the flow cell may include an inlet for delivering reagent(s) to the channel, and an outlet for removing reagent(s) from the channel. In some examples, the flow cell enables the detection of the reaction that occurs therein. For example, the flow cell can include one or more transparent surfaces allowing for the optical detection of arrays, optically labeled molecules, or the like.

[0033] *Flow channel*: An area that is defined between two bonded or otherwise attached components, or that is defined within a lane so that it is open to the surrounding environment. The flow channel can selectively receive a liquid sample. In some examples, the flow channel may be defined between two patterned sequencing surfaces or a patterned sequencing surface and a lid, and thus may be in fluid communication with one or more components of the sequencing surface(s).

[0034] *Fragment*: A portion or piece of the DNA sample. A "partially adapted fragment" is a portion or piece of the DNA sample that has been tagmented, and thus includes an adapter ligated to the 5' end of the DNA fragment. A "fully adapted fragment" is a portion or piece of the DNA sample that has adapters incorporated at both the 3' and 5' ends of the DNA fragment.

[0035] *Fragmentation*: The breaking of nucleic acid into shorter lengths. Fragmentation methods include enzymatic methods, physical methods (including sonication, nebulization, needle shearing, microwave, etc.), and chemical methods (including depurination, hydrolysis, oxidation, etc.). The terms "fragmenting enzymes" or "enzyme-based fragmentation" or "enzyme fragmentation," as used herein, refer to enzymes that fragment nucleic acids. The enzymes can be a single enzyme or two or more enzymes that work together to fragment the nucleic acid. Some enzymes work on single stranded nucleic acid whereas others work on double stranded nucleic acid and yet others work on one strand of a double stranded nucleic acid. Fragmenting enzymes can cut the nucleic acid randomly or specifically. Examples of fragmenting enzymes include transposase, restriction enzymes, Argonaute, CRISPR -associated nuclease (Cas), endonucleases, exonuclease, topoisomerase, FRAGMENTASE™ (New England Biolabs, Ipswich, MA). Preferred fragmentation examples include methods that fragment while retaining proximity information of the fragments.

[0036] *Methylated DNA*: A DNA strand that includes at least one modified cytosine. A modified cytosine refers to the nitrogenous base cytosine, which includes a methyl group or a hydroxymethyl group at the C5 position.

[0037] *Primer*: A single stranded nucleic acid molecule that can hybridize to a target sequence, such as an adapter attached to a fragment. As one example, a flow cell surface bound primer can serve as a starting point for fragment amplification and cluster generation. As another example, a flow cell surface bound primer can serve as a hybridization point for a spatial tag, and thus for targeting attachment of particular transposome complexes and DNA samples. As still another example, a primer (e.g., a sequencing primer) may be introduced that can hybridize to DNA fragments in order to prime synthesis of a new strand that is complementary to the fragments. Any primer can include any combination of nucleotides or analogs thereof. In some examples, the primer is a single-stranded oligonucleotide or polynucleotide. The primer length can be any number of bases long. In an example, each of the flow cell surface bound primer and the sequencing primer is a short strand, ranging from 10 to 60 bases, or from 20 to 40 bases.

[0038] *Nanoballs:* A concatemer comprising multiple copies of a target nucleic acid molecule. Rolling circle amplification/replication can be used to form nucleic acid nanoballs. These nucleic acid copies may be arranged one after another in a continuous linear strand of nucleotides. These nucleic acid copies may result in a nanoball folding configuration. The multiple copies of the target nucleic acid molecule in a nucleic acid nanoball may each contain an adaptor sequence of known sequence to facilitate amplification or sequencing. The adaptor sequence of each target nucleic acid molecule may be the same or different. The nucleic acid nanoball can be loaded on the surface of a solid support. The nanoball can be attached to the surface of solid support by any suitable method. Examples of such methods include nucleic acid hybridization, biotin streptavidin binding, thiol binding, photoactive binding, covalent binding, antibody-antigen, physical constraints via hydrogels or other porous polymers, etc., or combinations thereof. In some cases, the nanoball can be digested with an enzyme (nuclease, etc.) to produce a smaller nanoball or a fragment from the nanoball.

[0039] *Patterned / Random*: In some examples, the solid support comprises a patterned surface suitable for immobilization of molecules, such as enzymes, nucleic acids, and complexes thereof, in an ordered pattern. A "patterned surface" refers to an arrangement of different regions or features in or on an exposed layer of a solid support. The features can be separated by interstitial regions that contribute to the pattern. In some examples, the interstitial regions can be a different height, creating wells or raised platform patterns. In other examples, the interstitial regions can have different surface charges. In yet other examples, the interstitial regions can have different attachment moieties. In some examples, the pattern can be any suitable pattern, such as a grid patterns, radial patterns, and combinations thereof. In some examples, a patterned surface can contain pre-determined locations of features but the features are not arrayed in a repetitive pattern. Examples of grid patterns include rectangular patterns, hexagonal patterns, triangular patterns, and other suitable grid patterns. The regions for immobilization of molecules may be depressed regions, elevated regions, or planar regions relative to the interstitial regions. The regions may be fabricated as is generally known in the art using a variety of techniques, including,

but not limited to, photolithography, stamping techniques, molding techniques, microetching techniques, and combinations thereof. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the regions. For example, the regions for immobilization of molecules of a patterned surface may be wells, pits, channels, posts, pillars, ridges, stripes, swirls, lines, and other suitable topographies. For example, the wells may have any opening in any shape, such as circular, oval, polygonal (e.g., hexagonal, octagonal, square, rectangular, elliptical, etc.). Exemplary patterned surfaces that can be used in the methods and compositions set forth herein are described in U.S. Pat. No. 8,778,849 B2, which is incorporated herein by reference in its entirety.

[0040] In some examples, the solid support comprises a surface suitable for immobilization of molecules, such as enzymes, nucleic acids, and complexes thereof, in a random distribution over the solid support. Exemplary random distribution over a solid support is described in U.S. Pat. No. 8,241,573 B2, which is incorporated herein by reference in its entirety.

[0041] *Polonies*: Some examples further comprise rolling circle amplification/replication used to form polonies. The term "polony" or "polonies" used herein refers to a nucleic acid library molecule clonally amplified in-solution or on-support to generate an amplicon that can serve as a template molecule for sequencing. In some aspects, a linear library molecule can be circularized to generate a circularized library molecule, and the circularized library molecule can be clonally amplified in-solution or on-support to generate a concatemer. In some aspects, the concatemer can serve as a nucleic acid template molecule which can be sequenced. The concatemer is sometimes referred to as a polony. In some aspects, a polony includes nucleotide strands.

[0042] *Sequencing Procedures*: The term "read" or "sequence read" (or sequencing reads) refers to a sequence obtained from a portion of a nucleic acid sample. A read may be represented by a string of nucleotides sequenced from any part or all of a nucleic acid molecule. Typically, though not necessarily, a read represents a short sequence of contiguous base pairs in the sample. The read may be represented symbolically by the base pair sequence (in A, T, C, or G) of the sample portion. It may

be stored in a memory device and processed as appropriate to determine whether it matches a reference sequence or meets other criteria. A read may be obtained directly from a sequencing apparatus or indirectly from stored sequence information concerning the sample. In some cases, a read is a DNA sequence of sufficient length (such as at least about 25 bp) that can be used to identify a larger sequence or region, for example, that can be aligned and specifically assigned to a chromosome or genomic region or gene. For example, a sequence read may be a short string of nucleotides (such as 20-150 bases) sequenced from a nucleic acid fragment, a short string of nucleotides at one or both ends of a nucleic acid fragment, or the sequencing of the entire nucleic acid fragment that exists in the biological sample. Sequence reads may be obtained by any method known in the art. For example, a sequence read may be obtained in a variety of ways, such as using sequencing techniques or using probes, such as in hybridization arrays or capture probes, or amplification techniques.

[0043] Examples described herein can be used with any suitable sequencing chemistry, such as sequencing by synthesis (SBS), sequencing by binding, sequencing by ligation, or nanopore sequencing.

[0044] SBS can be performed with or without the use of reversible terminators. For example, SBS can be initiated by contacting the target nucleic acids with one or more nucleotides (e.g., labeled, synthetic, modified, or a combination thereof), DNA polymerase, etc. Those features where a primer is extended using the target nucleic acid as the template will incorporate a labeled nucleotide that can be detected. The incorporation time used in a sequencing run can be significantly reduced using altered polymerases. Optionally, the labeled nucleotides can further include a reversible termination property that terminates further primer extension once a nucleotide has been added to a primer. For example, a nucleotide analog having a reversible terminator moiety can be added to a primer such that subsequent extension cannot occur until a deblocking agent is delivered to remove the moiety. Thus, for examples that use reversible termination, a deblocking reagent can be delivered to the flow cell (before or after detection occurs). Washes can be carried out between the various delivery steps. The cycle can then be repeated n times to extend the primer by n

nucleotides, thereby detecting a sequence of length n. Exemplary SBS procedures, fluidic systems, and detection platforms that can be readily adapted for use with an array produced by the methods of the present disclosure are described, for example, in Bentley et al., *Nature* 456:53-59 (2008); WO 2004/018497 A2; WO 1991/006678 A1; WO 2007/123744 A1; U.S. Pat. Nos. 7,057,026 B2, 7,329,492 B2, 7,211,414 B2, 7,315,019 B2, 7,405,281 B2, and 8,343,746 B2. Sequence reads can be generated using instruments such as MINISEQ™, MISEQ™, NEXTSEQ™, HISEQX™, and NOVASEQ™ sequencing instruments from Illumina, Inc. (San Diego, CA).

[0045] One example of SBS is termed sequencing by binding. One implementation of sequencing by binding includes cycles of initiating sequencing of a template with a reversible blocker on the 3' end to prevent additional bases from incorporating, interrogating the template by flooding the flow cell with fluorescently tagged bases that do not include a blocker and measuring an emitted signal of bound bases, activating the 3' end via removal of the reversible blocker, and incorporating the complementary base from unlabeled, blocked nucleotides. Reads using sequencing by binding can be generated from using instruments such as ONSO™ sequencing instruments from Pacific Biosciences of California, Inc. (Menlo Park, CA). Another implementation of sequencing by binding could be sequencing by avidity. In sequencing by avidity, fluorescent dye labeled cores, termed avidites, are used. One potential cycle of sequencing by avidity includes providing a reagent of polymerase and reversibly terminated nucleotides to templates immobilized on a solid surface, de-blocking the incorporated nucleotides, flowing a set of four types of avidites, washing away unbound avidites, detecting the incorporated bases/nucleotides, and removing the bound avidites. The steps in the cycle of sequencing by avidity may be performed in other orders. Sequencing by avidity is described in Arslan, S., Garcia, F.J., Guo, M., et al. "Sequencing by avidity enables high accuracy with low reagent consumption." *Nat Biotechnol* 42, 132–138 (2024). https://doi.org/10.1038/s41587-023-01750-7, which is incorporated by reference in its entirety. Reads using sequencing by avidity can be generated using instruments such as AVITI™ sequencing instruments from Element Biosciences (San Diego).

[0046] One example of SBS using an open flow cell and without using reversible terminators is disclosed in Almogy, G. (2022) "Cost-efficient whole genome-sequencing using novel mostly natural sequencing-by-synthesis chemistry and open fluidics platform" https://doi.org/10.1101/2022.05.29.493900, which is incorporation by reference in its entirety. Sequence reads using an open flow cell can be generated using instruments such as UG 100TM Sequencer from Ultima Genomics, Inc. (Fremont, CA).

[0047] Some SBS examples include detection of a proton released upon incorporation of a nucleotide into an extension product. For example, sequencing based on detection of released protons can use an electrical detector and associated techniques that are described in U.S. Pat. Nos. 8,262,900 B2, 7,948,015 B2, 8,349,167 B2, and U.S. Pat. Pub. 2010/0137143 A1, each of which is incorporated by reference in its entirety.

[0048] Sequence reads can be generated using instruments such as DNBSEQTM sequencing instruments from MGI Tech Co., Ltd. (Shenzhen, China) and as SURFSeq™, FASTASeq™, and GenoLab™ sequencing instruments from GeneMind Biosciences Co., Ltd. (Shenzhen, China).

[0049] Some examples can use methods involving the real-time monitoring of DNA polymerase activity. For example, nucleotide incorporations can be detected through fluorescence resonance energy transfer (FRET) interactions between a fluorophore-bearing polymerase and γ-phosphate-labeled nucleotides, or with zeromode waveguides. Techniques and reagents for FRET-based sequencing are described, for example, in Levene et al., *Science* 299, 682-686 (2003); Lundquist et al., *Opt. Lett.* 33, 1026-1028 (2008); Korlach et al., *Proc. Natl. Acad. Sci.* USA 105, 1176-1181 (2008), each of which is incorporated by reference in its entirety. Techniques that sequence using zeromode waveguides are described in U.S. Pat. No. 6,917,726 B2, which is incorporated by reference in its entirety.

[0050] *Solid Support*: The terms "solid support," "solid surface," and other grammatical equivalents herein refer to any substrate that is appropriate for or can be modified to be appropriate for the attachment of enzymes, nucleic acids, and complexes thereof. As will be appreciated by those in the art, the number of possible

substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, polymers (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, polytetrafluoroethylene (e.g., TEFLON™ from Chemours), polyamides (i.e., nylon)), polysaccharides, nitrocellulose, ceramics, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, optical fiber bundles, quartz, metal oxides, inorganic oxides, other suitable transparent materials, other suitable non-transparent materials, other suitable translucent materials, and combinations thereof. The composition and geometry of the solid support can vary with its use.

[0051] In some examples, the solid support or solid surface is a planar structure, such as a flow cell, slide, chip, microchip, array, microarray, wafer, panel, charge pad, and/or web. The planar structure can be a single surface structure having a single surface of sample/reaction sites. The planar structure can be a dual surface structure. One example of a dual surface structure includes a top substrate having a top surface of sample/reactions sites, a bottom substrate having a bottom surface of sample/reactions sites, and a spacer layer separating the top substrate and the bottom substrate. The solid support or solid surface can be open to direct application of a fluid. One example of an open solid support or open solid surface is an open flow cell having a single surface structure without an inlet port. In some examples, the solid support is not necessarily planar, such as, for example, the surface of a well, tube, or other vessel. Nonlimiting examples include the surface of a microcentrifuge tube, a well of a multiwell plate, and the like.

[0052] In some examples, the solid support comprises one or more surfaces of a flowcell or flow cell. In accordance with definition set forth herein, the term “flowcell” or “flow cell” refers to a solid surface across which one or more fluid reagents can be flowed. Examples of flow cells and related fluidic systems and detection platforms that can be readily used in the methods of the present disclosure are described, for example, in Bentley et al., *Nature* 456:53-59 (2008), WO 2004/018497 A2; U.S. Pat. No. 7,057,026 B2; WO 1991/06678 A1; WO 2007/123744 A2; U.S. Pat. No. 7,329,492 B2; U.S. Pat. No. 7,211,414 B2; U.S. Pat. No. 7,315,019 B2; U.S. Pat. No. 7,405,281

B2, and U.S. Pat. Pub. 2008/0108082 A1, each of which is incorporated herein by reference in its entirety. In some examples, the flow cells can be one or more flow lanes. For flow cells having a plurality of flow lanes, each of the flow lanes can be independently accessed or two or more flow lanes can be accessed as a group.

[0053] In some examples, the solid support or solid surface is a non-planar structure, such as beads, microspheres, and/or inner and/or outer surface of a tube or vessel. The terms "beads", "microspheres," or "particles" or grammatical equivalents herein, refer to small discrete particles. Suitable bead compositions include, but are not limited to, plastics, ceramics, glass, polystyrene, methylstyrene, acrylic polymers, paramagnetic materials, thoria sol, carbon graphite, titanium dioxide, latex, polysaccharide (e.g., DEXTRAN™ , SEPHAROSE™, cellulose), polyamides, cross-linked micelles, TEFLON™, as well as any other materials outlined herein for solid supports may all be used. "Microsphere Detection Guide" from Bangs Laboratories, Fishers Ind. is a helpful guide. In certain examples, the microspheres are magnetic microspheres or beads. The beads need not be spherical; as irregular particles may be used. Alternatively or additionally, the beads may be porous. The bead sizes range from nanometers, i.e. 100 nm, to millimeters, i.e. 1 mm, with beads from about 0.2 micron to about 200 microns being preferred, and from about 0.5 to about 5 micron being particularly preferred, although in some examples smaller or larger beads may be used.

[0054] *Tagmentation*: A process in which the DNA sample strands are cleaved/fragmented and tagged (e.g., with the adapters) for analysis. Tagmentation is an *in vitro* transposition reaction.

[0055] *Transferred and Non-Transferred Strands*: The term "transferred strand" refers to a sequence that includes a transferred portion of a transposon end. Similarly, the term "non-transferred strand" refers to a sequence that includes the non-transferred portion of a transposon end. The 3'-end of a transferred strand is joined or transferred to a double stranded fragment during tagmentation. The non-transferred strand is not joined or transferred to the double stranded fragment during tagmentation. In an example, the transferred and non-transferred strands include at least partially complementary portions that are covalently bound together.

[0056] *Transposase or Transposase Enzyme*: An enzyme that is capable of forming a functional complex with a transposon end-containing composition (e.g., transposons, transposon ends, transposon end compositions) and catalyzing insertion or transposition of the transposon end-containing composition into the double-stranded DNA sample with which it is incubated, for example, in the *in vitro* transposition reaction (i.e., tagmentation). A transposase, as presented herein, can also include integrases from retrotransposons and retroviruses. Although many examples described herein refer to Tn5 transposase and/or hyperactive Tn5 transposase, it will be appreciated that any transposase that is capable of inserting a transposon end with sufficient efficiency to 5'-tag and fragment the DNA sample for its intended purpose can be used.

[0057] *Transposome / Transposome Complex*: An entity formed between a transposase enzyme and a nucleic acid. Typically, the nucleic acid is a double stranded nucleic acid including a transposase integration recognition site. For example, the transposome complex can be the product of incubating a transposase enzyme with double-stranded transposon DNA under conditions that support non-covalent complex formation. Double-stranded transposon DNA can include, for example, Tn5 DNA, a portion of Tn5 DNA, a transposon end composition, a mixture of transposon end compositions or other double-stranded DNAs capable of interacting with a transposase, such as the hyperactive Tn5 transposase.

[0058] *Transposon End*: A double-stranded nucleic acid strand that exhibits only the nucleotide sequences (the "transposon end sequences") that are necessary to form the complex with the transposase that is functional in tagmentation. The double-stranded nucleic acid strand of the transposon end can include any nucleic acid or nucleic acid analogue suitable for forming the functional complex with the transposase. For example, the transposon end can include natural DNA or DNA analogs (with modified bases and/or backbones), and can include nicks in one or both strands.

[0059] Transposases, transposomes and transposome complexes are generally known to those of skill in the art, as exemplified by the disclosure of U.S. Pat. Pub. 2010/0120098 A2, which is incorporated herein by reference in its entirety. Although many examples described herein refer to Tn5 transposase and/or hyperactive Tn5

transposase, it will be appreciated that any transposition system that is capable of inserting a transposon element with sufficient efficiency to tag a target nucleic acid can be used. In particular examples, a preferred transposition system is capable of inserting the transposon element in a random or in an almost random manner to tag the target nucleic acid.

[0060] ***Transposome Complexes***

[0061] The transposome complexes 10A, 10B used in the examples disclosed herein form dimers in solution. The dimer form is capable of attaching to the flow cell surface. An example of the transposome complexes 10A, 10B in dimer form is shown in Fig. 1.

[0062] In the example shown in Fig. 1, one of each complex 10A, 10B forms the dimer. This example dimer is a heterodimer because it includes one of each of the transposome complexes 10A, 10B. It is to be understood, however, that two of the same complexes 10A or 10B could form respective dimers. Thus, homodimers of the transposome complex 10A and homodimers of the transposome complex 10B may be formed and used together. The type of dimer(s) that form will depend upon the method used to create the dimers. In one example, the transposome complexes 10A and 10B are mixed in solution to form the dimers. In this example, heterodimers 10A-10B and homodimers 10A-10A and 10B-10B will form. In another example, homodimers of 10A and 10B are formed in separate solutions, and then the homodimers are introduced to a flow cell surface. It is to be understood that some transposome complexes 10A, 10B may not dimerize, and that these individual transposome complexes 10A, 10B can attach to the flow cell surface. The monomeric transposome complex(es) 10A, 10B will not participate in tagmentation.

[0063] In the example shown in Fig. 1, each of the transposome complexes 10A, 10B includes a transposase enzyme 12A, 12B non-covalently bound to the transposon end 14A, 14B. Each transposon end 14A, 14B is a double-stranded nucleic acid strand, one strand ME_A, ME_B of which is part of a transferred strand 16A, 16B and the other strand ME'_A, ME'_B of which is the non-transferred strand 18A, 18B. In other

words, each transposon end 14A, 14B includes a portion of the transferred strand 16A, 16B that is hybridized to the non-transferred strand 18A, 18B.

[0064] In the transposome complex 10A, the transferred strand 16A includes a 5' end functional group 20A, a first amplification domain 22A attached to the 5' end functional group 20A, and a sequencing primer sequence 24A that is attached to one strand ME_A of the transposon end 14A. As such, the strand ME_A of the transposon end 14A is positioned at the 3' end of the transferred strand 16A.

[0065] In the transposome complex 10B, the transferred strand 16B includes a second amplification domain 22B and a sequencing primer sequence 24B that is attached to one strand ME_B of the transposon end 14B. As such, the strand ME_B of the transposon end 14B is positioned at the 3' end of the transferred strand 16B.

[0066] The first and second amplification domains 22A, 22B of the transposome complexes 10A, 10B have different sequences from each other (e.g., P5 and P7), but have the same sequence, respectively, as first and second primers (shown as 26A, 26B in Fig. 2B) attached to the flow cell surface. The transposome complex 10A and the primer 26A together with the transposome complex 10B and the primer 26B enable the amplification of the tagmented and fully adapted DNA sample fragments. Examples of suitable sequences for the first amplification domain 22A/primer 26A and for the second amplification domain 22B/primer 26B include P5 and P7 sequences, examples of which are used on the surface of commercial flow cells sold by Illumina Inc. for sequencing on HISEQ™, HISEQX™, MISEQ™, MISEQDX™, MINISEQ™, NEXTSEQ™, NEXTSEQDX™, NOVASEQ™, GENOME ANALYZER™, ISEQ™, and other instrument platforms.

[0067] The P5 primer sequence is one of:

P5 #1: 5' → 3'
AATGATACGGCGACCACCGAGAUCTACAC (SEQ. ID. NO. 1);

P5 #2: 5' → 3'
AATGATACGGCGACCACCGAGAnCTACAC (SEQ. ID. NO. 2)

where “n” is inosine in SEQ. ID. NO. 2; or

P5 #3: 5’ → 3’
AATGATACGGCGACCACCGAGAnCTACAC (SEQ. ID. NO. 3)

where “n” is alkene-thymidine (i.e., alkene-dT) in SEQ. ID. NO. 3.

The P5’ sequence is the complement of any of the P5 examples.

The P7 primer sequence may be any of the following:

P7 #1: 5’ → 3’
CAAGCAGAAGACGGCATACGAnAT (SEQ. ID. NO. 4)

P7 #2: 5’ → 3’
CAAGCAGAAGACGGCATACnAGAT (SEQ. ID. NO. 5)

P7 #3: 5’ → 3’
CAAGCAGAAGACGGCATACnAnAT (SEQ. ID. NO. 6)

where “n” is 8-oxoguanine in each of SEQ. ID. NOS. 4-6. The P7’ sequence is the complement of any of the P7 examples.

[0068] It is to be understood that other sequences may be used for the amplification domains 22A, 22B and for the primers 26A, 26B, as long as the combination enables the desired amplification. As such, the designations P5, P5’, and P7, P7’ are provided as examples, and the corresponding domains 22A, 22B and/or primers 26A, 26B are not limited to the specific sequences set forth herein. As other examples, a P15, PA, PB, PC, or PD primer may be used.

The P15 primer sequence is:

P15: 5' → 3'
AATGATACGGCGACCACCGAGAnCTACAC (SEQ. ID. NO. 7)

where "n" is allyl-T (i.e., a thymine nucleotide analog having an allyl functionality).

The other primer sequences (PA-PD) mentioned above include:

PA 5' → 3'
GCTGGCACGTCCGAACGCTTCGTTAATCCGTTGAG (SEQ. ID. NO. 8)

PB 5' → 3'
CGTCGTCTGCCATGGCGCTTCGGTGGATATGAACT (SEQ. ID. NO. 9)

PC 5' → 3'
ACGGCCGCTAATATCAACGCGTCGAATCCGCAACT (SEQ. ID. NO. 10)

PD 5' → 3'
GCCGCGTTACGTTAGCCGGACTATTCGATGCAGC (SEQ. ID. NO. 11)

[0069] While not shown in the example sequences for PA-PD, it is to be understood that any of these sequences may include a cleavage site 38A, 38B, such as uracil, 8-oxoguanine, allyl-T, diols, etc. at any point in the strand. The sequences for the first amplification domain 22A/primer 26A and for the second amplification domain 22B/primer 26B may be selected to have orthogonal cleavage sites 38A, 38B (i.e., one cleavage site 38A is not susceptible to the cleaving agent used for the other cleavage site 38B, and vice versa), so that after amplification, forward or reverse strands can be cleaved, leaving the other of the reverse or forward strands on the flow cell surface for sequencing.

[0070] The primers 26A, 26B may also include a polyT sequence at the 5' end of the primer sequence. In some examples, the polyT region includes from 2 T bases to 20 T bases. As specific examples, the polyT region may include 3, 4, 5, 6, 7, or 10 T bases.

[0071] The sequencing primer sequences 24A, 24B of the respective transferred strands 16A, 16B have different sequences from each other that respectively bind to sequencing primers introduced into the flow cell 30 (see Fig. 2A) after tagmentation and amplification. As examples, the sequencing primer sequence 24A may bind a sequencing primer that primes synthesis of a new strand that is complementary to forward strand fragments and the sequencing primer sequence 24B may bind a sequencing primer that primes synthesis of a new strand that is complementary to reverse strand fragments.

[0072] While not shown in Fig. 1A, it is to be understood that the transferred strands 16A, 16B may further include an index sequence between the amplification domain 22A, 22B and the sequencing primer sequences 24A, 24B. In an example, the index sequence is a unique barcode sequence that can be used for DNA sample fragment identification and indexing. In another example, the index sequence is a unique molecular index (UMI). The index sequences may be desirable when the flow cell 30 is configured to attach different transposome complexes 10A, 10B with different index sequences at different areas of the flow cell 30 or when DNA is mixed in solution with the transposome complexes 10A, 10B prior to being introduced into the flow cell 30. The different transposome complexes 10A, 10B are used to tagment different samples, thus enabling individual samples to be uniquely indexed. Examples methods and flow cells that enable indexing of different DNA samples are described in U.S. Provisional Patent Application Serial No. 63/622,026 entitled "INDEXING TECHNIQUES FOR TAGMENTED DNA LIBRARIES" filed December 15, 2023, which is incorporated herein by reference in its entirety.

[0073] The transposon end 14A of the transposome complex 10A includes the strand ME_A hybridized to the strand ME'_A. As such, the strands ME_A and ME'_A are complementary. Similarly, the transposon end 14B of the transposome complex 10B includes the strand ME_B hybridized to the strand ME'_B. As such, the strands ME_B and

ME'_B are complementary. Each of the double stranded transposon ends 14A, 14B is respectively capable of complexing with the transposase enzyme 12A, 12B. As examples, the strands ME_A, ME'_A and ME_B, ME'_B of the transposon end 14A,14B may be the related but non-identical 19-base pair (bp) outer end (e.g., strand ME_A, ME_B) and inner end (e.g., strand ME'_A, ME'_B) sequences that serve as the substrate for the activity of the Tn5 transposase, or the mosaic ends recognized by a wild-type or mutant Tn5 transposase, or the R1 end (e.g., strand ME_A, ME_B) and the R2 end (strand ME'_A, ME'_B) recognized by the MuA transposase.

[0074] The transposome complexes 10A, 10B are configured for asymmetric attachment to the flow cell surface. For asymmetric attachment, one complex 10A includes a functional group 20A that is attached at a 5' end of its transferred strand 16A (referred to herein as the 5' end functional group 20A), and the other complex 10B includes a functional group 20B that is attached at a 3' end of its non-transferred strand 18B (referred to herein as the 3' end functional group 20B). In an example, each of the 5' and 3' end functional groups 20A, 20B may be any functional group that is capable of covalently or non-covalently attaching, directly or indirectly, to surface functional groups of a polymeric hydrogel present on a flow cell surface (see reference numeral 28 Fig. 2B and Fig. 3). Thus, in this example, the 5' and 3' end functional groups 20A, 20B will depend upon the surface functional groups of the polymer hydrogel 28. In one example, the polymeric hydrogel 28 includes azide or tetrazine surface groups, and the 5' and 3' end groups 20A, 20B each include a terminal alkyne (e.g., hexynyl) or an internal alkyne, where the alkyne is part of a cyclic compound (e.g., bicyclo[6.1.0]nonyne (BCN)). In another example, the polymeric hydrogel 28 is biotinylated, and each of the 5' and 3' end groups 20A, 20B is biotin. In these examples, additional streptavidin or avidin is added to indirectly attach the biotin groups to one another as described herein. Still other reactive pairs (e.g., R^A of the polymeric hydrogel and 5' and 3' end functional groups 20A, 20B) include tetrazine/TCO, amine/carboxylic acid, amines/alkyl halides, thiol/alkene, or thiol/carboxylic acid. In still another example, the 5' and 3' end functional groups 20A, 20B may be any functional group that can attach to a transposome capture mechanism positioned at a surface of the substrate (e.g., at the interstitial regions 34)

or to a substrate surface group positioned at the interstitial regions 34 as described in U.S. Prov. App. No. 63/752,277 entitled "TRANSPOSOME COMPLEX ATTACHMENT TO FLOW CELL SURFACES" filed January 31, 2025, which is incorporated herein by reference in its entirety. Examples are set forth in Tables 1A and 1B below.

TABLE 1A			TABLE 1B	
Transposome Capture Mechanism	**Transposome Complex Attachment Groups**		**Substrate Surface Groups**	**Transposome Complex Attachment Groups**
Biotinylated probe	-Biotin		Epoxide	-Biotin-bicyclononyne-methyl tetrazine-amino PEG
	-Biotin-Streptavidin		Azide	-Biotin
Hydrogel layer with biotinylated probes	-Biotin		(Activated) Carboxylic Acid	-Streptavidin
	-Biotin-Streptavidin			-Biotin-Streptavidin
Hydrophobic layer	-Biotin			-Amine
	-Biotin-Streptavidin			-Alcohol
Lysine-Streptavidin	-Biotin			-Thiol
Arginine-Streptavidin	-Biotin			-Amide
				-Lysine
Histidine-Streptavidin	-Biotin			Aspartic acid
tetrazine-PEG-methyl	-Biotin-Bicyclononyne		Silanol	-Biotin-Streptavidin
SiO_2	-Biotin-Linker-Silane			-Amide
Lysine	-Biotin-Streptavidin			-Alcohol
Arginine	-Biotin-Streptavidin			
Histidine	-Biotin-Streptavidin			
Streptavidin	-Biotin			
Nitriloacetic acid (NTA)	-Histidine			

[0075] ***Flow Cell***

[0076] Each of the method and kit disclosed herein uses or includes a flow cell. A top view of an example of the flow cell 30 is shown in Fig. 2A. As will be discussed in

reference to Fig. 2B, examples of the flow cell 30 include at least one substrate 40 or 42. In some examples, the substrate 40 or 42 is not bonded to another component, and thus is open to the surrounding environment. In other examples, a cover slip or other lid is bonded to a portion of the substrate 40 or 42. In still other examples, a second substrate (not shown) is bonded to a portion of the substrate 40 or 42.

[0077] As noted, two different substrates 40 or 42 are shown in Fig. 2B.

[0078] The substrate 40 is a single layered structure. Examples of suitable materials for the single layered substrate 40 include epoxy siloxane, glass, modified or functionalized glass, polymeric materials (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, polytetrafluoroethylene (such as TEFLON® from Chemours), cyclic olefins/cyclo-olefin polymers (COP) (such as ZEONOR® from Zeon), polyimides, nylon (polyamides), etc.), ceramics/ceramic oxides, silica, fused silica, or silica-based materials, aluminum silicate, silicon and modified silicon (e.g., boron doped p+ silicon), silicon nitride (Si_3N_4), silicon oxide (SiO_2), tantalum pentoxide (Ta_2O_5) or other tantalum oxide(s) (TaO_x), hafnium oxide (HfO_2), carbon, metals, or the like. In any of the examples disclosed herein, the substrate 40 may be selected to be transparent to visible light.

[0079] The substrate 42 is a multi-layered structure. The multi-layered substrate 42 includes a base support 46 and a patterned material 44 on the base support 46. In any of the examples disclosed herein, the components of the substrates 42 may be selected to be transparent to visible light.

[0080] The base support 46 may be any of the examples set forth herein for the single layered substrate 40. The patterned material 44 may be any material that is capable of being patterned with depressions 32.

[0081] In an example, the patterned material 44 may be an inorganic oxide that is selectively applied to the base support 46, e.g., via vapor deposition, aerosol printing, or inkjet printing, in the desired pattern. Examples of suitable inorganic oxides include tantalum oxide (e.g., Ta_2O_5), aluminum oxide (e.g., Al_2O_3), silicon oxide (e.g., SiO_2), hafnium oxide (e.g., HfO_2), etc. In another example, the patterned material 44 may be a resin matrix material that is applied to the base support 46 and then patterned.

Suitable deposition techniques include chemical vapor deposition, dip coating, dunk coating, spin coating, spray coating, puddle dispensing, ultrasonic spray coating, doctor blade coating, aerosol printing, screen printing, microcontact printing, etc. Suitable patterning techniques include photolithography, nanoimprint lithography (NIL), stamping techniques, embossing techniques, molding techniques, microetching techniques, printing techniques, etc. Some examples of suitable resins include a polyhedral oligomeric silsesquioxane-based resin, a non-polyhedral oligomeric silsesquioxane epoxy resin, a poly(ethylene glycol) resin, a polyether resin (e.g., ring opened epoxies), an acrylic resin, an acrylate resin, a methacrylate resin, an amorphous fluoropolymer resin (e.g., CYTOP® from Bellex), and combinations thereof.

[0082] In an example, the substrate 40 or 42 may be round and have a diameter ranging from about 2 mm to about 300 mm, or may be a rectangular sheet or panel having its largest dimension up to about 10 feet (~ 3 meters). In an example, the substrate 40 or 42 is a wafer having a diameter ranging from about 200 mm to about 300 mm. Wafers may subsequently be diced to form an individual flow cell substrate. In another example, the substrate 40 or 42 is a die having a width ranging from about 0.1 mm to about 10 mm. While example dimensions have been provided, it is to be understood that a substrate 40 or 42 with any suitable dimensions may be used. For another example, a panel may be used that is a rectangular support, which has a greater surface area than a 300 mm round wafer. Panels may subsequently be diced to form individual flow cells.

[0083] As shown Fig. 2A, the flow cell 30 also includes a flow channel 36. While several flow channels 36 are shown in Fig. 2A, it is to be understood that any number of flow channels 36 may be included in the flow cell 30 (e.g., a single channel 36, four channels 36, etc.). Each flow channel 36 may be isolated from each other flow channel 36 in a flow cell 30 so that fluid introduced into any particular flow channel 30 does not flow into any adjacent flow channel 36.

[0084] The flow channel 36 may be defined, at least in part, by an interposer (not shown) that is bonded to a portion 48 (see Fig. 2B) of the substrate 40 or 42. In the open configuration, the interposer may be positioned along the perimeter of the

substrate 40, 42 to define the sidewalls of a single flow channel 36 or along the perimeter and then intermittently along the length to define two or more flow channels 36. In the enclosed examples, the interposer may be positioned in a similar manner as described for the open configuration, but may also function to bond the substrate 40 or 42 to the cover slip, lid, or second substrate.

[0085] The flow channel 36 may alternatively be defined, at least in part, by a lane that is formed into the substrate 40 or 42. The lane may extend a predetermined depth from a surface of the substrate 40 or 42, and the sidewalls of the lane form the sidewalls of the flow channel 36. A lane may be etched, engraved, or imprinted into the substrate 40 or 42. When the lane is used, the depressions 32 may be patterned in the area that defines the lane.

[0086] In an example (similar to that shown in Fig. 1), the flow channel 36 has a substantially rectangular configuration with rounded ends. The length and width of the flow channel 36 may be smaller, respectively, than the length and width of the substrate 40 or 42 so that a portion of the substrate surface surrounding the flow channel 36 is available for attachment to another substrate 40 or 42 or to a lid or to define the perimeter of the open flow channel 36. In some instances, the width of each flow channel 36 can be at least about 1 mm, at least about 2.5 mm, at least about 5 mm, at least about 7 mm, at least about 10 mm, or more. In some instances, the length of each flow channel 36 can be at least about 10 mm, at least about 25 mm, at least about 50 mm, at least about 100 mm, or more. The width and/or length of each flow channel 36 can be greater than, less than or between the values specified above. In another example, the flow channel 36 is square (e.g., 10 mm x 10 mm).

[0087] The depth/height of each flow channel 36 can be as small as a few monolayers thick, for example, when microcontact, aerosol, or inkjet printing is used to deposit the interposer that partially defines the flow channel sidewalls. In other examples, the depth/height of each flow channel 36 can be about 1 μm, about 10 μm, about 50 μm, about 100 μm, or more. In an example, the depth/height may range from about 10 μm to about 100 μm. In another example, the depth/height is about 5 μm or less. It is to be understood that the depth/height of each flow channel 36 can also be greater than, less than or between the values specified above. The depth/height of the

flow channel 36 may also vary along the length and width of the flow cell 30 due to the depressions 32.

[0088] The flow cell architecture includes the depressions 32 separated by the interstitial regions 34, as shown in Fig. 2B. Many different layouts of the depressions 32 may be envisaged, including regular, repeating, and non-regular patterns. In an example, the depressions 32 are disposed in a hexagonal grid for close packing and improved density. Other layouts may include, for example, rectangular layouts, triangular layouts, and so forth. In some examples, the layout or pattern can be an x-y format in rows and columns. In some other examples, the layout or pattern can be a repeating arrangement of the depressions 32 and the interstitial regions 34. In still other examples, the layout or pattern can be a random arrangement of the depressions 32 and the interstitial regions 34.

[0089] The layout or pattern may be characterized with respect to the density (number) of the depressions 32 in a defined area. For example, the depressions 32 may be present at a density of approximately 2 million per mm^2. The density may be tuned to different densities including, for example, a density of about 100 per mm^2, about 1,000 per mm^2, about 0.1 million per mm^2, about 1 million per mm^2, about 2 million per mm^2, about 5 million per mm^2, about 10 million per mm^2, about 50 million per mm^2, or more, or less. It is to be further understood that the density can be between one of the lower values and one of the upper values selected from the ranges above, or that other densities (outside of the given ranges) may be used. As examples, a high density array may be characterized as having depressions 32 separated by less than about 100 nm, a medium density array may be characterized as having the depressions 32 separated by about 400 nm to about 1 μm, and a low density array may be characterized as having the depressions 32 separated by greater than about 1 μm.

[0090] The layout or pattern of the depressions 32 may also or alternatively be characterized in terms of the average pitch, or the spacing from the center of one depression 32 to the center of an adjacent depression 32 (center-to-center spacing) or from the right edge of one depression 32 to the left edge of an adjacent depression 32 (edge-to-edge spacing). The pattern can be regular, such that the coefficient of

variation around the average pitch is small, or the pattern can be non-regular in which case the coefficient of variation can be relatively large. In either case, the average pitch can be, for example, about 50 nm, about 0.1 μm, about 0.5 μm, about 1 μm, about 5 μm, about 10 μm, about 100 μm, or more or less. The average pitch for a particular pattern can be between one of the lower values and one of the upper values selected from the ranges above. In an example, the depressions 32 have a pitch (center-to-center spacing) of about 1.5 μm. While example average pitch values have been provided, it is to be understood that other average pitch values may be used.

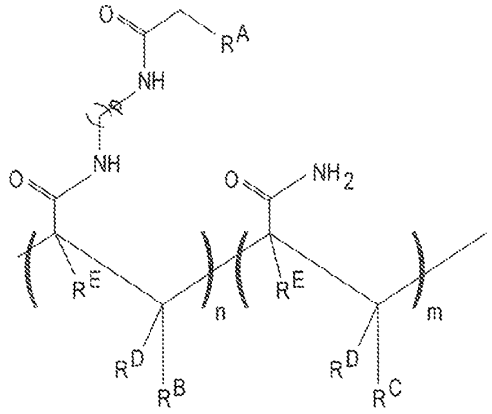
[0091] The size of each depression 32 may be characterized by its volume, opening area, depth, and/or diameter. For example, the volume can range from about 1×10^{-3} μm^3 to about 100 μm^3, e.g., about 1×10^{-2} μm^3, about 0.1 μm^3, about 1 μm^3, about 10 μm^3, or more, or less. For another example, the opening area can range from about 1×10^{-3} μm^2 to about 100 μm^2, e.g., about 1×10^{-2} μm^2, about 0.1 μm^2, about 1 μm^2, at least about 10 μm^2, or more, or less. For still another example, the depth can range from about 0.1 μm to about 100 μm, e.g., about 0.5 μm, about 1 μm, about 10 μm, or more, or less. For yet another example, the diameter or length and width can range from about 0.1 μm to about 100 μm, e.g., about 0.5 μm, about 1 μm, about 10 μm, or more, or less.

[0092] The flow cell architecture also includes the polymeric hydrogel 28 in each of the depressions 32.

[0093] The polymeric hydrogel 28 may be poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM) or another of the acrylamide copolymers disclosed herein, polyethylene glycol (PEG)-acrylate, PEG-diacrylate, PEG-amine, PEG-carboxylate, PEG-dithiol, PEG-epoxide, PEG-isocyanate, PEG-maleimide, crosslinked poly(methyl methacrylate) (PMMA), polyvinylpyrrolidone (PVPON), polyvinyl alcohol (PVA), polyethylene oxide-polypropylene oxide block copolymers (PEO-PPO), poly(hydroxyethyl methacrylate) (PHEMA), poly(N,N'-dimethylacrylamide) PAZNAM, poly(N-isopropylacrylamide) (PNIPAAm), poly(lactic acid)-poly(ethylene glycol) block copolymers, poly(ethylene glycol)-poly(lactic-co-glycolic acid) block copolymers, poly(acrylic-co-vinylsulfonic acid), poly(acrylamide-co-vinylsulfonic acid), poly(L-aspartic acid), poly(aspartamide),

adipic dihydrazide modified or aldehyde modified poly(L-glutamic acid), bisacrylamide, or hydrogels based on one or more of polylysine, starch, agar, agarose, heparin, alginate, alginate sulfate, dextran sulfate, hyaluronan, pectin, carrageenan, gelatin, chitosan, cellulose, and collagen, or combinations or mixtures thereof.

[0094] In one example, the polymeric hydrogel 28 includes an acrylamide copolymer. In this example, the acrylamide copolymer has a structure (I):



wherein:

R^A is an azide or a tetrazine or any other functional group that can attach to an alkyne, an amino, an alkenyl, an alkyne, a halogen, a hydrazone, a hydrazine, a carboxyl, a hydroxy, a tetrazole, nitrone, sulfate, or thiol;

R^B is H or optionally substituted alkyl;

R^C, R^D, and R^E are each independently selected from the group consisting of H and optionally substituted alkyl;

each of the $-(CH_2)_p-$ can be optionally substituted;

p is an integer in the range of 1 to 50;

n is an integer in the range of 1 to 50,000; and

m is an integer in the range of 1 to 100,000.

[0095] One specific example of the acrylamide copolymer represented by structure (I) is poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide, PAZAM.

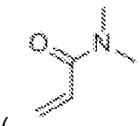
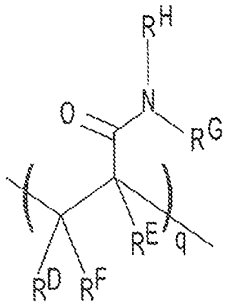
[0096] One of ordinary skill in the art will recognize that the arrangement of the recurring “n” and “m” features in structure (I) are representative, and the monomeric

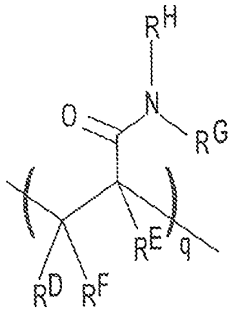
subunits may be present in any order in the polymer structure (e.g., random, block, patterned, or a combination thereof).

[0097] The molecular weight of the acrylamide copolymer may range from about 5 kDa to about 1500 kDa or from about 10 kDa to about 1000 kDa, or may be, in a specific example, about 312 kDa.

[0098] In some examples, the acrylamide copolymer is a linear polymer. In some other examples, the acrylamide copolymer is a cross-linked polymer with various degrees of cross-linking.

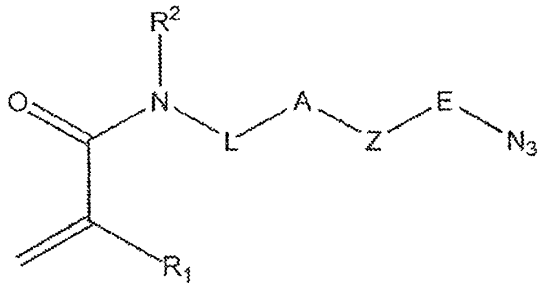
[0099] In some examples, the polymeric hydrogel 28 may be a variation of structure (I). In one example, the acrylamide unit may be replaced with N,N-dimethylacrylamide

(). In another example, the acrylamide unit in structure (I) may be replaced with,  where R^D, R^E, and R^F are each H or a C1-C6 alkyl, and R^G and R^H are each a C1-C6 alkyl (instead of H as is the case with the acrylamide). In this example, q may be an integer in the range of 1 to 100,000. In another example, the N,N-dimethylacrylamide may be used in addition to the



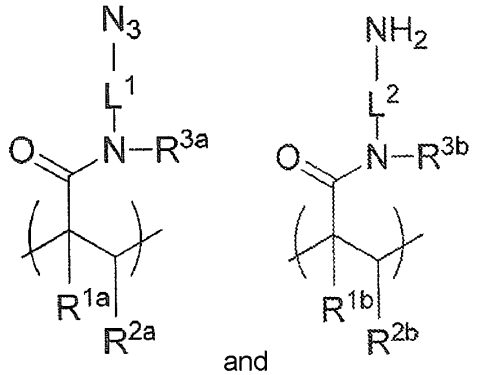
acrylamide unit. In this example, structure (I) may include in addition to the recurring "n" and "m" features, where R^D, R^E, and R^F are each H or a C1-C6 alkyl, and R^G and R^H are each a C1-C6 alkyl. In this example, q may be an integer in the range of 1 to 100,000.

[0100] As another example of the polymeric hydrogel 28, the recurring "n" feature in structure (I) may be replaced with a monomer including a heterocyclic azido group having structure (II):



wherein R^1 is H or a C1-C6 alkyl; R_2 is H or a C1-C6 alkyl; L is a linker including a linear chain with 2 to 20 atoms selected from the group consisting of carbon, oxygen, and nitrogen and 10 optional substituents on the carbon and any nitrogen atoms in the chain; E is a linear chain including 1 to 4 atoms selected from the group consisting of carbon, oxygen and nitrogen, and optional substituents on the carbon and any nitrogen atoms in the chain; A is an N substituted amide with an H or a C1-C4 alkyl attached to the N; and Z is a nitrogen containing heterocycle. Examples of Z include 5 to 10 carbon-containing ring members present as a single cyclic structure or a fused structure. Some specific examples of Z include pyrrolidinyl, pyridinyl, or pyrimidinyl.

[0101] As still another example, the polymeric hydrogel 28 may include a recurring unit of each of structure (III) and (IV):



and

wherein each of R^{1a}, R^{2a}, R^{1b} and R^{2b} is independently selected from hydrogen, an optionally substituted alkyl or optionally substituted phenyl; each of R^{3a} and R^{3b} is independently selected from hydrogen, an optionally substituted alkyl, an optionally substituted phenyl, or an optionally substituted C7-C14 aralkyl; and each L^1 and L^2 is independently selected from an optionally substituted alkylene linker or an optionally substituted heteroalkylene linker.

[0102] The R^A group of the polymeric hydrogel 28 is capable of attaching to a 5' end group of primers 26A, 26B of an amplification primer set. In some examples, the R^A group of the polymeric hydrogel 28 is capable of attaching to the 5' end group of primers 26A, 26B of an amplification primer set and to the end functional groups 20A, 20B of the transposome complexes 10A, 10B. In other examples, biotin is attached to the surface of the polymeric hydrogel (e.g., 28) through some of the R^A groups (e.g., the azide, tetrazine, or other functional group that can attach to an alkyne). In one specific example, the biotin is attached to a linker, such as bicyclo[6.1.0]nonyne (BCN), which can covalently attach to some of the R^A groups. Streptavidin may be attached to the hydrogel-bound biotin to attach biotinylated primers and biotinylated transposome complexes. When biotin is attached to the polymeric hydrogel 28, the biotin, and any linker used with the biotin, may be added to the polymeric hydrogel 28 before or after the polymeric hydrogel 28 is applied to the depressions 32.

[0103] The polymeric hydrogel 32 can be added to a liquid carrier and applied to the substrate 40, 42 using any suitable deposition technique. In an example, the polymeric hydrogel solution/mixture is blanketly deposited and then removed from the interstitial regions 34 using polishing. Polishing leaves the polymeric hydrogel 28 intact in the depressions 32.

[0104] As shown in Fig. 2B, the flow cell 30 includes the primers 26A, 26B attached to the polymeric hydrogel 28. Any of the primer sequences set forth herein for the amplification domain can be used, as long as together they enable the amplification of the fully adapted DNA fragments. As specific examples, the primers 26A, 26B may be P5 and P7 primers or P15 and P7 primers.

[0105] The 5' end of each primer 26A, 26B may also include a linker. Any linker that includes a terminal alkyne group or another suitable terminal functional group that can attach to the surface functional groups (e.g., R^A) of the polymeric hydrogel 28 may be used. In one example, the primers 26A, 26B are terminated with hexynyl functional groups.

[0106] The primers 26A, 26B may be added to a carrier fluid, and the fluid may be introduced to the flow cell 30 and allowed to incubate. The primers 26A, 26B may be included in a carrier liquid in a concentration ranging from about 0.5 μM to about 100 μM. In one example, the primer concentration ranges from about 5 μM to about 25 μM. The carrier liquid of the primer fluid may be water. A buffer and/or salt may be added to the carrier liquid for grafting the primers 26A, 26B to suitable functional groups of the polymeric hydrogel 28. The buffer has a pH ranging from 5 to 12, and the buffer used will depend upon the 5' end functional group of the primers 26A, 26B. As examples, a neutral buffer and/or salt may be added to the primer fluid for grafting BCN terminated primers, while an alkaline buffer may be added to the primer fluid for copper-assisted grafting methods (e.g., the click reaction). Any of the primer fluids used in copper-assisted grafting methods may also include a copper catalyst. Example of neutral buffers include Tris(hydroxymethyl) aminomethane (Tris or TRIS) buffers, such as Tris-HCl or Tris-EDTA, or a carbonate buffer (e.g., 0.25 M to 1 M). Sodium sulfate (e.g., 1 M to 2 M) is a suitable salt that may be used. Examples of alkaline buffers include Tris(hydroxymethyl) aminomethane (CHES), 3-

(Cyclohexylamino)-1-propanesulphonic acid (CAPS), and alkaline buffer solution (from Sigma-Aldrich).

[0107] Primer grafting may be performed at a temperature ranging from about 55°C to about 65°C for a time ranging from about 20 minutes to about 60 minutes. In one example, grafting is performed at 60°C for about 30 minutes or 60 minutes. It is to be understood that a lower temperature and a longer time or a higher temperature and a shorter time may also be used. During grafting, the 5' ends of the primers 26A, 26B attach to at least some of the surface groups of the polymeric hydrogel 28 and have no affinity for the interstitial regions 34 or other edge portions, e.g., portion 48, of the substrate 40 or 42.

[0108] The flow cell 30 also includes the transposome complexes 10A, 10B directly or indirectly (e.g., through the linker) attached to the R^A groups of the polymeric hydrogel 28, to a transposome capture mechanism at the surface of the substrate 40 or 42, and/or to a surface group of the substrate 40 or 42.

[0109] The transposome complexes 10A, 10B may be added to a carrier fluid, and the resulting transposome complex fluid may be introduced to the flow cell 30 and allowed to incubate. The transposome complexes 10A, 10B may be included in the carrier fluid in a concentration ranging from about 0.01 μM to about 1 μM. In this example, the carrier fluid may be water. A buffer and/or salt may be added to the carrier liquid for attaching (e.g., grafting or binding) the transposome complexes 10A, 10B to suitable functional groups of the polymeric hydrogel 28, the transposome capture mechanism, and/or the substrate 40 or 42. The buffer has a pH ranging from 5 to 12. Any of the neutral buffers and/or salts set forth herein may be added to the transposome complex fluid.

[0110] The transposome complex fluid may be introduced to the flow cell 30 using any suitable technique for the open or enclosed versions of the flow cell 30. Grafting may be performed at a temperature ranging from about 35°C to about 60°C for a time ranging from about 30 minutes to about 120 minutes. In one example, grafting is performed at 37°C for about 90 minutes or 120 minutes. Binding may be performed at a temperature ranging from about 20°C to about 45°C for a time ranging from about 30 minutes to about 120 minutes. During these processes, the transposome complexes

10A, 10B (in the form of dimers) attach to at least some of the surface groups of the polymeric hydrogel 28, the transposome capture mechanism, and/or the substrate 40 or 42. In some instances, the transposome complexes 10A, 10B have no affinity for the interstitial regions 34 or edge portions of the flow cell 30.

[0111] It is to be understood that transposome complex 10A, 10B attachment may take place during flow cell manufacturing. Alternatively, the transposome complex fluid may be contained in a reagent cartridge, and transposome complex 10A, 10B attachment may take place on the sequencing instrument prior to sample preparation.

[0112] ***Kit***

[0113] Any example of the flow cell 30 disclosed herein may be used in a kit. An example kit includes the flow cell 30, which includes the substrate 40 or 42 having depressions 32 separated by interstitial regions 34, first and second primers 26A, 26B immobilized within each of the depressions 32, and first and second transposome complexes 10A, 10B immobilized within each of the depressions 32 and/or over the interstitial regions 34; an extension mix; and an enzymatic methylation conversion mix.

[0114] The extension mix includes nucleotides, a polymerase, and a buffer agent. The buffer agent may include any of the neutral buffers set forth herein (e.g., Tris), ammonium sulfate, betaine, a metal co-factor (e.g., Mg^{2+}), a surfactant (e.g., TWEEN polysorbates, TRITON™ X-100 (a non-ionic surfactant from Dow)), and/or a co-solvent (e.g., dimethylsulfoxide). An example extension mix includes from about 0.1 mM to about 0.5 mM of the nucleotides, from about 40 U/mL to about 80 U/mL of the polymerase, from about 15 mM to about 25 mM of the neutral buffer, from about 5 mM to about 15 mM of ammonium sulfate (e.g., about 10 mM ammonium sulfate), from about 1.8 M to about 2.2 M of the betaine (e.g., about 2 M betaine), from about 2 mM to about 5.5 mM of the metal co-factor, from about 0.1% to about 0.4% of the surfactant, and from about 1.0% to about 2.0% of the co-solvent. The extension mix does not include a recombinase, as it is not desirable for the fully adapted DNA fragments to be amplified immediately upon being formed via extension.

[0115] The enzymatic methylation conversion mix includes a liquid carrier and an altered cytidine deaminase. Examples of this mix are described in International Patent

Application No. PCT/US2023/017846, entitled, "Altered Cytidine Deaminases and Methods of Use" (published as WO 2023/196572 A1) and International Patent Application No. PCT/IB2023/059798, entitled, "Helicase-Cytidine Deaminase Complexes and Methods of Use," each of which is incorporated herein by reference in its entirety.

[0116] The liquid carrier in the enzymatic methylation conversion mix may be a buffer having a pH lower than 7 (e.g., ranging from 5.1 to 6.5). Examples of suitable buffers include, but are not limited to: a citrate buffer, a sodium acetate buffer, Bis Tris-Propane HC1, and Tris-HCl Tris. Examples of other buffers include, but are not limited to, Bicine, DIPSO (3-[N,N-Bis(2-hydroxyethylamino)-2-hydroxy-1-propanesulfonic acid), glycylglycine, HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), imidazole, malonate, MES (2-(N-morpholino)ethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), phosphate, PIPES (1,4-Piperazinediethanesulfonic acid), SPG (succinic acid, sodium dihydrogen phosphate, and glycine in the molar ratio 2:7:7), succinate, TAPS (N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid), TAPSO (2-Hydroxy-3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid), trincine. In some examples, a reducing agent such as dithiothreitol (DTT) can be present. In some examples, a divalent cation is not included.

[0117] The enzymatic methylation conversion also includes the altered cytidine deaminase.

[0118] In the examples set forth herein, the type of altered cytidine deaminase that is used preferentially deaminates 5mC instead of C (i.e., converts 5mC to T at a greater rate than converting C to U) and thus has cytosine-defective deaminase activity or 5mC-enhanced or 5mC-selecting deaminase activity. In one example, the altered cytidine deaminase having cytosine-defective deaminase activity includes a substitution mutation at a position functionally equivalent to tyrosine at position 130 (Y130) in a member of the APOBEC3A subfamily (for instance, SEQ. ID. NO. 12). This substitution mutation can be a mutation to alanine (A), glycine (G), phenylalanine (F), histidine (H), glutamine (Q), methionine (M), asparagine (N), lysine (K), valine (V), aspartic acid (D), glutamic acid (E), serine (S), cysteine (C), proline (P), or threonine (T). For example, the altered cytidine deaminase can be SEQ. ID. NO. 13, wherein X

is selected from A, G, F, H, Q, M, N, K, V, D, E, S, C, P or T (and is not Y), or can comprise SEQ. ID. NO. 14, wherein X is selected from A, G, F, H, Q, M, N, K, V, D, E, S, C, P or T (and is not Y). In specific examples of SEQ. ID. NO. 13 or SEQ. ID. NO. 14, X is A or L. As one specific example, the substitution mutation at a position functionally equivalent to Y130 is a mutation to alanine (A), (e.g., SEQ. ID. NO. 15). Specific examples of altered cytidine deaminases having increased activity and preferentially acting on 5mC compared to cytosine include SEQ. ID. NO. 15 or a sequence having at least 90%, at least 95%, at least 98%, at least 99% sequence identity to SEQ. ID. NO. 15 and including Y130A.

[0119] The altered cytidine deaminase having cytosine-defective deaminase activity optionally includes a second substitution mutation at a position two, three, four, or five amino acids on the C-terminal side of the Y130 position, or functionally equivalent to the Y130 position. In one example, the second mutation is a tyrosine (Y), tryptophan (W), cysteine (C), histidine (H), or phenylalanine (F) at a position two, three, four, or five amino acids on the C-terminal side of the Y130 position, or functionally equivalent to the Y130 position. In one example, the second mutation is at a position functionally equivalent to tyrosine at position 132 (Y132) in a member of the APOBEC3A subfamily (for instance, SEQ. ID. NO. 12). An APOBEC protein, such as an APOBEC3A protein, containing substitution mutations at both the first site, a position functionally equivalent to Y130, and the second site, at a position two, three, four, or five amino acids on the C-terminal side of the Y130 position, increases the preferential activity to act on 5mC compared to the same APOBEC protein, such as an APOBEC3A protein, containing one substitution mutation at Y130. In one example, the substitution mutation at the second position is an amino acid having a positively charged side chain and selected from arginine (R), histidine (H), lysine (L), or a polar side chain selected from glutamine (Q). As a specific example, the substitution mutation at the second position is histidine (H), such as Y132 to histidine. The double mutant containing both first and second mutations can be any substitution mutation at a position functionally equivalent to Y130 described herein and any second substitution mutation at a position two, three, four, or five amino acids on the C-terminal side of the Y130 position described herein, in any combination. For example,

the altered cytidine deaminase can be SEQ. ID. NO.: 12, 16, or 15 and have a substitution at Y130 and Y132, or the position functionally equivalent to Y130 and Y132 as described herein. One example of an altered cytidine deaminase is SEQ. ID. NO. 17 including Y130X and Y132X, where Y130X is selected from (A), (L), or (W) (preferably (A)), and Y132X is selected from (R), (H), (L), or (Q), preferably (H). This encompasses examples including Y130A and Y132R, Y130A and Y132H, Y130A and Y132L, Y130A and Y132Q, Y130L and Y132R, Y130L and Y132H, Y130L and Y132L, Y130L and Y132Q, Y130W and Y132R, Y130W and Y132H, Y130W and Y132L, Y130W and Y130Q, or any suitable combinations therein. In one example, the double mutant includes substitution mutations Y130A and Y132H. Specific examples of altered cytidine deaminases having both substitution mutations and preferentially acting on 5mC compared to the APOBEC protein having just the single substitution mutation at cytosine include SEQ. ID. NO. 18 or a sequence having at least 90%, at least 95%, at least 98%, at least 99% sequence identity to SEQ. ID. NO. 18 and including Y130A and Y132H.

[0120] The enzymatic methylation conversion mix includes the modified cytidine deaminase at a concentration from at least 0.05 micromolar (μM) (i.e., 50 nM) to no greater than 5 μM. As examples instances, the concentration of the enzyme can be at least 0.5 μM, or at least 1 μM, or at least 2 μM, or at least 3 μM, or at least 4 μM, or 5 μM, and/or no greater than 5 μM, or no greater than 4 μM, or no greater than 3 μM, or no greater than 2 μM, or no greater than 1 μM. In some specific examples, the concentration of the enzyme can be about 0.4 μM, or about 0.5 μM, or about 0.8 μM.

[0121] Some examples of the kit also include a denaturation reagent. Any reagent that will denature the fully adapted DNA sample fragments and that is also inert toward an attachment mechanism of each of the first and second transposome complexes 10A, 10B may be used. In other words, the denature agent can separate the double stranded fully adapted DNA sample fragments into single stranded DNA sample fragments without deleteriously affecting the attachment of the first and second transposome complexes 10A, 10B to the polymeric hydrogel 28. An example denaturation reagent includes sodium hydroxide (NaOH).

[0122] Some examples of the kit also include a tagmentation buffer. The tagmentation buffer includes water, an optional co-solvent (e.g., dimethylformamide), a metal co-factor (e.g., magnesium acetate) for the transposase enzyme 12A, 12B, and a buffer salt (e.g., Tris(hydroxymethyl) aminomethane (Tris or TRIS) acetate salt, pH 7.6). In an example, the optional co-solvent may be present in an amount up to about 11%, the metal co-factor may be present in a concentration ranging from about 3 mM to about 25 mM, and the buffer salt may be present in a concentration ranging from about 7 mM to about 12 mM. In another example, the optional co-solvent may be present in an amount up to about 10%, the metal co-factor may be present in a concentration ranging from about 3 mM to about 5.5 mM or about 10 mM, and the buffer salt may be present in a concentration ranging from about 7 mM to about 10 mM.

[0123] Still other examples of the kit include a wash solution. The wash solution is an aqueous solution including a buffer agent (e.g., Tris), a salt (e.g., sodium chloride, sodium citrate, etc.), a surfactant (e.g., TWEEN polysorbates), and/or a chelating agent (e.g., EDTA). In one example, the wash solution includes water, the salt at a concentration ranging from about 25 mM to about 50 mM, the surfactant in an amount ranging from about 0.01 wt% to about 0.1 wt%, and optionally the chelating agent. The wash solution may have a relatively high pH, e.g., ranging from about 7 to about 10.

[0124] ***Method***

[0125] An example of the method disclosed herein is described in reference to Fig. 3 and Fig. 4. The method includes initiating tagmentation of a DNA sample 60 using first and second transposome complexes 10A, 10B asymmetrically attached in the flow cell 30, thereby forming partially adapted hybridized fragments 54 including a first partially adapted DNA fragment 50A that is immobilized, at its 5' end, to a substrate 40 or 42 of the flow cell 30, and a second partially adapted DNA fragment 50B that is removably attached to the substrate 40 or 42 (see Fig. 4, at A); removing a transposase enzyme 12A, 12B from each of the first and second transposome complexes 10A, 10B (see Fig. 4, at B); initiating an extension reaction of the partially

adapted hybridized fragments 54 to form fully adapted hybridized fragments 56 (see Fig. 4, at C); denaturing a second fully adapted DNA fragment 52B from a first fully adapted DNA fragment 52A of each of the fully adapted hybridized fragments 56 (see Fig. 4, at D); and initiating enzymatic methylation conversion of the first fully adapted DNA fragment 52A (see Fig. 4, at E).

[0126] Any example of the flow cell 30 disclosed herein may be used in the method.

[0127] At the outset of the method, the DNA sample 60 may be introduced into the flow cell 30 with a tagmentation buffer (not shown). In the examples disclosed herein, at least one strand of the DNA sample 60 is suspected of including at least one 5-methyl cytosine (5mC), at least one 5-hydroxymethyl cytosine (5hmC), at least one 5-formyl cytosine (5fC), at least one 5-carboxy cytosine (5CaC), or a combination thereof. In Fig. 4, the DNA sample 60 includes 5-methyl cytosine (shown as "mC").

[0128] The introduction of the DNA sample 60 is depicted, schematically, in Fig. 3. In Fig. 3, four depressions 32 of the flow cell 30 are depicted. Each depression 32 includes the polymeric hydrogel 28, at least one dimer of transposome complexes 10A, 10B, and primers 26A, 26B. As depicted, some of the dimers are homodimers including two of the same type of transposome 10A or 10B, and others of the dimers are heterodimers including two different types of transposomes 10A and 10B. In some examples, all of the dimers are homodimers. In other examples, the transposomes 10A and 10B are mixed to form the heterodimers. Some of the dimers are attached to the polymeric hydrogel 28 via the 5' end functional group 20A of the transposome complex 10A, and some other of the dimers are attached to the polymeric hydrogel 28 via the 3' end functional group 20B of the transposome complex 10B. While not shown, it is to be understood that the dimers may also be attached at the interstitial regions 34, via the transposome capture mechanism, and/or the substrate surface groups.

[0129] When the DNA sample 60 and the tagmentation buffer are introduced into the flow cell 30 including the transposome complexes 10A, 10B, tagmentation is initiated by bringing the flow cell 30 to a tagmentation temperature. Tagmentation, which includes fragmentation and attachment as described below, may take place at a

temperature at or above 30°C. In one example, the tagmentation temperature may range from 30°C to about 55°C. In another example, the tagmentation temperature may range from 35°C to about 45°C.

[0130] With the introduction of the tagmentation buffer and the temperature brought to the tagmentation temperature, the DNA sample 60 is tagmented using the transposome complex dimers, either in the same depression 32 or across neighboring depressions 32, as shown in Fig. 3. For ease of illustration, Fig. 4, at A, illustrates one set of partially adapted hybridized fragments 50A, 50B resulting from the tagmentation (dimers not shown for ease of illustration).

[0131] The partially adapted hybridized fragments formed from tagmentation include the first partially adapted DNA fragment 50A that is immobilized, at its 5' end, to the substrate 40 or 42, and the second partially adapted DNA fragment 50B that is removably attached to the substrate 40 or 42. As a result of tagmentation, the 5' ends of the fragmented strands 50A, 50B are attached to respective 3' ends of the transferred strands 16A, 16B of the transposome complexes 10A, 10B. The partially adapted DNA fragments 50A that are attached to the 3' end of the transferred strand 16A are considered to be "immobilized" to the substrate 40 or 42 because of the covalent or non-covalent attachment of the transferred strand 16A to the polymeric hydrogel 28. In contrast, the partially adapted DNA fragments 50B that are attached to the 3' end of the transferred strand 16B are considered to be "removably attached" to the substrate 40 or 42 because the transferred strand 16B is attached to the polymeric hydrogel 28 through its hybridization to the non-transferred strand 18B.

[0132] Also as a result of tagmentation (as shown in Fig. 4, at A), the 3' ends of each of the partially adapted DNA fragments 50A, 50B are not attached to the 5' ends of the non-transferred strands 18A, 18B. As such, a gap 55 exists between the 3' end of the partially adapted DNA fragment 50A and the 5' end of the non-transferred strand 18B, and a gap 55' exists between the 3' end of the partially adapted fragment 50B and the 5' end of the non-transferred strand 18A. In one example, each gap 55, 55' is nine (9) base pairs long.

[0133] As shown in Fig. 4, at B, the transposase enzymes 12A, 12B are then removed from the complexes 10A, 10B. Transposase 12A, 12B removal may be

accomplished, for example, using sodium dodecyl sulfate (SDS) or proteinase, or by heating the flow cell 30 to about 60°C. For transposase 12A, 12B removal after tagmentation, one example of the method involves introducing and removing, sequentially, the wash solution, sodium dodecyl sulfate (SDS) or proteinase, and more of the wash solution into and out of the flow cell 30. For transposase 12A, 12B removal after tagmentation, another example of the method involves introducing the wash solution into the flow cell 30; heating the flow cell 30, containing the wash solution, to about 60°C; and then removing the wash solution from the flow cell 30. Heating the flow cell 30 after tagmentation is sufficient to denature the transposase enzymes 12A, 12B without including additional buffers or reagents.

[0134] When heat is used to remove the transposase enzymes 12A, 12B, the method may further include reducing the temperature of the flow cell 30 to about 38°C.

[0135] As depicted in Fig. 4, at B, after transposase enzyme removal 12A, 12B, the partially adapted DNA fragments 50A, 50B remain hybridized to each other, and respectively attached to the flow cell 30 via the transferred strands 16A, 16B.

[0136] An extension reaction is then initiated using an example of the extension mix disclosed herein. To initiate the extension reaction, the extension mix is introduced into the flow cell 30. The flow cell 30 may be at a temperature up to about 55°C when the extension mix is introduced. The extension reaction is depicted in Fig. 4, at C.

[0137] The non-transferred strands 18A, 18B may be dehybridized prior to extension or displaced during extension, which allows the transferred strands 16A, 16B to be copied, thus forming fully extended (fully adapted) DNA fragments 52A, 52B.

[0138] When at least some of the strands 16A, 18A and 16B, 18B remain hybridized after transposase 12A, 12B removal, the extension reaction itself may displace the non-transferred strands 18A, 18B. In this example, a strand displacing polymerase may be used.

[0139] When heat is used to remove the transposase enzymes 12A, 12B, some of the strands 16A, 18A and 16B, 18B may dehybridize during transposase 12A, 12B removal. Because the non-transferred strands 18A, 18B are relatively short, their melting temperature may be low (e.g., from about 40°C to about 50°C); as such, the

transposase 12A, 12B removal temperature may be sufficient to dehybridize at least some of the transposon ends 14A, 14B. In contrast, the longer fragments 50A, 50B may remain hybridized to one another during transposase 12A, 12B removal. It is to be understood that the longer fragments 50A, 50B may dehybridize at regions (e.g., AT rich regions) that have a lower melting temperature, but the overall insert size includes regions with higher melting temperature that do not dehybridize. These regions keep the longer partially adapted hybridized fragments 54 from falling apart, thus enabling them to be extended.

[0140] During the extension reaction, additional sequences (adapters) are added to the 3' ends of partially adapted DNA fragments 50A, 50B. The extension reaction involves the addition of nucleotides in a template dependent fashion from the 3' ends of the partially adapted DNA fragments 50A, 50B using the respective transferred strands 16B, 16A as the template. As such, one DNA fragment, e.g., 50A, is extended along the transferred strand 16B to generate complementary sections of the sequencing primer sequence 24B and the second amplification domain 22B attached to the DNA fragment 50B; and the other DNA fragment 50B is extended along the transferred strand 16A to generate complementary sections of the sequencing primer sequence 24A and the first amplification domain 22A attached to the other DNA fragment 50A. The sequences resulting from the extension reaction render the partially adapted fragments 50A, 50B fully adapted and ready for further amplification and cluster generation. The fully adapted DNA fragments 52B that are generated along the transferred strand 16A of the transposome complex 10A include the second amplification domain 22B at one end and a complement of the first amplification domain 22A at the other end. The fully adapted DNA fragments 52A that are generated along the transferred strand 16B of the transposome complex 10B include the first amplification domain 22A at one end and a complement of the second amplification domain 22B at the other end.

[0141] It is noted that the extension mix does not enable the immediate amplification of the fully adapted DNA fragments 52A, 52B.

[0142] Upon completion of the extension reaction, the second fully adapted DNA fragment 52B is denatured from the first fully adapted DNA fragment 52A of each of

the fully adapted hybridized fragments 56. Denaturation may be accomplished by introducing the denaturation reagent into the flow cell 30 and allowing it to incubate at a suitable temperature for a suitable time. In an example, denaturing the second fully adapted DNA fragment 52A involves heating the flow cell 30 to a temperature ranging from about 20°C about 60°C. In an example, the denaturation reagent may be allowed to incubate in the flow cell 30 at a temperature ranging from about 20° about 60°C for a time ranging from about 30 seconds to about 30 minutes. In one specific example, 0.1 M NaOH is introduced into the flow cell 30 and is incubated at about 22°C for about 30 seconds.

[0143] As shown at C in Fig. 4, the fully adapted DNA fragments 52A are attached to the flow cell surface (specifically to the polymeric hydrogel 28) through covalent or non-covalent bonding at the 5' end functional group 20A, while the fully adapted DNA fragments 52B are attached to the flow cell surface through hybridization to the transferred strand 16A. As mentioned herein, the denaturation reagent does not deleteriously affect the covalent or non-covalent bonding at the 5' end functional group 20A, but it does denature the fully adapted hybridized fragments 56. As such, once denatured, the fully adapted DNA fragments 52B are no longer attached to the flow cell surface and can be removed from the flow cell 30. The unattached fully adapted DNA fragments 52B are removed with the denaturation reagent as it flows through the flow cell 30. In contrast, the fully adapted DNA fragments 52A remain attached to the flow cell surface. The remaining fragment 52A is shown in Fig. 4, at D.

[0144] In some instances, removal of the fully adapted DNA fragments 52B is followed by the introduction of a wash solution. The wash solution may be introduced into and removed from the flow cell 30 to ensure that the denaturation reagent is removed.

[0145] In other instances, however, it may not be desirable to introduce the wash solution between removal and methylation conversion. Without the additional wash, the fully adapted DNA fragments 52A are less likely to form secondary structures before methylation conversion.

[0146] The methylation conversion can then take place using an example of the enzymatic methylation conversion mix disclosed herein.

[0147] The enzymatic methylation conversion mix is introduced into the flow cell 30, now containing the fully adapted DNA fragments 52A, and is allowed to incubate in the flow cell 30 at a suitable temperature for a suitable time. In an example, the incubation temperature ranges from about 37°C to about 70°C and the incubation time ranges from about 5 minutes to about 60 minutes. In another example, the incubation temperature ranges from about 37°C to about 53°C and the incubation time ranges from about 15 minutes to about 45 minutes. The enzymatic methylation conversion mix enables the fragments 52A to remain single stranded and to remove secondary structures that may form.

[0148] During exposure of the fragments 52A to the enzymatic methylation conversion mix, the conversion of 5-methylcytosine (5mC, shown as ^{m}C in Fig. 4) to thymidine (T) by deamination takes place at a greater rate than conversion of cytosine (C) to uracil (U) by deamination, resulting result in a converted single-stranded DNA fragment 58 (shown at E in Fig. 4).

[0149] The converted single-stranded DNA fragments 58 can then be amplified and sequenced.

[0150] In an example, exclusion amplification may be used to amplify the converted single-stranded DNA fragments 58. In this example, an exclusion amplification mix may be introduced into the flow cell 30. The exclusion amplification mix is capable of rapidly making copies of the converted single-stranded DNA fragments 58 at amplification sites (e.g., surface bound primers 26A, 26B).

[0151] The exclusion amplification mix includes a polymerase and nucleoside triphosphates (NTPs). Any of a variety of polymerases known in the art can be used, but in some examples, it may be desirable to use a polymerase that is exonuclease negative. The NTPs can be deoxyribonucleoside triphosphates (dNTPs) for examples where DNA copies are made. The four native species of dNTPs, including dATP, dTTP, dGTP and dCTP, may be present in a DNA amplification reagent; however, analogs can be used if desired. The NTPs can be ribonucleoside triphosphates (rNTPs) for examples where RNA copies are made. The four native species of rNTPs, including rATP, rUTP, rGTP and rCTP, may be present in a RNA amplification reagent; however, analogs can be used if desired.

[0152] The exclusion amplification mix further includes components that facilitate amplicon formation and, in some cases, increase the rate of amplicon formation. An example is a recombinase. The recombinase can facilitate amplicon formation by allowing repeated invasion/extension. More specifically, the recombinase can facilitate invasion of the converted single-stranded DNA fragments 58 by the polymerase and extension of the primer 26A, 26B by the polymerase using the converted single-stranded DNA fragments 58 as a template for amplicon formation. This process can be repeated as a chain reaction where amplicons produced from each round of invasion/extension serve as templates in a subsequent round. The process can occur more rapidly than standard PCR since a denaturation cycle (e.g., via heating or chemical denaturation) is not required. As such, exclusion amplification can be carried out isothermally.

[0153] It may also be desirable to include other components in the exclusion amplification mix, such as dithiothreitol (DTT), CP, other enzymes (e.g., creatine kinase and/or Gp32), poly(ethylene glycol), magnesium acetate, surfactants (e.g., TWEEN 10), and glycerol.

[0154] Sequencing may then be performed. In one example, sequencing by synthesis is performed by introducing a sequencing primer followed by an incorporation mix including labelled nucleotides. Optical imaging may be used to detect each instance of nucleotide incorporation.

[0155] During two-channel base calling, image data extracted from two images may be used to determine the presence of one of four base types by encoding base identity as a combination of the intensities of the two images. For example, a first nucleotide type (e.g., A) includes a first label (e.g., configured to emit a first wavelength, such as green light) and a second label (e.g., configured to emit a second wavelength, such as red light), a second nucleotide type (e.g., G) does not include either the first label or the second label, a third nucleotide type (e.g., T) includes the first label (e.g., configured to emit the first wavelength, such as green light) and does not include the second label, and a fourth nucleotide type (e.g., C) does not include the first label, but includes the second label (e.g., configured to emit the second wavelength, such as red light). Two images can then be obtained, using detection channels for the first label

and the second label. For a given spot or location in each of the two images, base identity may be determined based on whether the combination of signal identities is [on, on], [on, off], [off, on], or [off, off]. Using the previous example, the first nucleotide type (e.g., A) is detectable in both a first channel (e.g., configured to detect the first wavelength, such as red light) and a second channel (e.g., configured to detect the second wavelength, such as green light) (i.e., [on, on]), the second nucleotide type (e.g., G) is not detectable in either of the first channel or the second channel (i.e., [off, off]), the third nucleotide type (e.g., T) is detectable in the first channel (e.g. configured to detect the first wavelength, such as red light), but is not detectable in the second channel (i.e., [on, off]), and the fourth nucleotide type (e.g., C) is not detectable in the first channel, but is detectable in the second channel (e.g., configured to detect the second wavelength, such as green light) (i.e., [off, on]). Although specific pairings of bases to signal types (e.g., wavelengths) and/or combinations of channels are described above, different signal types (e.g. wavelengths) and/or permutations may also be used. Examples are described in International Pub. No. WO 2023/175037 A2, incorporated herein by reference in its entirety, which is incorporated herein by reference in its entirety.

[0156] With the sequencing data, one can compare the converted single-stranded DNA fragments 58 (and copies thereof) with a corresponding untreated DNA fragment or a corresponding DNA fragment treated with a wild type cytidine deaminase. For instance, in the sequence of the converted single-stranded DNA fragments 58 can be compared to a reference sequence thereby permitting easy identification of point mutations and inference of modified cytosines. Thus, in examples where an altered cytidine deaminase having cytosine-defective deaminase activity (i.e., converts 5mC to T at a greater rate than converting C to U) is used, C to T point mutations can be easily identified, and these point mutations are inferred as 5mC positions.

[0157] In another example method, tagmentation of the DNA sample 60 and removal of the transposase enzyme 12A, 12B takes place as described in reference to Fig. 4 at A and B. Before or after the extension reaction is initiated as described in reference to Fig. 4 at C, the double stranded DNA sample 60 may be exposed to methylation conversion. The methylation conversion of the double stranded DNA

sample 60 may be performed as described in U.S. Provisional Patent Application Serial No. 63/715,069 entitled "METHODS OF IMPROVING SEQUENCING ACCURACY" filed November 1, 2024, which is incorporated herein by reference in its entirety. As an example, the double stranded DNA sample 60 may be exposed to a conversion reagent (e.g., cytidine deaminase or a wild-type or a mutant thereof). Upon completion of the conversion and the extension reaction (regardless of the order in which the processes take place), the second fully adapted and converted DNA fragment is denatured from the first fully adapted and converted DNA fragment of each of the fully adapted hybridized and converted fragments. The remaining fully adapted and converted DNA fragment can then be amplified and sequenced as described herein.

[0158] To further illustrate the present disclosure, examples are given herein. It is to be understood that these examples are provided for illustrative purposes and are not to be construed as limiting the scope of the present disclosure.

NON-LIMITING WORKING EXAMPLES

[0159] *Example 1*

[0160] Illumina NOVASEQ™ 6000 flow cells (each including 2 lanes patterned with depressions) were used in this example. The depressions of the flow cells contained PAZAM, and were grafted with bicyclononyne (BCN)-biotin-streptavidin linkers and biotinylated primers (P5 and P7). Asymmetrically biotinylated transposomes (including P5 and P7 amplification domains) were introduced into the lanes of the flow cells and were bound to the depressions through the grafted BCN-biotin-streptavidin linkers. Different DNA samples, namely Human gDNA (hg38), Lambda DNA (no methylation and thus a negative control), and pUC 19 (fully methylated and thus a positive control), were introduced into different lanes at different amounts (either 200 ng or 500 ng), and the flow cells were brought to a suitable temperature for tagmentation.

[0161] Following tagmentation, 1% sodium dodecyl sulfate (SDS) was introduced into each of the flow cells to remove the transposase enzymes from the transposomes.

[0162] The flow cell lanes were then washed with a wash solution.

[0163] The same extension mix was added into each lane of the flow cells to initiate extension reactions. The extension mix included Tris.HCl (pH8.8 @ 25°C), $(NH_4)_2SO_4$, $MgSO_4$, TRITON™ X-100, betaine, dimethylsulfoxide, deoxynucloetide triphosphates, and Bst Large Fragment DNA polymerase. The extension reaction took place at about 60°C.

[0164] The lanes of the flow cells were then washed with a wash solution.

[0165] 0.1 M NaOH was then was added into each of the lanes of the flow cell to denature the double stranded fragments. The NaOH denaturation reagent was removed with the denatured full adapted fragments that had been attached via hybridization (similar to the description in Fig. 4). The other fully adapted fragments (attached via the BCN-biotin-streptavidin linker) remained in the depressions.

[0166] An example of the enzymatic methylation conversion mix, including an altered cytidine deaminase having cytosine-defective deaminase activity, was added into each of the lanes of the flow cells. The enzymatic methylation conversion mix was allowed to incubate for about 30 minutes at about 53°C.

[0167] The converted samples were then amplified and sequenced. The percentage of reads passing filter and the percentage of methylated cytosines that were converted were evaluated. The percentage of converted methylated cytosines was determined by comparing the results with respective reference genomes. Table 2 illustrates the DNA amount, the %PF (passing filter), and the %methylation conversion. Passing filter (PF) is the metric used to describe clusters which pass a chastity threshold. All data was rounded to the nearest whole number.

TABLE 2

DNA Amount (ng)	%PF	hg38 (%Conversion)	Lambda (%Conversion)	pUC (%Conversion)
200	25	24	1	49
500	42	25	2	51
200	23	31	3	61
500	35	30	3	62

[0168] These results demonstrate partial conversion, and the ability to perform both tagmentation and methylation conversion on the flow cell surface.

[0169] *Example 2*

[0170] Illumina NEXTSEQ™ 2000 flow cells (including 1 lane patterned with depressions) were used in this example. The depressions in each flow cell contained PAZAM, and were grafted with bicyclononyne (BCN)-biotin-streptavidin linkers and biotinylated primers (P5 and P7). Asymmetrically biotinylated transposomes (including P5 and P7 amplification domains) were introduced into each flow cell and were bound to the depressions through the grafted BCN-biotin-streptavidin linkers. 200 ng of each DNA sample, namely Human gDNA (hg38), Lambda DNA (no methylation and thus a negative control), and pUC 19 (fully methylated and thus a positive control), was introduced into a different flow cell, and each flow cell was brought to a suitable temperature for tagmentation.

[0171] Following tagmentation, the temperature of each of the flow cells was increased to about 60°C to remove the transposase enzymes from the transposomes.

[0172] The flow cells were then washed with a wash solution.

[0173] The same extension mix was added into each flow cell to initiate extension reactions. The extension mix included Tris.HCl (pH8.8 @ 25°C), $(NH_4)_2SO_4$, $MgSO_4$, TRITON™ X-100, betaine, dimethylsulfoxide, deoxynucloetide triphosphates, and Bst Large Fragment DNA polymerase. The extension reaction took place at about 60°C.

[0174] The flow cells were then washed with a wash solution.

[0175] 0.1M NaOH was then was added into each of the flow cells to denature the double stranded fragments. The NaOH denaturation reagent was removed with the denatured full adapted fragments that had been attached via hybridization (similar to the description in Fig. 4). The other fully adapted fragments (attached via the BCN-biotin-streptavidin linker) remained in the depressions.

[0176] An example of the enzymatic methylation conversion mix, including 800 nM of an altered cytidine deaminase having cytosine-defective deaminase activity and 2M betaine, was added into each of the flow cells. The enzymatic methylation conversion mix was allowed to incubate for about 30 minutes at about 45°C.

[0177] The converted samples were then amplified and sequenced. The percentage of reads passing filter and the percentage of methylated cytosines that

were converted were evaluated, and the results are shown in Table 3. All data was rounded to the nearest whole number.

TABLE 3

DNA Amount (ng)	%PF	hg38 (%Conversion)	Lambda (%Conversion)	pUC (%Conversion)
200	30	42	4	84

[0178] These results demonstrate partial conversion, and the ability to perform both tagmentation and methylation conversion on the flow cell surface.

[0179] ***Additional Notes***

[0180] It should be appreciated that all combinations of the foregoing concepts and additional concepts discussed in greater detail below (provided such concepts are not mutually inconsistent) are contemplated as being part of the inventive subject matter disclosed herein. In particular, all combinations of claimed subject matter appearing at the end of this disclosure are contemplated as being part of the inventive subject matter disclosed herein. It should also be appreciated that terminology explicitly employed herein that also may appear in any disclosure incorporated by reference should be accorded a meaning most consistent with the particular concepts disclosed herein.

[0181] Reference throughout the specification to "one example", "another example", "an example", and so forth, means that a particular element (e.g., feature, structure, and/or characteristic) described in connection with the example is included in at least one example described herein, and may or may not be present in other examples. In addition, it is to be understood that the described elements for any example may be combined in any suitable manner in the various examples unless the context clearly dictates otherwise.

[0182] While several examples have been described in detail, it is to be understood that the disclosed examples may be modified. Therefore, the foregoing description is to be considered non-limiting.

What is claimed is:

1. A kit, comprising:

a flow cell including:

a substrate having depressions separated by interstitial regions;

first and second primers immobilized within each of the depressions; and

first and second transposome complexes immobilized within each of the depressions, over the interstitial regions, or both within each of the depressions and over the interstitial regions;

an extension mix; and

an enzymatic methylation conversion mix.

2. The kit as defined in claim 1, further comprising a denaturation reagent that is inert toward an attachment mechanism of each of the first and second transposome complexes.

3. The kit as defined in one of claims 1-2, wherein:

the first transposome complexes are immobilized at a 5' end of its transferred strand; and

the second transposome complexes are immobilized at a 3' end of its non-transferred strand.

4. The kit as defined in one of claims 1-3, wherein the extension mix includes a liquid carrier, a polymerase, and nucleotides.

5. The kit as defined in one of claims 1-4, wherein the enzymatic methylation conversion mix includes a liquid carrier and an altered cytidine deaminase.

6. A method, comprising:

initiating tagmentation of a DNA sample using first and second transposome complexes asymmetrically attached in a flow cell, thereby forming partially adapted hybridized fragments including a first partially adapted DNA fragment that is

immobilized, at its 5' end, to a substrate of the flow cell, and a second partially adapted DNA fragment that is removably attached to the substrate;

removing a transposase enzyme from each of the first and second transposome complexes;

initiating an extension reaction of the partially adapted hybridized fragments to form fully adapted hybridized fragments;

denaturing a second fully adapted DNA fragment from a first fully adapted DNA fragment of each of the fully adapted hybridized fragments; and

initiating enzymatic methylation conversion of the first fully adapted DNA fragment.

7. The method as defined in claim 6, wherein initiating the tagmentation involves:

introducing the DNA sample into the flow cell with a tagmentation buffer;

introducing a tagmentation buffer into the flow cell; and

raising a temperature of the flow cell to a tagmentation temperature.

8. The method as defined in one of claims 6-7, wherein:

removing the transposase enzyme involves introducing sodium dodecyl sulfate (SDS) or proteinase to the flow cell; and

the method further comprises flushing a wash solution through the flow cell to remove the transposase enzyme and the sodium dodecyl sulfate (SDS) or proteinase from the flow cell.

9. The method as defined in one of claims 6-7, wherein removing the transposase enzyme involves heating the flow cell to about 60°C.

10. The method as defined in one of claims 6-9, wherein initiating the extension reaction involves introducing an extension mix into the flow cell, the extension mix including a liquid carrier, a polymerase, and nucleotides.

11. The method as defined in one of claims 6-10, wherein denaturing the second fully adapted DNA fragment involves heating the flow cell to a temperature ranging from about 20°C about 60°C.

12. The method as defined in one of claims 6-11, wherein initiating the enzymatic methylation conversion involves introducing an altered cytidine deaminase to the flow cell under conditions suitable for (i) conversion of 5-methylcytosine (5mC) to thymidine (T) by deamination at a greater rate than conversion of cytosine (C) to uracil (U) by deamination, to result in converted single-stranded DNA, or (ii) conversion of C to U by deamination and 5mC to T by deamination at a greater rate than conversion of 5-hydroxymethyl cytosine (5hmC) to 5-hydroxymethyl uracil (5hmU) by deamination.

13. The method as defined in one of claims 6-12, wherein the enzymatic methylation conversion generates converted fully adapted DNA fragments, and the method further comprises processing the converted fully adapted DNA fragments to produce a sequencing library.