whole genome sequencing systems such as the Roche 454 require several days of sample preparation and several days for sequencing. Figure 1 shows a flow chart demonstrating the lengthy process now involved in sample preparation for next-generation sequencing techniques, such as the Roche 454 method. As shown in this figure, it can take one day for sample pretreatment; cell lysis; nucleic acid extraction; and whole genome amplification (WGA). It can then take one to five days for generating DNA libraries, which involves the following steps: DNA fragmentation; DNA end repair; adaptor ligation; fragment immobilization; nick repair; single-strand DNA isolation; and emulsion PCR titration. Finally, it can then take one to four days to prepare the sample for sequencing and the sequencing itself using the following steps: bulk emulsion PCR; break emulsion PCR; purify PCR positive beads; prepare beads for sequencing; and performing the sequencing reaction.

What is needed are methods for preparing DNA sequencing libraries that are faster and easier to perform and the integration of these methods with the sequencers.

SUMMARY ~~OF THE INVENTION~~

The present ~~invention~~disclosure provides integrated sample preparation/nucleic acid sequencing systems and stabilized enzyme mixtures. In particular, the present ~~invention~~disclosure provides microfluidic cards configured for processing a sample and generating DNA libraries that are suitable for use in sequencing methods (e.g., next generation sequencing methods) or other suitable nucleic acid analysis methods. The present ~~invention~~disclosure also provides stabilized enzyme mixtures containing an enzyme (e.g., an enzyme used in whole genome amplification), BSA, and a sugar. Such enzyme mixtures may be lyophilized and stored at room temperature without significant loss of enzyme activity for months.

In certain embodiments, the present ~~invention~~disclosure provides microfluidic cards comprising: a) a loading port configured for introduction of a biological sample; b) a fragmentation sub-circuit comprising: i) a reagent mixture configured for digesting nucleic acid (e.g., amplified nucleic acid) to generate fragmented nucleic acid, and/or ii) a fragmentation component configured for mechanically fragmenting nucleic acid to generate fragmented nucleic acid; and c) a linker ligation sub-circuit operably linked to

said fragmentation sub-circuit, wherein said linker ligation sub-circuit comprises: i) nucleic acid linkers configured for use in sequencing methods or other methods, and ii) a ligation enzyme mixture configured for ligating said nucleic acid linkers to said fragmented nucleic acid to generate a nucleic acid sequencing library.

In certain embodiments, the present ~~invention~~disclosure provides microfluidic cards comprising: a) a loading port configured for introduction of a biological sample; b) a cell lysis subcircuit; c) as nucleic acid extraction subcircuit; d) a nucleic acid amplification subcircuit wherein said amplification subcircuit comprises i) a target sequence specific amplification such as PCR or ii) a total nucleic acid amplification subcircuit such as whole genome amplification by multiple displacement amplification; e) a fragmentation sub-circuit comprising: i) a reagent mixture configured for digesting nucleic acid (e.g., amplified nucleic acid) to generate fragmented nucleic acid, and/or ii) a fragmentation component configured for physically fragmenting nucleic acid to generate fragmented nucleic acid; f) end polishing of fragmented nucleic acid; g) a linker ligation sub-circuit operably linked to said fragmentation sub-circuit, wherein said linker ligation sub-circuit comprises: i) nucleic acid linkers configured for use in sequencing methods or other methods, and ii) a ligation enzyme mixture configured for ligating said nucleic acid linkers to said fragmented nucleic acid to generate a nucleic acid sequencing library; h) methods for the removal of non-ligated or partially ligated nucleic acids; i) This can be performed enzymatically using for example exonucleases and or ii) by bind and elute extraction or iii) by affinity isolations using affinity tags as biotin labeled ligation products; i) final library purification by enzymatic or bind elute or affinity or size exclusion etc; j) integration with a sequencing system.

In particular embodiments, the microfluidics cards further comprise at least one additional sub-circuit selected from the following group: 1) a lysis sub-circuit (e.g., operably linked to said loading port) comprising: i) a mixing chamber and, ii) lysis buffer (e.g., in a sealed package); 2) a nucleic acid extraction sub-circuit (e.g., operably linked to both said lysis sub-circuit and a waste chamber), wherein said nucleic acid extraction sub-circuit comprises: i) a nucleic acid extraction component configured to bind nucleic acids present in a lysed sample, ii) a wash buffer (e.g., in a sealed package), iii) an elution buffer (e.g., in a sealed package), and iv) a pump component (e.g., configured for

pumping the elution buffer over the nucleic acid extraction component to generate a mixture of extracted nucleic acids); 3) an amplification sub-circuit (e.g., operably linked to the nucleic acid extraction sub-circuit) comprising a stabilized enzyme mixture, wherein said stabilized enzyme mixture comprises at least one amplification-related enzyme useful for performing amplification on the extracted nucleic acid to generate amplified nucleic acid; and 4) a waste chamber;

In certain embodiments, the present ~~invention~~disclosure provides microfluidic cards comprising: a) a loading port configured for introduction of a biological sample; b) an amplification sub-circuit (e.g., operably linked to the nucleic acid extraction sub-circuit) comprising a stabilized enzyme mixture, wherein said stabilized enzyme mixture comprises at least one amplification-related enzyme useful for performing whole genome amplification on nucleic acids to generate amplified nucleic acid.

In some embodiments, the present ~~invention~~disclosure provides microfluidic cards comprising: a) a loading port configured for introduction of a biological sample; b) a waste chamber; c) a cell lysis sub-circuit operably linked to the loading port, wherein the cell lysis sub-circuit comprises: i) a mixing chamber and, ii) a first sealed package containing lysis buffer; wherein the lysis sub-circuit is configured to lyse the biological sample in the mixing chamber with the lysis buffer to generate a lysed sample; d) a nucleic acid extraction sub-circuit operably linked to both the cell lysis sub-circuit and the waste chamber, wherein the nucleic acid extraction sub-circuit comprises: i) a nucleic acid extraction component configured to bind nucleic acids present in the lysed sample, ii) a second sealed package containing wash buffer, iii) a third sealed package containing elution buffer, and iv) a pump component configured for pumping the elution buffer over the nucleic acid extraction component to generate a mixture of extracted nucleic acids; e) an amplification sub-circuit operably linked to the nucleic acid extraction sub-circuit, wherein the amplification sub-circuit comprises a stabilized enzyme mixture, wherein the stabilized enzyme mixture comprises at least one amplification-related enzyme useful for performing amplification on the extracted nucleic acid to generate amplified nucleic acid; f) a fragmentation sub-circuit operably linked to the amplification sub-circuit, wherein the fragmentation sub-circuit comprises: i) a reagent mixture configured for digesting the amplified nucleic acid to generate fragmented nucleic acid, and/or ii) a fragmentation

component configured for mechanically fragmenting the amplified nucleic acid to generate fragmented nucleic acid; and g) a linker ligation sub-circuit operably linked to the fragmentation sub-circuit, wherein the linker ligation sub-circuit comprises: i) nucleic acid linkers (adaptors) configured for use in sequencing methods, and ii) a ligation enzyme mixture configured for ligating the nucleic acid linkers to the fragmented nucleic acid to generate a nucleic acid sequencing library.

In an aspect, there is provided a microfluidic card comprising:

a) a loading port configured for introduction of a biological sample; and

b) a lysis sub-circuit operably linked to said loading port, wherein said lysis sub-circuit comprises:

i) a lysis chamber; and

ii) a lysis buffer wherein said lysis sub-circuit is configured to lyse said biological sample with said lysis buffer to generate a lysed sample;

c) a nucleic acid extraction sub-circuit operably linked to said lysis sub-circuit wherein said nucleic acid extraction sub-circuit comprises:

i) a nucleic acid extraction component configured to bind nucleic acids present in said lysed sample;

ii) a wash buffer;

iii) an elution buffer; and

iv) a pump component configured for pumping said elution buffer over said nucleic acid extraction component to generate a mixture of extracted nucleic acids; and

d) an amplification sub-circuit operably linked to said nucleic acid extraction sub-circuit, wherein said amplification sub-circuit comprises a stabilized enzyme mixture, wherein said stabilized enzyme mixture comprises at least one amplification-related enzyme useful for performing amplification on said extracted nucleic acid to generate amplified nucleic acid,

e) a fragmentation sub-circuit operably linked to said amplification sub-circuit, wherein said fragmentation sub-circuit comprises:

i) a reagent mixture configured for digesting said amplified nucleic acid to generate fragmented nucleic acid; and/or

ii) a fragmentation component configured for mechanically fragmenting said amplified nucleic acid to generate fragmented nucleic acid; and

iii) at least one type of enzyme configured for polishing the ends of said fragmented nucleic acid; and

f) a linker ligation sub-circuit operably linked to said fragmentation sub-circuit, wherein said linker ligation sub-circuit comprises:

i) nucleic acid linkers configured for use in sequencing methods;

ii) a ligation enzyme mixture configured for ligating said nucleic acid linkers to said fragmented nucleic acid to generate a nucleic acid sequencing library; and

iii) at least one type of enzyme configured for polishing the ends of said nucleic acid sequencing library; and

g) a purification sub-circuit operably linked to said linker ligation sub-circuit wherein said purification sub-circuit comprises a nucleic acid purification component.

In certain embodiments, the at least one amplification-related enzyme comprises an enzyme useful for performing whole genome amplification (WGA) (e.g., multiple displacement amplification), or useful for performing PCR, or useful for performing transcription mediated amplification (TMA). In some embodiments, the at least one amplification-related enzyme is selected from the group consisting of: Phi-29 polymerase, E. coli DNA polymerase I, inorganic pyrophosphatase, or any combination thereof. In certain embodiments, the amplification related enzymes are combined with linkers (adapters) that are used in next-generation sequencing, such as the next-generation sequencing methods described herein. In particular embodiments, the primer used for any amplification step (e.g., PCR) comprise linkers that are useful in next-generation sequencing methods (e.g., for tethering the amplicons to a solid support).

In particular embodiments, the stabilized enzyme mixture further comprises: i) BSA, ii) a sugar, and iii) at least one additional component selected from the group consisting of: an inorganic salt, a divalent metal cation, a buffering agent, an emulsifier, and a reducing agent. In other embodiments, the stabilized enzyme mixture further comprises: i) BSA, ii) a sugar, iii) an inorganic salt, iv) a divalent metal cation, v) a buffering agent, vi) an emulsifier, and vii) a reducing agent.

In some embodiments, the microfluidic cards further comprise: h) a purification sub-circuit operably linked to the linker ligation component, wherein the purification sub-circuit comprises a nucleic acid purification component, wherein the nucleic acid purification component comprises anchored nucleic acid sequences configured to hybridize to the linkers on the nucleic acid sequencing library. In other embodiments, the microfluidic cards further comprise an outlet port configured to allow a user to withdraw at least a portion of the nucleic acid sequencing library. In certain embodiments, the fragmentation subcircuit further comprises: at least one type of enzyme configured for

polishing the ends of the fragmented nucleic acid. In particular embodiments, the linker ligation sub-circuit further comprises: at least one type of enzyme configured for polishing the ends of the nucleic acid sequencing library. In other embodiments, the stabilized enzyme mixture is present in a dried format. In some embodiments, the reagent mixture is present in a dried format.

In further embodiments, the pump component comprises a bellows. In other embodiments, the nucleic acid extraction components comprises a membrane. In additional embodiments, the nucleic acid extraction components comprises a filter. In particular embodiments, the microfluidics cards comprise a plurality of valves. In additional embodiments, the card is configured to operably link to, and be operated by, a processing instrument. In other embodiments, the microfluidics cards further comprise a plurality of air ports configured to operably link with a pneumatic interface on the processing instrument. In some embodiments, the nucleic acid linkers are configured for use in a sequencing method selected from the group consisting of: ABI SOLID, ILLUMINA SOLEXA, ROCHE 454, ION TORRENT, Lifetechnologies STARLITE, and PACIFIC BIOSCIENCES SMRT sequencing. In additional embodiments, the microfluidics cards further comprise a sensor configured to determine if the mixture of extracted nucleic acids requires amplification or not.

The sample preparation systems of the present ~~invention~~disclosure may be integrated with any type of sequencing system (e.g., as a re-usable card or disposable card). In certain embodiments, the sample preparation systems of the present ~~invention~~disclosure are integrated with ILLIMINA SOLEXA sequencers including HiSeq 2000, HiSeq 1000, HiScanSQ, Genome Analyzer IIx, MiSeq, where the sample preparation systems may be configured to work with the related Illumina software, such as the PIPELINE and/or CASAVA software packages. In other embodiments, the sample preparation systems of the present ~~invention~~disclosure are integrated with the ROCHE 454 sequencers, including the Genome Sequencer FLX System and GS Junior System, where the sample preparation systems may be configured to work with the related GS Run Browser Software, GS De Novo Assembler Software, GS Reference Mapper Software, GS Amplicon Variant Analyzer Software, and the GS FLX Titanium Cluster. In some embodiments, the sample preparation systems of the present ~~invention~~disclosure are integrated with the ION TORRENT

sequencers, including the Ion Personal Genome Machine (PGM) sequencer, where the sample preparation systems may be configured to work with the related DNASTAR® SeqMan® NGen® Software, Partek® Genomics Suite™ Software, NextGENe® software for Ion PGM platform by SoftGenetics, or Avadis NGS Software. In additional embodiments, the sample preparation systems of the present ~~invention~~disclosure are integrated with the PACIFIC BIOSCIENCES sequencers, including the PacBio RS sequencer, where the sample preparation systems may be configured to work with the related RS remote software, RS touch software, Primary Analysis software, SMRT Portal software, and SMRT View software.

In some embodiments, the present ~~invention~~disclosure provides systems comprising: a microfluidic card as described herein; and b) a processing instrument configured to receive and operate the microfluidics card. In certain embodiments, the processing instrument comprises at least one component selected from: a pressure reservoir, a vacuum reservoir, at least one pump, a plurality of valves, at least one heater, a pneumatic interface, and an input-output computer connection. In additional embodiments, the processing instrument comprises: a pressure reservoir, a vacuum reservoir, at least one pump, a plurality of valves, at least one heater, a pneumatic interface, and an input-output computer connection.

In some embodiments, the present ~~invention~~disclosure provides stabilized enzyme mixtures comprising, or consisting essentially of, or consisting of: a) at least one type of enzyme; b) bovine serum albumin (BSA); c) a sugar; and d) a buffering agent; and optionally water. In certain embodiments, the stabilized enzyme mixture further comprises, or consists essentially of, or consists of: at least one reagent selected from the group consisting of: an inorganic salt; a divalent metal cation; an emulsifier; and a reducing agent.

In particular embodiments, the present ~~invention~~disclosure provides stabilized enzyme mixtures comprising, or consisting essentially of, or consisting of: a) at least one type of enzyme; b) bovine serum albumin (BSA); c) a sugar; d) an inorganic salt; e) a divalent metal cation; f) a buffering agent; g) an emulsifier; and h) a reducing agent.

In certain embodiments, the mixtures further comprise polyethylene glycol (PEG) (e.g., PEG-8000 or other weights). In further embodiments, the stabilized enzyme

mM ... 5 mM ... 10 mM). In certain embodiments, the reducing agent is dithiothreitol (DTT).

In some embodiments, the present ~~invention~~disclosure provides compositions for stabilizing an enzyme comprising, consisting essentially of, or consisting of: a) bovine serum albumin (BSA); b) a sugar; c) an inorganic salt; and optionally 1) a divalent metal cation; 2) a buffering agent; 3) an emulsifier; 4) a reducing agent; and 5) water.

In further embodiments, the present ~~invention~~disclosure provides compositions comprising, or consisting essentially of, or consisting of: *E. coli* polymerase I and bovine serum albumin (BSA), wherein the composition is a lyophilized composition. In particular embodiments, the lyophilized composition allows the *E. coli* polymerase I to retain at least 70% of its activity (e.g., 70% ... 90% ... 100%) upon hydration at 20-25 degrees Celsius after storage for at least one, two, or three months at 20-40 degrees Celsius.

In certain embodiments, the present ~~invention~~disclosure provides compositions comprising, or consisting essentially of, or consisting of: inorganic phosphatase and bovine serum albumin (BSA), wherein the composition is a lyophilized composition. In some embodiments, the lyophilized composition allows the inorganic phosphatase to retain at least 70% (e.g., 70% ... 90% ... 100%) of its activity upon hydration at 20-25 degrees Celsius after storage for two months at 20-40 degrees Celsius.

In further embodiments, the present ~~invention~~disclosure provides methods of storing a lyophilized composition comprising: a) providing a lyophilized composition comprising, or consisting essentially of, or consisting of: i) at least one type of enzyme; ii) bovine serum albumin (BSA); iii) a sugar; iv) an inorganic salt; v) a divalent metal cation; vi) a buffering agent; vii) an emulsifier; and viii) a reducing agent; and b) storing the lyophilized composition at a storage temperature of 15-45 degrees Celsius for at least 15 days such that the at least one enzyme retains at least 70% of its activity upon hydration at 20-25 degrees Celsius. In further embodiments, the at least 15 days is at least 30 days (e.g., at least 30 days ... 60 days ... 90 days ... 120 days ... or longer). In certain embodiments, the storage temperature is 20-25 degrees Celsius. In some embodiments, the at least one enzyme is selected from *E. coli* polymerase I, Phi-29 polymerase, and inorganic pyrophosphatase.

In particular embodiments, the present ~~invention~~disclosure provides methods of generating a stabilized enzyme composition comprising: a) providing: i) an aqueous composition comprising, or consisting essentially of, or consisting of: A) at least one type of enzyme; B) bovine serum albumin (BSA); C) a sugar; D) an inorganic salt; E) a divalent metal cation; F) a buffering agent; G) an emulsifier; and H) a reducing agent; and ii) a dializing membrane; and b) dialyzing the aqueous composition with the dialzying membrane into a solution comprising or consisting essentially of: i) the sugar; ii) the inorganic salt; iii) the divalent metal cation; iv) the buffering agent; v) the emulsifier; and vii) the reducing agent; c) freezing the aqueous composition to generate a frozen composition; and d) subjecting the frozen composition to high vacuum to remove water via sublimation such that a lyophilized composition is generated.

DESCRIPTION OF THE FIGURES

Figure 1 shows a flow chart demonstrating the lengthy process now involved in sample preparation for next-generation sequencing techniques, such as the Roche 454 method.

Figure 2 shows an exemplary injected molded microfluidic card and the various layers that may make up a card. Figure 2 also describes the first three sub-circuits of cell lysis, DNA extraction, and whole genome amplification.

Figure 3 shows an exemplary microfluidic card with various sub-circuits labeled, including a sample inlet port, a DNA extraction circuit, and an amplification circuit which includes lyophilized enzymes (e.g., such as those described below in Example 1).

Figure 4 shows step 1 of a sample preparation process utilizing a microfluidic card. This figure specifically shows a sample inlet port and a mixing chamber.

Figure 5 shows step 2 which is a lysis step. Lysis buffer from a blister pack flows into a mixing chamber with cells from the sample such that the cells are lysed.

Figure 6 shows step 3 where the lysed mixture is passed over a capture filter and then to a waste chamber. A first wash buffer from a blister pack is then passed over the filter before going into the waste chamber.

Figure 7 shows step 4 where a second wash is passed through the capture filter and then on to the waste chamber. The filter is then air dried to remove the wash buffer.