

Next Gen DNA Library Kit & Next Gen Indexing Kit

Powered by Swift Biosciences

(version A2)

Catalog Nos. 53216 & 53264

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Revision History

Revision	Date	Description of Change
A2	Sept 2018	Corrected Kit Components and Storage quantities for Alu115 and Alu247

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Overview

Active Motif's Next Gen DNA Library Kit is designed for the preparation of high complexity Next-generation sequencing (NGS) libraries from double-stranded input DNA for use with Illumina® platforms, including MiSeq®, HiSeq® 2500 and NextSeq® 500. Libraries can be generated from as little as 10 pg high quality DNA, or from as low as 100 ng DNA if preparing PCR-free libraries.

The advantage of the Next Gen DNA Library Kit is the inclusion of molecular identifiers (MIDs) to enable accurate de-duplication from single read sequencing by distinguishing PCR duplicates from fragmentation duplicates. This helps to increase the number of unique alignments from sequencing for more accurate data.

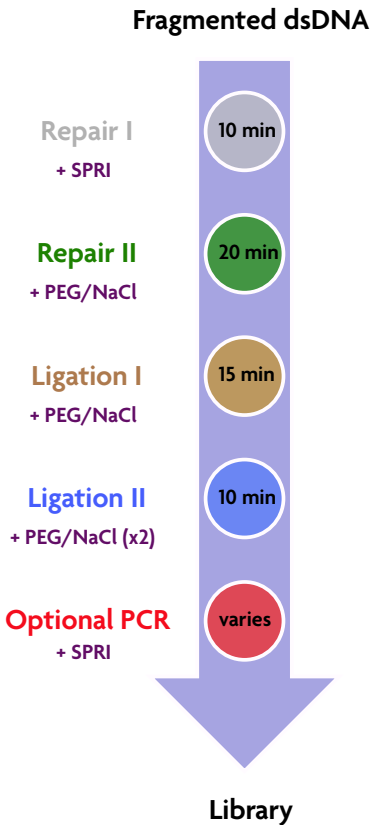
The Next Gen DNA Library Kit contains enough reagents to prepare 16 libraries. In addition to the Library Preparation reagents, customers also need to purchase a Next Gen Indexing Kit, which contains 16 unique index adapters that may be used to multiplex different samples together during cluster generation for co-sequencing on the same Illumina flow cell.

product	format	catalog no.
Next Gen DNA Library Kit	16 rxns	53216
Next Gen Indexing Kit (16 indices)	64 rxns	53264

The Next Gen DNA Library and Next Gen Indexing Kits are powered by Swift Biosciences.

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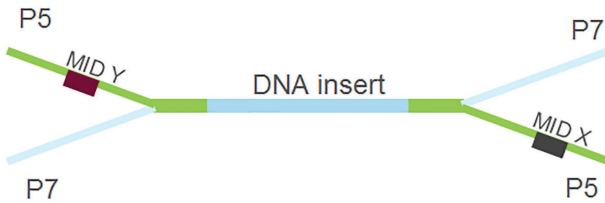
Flow Chart



- Using four incubations, this protocol repairs both 5' and 3' termini and sequentially attaches Illumina adapter sequences to the ends of fragmented dsDNA.
- Bead-based SPRI clean-ups are used to remove oligonucleotides and small fragments and to change enzymatic buffer composition between steps. Different SPRIselect bead-to-sample ratios are utilized for different input quantities and insert sizes.
- For PCR-free applications, the resulting functional library is ready for library quantification and sequencing on the Illumina platform.
- Alternatively, an optional PCR step may be used to increase yield of indexed libraries, which then may be quantified and sequenced. Please refer to page 4 for recommended input requirements.

Introduction

The Next Gen DNA Library Kit is designed to generate high complexity DNA libraries for Next generation sequencing (NGS). The Next Gen DNA Library Kit offers the advantage of including molecular identifiers (MIDs) during library generation to enable removal of true PCR duplicates from the sequencing analysis. This helps to distinguish PCR duplicates from fragmentation duplicates, thereby, increasing the number of unique alignments for more accurate data analysis.



The Next Gen DNA Library Kit works by adding standard low throughput (LT) P7 adapters containing a single index for multiplexed sequencing (index 1 position) during Ligation I. Following a PEG NaCl clean-up, a high throughput (HT) P5 adapter containing the MID is incorporated during Ligation II. The MID is a 9 base random N sequence at the [i5] (index 2 position) of the P5 adapter. Addition of the MID is strand-specific with each dsDNA insert receiving two MIDs (X and Y). The two MIDs cluster and sequence independently. Bioinformatically, PCR duplicates can be removed from the data set, while fragmentation duplicates are preserved.

Given the usefulness of NGS data in understanding biological function and disease, it is important to prepare high quality sequencing libraries. Active Motif's Next Gen DNA Library Kit in combination with the Next Gen Indexing Kit provide a complete solution for multiplexed sequencing of up to 16 samples on the Illumina platforms while also offering the added value of MIDs to improve data analysis.

Kit Performance and Benefits

The Next Gen DNA Library Kit can be used to prepare high quality NGS libraries from dsDNA. It is designed to be used in combination with Active Motif's Next Gen Indexing Kit (Catalog No. 53264) which contains 16 unique index adapters.

Input amounts: We recommend using between 10 pg – 250 ng input DNA per library preparation. Input DNA should be fragmented dsDNA (e.g. genomic DNA, CHIP DNA, methyl-enriched dsDNA, FFPE DNA or cell-free DNA). Input DNA should be resuspended in Low EDTA TE buffer to reach a final volume of 40 μ l.

PCR-free library preparation: Minimum input of 100 ng DNA

Library amplification by PCR: Minimum input of 10 pg DNA

Expected yield: Yields will vary between sample types and input amounts but > 4 nM is expected.

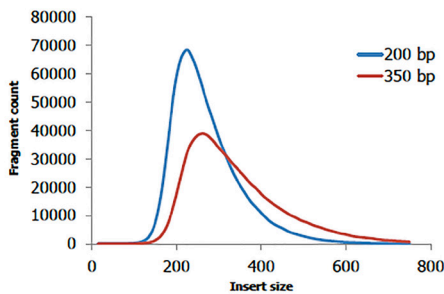


Figure 1: Insert size based on Paired-End alignment of PCR-free libraries prepared from 100 ng Coriell Human Genomic DNA (NA 12878).

Next Gen DNA Library Kit

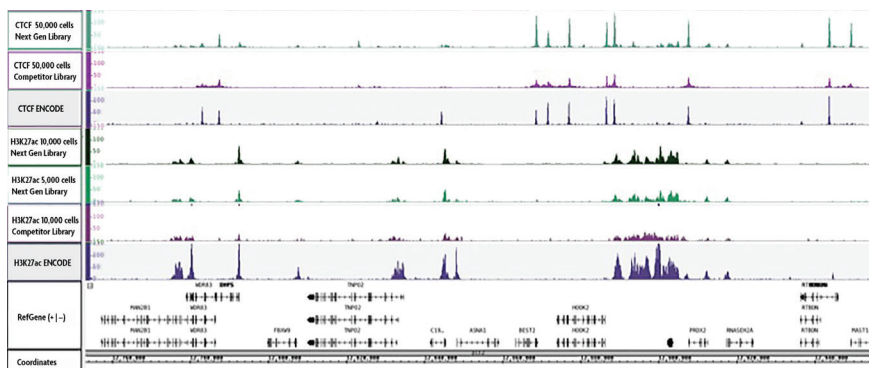


Figure 2: Next Gen DNA Library Kit prepares high quality ChIP-seq libraries, even with lower cell numbers.

CTCF and Histone H3K27ac ChIP-Seq libraries from GM12878 cells were prepared using either Next Gen DNA Library and Next Gen Indexing Kits or competitor library preparation reagents and sequenced using the Illumina NextSeq 500. Sequencing results were compared to published ENCODE data sets available on the UCSC genome browser. Results show high quality sequencing results with strong peaks that match the ENCODE data sets for the libraries prepared using the Next Gen DNA Library Kit, even when using lower cell numbers.

Next Gen DNA Library Kit (Cat. No. 53216) Components & Storage

Next Gen DNA Library Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Buffer W1	106 µl	-20°C
Enzyme W2	18 µl	-20°C
Buffer G1	88 µl	-20°C
Reagent G2	229 µl	-20°C
Enzyme G3	18 µl	-20°C
Enzyme G4	18 µl	-20°C
Buffer Y1	53 µl	-20°C
Enzyme Y3	36 µl	-20°C
Buffer B1	88 µl	-20°C
Reagent B3	159 µl	-20°C
Enzyme B4	18 µl	-20°C
Enzyme B5	36 µl	-20°C
Enzyme B6	18 µl	-20°C
Reagent R2	71 µl	-20°C
Buffer R3	176 µl	-20°C
Enzyme R4	18 µl	-20°C
Alu 115	100 µl	-20°C
Alu 247	100 µl	-20°C
PEG Solution	10 ml	RT
Low EDTA TE	10 ml	RT

Additional materials required

- Next Gen Indexing Kit (Catalog No. 53264) which contains 16 unique indices that are compatible with Illumina® sequencing
- Magnetic SPRI beads for clean-up steps (e.g. Beckman Coulter, Catalog Nos. B23317, B23318, B23319)
- Magnetic rack or bar magnet
- qPCR-based library quantification kit (e.g. Kapa Biosystems, Cat. KR0405)
- Qubit®, NanoDrop® or other device for determining DNA concentration

- Method for fragmentation of input DNA by mechanical or enzymatic shearing
- Multichannel pipette and aerosol-resistant pipette tips from 2- 1000 μ l
- Microcentrifuge
- 1.5 ml low retention microcentrifuge tubes
- 0.2 ml PCR tubes
- Thermal cycler
- Absolute ethanol (200-proof)
- Nuclease-free water

Next Gen Indexing Kit (Cat. No. 53264) Components & Storage

Next Gen Indexing Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Reagent Y2 Index 1	22 μ l	-20°C
Reagent Y2 Index 2	22 μ l	-20°C
Reagent Y2 Index 3	22 μ l	-20°C
Reagent Y2 Index 4	22 μ l	-20°C
Reagent Y2 Index 5	22 μ l	-20°C
Reagent Y2 Index 6	22 μ l	-20°C
Reagent Y2 Index 7	22 μ l	-20°C
Reagent Y2 Index 8	22 μ l	-20°C
Reagent Y2 Index 9	22 μ l	-20°C
Reagent Y2 Index 12	22 μ l	-20°C
Reagent Y2 Index 13	22 μ l	-20°C
Reagent Y2 Index 14	22 μ l	-20°C
Reagent Y2 Index 15	22 μ l	-20°C
Reagent Y2 Index 16	22 μ l	-20°C
Reagent Y2 Index 18	22 μ l	-20°C
Reagent Y2 Index 19	22 μ l	-20°C
Reagent R1	141 μ l	-20°C
Reagent B2 MID	352 μ l	-20°C

Protocols

Buffer Preparation and Recommendations

Input Material

Please consider genome complexity and sample quality when choosing input DNA quantity. Although libraries may be successfully prepared from ultra-low inputs, reduced representation of genome complexity may occur. 10 pg of input DNA is the supported minimum input with PCR and 100 ng is the supported minimum input for PCR-free library preparation.

This kit is designed to make libraries from double-stranded DNA. It is recommended to determine dsDNA concentration and purity using Qubit, or a similar fluorometric method, as it will more accurately represent the dsDNA content of your sample. For low quality DNA, including cfDNA or FFPE samples, it is important to use a quality control metric to analyze DNA integrity and purity. A qPCR will provide the best result as it will quantify the usable amount of DNA in the sample.

Starting material should be in 1-40 μ l of Low EDTA Buffer. Please contact Technical Support if you need to work with larger volumes.

This kit has two bead purification options based on input amounts: Low input (10 pg – 10 ng) or High input (10 ng – 250 ng). Refer to the table for each bead clean-up step to use the correct SPRIselect bead ratio.

Enzyme Reagents

To maximize efficient use of enzyme reagents, remove enzymes from -20°C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow reagents to reach 4°C prior to pipetting. After thawing reagents, invert to mix well (do not vortex enzymes). Spin tubes in a microcentrifuge to collect contents prior to opening. **Attempting to pipette enzymes at -20°C, or failure to spin enzyme contents prior to opening may result in a shortage of enzyme reagents.**

80% Ethanol Solution

Before starting the assay, prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Calculate the amount of 80% ethanol solution to prepare based on need—approximately 2.0 ml per sample. Discard after use.

Recommendations

To reduce the risk of DNA and library contamination, particularly with ultra-low input samples, we suggest to physically separate the laboratory space, equipment and supplies where pre-PCR and post-PCR processes are performed. We also suggest to clean lab areas using 0.5% Sodium Hypochlorite (10% bleach). The use of specialty barrier pipette tips also helps to avoid exposure to potential contaminants.

Next Gen DNA Library Protocol

Read the entire protocol before use.

For best results we recommend following the instructions in the Buffer Preparation and Recommendations section on page 8 for working with enzyme reagents. Assemble reagent master mixes on ice and scale volumes as needed, using a 5% excess volume to compensate for pipetting loss. We suggest preparing reagent mixes in advance to ensure the SPRIselect beads do not dry out. **Always add reagents in the specified order.**

(Optional) DNA Fragmentation

If working with intact genomic DNA, the DNA will need to be fragmented prior to library preparation. Fragmentation may be performed through mechanical methods such as sonication, or through enzymatic digestion methods to produce DNA fragments in the range of 150 - 1000 bp. Probe tip sonication, such as Active Motif's EpiShear Probe Sonicator (Catalog No. 53051) or focused-ultrasonicators, such as Covaris®, can be used for mechanical fragmentation of DNA. For enzymatic digestion, NEBNext® DNA Fragmentase® can be used.

If working with ChIP DNA, highly fragmented FFPE samples or dsDNA samples that have already undergone mechanical or enzymatic fragmentation, this step may be omitted. Cell-free DNA (cfDNA) samples also do not require fragmentation as enzymatic cleavage of cfDNA in the blood produces fragments of approximately 165 bp in size.

Section A: Input DNA Quantification

1. Determine dsDNA concentration and purity of the input DNA sample. We recommend the use of fluorometric-based (Qubit®) methods when working with high quality DNA. For low quality DNA samples we recommend quantification by qPCR using Alu primer sets to accurately assess DNA damage and to determine the concentration and quality of the DNA.

gDNA	Quantify with Qubit or similar fluorometric method.
ChIP DNA	Quantify with Qubit or similar fluorometric method.
cfDNA	Quantify by qPCR with Alu primer sets (See Appendix Section M)
FFPE DNA	Quantify by qPCR with Alu primer sets (See Appendix Section M)

2. We recommend using between 10 pg - 250 ng input DNA per library preparation. Input DNA should be resuspended in Low EDTA buffer to reach a final volume of 40 µl.

PCR-free library preparation: Minimum input of 100 ng DNA

Library amplification by PCR: Minimum input of 10 pg DNA

Section B: Repair I

1. Transfer the dsDNA sample (e.g. ChIP DNA) to a 0.2 ml PCR tube and adjust the volume of the sample to a final volume of 40 μ l using Low EDTA TE, if necessary.
2. Place a new 0.2 ml PCR tube on ice and prepare the Repair I reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	13 μ l	54.6 μ l
Buffer W1	6 μ l	25.2 μ l
Enzyme W2	1 μ l	4.2 μ l
Total Volume	20 μl	84 μl

3. Add 20 μ l of the pre-mixed Repair I reaction mix to each PCR tube containing the 40 μ l DNA sample. Mix by pipetting. Place in the thermal cycler and run the Repair I thermal cycler program as described below.

Repair I Thermal Cycler Program	37°C for 10 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.
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Section C: Post-Repair I SPRI

1. Clean up the Repair I reaction using SPRIselect beads and freshly prepared 80% ethanol. See Appendix, Section N for instructions.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	60 μ l	84 μ l (ratio: 1.4)	N/A
10 ng – 250 ng	200 bp 350 bp	60 μ l 60 μ l	60 μ l (ratio: 1.0) 54 μ l (ratio: 0.9)	N/A N/A

Section D: Repair II

1. Place a new 0.65 ml tube on ice and prepare the Repair II reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	30 μ l	126 μ l

Buffer G1	5 μ l	21 μ l
Reagent G2	13 μ l	54.6 μ l
Enzyme G3	1 μ l	4.2 μ l
Enzyme G4	1 μ l	4.2 μ l
Total Volume	50 μl	210 μl

- Add 50 μ l of the pre-mixed Repair II reaction mix to the beads for each sample and resuspend by pipetting.
- Place in the thermal cycler and run the Repair II thermal cycler program as described below.

Repair II Thermal Cycler Program	20°C for 20 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.
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Section E: Post-Repair II SPRI

- Clean up the Repair II reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section N for instructions.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	50 μ l	N/A	60 μ l (ratio: 1.2)
10 ng – 250 ng	200 bp	50 μ l	N/A	42.5 μ l (ratio: 0.85)
	350 bp	50 μ l	N/A	37.5 μ l (ratio: 0.75)

Section F: Ligation I

- Place a new 0.2 ml PCR tube on ice and prepare the Ligation I reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	20 μ l	84 μ l
Buffer Y1	3 μ l	12.6 μ l
Enzyme Y3	2 μ l	8.4 μ l
Total Volume	25 μl	105 μl

- Add 25 μ l of the pre-mixed Ligation I reaction mix to the beads for each sample.

- Add 5 µl of the desired Reagent Y2 Index to each sample for a total volume of 30 µl. Resuspend by pipetting. Record the identity of the index used for each sample to ensure proper de-multiplexing after sequencing.

Note: Reagent Y2 Index samples are part of Active Motif's Next Gen Index Kit, Catalog No. 53264. Index primer sequences can be found in the Appendix Section O. All 16 unique indices provided are compatible within the same sequencing reaction.

- Place in the thermal cycler and run the Ligation I thermal cycler program as described below.

Ligation I Thermal Cycler Program	25°C for 15 minutes, lid heating OFF* *Alternatively, the thermal cycler lid may be left open.
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Section G: Post-Ligation I SPRI

- Clean up the Ligation I reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section N for instructions.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	30 µl	N/A	25.5 µl (ratio: 0.85)
10 ng – 250 ng	All sizes	30 µl	N/A	36 µl (ratio: 1.2)

Section H: Ligation II

- Place a new 0.65 ml tube on ice and prepare the Ligation II reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	30 µl	126 µl
Buffer B1	5 µl	21 µl
Reagent B2-MID	2 µl	8.4 µl
Reagent B3	9 µl	37.8 µl
Enzyme B4	1 µl	4.2 µl
Enzyme B5	2 µl	8.4 µl
Enzyme B6	1 µl	4.2 µl
Total Volume	50 µl	210 µl

2. Add 50 µl of the pre-mixed Ligation II reaction mix to the beads for each sample and resuspend by pipetting.
3. Place in the thermal cycler and run the Ligation II thermal cycler program as described below.

Ligation II Thermal Cycler Program	40°C for 10 minutes, lid heating OFF* 25°C Hold *Alternatively, the thermal cycler lid may be left open.
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Section I: Post-Ligation II SPRI

1. Clean up the Ligation II reaction using PEG NaCl solution and freshly prepared 80% ethanol. See Appendix, Section N for instructions.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	N/A	42.5 µl (ratio: 0.85)	50 µl
10 ng – 250 ng	All sizes	50 µl	N/A	60 µl (ratio: 1.2)	50 µl

2. At the end of the first Ligation II SPRI clean up, resuspend the beads in 50 µl Low EDTA TE buffer.
3. To ensure optimal removal of the unincorporated Reagent B2 MID adapter, perform a second clean up using PEG NaCl solution and freshly prepared 80% ethanol. Resuspend the beads in 20 µl Low EDTA TE buffer.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	N/A	42.5 µl (ratio: 0.85)	20 µl
10 ng – 250 ng	All sizes	50 µl	N/A	60 µl (ratio: 1.2)	20 µl

4. Using a magnetic rack, collect the beads to the side of the tube. Allow to sit for 1 minute to capture all of the beads
5. Carefully transfer the supernatant containing your adapted library to a new 1.5 ml low retention microcentrifuge tube.
6. Libraries can be stored at 4°C for short-term storage or -20°C for long-term storage.
7. For library amplification, please proceed with Sections J-K. If performing PCR-free library preparation, the library is now ready for quantification by qPCR and sequencing. PCR-free libraries cannot be accurately quantified or assessed for library size using the Bioanalyzer.

(Optional) Section J: Library Amplification

- Place a new 0.2 ml PCR tube on ice and prepare the Library PCR reaction mix by adding reagents in the order listed below.

Reagent	One rxn	4 rxns
Low EDTA TE	10 μ l	42 μ l
Reagent R1	5 μ l	21 μ l
Reagent R2	4 μ l	16.8 μ l
Buffer R3	10 μ l	42 μ l
Enzyme R4	1 μ l	4.2 μ l
Total Volume	30 μl	126 μl

- If qPCR analysis of the PCR-free library is desired, set aside 3 μ l of the library eluate for PCR evaluation. Then add 3 μ l Low EDTA TE Buffer to the remaining 17 μ l eluate to bring the library to a 20 μ l final volume. Alternatively, the entire 20 μ l eluted library can be used in the PCR.
- Add 30 μ l of the pre-mixed Library PCR reaction mix to the 20 μ l eluted library and mix by pipetting. The total volume of the PCR reaction is 50 μ l.
- Place in the thermal cycler and run the Library PCR thermal cycler program as described below. Recommendations are provided for the number of PCR cycles based on input amount.

Library PCR Thermal Cycler Program	98°C for 30 seconds
	98°C for 10 seconds
	60°C for 30 seconds x PCR cycles
	68°C for 60 seconds
	4°C Hold
Proceed immediately to Post-PCR SPRI	

Input	# PCR Cycles
10 ng	6
1 ng	9
100 pg	12
10 pg	15

Note: The number of cycles required to produce enough library for sequencing will depend on input quantity and quality. In the case of low quality samples, including FFPE, the number of cycles required may vary based on the quality of the sample and the amount of usable DNA present. Approximate guidelines for high quality DNA are indicated in the table above, but the exact number of cycles required must be determined by the user.

Section K: Post-Library PCR SPRI

1. Clean up the Library PCR reaction using SPRIselect beads and freshly prepared 80% ethanol. See Appendix, Section N for instructions.

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	37.5 µl (ratio: 0.75)	N/A	20 µl
10 ng – 250 ng	All sizes	50 µl	70 µl (ratio: 1.4)	N/A	20 µl

2. Resuspend the beads in 20 µl Low EDTA TE buffer.
3. Use a library quantification kit for Next-generation sequencing to quantify the library (e.g. Kapa Biosystems, Catalog No.KR0405). PCR amplified libraries can also be analyzed with a Bioanalyzer to assess size distribution. Please note the sensitivity limits specified by Agilent for the Bioanalyzer and consult the application note titled “Analysis of DNA Fragments Using Agilent 2100 Bioanalyzer” by Covaris to ensure proper analysis of library size.
4. Libraries can be stored at 4°C for short-term storage or -20°C for long-term storage. Libraries are now ready for sequencing on the Illumina platforms.

Section L: Sequencing Guidelines

1. Guidelines for the run set-up and sample sheet preparation for use with Illumina MiSeq, HiSeq2500 and NextSeq 500 platforms can be found at www.activemotif.com/nextgen. Select the Documents tab to download a copy of the Sequencing Guidelines. The Sequencing Guidelines will also include instructions for the use of MIDs, including recommendations for data analysis.

Appendix

Section M: Input DNA Quantification

Spectrophotometric-based (NanoDrop®) or fluorometric-based (Qubit®) quantification will provide accurate DNA concentrations for samples with high quality DNA but is not recommended for low quality samples such as cell-free DNA (cfDNA) and formalin-fixed, paraffin-embedded (FFPE) samples. If working with limited quantity samples, we recommend quantification by qPCR using both short and long amplicons to accurately determine the concentration and quality of the sample DNA. The qPCR method quantifies the DNA and also provides a metric to assess DNA damage.

Alu sequences are highly abundant in the human genome and can be used for the sensitive quantification of human genomic DNA. Included in this kit are qPCR primers that can be used to amplify two differently sized amplicons: short (115 bp; Alu 115) and long (247 bp; Alu 247) amplicons from genomic Alu repeats. Following input analysis, the appropriate amount of sample DNA can be used as input for NGS library preparation.

The following protocol describes the quantification of low quality dsDNA samples using the Alu repeat primers for qPCR quantification.

1. Prepare a standard curve using serial dilutions of human genomic DNA of known quantities (11 ng, 1.1 ng, 0.11 ng, 0.011 ng, 0.0011 ng) for each Alu primer set (in triplicate).
2. Run your sample and a no template control in triplicate for sample quantification
3. Place a 0.65 ml tube on ice and prepare the qPCR reaction by adding reagents in the order listed below. We suggest the use of iTaq™ Universal SYBR Green Supermix (Bio-Rad, 172-5120).

Reagent	One rxn	24 rxns
iTaq™ Universal SYBR Green Supermix	10 µl	252 µl
Alu 115 or Alu 247 primers	2 µl	50.4 µl
DNA	X µl	X µl
Low EDTA TE	up to 20 µl	up to 504 µl
Total Volume	20 µl	504 µl

4. Place in the thermal cycler and run the Alu Primer PCR Quantification program as described below.

Alu Primer qPCR Quantification Thermal Cycler Program	95°C for 3 minutes, lid heating ON
	95°C for 5 seconds, lid heating ON 62°C for 30 seconds, lid heating ON x 35 cycles

- Plot Ct values (y-axis) vs. DNA quantity of the serial dilutions (x-axis) on a log scale to produce the standard curve. Identify the slope and the y-intercept. Solve using the following formula to determine the sample DNA concentration.

$$\text{ng}/\mu\text{l} = 10^{(\text{Ct} - \text{y-intercept}) / \text{slope}}$$

- The concentration for the Alu 115 amplicon can be used to determine the total quantity of usable DNA in ng/ μ l.
- Use a ratio of the Alu 247 and Alu 115 amplicons to calculate a DNA integrity score. High quality DNA is expected to have a DNA integrity score of 1.0, while lower quality DNA will have a score between 0 and 1.0. The DNA integrity score is intended to be used as an indicator of probability of successful library construction. Due to the diversity of sample types and protocols, specific recommendations concerning library construction and sequencing metrics are difficult to define in terms of the DNA integrity score. Use your best judgement.

$$\text{DNA integrity score} = (\text{ng}/\mu\text{l of Alu 247}) / (\text{ng}/\mu\text{l of Alu 115})$$

Additional Notes:

DNA extracted from FFPE samples can exhibit varying degrees of DNA damage and the consequence of this damage will be a more pronounced amplification of the longer (Alu 247) amplicon. Therefore, with FFPE samples, the Alu 115 qPCR results accurately detect the total quantity of usable DNA (ng/ μ l).

As cfDNA exhibits a narrow size distribution around 165 bp, the Alu 115 qPCR result will accurately detect the total quantity of cfDNA and high molecular weight genomic DNA. Alu 247 qPCR results indicate only the presence of high molecular weight genomic DNA. Subtract the DNA mass in the Alu 247 value from the DNA mass in the Alu 115 value to obtain the quantity of cfDNA in the sample.

Alu 115 Forward: 5' - CCTGAGGTCAGGAGTTCGAG - 3'
Reverse: 5' - CCCGAGTAGCTGGGATTACA - 3'

ALU 247 Forward 5' - GTGGCTCACGCCTGTAATC - 3'
Reverse 5' - CAGGCTGGAGTGCAGTGG - 3'

Section N: SPRIselect Clean-up Protocol

Please use the following protocol for each SPRI step, substituting the correct **Bead Volume**, **PEG NaCl Volume** and **Elution Volume** based on the table provided for each section.

1. Ensure the SPRI beads are at room temperature and briefly vortex beads to homogenize the suspension before use.
2. Add the specified Bead Volume, or PEG NaCl Volume to each sample. Mix by vortexing. Quick spin the samples in a tabletop microcentrifuge.
3. Incubate the samples for 5 minutes at room temperature.
4. Place the sample on a magnetic rack until the solution clears and a pellet is formed (-5 min).
5. Remove and discard the supernatant without disturbing the pellet (<5 µl may be left behind).
6. Add 180 µl of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, and then carefully remove the ethanol solution.
7. Repeat step 6 once more for a second wash with the 80% ethanol solution.
8. Quick spin the samples in a tabletop microcentrifuge and place back onto the magnet. Remove any residual ethanol solution from the bottom of the tube.
9. Air dry the pellet for no more than 5 minutes. Watch the pellet to avoid cracking or over-drying, which could result in reduced DNA recovery.
10. Add the specified volume of each reaction mix (Repair II, Ligation I and Ligation II) or elution volume (Post-Ligation II and Post-Library PCR) of Low EDTA TE buffer and resuspend the pellet. Mix well by pipetting up and down until homogenous.

Post-Repair I SPRI

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	60 µl	84 µl (ratio: 1.4)	N/A
10 ng – 250 ng	200 bp	60 µl	60 µl (ratio: 1.0)	N/A
	350 bp	60 µl	54 µl (ratio: 0.9)	N/A

Post-Repair II SPRI

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	50 µl	N/A	60 µl (ratio: 1.2)
10 ng – 250 ng	200 bp	50 µl	N/A	42.5 µl (ratio: 0.85)
	350 bp	50 µl	N/A	37.5 µl (ratio: 0.75)

Post-Ligation I SPRI

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume
< 10 ng	All sizes	30 µl	N/A	25.5 µl (ratio: 0.85)
10 ng – 250 ng	All sizes	30 µl	N/A	36 µl (ratio: 1.2)

Post-Ligation II SPRI

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	N/A	42.5 µl (ratio: 0.85)	50 µl
10 ng – 250 ng	All sizes	50 µl	N/A	60 µl (ratio: 1.2)	50 µl

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	N/A	42.5 µl (ratio: 0.85)	20 µl
10 ng – 250 ng	All sizes	50 µl	N/A	60 µl (ratio: 1.2)	20 µl

Post-Library PCR SPRI

Input	Insert Size	Sample Volume	Bead Volume	PEG NaCl Volume	Elution Volume
< 10 ng	All sizes	50 µl	37.5 µl (ratio: 0.75)	N/A	20 µl
10 ng – 250 ng	All sizes	50 µl	70 µl (ratio: 1.4)	N/A	20 µl

Section O: Next Gen Indexing Kit (Catalog No. 53264)

During Ligation I in the protocol, you must use a unique indexed adapter “Reagent Y2 Index” to label each library. If no multiplex sequencing is being performed, all libraries may be labeled with the same Index. Libraries made with unique Index adapters may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell.

Below are the unique sequences for the 16 Reagent Y2 Index adapters provided in the Next Gen Indexing Kit (Active Motif, Catalog No. 53264). Use 5 µl “Reagent Y2 Index” in the Ligation I protocol on pages 11-12. Make sure to note which indexed adapter was used with each sample. Do not use the same index adapter on two different samples you plan to multiplex together.

Indexed Adapters	Sequence*
Reagent Y2 Index 1	ATCACG(A)
Reagent Y2 Index 2	CGATGT(A)
Reagent Y2 Index 3	TTAGGC(A)
Reagent Y2 Index 4	TGACCA(A)
Reagent Y2 Index 5	ACAGTG(A)
Reagent Y2 Index 6	GCCAAT(A)
Reagent Y2 Index 7	CAGATC(A)
Reagent Y2 Index 8	ACTTGA(A)
Reagent Y2 Index 9	GATCAG(A)
Reagent Y2 Index 12	CTTGTA(A)
Reagent Y2 Index 13	AGTCAA(C)
Reagent Y2 Index 14	AGTTCC(G)
Reagent Y2 Index 15	ATGTCA(G)
Reagent Y2 Index 16	CCGTCC(C)
Reagent Y2 Index 18	GTCCGC(A)
Reagent Y2 Index 19	GTGAAA(C)

* The base pair in parentheses is read during a seventh cycle, but is not considered part of the index sequence.

Section P: Troubleshooting Guide

Problem/question	Possible cause	Recommendation
Library migrates unexpectedly on Bioanalyzer	When analyzed on the Agilent High Sensitivity chip, migration behavior overestimates library size of PCR-free libraries made from DNA fragmented to the 200-300 bp range.	<p>“200 bp insert” PCR-free libraries should migrate to a -500 bp peak and “350 bp insert” PCR-free libraries should migrate to a -800 bp peak on the High Sensitivity chip.</p> <p>Consult the Expected results section and the application note by Covaris titled “Analysis of DNA Fragments Using the Agilent 2100 Bioanalyzer” to ensure proper analysis of library size.</p>
	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally.	<p>Quantify library by qPCR as other quantification methods will not accurately detect heteroduplex library molecules.</p> <p>Perform the minimum number of PCR cycles necessary to avoid over-amplification.</p>
DNA does not fragment properly; broad or lopsided (high MW) sonication profile of fragmented DNA	Impure DNA.	Use isopropanol purification, bead clean-up, column purification or other method to purify DNA before fragmentation.
	Fragmentation device malfunction.	Ensure fragmentation device is functioning within the manufacturer's parameters.
Incomplete resuspension of beads after ethanol wash during SPRI steps	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
Shortage of enzyme reagents	Pipetting enzymes at -20°C instead of placing enzymes at 0-4°C before use.	Allow enzyme reagents to equilibrate to 0-4°C for 10 minutes prior to pipetting. Place enzymes on ice prior to use, do not use a cryocooler.
Retention of liquid in pipette tip	Viscous reagents may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to ensure all liquid and/or beads are released from the pipette tip.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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