ACTIVE MOTIF®

Enabling Epigenetics Research

DNA Library Prep Kit for Illumina®

Catalog No. 53220

(Version A1)

Information in this manual is subject to change without notice and does not constitute a commitment on the part of Active Motif, Inc. It is supplied on an “as is” basis without any warranty of any kind, either explicit or implied. Information may be changed or updated in this manual at any time.

The manufacturer of this documentation is Active Motif, Inc.

All trademarks, trade names, service marks or logos referenced herein belong to their respective companies.

For research use only. Not for use in diagnostic procedures.

Contents

Overview 1

Flowchart of Process 2

Kit Components and Storage 3

Additional Materials Required 5

Protocols 6

Prepare Samples and Reagents 6

Samples 6

Reagents 7

One-Tube End Repair 8

Illumina Adapter Ligation 9

Purification of Illumina Adapter Ligated DNA 9

Purification of Library 10

Downstream Analysis 11

Appendix 12

Notes and Expected Results 14

Troubleshooting Guide 15

Technical Services 16

Overview

The Active Motif DNA Library Prep Kit for Illumina® is designed to generate high complexity DNA libraries for NGS sequencing on Illumina® platforms from genomic DNA fragments generated from assays such as ChIP, ChIC/CUT&RUN, and MBD Capture.

In the protocol, sheared or enzymatically digested genomic DNA (gDNA) is first end-repaired to allow for ligation with truncated Illumina adapters. Following clean-up of the adapter ligated DNA using the included SPRI beads, PCR enrichment is performed to add indexes and generate full-length P5 and P7 sequences. SPRI beads are used again to create a sequencing ready library.

This kit has been optimized for fragmented double-stranded DNA. DNA fragments > 800 bp are not recommended as the quality of sequencing on Illumina® Sequencers will significantly be affected. Single stranded DNA (ssDNA) is not compatible with this protocol.

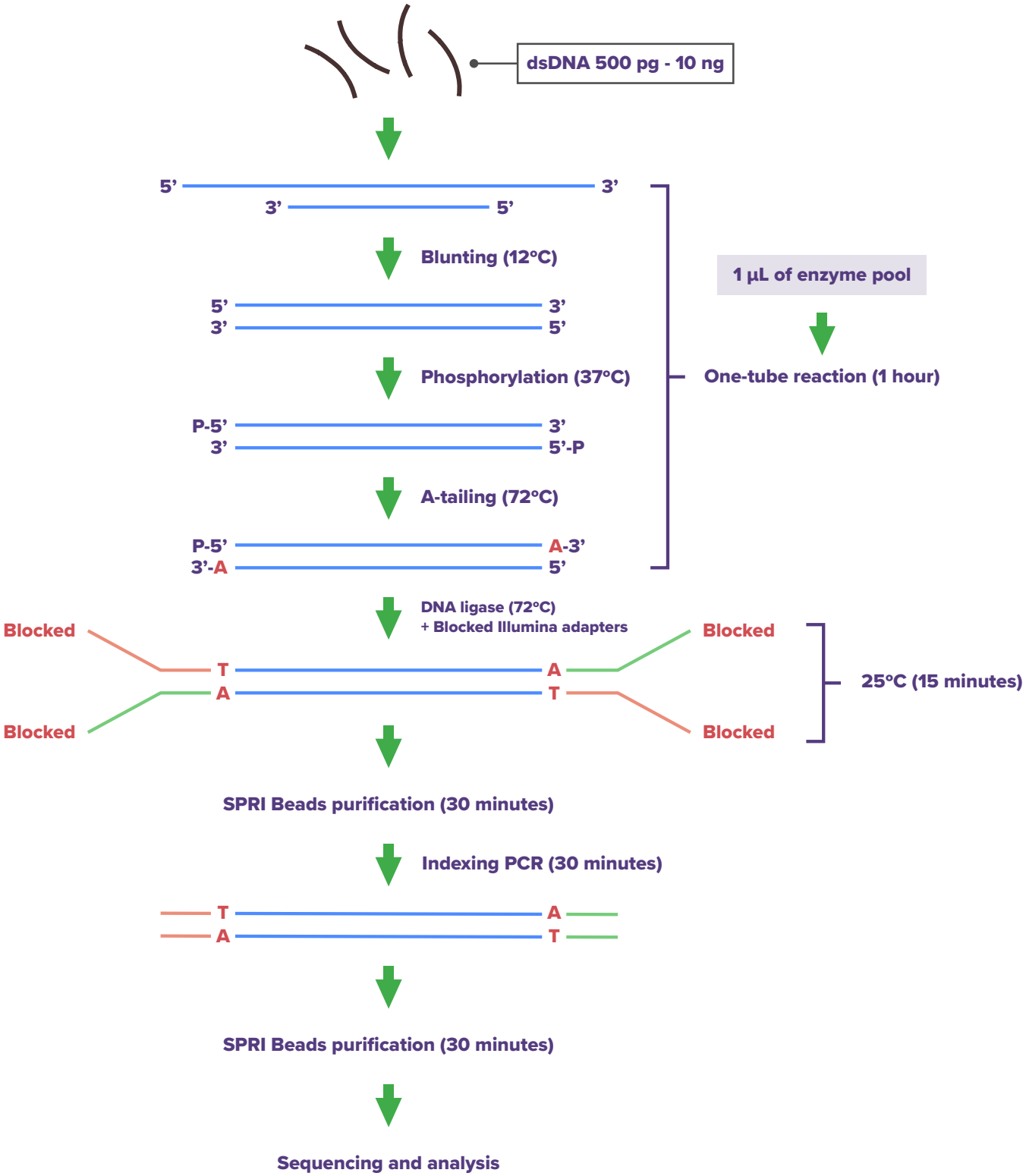
Recommended dsDNA Input Range: 500 pg - 10 ng

Recommend dsDNA Size Range: 150 bp - 600 bp

The Dual Index Primers Set 1 for Illumina® and Dual Index Primers Set 2 for Illumina® each include enough combinatorial dual indexes for multiplexing 48 samples respectively. When combined, they can be used to multiplex up to 96 unique samples.

Product	Format	Catalog No.
DNA Library Prep Kit for Illumina®	48 rxns	53220
Dual Index Primers Set 1 for Illumina®	48 rxns	53221
Dual Index Primers Set 2 for Illumina®	48 rxns	53222

Flowchart of Process



Kit Components and Storage

The Active Motif DNA Library Prep Kit for Illumina® is shipped in two separate boxes, one on dry ice and one at ambient temperature. Upon receipt, please store the box on dry ice at -20°C and the other box at 4°C. The TT Buffer ships in -20°C box, it does not need to be kept frozen.

Important: Do not freeze SPRI Beads.

The Dual Index Primers Set 1 for Illumina® and Dual Index Primers Set 2 for Illumina® are shipped on dry ice. Upon receipt, please store at -20°C.

Kits and components are guaranteed for 6 months after receipt when stored properly.

DNA Library Prep Kit for Illumina® (Catalog No. 53220)

Reagents	Quantity	Storage
End Repair Buffer	550 µL	-20°C
End Repair Enzyme Mix	60 µL	-20°C
Illumina Adapter	35 µL	-20°C
T4 DNA Ligase	75 µL	-20°C
Q5 Polymerase NGS MM	1350 µL	-20°C
SPRI Beads	5 mL	4°C
PEG	850 µL	4°C
TT Buffer	4 mL	RT

Dual Index Primers Set 1 for Illumina® (Catalog No. 53221)

Reagents	Concentration	Quantity	Storage
AM i5-001 Primer	10 µM	20 µL	-20°C
AM i5-002 Primer	10 µM	20 µL	-20°C
AM i5-003 Primer	10 µM	20 µL	-20°C
AM i5-004 Primer	10 µM	20 µL	-20°C
AM i5-005 Primer	10 µM	20 µL	-20°C
AM i5-006 Primer	10 µM	20 µL	-20°C
AM i7-001 Primer	10 µM	15 µL	-20°C
AM i7-002 Primer	10 µM	15 µL	-20°C
AM i7-003 Primer	10 µM	15 µL	-20°C
AM i7-004 Primer	10 µM	15 µL	-20°C
AM i7-005 Primer	10 µM	15 µL	-20°C
AM i7-006 Primer	10 µM	15 µL	-20°C
AM i7-007 Primer	10 µM	15 µL	-20°C
AM i7-008 Primer	10 µM	15 µL	-20°C

Dual Index Primers Set 2 for Illumina® (Catalog No. 53222)

Reagents	Concentration	Quantity	Storage
AM i5-007 Primer	10 µM	20 µL	-20°C
AM i5-008 Primer	10 µM	20 µL	-20°C
AM i5-009 Primer	10 µM	20 µL	-20°C
AM i5-010 Primer	10 µM	20 µL	-20°C
AM i5-011 Primer	10 µM	20 µL	-20°C
AM i5-012 Primer	10 µM	20 µL	-20°C
AM i7-001 Primer	10 µM	15 µL	-20°C
AM i7-002 Primer	10 µM	15 µL	-20°C
AM i7-003 Primer	10 µM	15 µL	-20°C
AM i7-004 Primer	10 µM	15 µL	-20°C
AM i7-005 Primer	10 µM	15 µL	-20°C
AM i7-006 Primer	10 µM	15 µL	-20°C
AM i7-007 Primer	10 µM	15 µL	-20°C
AM i7-008 Primer	10 µM	15 µL	-20°C

Additional Materials Required

- Sample DNA that has been fragmented between 150-600 bp in size
- Thermal cycler
- DNase-free sterile water
- 10 mM Tris-HCl, pH 8.0 or low EDTA TE Buffer, pH 8.0
- 80% Ethanol (made fresh each time)
- Magnet for 200 µL PCR tubes
- 1.5 or 2 mL microcentrifuge tubes (optional)
- 0.2 mL PCR tubes
- Microcentrifuge for PCR tube adapter

Prepare Samples and Reagents

Samples

Recommended dsDNA Input Range: 500 pg - 10 ng

Recommend dsDNA Size Range: 150 bp - 600 bp

While Active Motif's DNA Library Prep Kit for Illumina® can be performed using 500 pg - 10 ng of double-stranded fragmented genomic DNA, for the most optimal results 5 ng - 10 ng is recommended. Fragments should range in size from 150 bp - 600 bp.

We recommend determining the size of the genomic DNA by TapeStation™ or agarose gel electrophoresis and concentration by fluorometry (e.g. Qubit™). DNA quantification using a fluorometric assay such as Qubit is recommended.

The DNA sample should be diluted to a final volume of 39 µL in either 10 mM Tris-HCl or low EDTA TE buffer before use in the One-Tube End Repair reaction.

Use with Active Motif Kits

If using one of the Active Motif kits listed below, measuring the quantity and size distribution may not be required. Fragmentation has already been performed before chromatin/DNA precipitation by using mechanical fragmentation (e.g. sonication) or restriction digestion. The kit eluate DNA should already be in an ideal size range and concentration for library prep. For convenience, a recommended volume of kit eluate and 10 mM Tris-HCl is given below for creating the required final volume of 39 µL used in the One-Tube End Repair reaction.

For other kits please contact Active Motif Technical Support.

Active Motif Kit	Catalog no.	Kit Eluate DNA	10 mM Tris-HCl	Total Volume
ChIC/CUT&RUN	53180	39 µL	0 µL	39 µL
Low Cell ChIP Kit	53086	39 µL	0 µL	39 µL
ChIP-IT High Sensitivity	53040	36 µL	3 µL	39 µL
5hmC Profiling Kit	55023	20 µL	19 µL	39 µL

Reagents

Dilution of Illumina Adapter

The Illumina Adapter needs to be freshly diluted in TT Buffer prior to using in the Illumina Adapter Ligation reaction. The dilution of the Illumina Adapter will depend on the amount of DNA input material. Note that if the DNA sample is not easily quantifiable or if using DNA input volume from one of the kits listed above, we suggest following the adapter dilution recommendation for < 1 ng DNA.

Enough reagents are included in the kit to create a 4-reaction stock twelve times. Please follow the guidelines below for the appropriate DNA sample input.

Option 1: Dilution of Illumina Adapter for sample DNA input > 1 ng

If using 1 ng of DNA sample, create a 1:7 dilution stock of the Illumina Adapter before using in the ligation reaction. You will be using 2.5 µL of diluted Illumina Adapter for each ligation reaction. Example dilutions below are for different numbers of reactions and include overage.

Dilution of Illumina Adapter for sample DNA inputs >1 ng (1:7 dilution)

Reagents	1-4 rxns	5-24 rxns	25-48 rxns
Illumina Adapter	2 µL	10 µL	20 µL
TT Buffer	12 µL	60 µL	120 µL
Total Volume	**14 µL**	**70 µL**	**140 µL**

Option 2: Dilution of Illumina Adapter for sample DNA input < 1 ng DNA

If using less than 1 ng of DNA sample, create a 1:42 dilution stock of the Illumina Adapter before using in the ligation reaction. You will be using 2.5 µL of diluted Illumina Adapter to each ligation reaction. Example dilutions below are for different numbers of reactions and include overage.

Dilution of Illumina Adapter for sample DNA inputs <1 ng DNA (1:42 dilution)

Reagents	1-24 rxns	25-48 rxns
Illumina Adapter	2 µL	4 µL
TT Buffer	82 µL	164
Total Volume	**84 µL**	**168 µL**

SPRI Beads

Bring to room temperature for 30 minutes before use. Vortex 10 seconds.

Important: Do not freeze the SPRI Beads. Store at 4°C.

One-Tube End Repair

1. Set up a 200 µL PCR tube for each end-repair reaction to be performed.
2. Fragmented DNA Sample: Recommended range between 0.5 ng to 10 ng.
3. Following the guidelines under the section Prepare samples and reagents, dilute the fragmented DNA Sample in 10 mM Tris-HCl, pH 8 to a final volume of 39 µL.

   Important: Your sample cannot exceed 39 µL.

4. Add reagents in the order listed below to each PCR tube.

Reagents	Sample
End Repair buffer	10 µL
End Repair Enzyme Mix	1 µL
Fragmented DNA Sample	39 µL
Total Volume	**50 µL**

5. Pipette up and down to ensure complete mixture. Cap the PCR tubes tightly. Quick spin the tubes to collect the material to the bottom.

Note: Keep samples on ice until thermal cycler reaches 12°C.

6. Incubate the reaction in a thermal cycler with the following parameters:

   In thermal cycler:

   15 minutes @ 12°C,
   15 minutes @ 37°C,
   30 minutes @ 72°C,
   Hold at 4°C.

7. Immediately proceed to Illumina Adapter Ligation section.

Illumina Adapter Ligation

1. Quick spin the One-Tube Repair Reaction to collect the contents at the bottom.
2. Add the following components in the order listed in the table to the One-Tube Repair Reaction.

Note: After PEG is added, pippette gently to mix before adding the next reagents. PEG is highly viscous. Pipette slowly.

Reagents	Sample
Diluted Illumina Adapter*	2.5 µL
PEG	15 µL
T4 DNA Ligase	1.25 µL
End-Repair Reaction	50 µL
Total	**68.75 µL**

*Refer to the Dilution of Illumina Adapter section.

3. Pipette up and down 10 times to ensure complete mixture. Cap the PCR tubes tightly. Quick spin the tubes to collect the material to the bottom.
4. Incubate the reaction for 15 minutes at 25°C in a thermal cycler (no heated lid required).

Purification of Illumina Adapter Ligated DNA

Perform clean-up of adapter-ligated DNA using 55 µL of SPRI Beads. The SPRI beads should be brought to room temperature for 30 minutes and then vortexed for 10 seconds before using. The 80% ethanol should be made fresh each time.

Important: Do not freeze the SPRI Beads. Store at 4°C.

1. Add 55 µL of room temperature SPRI Beads to adapter-ligated DNA.
2. Pipet up and down 5-10 times.
3. Incubate the tubes for 5 minutes.
4. Place on magnet to collect beads for 10 minutes.
5. Aspirate the supernatant and discard.
6. Repeat the following wash step 2 times.
    a. With the magnet still applied, add 200 µL 80% ethanol.
    b. Incubate for 30 seconds at room temperature.
    c. Aspirate the supernatant and discard.
7. Allow tubes to sit at room temperature so that residual ethanol can evaporate. Once the beads transition from shiny to matte (2-5 minutes) proceed to the next step.

8. With the tubes off the magnet, resuspend the beads using 22 µL of TT Buffer. Wait 2 minutes.
9. Place the tubes on the magnet for 2 minutes.
10. Transfer 20 µL of purified ligated DNA off the beads to fresh 200 µL PCR tube.

Note: This DNA can be safely stored in tubes at -20°C.

PCR Enrichment of Adapter-ligated DNA

Refer to the Appendix Section Indexing Primer Sets, for i5 and i7 information. Use a unique combination of i5 and i7 adapters for each sample.

1. Add reagents in the order listed below to each PCR tube of purified adapter-ligated DNA.

Reagents	Sample
Purified adapter-ligated DNA	20 µL
AM i5-XXX Primer	2.5 µL
AM i7-XXX Primer	2.5 µL
Q5 Polymerase NGS MM	25 µL
Total Volume	**50 µL**

2. Pipette up and down 10x to ensure complete mixture. Cap the PCR tubes tightly. Quick spin the tubes to collect the material to the bottom.
3. Incubate the reaction in a thermal cycler with heated lid at 105°C using the following parameters, for 12 cycles of amplification for all input amounts:

In thermal cycler:

2 minutes @ 98°C
10 seconds @ 98°C
75 seconds @ 65°C — ***12 cycles***
2 minutes @ 65°C
Hold @ 4°C

Purification of Library

Perform SPRI clean-up of PCR enriched adapter-ligated DNA using 37.5 µL of SPRI Beads.

Note: SPRI beads should be brought to room temperature for 30 minutes and then vortexed for 10 seconds before using. 80% ethanol should be made fresh each time.

Important: Do not freeze the SPRI Beads. Store at 4°C.

1. Add 37.5 µL of room temperature SPRI Beads to enriched library DNA.
2. Pipet up and down 5-10 times.

3. Incubate the tubes for 5 minutes.
4. Place on magnet to collect beads for 10 minutes.
5. Aspirate the supernatant and discard.
6. Repeat the following wash step 2 times.
    a. With the magnet still applied, add 200 µL 80% ethanol.
    b. Incubate for 30 seconds at room temperature.
    c. Aspirate the supernatant and discard.
7. Allow tubes to sit at room temperature so that residual ethanol can evaporate. Once the beads transition from shiny to matte (2-5 minutes) proceed to the next step.
8. With the tubes off the magnet, resuspend the beads using 17 µL of TT Buffer.
9. Place the tubes on the magnet for 2 minutes.
10. Transfer 15 µL of library DNA off the beads to fresh 200 µL PCR tube.

Note: This library DNA can be safely stored in tubes at -20°C until sequencing.

Downstream Analysis

DNA libraries can be further quantified using Qubit® dsDNA HS (High Sensitivity) and sizes verified with TapeStation® and High Sensitivity DNA ScreenTape. Libraries can then be pooled together and sequenced on an Illumina sequencer. It is important to note that each sample must have a unique barcode to allow for correct demultiplexing.

Appendix

I5 Primer

5´-AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3´

I7 Primer

5´- CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTGTGCTCTTCCGA*T-3´

Dual Index Primer Set 1 for Illumina®

	MiSeq, HiSeq 2000/2500, NovaSeq v1.0 Reagent Kits	iSeq, MiniSeq, NextSeq, HiSeq 3000/4000, NovaSeq v1.5 Reagent Kits
Index Primer	**Sample Sheet**	**Sample Sheet**
AM i5-001 Primer	AGTTGAAT	ATTCAACT
AM i5-002 Primer	ACCGGCCA	TGGCCGGT
AM i5-003 Primer	CTGAACCG	CGGTTCAG
AM i5-004 Primer	GCGTGCTC	GAGCACGC
AM i5-005 Primer	ATACCGTT	AACGGTAT
AM i5-006 Primer	CGGTCCTA	TAGGACCG
AM i7-001 Primer	GCAGTCTT	GCAGTCTT
AM i7-002 Primer	TGATCAGT	TGATCAGT
AM i7-003 Primer	GTCGGCAC	GTCGGCAC
AM i7-004 Primer	TGGAAGAG	TGGAAGAG
AM i7-005 Primer	CGGAAGGT	CGGAAGGT
AM i7-006 Primer	AATCTGGT	AATCTGGT
AM i7-007 Primer	ATGTGCCT	ATGTGCCT
AM i7-008 Primer	GTGTCCTG	GTGTCCTG

Dual Index Primer Set 2 for Illumina®

	MiSeq, HiSeq 2000/2500, NovaSeq v1.0 Reagent Kits	iSeq, MiniSeq, NextSeq, HiSeq 3000/4000, NovaSeq v1.5 Reagent Kits
Index Primer	**Sample Sheet**	**Sample Sheet**
AM i5-007 Primer	TGCGCGAC	GTCGCGCA
AM i5-008 Primer	TGCATTGT	ACAATGCA
AM i5-009 Primer	CAGCGAGT	ACTCGCTG
AM i5-010 Primer	GATTCAGA	TCTGAATC
AM i5-011 Primer	GTGATGCA	TGCATCAC
AM i5-012 Primer	CTGATTGT	ACAATCAG
AM i7-001 Primer	GCAGTCTT	GCAGTCTT
AM i7-002 Primer	TGATCAGT	TGATCAGT
AM i7-003 Primer	GTCGGCAC	GTCGGCAC
AM i7-004 Primer	TGGAAGAG	TGGAAGAG
AM i7-005 Primer	CGGAAGGT	CGGAAGGT
AM i7-006 Primer	AATCTGGT	AATCTGGT
AM i7-007 Primer	ATGTGCCT	ATGTGCCT
AM i7-008 Primer	GTGTCCTG	GTGTCCTG

Notes and Expected Results

Upon ligating Illumina adapters, DNA fragments can be further sequenced and analyzed using regular alignment packages such as Bowtie[2] or BWA. This kit has been benchmarked against other commercial competitors and showed good reproducibility.

The DNA library prep kit can be used with a broad variety of starting inputs, for example double-stranded DNA resulting from a CUT&RUN, ChIP from as low as 500 pg -10 ng of starting material. We do not recommend using less than 500 pg of starting material as this will increase the likelihood of obtaining adapter dimers and PCR duplicates that can be deleterious for sequencing and further analyses. It is crucial to start with double-stranded DNA, single-stranded DNAs (such as those obtained from MeDIP) are not compatible with this kit.

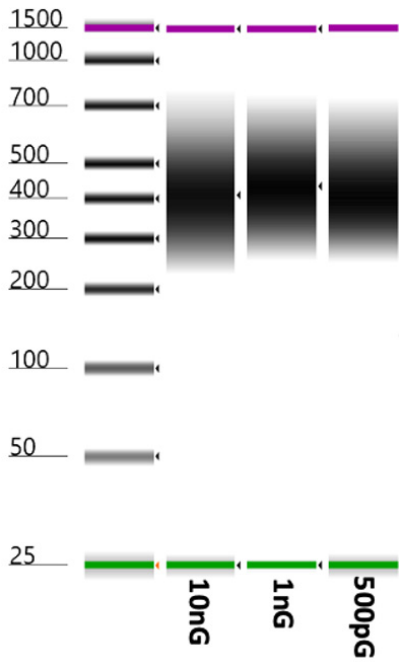


Figure 1.
TapeStation profiles obtained with 10 ng, 1 ng, or 500 pg of sonicated genomic DNA from *E. coli*

Troubleshooting Guide

Problem/Question	Recommendation
Library migrates unexpectedly on Tapestation	A ~150bp band often results from adapter dimers. Increase the input material and try to keep the PCR amplification cycles to a maximum of 12 cycles.
DNA does not fragment properly; broad or lopsided (high MW) sonication profile of fragmented DNA	Use isopropanol purification, bead clean-up, column purification or other method to purify DNA before fragmentation. Ensure fragmentation device is functioning within the manufacturer's parameters.
Storage of DNA	Once the library is created, samples may be stored at -20°C prior to sequencing.
Incomplete resuspension of beads after ethanol wash during SPRI steps	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

North America	Toll free:	877 222 9543
	Direct:	760 431 1263
	Fax:	760 431 1351
	Email:	tech_service@activemotif.com
Europe	Direct:	+32 (0)2 653 0001
	Fax:	+32 (0)2 653 0050
	Email:	eurotech@activemotif.com
Japan	Direct:	+81 (0)3 5225 3638
	Fax:	+81 (0)3 5261 8733
	Email:	japantech@activemotif.com
China	Direct:	(86)-21-20926090
	Cell Phone:	18521362870
	Email:	techchina@activemotif.com