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APPLICATION NOTE No. 275

Automation of ChIP-Seq Library Preparation for Next Generation Sequencing on the ep*Motion*® 5075t

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Abstract

ChIP-Seq library preparation can be a challenging procedure to automate because of its low ChIP DNA input. This Application Note describes the successful automation of Illumina ChIP-Seq library preparation on the Eppendorf ep*Motion* 5075t, using the KAPA High-Throughput Library Preparation Kit from KAPA Biosystems. Size-selected libraries were prepared from as little as 1 ng of fragmented ChIP DNA. To obtain the recommended 100 ng of library material for sequencing on the Illumina Genome Analyzer, only 18 amplification cycles were needed for 1 ng of input DNA, or 9 cycles when starting library construction with 10 ng of ChIP DNA.

Introduction

The Eppendorf ep*Motion* is a multi-purpose liquid handling workstation that is suitable for many laboratory procedures. The ep*Motion* 5075t and other models in the family are ideal walk-away companions for labs that demand high efficiency, accuracy and automated workflow. The system can be easily programmed to perform entire procedures such as nucleic acid extractions based on a wide selection of kits and reagents. Equipped with twelve positions on the worktable, it is capable of working with virtually any type of tube or plate. What's more, its built-in expandability allows integration of any labware of your choice on the fly. The on-deck mixer has an incorporated thermo unit, providing incubation capability needed for the NGS library preparation. Six dispensing tools covering 1-1000 µL range are available in both single- and 8-channel formats to meet different throughput requirements. Additionally, a gripper can be added to transport plates around the worktable with ease.
We have previously demonstrated successful automation of a wide variety of common nucleic acid purification procedures from Promega, Macherey-Nagel, Invitrogen and others.

The rapid growth of Next Generation Sequencing (NGS) in recent years has created new applications for automated liquid handling, including library construction and quantification. To meet the growing demand for quality and throughput in this field, we have developed ep*Motion* protocols that are compatible with library preparation chemistries utilized by major NGS platforms from Illumina, Life Technologies and Roche. Our most recent addition to this expanding application family involved automation of library construction using the KAPA High-Throughput Library Preparation Kit, and validation of this automated protocol for the construction of ChIP-Seq libraries.

NGS library construction workflows are comprised of repetitive liquid handling and incubation steps, and are therefore ideal for automation. The ep*Motion* 5075t has been optimized to carry out a full library construction workflow - including end repair, A-tailing, adapter ligation, bead-based cleanups, dual-SPRI (Solid Phase Reversible Immobilization) size selection and all on-deck incubations for 24 samples in less than 6 hours without the need for user interference.

The same protocol can be quickly expanded to process up to 96 samples. The system is carefully calibrated to minimize the consumption of expensive reagents. Its high-precision pipetting tools[1] further assure the production of high-quality libraries for downstream sequencing.

The successful implementation of a high-throughput NGS construction pipeline requires a reliable automation platform, combined with appropriate reagent formulation, packaging, and an optimized protocol. Many library preparation kits that are labeled "automation-friendly" are nevertheless poorly suited for automation, for reasons including: 1) impractical reaction setups, 2) suboptimal cleanup parameters, 3) unsuitable recommendations with respect to user-supplied consumables, particularly with regard to quality (affinity for nucleic acids[2]) and compatibility with the overall process (e.g. microtiter plates are incompatible with thermocycler incubation), and 4) the requirement for off-instrument size selection.

Kapa Biosystems (www.kapabiosystems.com) has recently upgraded its NGS Library Preparation Kit for Illumina sequencing to accommodate the specific requirements of automated liquid handling on the ep*Motion*. Specifically, 1) reagent volumes have been tailored for processing 96 samples per kit (which facilitates inventory management), and generous excesses of all reagents are supplied to provide for the dead volumes required by automated liquid handling; 2) reaction setups have been optimized to avoid pipetting of viscous enzyme solutions and volumes less than 5 µL; 3) total reaction and cleanup volumes never exceed 200 µL to ensure compatibility with standard 96-well plates; 4) efficient, cost-effective, and automation-friendly reaction cleanups are achieved through implementation of the "with-bead" strategy developed at the Broad Institute of MIT & Harvard and Foundation Medicine[3], and the kit includes the PEG/NaCl SPRI solution required for re-using beads. This "with-bead" strategy significantly reduces the consumption of beads and plasticware, and eliminates sample loss associated with the physical transfer of DNA solutions from one plate to the next. Combined with the Eppendorf twin.tec semi-skirted PCR plates that exhibit very low affinity to nucleic acids, these improvements present an ideal solution for low-input library preparation such as ChIP-Seq. Because the automated "with-bead" protocol leads to a higher yield of adapter-ligated library fragments, fewer cycles of library amplification with the engineered KAPA HiFi HotStart DNA Polymerase are likely to be required, thereby further reducing the risk of bias[4,5] and other amplification artifacts. In addition, the automated protocol provides fully validated dual-SPRI size selection, which is performed on the ep*Motion* without any user intervention.

Materials and Methods

Kits and Reagents

> Fragmented DNA (sheared to ~200 bp). Libraries were prepared from various quantities of input DNA, ranging between 1 ng and 200 ng. 1, 5, 10 and 20 ng samples were ChIP DNA, whereas 50 and 200 ng samples were cDNA.
> Illumina™ TruSeq® Y-adapters
> KAPA High-Throughput Library Preparation Kit (Cat. No. KK8234)
> Agencourt® AMPure® XP beads (Beckman Coulter Cat. No. A63880)
> Elution buffer (1X TE)
> Freshly prepared 80% ethanol

Consumables

> 1.5 mL Eppendorf DNA LoBind tubes
> 0.1 mL Eppendorf PCR tubes
> Eppendorf PCR Film (self-adhesive)
> Eppendorf twin.tec PCR plates, semi-skirted
> Eppendorf epT.I.P.S. Motion Filter 1-50 µL
> Eppendorf epT.I.P.S. Motion Filter 20-300 µL
> ep*Motion* Reservoir 30 mL
> ep*Motion* Reservoir 100 mL

Equipment

> ep*Motion* 5075t automated pipetting system with gripper
> Dispensing tool TS 50
> Dispensing tool TM 50-8
> Dispensing tool TM 300-8
> Reservoir Rack
> TC module for 1.5/2.0 mL tubes
> Thermorack for 24x1.5/2.0 mL Safe-Lock tubes
> Thermoblock for 96 well PCR plates
> Eppendorf Mastercycler Pro
> Agilent Technologies 2100 Bioanalyzer
> Agencourt® SPRIPlate® Super Magnet Plate (Beckman Coulter Cat. No. A32782)

Procedure

Figure 1 outlines the essential steps of the workflow. Additional details are provided below.

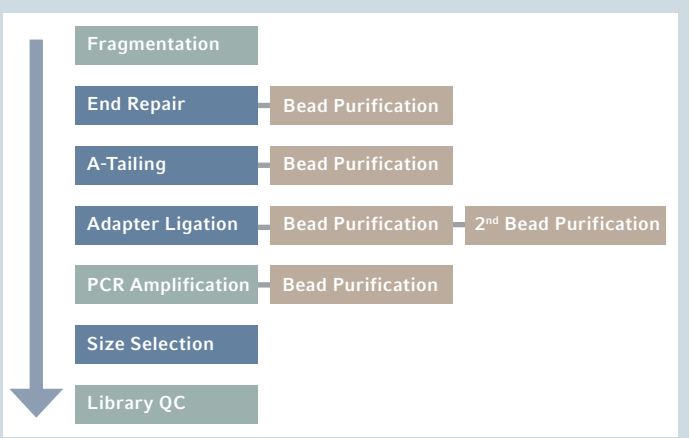

Figure 1: Library preparation workflow for the KAPA High-Throughput Library Preparation Kit on the ep*Motion* 5075t. Blue and orange steps can be performed on the ep*Motion* 5075t without any user intervention. Green steps are performed off-deck using other equipment. PCR amplification and size selection steps are interchangeable depending on user needs.

> The ep*Motion* was programmed according to the instructions provided in the KAPA High-Throughput Library Preparation Kit Technical Data Sheet. The ep*Motion* worktable setup is shown in Figure 2.

> The recommended second post-ligation cleanup was performed to completely remove un-ligated adapters (see section 6 of KAPA HTP Library Preparation Kit Technical Data Sheet). DNA was eluted into 30 µL of elution buffer after this step. About 25% (7 µL) was set aside for QC and/or troubleshooting, and the remaining 23 µL were used for library amplification.

> PCR library amplification was performed prior to size selection in order to preserve the diversity of the library. Amplified library was eluted in 50 µL of elution buffer. 10% (5 µL) was set aside for QC and/or troubleshooting, and the remaining 45 µL were used for size selection.

> The number of PCR cycles used to amplify each library is listed in Table 1.

> To obtain size-selected library material in the 200-500 bp range, 0.6 volumes of SPRI beads were used to remove fragments larger than 500 bp. To the supernatant (containing fragments <500 bp), 0.2 volumes of beads (relative to the starting volume of 45 µL) were added, to exclude fragments <200 bp.

> Sequencing-ready library was eluted in 30 µL of elution buffer, after which 1 µL of each library was used to assess quality and concentration on the Agilent 2100 Bioanalyzer.

Table 1: Number of PCR cycles used for different DNA input quantities.

Template Qty (ng)	Number of PCR cycles
1	12
5	7
10	6
20	5
50	4
200	2

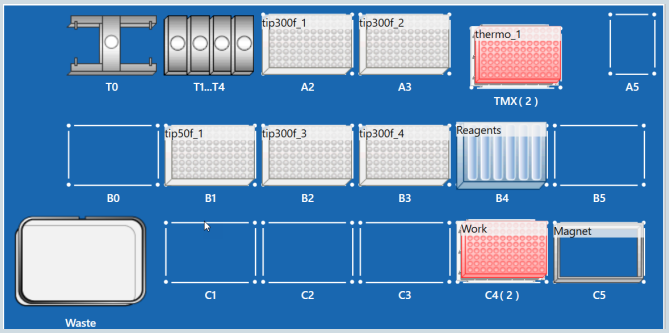

Figure 2: Screenshot of the epBlue™ software showing the setup of ep*Motion* 5075t worktable for use with the KAPA High-Throughput Library Preparation Kit.

Table 2: ep*Motion* 5075t worktable details for the KAPA High-Throughput Library Preparation Kit

Position	Labware	Comment
A2	epT.I.P.S Motion 300 µL, filtered	
A3	epT.I.P.S Motion 300 µL, filtered	
TMX	Thermoblock for 96 well PCR plates	Support for semi-skirted plate
B1	epT.I.P.S Motion 50 µL, filtered	Waste collector
B2	epT.I.P.S Motion 300 µL, filtered	
B3	epT.I.P.S Motion 300 µL, filtered	
B4	Reservoir Rack Position 1/A: End Repair Mix Position 1/B: A-Tailing Mix Position 1/C: Ligation Mix Position 1/D: Adapter[1] Position 2: AMPure® XP Beads Position 3: 80% Ethanol Position 4: PEG/NaCl Position 5: Empty Position 6: Empty Position 7: Waste	 TC module for 1.5/2.0 mL tubes 30 mL reservoir 30 mL reservoir 30 mL reservoir 30 mL reservoir 100 mL reservoir
C3	Thermoblock for 96 well PCR plates Eppendorf twin.tec PCR plates, semi-skirted	Support for semi-skirted plate Work Plate for all steps
C4	Agencourt® SPRIPlate® Super Magnet	
T0	Gripper	
T1-T3	TM 50, TM 50-8, TM 300-8	Pipetting tools

[1] Use a 24 x 1.5/2.0 mL thermorack for more than one adapter.

Results

Bioanalyzer data obtained from the analysis of 1 µL of each of the amplified, size-selected libraries are shown in Figure 3. Libraries were obtained from fragmented ChIP DNA or cDNA, with library construction input ranged between 1 and 200 ng. The maximum number of PCR cycles used during library amplification was 12 cycles (for 1 ng ChIP DNA). The number of PCR cycles was progressively reduced to compensate for increasing amounts of input DNA (Table 1). These data demonstrated that the Eppendorf ep*Motion* 5075t is capable of fully automating the NGS library preparation process (from end repair to sequencing-ready library). Overlaid electropherograms showed that efficient size selection was achieved across the whole range of libraries, despite the variable amounts of amplified DNA in each sample.

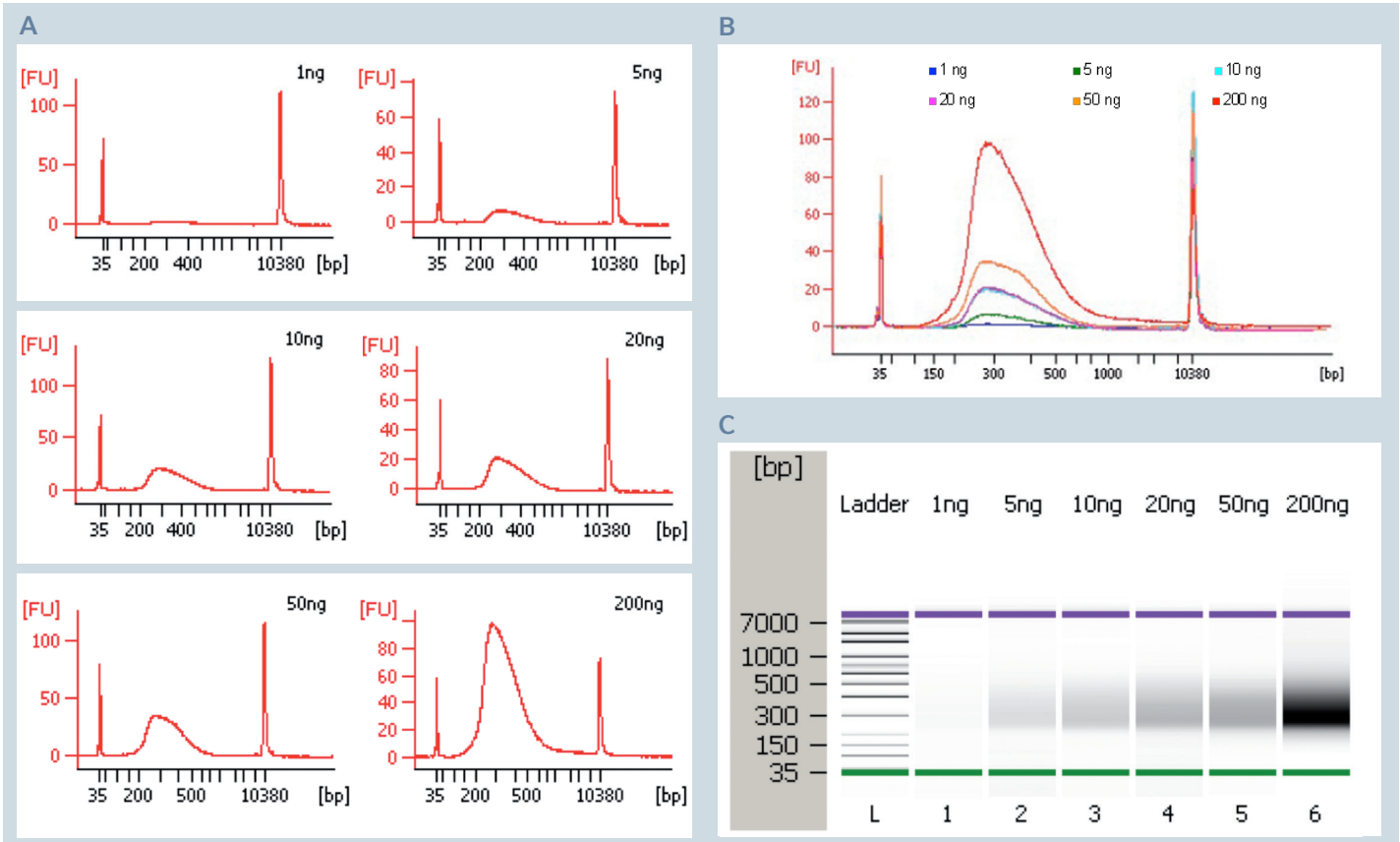

Figure 3: Bioanalyzer analysis of amplified, size-selected libraries prepared from 1 ng to 200 ng input DNA.
(A) Electropherograms for individual libraries. (B) Overlay of all libraries. (C) Virtual gel. All libraries displayed a consistent fragment length distribution, with an average peak size at ~265 bp.

Yields of amplified, size-selected libraries are summarized in Table 3. These numbers represent net yields, after all unavoidable sample losses, attributable to one or more of the following possible factors:

> DNA adsorption to plastic surfaces during pipetting and incubations
> Liquid handling (i.e. viscous solution carry-over)
> Imperfect efficiency of enzymatic reactions
> Incomplete elution of DNA from SPRI beads (five bead cleanup steps in total)
> Imperfect PCR efficiency
> Loss of DNA due to size-selection

In this experiment, sample losses throughout the library construction process were minimized by 1) carefully optimizing dispensing and aspiration steps, 2) employing a "with-bead" strategy that obviates the transfer of DNA between plates for consecutive enzymatic steps, and 3) shearing input DNA to a size (~200 bp) that overlaps well with the range of adapter ligated fragment sizes (200–500 bp) captured during size selection. Unlike common ChIP-Seq protocols that recommend 18 cycles of amplification, we varied the number of cycles based on DNA input, and calculated the number of amplification cycles required to produce 100 ng of library for sequencing on the Illumina Genome Analyzer. As shown in Table 3, 18 cycles of enrichment are expected to produce sufficient library material when 1 ng input ChIP DNA is used for library construction. For the 10 ng of input recommended in the Illumina protocol[6], only 9 cycles should be needed to produce the desired quantity of material for sequencing.

Table 3: Yields of amplified, size-selected libraries, and predicted number of amplification cycles required to obtain 100 ng of library.

Template	Cycle Num.	Con. (pg/µL)	Yield (ng)[1]	Adj. Yield (ng)[2]	Cycles to 100 ng[3]
1	12	40.16	1.20	1.74	18
5	7	210.40	6.31	9.14	11
10	6	372.42	11.17	16.19	9
20	5	559.62	16.79	24.33	7
50	4	730.03	21.90	31.74	6
200	2	3106.37	93.19	135.06	2

[1] In total of 30 µL of size-selected DNA recovered for each library.
[2] Adjusted yield to account for procedural losses (see second and third bullet points in "Procedure").
[3] Predicted number of PCR cycles necessary to meet the requirement of sequencing on the Illumina Genome Analyzer. (assumes 100% PCR efficiency throughout the specified number of cycles).

Discussion

NGS library preparation has become a standard procedure for many sequencing labs. Preparing high-quality libraries from limited amounts of input DNA remains a challenge, particularly when automated liquid handling is required to achieve high throughput. Whilst adapter-ligated, size-selected DNA can easily be amplified to levels required for sequencing, it is important to limit the number of PCR amplification cycles in any library construction workflow as much as possible to ensure the lowest possible incidence of duplicate reads, amplification bias (leading to uneven coverage), chimeric library inserts, nucleotide substitutions, and heteroduplex formation (due to thermocycling after substrate depletion) which can lead to inaccurate library quantification.

This Application Note demonstrates the successful automation of low-input library construction, using the KAPA High-Throughput Library Preparation Kit on the Eppendorf ep*Motion* 5075t. Our data have shown that it is possible to obtain sufficient amounts of size-selected library material for Illumina sequencing from as little as 1 ng of input DNA after 18 cycles of amplification with the engineered KAPA HiFi DNA Polymerase, or only 9 cycles beginning with 10 ng of input DNA. This presents a significant improvement over competitor solutions for the preparation of ChIP-Seq libraries.

In summary, the following factors are critical for successful, automated library construction from low-input DNA:

> A reliable, optimized liquid handling workstation that requires minimal human intervention
> High-quality Eppendorf consumables (tubes, tips and PCR plates) that offer ultra-low affinity to nucleic acid, which is vital for samples that can be obtained only in trace amounts
> High-quality library construction reagents for efficient enzymatic reactions, combined with the optimized cleanup and dual-SPRI size selection parameters provided by the KAPA HTP Library Preparation Kit
> The "with-bead" strategy, which allows for recycling of SPRI beads in consecutive reaction cleanups, thereby minimizing DNA losses and reducing per sample cost
> Highly efficient, low bias library amplification with the engineered high-fidelity KAPA HiFi DNA Polymerase[4,5]

The combined advantages of the Eppendorf ep*Motion* 5075t and the KAPA High-Throughput Library Preparation Kit offer an industry-leading solution for standard and low-input library preparation, particularly for ChIP-Seq. The automated workflow on the ep*Motion* is easily scalable, to provide for the processing of up to 96 samples per run. Moreover, the spectrum of applications can be expanded to any other NGS library construction workflows that share the same basic steps. Data obtained from the 50 and 200 ng cDNA samples are good examples of utility in DNA and RNA sequencing applications.

Literature

[1] Eppendorf Application Note 168: Measuring the Accuracy and Precision of the ep*Motion* 5070 Workstation using the Artel® Multichannel Verification System (MVS®)
[2] Eppendorf Application Note 226: Use of Eppendorf LoBind Tubes to consistently prepare and store standard panels for real-time PCR absolute quantifications.
[3] S. Fisher *et al.*, 2011. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biology*, 12(1), p.R1.
[4] M.A. Quail *et al.*, 2012. Optimal enzymes for amplifying sequence libraries. *Nature Methods*, 9(1), p.10.
[5] Quail *et al.* 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics*, 13:341.
[6] Illumina Specification Sheet "Genome Analyzer*IIx* System".

Ordering information

Description	Order no. International
ep*Motion*® 5075t, basic device incl. Eppendorf ThermoMixer®, epBlue™ software, mouse, waste box, 100–240 V ±10%/50–60 Hz ±5%, 0.2 µL–1 mL	5075 000.302
Completely contained housing	5075 751.623
Eppendorf MultiCon™ PC controller incl. keyboard	5075 001.101
Gripper for labware transport, incl. holder	5282 000.018
Dispensing tool TS 50, 1–50 µL	5280 000.010
Dispensing tool TM 50-8, 1–50 µL	5280 000.215
Dispensing tool TM 300-8, 20–300 uL	5280 000.231
ReservoirRack, for ReservoirRack Modules TC and max. 7 of 10 mL, 30 mL and 100 mL reagent reservoirs	5075 754.002
Thermoblock for PCR plates, 96-well, for heating or cooling of PCR plates; exchangeable using the gripper	5075 766.000
Reservoir rack module TC Safe-Lock Tubes, for 4 × 0.5/1.5/2 mL tubes	5075 799.081
Thermorack for 24 . 1.5/2 mL Safe-Lock Tubes, for a supply of 24 test tubes, temperature control	5075 771.004
epT.I.P.S. Motion Filtertip 1–50 µL PCR Clean	0030 014.413
epT.I.P.S. Motion Filtertip 20–300 µL PCR Clean	0030 014.456
ep*Motion* Reservoir 30 mL	0030 126.505
ep*Motion* Reservoir 100 mL	0030 126.513
twin.tec PCR plate 96, semi-skirted	0030 128.575
PCR tube strips 0.1 mL plus cap strips	0030 124.820

KAPA Biosystems	
KAPA HTP Library Preparation Kit (96 libraries)	KK8234
KAPA Library Amplification Kit (50 x 50 µL reactions)	KK2611
KAPA Library Amplification Kit (250 x 50 µL reactions)	KK2612

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