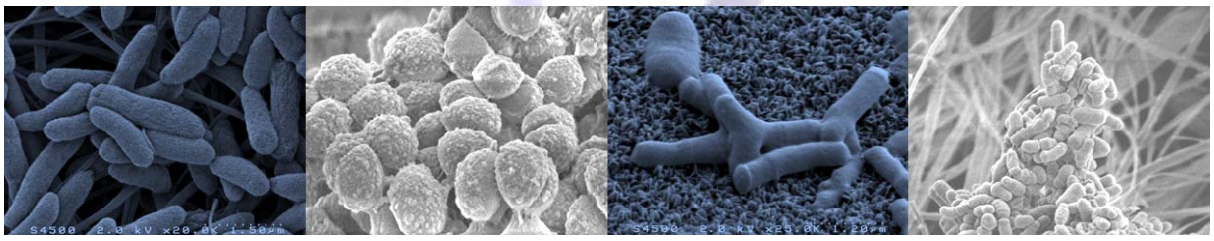
IHMS – QUALITY PROCEDURE
International Human Microbiome Standards
http://www.microbiome-standards.org

IHMS SOP 09 V1: STANDARD OPERATING PROCEDURE FOR METAGENOMIC SHORT READS



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Introduction

IHMS seeks to coordinate development of standard operating procedures (SOPs) and protocols that will optimize data comparisons in the human microbiome field. The IHMS project concentrates on following objectives:

- Coordinate standardization of procedures and protocols within the existing Human Microbiome research programs and those yet to come,

- Gather and compare the protocols used to collect, identify and process human samples and aid to develop the standard operating procedures for sample collection, identification and processing,

- Compare sequences of genes and genomes of human-associated microorganisms generated by various methodologies and approaches, and to develop standards to define sequence quality and recommend procedures to reach the standards,

- Assess the approaches and procedures used to analyze the sequence data and the associated metadata and recommend standards for data analysis.

Beside these objectives, IHMS project aims at ensuring the optimal public access and use of the data generated by various microbiome projects. The project is supported by the European Commission under the 7th Framework Programme. The consortium gathers 8 partners and 15 contributors across 12 different countries. Its total cost has been evaluated at 2,3 million €, the funding from the European Commission has been set with an upper limit of almost 2 million € and a duration of 4 years, beginning in February 1st, 2011.

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SEVENTH FRAMEWORK PROGRAMME

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Summary

1. OBJECTIVE:

Optimize data comparisons in the human microbiome field by the standardization of the procedure for sequencing metagenomic fecal samples.

This SOP is of first interest for efficient and reproducible library preparation for Illumina sequencing of metagenomic samples.

2. PRINCIPLE:

This SOP aims to standardize library preparation for Illumina sequencing of metagenomic DNA from fecal extractions. The library preparation includes all the steps necessary to adapt DNA for sequencing with Illumina technology.

3. RELATED DOCUMENTS:

Titles	Codes	Localizations
Illumina Cbot User Guide	Part # 15006165	www.illumina.com
Illumina HiSeq2000 System User Guide	Part # 15011190	www.illumina.com
Sequencing Library qPCR quantification Guide	Part # 11322363	www.illumina.com
Agilent 2100 Bioanalyzer 2100 Expert User's Guide	Part #G2946-90004	www.genomics.agilent.com
MxPro QPCR Software Instruction Manual For Mx3000P and Mx3005P QPCR Systems	IN#70224D IN#70225J.0	www.genomics.agilent.com

SEVENTH FRAMEWORK PROGRAMME

4. Persons entitled to use the procedure:

This SOP applies to any laboratory personal involved in preparation of DNA, issued from extraction of fecal samples, for sequencing on an Illumina instrument.

5. Preliminary steps, specificities:

We strongly recommend to check DNA quantity and integrity following the quality control evaluation procedure described in IHMS_SOP 08. The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA, particularly when starting quantities are <1 µg. Thus, correct quantitation of genomic DNA is essential. DNA integrity is also important for successful library generation. If degraded genomic DNA is used, the fragmentation step of the protocol is likely to fragment the DNA to a size below the desired size range. Furthermore, damaged and degraded DNA samples will PCR amplify less efficiently, leading to diminished library yields and diversity.

6. Conditions and usage constraints to follow:

1-Avoid Cross-Contamination- Pipettes and work surfaces must be cleaned before and after the procedure

2- Temperature Considerations - During the process le libraries must be kept at temperatures ≤ 37°C and placed at stopping point in an accurate storage

3- Handling Liquids - Ensure that pipettes are correctly calibrated. Prepare reagents for multiple samples simultaneously to minimize pipetting errors

4- Pre-PCR and post-PCR areas should be identified (ideally in two separate rooms)

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7. Materials, products, kits, solutions:

Materials:

Materials	Suppliers Names and references
Covaris™ AFA Ultrasonicator	Covaris, E210 ultrasonicator device
Covaris 6-mm × 16-mm AFA microtubes and snap caps	Covaris, ref.520045
Covaris 96 microTUBE Plate	Covaris, ref. 520078
2100 Bioanalyser	Agilent Technologies, ref. G2938C
Stratagene Mx3005P	Agilent Technologies ref. 401457
1000µl single chanel pipette	General lab supplier
200 µl single chanel pipette	General lab supplier
100 µl single chanel pipette	General lab supplier
20 µl single chanel pipette	General lab supplier
10 µl single chanel pipette	General lab supplier
Filtered pipette tips	General lab supplier
Heat blocks	General lab supplier
Vortexer	General lab supplier

1.5 mL nuclease free non-sticky tubes	VWR, Ref. 525-0130	
0,2 ml thin wall PCR tubes	VWR, Ref. 732-0097	
Qubit™ fluorometer 2.0	Life Technologies,	Ref. Q32866
Microcentrifuge for individual 1.5 ml and 0.2 ml tubes	General lab supplier	
Magnetic separation device	General lab supplier	
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier	
96-well 0.3 ml PCR plates	General lab supplier	
96 well thermal cycler with heated lid	General lab supplier	
QPCR 96 well Plates, Semi-Skirted	Stratagene, ref.401334	
Mx3000P/Mx3005P Optical Strip Caps	Stratagene, ref.401425	
Microseal 'B' Adhesive Seals	Bio-Rad,	ref. MSB-1001
Disposable gloves	General lab supplier	

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Kits:

Materials	Risks	Prevention	Suppliers Names and references
Agilent High Sensivity DNA Kit	 H320 327-373	 S26 S36/39	Agilent, ref. 5067-4626
Quant-iT™ dsDNA HS assay kit	 H327 R25 R36/37	 S26 S36/39	Invitrogen, ref. Q32851

Risk and Safety statements:
H320 Flammable.
H327 Risk of serious damage to eyes and irritating to eyes.
H373 May cause damage to organs through prolonged or repeated exposure
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S36/39 Wear suitable protective clothing and eye/face protection.
R25 Toxic if swallowed
R36/37 Irritating to eyes and respiratory system.

Products:

Products	Risks	Prevention	Suppliers Names and references
Agencourt® AMPure® XP	NA*		Beckmann Coulter, ref. A63881
Resuspension buffer (10 mM Tris-Cl, pH 8.0)	NA*		General lab supplier
Ethanol absolute for molecular biology	 R11	 S2-7-16	VWR, ref. 20821.296
Kapa Hifi Hotstart NGS Library Amplification Kit	 R 21-25 36/37/38	 S 28-26-45 36/37	KapaBiosystems, ref. KK2612
KAPA Library Quantification Kit	 R 21- 25 R 36/37/38	 S 28-26-45 S 36/37	KapaBiosystems, Ref. KK4824
NEBNext® End Repair Enzyme Mix		S 23 S24/25	NEB, ref. E6041

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NEBNext® End Repair Reaction Buffer		S 23 S24/25	NEB, ref. E6042
Klenow Fragment (3'-5' exo-)		S 23 S24/25	NEB, ref. E6054
NEBNext dA-Tailing Reaction Buffer		S 23 S24/25	NEB, ref. E6055
Quick Ligation Reaction Buffer		S 23 S24/25	NEB, ref. E2200
Quick T4 DNA Ligase		S 23 S24/25	NEB, ref. M2200
Poly(ethylene glycol) BioUltra 8000		Use equipment for eye protection Handle with gloves	Sigma, ref. 89510
NaCl 5M		Avoid contact with skin and eyes. Avoid inhalation of vapor or mist.	Sigma, ref. S5150-1L
NEXTflex™ PCR-Free Barcodes-48. Indexing of DNA samples for NGS on Illumina platforms.	NA*		BioScientific, ref. 514110
Nuclease free ultrapure water	NA*		Generallab supplier
RNAse/DNAse zapper (to decontaminate surfaces)	 R 36/38		General lab supplier

Primer PCR P5 (HPLC purified)	NA*	Oligonucleotide supplier
Primer PCR P7 (HPLC purified)	NA*	Oligonucleotide supplier

Risk and Safety statements:
NA Not applicable.
R11 Highly flammable.
R36/38 Irritating to eyes and skin
R36/37/38 Irritating to eyes, respiratory system and skin.
R21 Harmful in contact with skin.
R25 Toxic if swallowed.
S2 Keep out of the reach of children
S7 Keep container tightly closed.
S16 Keep away from sources of ignition - No smoking.
S23 3 Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer)
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S28 After contact with skin, wash immediately with plenty of ... (to be specified by the manufacturer).
S45 In case of accident or if you feel unwell seek medical advice immediately (show the label where possible).
S24/25 Avoid contact with skin and eyes.
S36/37 Wear suitable protective clothing and gloves.

Solutions:

I/ Solution n° 1: PEG/NaCl solution

Title: PEG/NaCl solution

Quantity to prepare: 200ml

Ingredients:

Poly(ethylene glycol) BioUltra 8000

NaCl 5M

Material needed: general lab vessels

Prevention: none

Recipe:

Add 40g of Poly(ethylene glycol) BioUltra 8000 to 100ml of NaCl 5M

Adjust total volume to 200ml with ultrapure water

Mix until complete dissolution

Storage: room temperature

I/ Solution n° 2: 80% ethanol

Title: 80% ethanol

Quantity to prepare: 2 ml/sample

Ingredients: Ethanol absolute for molecular biology, nuclease free water

Material needed: General lab vessels

Prevention: S2, S7, S16

Recipe for 100 mL: mix 80 mL absolute ethanol with 20 mL ultrapure nuclease free water *Storage:* to be used freshly

8. Step by step procedure:

Library preparation overview

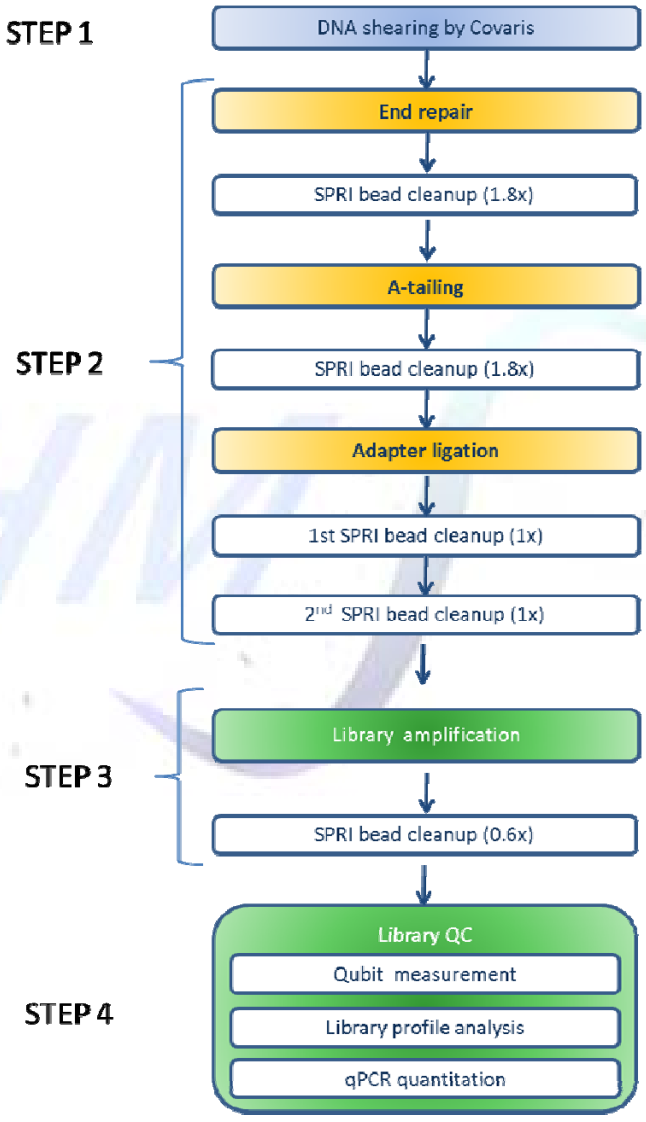
The purpose of this procedure is to prepare indexed paired end libraries with 180 - 680 bp insert size DNA library that will be used for sequencing on the Illumina HiSeq2000 on 100 bp paired end lengths. Starting material is 250 ng genomic DNA extracted from fecal samples.

This protocol is intended for low-throughput, manual library construction, but it is designed to be automation-friendly, easing the transition to automation if needed by throughput requirements.

Genomic DNA is broken into smaller fragments via Covaris E210 instrument. Then end repair, A tailing and barcoded adaptors ligation are performed manually or by a liquid handling system. After PCR enrichment, library purification, qualitative and quantitative assessment of the library are performed.

Quality control includes a Qubit measurement, library profile analysis by capillary electrophoresis and qPCR. These last steps as well as library purification are also easily amenable to automation by means of liquid handling systems.

Workflow summary:



General considerations

- This protocol has been validated starting from 250 ng metagenomics DNA extracted from feces but libraries can be successfully constructed using lower input amounts if the sample represents sufficient copies to ensure the requisite coverage and complexity in the final library.

Procedure

STEP 1: DNA fragmentation using Covaris

Important considerations specific to this step

- This step involves sonicating the input DNA into fragments with a mean fragment size of 600 bp and a fragment size range of 100-1000 bp using the Covaris E210 System. The conditions have been tested for shearing 100 to 500 ng DNA in a total volume of 50 µl.
- EDTA and strong buffer solutions (>10 mM Tris-HCl) may negatively affect the subsequent end repair reaction, and should be absent from the starting DNA sample.
- Follow the manufacturer recommendations for correct use of the instrument.

1) Allow the Covaris chiller to reach 4 °C, and degas at least 45 min.
2) During this time, prepare the DNA sample:
   Dilute 250 ng DNA to 50 µl with resuspension buffer and transfer the DNA sample to the Covaris microTUBE or microTUBE plate.
3) Insert the tube or plate on the rack and run the Covaris with the following settings:

   Mode: frequency sweeping
   Duty cycle: 10%
   Intensity: 2
   Cycles per burst: 200
   Time: 45 sec.

4) QC step: remove 1µl of the sample to test fragmentation size on a High Sensitivity DNA Chip on Agilent Bioanalyzer or equivalent instrument. The expected DNA fragment range is 100bp to 1kb with a peak around 600 bp

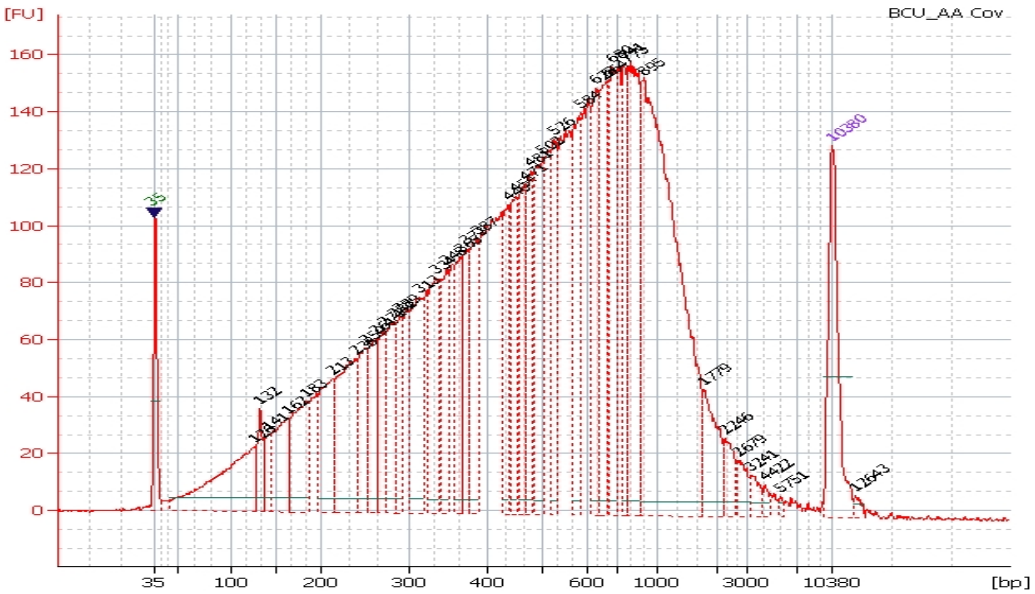


Figure 1: example of DNA size profile after fragmentation using the above Covaris setting

STEP 2: End repair, A tailing and barcoded adaptors ligation

This step is performed with NEBNext End Repair, A tailing and Ligation enzymes (New England Biolabs). End repair enzymes are used to convert DNA that has been damaged or with incompatible 5'-protruding and/or 3'- protruding ends to 5'-phosphorylated blunt–ended DNA. A tailing enzyme adds dAMP to the 3' ends of the fragments. Finally, ligase adds Y-shaped ds DNA adapters with 3'-dTMP overhangs to the A tailed library fragments. Adapters are Illumina-compatible and contain a unique 6-bases barcode which allow pooling multiple libraries preparations in a single flow cell lane.

Important considerations specific to this step

- Reactions setup
  - This protocol is intended for low-throughput, manual library construction, but it is designed to be automation-friendly, easing the transition to automation if needed by throughput requirements. For this reason, reaction components may be combined into master mixes, rather than being dispensed separately into individual tubes. The reactions cleanup is based on a cost-efficient "on-beads" strategy which facilitates automation on a liquid handling system.
  - Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you might not have enough reagents for the full number of reactions over multiple uses.
  - When processing multiple samples, prepare 5 – 10% excess of each master mix, to allow for small inaccuracies during dispensing.
  - Libraries may be prepared in any standard reaction vessels including 1.5 ml microtubes, PCR tubes, strip tubes, or PCR plates. When selecting the most appropriate plasticware for your workflow, consider the following points:
    • Compatibility with the magnetic stand used to collect AMPure XP beads.
    • Compatibility with vortex mixers and centrifuges, where appropriate.
    • Compatibility with thermocyclers or heating blocks used for reaction incubations

- Safe stopping points
  If necessary, the protocol may be paused safely for up to 2 hours after any of the bead cleanup steps by storing the mixture DNA/beads at 4°C.

- Paramagnetic SPRI beads and reaction cleanups
  - Cleanups should be performed timeously to ensure that enzyme reactions do not proceed beyond optimal incubation times.
  - Prior to use, allow the beads to come to room temperature.
  - Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.

- This protocol has been validated using Agencourt AMPure XP beads (Beckman Coulter). Solutions and conditions for DNA binding may differ if other beads are used.
- Observe all manufacturer's storage and handling recommendations.
- Beads will gradually settle: ensure that they are fully resuspended at the time of pipetting. The incubation times provided in the protocol for reaction cleanups should eventually be modified/adapted according to your current protocols, previous experience, and specific equipment in order to maximize library construction efficiency and throughput.
- The time required to completely capture magnetic beads varies according to the plates and magnet used. It is important that no beads are inadvertently discarded with the removal of the supernatant. Capture times should be optimized accordingly.
- Prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore fresh 80% ethanol should be prepared for optimal results.
- The volumes of 80% ethanol used for the bead wash steps may be adjusted to accommodate smaller tubes and/or limited pipetting capacity, but it is important that the beads are entirely submerged during the wash steps.
- It is important that all the ethanol is removed before proceeding with subsequent reactions, but over-drying the beads may make them difficult to resuspend, and may result in dramatic yield loss.
- Drying of beads at 37 °C is not recommended.
- The protocol provides for 1.8X SPRI cleanups after end repair and A-tailing. This ratio of PEG/NaCl to sample volume will retain all DNA fragments larger than ~75 bp.

o Adapter design and concentration

- This protocol has been validated using standard, indexed Y-adapters provided by Bioo Scientific, but the protocol is compatible with other similar adapter strategies.
- While it is not necessary to adjust adapter concentrations to accommodate moderate sample-to-sample variations, we recommend using an adapter concentration that is appropriate for the expected range of input DNA amounts.
- We have validated our protocol using an adapter: insert molar ratio of approximately 2 : 1. For libraries from 250 ng DNA input and with average insert sizes of ~500 bp, this ratio corresponds to 150 nM concentration of adapters in the final ligation reaction. However, depending on the adapter source and /or starting material, the adapters may have to be titrated relative to starting

material. Excess adapters concentration affects ligation efficiency as well as adapter and adapter-dimer carry-over in post-ligation cleanup and can interfere with sequencing.

1) End repair reaction setup

1.1) Combine and mix the following components in a LoBind tube

Fragmented DNA	50 µl
NEBNext End Repair Reaction Buffer (10X)	6 µl
NEBNext End Repair Enzyme Mix	1.25 µl
Resuspension buffer	2.75 µl
Total volume	**60 µl**

1.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
1.3) Incubate for 30 minutes at 20 °C.

2) End repair reaction cleanup

2.1) To each 60 µl end repair reaction, add 108 µl AMPure XP beads.
2.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
2.3) Incubate the plate/tube at room temperature for 5 min to allow the DNA to bind to the beads.
2.4) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
2.5) Remove and discard the supernatant.
2.6) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
2.7) Incubate the plate/tube at room temperature for≥30 sec.
2.8) Remove and discard the ethanol.
2.9) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
2.10) Incubate the plate at room temperature for ≥30 sec.
2.11) Remove and discard the ethanol.

2.12) Allow the beads to dry 5-15 minutes, sufficiently for all the ethanol to evaporate.
Caution: over-drying the beads may result in dramatic yield loss.
2.13) Resuspend the beads in 21.75 µl resuspension buffer by pipetting up and down multiple times to elute DNA.
2.14) Proceed to step 3. The mixture DNA/beads can be kept at 4°C for to 2 hours maximum.

3) A-tailing reaction setup

3.1) To each well/tube containing the mixture beads/DNA, add:

NEBNext dA-Tailing Reaction Buffer (10X)	2.5 µl
Klenow exo$^-$	0.75 µl
Total volume	**25 µl**

3.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
3.3) Incubate for 30 minutes at 37 °C.

4) A-tailing reaction cleanup

4.1) To each well/tube containing the 25 µl A-tailing reaction with beads, add 45 µl PEG/NaCl solution.
4.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
4.3) Incubate the plate/tube at room temperature for 5 min to allow the DNA to bind to the beads.
4.4) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
4.5) Remove and discard the supernatant.
4.6) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
4.7) Incubate the plate/tube at room temperature for≥30 sec.
4.8) Remove and discard the ethanol.

4.9) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
4.10) Incubate the plate at room temperature for ≥30 sec.
4.11) Remove and discard the ethanol.
4.12) Allow the beads to dry 5-15 minutes, sufficiently for all the ethanol to evaporate.
Caution: over-drying the beads may result in dramatic yield loss.
4.13) Resuspend the beads in 17.75 µl resuspension buffer by pipetting up and down multiple times to elute DNA.
4.14) Proceed to step 5. The mixture DNA/beads can be kept at 4°C for to 2 hours maximum

5) Adapter ligation reaction setup

5.1) Depending on the initial adapter concentration, dilute adapter stock solution with resuspension buffer in order to adjust for the initial quantity of DNA.
5.2) To each well/tube containing the beads and A-tailed DNA, add:

Quick Ligation Reaction Buffer (5X)	5 µl
Diluted adapter (final concentration 150nM)	1 µl
Quick T4 DNA Ligase	1.25 µl
Total volume	**25 µl**

5.3) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
5.4) Incubate for 30 minutes at 25 °C.

6) Two rounds adapter ligation reaction cleanups

6.1) To each well/tube containing the 25 µl ligation reaction with beads, add 25 µl PEG/NaCl solution.

6.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
6.3) Incubate the plate/tube at room temperature for 5 min to allow the DNA to bind to the beads.
6.4) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
6.5) Remove and discard the supernatant.
6.6) Resuspend the beads in 50 µl resuspension buffer by pipetting up and down multiple times to elute DNA.
6.7) Incubate the plate/tube at room temperature for 2 min.
6.8) Add 50 µl PEG/NaCl solution.
6.9) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
6.10) Incubate the plate/tube at room temperature for 5 min to allow the DNA to bind to the beads.
6.11) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
6.12) Remove and discard the supernatant.
4.13) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
6.14) Incubate the plate/tube at room temperature for≥30 sec.
6.15) Remove and discard the ethanol.
6.16) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
6.17) Incubate the plate at room temperature for ≥30 sec.
6.18) Remove and discard the ethanol.
6.19) Allow the beads to dry 5-15 minutes, sufficiently for all the ethanol to evaporate.
Caution: over-drying the beads may result in dramatic yield loss.
6.20) Resuspend the beads in 40 µl resuspension buffer by pipetting up and down multiple times to elute DNA.
6.21) Incubate the plate/tube at room temperature for 2 min
6.22) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
6.23) Transfer the clear supernatant to a new plate/tube.
6.24) Proceed with library amplification or store DNA to -20°C for 7 days maximum.

STEP 3: Library amplification

This step allows to amplify DNA containing adapters in order to obtain sufficient quantity for sequencing reactions.

Important considerations specific to this step

- The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.
  Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:
  - Physically separate laboratory space where pre-PCR processes are performed from the laboratory space where PCR products are made and processed during the post-PCR processes.
  - Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes.
  - We recommend to perform PCR reaction cleanup under a PCR hood and to clean the area after each cleanup using DNA decontamination solutions (i.e. DNAaway). To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before you begin any processes.
- Reactions setup
  - This protocol has been validated with Kapa Hifi Hotstart NGS library amplification kit but other high fidelity Taq polymerases can be used. The volume of purified ligation used for PCR corresponds to about 10 ng DNA and the cycling parameters have been optimized in order that the final library yields ranges between 0.5 and 1 µg amplified DNA sample.
- Excessive library amplification can result in unwanted artefacts such as PCR duplicates, chimeric library inserts, amplification bias, etc. Especially if using other enzymes or other DNA samples types, we recommend that PCR cycling and DNA template volume should be adjusted to produce ~0.5 – 1.5 µg purified PCR product. It is generally best to limit the extent of library amplification as far as possible, while ensuring that sufficient material is generated for QC and sequencing.
- The protocol provides for 0.6x SPRI cleanup after PCR. This ratio of beads to sample volume will retain DNA fragments larger than ~300 bp. If retention of

smaller fragments is needed, AMPure volume has to be adjusted. However, the ratio should not be > 1x in order to allow for efficient removal of adapters dimers eventually produced during PCR amplification..

- Use the following primers sequence for PCR:
  Primer P5
  5' AATGATACGGCGACCACCGAG

  Primer P7
  5'CAAGCAGAAGACGGCATACGAG

1) PCR reaction setup and running

1.1) Combine and mix the following components in each well/tube:

Ligated DNA (~ 10 ng)	4 µl
KAPA HiFi HotStart Ready Mix (2x)	25 µl
P5 primer 50 µM	1 µl
P7 primer 50 µM	1 µl
H_2O	19 µl
Total volume	50 µl

1.2) Amplify using the following PCR cycling conditions:

45sec at 98 °C
[15 sec at 98 °C, 30 sec at 60 °C, 30 sec at 72 °C] 12 cycles total
1 min at 72 °C
Hold at 4 °C

2) PCR reaction cleanup

2.1) To each well/tube containing the 50 µl PCR reaction, add 30 µl AMPure beads solution.
2.2) Mix thoroughly by pipetting up and down multiple times and/or by vortexing.
2.3) Incubate the plate/tube at room temperature for 5 min to allow the DNA to bind to the beads.
2.4) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
2.5) Remove and discard the supernatant.
2.6) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
2.7) Incubate the plate/tube at room temperature for≥30 sec.
2.8) Remove and discard the ethanol.
2.9) Keeping the plate/tube on the magnet, add 200 µl of 80% ethanol.
2.10) Incubate the plate at room temperature for ≥30 sec.
2.11) Remove and discard the ethanol.
2.12) Allow the beads to dry 5-15 minutes, sufficiently for all the ethanol to evaporate.
Caution: over-drying the beads may result in dramatic yield loss.
2.13) Resuspend the beads in 25 µl resuspension buffer by pipetting up and down multiple times to elute DNA.
2.14) Incubate the plate/tube at room temperature for 2 min
2.15) Place the plate/tube on a magnet to capture the beads at least 2 minutes or until the liquid is clear.
2.16) Transfer the clear supernatant to a new plate/tube and proceed to library QC.

STEP 4: Quantitative and qualitative assessment of the library

Important considerations specific to this step

- In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. This requires accurate quantitation of DNA library templates. It is possible to use the Bioanalyzer quantification as a rough quantitation of libraries, however for more accurate results, we recommend quantifying your library using qPCR according to the *Illumina Sequencing Library qPCR Quantification Guide*. We use the KAPA Library Quantification Kit for Illumina libraries but other kits can be used instead.

- If needed by throughput requirements, various steps in this protocol can be implemented in order to optimize throughput:
  - Qubit quantification can be replaced by PicoGreen method on 96-wells plates.
  - Library profile analysis can be performed by 96-well compatible automated capillary electrophoresis systems (various instruments are commercially available).
  - PCR reagents mix preparation and aliquoting can be performed by a high throughput liquid handling system.

1) Quality control

1.1) Measure the concentration of the library by using 1 µl on a Qubit fluorometer with the dsDNA HS Assay kit.

1.2) Load 1 ng of sample diluted in 1µl volume on a High Sensitivity DNA LabChip and run on a Bioanalyzer instrument. The expected library size range is 300 bp to 1000 bp.

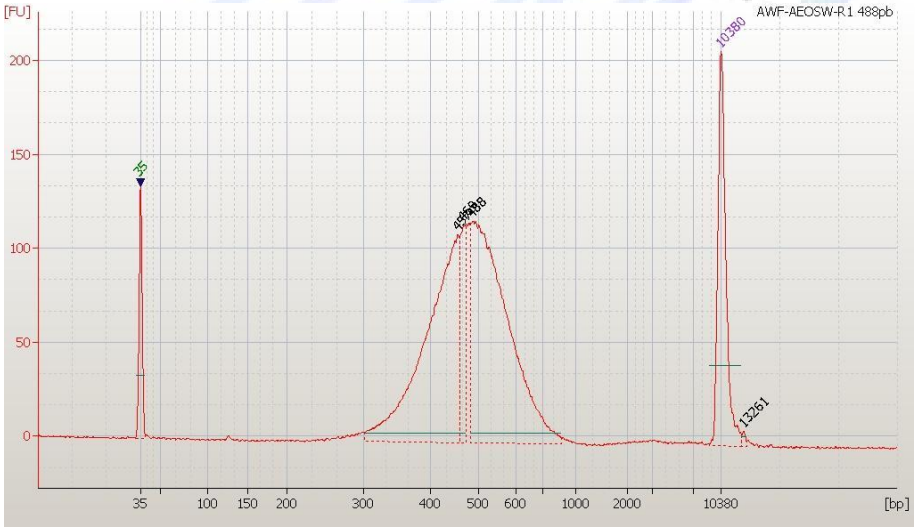


Figure 2. Example of library profile. Library size range varies between 300 and 800 bp. However, as the adapters size is 120 bp, the DNA fragments size is 180-680 bp.

2) Library quantification

2.1) Quantify libraries by qPCR according to the *Illumina qPCR Quantification Protocol Guide*.
2.2) Calculate concentration of library on the basis of qPCR results.
2.3) Normalize the libraries to 10 nM by diluting with resuspension buffer.

3) Pool libraries and sequencing

In this step libraries are pooled and stock solutions are prepared. . According to qPCR quantitation, 8 libraries are pooled equimolarly. Libraries are then sequenced on an HiSeq 2000 instrument in a paired end mode with 100 bp length.

3.1) Combine 10 µl each library to be pooled into a 1.5 ml tube.
3.2) Vortex briefly to mix and spin to collect solution at bottom of tube
3.3) Proceed to cluster generation and sequencing according to the following Illumina User guides:

- For cluster generation: Cbot User Guide (Part # 15006165)
- For sequencing: HiSeq2000 System User Guide (Part # 15011190)

9. Contacts:

If you have any question regarding this SOP please contact us at

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