Library Preparation for Illumina Sequencing

This protocol mainly uses the KAPA Hyper Prep kit to prepare libraries for the Illumina platform. Please look at the manufacturer's instructions and product information for more details.

The following kits are required:

i) KAPA Hyper Prep Kit (ROCHE, cat# KK8500, KK8502, KK8504)
ii) KAPA HiFi HotStart ReadyMix (ROCHE, cat# KK2601)
iii) KAPA Library Quantification Kit (ROCHE, cat# KK4854)
iv) AMPure XP beads (Beckman, cat# A63881)
v) Fragment Analyzer kit DNF-474-0500
vi) Qubit™ dsDNA HS (ThermoFisher, cat# Q32854)

I. End Repair and A-tailing

1) Assemble each end repair and A-tailing reaction in a tube or well of a PCR plate as follows:

End Repair & A-Tailing Mix	
Reagent	**Volume (µL)**
Fragmented, double-stranded DNA	50
End Repair & A-Tailing Buffer	7
End Repair & A-Tailing Enzyme Mix	3
TOTAL	**60**

2) Vortex gently and spin down briefly.
3) Incubate in a thermocycler programmed as outlined below:

Thermocycler Conditions		
Stage 1	20	30:00
	65	30:00
	4	∞

- ❖ A heated lid is required for this incubation. If possible, set the temperature of the lid at 85°C.
- ❖ If proceeding directly to the adapter ligation reaction setup, the reaction may be cooled to 20°C instead of 4°C.

4) Proceed to adaptor ligation

II. Adapter Ligation

Recommended Adaptor Concentrations for Libraries		
Fragmented DNA per 50 µL ER & AT reaction	Adapter stock concentration	Adapter:insert molar ratio
1 µg	15 µM	10:1
500 ng	15 µM	20:1
250 ng	15 µM	40:1
100 ng	15 µM	100:1
50 ng	15 µM	200:1
25 ng	7.5 µM	200:1
10 ng	3 µM	200:1
5 ng	1.5 µM	200:1
2.5 ng	750 nM	200:1
1 ng	300 nM	200:1

1) Dilute adapter stocks to the appropriate concentration (listed above).
2) In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

Ligation Mix	
Reagent	**Volume (µL)**
End repair and A-tailing reaction	60
Adaptor Stock	5
H2O	5
Ligation Buffer	30
DNA Ligase	10
TOTAL	**110**

3) Mix thoroughly and centrifuge briefly.
4) Incubate at 20°C for 15 min.
   - ❖ to achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time—to a maximum of 4 hrs at 20°C, or overnight at 2°C to 8°C. Please note that longer ligation times may lead to increased levels of adapter-dimer.

III. Post-ligation Cleanup

1) In the same plate/tube(s), perform a 0.8X bead based cleanup by adding 88 µL AMPure beads to your 110 µL ligation reaction (total vol = 198 µL)
2) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
4) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5) Carefully remove and discard the supernatant.
6) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
7) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
8) Carefully remove and discard the ethanol.
9) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
10) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
11) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
13) Remove the plate/tube(s) from the magnet.
14) Thoroughly resuspend the beads in 25 µL of elution buffer or 10 mM Tris-HCl, pH 8.0
15) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
16) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
17) Transfer the clear supernatant to a new plate/tube(s)

IV. Cycle Determination for Library Amplification

1) Dilute each library to 1:10, 1:100, and 1:1000 in water or dilution buffer
2) Make enough qPCR master mix for each sample at 3 different concentrations in triplicate and 6 standards in triplicate (#samples x 3 x 3 + 6 standards x 3):

KAPA qPCR Mix	
Reagent	**Volume (µL)**
KAPA Sybr Fast Mix (2X)	5
KAPA Library Amplification Primer Mix (10X)	1
H2O	2
diluted Adapter-ligated library	2
TOTAL	**10**

3) Place 8 µL of master mix into each well of a 384 well qPCR plate
4) Add in 2 µL of standard or diluted sample in each well
5) Cover the top securely with clear film
6) Spin down in a centrifuge
7) Run on the Roche Lightcycler 480 with the following program

Program Name	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
Pre-Incubation	1	None	95	None	00:05:00	4.8	---	0	0	0
Amplification	35	Quantification	95	None	00:00:30	4.8	---	0	0	0
			60	Single	00:00:45	2.5	---	0	0	0
Melting Curve	1	Melting Curves	95	None	00:00:05	4.8	---	---	---	---
			65	None	00:01:00	2.5	---	---	---	---
			95	Continuous	---	0.11	5	---	---	---
Cooling	1	None	40	None	00:00:30	2.5	---	0	0	0

8) Use Ct values to calculate optimal number of cycles

V. Library Amplification

1) Assemble each library amplification reaction as follows:

PCR Mix	
Reagent	**Volume (µL)**
KAPA HiFi HotStart ReadyMix (2X)	25
KAPA Library Amplification Primer Mix (10X)	5
Adapter-ligated library	20
TOTAL	**50**

2) Mix thoroughly and centrifuge briefly.
3) Amplify using the following cycling protocol:

Thermocycler Conditions			
Stage 1	98	00:45	
Stage 2	98	00:15	Calculated # of cycles
	60	00:30	
	72	00:30	
Stage 3	72	01:00	
	4	∞	

4) Clean up PCR samples using beads
   - ❖ Can do a single or double size selection or both

VI. Post-amplification Cleanup: Single Size Selection

1) Add 50 µL H2O to each of your samples for a total vol of 100 µL
2) In the same plate/tube(s), perform a 0.8X bead based cleanup by adding 80 µL AMPure beads to your 100 µL PCR reaction (total vol = 180 µL)
3) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
4) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
5) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6) Carefully remove and discard the supernatant.
7) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
8) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
9) Carefully remove and discard the ethanol.
10) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
11) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
13) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
14) Remove the plate/tube(s) from the magnet.
15) Thoroughly resuspend the beads in 25 µL of elution buffer or 10 mM Tris-HCl, pH 8.0
16) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
17) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
18) Transfer the clear supernatant to a new plate/tube(s)

VII. Post-amplification Cleanup: Double Size Selection

1) Add 50 µL H2O to each of your samples for a total vol of 100 µL
2) In the same plate/tube(s), perform a 0.7X bead based cleanup by adding 70 µL AMPure beads to your 100 µL PCR reaction (total vol = 170 µL)
3) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
4) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
5) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6) Carefully remove and transfer the supernatant to a new PCR tube.
7) To the supernatant, add 0.2x AMPure beads (20 µL) to your 170 µL PCR reaction (total vol = 190 µL)
8) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
9) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
10) Carefully remove and discard the supernatant.
11) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
12) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
13) Carefully remove and discard the ethanol.
14) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
15) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
16) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
17) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
18) Remove the plate/tube(s) from the magnet.
19) Thoroughly resuspend the beads in 25 µL of elution buffer or 10 mM Tris-HCl, pH 8.0
20) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
21) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
22) Transfer the clear supernatant to a new plate/tube(s)

VIII. Library Quantification: Qubit

1) Mix enough components A and B (1:200) together for all your samples and 2 standards
2) For standards, add 190 µL mix with 10 µL of standard in a Qubit tube
3) For samples, add 199 µL mix with 1 µL sample in a Qubit tube
4) Vortex to mix and incubate in the dark at room temp for 2min
5) Quantify using Qubit machine

IX. Average Size Determination: Fragment Analyzer

1) From the 5200 Fragment Analyzer DNF-474 High Sensitivity NGS 1-6000bp kit, mix gel and intercalating dye together (1 µL dye to 10 mL gel)
2) Pipette 22 µL diluent marker to the wells of the 96 well plate that will contain samples or ladder
    a. FA runs row by row
    b. The 12^{th} well always contains the ladder
    c. Do not go over 5ng of sample
3) Add in 2 µL of sample or ladder
4) Add Blank Solution to the unused wells
5) Cover the row securely with a strip of clear film
6) Spin down in a centrifuge
7) Take gel and sample plate and run on FA

X. Library Quantification: qPCR

1) Dilute each library to 1:1000, 1:10,000, and 1:100,000 in water or dilution buffer
2) Make enough qPCR master mix for each sample at 3 different concentrations in triplicate and 6 standards in triplicate (#samples x 3 x 3 + 6 standards x 3):

KAPA qPCR Mix	
Reagent	**Volume (µL)**
KAPA HiFi HotStart ReadyMix (2X)	25
KAPA Library Amplification Primer Mix (10X)	5
Adapter-ligated library	20
TOTAL	**50**

3) Place 8 µL of master mix into each well of a 384 well qPCR plate
4) Add in 2 µL of standard or diluted sample in each well
5) Cover the top securely with clear film
6) Spin down in a centrifuge
7) Run on the Roche Lightcycler 480 with the following program

Program Name	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
Pre-Incubation	1	None	95	None	00:05:00	4.8	---	0	0	0
Amplification	35	Quantification	95	None	00:00:30	4.8	---	0	0	0
			60	Single	00:00:45	2.5	---	0	0	0
Melting Curve	1	Melting Curves	95	None	00:00:05	4.8	---	---	---	---
			65	None	00:01:00	2.5	---	---	---	---
			95	Continuous	---	0.11	5	---	---	---
Cooling	1	None	40	None	00:00:30	2.5	---	0	0	0

8) Use Ct values to determine sample concentration
9) Pool together samples that need to be sequenced
10) Qubit (optional) and qPCR to quantify the pooled library