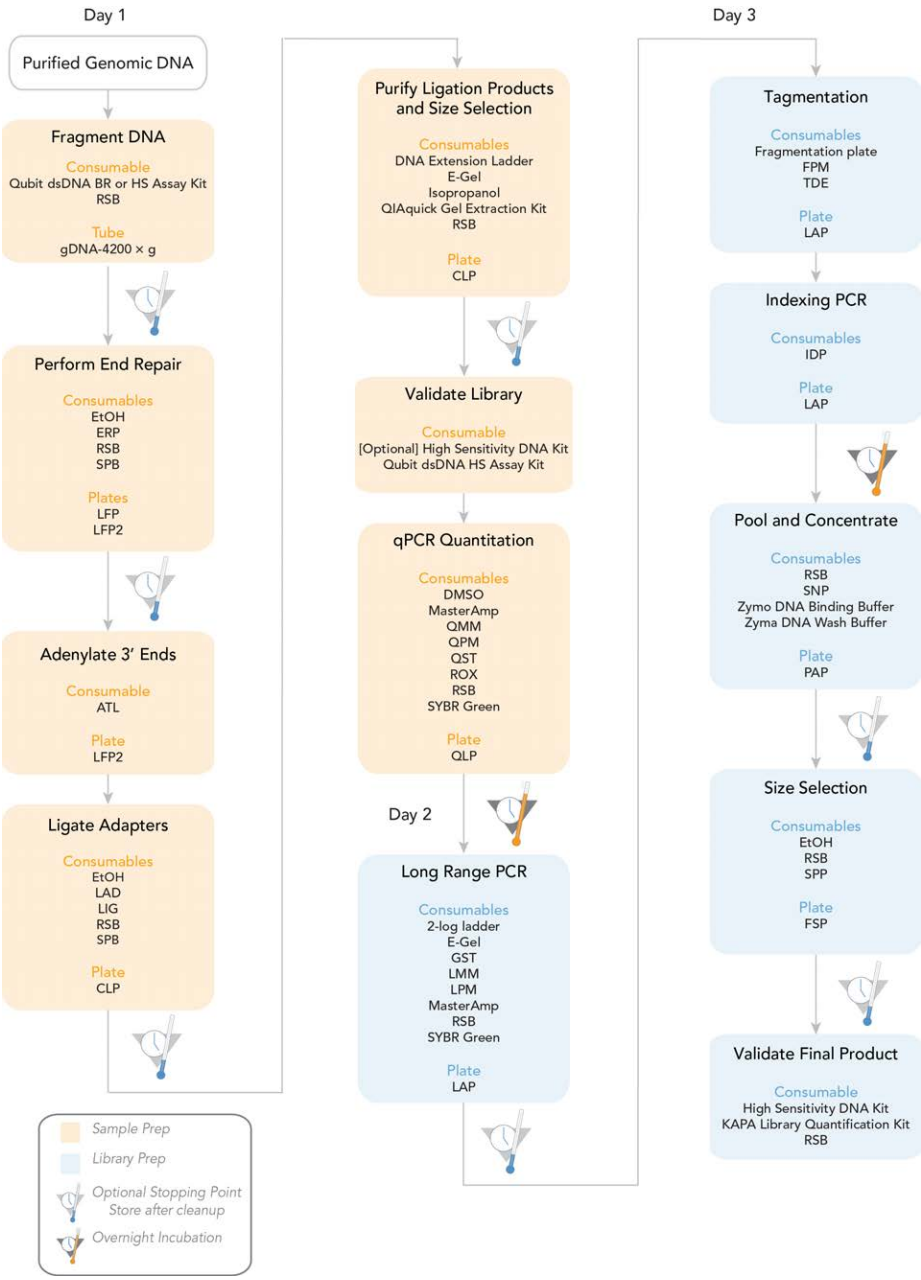
TruSeq Synthetic Long-Read DNA Library Prep

Experienced User Card and Lab Tracking Form

FOR RESEARCH USE ONLY

Date: _____ Description: _____

Illumina Kit Lot #: _____ _____



NOTE

New or less experienced users are advised to follow the protocol in the *TruSeq Synthetic Long-Read DNA Library Prep Guide (part # 15047264)* before using this document.

Consumables

Library Prep Kit Item	Lot Number
A-Tailing Mix (ATL)	Lot #: _____
End Repair Mix (ERP)	Lot #: _____
Ligation Mix (LIG)	Lot #: _____
Long Fragment Adapter (LAD)	Lot #: _____
MasterAmp Extra-Long DNA Polymerase Mix	Lot #: _____
qPCR Long-amp Primer Mix (QPM)	Lot #: _____
qPCR Master Mix (QMM)	Lot #: _____
qPCR Standard (QST)	Lot #: _____
Resuspension Buffer (RSB)	Lot #: _____
Sample Purification Beads (SPB)	Lot #: _____

Barcode Kit Item	Lot Number
Fragmentation Pre-Mix (FPM)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Gel Standard (GST)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Indexing Plate (IDP)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Long-amp Master Mix (LMM)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____

Barcode Kit Item	Lot Number
Long-amp Primer Mix (LPM)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
MasterAmp Extra-Long DNA Polymerase Mix	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Resuspension Buffer (RSB)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Sample Neutralization Buffer (SNB)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Sample Purification Beads (SPB)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____
Tagment DNA Enzyme (TDE)	Lot #: _____
	Lot #: _____
	Lot #: _____
	Lot #: _____

Item	Date Prepared
80% Ethanol	Date Prepared: _____

Fragment DNA

This process describes how to optimally fragment the gDNA using a g-TUBE for phasing and long-read workflows.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C (2°C to 8°C after initial thaw)	Illumina
gDNA samples	500 ng at 10 ng/µl per sample	-25°C to -15°C	User
g-TUBE	1	15°C to 30°C	User
Microcentrifuge tubes	2	15°C to 30°C	User
Qubit dsDNA BR or HS assay kit	1	As specified by manufacturer	User

Use IEM to create a sample sheet before beginning library preparation.

Sample Sheet Name: _____

Procedure

[_] 1 Quantify the gDNA sample using the Qubit dsDNA BR or HS assay kit.

[_] 2 Normalize the gDNA sample with Resuspension Buffer to a final volume of 50 µl at 10 ng/µl in a new microcentrifuge tube.

[_] 3 Transfer 50 µl normalized gDNA to a g-TUBE.

[_] 4 Centrifuge the g-TUBE, with the blue cap up, to 4200 × g for 1 minute with a balance.

[_] 5 Flip the g-TUBE over, so that the blue cap is down, and centrifuge the tube one more time to 4200 × g for 1 minute with a balance.

[_] 6 Immediately remove the g-TUBE from the centrifuge.

[_] 7 Use a g-TUBE cap holder to transfer all of the fragmented DNA from the blue cap to the microcentrifuge tube labeled **gDNA-4200 × g** along with the experiment date.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Perform End Repair* on page 7, you can safely stop the protocol here. If you are stopping, store the **gDNA-4200 × g** tube at 2°C to 8°C for up to 30 days.

Comments

_____

_____

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix (ERP)	1 tube per 4 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 4 reactions	2°C to 8°C	Illumina
Barcode labels for: • LFP (Long Fragment Plate) • LFP2 (Long Fragment Plate 2)	1 label per plate	15°C to 30°C	Illumina
96-well PCR plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User

Make LFP

[_] 1 Centrifuge the thawed End Repair Mix tube at 600 × g for 5 seconds.

[_] 2 Add 30 µl fragmented DNA sample from each **gDNA-4200 × g** tube to a separate well of the new PCR plate labeled with the LFP barcode.

[_] 3 Add 20 µl End Repair Mix to each sample well of the plate. Set a 200 µl pipette to 40 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 4 Seal the plate with a Microseal 'B' adhesive seal, then centrifuge the plate at 280 × g for 1 minute.

[_] 5 Return the End Repair Mix tube to -25°C to -15°C storage.

Incubate LFP

[_] 1 Place the sealed plate on the pre-programmed thermal cycler. Close the lid then select and run the **ERP** program.

- [_] a Choose the thermal cycler pre-heat lid option and set to 100°C
- [_] b 30°C for 30 minutes
- [_] c Hold at 4°C

[_] 2 Remove the plate from the thermal cycler when the program reaches 4°C.

[_] 3 Centrifuge the plate at 280 × g for 1 minute.

Clean Up LFP

[_] 1 Remove the adhesive seal from the plate.

[_] 2 Vortex the Sample Purification Beads until they are well dispersed.

[_] 3 Add 80 µl well-mixed Sample Purification Beads to each well of the plate containing 50 µl of the end repaired sample. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 4 Incubate the plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 5 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 6 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of supernatant from each well of the plate.

[_] 7 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 8 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 9 Repeat steps 7 and 8 one time for a total of two 80% EtOH washes.

[_] 10 Remove and discard any remaining EtOH from each well of the plate with a 10 µl pipette.

[_] 11 Let the plate stand at room temperature for 5 minutes to dry, and then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 12 Add 20 µl Resuspension Buffer to each well of the plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Start time: _____ Stop time: _____

[_] 13 Incubate the plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 14 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 15 Transfer 17.5 µl of supernatant from each well of the LFP plate to the corresponding well of the new PCR plate labeled with the LFP2 plate barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 9, you can safely stop the protocol here. If you are stopping, seal the LFP2 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

_____

_____

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	1 tube per 4 reactions	-25°C to -15°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seal	1	15° to 30°C	User

Add ATL

[_] 1 Centrifuge the thawed A-Tailing Mix tube at 600 × g for 5 seconds.

[_] 2 Add 12.5 µl thawed A-Tailing Mix to each well of the LFP2 plate. Set a 20 µl pipette to 20 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 3 Seal the plate with a Microseal 'B' adhesive seal.

[_] 4 Return the A-Tailing Mix tube to -25°C to -15°C storage.

Incubate 1 LFP2

[_] 1 Centrifuge the plate at 280 × g for 1 minute.

[_] 2 Place the sealed plate, containing 30 µl of each sample, on the pre-programmed thermal cycler. Close the lid, then select and run the **ATAIL** program.

- [_] a Choose the pre-heat lid option and set to 100°C
- [_] b 37°C for 30 minutes
- [_] c Hold at 4°C

[_] 3 When the thermal cycler temperature is 4°C, remove the LFP2 plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 11.

Comments

_____

_____

Ligate Adapters

This process ligates adapters to the ends of the long DNA fragments. These adapters are used as markers in downstream data analysis processes, to denote the end of a contig.

Consumables

Item	Quantity	Storage	Supplied By
Ligation Mix (LIG)	1 tube per 4 reactions	-25°C to -15°C	Illumina
Long Fragment Adapters (LAD)	1 tube per 4 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 4 reactions	2°C to 8°C	Illumina
CLP (Cleaned Long Fragment Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well PCR plate	1	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User

Add LIG

[_] 1 Centrifuge the Long Fragment Adapters tube at 600 × g for 5 seconds.

[_] 2 Immediately before use, remove the Ligation Mix tube from -25°C to -15°C storage.

[_] 3 Centrifuge the LFP2 plate at 280 × g for 1 minute.

[_] 4 Remove the adhesive seal from the plate.

[_] 5 Add 5 µl Long Fragment Adapters to each sample well of the plate.

[_] 6 Add 2.5 µl Ligation Mix to each sample well of the plate. Set a 200 µl pipette to 30 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 7 Return the Ligation Mix tube back to -25°C to -15°C storage immediately after use.

[_] 8 Seal the plate with a Microseal 'B' adhesive seal, then centrifuge the plate at 280 × g for 1 minute.

Incubate 2 LFP2

[_] 1 Place the sealed plate, containing 37.5 µl of each sample, on the pre-programmed thermal cycler. Close the lid then select and run the **LIG** program.

- [_] a Choose the thermal cycler pre-heat lid option and set to 100°C
- [_] b 30°C for 10 minutes
- [_] c Hold at 4°C

[_] 2 Remove the plate from the thermal cycler when the program reaches 4°C.

[_] 3 Centrifuge the plate at 280 × g for 1 minute.

Clean Up LFP2

[_] 1 Remove the adhesive seal from the plate.

[_] 2 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

[_] 3 Add 37.5 µl well-mixed Sample Purification Beads to each sample well of the plate. Set a 200 µl pipette to 65 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 4 Incubate the plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 5 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 6 Remove and discard 70 µl of the supernatant from each well of the plate.

[_] 7 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each sample well without disturbing the beads.

[_] 8 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 9 Repeat steps 7 and 8 one time for a total of two 80% EtOH washes.

[_] 10 Remove and discard any remaining EtOH from each well of the plate with a 10 µl pipette.

[_] 11 With the plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 12 With the plate on the magnetic stand, add 22.5 µl Resuspension Buffer to each sample well of the plate.

[_] 13 Remove the plate from the magnetic stand.

[_] 14 Resuspend the beads in each well of the plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 15 Incubate the plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 16 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 17 Transfer 20 µl of the supernatant from each well of the LFP2 plate to the corresponding well of the new PCR plate labeled with the CLP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Purify Ligation Products and Size Selection* on page 13, you can safely stop the protocol here. If you are stopping, seal the CLP plate with a Microseal 'B' adhesive seal and store at 2°C to 8°C overnight.

Purify Ligation Products and Size Selection

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, as well as any adapters that might have ligated to one another. Long adapter ligated fragments of DNA of 8–10 kb in size are selected for Long Range PCR and subsequent Tagmentation procedures.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
1 Kb DNA Extension Ladder	0.5 µl per sample	15°C to 30°C	User
2-propanol (Isopropanol)	1 µl × the mg weight of each gel slice	15°C to 30°C	User
E-Gel NGS, 0.8% Agarose	1 per sample	15°C to 30°C	User
Fragmented gDNA-4200 × g	5 µl per sample	15°C to 30°C	User
Lab pen	1	15C° to 30°C	User
Microcentrifuge tubes	2 per sample + 2	15°C to 30°C	User
QIAquick Gel Extraction Kit	1	15°C to 30°C	User
Ruler	1	15C° to 30°C	User
X-tracta Gel Extraction Tool	1 per sample	15C° to 30°C	User

Size Separate

[_] 1 Place one E-Gel NGS, 0.8% Agarose per sample into an E-Gel iBase Power System according to manufacturer instructions.

[_] 2 Add 0.5 µl 1 Kb DNA Extension Ladder to 19.5 µl Resuspension Buffer in a microcentrifuge tube to dilute the DNA ladder. Multiply each reagent volume by the number of gels being prepared. Gently pipette the entire volume up and down 6-8 times to mix thoroughly.

[_] 3 Add 5 µl of 10 ng/µl fragmented DNA sample from the tube labeled **gDNA-4200 × g** to 15 µl Resuspension Buffer in a microcentrifuge tube to dilute the fragmented gDNA. Multiply each reagent volume by the number of gels being prepared. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

[_] 4 Centrifuge the diluted fragmented gDNA tube at 280 × g for 1 minute.

[_] 5 Load 20 µl sample from one well of the CLP plate into lane 4 of one gel.

[_] 6 Repeat step 5 for each sample, loading a single sample into lane 4 of each gel.

[_] 7 Load 20 µl diluted 1 Kb DNA Extension Ladder into lane 6 of each gel.

[_] 8 Load 20 µl diluted fragmented gDNA sample into lane 8 of each gel.

[_] 9 Load each empty well of each gel with 20 µl Resuspension Buffer.

[_] 10 On the E-Gel iBase Power System, select and run the **E-Gel 0.8-2%** program. Set the run time to 26 minutes.

[_] 11 View the gel on a Dark Reader transilluminator.

[_] 12 Use a lab pen and ruler to mark the 8–10 kb region of interest for precise gel excision, as follows:

- [_] a Draw two vertical lines on the plastic gel cassette to mark the left and right side of the sample well.
- [_] b Draw two horizontal lines on the plastic gel cassette to mark the position of the 10 kb and 8 kb bands of the ladder.
- [_] c Repeat step a and b on the other side of plastic cassette, because the gel can stick to either side of cassette when it is opened.

Purify Gel

[_] 1 Carefully open the gel with the Novex Gel Knife.

[_] 2 View the gel on a Dark Reader transilluminator to confirm the correct placement of the lines drawn on the plastic gel cassette at the 8-10 kb region of interest. Carefully adjust the position of the gel if it has shifted relative to the marked region.

[_] 3 Place an x-tracta tool on the gel between the marked region of interest on the plastic gel cassette and press the tool into the gel.

[_] 4 Rock the x-tracta tool side to side to extract the desired gel slice.

[_] 5 Place the x-tracta tool over the appropriately labeled microcentrifuge tube and expel the extracted gel band from the tool into the tube with a quick squeeze.

[_] 6 Weigh the tube containing the gel slice. Subtract the weight of the empty tube to determine weight of the gel slice in milligrams (mg).

Sample	Tube Weight	Empty Tube Weight	Gel Slice Weight (mg)
1			
2			
3			
4			

[_] 7 For each sample, add X µl QIAGEN Buffer QG, with X equaling three times the mg weight of the gel slice, to the tube containing the gel slice.

[_] 8 Place the tube containing the gel and QIAGEN Buffer QG mixture on the pre-heated microheating system or water bath. Close the lid and incubate at 50°C for 10 minutes to melt the gel. Gently flick the tube periodically until the gel is fully melted.

Start time: _____ Stop time: _____

[_] 9 Add 1 µl Isopropanol × the mg weight of the gel slice to the gel and QIAGEN Buffer QG mixture.

[_] 10 Add the dissolved gel, QIAGEN Buffer QG, and Isopropanol mixture to a QIAquick column.

[_] 11 Centrifuge the QIAquick column to 13,000 rpm for 1 minute.

[_] 12 Remove and discard the eluate from the QIAquick column.

[_] 13 Add 750 µl PE buffer (with ethanol added) to the QIAquick column.

[_] 14 Centrifuge the QIAquick column to 13,000 rpm for 1 minute.

[_] 15 Remove and discard the supernatant from the QIAquick column.

[_] 16 Centrifuge the QIAquick column to 13,000 rpm for 1 minute.

[_] 17 Remove and discard the supernatant from the QIAquick column.

[_] 18 Remove the QIAquick column from the collection tube and place it in the new microcentrifuge tube labeled **size-selected** [sample name].

[_] 19 Add 52 µl Resuspension Buffer to the QIAquick column in the microcentrifuge tube.

[_] 20 Incubate the microcentrifuge tube at room temperature for 1 minute.

[_] 21 Centrifuge the QIAquick column in the microcentrifuge tube at 13,000 rpm for 1 minute.

[_] 22 Discard the QIAquick column.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 17, you can safely stop the protocol here. If you are stopping, cap the **size-selected** [sample name] tube and store at 2°C to 8°C for up to 3 months. Avoid a freeze-thaw cycle.

Comments

_____

_____

Validate Library

Perform the following procedures for quality control analysis and quantification of the long DNA fragments.

Quantify Libraries

Quantify 2 µl of the library using the Qubit dsDNA HS Assay Kit. The library should yield > 0.05 ng/µl.

[Optional] Quality Control

To verify the size of your fragments, check the template size distribution.

Run 1 µl of the DNA library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip. The peak partially overlaps with 10 kb upper marker.

Comments

_____

_____

qPCR Quantitation

This process quantifies the long DNA fragments to make sure that the appropriate amount of DNA is used for the Long Range PCR and subsequent Tagmentation procedures.

Consumables

Item	Quantity	Storage	Supplied By
MasterAmp Extra-Long DNA Polymerase Mix	0.4 µl per reaction	-25°C to -15°C	Illumina
qPCR Long-amp Primer Mix (QPM)	2 µl per reaction	-25°C to -15°C	Illumina
qPCR Master Mix (QMM)	11.6 µl per reaction	-25°C to -15°C	Illumina
qPCR Standard (QST)	5 µl per standard curve	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
QLP (Quantification Long Fragment Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
Dimethyl sulfoxide (DMSO)	1 ml	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
PCR-grade water	1 ml	15°C to 30°C	User
RNase/DNase-free eight-tube strip with caps	1	15°C to 30°C	User
Microcentrifuge tubes	1 per sample + 3	15°C to 30°C	User
qPCR plate and seal	1	15°C to 30°C	User
ROX Reference Dye 50x	As specified by manufacturer	-25°C to -15°C	User
SYBR Green 10,000x	5 µl	-25°C to -15°C	User

Prepare SYBR Green

[_] 1 Vortex the thawed SYBR Green 10,000x to mix thoroughly.

[_] 2 Add 5 µl SYBR Green 10,000x and 495 µl of DMSO to a microcentrifuge tube to dilute the SYBR Green to 100x. Vortex the solution to mix thoroughly.

[_] 3 Measure the absorbance of 100x diluted SYBR Green on a NanoDrop instrument. The ideal $Abs_{494\pm3\ nm}$ of 100x SYBR Green stock is 0.5–0.6, which indicates that the concentration is 100x. Adjust the concentration if necessary. For more information, see *Calibrate Diluted SYBR Green* in the *TruSeq Synthetic Long-Read DNA Library Prep Guide (part # 15047264)*.

Dilute qPCR Standard

[_] 1 Using a 200 µl single channel pipette set to 20 µl, gently pipette the qPCR Standard up and down 10 times to mix thoroughly, then centrifuge briefly.

[_] 2 Add Resuspension Buffer to the labeled tubes as follows:

Tube Label	Tube Type	Resuspension Buffer Volume (µl)
QST 1:100 Dilution	Microcentrifuge	495
Std2	Eight-tube strip	45
Std3	Eight-tube strip	45
Std4	Eight-tube strip	45
NTC (no template control)	Eight-tube strip	50
[Sample name] 1:100 Dilution	Microcentrifuge	495

[_] 3 Add 5 µl qPCR Standard to the **QST 1:100 Dilution** tube for a total of 10 pg/µl (10,000 fg/µl). Using a 1000 µl single channel or multichannel pipette, gently pipette the entire volume up and down 6–8 times to mix thoroughly.

[_] 4 Centrifuge the **QST 1:100 Dilution** tube at 600 × g for 5 seconds.

[_] 5 Transfer 50 µl from the **QST 1:100 Dilution** tube to the **Std 1** tube in the eight-tube strip. Change the tip.

[_] 6 Transfer 5 µl from the **Std1** tube to the **Std2** tube for a total of 1 pg/µl (1000 fg/µl). Using a 200 µl single channel pipette set to 45 µl, gently pipette the entire volume up and down 6–8 times to mix thoroughly. Change the tip.

[_] 7 Transfer 5 µl from the **Std2** tube to the **Std3** tube for a total for a total of 0.1 pg/µl (100 fg/µl). Using a 200 µl single channel pipette set to 45 µl, gently pipette the entire volume up and down 6–8 times to mix thoroughly. Change the tip.

[_] 8 Transfer 5 µl from the **Std3** tube to the **Std4** tube for a total of 0.01 pg/µl (10 fg/µl). Using a 200 µl single channel pipette set to 45 µl, gently pipette the entire volume up and down 6–8 times to mix thoroughly. Discard the tip.

[_] 9 Cap the eight-tube strip that contains the serial diluted qPCR Standard, then centrifuge briefly. This serves as the qPCR standard in the *Long Range PCR* procedure.

Dilute Sample

[_] 1 Add 5 µl size-selected DNA from the microcentrifuge tube from step 21 of *Purify Gel* on page 14 to each tube labeled with the [sample name] **1:100 Dilution**. Using a 1000 µl single channel or multichannel pipette, gently pipette the entire volume up and down 6–8 times to mix thoroughly.

[_] 2 Cap and store the **size-selected** [sample name] tubes at 2°C to 8°C for up to 90 days.

Prepare Master Mix

[_] 1 Prepare a fresh dilution of 1.5x SYBR Green from 100x SYBR Green stock (3 µl 100x SYBR Green in 197 µl PCR-grade water) to create a dye mix. If ROX is required for your qPCR instrument, dilute SYBR Green and ROX dye together to make a 1.5x SYBR Green/10x ROX dye mixture.

[_] 2 Set up a master mix in a sterile, nuclease-free microcentrifuge tube on ice using the following. Using a 1000 µl single channel or multichannel pipette, gently pipette the entire volume up and down 6–8 times to mix thoroughly.

Reagent	1 Sample	2 Samples	3 Samples	4 Samples
qPCR Master Mix	255 µl	302 µl	336 µl	394 µl
qPCR Long-amp Primer Mix	44 µl	52 µl	58 µl	68 µl
Dye Mix (1.5x SYBR Green with optional 10x ROX)	44 µl	52 µl	58 µl	68 µl
MasterAmp Extra-long DNA Polymerase Mix	9 µl	10.5 µl	11.5 µl	13.5 µl
Total volume	**352 µl**	**416.5 µl**	**463.5 µl**	**543.5 µl**

[_] 3 Add 16 µl master mix to each required well of the plate labeled with the QLP barcode.

[_] 4 Remove the cap from the standard eight-strip tube from step 9 of *Dilute qPCR Standard* on page 20.

[_] 5 Add 4 µl **Std 1** to each well in rows C–E, column 4.

[_] 6 Add 4 µl **Std 2** to each well in rows C–E, column 5. Change the tip.

[_] 7 Add 4 µl **Std 3** to each well in rows C–E, column 6 Change the tip.

[_] 8 Add 4 µl **Std 4** to each well in rows C–E, column 7. Change the tip.

[_] 9 Add 4 µl **NTC** to each well in rows C–E, column 8. Change the tip.

[_] 10 Remove the cap from the microcentrifuge tube that contains each one **1:100 Dilution** sample.

[_] 11 Add 4 µl of one **1:100 Dilution** sample to each well in rows C–E, column 9.

[_] 12 Add 4 µl of each additional **1:100 Dilution** sample to each well in rows C–E, adding one column per sample.

[_] 13 Cap and store the [sample name] **1:100 Dilution** tubes at 2°C to 8°C for subsequent use in this protocol. The samples can be stored for up to 30 days.

[_] 14 Mix the plate thoroughly as follows:

- [_] a Seal the plate with an appropriate adhesive seal for the plate.
- [_] b Shake the plate on a microplate shaker at 1600 rpm for 30 seconds.

[_] 15 Centrifuge the plate at 280 × g for 1 minute.

[_] 16 Place the sealed plate on the qPCR instrument. Close the lid then and run the instrument as follows:

- [_] a 94°C for 1 minute
- [_] b 40 cycles of:
  - 94°C for 30 seconds
  - 65°C for 30 seconds
  - 68°C for 10 minutes
- [_] c [Optional] Melting Curve setting suggested by qPCR instrument

[_] 17 Remove the plate from the qPCR instrument.

Analysis

Do one of the following:

[_] 1 If you are using qPCR instrument software to annotate standards and sample concentration:

[_] a Calculate the average Cq value of the qPCR standards and 1:100 dilution of sample from triplicate wells in the QLP plate.

[_] b Use the qPCR instrument software to annotate standards as follows:

	Concentration (pg/µl)
Std1	10
Std2	1
Std3	0.1
Std4	0.01

[_] c Confirm that the qPCR reaction efficiency is 50–100%, which is a typical reaction efficiency of a long qPCR amplicon.

[_] d Confirm that the R^2 of the best fit line is > 0.97. Poor R^2 values can indicate a dilution error in the standard curve or poor amplification of one or more of the standards.

[_] e Use the average of the triplicate data points corresponding 1:100 sample dilution to calculate the concentration of the sample.

[_] 2 If you are using a graphing program to manually calculate sample concentration:

[_] a Calculate the average Cq value of the qPCR standards and 1:100 dilution of sample from triplicate wells in the QLP plate.

[_] b Create a scatter plot of the average Cq of the qPCR standards on the X-axis and the log base 2 value of the DNA concentration (pg/µl) of the qPCR standards on the Y-axis.

[_] c Determine the equation of the best fit line for the qPCR standard curve values, which is in the format of y = mx + b. This is equivalent to: log base 2 DNA concentration = (slope × Cq) + y_int.

[_] d Confirm that the qPCR reaction efficiency (the slope in the equation in step c) is 50-100%, which is a typical reaction efficiency of a long qPCR amplicon.

[_] e Confirm that the R^2 of the best fit line is > 0.97. Poor R^2 values can indicate a dilution error in the standard curve or poor amplification of one or more of the standards.

[_] f Determine the value of y in y = mx + b by using the average Cq of each 1:100 dilution of sample for x in the equation.

[_] g Calculate the concentration of each 1:100 dilution of sample in pg/µl, using the following equation, where Concentration (pg/µl) = 2 ^ y:
Sample average Cq = 14.6
y = (-0.935 × 14.6) + 12.431 = -1.226
Concentration of 1:100 dilution of sample = 2 ^ -1.226 = 0.428 pg/µl

Comments

_____
_____

Long Range PCR

This process enriches long DNA fragments with the appropriate adapters. The PCR starting material is diluted in a 384-well plate to limit the number of molecules in each well, which enables downstream data-analysis applications. The PCR-amplified material is subject to gel quality control to make sure that the material is not over- or under-amplified.

Consumables

Item	Quantity	Storage	Supplied By
Gel Standard (GST)	1 tube	-25°C to -15°C	Illumina
Long-amp Master Mix (LMM)	1 tube	-25°C to -15°C	Illumina
Long-amp Primer Mix (LPM)	1 tube	-25°C to -15°C	Illumina
MasterAmp Extra-Long DNA Polymerase Mix	1 tube	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
LAP (Long Fragment Amplification Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
E-Gel EX Agarose Gel, 1%	1	15°C to 30°C	User
2-Log DNA Ladder	1	15°C to 30°C	User
15 ml conical tube	1	15°C to 30°C	User
96-well PCR plate or RNase/DNase-free eight-tube strip with caps	1	15°C to 30°C	User
384-well PCR plate	1	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microcentrifuge tubes	6	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
Needle (22 1/2 gauge)	1 per sample	15°C to 30°C	User
RNase/DNase-free eight-tube strips with caps	2	15°C to 30°C	User
RNase/DNase-free reagent reservoir	1	15°C to 30°C	User

Dilute Template

[_] 1 Determine if the 1:100 diluted library template is of sufficient quantity for Long Range PCR.

- For the Phasing workflow, 75 fg library is required per well. A total of 37,500 fg library per plate.
- For the Long-Read workflow, 3 fg library is required per well. A total of 1500 fg library per plate.

[_] 2 If there is not enough library template to make the dilution, use the undiluted template from step 2 of *Dilute Sample* on page 20.

[_] 3 Dilute the library template with Resuspension Buffer to the following concentration with a total volume of 750 µl.

- For the Phasing workflow, dilute the library template to 50 fg/µl.
- For the Long-Read workflow, dilute the library template to 2 fg/µl.

Sample	1:100 Diluted Library (fg/µl)	Diluted Library (µl)	Resuspension Buffer (µl)	Total Volume (µl)
1				
2				
3				
4				

Prepare PCR Master Mix

[_] 1 Set up a PCR master mix in a sterile, nuclease-free 15 ml conical tube on ice using the following:

Reagent	Volume (µl)
Diluted template	750
Long-amp Master Mix	1450
Long-amp Primer Mix	250
MasterAmp Extra-long DNA Polymerase Mix	50
Total Volume	**2500**

[_] 2 Cap the tube and gently invert the tube several times to mix.

[_] 3 Aliquot 280 µl PCR master mix into each well of an eight-tube strip.

[_] 4 Set a 200 µl electronic eight-channel pipette to 120 µl and 5 µl per dispense, for a total of 24 dispenses.

[_] 5 Add 5 µl PCR master mix to each well of the new 384-well PCR plate labeled with the LAP barcode.

[_] 6 Repeat steps 4 and 5 one time.

[_] 7 Quickly seal the plate with a Microseal 'B' adhesive seal, then centrifuge the plate at 500 × g for 1 minute.

[_] 8 Cap the PCR master mix eight-tube strip and keep the strip on ice

Long Amp Plate

[_] 1 Place the sealed plate on the 384-well thermal cycler and place a compression mat on top of the plate. Close the lid then select and run the **Phasing15** or **LongRead21** program, depending on the workflow.

[_] 2 While the thermal cycler is running, proceed to *Long Amp Quality Control*.

Long Amp Quality Control

[_] 1 Add 50 µl PCR master mix to one well of a new PCR plate or an eight-tube strip.

[_] 2 Seal the plate with a Microseal 'B' adhesive seal or cap the eight-tube strip.

[_] 3 Place the sealed plate or capped tube on the 96-well thermal cycler. Close the lid then select and run the **Phasing20QC** or **LongRead26QC** program, depending on the workflow.

[_] 4 Remove the LAP plate and 96-well PCR plate or eight-tube strip from both thermal cyclers and place them on ice.

Gel Quality Control

[_] 1 Add Resuspension Buffer to the labeled microcentrifuge tubes as follows:

Tube	Resuspension Buffer Volume (µl)
GST1	36
GST2	20
GST3	20
GST4	20

[_] 2 Add 4 µl undiluted Gel Standard to the **GST1** tube for a total of 0.1 ng/µl. Gently pipette the entire volume up and down 6–8 times to mix thoroughly. Change the tip.

[_] 3 Transfer 20 µl from the **GST1** tube to the **GST2** tube for a total of 0.05 ng/µl. Gently pipette the entire volume up and down 6–8 times to mix thoroughly. Change the tip.

[_] 4 Transfer 20 µl from the **GST2** tube to the **GST3** tube for a total of 0.025 ng/µl. Gently pipette the entire volume up and down 6–8 times to mix thoroughly. Change the tip.

[_] 5 Transfer 20 µl from the **GST3** tube to the **GST4** tube for a total of 0.0125 ng/µl. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

[_] 6 Centrifuge the LAP plate at 500 × g for 1 minute.

[_] 7 Using 22 1/2 gauge needle, carefully pierce a hole in the plate seal above four randomly selected wells of the plate.

[_] 8 Transfer 5 µl from each of four randomly selected plate wells to pool in one well of an eight-tube strip. Note which wells were selected from the plate.

Sample	LAP Plate Well
1	
2	
3	
4	

[_] 9 Place the LAP plate on ice or store the plate at 2°C to 8°C for up to 24 hours or until this *Long Range PCR* procedure is complete.

[_] 10 Cap the eight-tube strip that contains the pooled samples, briefly centrifuge the strip at 500 × g.

[_] 11 Add 0.5 µl 2-Log DNA Ladder and 19.5 µl Resuspension Buffer to the tube labeled **2-Log Ladder** to dilute. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

[_] 12 Load all of the Diluted 2-Log DNA Ladder into the well of lane 1 of a E-Gel EX 1%.

[_] 13 Load 20 µl from the **GST1** tube into the well of lane 2 of the gel.

[_] 14 Load 20 µl from the **GST2** tube into the well of lane 3 of the gel.

[_] 15 Load 20 µl from the **GST3** tube into the well of lane 4 of the gel.

[_] 16 Load 20 µl from the **GST4** tube into the well of lane 5 of the gel.

[_] 17 Load 20 µl pooled sample into the well of lane 6 of the gel.

[_] 18 Load 20 µl quality control sample, from the conclusion of *Long Amp Quality Control* on page 24, into the well of lane 7 of the gel.

[_] 19 Load each of the empty wells with 20 µl Resuspension Buffer.

[_] 20 Select and run the **E-Gel EX 1–2%** program.

[_] 21 View the gel on a Dark Reader transilluminator.
The pooled sample and QC sample bands should migrate the same distance and the pooled sample intensity should be between the intensity of GST1 (0.1 ng/µl) and GST4 (0.0125 ng/µl). The Gel Standard migrates in the gel as a single band at 10 kb.

SAFE STOPPING POINT
If you do not plan to proceed immediately to *Tagmentation* on page 27, you can safely stop the protocol here. If you are stopping, seal the LAP plate with a Microseal 'B' adhesive seal and store at 2°C to 8°C for ***up to 24 hours***.

Comments

_____
_____

Tagmentation

This process tagments (tags and fragments) PCR amplified long DNA fragments by adding the Nextera transposome to the 384-well plate. The Nextera transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends, allowing for amplification by PCR in subsequent procedures.

Consumables

Item	Quantity	Storage	Supplied By
Fragmentation plate	1	15°C to 30°C	Illumina
Fragmentation Pre-Mix (FPM)	1 tube per LAP plate	-25°C to -15°C	Illumina
Tagment DNA Enzyme (TDE)	1 tube per LAP plate	-25°C to -15°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microcentrifuge tube	1	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User

Procedure

[_] 1 Centrifuge the LAP plate at 500 × g for 1 minute.

[_] 2 Remove the seal from the LAP plate, then place the Fragmentation plate on top of LAP plate.

[_] 3 Add 36 µl Tagment DNA Enzyme and 1464 µl Fragmentation Pre-Mix to a microcentrifuge tube.

[_] 4 Invert the tube 10 times to mix thoroughly, then centrifuge briefly.

[_] 5 Transfer 180 µl of the mixture to each well of an eight-tube strip.

[_] 6 Set a 200 µl electronic eight-channel pipette to 144 µl and 3 µl per dispense.

[_] 7 Add 3 µl Tagment DNA Enzyme and Fragmentation Pre-Mix to each well of the Fragmentation plate that is on top of the LAP plate.

[_] 8 Centrifuge the stacked Fragmentation plate and LAP plates to 500 × g for 1 minute.

[_] 9 Place the stacked Fragmentation plate and LAP plates on the benchtop, with the LAP plate on the bottom.

[_] 10 Carefully remove the Fragmentation plate from the LAP plate and discard the Fragmentation plate.

[_] 11 Mix the LAP plate thoroughly as follows:

- [_] a Seal the plate with a Microseal 'B' adhesive seal.
- [_] b Shake the plate on a microplate shaker at 1600 rpm for 30 seconds.

[_] 12 Centrifuge the plate at 500 × g for 1 minute.

[_] 13 Place the sealed plate on the thermal cycler and place a compression mat on top of the plate. Close the lid and then select and run the **Tag** program.

- [_] a Choose the thermal cycler pre-heat lid option and set to 100°C
- [_] b 55°C for 15 minutes
- [_] c 4°C for 5 minutes
- [_] d 72°C for 4 minutes
- [_] e Hold at 4°C

[_] 14 Remove the LAP plate from the thermal cycler.

Comments

_____

_____

Indexing PCR

This process amplifies tagmented DNA by PCR. A unique index and the P5 and P7 adapters are added to the tagmented DNA in each well of the 384-well plate. The P5 and P7 adapters are required for cluster generation and sequencing.

Consumables

Item	Quantity	Storage	Supplied By
Indexing Plate (IDP)	1	-25°C to -15°C	Illumina
[Optional] Alignment ring	1 per LAP plate	15°C to 30°C	Illumina
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Procedure

[_] 1 Centrifuge the LAP plate at 500 × g for 1 minute.

[_] 2 Centrifuge the IDP plate at 500 × g for 1 minute.

[_] 3 Make sure that the droplets are at the bottom of each well of the IDP plate.

[_] 4 Remove the adhesive seal from the LAP plate.

[_] 5 Remove the foil seal from the IDP plate.

[_] 6 [Optional] Place the alignment ring on the LAP plate so that the notched corners align.

[_] 7 Invert the IDP plate.

[_] 8 Carefully place the inverted IDP plate on top of the LAP plate, so that the corner notches and wells of both plates align.

[_] 9 Centrifuge the stacked IDP and LAP plates to 500 × g for 1 minute. Make sure that the LAP plate is on the bottom.

[_] 10 Place the stacked IDP and LAP plates on the benchtop, with the LAP plate on the bottom.

[_] 11 Carefully remove the IDP plate from the LAP plate and place it, the top side facing up, on the benchtop.

[_] 12 Mix the LAP plate thoroughly as follows:

- [_] a Seal the plate with a Microseal 'B' adhesive seal.
- [_] b Shake the plate on a microplate shaker at 1600 rpm for 30 seconds.

[_] 13 Centrifuge the plate at 500 × g for 1 minute.

[_] 14 Place the sealed plate on the thermal cycler and place a compression mat on top of the plate. Close the lid then select and run the **PostTagAmp** program.

- [_] a Choose the thermal cycler pre-heat lid option and set to 100°C
- [_] b 94°C for 1 minute
- [_] c 10 cycles of:
  - 94°C for 15 seconds
  - 65°C for 4 minutes
- [_] d Hold at 4°C for up to one hour

[_] 15 Remove the LAP plate from the thermal cycler.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Pool and Concentrate* on page 31, you can safely stop the protocol here. If you are stopping, store the LAP plate at -25°C to -15°C for up to 7 days or at 2°C to 8°C for up to 24 hours.

Comments

_____

_____

Pool and Concentrate

This process collects and concentrates the PCR-amplified and indexed DNA from the 384-well plate into a single sample.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Neutralization Buffer (SNB)	1 tube per 1 reaction	2°C to 8°C	Illumina
Collection plate	1 per sample	15°C to 30°C	Illumina
PAP (Pooled Amplicon Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
50 ml conical tube	1 per sample	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Microcentrifuge tube	2 per sample	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strip and caps	1 per sample	15°C to 30°C	User
Zymo DNA Binding Buffer	1	15°C to 30°C	User
Zymo DNA Wash Buffer (with ethanol added)	1	15°C to 30°C	User
Zymo-Spin V Column with Reservoir	1	15°C to 30°C	User

Procedure

[_] 1 Centrifuge the LAP plate at 500 × g for 1 minute.

[_] 2 Remove the adhesive seal from the LAP plate, then attach the collection plate to the LAP plate, so that the LAP plate is covered with the collection plate.

[_] 3 Invert the attached collection and LAP plates so that the sample plate wells face down into the collection plate.

[_] 4 Centrifuge the collection and LAP plates with a balance to 500 × g for 30 seconds.

[_] 5 Set up a master mix in a new, sterile, nuclease-free 50 ml conical tube using the following:

Reagent	Volume
All of the pooled library from the collection plate	4–5 ml
Sample Neutralization Buffer	200 µl
Zymo DNA Binding Buffer	20 ml
Total Volume	**~24.5 ml**

[_] 6 Cap the master mix tube and invert the tube several times to mix.

[_] 7 Set up a Zymo-Spin V column with reservoir on a vacuum manifold.

[_] 8 Turn on the vacuum and leave it on.

[_] 9 Add 12 ml master mix to the Zymo-Spin V column.

[_] 10 Run the master mix through the vacuum until all of the liquid has passed through the Zymo-Spin V column and into the vacuum manifold.

[_] 11 Add the remaining master mix to the Zymo-Spin V column.

[_] 12 Run the master mix through the vacuum until all of the liquid has passed through the Zymo-Spin V column and into the vacuum manifold.

[_] 13 Add 4 ml Zymo DNA Wash Buffer (with ethanol added) to the Zymo-Spin V column to wash the sample while it is on the vacuum.

[_] 14 Remove the Zymo-Spin V column from the vacuum manifold and unattach the reagent reservoir from the column.

[_] 15 Discard reagent reservoir

[_] 16 Centrifuge Zymo-Spin V column at 11,000 × g for 1 minute in a microcentrifuge tube to remove any residual Zymo DNA Wash Buffer.

[_] 17 Place the Zymo-Spin V column into a new microcentrifuge tube, then add 160 µl Resuspension Buffer to the column.

[_] 18 Centrifuge the microcentrifuge tube at 10,000 × g for 1 minute to collect the eluate.

[_] 19 Transfer 150 µl from the microcentrifuge tube to a single well of the new MIDI plate labeled with the PAP barcode.

[_] 20 Transfer 5 µl from the microcentrifuge tube to the new eight-tube strip labeled **QC1: Pre Size Selection**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Size Selection* on page 33, you can safely stop the protocol here. If you are stopping, seal the PAP plate with a Microseal 'B' adhesive seal. Store the plate at -25°C to -15°C for up to 7 days or at 2°C to 8°C for up to 24 hours.

Comments

_____

_____

Size Selection

This process removes adapter dimers and DNA fragments that are either too small or too large, selecting for tagmented DNA in the optimal range for cluster formation.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
FSP (Final Sample Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well MIDI plate	1	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Procedure

[_] 1 Vortex the Sample Purification Beads until they are well dispersed.

[_] 2 Add 67.5 µl well-mixed Sample Purification Beads to each sample well of the PAP plate. Mix thoroughly as follows:

- [_] a Seal the plate with a Microseal 'B' adhesive seal.
- [_] b Shake the plate on a microplate shaker at 1600 rpm for 2 minutes or until the beads are well dispersed.

[_] 3 Incubate the plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 4 Centrifuge the plate at 280 × g for 1 minute.

[_] 5 Remove the adhesive seal from the plate, then place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 6 Using a 200 µl single channel or multichannel pipette set to 106 µl, transfer 106 µl of the supernatant, containing the DNA of interest, from each sample well of the plate to an empty well in the same plate.

[_] 7 Repeat step 6 one time, transferring each sample to the same well that the sample was transferred to in step 6. **Each plate sample well now contains a total of 212 µl of DNA of interest.**

[_] 8 Remove the plate from the magnetic stand.

[_] 9 Add 30 µl well-mixed Sample Purification Beads to each well of the plate. Mix thoroughly as follows:

- [_] a Seal the plate with a Microseal 'B' adhesive seal.
- [_] b Shake the plate on a microplate shaker at 1600 rpm for 2 minutes.

[_] 10 Incubate the plate at room temperature for 5 minutes.

Start time: _____ Stop time: _____

[_] 11 Centrifuge the plate at 280 × g for 1 minute.

[_] 12 Remove the adhesive seal from the plate.

[_] 13 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 14 Remove and discard all of the supernatant from each well of the plate.

[_] 15 With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

[_] 16 Incubate the plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

[_] 17 Repeat steps 15 and 16 one time for a total of two 80% EtOH washes.

[_] 18 Remove and discard any remaining EtOH from each well of the plate with a 10 µl pipette.

[_] 19 Let the plate stand at room temperature for 5 minutes to dry, and then remove the plate from the magnetic stand.

Start time: _____ Stop time: _____

[_] 20 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Start time: _____ Stop time: _____

[_] 21 Incubate the plate at room temperature for 2 minutes.

Start time: _____ Stop time: _____

[_] 22 Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.

Start time: _____ Stop time: _____

[_] 23 Transfer 30 µl of supernatant from each well of the PAP plate to the corresponding well of the new MIDI plate labeled with the FSP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Final Product* on page 35, you can safely stop the protocol here. If you are stopping, seal the FSP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Comments

_____

_____

Validate Final Product

Perform the following procedures for quality control analysis on your sample library and quantification of the final library.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
KAPA Library Quantification Kit - Illumina/Universal	1	As specified by manufacturer	User
High Sensitivity DNA Kit	1	As specified by manufacturer	User
Qubit dsDNA HS Assay Kit	1	As specified by manufacturer	User

Quantify Libraries

Illumina recommends that you quantify your libraries by qPCR.

Follow qPCR instructions included in the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* using the KAPA standard, with the following modification:

Perform a size adjustment calculation to account for the difference in size between the average fragment length of the library and the KAPA DNA Standard (452 bp). Determine the average fragment length of the library between 200–2000 bp using an Agilent Technologies 2100 Bioanalyzer or equivalent. Use this average fragment length for the size adjustment calculation.

Quality Control

[_] 1 Dilute the **Final** DNA library from the FSP plate to an optimal concentration for the Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip as follows:

- [_] a Quant the **Final** DNA library using a Qubit dsDNA HS Assay Kit.
- [_] b Dilute 2 µl of the **Final** DNA library to 1 ng/µl with Resuspension Buffer.

[_] 2 Load 1 µl of the diluted **Final** DNA library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

[_] 3 Prepare a 1:5 dilution of the **QC1:Pre-size selection** DNA library, from step 20 of *Pool and Concentrate* on page 31, with Resuspension Buffer.

[_] 4 Load 1 µl of the diluted **QC1:Pre-size selection** DNA library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.
Check the size of the sample for a broad distribution of DNA fragments with a size range from approximately 200–3000 bp.

[_] 5 Do one of the following:

- Proceed to cluster generation.
- Store the sealed FSP plate at -25°C to -15°C.

Comments

Illumina
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

Part # 15047265 Rev. B