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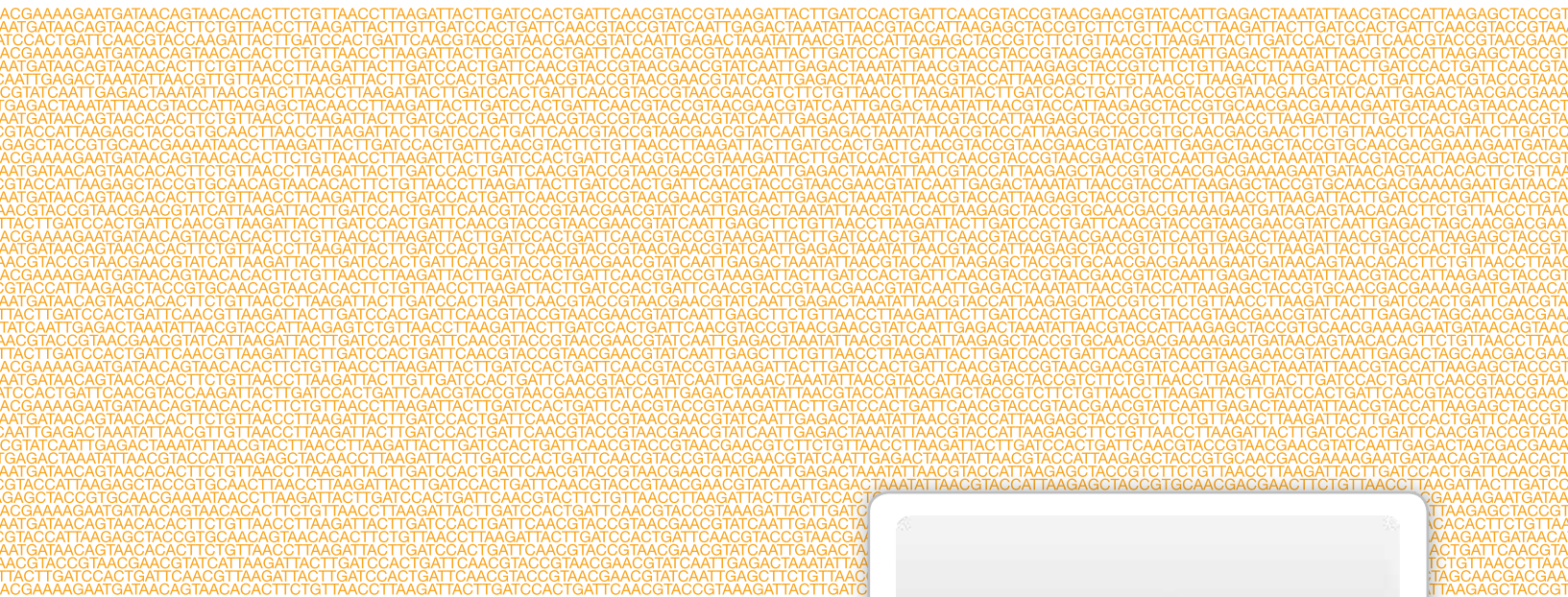
TruSeq® Synthetic Long-Read DNA Library Prep Guide



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Revision History

Part #	Revision	Date	Description of Change

15047264	B	September 2014	• Updated *Additional Resources* to remove web navigation instructions and written urls
			• Removed use of plate name (e.g., LFP plate), except for first instance and last instance in each procedure
			• Modified the *Purify Ligation Products and Size Selection* protocol to use the E-Gel NGS, 0.8% Agarose, E-Gel iBase Power System, and run the E-Gel 0.8-2% program.
			• Removed the following *Consumables and Equipment*:
			  • E-Gel CloneWell 0.8% SYBR Safe gels
			  • PCR Tube Plate 384-well Prism
			  • Electrophoresis power supply
			  • Gel Opener
			• Added a *BluePippin Size Selection* protocol option and the necessary *Consumables and Equipment*.
			• Corrected the Collection Plate and Fragmentation Plate part numbers in *Kit Contents*.
			• Clarified thermal cycler and qPCR instrument requirements
			• Updated SDS link to support.illumina.com/sds.html

15047264	A	June 2014	Initial Release

Table of Contents

Overview

Chapter 1

Introduction

This protocol explains how to prepare up to four libraries of genomic DNA (gDNA) using the reagents provided in the Illumina® TruSeq® Synthetic Long-Read DNA Library Prep Kit and TruSeq Synthetic Long-Read DNA Barcode Kit. The libraries are prepared for subsequent cluster generation and DNA sequencing. The kits are designed for two applications, preparing DNA libraries for long-read assembly and phasing analysis from whole human genome sequencing data.

TruSeq Synthetic Long-Read DNA Library Prep leverages TruSeq and Nextera® chemistries with the high accuracy of short sequencing reads to construct long synthetic fragments with high assembly accuracy or efficient phasing of whole human genome sequencing data. It enables phasing of *de novo* mutations and the identification of co-inherited alleles in a population, providing greater insight into the human genome.

The long-read application generates synthetic long-read fragments that can improve the accuracy of genome construction by providing data on traditionally challenging regions, such as repetitive content. This application enables more accurate, long contigs for de novo assembly, genome finishing, or metagenomics applications.

The phasing application is designed for preparing human DNA libraries for phasing analysis. Combined with whole human genome sequencing variant data, this method assigns highly accurate shorter reads into long haplotype fragments for allele-specific analysis.

DNA Input Recommendations

It is important to quantitate the input DNA and assess the DNA quality before performing TruSeq Synthetic Long-Read DNA Library Prep.

Input DNA Quantitation

Follow these DNA input recommendations:

- 50 µl input DNA at 10 ng/µl is required to prepare one sample library.
- The ultimate success or failure of library preparation strongly depends on using an accurately quantified amount of input DNA.
- Use multiple methods of quantification to verify results.
- Illumina recommends using fluorometric based methods for quantification, such as Qubit or PicoGreen, to provide accurate quantification of dsDNA. UV spectrophotometric-based methods, such as Nanodrop, measure any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides. This can give an inaccurate measurement of gDNA.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to the presence of excess nucleic acids.
  - These methods require the preparation of calibration curves and are highly sensitive to pipetting error.
  - Make sure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

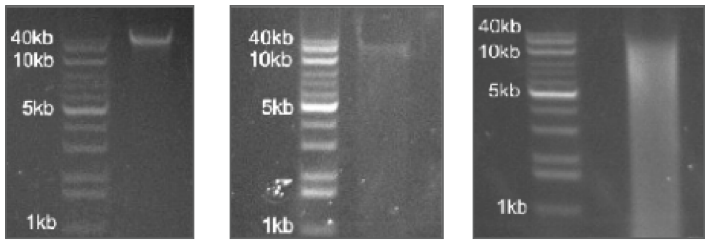
Assessing DNA Quality

Genomic DNA integrity is critical for the success of TruSeq Synthetic Long-Read DNA Library Prep. The DNA must be phenol free, with a size ≥ 40 kb. Illumina recommends a gDNA quality check, using an agarose gel or other instrument, before proceeding with the protocol. To assess quality on agarose gel, Illumina recommends running 100–500 ng of gDNA on a 0.8% Agarose gel with 200 ng of 1 kb DNA extension ladder. Compare the results to the Figure 1 examples of 0.8% CloneWell gels run for 30 minutes.

Table 1 Quality Assessment Guidelines

	Pass	Intermediate	Fail
gDNA gel QC result	All gDNA migrates in a discreet band > 40 kb	Some gDNA migrates in a discreet band > 40 kb, some gDNA migrates as a smear < 40 kb	All gDNA migrates as a smear < 40 kb
% Success in TruSeq Synthetic Long-Read DNA Library Prep	> 95%	~ 60%	0%
Mode of failure	N/A	Insufficient yield in Long Fragment qPCR quantitation	Insufficient yield in Long Fragment qPCR quantitation
Recommendation for a second library prep attempt	N/A	< 20% of gDNA samples succeed in second attempt	0% of gDNA samples succeed in second attempt

Figure 1 Examples of DNA Quality



A Pass
B Intermediate
C Fail

Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
TruSeq Synthetic Long-Read Library Prep Experienced User Card and Lab Tracking Form (part # 15047265)	Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.**
Illumina Experiment Manager Guide (part # 15031335) and *IEM TruSeq Synthetic Long-Read DNA Quick Reference Card (part # 15056316)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace User Guide (part # 15044182)	Provides information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.

Visit the TruSeq Synthetic Long-Read DNA Library Prep support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Protocol

Chapter 2

Introduction

This chapter describes the TruSeq Synthetic Long-Read DNA Library Prep protocol.

- Use IEM to create a sample sheet for Illumina sequencing systems and analysis software. See *Additional Resources* on page 5 for information about IEM documentation on the Illumina website.
- Use BaseSpace to organize samples, libraries, pools, and runs for Illumina sequencing systems and analysis software. See *Additional Resources* on page 5 for information about BaseSpace documentation on the Illumina website.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

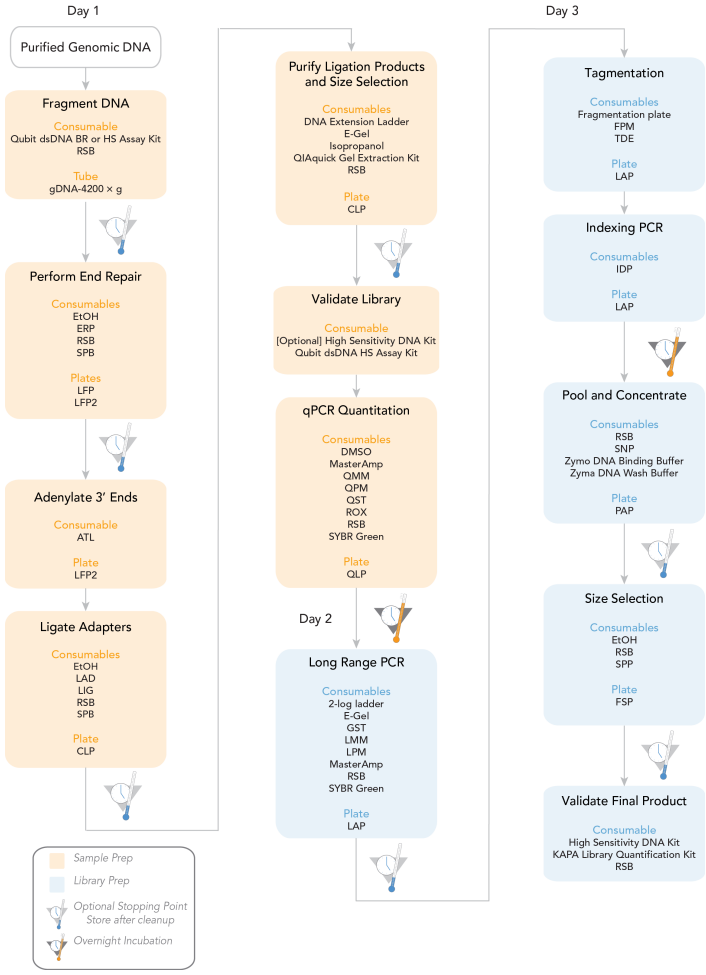
  NOTE
  The library prep procedures are described using a 96-well PCR plate. However, due to the small number of samples, they can be performed with an eight-tube strip or a previously unused well of a plate.

- Review best practices before proceeding. See *Additional Resources* on page 5 for information about TruSeq Synthetic Long-Read DNA Library Prep best practices on the Illumina website.
- Review Appendix A Supporting Information before proceeding, to confirm your kit contents and make sure that you have obtained all of the requisite consumables and equipment.

Library Prep Workflow

The following figure illustrates the processes of the TruSeq Synthetic Long-Read DNA Library Prep protocol.

Figure 2 TruSeq Synthetic Long-Read DNA Library Prep Workflow



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
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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

Preparation

- Review *DNA Input Recommendations* on page 3.
- Remove the Resuspension Buffer from -25°C to -15°C storage and thaw it at room temperature.

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- Label a new microcentrifuge tube **gDNA-4200 × g** and the experiment date with a smudge resistant pen.

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.

   NOTE
   Steps 4–7 must be performed within 15 minutes of the gDNA being added to the g-TUBE, according to manufacturer instructions.

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 12, 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.

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
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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

NOTE
This procedure is described using 96-well PCR plates, however, RNase/DNase-free eight-tube strips with caps can be used in this procedure in place of the plates.

Preparation

- Prepare an ice bucket.

- Remove the End Repair Mix from -25°C to -15°C storage. Thaw it at room temperature and then place it on ice.
- Review best practices for handling magnetic beads. See *Additional Resources* on page 5 for information about TruSeq Synthetic Long-Read DNA Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the gDNA-4200 × g tube from 2°C to 8°C storage, if it was stored at the conclusion of *Fragment DNA* on page 10.
  - Let the tube stand to bring it to room temperature.
  - Centrifuge the tube at 280 × g for 1 minute.
  - Remove the cap from the tube.
- Pre-program the thermal cycler with the following program and save as **ERP**:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 30°C for 30 minutes
  - Hold at 4°C
- Apply an LFP barcode label to a new 96-well PCR plate.
- Apply an LFP2 barcode label to a new 96-well PCR plate.

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.

   NOTE
   Place the **gDNA-4200 × g** tubes in -25°C to -15°C storage for use later in the protocol.
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.
5. Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.
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.

NOTE
Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (7–9).

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. Take care not to disturb the beads.
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.

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.

13 Incubate the plate at room temperature for 2 minutes.

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

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 16, 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.

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
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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

Preparation

- Prepare an ice bucket.
- Remove the A-Tailing Mix from -25°C to -15°C storage and thaw it at room temperature. Place the tube on ice.
- Remove the LFP2 plate from 2°C to 8°C storage, if it was stored at the conclusion of *Perform End Repair* on page 12.
  - Let the LFP2 plate stand at room temperature.
  - Centrifuge the LFP2 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the LFP2 plate.
- Pre-program the thermal cycler with the following program and save as **ATAIL**:
  - Choose the pre-heat lid option and set to 100°C
  - 37°C for 30 minutes
  - Hold at 4°C

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 18.

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
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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

NOTE
This procedure is described using 96-well PCR plates, however, RNase/DNase-free eight-tube strips with caps can be used in this procedure in place of the plates.

Preparation

- Prepare an ice bucket.

- Remove the Long Fragment Adapters from -25°C to -15°C storage and thaw at room temperature. Place the tube on ice.

  NOTE
  Do not remove the Ligation Mix tube from -25°C to -15°C storage until instructed to do so in the procedures.

- Review best practices for handling magnetic beads. See *Additional Resources* on page 5 for information about TruSeq Synthetic Long-Read DNA Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and bring them to room temperature.
- Pre-program the thermal cycler with the following program and save as **LIG**:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 30°C for 10 minutes
  - Hold at 4°C
- Apply a CLP barcode label to a new 96-well PCR plate.

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.
5. Place the plate on the magnetic stand for 5 minutes or until the liquid is clear.
6. Remove and discard 70 µl of the supernatant from each well of the plate. Take care not to disturb the beads.

> NOTE
> Leave the plate on the magnetic stand while performing the following steps 7–12.

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. Take care not to disturb the beads.
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.

12 With the plate on the magnetic stand, add 22.5 µl Resuspension Buffer to each sample well of the plate. Make sure the Resuspension Buffer runs over the beads.

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.

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

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. Take care not to disturb the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Purify Ligation Products and Size Selection* on page 22, 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.

NOTE
TruSeq Synthetic Long-Read DNA size selection is performed using agarose gel electrophoresis. However, an alternative method using the BluePippin System can be performed in place of the procedures in this section. To perform the alternative method, see *BluePippin Size Selection* on page 95.

Consumables

Item	Quantity	Storage	Supplied By
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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 (from *Fragment DNA* on page 10)	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

Preparation

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the CLP plate from 2°C to 8°C storage, if it was stored at the conclusion of *Clean Up LFP2* on page 20.
  - Let the CLP plate stand to bring it to room temperature.
  - Centrifuge the CLP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the CLP plate.
- Prepare E-Gel NGS, 0.8% Agarose by removing the comb. One gel is recommended per sample to avoid cross-contamination.
- Pre-heat the microheating system or water bath to 50°C.
- Label one new microcentrifuge tube for each sample with the name of the sample, using a smudge resistant pen.
- Weigh each microcentrifuge tube labeled with the sample name and record the weight.

Sample	Sample Name	Empty Tube Weight
1		
2		
3		
4		

- Label one new microcentrifuge tube for each sample with **size-selected** [sample name], using a smudge resistant pen.

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.

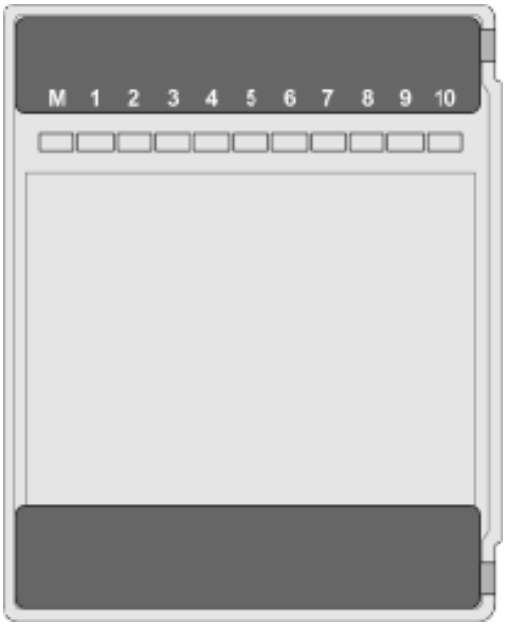
NOTE
The fragmented DNA sample in the tube labeled **gDNA-4200 × g** is from the conclusion of *Fragment DNA* on page 10 and is used as a control sample.

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

NOTE
Reference Figure 3 while performing steps 5–9.

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

Figure 3 E-Gel NGS, 0.8% Agarose Loading Layout



Lane M—Resuspension Buffer
Lane 1—Resuspension Buffer
Lane 2—Resuspension Buffer
Lane 3—Resuspension Buffer
Lane 4—Sample (from CLP plate)
Lane 5—Resuspension Buffer
Lane 6—Diluted 1 Kb DNA Extension Ladder
Lane 7—Resuspension Buffer
Lane 8—Diluted fragmented gDNA
Lane 9—Resuspension Buffer
Lane 10—Resuspension Buffer

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.

NOTE
Do not overload the DNA ladder. Without clear and distinct bands, it is difficult to excise the correct fragment size. Also, an overloaded ladder might run faster than the DNA sample library.

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 (lanes M, 1, 2, 3, 5, 7, 9 and 10).

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.

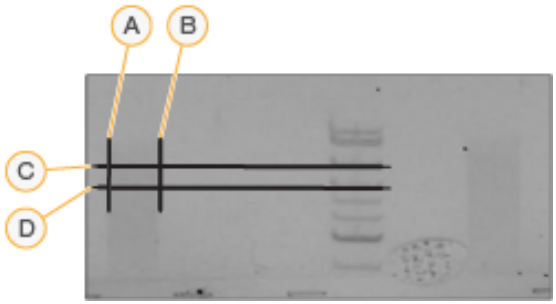
12 Use a lab pen and ruler to mark the 8–10 kb region of interest for precise gel excision, as follows:

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.

Figure 4 1 kb Extension Ladder



A Line drawn to the left of the sample
B Line drawn to the right of the sample
C Line drawn at 10 kb band
D Line drawn at 8 kb band

Purify Gel

NOTE
The following procedures are from the QIAquick Gel Extraction Kit handbook. Make sure to use Isopropanol as specified. Contact the kit manufacturer for technical support.

1 Carefully open the gel with the Novex Gel Knife.

NOTE
For information on how to open the gel, see the Novex Gel Knife manufacturer instructions.

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.

NOTE
View the gel on a Dark Reader transilluminator to confirm that the entire region of interest is extracted.

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). Use the following table as an example for tracking and calculating the weight of the gel.

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. For example, if the gel slice weights 100 mg, add 300 µl QIAGEN Buffer QG to the tube.

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.

9 Add 1 µl Isopropanol × the mg weight of the gel slice to the gel and QIAGEN Buffer QG mixture. For example, if the gel slice weighs 100 mg, then add 100 µl Isopropanol to the 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 29, 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.

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.

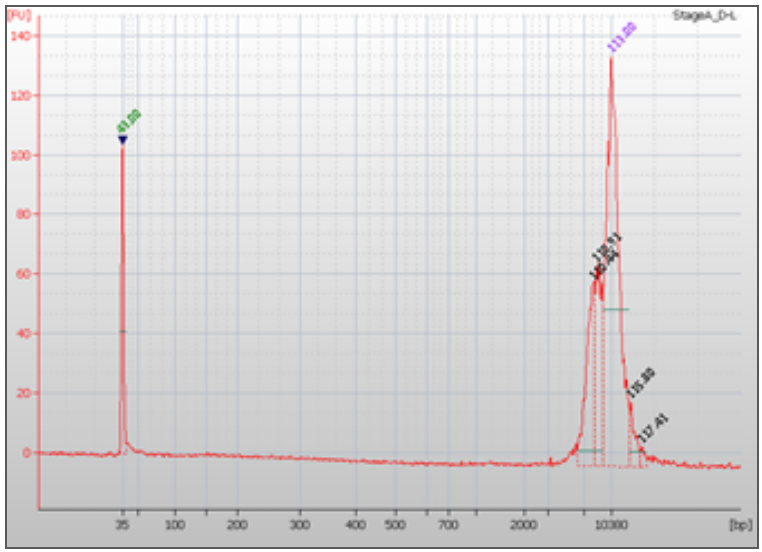
NOTE
For information on how to perform the Qubit dsDNA HS Assay, see manufacturer instructions.

[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.

Figure 5 Example TruSeq Synthetic Long-Read DNA Library Distribution



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
TruSeq Synthetic Long-Read DNA Library Prep Kit contents:			
• 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

Item	Quantity	Storage	Supplied By
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

Preparation

- Prepare an ice bucket.
- Remove the following from -25°C to -15°C storage and thaw them at room temperature. Place the tubes on ice.
  - MasterAmp Extra-Long DNA Polymerase Mix
  - qPCR Long-amp Primer Mix
  - qPCR Master Mix
  - qPCR Standard
- Remove the SYBR Green 10,000x from -25°C to -15°C storage and thaw it room temperature. Do not place it on ice.

  NOTE
  DMSO thaws slowly, so make sure that the SYBR Green 10,000x is completely thawed before using.

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Set up your qPCR instrument. See *qPCR Systems* on page 87 for a list of validated qPCR systems for this protocol.
  - Select the SYBR/DNA Binding Dye Assay workflow.
  - Determine if ROX is needed for your instrument.
  - See your instrument guide for the standard quantification process.
  - Pre-program the qPCR instrument with the following program:
    - 94°C for 1 minute
    - 40 cycles of:
      - 94°C for 30 seconds
      - 65°C for 30 seconds
      - 68°C for 10 minutes
    - [Optional] Melting Curve setting suggested by qPCR instrument

- Apply a QLP barcode label to a new plate for the qPCR instrument.
- Label new microcentrifuge tubes with a smudge resistant pen as follows:
  - **QST 1:100 Dilution**
  - One tube for each sample with the [sample name] **1:100 Dilution**
- Label five tubes of an eight-tube strip with a smudge resistant pen as follows:
  - **Std1**
  - **Std2**
  - **Std3**
  - **Std4**
  - **NTC**

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* on page 98.

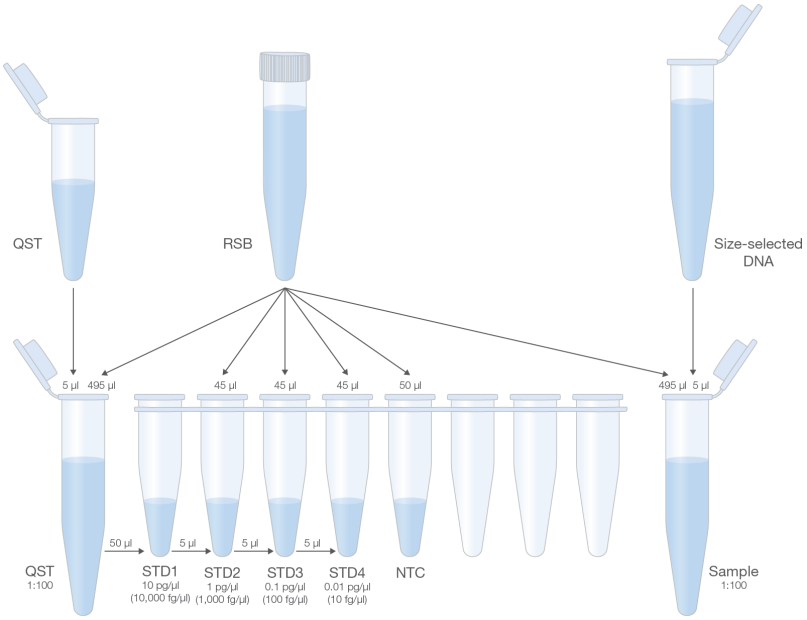
NOTE

- Protect the 100x diluted SYBR Green from light.
- You can store the 100x diluted SYBR Green at -25°C to -15°C for up to six months. When removing the dilution from storage, thaw it completely, then mix thoroughly, while protecting it from light.

Dilute qPCR Standard

NOTE
Reference Figure 6 while performing the dilution procedures.

Figure 6 Dilute qPCR Standard and Sample



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 27 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.

CAUTION

This qPCR procedure is sensitive to the SYBR Green concentration. Make sure that you have calibrated the 100x diluted SYBR Green on a NanoDrop instrument.

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.

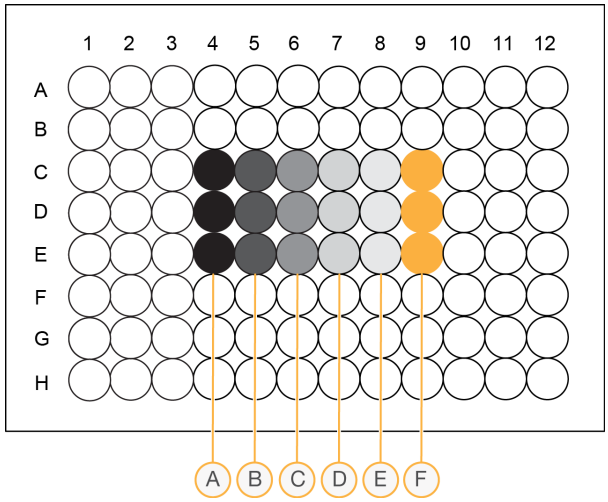
Reagent	1 Sample	2 Samples	3 Samples	4 Samples
qPCR Master Mix	255 µl	302 µl	336 µl	394 µl
qPCR Long-amp Primer Mix	44 µl	52 µl	58 µl	68 µl
Dye Mix (1.5x SYBR Green with optional 10x ROX)	44 µl	52 µl	58 µl	68 µl
MasterAmp Extra-long DNA Polymerase Mix	9 µl	10.5 µl	11.5 µl	13.5 µl
Total volume	**352 µl**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

NOTE

Reference Figure 7 while performing steps 3–12.

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

Figure 7 Example: QLP Plate Setup for 1 Sample



A Std 1
B Std 2
C Std 3
D Std 4
E NTC
F **1:100 Dilution** sample

4 Remove the cap from the standard eight-strip tube from step 9 of *Dilute qPCR Standard* on page 33.

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

Assess the quality of the qPCR run and calculate the DNA concentration of your unknown samples using the Cq values from the qPCR run. 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. If one of the three replicates appears to be an outlier, it can be omitted from the calculation. If more than one of the three replicates appear to be outliers, repeat the protocol.
   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. Successive 10-fold dilutions of the Standard should have Cq values evenly spaced approximately 3.2 cycles apart. Pay particular attention to the spacing between Std1 and Std2 and Sample dilutions in this concentration range. Examine the amplification plots to make sure that early amplification has not interfered with the automatic baseline determination/subtraction on your instrument. For more information, see your qPCR instrument-specific instructions.

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. If so, repeat the protocol.

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

CAUTION

Unexpected results, such as delayed amplification, no amplification, or poor R^2, can be due to the inhibition of qPCR by SYBR Green. If you get unexpected results, Illumina recommends diluting the 100x SYBR Green to 50x and repeating *qPCR Quantitation*.

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. If one of the three replicates appears to be an outlier, it can be omitted from the calculation. If more than one of the three replicates appear to be outliers, repeat the assay.

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. For example:

	Concentration (pg/µl)	$\log_2$ Concentration	Example Avg. Cq
Std1	10	3.321928	9.98
Std2	1	0	13.03
Std3	0.1	-3.32193	16.69
Std4	0.01	-6.64386	20.56

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.

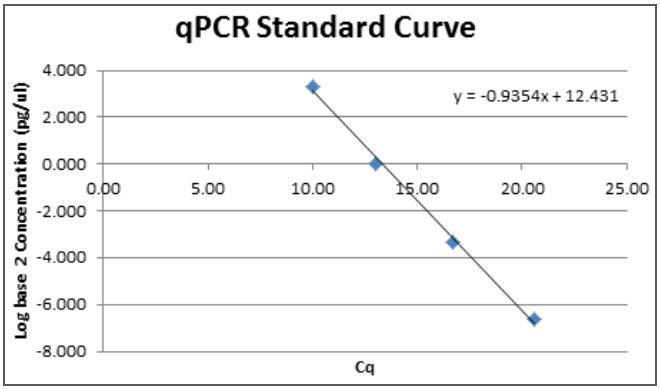
d Confirm that the qPCR reaction efficiency (the slope in the equation in step c) is 50-100%, which is a typical reaction efficiency of a long qPCR amplicon. Successive 10-fold dilutions of Standard should have Cq values evenly spaced approximately 3.2 cycles apart. Pay particular attention to the spacing between Std1 and Std2 and Sample dilutions in this concentration range. Examine the amplification plots to make sure that early amplification has not interfered with the automatic baseline determination/subtraction on your instrument. For more information, see your qPCR instrument-specific instructions.

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. If so, repeat the protocol.

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:

Figure 8 Example: qPCR Standard Curve



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

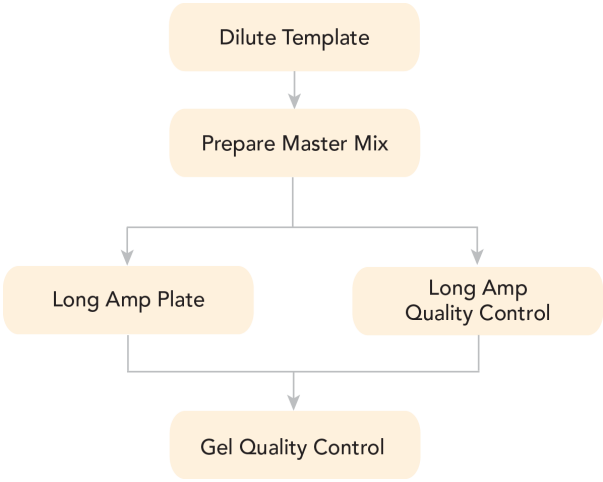
CAUTION

Unexpected results, such as delayed amplification, no amplification, or poor R^2, can be due to the inhibition of qPCR by SYBR Green. If you get unexpected results, Illumina recommends diluting the 100x SYBR Green to 50x and repeating *qPCR Quantitation*.

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.

Figure 9 Long Range PCR Workflow



Consumables

Item	Quantity	Storage	Supplied By
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• 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

Item	Quantity	Storage	Supplied By
• 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

Preparation

- Prepare an ice bucket.
- Remove the following from -25°C to -15°C storage and thaw at room temperature. Place the tubes on ice.
  - Gel Standard

- MasterAmp Extra-Long DNA Polymerase Mix
- Long-amp Master Mix
- Long-amp Primer Mix

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- For the Phasing workflow, pre-program your thermal cyclers as follows:

Program Name	Phasing15	Phasing20QC
Thermal cycler	384-well	96-well

Program	• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
	• 94°C for 1 minute
	• 15 cycles of:
	  • 94°C for 30 seconds
	  • 65°C for 30 seconds
	  • 68°C for 10 minutes
	• 68°C for 10 minutes
	• Hold at 4°C
		• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
		• 94°C for 1 minute
		• 20 cycles of:
		  • 94°C for 30 seconds
		  • 65°C for 30 seconds
		  • 68°C for 10 minutes
		• 68°C for 10 minutes
		• Hold at 4°C

- For the Long-Read workflow, pre-program your thermal cyclers as follows:

Program Name	LongRead21	LongRead26QC
Thermal cycler	384-well	96-well

Program	• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
	• 94°C for 1 minute
	• 21 cycles of:
	  • 94°C for 30 seconds
	  • 65°C for 30 seconds
	  • 68°C for 10 minutes
	• 68°C for 10 minutes
	• Hold at 4°C
		• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
		• 94°C for 1 minute
		• 26 cycles of:
		  • 94°C for 30 seconds
		  • 65°C for 30 seconds
		  • 68°C for 10 minutes
		• 68°C for 10 minutes
		• Hold at 4°C

- Label five new microcentrifuge tubes with a smudge resistant pen as follows:
  - **GST1**
  - **GST2**
  - **GST3**
  - **GST4**
  - **2-Log Ladder**
- Apply a LAP barcode label to a new 384-well PCR plate.

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 34.

3 Dilute the library template with Resuspension Buffer to the following concentration with a total volume of 750 µl. Use the table as an example for tracking and calculating the dilution.
   - 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. Make sure that each well contains 5 µl PCR master mix.

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.

Workflow	Phasing	Long-Read
Program Name	Phasing15	LongRead21

Program	• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
	• 94°C for 1 minute
	• 15 cycles of:
	  • 94°C for 30 seconds
	  • 65°C for 30 seconds
	  • 68°C for 10 minutes
	• 68°C for 10 minutes
	• Hold at 4°C
		• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
		• 94°C for 1 minute
		• 21 cycles of:
		  • 94°C for 30 seconds
		  • 65°C for 30 seconds
		  • 68°C for 10 minutes
		• 68°C for 10 minutes
		• Hold at 4°C

Hours to Complete	3	4.5

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

Long Amp Quality Control

1 Add 50 µl PCR master mix (from *Prepare 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.

Workflow	Phasing	Long-Read
Program Name	Phasing20QC	LongRead26QC

Program	• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
	• 94°C for 1 minute
	• 20 cycles of:
	  • 94°C for 30 seconds
	  • 65°C for 30 seconds
	  • 68°C for 10 minutes
	• 68°C for 10 minutes
	• Hold at 4°C
		• Choose the thermal cycler pre-heat lid option and set to 95°C–100°C
		• 94°C for 1 minute
		• 26 cycles of:
		  • 94°C for 30 seconds
		  • 65°C for 30 seconds
		  • 68°C for 10 minutes
		• 68°C for 10 minutes
		• Hold at 4°C

Hours to Complete	4	5.5

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.

NOTE

- Select samples from across the entire plate and not confined to the perimeter or to a single region of the plate.
- Avoid selecting corner wells.

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.

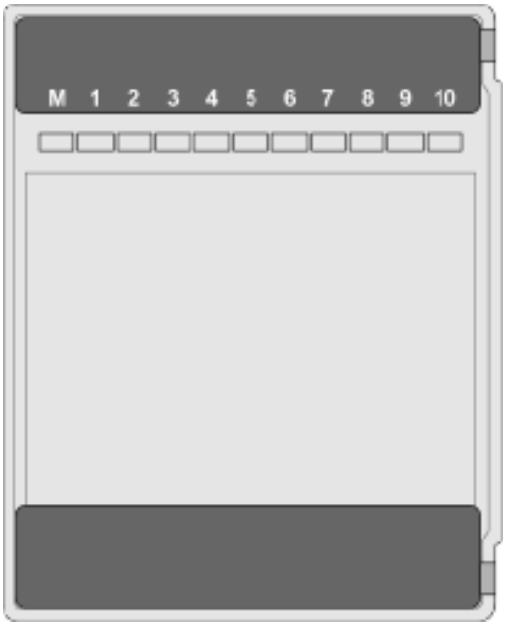
10 Cap the eight-tube strip that contains the pooled samples, briefly centrifuge the strip at 500 × g.

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.

NOTE
Reference Figure 12 while performing steps 12–19.

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

Figure 10 E-Gel EX Agarose Gel, 1% Loading Layout



Lane M—Resuspension Buffer
Lane 1—Diluted 2-Log DNA Ladder
Lane 2—GST1
Lane 3—GST2
Lane 4—GST3
Lane 5—GST4
Lane 6—Pooled sample
Lane 7—QC sample
Lane 8—Resuspension Buffer
Lane 9—Resuspension Buffer
Lane 10—Resuspension Buffer

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 45, into the well of lane 7 of the gel.

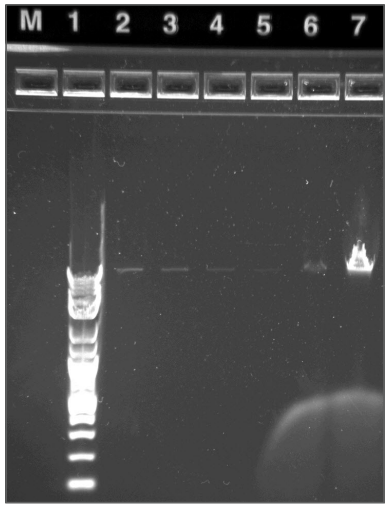
19 Load each of the empty wells with 20 µl Resuspension Buffer (lanes M, 8–10).

20 Select and run the **E-Gel EX 1–2%** program. The run time is 10 minutes.

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.

NOTE
If the band is dimmer than GST4 or brighter than GST1, the quantification is probably inaccurate for the long fragment. To ensure optimal tagmentation performance, reevaluate the *qPCR Quantitation* and repeat *Long Range PCR* using the correct dilution.

Figure 11 Gel Quality Control



A Pooled sample
B QC sample

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Tagmentation* on page 51, 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***.

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
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• 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

Preparation

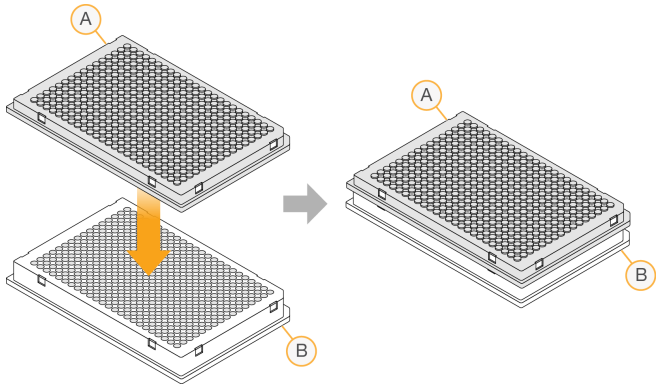
- Prepare an ice bucket.
- Remove the Fragmentation Pre-Mix from -25°C to -15°C storage and thaw at room temperature.
- Remove the LAP plate from 2°C to 8°C storage if it was stored at the conclusion of *Long Range PCR* on page 40.
- Remove the Fragmentation plate from the kit box.

- Pre-program the thermal cycler with the following program and save as **Tag**:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 55°C for 15 minutes
  - 4°C for 5 minutes
  - 72°C for 4 minutes
  - Hold at 4°C

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. Line up the keyed corners and make sure that the wells of the Fragmentation plate are centered in the wells of the LAP plate.

Figure 12 Fragmentation plate on LAP



A Fragmentation plate
B LAP

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. Make sure that each well contains liquid.

8 Centrifuge the stacked Fragmentation plate and LAP plates to 500 × g for 1 minute. Make sure that the LAP plate is on the bottom.

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. The total volume of the mixture is 8 µl.
   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.

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
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• Indexing Plate (IDP)	1	-25°C to -15°C	Illumina
[Optional] TruSeq Synthetic Long-Read DNA Accessory Kit contents:			
• Alignment ring	1 per LAP plate	15°C to 30°C	Illumina
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Preparation

- Remove the IDP plate from -25°C to -15°C storage and thaw at room temperature for at least 10 minutes.
- Pre-program the thermal cycler with the following program and save as **PostTagAmp**
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 94°C for 1 minute
  - 10 cycles of:
    - 94°C for 15 seconds
    - 65°C for 4 minutes
  - Hold at 4°C

Procedure

1. Centrifuge the LAP plate at 500 × g for 1 minute.
2. Centrifuge the IDP plate at 500 × g for 1 minute.

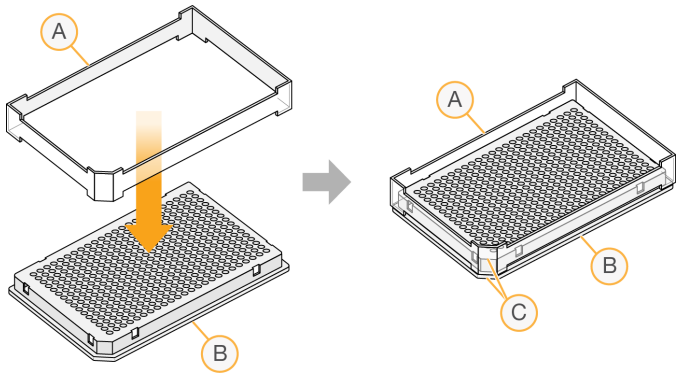
3 Make sure that the droplets are at the bottom of each well of the IDP plate.

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.

Figure 13 Alignment Ring on LAP

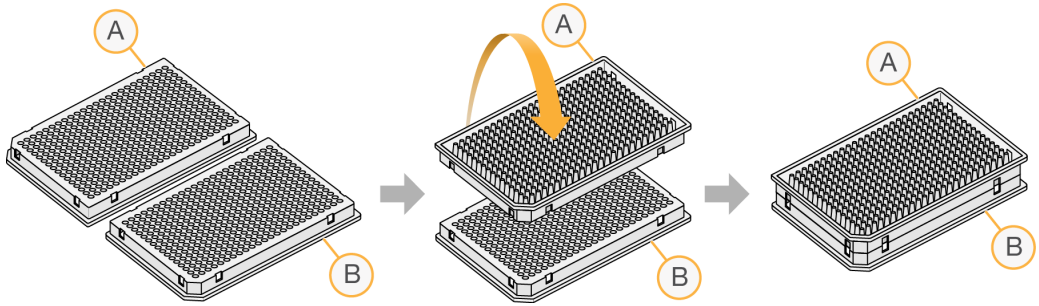


A Alignment ring
B LAP
C Aligned corner notches

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. Make sure that both plates snap together tightly.

Figure 14 IDP on LAP



A IDP
B LAP

NOTE
Surface tension holds the liquid in the IDP plate, so liquid does not come out of the plate when it is turned upside down.

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.

NOTE
Make sure that all wells of the IDP plate are empty. Transfer any remaining supernatant to the corresponding well of the LAP plate using a single channel pipette.

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. The total volume of the mixture is 13 µl.

   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 58, 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.

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
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• 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

NOTE
This procedure is described using a 96-well MIDI plate. However, a microcentrifuge tube can be used instead of the plate.

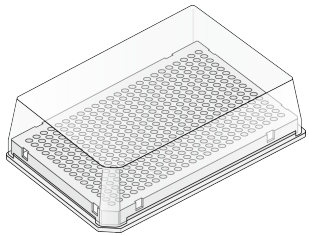
Preparation

- Remove the following from 2°C to 8°C storage and bring it to room temperature:
  - Resuspension Buffer ja
  - Sample Neutralization Buffer
- Apply a PAP barcode label to a new 96-well MIDI plate.
- Label a new eight-tube strip **QC1: Pre Size Selection** with a smudge resistant pen.

Procedure

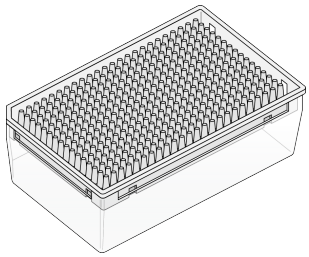
1. Centrifuge the LAP plate at 500 × g for 1 minute.
2. Remove the adhesive seal from the LAP plate, then attach the collection plate to the LAP plate, so that the LAP plate is covered with the collection plate.

Figure 15 Collection Plate Attached to LAP Plate



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

Figure 16 Invert Collection Plate and LAP Plate



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

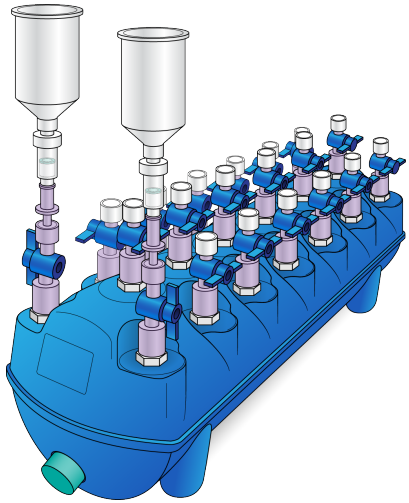
5 Set up a master mix in a new, sterile, nuclease-free 50 ml conical tube using the following:

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.

Figure 17 Zymo-Spin V Column with Reservoir on Vacuum Manifold



8 Turn on the vacuum and leave it on.

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

NOTE
See manufacturer instructions for recommendations for syringe or centrifuge base purification.

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.

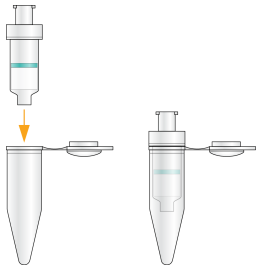
14 Remove the Zymo-Spin V column from the vacuum manifold and unattach the reagent reservoir from the column.

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.

Figure 18 Place Zymo-Spin V Into Microcentrifuge Tube



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 63, 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.

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
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• 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

NOTE
This procedure is described using a 96-well MIDI plate. However, a microcentrifuge tube or 96-well PCR plate can be used instead of the 96-well MIDI plate, with a corresponding magnet.

Preparation

- Remove the PAP plate from -25°C to -15°C storage and thaw at room temperature or from 2°C to 8°C storage and let stand at room temperature, if it was stored at the conclusion of *Pool and Concentrate* on page 58.
  - Centrifuge the PAP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the PAP plate.

- Review best practices for handling magnetic beads. See *Additional Resources* on page 5 for information about TruSeq Synthetic Long-Read DNA Library Prep best practices on the Illumina website.
- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and bring them to room temperature.
- Apply an FSP barcode label to a new 96-well MIDI plate.

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.
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.
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. Take care not to disturb the beads.

   NOTE
   Transfer, do not discard, the supernatant. It contains the DNA of interest.

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.

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.

14 Remove and discard all of the supernatant from each well of the plate. Take care not to disturb the beads.

NOTE

Leave the plate on the magnetic stand while performing the following 80% EtOH wash steps (15–17).

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. Take care not to disturb the beads.

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.

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.

21 Incubate the plate at room temperature for 2 minutes.

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

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 66, 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.

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
TruSeq Synthetic Long-Read DNA Barcode Kit contents:			
• 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

Preparation

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the FSP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Size Selection* on page 63.
  - Let the FSP plate thaw at room temperature.
  - Centrifuge the FSP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the FSP plate.

Quantify Libraries

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Illumina recommends that you quantify your libraries by qPCR.

NOTE
TruSeq Synthetic Long-Read DNA Library Prep library quantitation has been validated using the KAPA Library Quantification Kit specified in the *Consumables and Equipment* on page 82. Follow the KAPA instructions with the KAPA standard.

Follow qPCR instructions included in the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* using the KAPA standard (www.kapabiosystems.com/), with the following modification:

NOTE
You can download the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* from the Kapa Biosystems website (www.kapabiosystems.com).

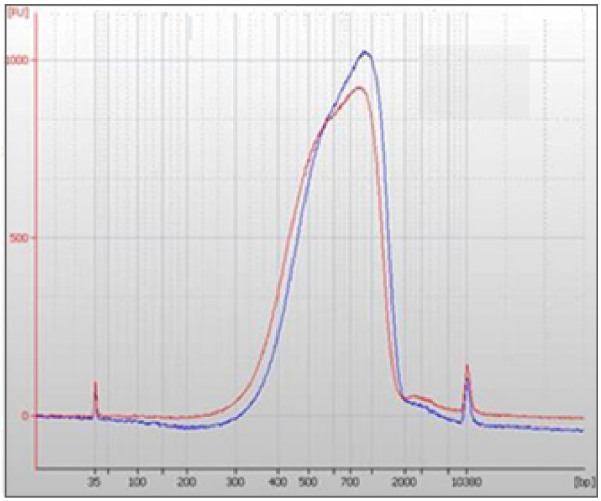
Perform a size adjustment calculation to account for the difference in size between the average fragment length of the library and the KAPA DNA Standard (452 bp). Determine the average fragment length of the library between 200–2000 bp using an Agilent Technologies 2100 Bioanalyzer or equivalent. Use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

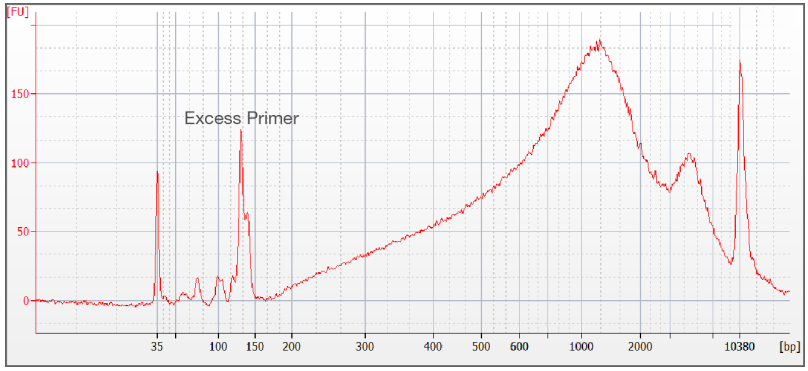
Figure 19 Example TruSeq Synthetic Long-Read DNA Library Prep Final Library Distribution for Human DNA



3 Prepare a 1:5 dilution of the **QC1:Pre-size selection** DNA library, from step 20 of *Pool and Concentrate* on page 58, 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.

Figure 20 Example TruSeq Synthetic Long-Read DNA Library Prep QC1: Pre-Size Selection Library Distribution for Human DNA



5 Do one of the following:
- Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
- Store the sealed FSP plate at -25°C to -15°C.

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Acronyms

Table 2 TruSeq Synthetic Long-Read DNA Library Prep Acronyms

Acronym	Definition
ATL	A-Tailing Mix
CLP	Cleaned Long Fragment Plate
DMSO	Dimethyl sulfoxide
ERP	End Repair Mix
EUC	Experienced User Card
FPM	Fragmentation Pre-Mix
FSP	Final Sample Plate
gDNA	Genomic DNA
GST	Gel Standard
IDP	Indexing Plate
LAD	Long Fragment Adapter
LAP	Long Fragment Amplification Plate
LFP	Long Fragment Plate
LFP2	Long Fragment Plate 2
LIG	Ligation Mix
LMM	Long-amp Master Mix
LPM	Long-amp Primer Mix
NTC	No Template Control

Acronym	Definition
PAP	Pooled Amplicon Plate
PCR	Polymerase Chain Reaction
QLP	Quantification Long Fragment Plate
QMM	qPCR Master Mix
QPM	qPCR Long-amp Primer Mix
QST	qPCR Standard
RSB	Resuspension Buffer
SNB	Sample Neutralization Buffer
SPB	Sample Purification Beads
TDE	Tagment DNA Enzyme

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the protocol.

Table 3 TruSeq Synthetic Long-Read DNA Library Prep Kits and Accessories

Name	Catalog #
TruSeq Synthetic Long-Read DNA Library Prep Kit (4 Samples)	FC-126-1001
TruSeq Synthetic Long-Read DNA Barcode Kit (1 Sample)	FC-126-1002
TruSeq Synthetic Long-Read DNA Barcode Kit (4 Samples)	FC-126-1003
TruSeq Synthetic Long-Read DNA Accessory Kit	FC-126-1004

TruSeq Synthetic Long-Read DNA Library Prep Kit (4 Samples)

The TruSeq Synthetic Long-Read DNA Library Prep Kit contains one Box A and one Box B.

Library Prep Kit - Box A

Store at 2°C to 8°C

This box is shipped at room temperature. As soon as you receive it, store the component at 2°C to 8°C. This box also contains plate barcode labels.

TruSeq Synthetic Long-Read DNA Library Prep Kit (4 Samples) Box A, part # 15048200

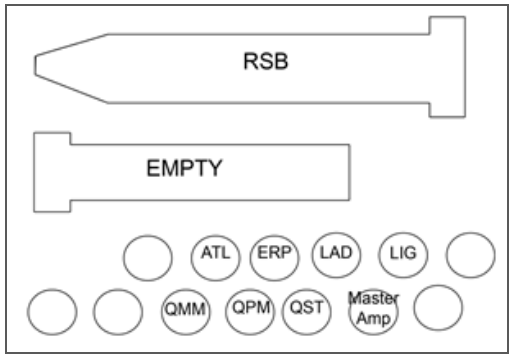
Quantity	Reagent	Part #	Description
1	SPB	15047458	Sample Purification Beads

Library Prep Kit - Box B

Store at -25°C to -15°C

This box is shipped on dry ice. As soon as you receive it, store the components at -25°C to -15°C.

Figure 21 TruSeq Synthetic Long-Read DNA Library Prep Kit (4 Samples) Box B, part # 15048201



Quantity	Reagent	Part #	Description
1	ATL	15046466	A-Tailing Mix
1	ERP	15046463	End Repair Mix
1	LAD	15046469	Long Fragment Adapter
1	LIG	15046468	Ligation Mix
1	MasterAmp	QU13150	MasterAmp Extra-Long DNA Polymerase Mix
1	QMM	15046470	qPCR Master Mix
1	QPM	15046472	qPCR Long-amp Primer Mix
1	QST	15048790	qPCR Standard
1	RSB	15047457	Resuspension Buffer

TruSeq Synthetic Long-Read DNA Barcode Kit (1 Sample)

The TruSeq Synthetic Long-Read DNA Barcode Kit (1 Sample) contains one Box A, one Box B, and one Box C.

Barcode Kit (1 Sample) - Box A

Store at 2°C to 8°C

This box is shipped at room temperature. As soon as you receive it, store the component at 2°C to 8°C. This box also contains plate barcode labels.

TruSeq Synthetic Long-Read DNA Barcode Kit (1 Sample) Box A, part # 15051787

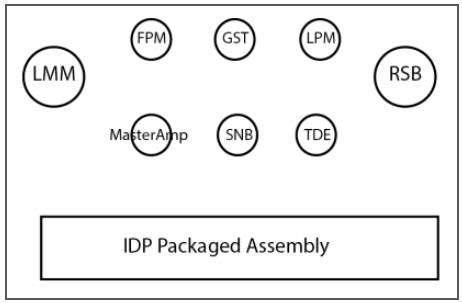
Quantity	Reagent	Part #	Description
1	SPB	15044759	Sample Purification Beads

Barcode Kit - Box B

Store as specified

This box is shipped on dry ice. As soon as you receive it, store the components as specified.

Figure 22 TruSeq Synthetic Long-Read DNA Barcode Kit Box B, part # 15048203



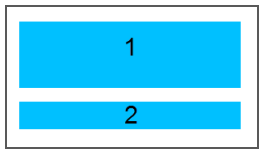
Quantity	Reagent	Part #	Description	Storage Temperature
1	FPM	15046475	Fragmentation Pre-Mix	-25°C to -15°C
1	GST	15048792	Gel Standard	-25°C to -15°C
1	IDP	15049893	Indexing Plate	-25°C to -15°C
1	LMM	15046471	Long-amp Master Mix	-25°C to -15°C
1	LPM	15046473	Long-amp Primer Mix	-25°C to -15°C
1	MasterAmp	QU13150	MasterAmp Extra-Long DNA Polymerase Mix	-25°C to -15°C
1	RSB	15047457	Resuspension Buffer	-25°C to -15°C
1	SNB	15046474	Sample Neutralization Buffer	2°C to 8°C
1	TDE	15047460	Tagment DNA Enzyme	-25°C to -15°C

Barcode Kit (1 Sample) - Box C

Store at room temperature

This box is shipped at room temperature. As soon as you receive it, store the components at room temperature.

Figure 23 TruSeq Synthetic Long-Read DNA Barcode Kit (1 Sample) Box C, part # 15048202



Slot	Part #	Description
1	15049001	Collection Plate
2	15044207	Fragmentation Plate

TruSeq Synthetic Long-Read DNA Barcode Kit (4 Samples)

The TruSeq Synthetic Long-Read DNA Barcode Kit (4 Samples) contains one Box A, four Box Bs, and one Box C.

Barcode Kit (4 Samples) - Box A

Store at 2°C to 8°C

This box is shipped at room temperature. As soon as you receive it, store the components at 2°C to 8°C. This box also contains plate barcode labels.

TruSeq Synthetic Long-Read DNA Barcode Kit (4 Samples) Box A, part # 15051788

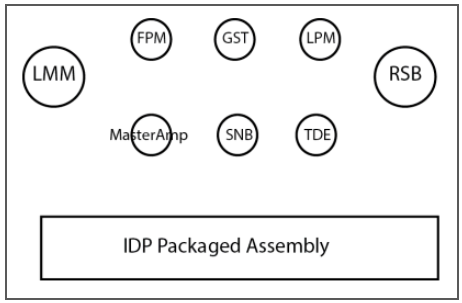
Quantity	Reagent	Part #	Description
4	SPB	15044759	Sample Purification Beads

Barcode Kit - Box B

Store as specified

This box is shipped on dry ice. As soon as you receive it, store the components as specified.

Figure 24 TruSeq Synthetic Long-Read DNA Barcode Kit Box B, part # 15048203



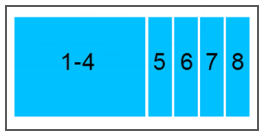
Quantity	Reagent	Part #	Description	Storage Temperature
1	FPM	15046475	Fragmentation Pre-Mix	-25°C to -15°C
1	GST	15048792	Gel Standard	-25°C to -15°C
1	IDP	15049893	Indexing Plate	-25°C to -15°C
1	LMM	15046471	Long-amp Master Mix	-25°C to -15°C
1	LPM	15046473	Long-amp Primer Mix	-25°C to -15°C
1	MasterAmp	QU13150	MasterAmp Extra-Long DNA Polymerase Mix	-25°C to -15°C
1	RSB	15047457	Resuspension Buffer	-25°C to -15°C
1	SNB	15046474	Sample Neutralization Buffer	2°C to 8°C
1	TDE	15047460	Tagment DNA Enzyme	-25°C to -15°C

Barcode Kit (4 Samples) - Box C

Store at room temperature

This box is shipped at room temperature. As soon as you receive it, store the components at room temperature.

Figure 25 TruSeq Synthetic Long-Read DNA Barcode Kit (4 Samples) Box C, part # 15048205



Slot	Part #	Description
1–4	15052780	Collection Plate
5–8	15055281	Fragmentation Plate

TruSeq Synthetic Long-Read DNA Accessory Kit

Store at room temperature

The TruSeq Synthetic Long-Read DNA Accessory Kit contains one box shipped at room temperature. As soon as you receive it, store the components at room temperature.

TruSeq Synthetic Long-Read DNA Accessory Box, part # 15048204

Quantity	Part #	Description
4	15044644	Alignment Ring Fixture

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to the TruSeq Synthetic Long-Read DNA Library Prep protocol.

NOTE

The TruSeq Synthetic Long-Read DNA Library Prep protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 4 User-Supplied Consumables

Consumable	Supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl electronic eight-channel pipette	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1 Kb DNA Extension Ladder	Invitrogen, part # 10511-012

Consumable	Supplier
2-Log DNA Ladder (0.1–10.0 kb)	NEB, part # N3200L
2-propanol (Isopropanol)	General lab supplier
15 ml conical tube	General lab supplier
15 ml RNase/DNase-free reagent reservoirs	General lab supplier
50 ml conical tube	General lab supplier
96-well 0.3 ml skirtless PCR plates or Twin.Tec 96-well PCR plates	E&K Scientific, part # 480096 Eppendorf, part # 951020303
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
Axygen 384 well PCR microplate	VWR, part # 10011-194 or 47744-810
Compression Mats for PCR Plates	VWR, part # 10011-006
Dimethyl sulfoxide (DMSO)	General lab supplier
DNA Binding Buffer	Zymo Research, part # D4003-1-25
DNA Wash Buffer	Zymo Research, part # D4003-2-24
E-Gel EX Agarose Gels, 1%	Invitrogen, catalog # G4020-01
E-Gel NGS 0.8% Agarose Gels	Invitrogen, catalog # A25798
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
g-TUBE	Covaris, part # 520079
Ice bucket	General lab supplier
KAPA Library Quantification Kit - Illumina/Universal	KAPA Biosystems, part # KK4824

Consumable	Supplier
Lab pen	General lab supplier
Lab tissue, low-lint	VWR, part # 21905-026 or equivalent
Microcentrifuge Tubes with Attached Flat Caps, Neptune (1.6 ml)	VWR, part # 89126-722
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Needles (22 1/2 gauge)	General lab supplier
PCR-grade water	General lab supplier
QIAquick Gel Extraction Kit	QIAGEN, part # 28704
qPCR plate and seal	General lab supplier
Qubit dsDNA BR Assay Kit	Life Technologies catalog # Q32850
Qubit dsDNA HS Assay Kit	Life Technologies catalog # Q32851
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNaseZap (to decontaminate surfaces)	General lab supplier
ROX Reference Dye	Invitrogen, part # 12223012
Ruler	General lab supplier
SYBR Green Nucleic Acid Gel Stain	Invitrogen, part # S7585
x-tracta gel extractor	USA Scientific, catalog # 5454-0100

Consumable	Supplier
Zymo-Spin V with Reservoir	Zymo Research, part # C1016-25 or C1016-50
[Optional - for BluePippin size selection] 0.75% Agarose gel cassettes , Dye Free, Low Range 10/pk S1	Sage Science, catalog # BLF7510
[Optional - for BluePippin size selection] TE buffer	General lab supplier

Table 5 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler with heated lid	Bio-Rad, part # ALS-1296G or equivalent
384-well thermal cycler	Bio-Rad, part # 185-1138 or equivalent
2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
Agilent High Sensitivity DNA Kit	Agilent, part # 5067-4626
Dark reader transilluminator	Clare Chemical Research, part # DR195M
E-Gel iBase Power System	Life Technologies, catalog # G6400
High-Speed Microplate Shaker	VWR, catalog # 13500-890 (110 V/120 V) VWR, catalog # 14216-214 (230 V)
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
Novex Gel Knife	Life Technologies catalog # EI9010

Equipment	Supplier
qPCR system See *qPCR Systems* on page 87.	General lab supplier
Qubit 2.0 Fluorometer	Life Technologies, catalog # Q32866
Vacuum manifold	Promega, catalog # A7231 or QIAGEN, part # 19413
[Optional - for BluePippin size selection] BluePippin Size Selection System	Sage Science, catalog # BLU0001
[Optional - for SYBR Green calibration] NanoDrop Spectrophotometer	Thermo Scientific, catalog # ND-1000 or ND-2000

qPCR Systems

The following table lists the validated qPCR systems for the TruSeq Synthetic Long-Read DNA Library Prep protocol. Either a 96-well or 384-well qPCR system is required.

Equipment	Supplier
CFX96 Touch Real-Time PCR Detection System[1]	Bio-Rad, part # 185-5195
CFX384 Touch Real-Time PCR Detection System	Bio-Rad, part # 185-5484
7900HT Fast Real-Time PCR System with 384-Well Block Module[2]	Life Technologies, part # 4329001
Mx3000P qPCR System	Agilent, part # 401511
LightCycler 480 Instrument II (384-well version)	Roche, part # 05015243001

1. Illumina recommends using CFX Manager software version 3.0 with Cq Determination mode: Single Threshold; Baseline Setting:Baseline Subtracted Curve Fit and Apply Fluorescent Drift Correction for data analysis. This can correct for abnormalities in fluorescence intensity of the standard curve caused by the instrument. For software installation, contact Bio-Rad.
2. When setting up the SDS software before running the qPCR, select only the wells with samples. If the entire plate is selected, the resulting file is too large to open by the SDS software. For more information, contact Life Technologies.

Barcode Sequences

TruSeq Synthetic Long-Read DNA Barcode Kits contain the following the barcodes.

Name	Barcode	Name	Barcode	Name	Barcode
BC001	TGAACGTG	BC020	TCCTTGGT	BC039	TGCGCTAT
BC002	TCATGTCC	BC021	GAACCAGT	BC040	GAGATGTC
BC003	TGTGAGGT	BC022	CTGAGGAT	BC041	CGTTCGTT
BC004	GAGTGAAC	BC023	TTCGAGTG	BC042	GTCAATGC
BC005	TGGATCAG	BC024	TTGTCCAG	BC043	GTTGTTCC
BC006	CTCTCCTA	BC025	GTTACTGG	BC044	CCAGAACA
BC007	TAAGGAGC	BC026	GCCATTCA	BC045	TTGCATGG
BC008	CGTAAGTG	BC027	CTATGGAC	BC046	CTACCGTT
BC009	CCATCTTC	BC028	CCTAGTTG	BC047	GACGTAAG
BC010	TACAAGGC	BC029	TCCAACGT	BC048	TCGCTACA
BC011	GTCTTGAC	BC030	TTACGGCT	BC049	CTTGACGA
BC012	TCTGGATC	BC031	CGATTCGT	BC050	TCAATCCG
BC013	GCAGTAGA	BC032	CTTAGACC	BC051	CCGGATTA
BC014	TAAGGTCG	BC033	CTTGGTTC	BC052	CGGTATTG
BC015	TTCCAAGC	BC034	CGAACTGA	BC053	GGATTACC
BC016	CAACAGGA	BC035	CGGATACA	BC054	TGAGGACT
BC017	GATCTGGT	BC036	GACTAACC	BC055	GTGTCGAA
BC018	GAGAACCT	BC037	GAACAGTG	BC056	TTGCCGAT
BC019	TTCGGAAG	BC038	TTCACGAG	BC057	CGACTGAA

Name	Barcode	Name	Barcode	Name	Barcode
BC058	GTTCAAGG	BC079	GAAGACTC	BC100	TGTCGAGT
BC059	GGCCTAAT	BC080	CTGGTTAG	BC101	TCTCAGCT
BC060	TGATGCAC	BC081	GCAGGTTA	BC102	GGAATTGC
BC061	TGGTGGTA	BC082	CTAGACCT	BC103	CTGTTGTG
BC062	CCGTACTT	BC083	CACTTCCT	BC104	GTACGACA
BC063	GCCGAATT	BC084	GCCATAAC	BC105	CTTGCACT
BC064	CGTGTTCT	BC085	CAATCCTG	BC106	TAGGTTCC
BC065	TTGAGCTC	BC086	GGTTGTCT	BC107	TCTGACCA
BC066	GTTGGAGA	BC087	CGTACCAT	BC108	GGTCAGAT
BC067	GAAGTGCA	BC088	GTTAGCGT	BC109	CTCATGGT
BC068	TCAACGCA	BC089	GTGTTCTC	BC110	TTAGCGGA
BC069	GCTAGTGA	BC090	CACTAGTG	BC111	TCACAACG
BC070	CAGGTAGT	BC091	CCACTATC	BC112	CAGCCATT
BC071	TGACTTGG	BC092	GCTAACTG	BC113	TGTGCGAA
BC072	GGACGATT	BC093	TAACTCGC	BC114	CTCCATGA
BC073	GTATCAGC	BC094	CGGTCAAT	BC115	CGACTACT
BC074	GGTGATTG	BC095	GCGTACAA	BC116	TGATAGCG
BC075	CTGTAGCA	BC096	CCTGGTAT	BC117	GGACAAGA
BC076	CATAGGTC	BC097	CCTGAATG	BC118	TGTTCCTC
BC077	GAGTCCTA	BC098	CGTTACCA	BC119	GATGCATG
BC078	TCCATCTC	BC099	GTTCTCCT	BC120	CTTCCGAA

Name	Barcode	Name	Barcode	Name	Barcode
BC121	CTGTCTGA	BC142	TCGGTATG	BC163	CGAGTGTT
BC122	GGCAATCT	BC143	CTTCGTCT	BC164	CTATCTCG
BC123	TGGTTAGG	BC144	GGTATGGA	BC165	GTTACCAC
BC124	GAGTTCGT	BC145	CACATTGG	BC166	CGATACAG
BC125	CGCAATAC	BC146	TACTGTGC	BC167	TGGTCTCA
BC126	TCTCGCTA	BC147	CTTGATCG	BC168	TTAGGCTG
BC127	TCATCTGG	BC148	TCCTAAGG	BC169	GCCTCATA
BC128	CGAAGATC	BC149	TCGCAGAA	BC170	CCATGGTT
BC129	TTACCTCC	BC150	CTCTGTAG	BC171	TAGACGCT
BC130	CAAGTCGA	BC151	CAAGCCAT	BC172	TTGGCAAC
BC131	CAAGTGAC	BC152	GCGAATAG	BC173	TCGAGACT
BC132	GTGTTACG	BC153	TCGTTGAG	BC174	TTGACCGT
BC133	GCACTCAA	BC154	TGACACTG	BC175	CATCTACC
BC134	TGAAGAGG	BC155	GATGGACT	BC176	GCATTGTC
BC135	TGAGACGA	BC156	CCTTGACT	BC177	GTGGATGT
BC136	CATCTGAG	BC157	TCTGTTGG	BC178	CTTGAGAC
BC137	GTCTGGTA	BC158	CCAACTAG	BC179	TCGTCAGT
BC138	GGATGTTC	BC159	CATCACTC	BC180	TTCGTGCA
BC139	GAAGCTGA	BC160	GGATCTAG	BC181	GACTAGGA
BC140	CCGTTAAC	BC161	TCAGGCAA	BC182	GGTCGTAA
BC141	TGGCAATC	BC162	CGCTTATG	BC183	GATAGGCA

Name	Barcode	Name	Barcode	Name	Barcode
BC184	CACAGCAT	BC205	TAACGGAC	BC226	TGCTGATC
BC185	TGTGGCTT	BC206	TAGGCGTA	BC227	TTCCGTGT
BC186	TTCGTTGC	BC207	GGTCCATA	BC228	GATTACGG
BC187	GTTGTCAG	BC208	TGCACACT	BC229	CAGTAGGT
BC188	TTGGCTCT	BC209	GATTCGAG	BC230	GGTATTCG
BC189	GATCGATC	BC210	CGGAACAA	BC231	CGGTTCTA
BC190	CCTAGGAA	BC211	TGGAGAAC	BC232	TTAGAGCC
BC191	CCTCTTCA	BC212	CCACATCT	BC233	CGCGAATA
BC192	GGAAGGTA	BC213	CATTCCGT	BC234	TCTACCAG
BC193	TCACGTGA	BC214	GTGGTGTT	BC235	TTGCGATG
BC194	CACCAGAT	BC215	CTCTAACG	BC236	CCATCCAA
BC195	TCTGAGAG	BC216	TGCTATCC	BC237	CTTCCATC
BC196	TATAGCGG	BC217	GTCGGTAA	BC238	TGTAAGCC
BC197	TTGCGTAC	BC218	GCTTCTTG	BC239	CTCAAGTC
BC198	GGTAGAAG	BC219	CCAAGAGT	BC240	CCTGTGTA
BC199	CAAGCATC	BC220	CTGATCCT	BC241	CATGAAGC
BC200	CACGACTT	BC221	GCAACATC	BC242	GGATTCTG
BC201	GAAGAAGG	BC222	TCCAGGTA	BC243	GAATGCCA
BC202	GACCATAG	BC223	TGCCAGTA	BC244	GTGATGCA
BC203	GACGGATA	BC224	GTCAACAG	BC245	TCTTGGCA
BC204	CAGGAGAA	BC225	GCGTCTAT	BC246	CGTCTAGA

Name	Barcode	Name	Barcode	Name	Barcode
BC247	TCGTGTTG	BC268	TCCGATTC	BC289	CATTGCAG
BC248	GTCTCCAT	BC269	CTAACCTC	BC290	TGGAATCG
BC249	TGTCCTGA	BC270	GCATCACT	BC291	GTGAACGA
BC250	CCTATCGT	BC271	TACGATGG	BC292	TGCAGTAG
BC251	GTCTTCGA	BC272	CTACAACC	BC293	GCGTATTC
BC252	GTACCAAG	BC273	CTAGCTAC	BC294	CGGTAAGA
BC253	CCTTATCC	BC274	TAGCGCTT	BC295	GGTTCAAC
BC254	TATTCGCC	BC275	CAGGATCT	BC296	TAGAGGTG
BC255	CACTGGAA	BC276	CACTTAGC	BC297	TGGAAGGA
BC256	CTCAACCA	BC277	TTGGTCGA	BC298	CTACGCTA
BC257	TTCCTACG	BC278	CCAATGGA	BC299	TGGTTGCT
BC258	TTGTAGGC	BC279	TAGGCTAG	BC300	CTGGAATC
BC259	GCAGTCTT	BC280	TGTTGGAG	BC301	CGTGTAAG
BC260	GATCATGC	BC281	GGTACTTC	BC302	TCGCTTGT
BC261	GCGTGATT	BC282	GCTTACCT	BC303	GGAATGCT
BC262	TGAGTGAG	BC283	CGAGGTAA	BC304	TCCACATG
BC263	CTCACAAC	BC284	CTCGTCAA	BC305	TGAGCTTC
BC264	CGCTGTTA	BC285	CAATGTGG	BC306	CTCTACGT
BC265	CTAAGGCA	BC286	CAACCACA	BC307	GTTAAGCG
BC266	CAACTAGG	BC287	TACGTCAC	BC308	GAAGGTAC
BC267	GTGCTTGA	BC288	CAGACTTC	BC309	GAGCGTTA

Name	Barcode	Name	Barcode	Name	Barcode
BC310	GTGAAGAC	BC331	CGAGAAGT	BC352	GTTGCGAT
BC311	GATCACCA	BC332	CCTTCAGA	BC353	GCATAGGT
BC312	GAACCTTC	BC333	CTGACGTA	BC354	CGTTGTAC
BC313	GCTCGAAT	BC334	TCCGTTCT	BC355	TTCCGCAA
BC314	CCTAACAC	BC335	GTCTCTTC	BC356	TCAAGGAG
BC315	GACCTAGA	BC336	GCAAGTCT	BC357	TTCCTGTC
BC316	CATGGTGA	BC337	TACCTCCA	BC358	GAGGCAAT
BC317	GGCAAGAA	BC338	TGAAGCCA	BC359	TCGGACAT
BC318	CGAATCAC	BC339	CATCCTAC	BC360	TATGTCCG
BC319	GTTCCTCA	BC340	CCTATACG	BC361	TAGACCAC
BC320	TATCGACG	BC341	CTCACTCT	BC362	GTAGTTGG
BC321	TCCTTACC	BC342	GAGCTGAA	BC363	TGGACCTA
BC322	GAAGCGTT	BC343	CCATGAAG	BC364	GATCTCAC
BC323	GTAGGATC	BC344	TCACCAAC	BC365	GCTAGGTT
BC324	GTGCTAAC	BC345	GGAACCAA	BC366	GACGACAA
BC325	CTAACAGG	BC346	CCGTGTAA	BC367	CAATCGCT
BC326	GGCTTGTT	BC347	GGTGAACA	BC368	TGTTGCGA
BC327	TTGCCAGA	BC348	GTGAGTTG	BC369	TTGTGACC
BC328	GAATCCAC	BC349	CAGTGATG	BC370	TGTCTGCA
BC329	CTGATTGC	BC350	TGGTACGT	BC371	CCAATTCC
BC330	CCAGTTGT	BC351	GGACTTCA	BC372	TGTGTACC

Name	Barcode	Name	Barcode	Name	Barcode
BC373	TTGCACCT	BC377	TGTGGTCA	BC381	TAGGACTG
BC374	TACTTGCG	BC378	GGCTACTA	BC382	TGTCCAAG
BC375	TGATTGGC	BC379	GTCATGTG	BC383	TATGTGGC
BC376	TTCAGTCC	BC380	TCGGAAGA	BC384	GCTTAAGC

BluePippin Size Selection

Gel-based size selection of ligated products is required to ensure stringent purification of long fragments and the elimination of smaller fragments that can bias the results. The BluePippin System can be used as an alternative to the gel-based method of size selection of ligated products described in *Purify Ligation Products and Size Selection* on page 22.

The BluePippin system performs pulsed-field electrophoresis for resolving and automatically collecting high molecular weight DNA. It has the advantage of automated size selection and purification and little risk to cross-contamination from sample to sample in the same cassette. For detailed instructions on operating the instrument, see BluePippin documentation or contact Sage Science.

NOTE

This procedure requires using 1 µg of genomic DNA normalized to 20 ng/ul as input. This is to compensate for lower DNA recovery with this alternate protocol than the E-Gel based size selection.

User-supplied Consumables

Item	Quantity	Storage
0.75% Agarose gel cassette, low range S1	1 per 4 samples	15°C to 30°C
TE buffer	10 µl per sample	15°C to 30°C
Lab pen	1	15°C to 30°C
Microcentrifuge tubes	1 per sample	15°C to 30°C

Preparation

- Remove the CLP plate from 2°C to 8°C storage, if it was stored at the conclusion of *Clean Up LFP2* on page 20.
  - Let the plate stand to bring it to room temperature.
  - Centrifuge the plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the plate.
- Bring the loading solution that comes with the cassette to room temperature.
- Prepare the BluePippin instrument and cassette according to manufacturer instructions.

- Use BluePippin software v.6.0 with cassette definition 12 or higher.
- Pre-program the BluePippin with the following program and save as **HF 7-11kb, Lane 1: S1 Marker**:

Option	Setting
Run Time	8:00 hr
Ref Lane	1
BP Target	9000 (bp)
BP Start	7000 (bp)
BP End	11000 (bp)
BP Pause	0
BP Range Flag	broad

  - Apply references to all lanes.
  - End the run when elution is complete.
  - Select the **0.75% DF 3-10kb Marker S1 - Improved Recovery** cassette.
- Label one new microcentrifuge tube for each sample with **size-selected** [sample name], using a smudge resistant pen.

Procedure

1. Add 10 µl TE buffer to each well of the CLP plate.
2. Add 10 µl loading solution to each well of the plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Perform steps 3–9 according to manufacturer instructions in the *BluePippin Quick Guide BLF7510 marker S1*.

3. Calibrate the optics.
4. Inspect the gel cassette.
5. Prepare the cassette for loading.
6. Load the DNA marker S1 into well 1.

7 Transfer 40 µl from each well of the plate to wells 2–5.

8 Close the lid, then select and run the **HF 7-11kb, Lane 1: S1 Marker** program.

9 Allow the samples to remain on the cassette after the run for 16–18 hours. Make sure that the wells are sealed to prevent evaporation.

CAUTION

Illumina recommends overnight elution for maximal recovery of DNA. Illumina has validated the longer elution time and it has been confirmed by Sage Science as a safe protocol.

10 Transfer each sample from the cassette to a new microcentrifuge tube labeled **size-selected** [sample name].

11 Proceed to *Validate Library* on page 29.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library*, 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 1 month. Avoid a freeze-thaw cycle.

Calibrate Diluted SYBR Green

This process measures the absorbance of 100x diluted SYBR Green on a NanoDrop instrument for the preparation of *qPCR Quantitation* on page 30.

NOTE
Perform these procedures according to NanoDrop manufacturer instructions.

User-supplied Consumables

Item	Quantity	Storage
Dimethyl sulfoxide (DMSO)	as needed	15°C to 30°C
Lab tissue, low-lint	as needed	15°C to 30°C
PCR-grade water	as needed	15°C to 30°C
100x SYBR Green stock (from *qPCR Quantitation* on page 30 preparation)	1 µl	-25°C to -15°C

Preparation

- Remove 100x SYBR Green from -25°C to -15°C storage and thaw to room temperature.

NOTE
When removing 100x SYBR Green from storage, thaw it completely, then mix thoroughly, while protecting it from light.

Measure Absorbance

1. Open the **Nanodrop ND1000** software and select **UV-Vis** from the method panel.
2. Initialize the NanoDrop instrument.
3. Blank the NanoDrop instrument with 100% DMSO.
4. Adjust the NanoDrop instrument settings:
   - For the NanoDrop ND-1000, do not select **Normalize**.
   - For the NanoDrop ND-2000, do not check **Baseline correction.**

5 Using 1 µl 100x SYBR Green stock, measure its absorbance at wavelengths 480 nm, 490 nm, 494 nm, 500 nm, and 510 nm and record the values.

6 Wipe out the sample on the stage with a low-lint lab tissue and then clean the stage with a water wetted low-lint lab tissue. Clean the stage again with a dry low-lint lab tissue.

7 Repeat step 5–6 two times to collect two additional replicate readings of the 100x SYBR Green stock.

Adjust Concentration

1 Calculate the average absorption of the three replicates for each wavelength. The maximum absorption value recorded should be at one wavelength 490–510 nm.

2 The ideal $Abs_{494\pm3\ nm}$ of 100x SYBR Green stock is 0.5–0.6, which indicates that the concentration is 100x. If the maximum absorbance reading is in the 0.5–0.6 range, clean the instrument, then return to the preparation of *qPCR Quantitation* on page 30.

3 If the maximum absorbance reading is out of the 0.5–0.6 range, calculate the real concentration of the 100x SYBR Green stock using the following equation.

$$\frac{0.55}{100} = \frac{\text{maximum Abs}}{\text{real concentration of SYBR}}$$

For example, if the maximum Abs of the 100x SYBR Green is 0.8, the real concentration of 100x SYBR Green is 145.5x.

4 Adjust the concentration of the 100x SYBR Green stock to 100x based on the calculation in step 3.

- For concentrations > 100x, dilute 100x SYBR Green stock with DMSO.
- For concentrations < 100x, make new 100x SYBR Green stock from SYBR Green 10,000x concentrate as follows:
  a Mix thawed SYBR Green 10,000x thoroughly
  b Add 5 µl SYBR Green to 495 µl of DMSO to dilute

5 Repeat *Measure Absorbance* on page 98 to measure the adjusted or new 100x SYBR Green stock until the maximum absorption is 0.5–0.6.

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 6 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 7 Illumina Customer Support Telephone Numbers

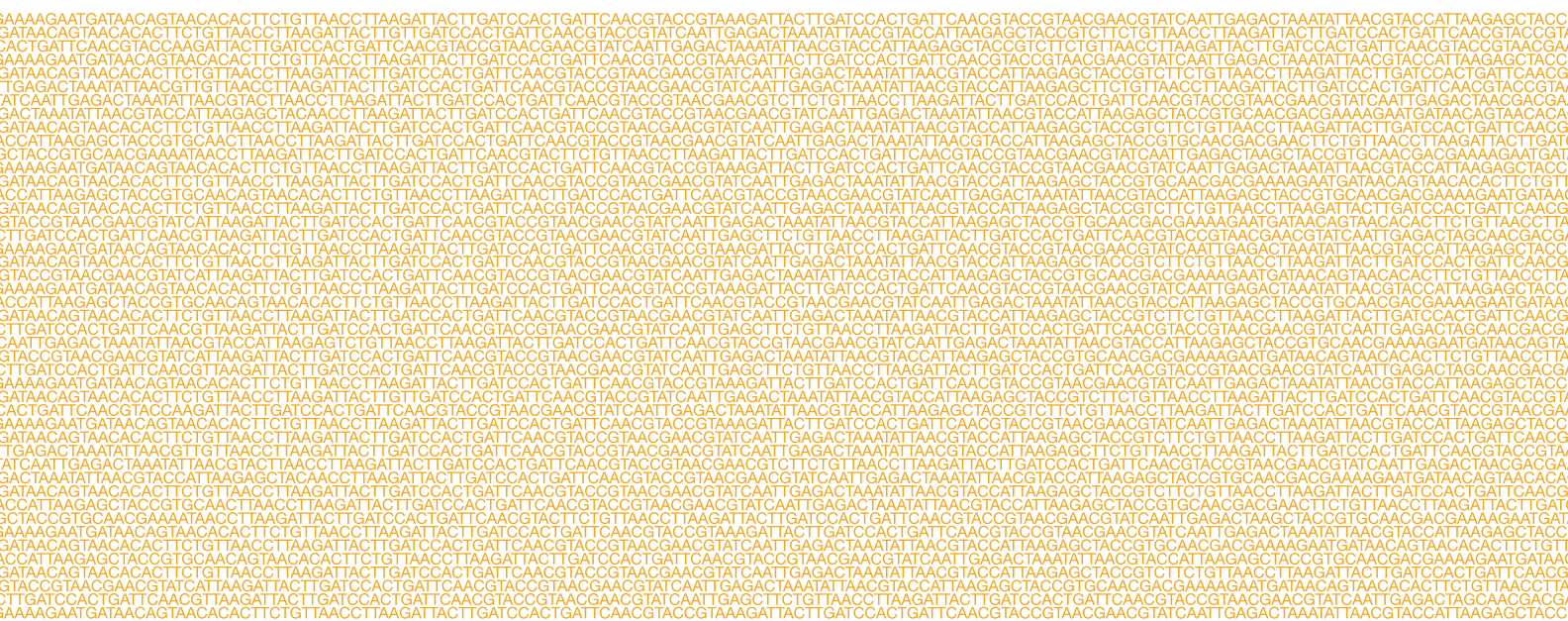
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to support.illumina.com, select a product, then click **Documentation & Literature**.



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