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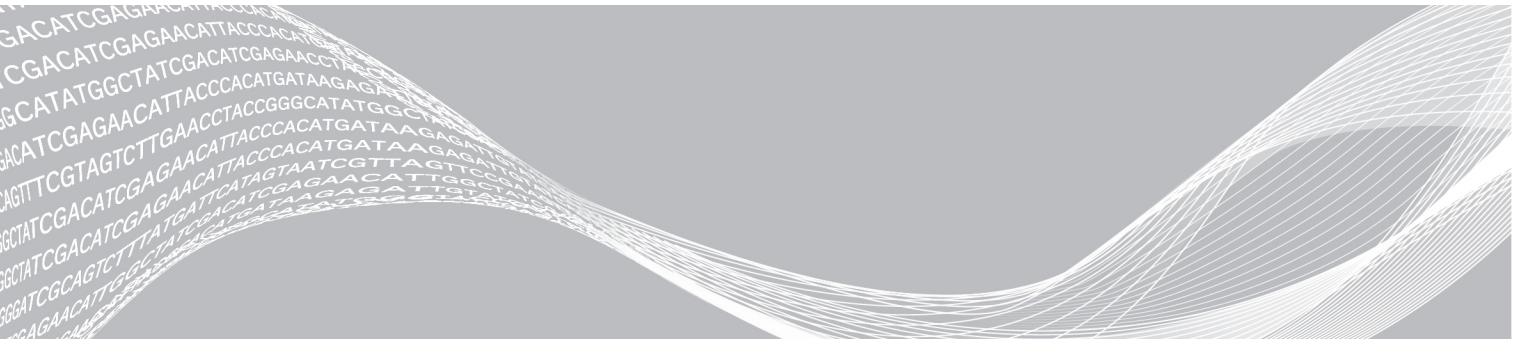
TruSeq DNA PCR-Free

Reference Guide



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Chapter 1 Overview

Introduction

This protocol explains how to prepare up to 96 libraries starting from genomic DNA (gDNA) using the Illumina® TruSeq® DNA PCR-Free library prep workflow. The goal is to add adapter sequences to DNA fragment ends to create indexed libraries for single-read or paired-end sequencing.

The TruSeq DNA PCR-Free Library Prep workflow protocol includes the following features.

- Streamlined workflow:
  - Size-selection beads and master-mixed reagents reduce reagent containers and pipetting.
  - Universal adapter to prepare DNA libraries for single-read, paired-end, and indexed sequencing.
  - One workflow with options for processing low sample (LS) and high sample (HS) numbers.
- Flexible throughput:
  - 24- and 96-sample workflow configurations accommodate a range of experiments.
  - Support for non-indexed sequencing and low-plexity pooling.
  - Optimized shearing for whole-genome resequencing with insert sizes of 350 bp or 550 bp.
- Inclusive components:
  - Library Prep components include library prep reagents excluding index adapters.
  - Index adapter components must be purchased separately. See *Supporting Information* on page 21 for more details.

DNA Input Recommendations

Quantify the input gDNA and assess the quality before starting library preparation. For best results, use the following input amounts.

Insert Size	Input gDNA
350 bp	1 µg
550 bp	2 µg

Lower input amounts result in low yield and increased duplicates.

Quantify Input DNA

Quantify input DNA per the following recommendations:

- Successful library prep depends on accurate quantification of input DNA.
- Use fluorometric-based methods for quantification, such as Qubit or PicoGreen to provide accurate quantification for dsDNA. UV spectrophotometric based methods, such as the Nanodrop, measures any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides, which can give an inaccurate measurement of gDNA.
- Quantification methods depend on accurate pipetting methods. Do not use pipettes at the extremes of volume specifications. Make sure that pipettes are calibrated.

Assess DNA Quality

Absorbance measurements at 260 nm are commonly used to assess DNA quality:

- The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity. Values from 1.8 through 2.0 indicate relatively pure DNA.
- The presence of RNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
- Make sure that samples are free of contaminants.

Additional Resources

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

Resource	Description
Custom Protocol Selector	A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSeq DNA PCR-Free Checklist (document # 1000000040870)	Provides a checklist of the protocol steps, and is intended for experienced users.
Index Adapter Pooling Guide (document # 1000000041074)	Provides pooling guidelines for preparing libraries for sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Illumina Experiment Manager Guide (document # 15031335) and *IEM TruSeq DNA, RNA, or ChIP Quick Reference Card (document # 15037152)*	Provides information about creating and editing sample sheets.
BaseSpace Sequence Hub help	Provides information about BaseSpace® Sequence Hub, a data analysis tool.
Local Run Manager Software Guide (document # 1000000002702)	Provides an overview of the Local Run Manager (LRM) software, instructions for using software features, and instructions for installing analysis modules on the instrument computer.

Visit the TruSeq DNA PCR-Free workflow support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Chapter 2 Protocol

Introduction

This chapter describes the TruSeq DNA PCR-Free Library Prep workflow protocol.

- Follow the steps in the order shown, using the specified volumes and incubation parameters.
- Before proceeding, confirm the delivered contents and make sure that you have the required equipment and consumables.
- Review Best Practices from the TruSeq DNA PCR-Free Library Prep workflow support page on the Illumina website.

This protocol provides one workflow with variations for differences in sample numbers. [HS] and [LS] identify the appropriate option for your number of samples. Expect equivalent results from either option, but the HS option can yield more consistent results between samples.

Table 1 Workflow Variations

Workflow Variable	HS	LS
24-Sample Workflow	Process > 24 samples with index adapter tubes*	Process ≤ 24 samples with index adapter tubes*
96-Sample Workflow	Process > 24 samples with index adapter plate	Process ≤ 24 samples with index adapter plate
Plate Type	96-well Hard-Shell PCR plate 96-well midi plate	96-well 0.3 ml PCR plate 96-well midi plate
Incubation Equipment	Microheating systems	96-well thermal cycler
Mixing Method	Microplate shaker	Pipetting

* Combine the Set A and Set B indexes to pool up to 24 libraries.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between *each sample*.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate, and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

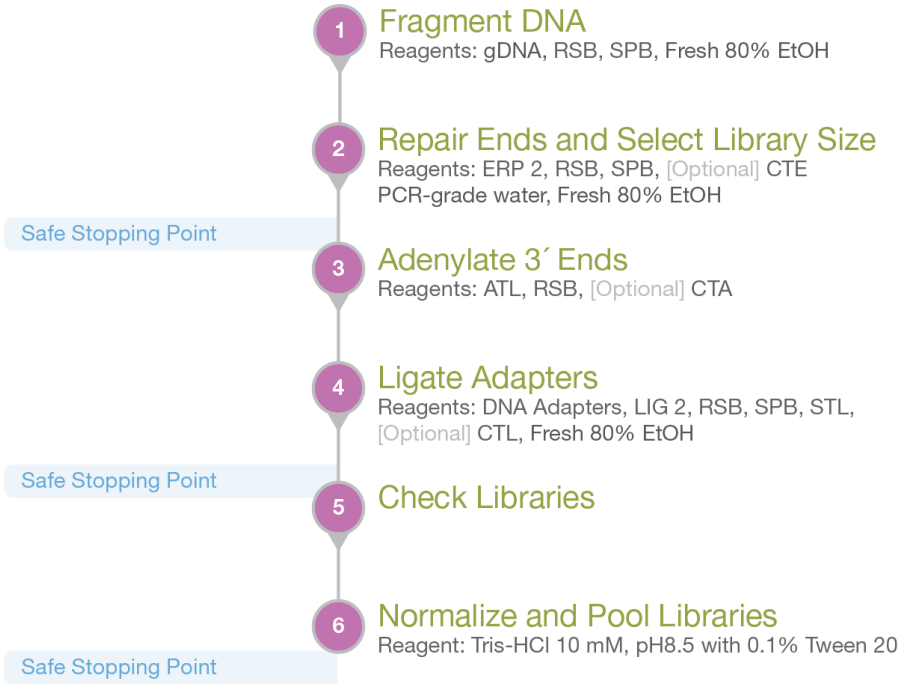
Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the specified magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.

Library Prep Workflow

The following diagram illustrates the workflow using a TruSeq DNA PCR-Free Library Prep workflow. Safe stopping points are marked between steps.

Figure 1 TruSeq DNA PCR-Free Workflow



Prepare for Pooling

When pooling samples for sequencing, use IEM, LRM, or BaseSpace Prep Tab to record information about your samples before beginning library preparation.

- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use LRM and BaseSpace Prep Tab to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

Review the planning steps in the *Index Adapter Pooling Guide (document # 1000000041074)* when preparing libraries that require balanced index combinations.

Fragment DNA

This step fragments to an insert size of 350 bp or 550 bp. Covaris shearing generates double-stranded DNA (dsDNA) fragments with 3′ or 5′ overhangs.

Consumables

- gDNA samples
  - [350 bp insert size] 1 µg per sample
  - [550 bp insert size] 2 µg per sample
- RSB (Resuspension Buffer)

- SPB (Sample Purification Beads)
- Barcode labels
  - CFP (Covaris Fragmentation Plate)
  - CSP (Clean Up Sheared DNA Plate)
  - DNA (DNA Plate)
  - IMP (Insert Modification Plate)
- Freshly prepared 80% ethanol (EtOH)
- Plates
  - [HS] 96-well midi plates (3)
  - [HS] 96-well Hard-Shell 0.3 ml PCR plate (1)
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (4)
- Covaris tubes (1 per sample)
- Microseal 'B' adhesive seal

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
RSB	-25°C to -15°C	Thaw at room temperature. After the initial thaw, store at 2°C to 8°C.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Turn on and set up the Covaris instrument per manufacturer guidelines.

3 [HS] Calibrate the microplate shaker with a stroboscope and set to 1800 rpm.

4 Apply barcode labels to plates.

Barcode Label	Plate for HS	Plate for LS
DNA	Midi	PCR
CFP	Hard-Shell PCR	PCR
CSP	Midi	PCR
IMP	Midi	PCR

Procedure

Normalize gDNA

1 Quantify gDNA using a fluorometric-based method.

2 Normalize gDNA samples with RSB to a final volume of 55 µl in the DNA plate.
  - 1 ug for a 350 bp insert size
  - 2 ug for a 550 bp insert size

3 [HS] Mix and centrifuge as follows.

   a Shake at 1800 rpm for 2 minutes.
   b Centrifuge at 280 × g for 1 minute.

4 [LS] Pipette to mix, and then centrifuge briefly.

Fragment DNA

1 Transfer 52.5 µl DNA samples to separate Covaris tubes. Use the wells of the CFP plate to hold the tubes upright.

2 Centrifuge at 280 × g for 5 seconds.

3 Fragment using the appropriate Covaris settings:

Table 2 350 bp Insert

Setting	M220	S220	S2	E210
Duty Cycle (%)	20	5	10	
Intensity	—	—	5.0	
Peak/Displayed Power (W)	50	175	23	14
Cycles/Burst	200			
Duration (seconds)	65	50	45	
Mode	—	Frequency sweeping		
Temperature (°C)	20	5.5–6		

Table 3 550 bp Insert

Setting	M220	S220	S2	E210
Duty Cycle (%)	20	5	10	
Intensity	—	—	2.0	
Peak/Displayed Power (W)	50	175	9	7
Cycles/Burst	200			
Duration (seconds)	45	25	45	
Mode	—	Frequency sweeping		
Temperature (°C)	20	5.5–6		

4 Centrifuge at 280 × g for 5 seconds.

5 Transfer 50 µl sample from each Covaris tube to the corresponding well of the CSP plate.

Clean Up Fragmented DNA

1 Vortex SPB until well-dispersed.

2 Add 80 µl SPB to each well.

3 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

4 Incubate at room temperature for 5 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on a magnetic stand and wait until the liquid is clear (~8 minutes).

7 Remove and discard all supernatant from each well.

8 Wash two times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

9 Use a 20 µl pipette to remove residual EtOH from each well.

10 Air dry on the magnetic stand for 5 minutes.

11 Add RSB to each well, and then remove from the magnetic stand.

12 Mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down.

13 Incubate at room temperature for 2 minutes.

14 Centrifuge at 280 × g for 1 minute.

15 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

16 Transfer 50 µl supernatant to the corresponding well of the IMP plate.

Repair Ends and Select Library Size

This step uses End Repair Mix 2 to convert the overhangs resulting from fragmentation into blunt ends. A 3′ to 5′ exonuclease activity removes the 3′ overhangs. A 5′ to 3′ polymerase activity completes the 5′ overhangs.

After end repair, different ratios of Sample Purification Beads are used to select the appropriate library size.

Consumables

- ERP 2 or ERP 3 (End Repair Mix)
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Barcode labels
  - ALP (Adapter Ligation Plate)
  - CEP (Clean Up End Repair Plate)
- Freshly prepared 80% ethanol (EtOH)
- PCR-grade water
- Tube
  - [≤ 6 samples] 1.7 ml microcentrifuge tube
  - [> 6 samples] 15 ml conical tube
- Plates
  - [HS] 96-well midi plates (2)
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (2)
- Microseal 'B' adhesive seals

About Reagents

- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ERP 2 or ERP 3	-25°C to -15°C	Thaw at room temperature, and then set aside on ice. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 [HS] Preheat the microheating system to 30°C.

3 [LS] Save the following ERP program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 30 minutes
- Hold at 4°C

4 Label plates as follows.
- Apply an ALP barcode label to a midi or PCR plate.
- Apply a CEP barcode label to a midi or PCR plate.

Procedure

Convert Overhangs

1 Centrifuge CTE at 600 × g for 5 seconds.

2 Add 10 µl CTE or RSB to each well.

3 Add 40 µl ERP 2 or ERP 3 to each well.

4 [HS] Mix, centrifuge, and incubate as follows.

a Shake at 1800 rpm for 2 minutes.
b Centrifuge at 280 × g for 1 minute.
c Place on the 30°C microheating system, lid closed, for 30 minutes.
d Place on ice.

5 [LS] Pipette to mix, centrifuge, and then place on the thermal cycler and run the ERP program. Each well contains 100 µl.

Remove Large DNA Fragments

1 Vortex SPB until well-dispersed.

2 Using the following formulas, determine the appropriate volumes of SPB and PCR-grade water for diluting SPB.
The formulas include 15% excess for multiple samples.

Table 4 Diluted SPB for a 350 bp Insert Size

Reagent	Formula	Example Volume for 12 Samples	Your Calculation
SPB	# of samples × 109.25 µl	1311 µl	
PCR-grade water	# of samples × 74.75 µl	897 µl	

Table 5 Diluted SPB for a 550 bp Insert Size

Reagent	Formula	Example Volume for 12 Samples	Your Calculation
SPB	# of samples × 92 µl	1104 µl	
PCR-grade water	# of samples × 92 µl	1104 µl	

3 Using your calculations from the previous step, dilute SPB with PCR-grade water.
- For ≤ 6 samples, dilute in a new 1.7 ml microcentrifuge tube.
- For > 6 samples, dilute in a new 15 ml conical tube.

4 Vortex diluted SPB until well-dispersed.

5 Add 160 µl diluted SPB to each well.

6 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

7 Incubate at room temperature for 5 minutes.

8 Centrifuge at 280 × g for 1 minute.

9 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

10 Transfer 250 µl supernatant to the corresponding well of the CEP plate.

11 Discard remaining diluted SPB.

Remove Small DNA Fragments

1 Vortex undiluted SPB until well-dispersed.

2 Add 30 µl undiluted SPB to each well.

3 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

4 Incubate at room temperature for 5 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

7 Remove and discard all supernatant from each well.

8 Wash two times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

9 Use a 20 µl pipette to remove residual EtOH from each well.

10 Air dry on the magnetic stand for 5 minutes.

11 Add 17.5 µl RSB to each well, and then remove from the magnetic stand.

12 Mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down.

13 Incubate at room temperature for 2 minutes.

14 Centrifuge at 280 × g for 1 minute.

15 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

16 Transfer 15 µl supernatant to the corresponding well of the ALP plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Adenylate 3′ Ends

One adenine (A) nucleotide is added to the 3′ ends of the blunt fragments to prevent them from ligating to each other during adapter ligation reaction. One corresponding thymine (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

- RSB (Resuspension Buffer)
- [Optional] CTA (A-Tailing Control)
- Microseal 'B' adhesive seals

About Reagents

- Using CTA is optional. Use equal volume of RSB as a substitute.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ATL or ATL 2	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
CTA	-25°C to -15°C	Thaw at room temperature, and then place on ice.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 [HS] Preheat two microheating systems, one to 37°C and the other to 70°C.

3 [LS] Save the following ATAIL70 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 37°C for 30 minutes
   - 70°C for 5 minutes
   - Hold at 4°C

Procedure

1 Centrifuge CTA at 600 × g for 5 seconds.

2 Add 2.5 µl CTA to each well.

3 Centrifuge ATL or ATL 2 at 600 × g for 5 seconds.

4 Add 12.5 µl ATL or ATL 2 to each well.

5 [HS] Mix and incubate as follows.

   a Shake at 1800 rpm for 2 minutes.
   b Place on the 37°C microheating system, lid closed, for 30 minutes.
   c Move to the 70°C microheating system, lid closed, for 5 minutes.
   d Place on ice for 5 minutes.

6 [LS] Pipette to mix, and then place on the thermal cycler and run the ATAIL70 program. Each well contains 30 µl.

Ligate Adapters

This step ligates index adapters to the DNA fragment ends, preparing them for hybridization to a flow cell.

Index adapters must be ordered separately from the Library Prep components. For information on compatible index adapters, see *Supporting Information* on page 21.

Consumables

- DNA Adapters (tubes or index adapter plate)
- LIG 2 (Ligation Mix 2)
- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- STL (Stop Ligation Buffer)
- [Optional] CTL (Ligation Control)
- Barcode labels
  - CAP (Clean Up ALP Plate)
  - Index Adapter Plate
  - TSP1 (Target Sample Plate)
- Freshly prepared 80% ethanol (EtOH)
- Plates
  - [HS] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate (1)
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (2)
- Microseal 'B' adhesive seals

About Reagents

- Using CTL is optional. Use RSB as a substitute.
- Do not remove LIG 2 from storage until instructed to do so in the procedure.
- Return LIG 2 to storage immediately after use.
- Vortex SPB before each use.
- Vortex SPB frequently to make sure that beads are evenly distributed.
- Aspirate and dispense SPB slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CTL	-25°C to -15°C	Thaw at room temperature, and then place on ice.
DNA Adapters	-25°C to -15°C	Thaw at room temperature for 10 minutes. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
STL	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

2 [HS] Preheat a microheating system to 30°C.

3 [LS] Save the following LIG program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 10 minutes
- Hold at 4°C

4 Label plates as follows.
- Apply a CAP barcode label to a midi or PCR plate.
- Apply a barcode label to a Hard-Shell PCR or PCR plate.

Procedure

Add Index Adapters

1 [HS] Prepare the appropriate Index Adapter Plate as follows.

a Remove the tape seal.
b Centrifuge at 280 × g for 1 minute.
c Remove the plastic cover. If you are not processing the entire plate, save the cover.
d Apply the index adapter plate barcode label.

2 [LS] Centrifuge the adapter tubes at 600 × g for 5 seconds.

3 Remove LIG 2 from -25°C to -15°C storage.

4 In the order listed, add the following reagents to each well:
- CTL (2.5 µl)
- LIG 2 (2.5 µl)

- DNA adapters (2.5 µl)

5 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

6 Centrifuge at 280 × g for 1 minute.

7 Incubate as follows.
- [HS] Place on the 30°C microheating system, lid closed, for 10 minutes. Set aside on ice.
- [LS] Place on the thermal cycler and run the LIG program.
  Each well contains 37.5 µl.

8 Centrifuge the STL at 600 × g for 5 seconds.

9 Add 5 µl STL to each well.

10 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

11 Centrifuge at 280 × g for 1 minute.

Clean Up Ligated Fragments

Steps 1 through 14 are performed one time using the Round 1 volumes, then repeated using the Round 2 volumes.

1 Add the appropriate volume of SPB to each well.
- **Round 1**—42.5 µl
- **Round 2**—50 µl

2 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

3 Incubate at room temperature for 5 minutes.

4 Centrifuge at 280 × g for 1 minute.

5 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

6 Remove and discard all supernatant from each well.

7 Wash two times as follows.

a Add 200 µl fresh 80% EtOH to each well.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well.

8 Use a 20 µl pipette to remove residual EtOH from each well.

9 Air dry on the magnetic stand for 5 minutes.

10 Add the appropriate volume of RSB to each well.
- **Round 1**—52.5 µl
- **Round 2**—22.5 µl

11 Remove from the magnetic stand, and then mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.

- [LS] Pipette up and down.

12 Incubate at room temperature for 2 minutes.

13 Centrifuge at 280 × g for 1 minute.

14 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

15 Transfer 50 µl supernatant to the corresponding well of the CAP plate.

16 Repeat steps 1 through 14 using the new plate and the **Round 2** volumes.

17 Transfer 20 µl supernatant to the corresponding well of the TSP1 plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Check Libraries

Quantify Libraries

Achieving high-quality data on Illumina sequencing systems requires optimum cluster density across every lane of the flow cell. Optimizing cluster densities requires accurate quantification of DNA libraries using qPCR.

- Quantification of TruSeq DNA PCR-Free libraries has been validated with the KAPA Library Quantification Kit – Illumina/Universal.
- Non-qPCR methods quantify molecules that do not have adapters on both ends and do not form clusters. More of these nonclustering molecules can be present due to the absence of PCR enrichment. Thus, quantification by methods other than qPCR might be inaccurate.

NOTE

For information on handling small liquid volumes, see Best Practices on the support page for your workflow.

1 Follow qPCR instructions for the KAPA kit, with the following modifications:
   - For the library dilution step, use at least 2 µl of the original library stock to ensure accurate and reproducible quantification.
   - Using at least 2 µl of initial diluted libraries, perform two additional independent (nonserial) 1:10,000 and 1:20,000 dilutions to evaluate quantification precision. The concentration of each library is calculated as shown in the following tables.

Table 6 350 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR Instrument (pM)*		Average Diluted Library (pM)	Size-Adjusted Diluted library (pM)	Undiluted Library (pM)*	Undiluted Library (pM)
1:10,000	A1	A2	A = (A1 + A2)/2	W1 = A × (452/470)	C1 = W1 x 10,000	(C1 + C2)/2
1:20,000	B1	B2	B = (B1 + B2)/2	W2 = B × (452/470)	C2 = W2 × 20,000	

Table 7 550 bp Library Concentration Calculation

Dilution Factor	Calculated by qPCR Instrument (pM)*		Average Diluted Library (pM)	Size-Adjusted Diluted Library (pM)	Undiluted Library (pM)*	Undiluted Library (pM)
1:10,000	C1	C2	C = (C1 + C2)/2	W3 = C × (452/670)	C3 = W3 × 10,000	(C3 + C4)/2
1:20,000	D1	D2	D = (D1 + D2)/2	W4 = D × (452/670)	C4 = W4 × 20,000	

- Obtain the calculated concentration of the 1:10,000 and 1:20,000 library dilutions, as determined by qPCR, in relation to the concentrations of the annotated KAPA DNA Standards 1–6. Use the average of the replicate data points to determine the concentration of the diluted library.
- Make a size adjustment calculation to account for the size difference between the average fragment length of the library and the KAPA DNA Standard (452 bp).

> **NOTE**
>
> Do not use the average fragment length of the library insert size based on the Bioanalyzer results. Fragment sizes of TruSeq DNA PCR-Free libraries measured on the Bioanalyzer are much larger than sequencing data can indicate.

- Calculate the concentration of the undiluted library by accounting for the relevant dilution factor (1:10,000 and 1:20,000). To calculate the concentration of the undiluted library, use the average of the replicate data points corresponding to each library DNA dilution.
- If a replicate is an outlier, it can be omitted from the calculation. If multiple replicates are outliers, repeat the assay.

Quality Control

1 Verify fragment size by checking the library size distribution. Run on an Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit or an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit for qualitative purposes only.

a Dilute the DNA library 1:5 with water.
b Run 1 µl diluted DNA library on a High Sensitivity DNA chip or NGS kit.

Figure 2 350bp Library Run on Fragment Analyzer Using High Sensitivity NGS Kit

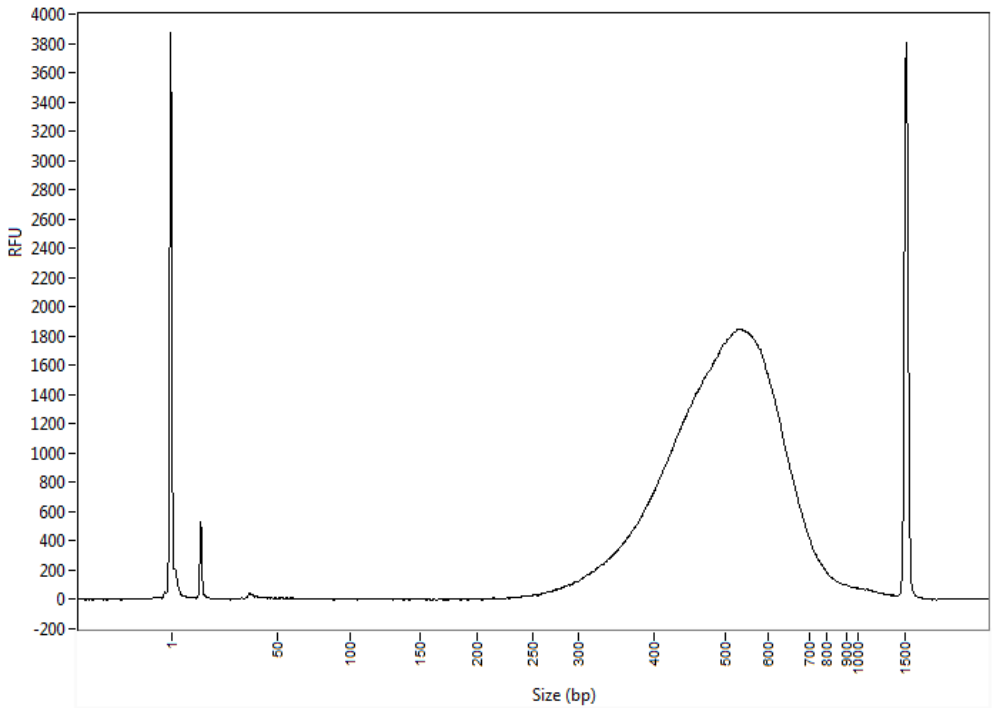
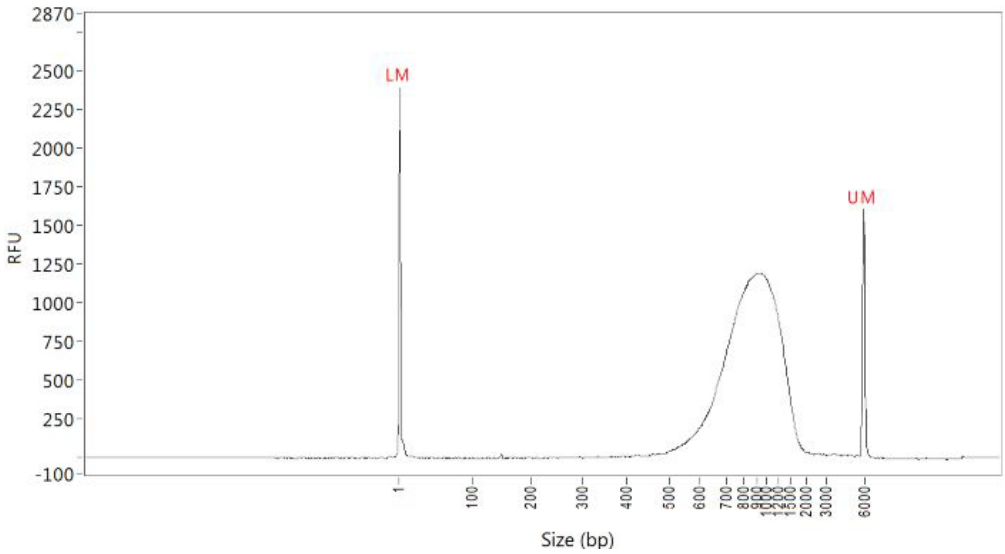
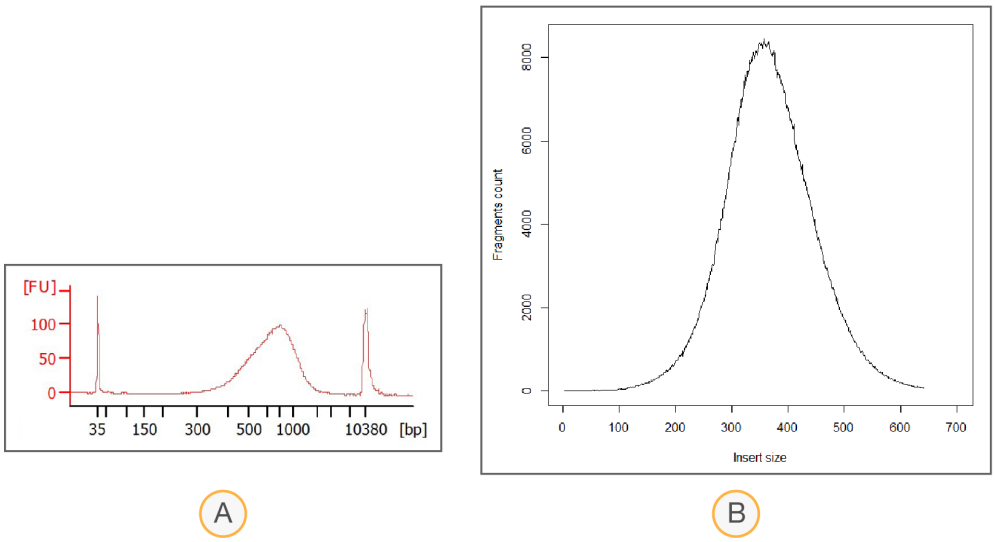


Figure 3 550bp Library Run on Fragment Analyzer Using High Sensitivity NGS Kit



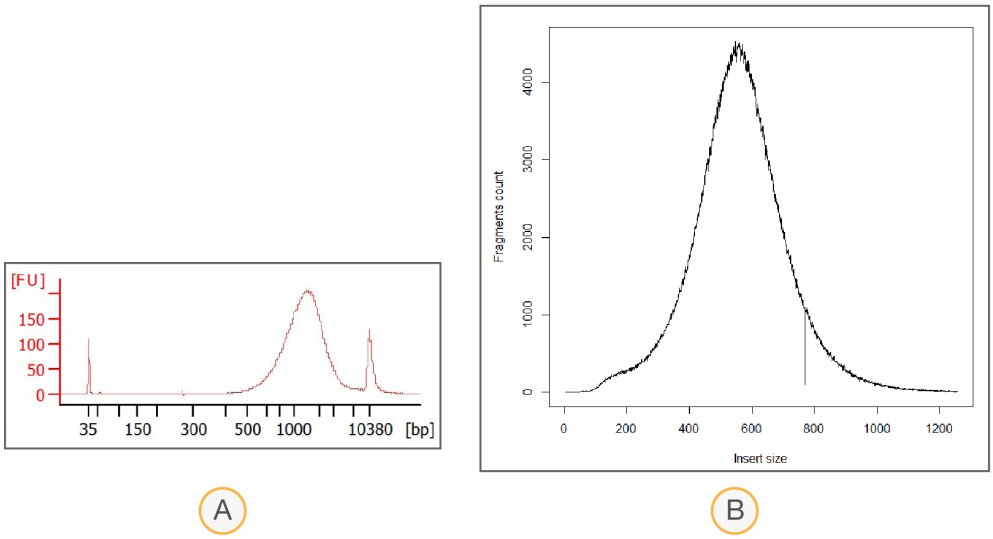
Library fragments measured on the Bioanalyzer are unexpectedly large due to the presence of certain structural features, which are normally removed during subsequent PCR enrichment. The following figures show example comparisons between library fragment sizes from a Bioanalyzer and the corresponding insert sizes from the alignment of paired-end reads to a reference sequence.

Figure 4 350bp Library Run on Bioanalyzer Using High Sensitivity DNA Kit



A Bioanalyzer
B Paired-End Alignment

Figure 5 550 bp Library Run on Bioanalyzer Using High Sensitivity DNA Kit



A Bioanalyzer
B Paired-End Alignment

Normalize and Pool Libraries

This step prepares DNA template for cluster generation. Non-indexed DNA libraries are normalized to 4 nM in the DCT plate. Indexed DNA libraries are normalized to 4 nM in the DCT plate and then pooled in equal volumes in the PDP plate.

NOTE

For best practice, perform normalization and pooling directly prior to sequencing. To minimize index hopping, do not store libraries in the pooled form. For more information, see *Minimize index hopping in multiplexed runs* on the Illumina website.

Consumables

- Barcode labels
  - DCT (Diluted Cluster Template)
  - PDP (Pooled DCT Plate) (for pooling only)
- Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20
- Plates
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (2) (second plate for pooling ≤ 40 samples)
- Microseal 'B' adhesive seals

Preparation

1 Label plates as follows.
   - Apply a DCT barcode label to a Hard-Shell PCR or PCR plate.
   - For pooling only, apply a PDP barcode label to the appropriate plate:
     - [> 40 samples] Midi plate

- [≤ 40 samples] Hard-Shell PCR or PCR plate

Procedure

Normalize Libraries

1 Transfer 5 µl library to the corresponding well of the DCT plate.

2 Normalize the library concentration to 4 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

3 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

Depending on the yield quantification data of each library, the final volume of each well can vary from 5–100 µl.

4 Centrifuge at 280 × g for 1 minute.

5 Proceed to pooling or clustering:
- To pool libraries, proceed to *Pool Libraries*.
- To leave libraries unpooled, skip the remaining library prep steps and proceed to cluster generation. For instructions, see the system guide for your instrument.

Pool Libraries

The pooling procedure depends on the number of libraries being pooled2–24 or 25–29.

Pool 2–24 Libraries

1 Transfer 5 µl of each normalized library to one well of the PDP plate.

2 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

3 Centrifuge at 280 × g for 1 minute.

4 Proceed to cluster generation.
For instructions, see the system guide for your Illumina instrument.

Pool 25–96 Libraries

1 Transfer 5 µl of each column of normalized library to column 1 of the PDP plate.

2 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

3 Centrifuge at 280 × g for 1 minute.

4 Transfer the contents from each well of column 1 to well A2.

5 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

6 Centrifuge at 280 × g for 1 minute.

7 Proceed to cluster generation.
For instructions, see the system guide for your Illumina instrument.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C.

Supporting Information

Product Contents

Make sure that you have all reagents identified in this section before starting the protocol.

The following library prep and index adapter components are available to order through Illumina to support the TruSeq DNA PCR-Free Library Prep workflow.

From Illumina, order one catalog number for the library prep component and one catalog number for the index adapter component depending on the number of samples for your experiment.

Library Prep Component	Catalog #
TruSeq DNA PCR-Free Library Prep (24 Samples)	20015962
TruSeq DNA PCR-Free Library Prep (96 Samples)	20015963

Index Adapter Component	Catalog #
IDT for Illumina-TruSeq DNA UD Indexes (24 indexes, 96 samples)	20020590
IDT for Illumina-TruSeq DNA UD Indexes (96 indexes, 96 samples)	20022370
TruSeq DNA Combinatorial Dual Indexes (96 indexes, 96 samples)	20015949
TruSeq DNA Single Indexes (12 indexes, 24 samples) Set A	20015960
TruSeq DNA Single Indexes (12 indexes, 24 samples) Set B	20015961

TruSeq DNA PCR-Free Library Prep (24 Samples)

This workflow contains two boxes: Box 1 and an SPB (Sample Purification Beads) box.

Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	RSB	Resuspension Buffer
1	ERP 2 or ERP 3	End Repair Mix
1	ATL or ATL 2	A-Tailing Mix
1	LIG 2	Ligation Mix 2
1	CTE	End Repair Control
1	CTA	A -Tailing Control
1	CTL	Ligation Control
1	STL	Stop Ligation Buffer

SPB Box, Store at 2°C to 8°C

Quantity	Reagent	Description
1	SPB	Sample Purification Beads

TruSeq DNA PCR-Free Library Prep (96 Samples)

This workflow contains two boxes: Box 1 and an SPB (Sample Purification Beads) box.

Box 1, Store at -25°C to -15°C

This box also contains plate barcode labels.

Quantity	Reagent	Description
2	RSB	Resuspension Buffer
2	ERP 2 or ERP 3	End Repair Mix
2	ATL or ATL 2	A-Tailing Mix
2	LIG 2	Ligation Mix 2
2	CTE	End Repair Control
2	CTA	A -Tailing Control
2	CTL	Ligation Control
2	STL	Stop Ligation Buffer

SPB Box, Store at 2°C to 8°C

Quantity	Reagent	Description
4	SPB	Sample Purification Beads

Inline Control DNA [Optional]

The use of Inline Control DNA provided with this workflow is optional, and only recommended if a custom analysis pipeline is available.

End Repair Control (CTE), A-Tailing Control (CTA), and Ligation Control (CTL) contain fragments used as controls for the enzymatic activities of End Repair Mix (ERP 2 or ERP 3), A-Tailing Mix (ATL or ATL 2), and Ligation Mix 2 (LIG 2). The inline controls contain dsDNA fragments that report the success or failure of a specific enzymatic activity in a custom analysis pipeline. If no such pipeline is available, it is recommended to omit these controls from the prep.

Controls are added to reactions before the corresponding protocol step. The end structures of the controls match the end structures of a DNA molecule that has not undergone the protocol step. If the step is successful, the control molecule is modified to participate in downstream reactions of library generation and result in sequencing data. If the step fails, the control molecule does not advance and sequencing data are not generated.

Table 8 Inline Control Functions

Reagent	Function	Control Reagent	Structure of Control DNA Ends
ERP 2 or ERP 3	End repair: Generate blunt ended fragments by 3′—>5′ exonuclease and 5′—>3′ polymerase activities.	CTE 1*	5′ overhang at one end, 3′ overhang at the other end
ERP 2 or ERP 3	End repair: Add 5′-phosphate groups needed for downstream ligation.	CTE 2*	Blunt with 5′-OH group
ATL or ATL 2	A-tailing: Make fragments compatible with adapters and prevent self-ligation by adding a 3′-A overhang	CTA	Blunt with 5′-phosphate group
LIG 2	Ligation: Join 3′-T overhang adapters to 3′-A overhang inserts	CTL	Single-base 3′ A base overhang

*CTE 1 and CTE 2 are separate controls included in the CTE reagent.

Inline controls can be used for various insert sizes. Each control is provided in ladders ranging from about 150 bp through 850 bp in 100 bp increments. Each control molecule has a unique DNA sequence that indicates function and size. Because the size selection step precedes A-tailing, CTE 1 and CTE 2 show a narrow size distribution while CTA and CTL show a broad size distribution.

Inline controls are optional for identifying a specific mode of failure and other troubleshooting. You can replace inline controls with equal volumes of RSB. When sequencing data are not generated from a library, inline controls are uninformative.

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol. Items that are unique to the HS or LS workflow are indicated.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 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 multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier

Consumable	Supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well storage plates, round well, 0.8 ml (midi plate)	Thermo Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part E7023
Ice bucket	General lab supplier
KAPA Library Quantification Kit - Illumina/Universal	KAPA Biosystems, part # KK4824
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
microTUBE AFA Fiber 6x16mm with: • Crimp-Cap or • Pre-Slit Snap-Cap (for use with Covaris M220)	Covaris, part # • 520052 or • 520045
PCR-grade water	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
Tween 20	Sigma-Aldrich, part # P7949
[Optional] High Sensitivity NGS Fragment Analysis Kit	Advanced Analytical, catalog # DNF-474
[Optional] High Sensitivity DNA Kit	Agilent Technologies, part # 5067-4626

Additional Consumables for HS Workflow

Consumable	Supplier
96-well Hard-Shell 0.3 ml PCR plate	Bio-Rad, part # HSP-9601

Additional Consumables for LS Workflow

Consumable	Supplier
96-well 0.3 ml skirtless PCR plates or Twin.tec 96 well PCR plates	E&K Scientific, part # 480096 or Eppendorf, part # 951020303

Equipment

Equipment	Supplier
[Optional] Fragment Analyzer™	Advanced Analytical, catalog # FSV2CE2F
[Optional] 2100 Bioanalyzer Desktop System	Agilent Technologies, part # G2940CA
One of the following Covaris systems: • S2 • S220 • E210 • M220	Covaris M220, part # 500295*
Magnetic stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier
qPCR system	General lab supplier

* Contact Covaris for all other models.

Additional Equipment for HS Workflow

Equipment	Supplier
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
SciGene TruTemp Heating System[1]	Illumina, catalog # • SC-60-503 (110 V) or • SC-60-504 (220 V)
Midi plate insert for heating system[2]	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier

[1] Two systems are recommended to support successive heating procedures.

[2] Two inserts are recommended to support successive heating procedures.

Additional Equipment for LS Workflow

Equipment	Supplier
96-well thermal cycler with heated lid	General lab supplier

Thermal Cyclers

The following table lists the recommended specifications for the thermal cycler. If your lab has a thermal cycler that is not listed, validate it before starting the protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, constant at 100°C	Plate
MJ Research PTC-225 DNA Engine Tetrad	Calculated	Heated, constant at 100°C	Plate
Bio-Rad S1000	N/A	Heated, constant at 100°C	Plate

qPCR Systems

The following table lists the validated qPCR systems for the TruSeq DNA PCR-Free protocol.

Equipment	Supplier
CFX96 Touch Real-Time PCR Detection System*	Bio-Rad, part # 185-5195
Mx3000P qPCR System	Agilent, part # 401511

* Use 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 setting can correct for abnormalities in fluorescence intensity of the standard curve caused by the instrument. For software installation, contact Bio-Rad.

Index Adapter Sequences

For information on index adapter sequences, see *Illumina Adapter Sequences (document # 1000000002694)* which provides information regarding the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CEP	Clean Up End Repair Plate
CFP	Covaris Fragmentation Plate
CSP	Clean Up Sheared DNA Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DCT	Diluted Cluster Template Plate
DNA	Customer Sample DNA Plate
ERP	End Repair Mix
HS	High Sample
IEM	Illumina Experiment Manager
IMP	Insert Modification Plate
LIG	Ligation Mix
LRM	Local Run Manager
LS	Low Sample
PDP	Pooled Dilution Plate
RSB	Resuspension Buffer
SPB	Sample Purification Beads
STL	Stop Ligation Buffer
TSP1	Target Sample Plate 1

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.635.9898	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

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

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

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