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Illumina Stranded mRNA Prep

Reference Guide

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Revision History

Document	Date	Description of Change
Document # 1000000124518 v02	April 2021	Added dilution and quantitation information for the NextSeq 1000/2000 Sequencing System. Removed Agencourt RNAClean XP Beads. Corrected EPH3 and FSA definitions.
Document # 1000000124518 v01	August 2020	Updated workflow diagram description to include the number of samples used to calculate processing times. Corrected formatting of index kit names.
Document # 1000000124518 v00	June 2020	Initial release.

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Overview

Introduction

The Illumina® Stranded mRNA Prep, Ligation kit converts the messenger (mRNA) in total RNA into up to 384 dual-indexed libraries.

Oligo(dT) magnetic beads purify and capture the mRNA molecules containing polyA tails. The purified mRNA is fragmented and copied into first strand complimentary DNA (cDNA) using reverse transcriptase and random primers. In a second strand cDNA synthesis step, dUTP replaces dTTP to achieve strand specificity. The final steps add adenine (A) and thymine (T) bases to fragment ends and ligate adapters. The resulting products are purified and selectively amplified for sequencing on an Illumina system.

The kit offers the following features:

- Capture of both coding RNA and multiple forms of noncoding RNA that are polyadenlyated
- A polyA capture to selectively sequence mRNA
- Unique dual (UD) indexing with the IDT for Illumina RNA UD Indexes

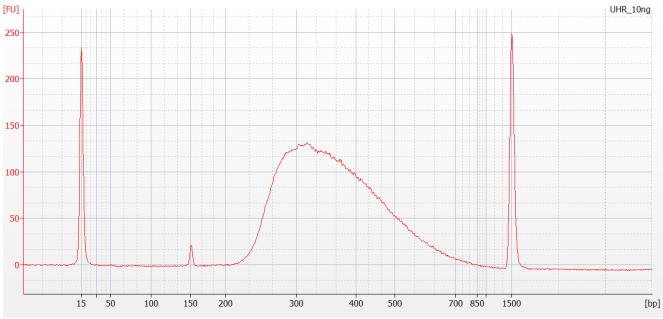
RNA Input Recommendations

The protocol is optimized for 25–1000 ng of high-quality total human RNA. Lower amounts can compromise ligation and lower yield. Determining the input amount for other species and quality levels requires further optimization.

Include a DNase treatment with the RNA isolation method. The DNase treatment ensures sample purity and accurate quantification. Before starting the protocol, quantify the total RNA using standard methods and assess quality using a fragment analysis method.

The following figure provides an example trace for Universal Human Reference (UHR) total RNA input. The trace is from a 2100 Bioanalyzer System using an RNA 6000 Pico Kit.

Figure 1 Example Trace of UHR Input



Additional Resources

The following resources provide instructions and guidelines for using the Illumina Stranded mRNA kit to prepare libraries. Visit the Illumina Stranded mRNA support pages for additional information:

- Compatible products and requirements for recording sample information, sequencing libraries, and analyzing data
- Questions and answers about using the kit
- Courses for related products and subjects
- The latest versions of the kit documentation

Resource	Description
Custom Protocol Selector	A tool for generating end-to-end instructions tailored to your library prep method, run parameters, and analysis method, with options to refine the level of detail.
Illumina Stranded mRNA Prep, Ligation Checklist (document # 1000000124519)	Provides a checklist of steps for the experienced user.
Illumina Stranded mRNA Prep, Ligation Consumables & Equipment (document # 1000000124520)	Provides an interactive checklist of user-provided consumables and equipment.
Index Adapters Pooling Guide (document # 1000000041074)	Provides guidelines for preparing dual-indexed libraries with balanced index combinations for sequencing on Illumina systems.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

Protocol

Introduction

This section describes the Illumina Stranded mRNA Prep, Ligation protocol with step-by-step instructions.

- Review the complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Confirm kit contents and make sure that you have the required consumables and equipment, including library prep reagents and index anchors and adapters. For a complete list, see *Supporting Information* on page 25.

Pooling Preparation

When pooling libraries, record information about your samples before starting library prep. Use a recording tool compatible with your sequencing system and libraries. For compatibility information, see the Illumina Stranded mRNA support pages or the support pages for your system.

The protocol uses IDT for Illumina RNA UD Indexes to index libraries in two separate steps, first adding anchors and then the UD indexes. The UD index primers add distinct Index 1 (i7) and Index 2 (i5) sequences to each end of a fragment. Each index sequence is 10 bp long.

- For strategies on forming low-plex, color-balanced pools, see the *Index Adapters Pooling Guide (document # 1000000041074)*.
- For index adapter sequences and how to record them, see *Illumina Adapter Sequences (document # 11000000002694)*.

Handling Beads

The protocol uses two types of beads: RNA Purification Beads and Agencourt AMPure XP. Each bead type has a specific technical application and cannot be substituted.

Apply the following techniques when handling beads:

- Use beads at room temperature.
- ***Do not*** use beads that have been stored < 2°C.
- Vortex beads before each use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- Dispense liquid directly onto bead pellets so that beads on the side of the wells are wetted.

- When the plate is on the magnetic stand, do not agitate the plate or disturb the bead pellet.
- 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).
- If beads adhere to well walls, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.

Tips and Techniques

Protocol Continuity

- Follow the protocol in the order described using the specified parameters.
- Avoid extended pauses until RNA is converted into double-stranded cDNA.
- 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***.
- When adding adapters or primers, change tips between ***each well***.
- Remove unused index adapter plates from the working area.

Handling Reagents and RNA

- Avoid multiple freeze-thaw cycles of input RNA.
  - You can store RNA in RNase-free water or TE buffer at -85°C to -65°C for up to 1 year.
  - If you must reuse the sample, aliquot into separate tubes for single-use.
- Keep thawed reagents on ice until needed. Promptly return all reagents to storage after use.
- When not in use, seal plates and close lids to limit contamination.

Sealing the Plate

- Use Microseal 'B' adhesive seals throughout the protocol. The seals are effective at -40°C to 110°C.
- Cover the plate with the seal, and seal with a rubber roller or wedge.
- After each use, discard seals from plates.

Plate Transfers

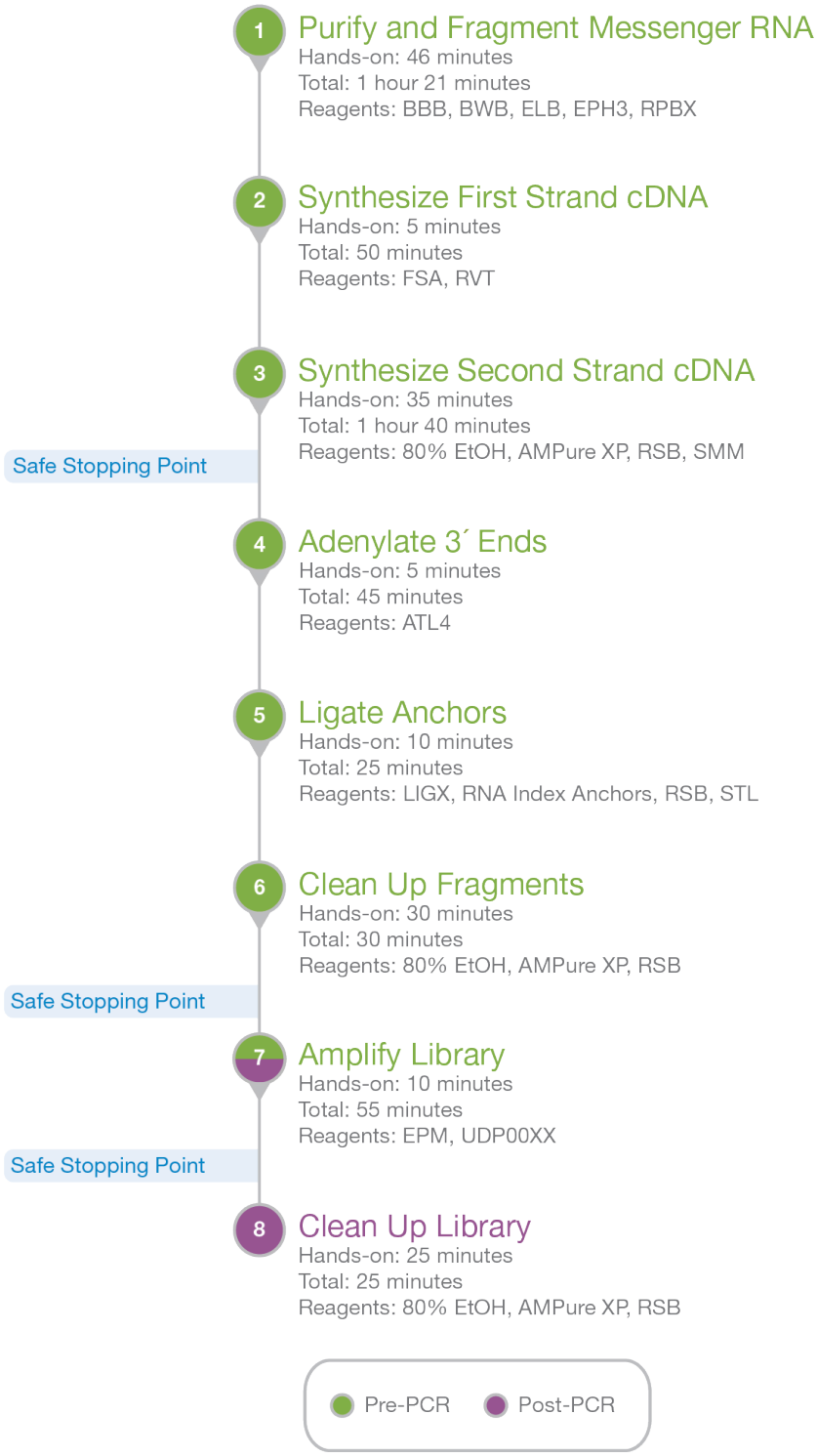
- When transferring volumes between plates, transfer the specified volume from each well of the first plate to the corresponding well of the second plate.

Mixing and Centrifugation

- The protocol includes the choice to mix by shaking or pipetting. Use the applicable magnetic stand:
  - If you choose to mix by pipette, use a Magnetic Stand-96.
  - If you choose to mix by shaking, use a DynaMag-96 Side Magnet.
- At any step, centrifuge at 280 × g for 10 seconds to consolidate liquid or beads in the bottom of the well and prevent sample loss.

Library Prep Diagram

The following diagram provides an overview of the Illumina Stranded mRNA protocol when using 24 samples. Safe stopping points are marked between steps.



Purify and Fragment mRNA

This step uses oligo(dT) magnetic beads to capture messenger RNAs (mRNAs) with polyA tails. The RNA is then fragmented and primed for cDNA synthesis.

Consumables

- BBB (Bead Binding Buffer)
- BWB (Bead Washing Buffer)
- ELB (Elution Buffer)
- EPH3 (Elute, Prime, Fragment 3HC Mix)
- RPBX (RNA Purification Beads)
- Nuclease-free ultrapure water
- 1.7 ml microcentrifuge tube, RNase-free
- 96-well PCR plates, semiskirted (2)
- Microseal 'B' adhesive film
- Prepare for a later procedure:
  - FSA (First Strand Synthesis Mix)

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
BBB	2°C to 8°C	Vortex and invert to mix.
BWB	2°C to 8°C	Vortex and invert to mix.
ELB	2°C to 8°C	Vortex and invert to mix.
EPH3	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
FSA	-25°C to -15°C	Thaw on ice. Vortex to mix, and then centrifuge briefly.
RPBX	2°C to 8°C	Let stand for 10 minutes to bring to room temperature. Vortex and invert to mix.

2. Save the following mRNA_CAP program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C

- Reaction volume is 50 µl
- 65°C for 5 minutes
- 4°C for 30 seconds
- 23° for 5 minutes
- Hold at 23°C

3. Save the following mRNA_ELT program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 25 µl
   - 80°C for 2 minutes
   - Hold at 25°C
4. Save the following DEN94_8 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 19 µl
   - 94°C for 8 minutes
   - Hold at 4°C

Procedure

Capture mRNA

1. In each well of a new PCR plate, dilute 25–1000 ng total RNA in nuclease-free ultrapure water to a volume of 25 µl.
2. Vortex RPBX to resuspend.
3. Add 25 µl RPBX to each well.
4. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Pipette 10 times, and then seal.
5. Place on the preprogrammed thermal cycler and run the mRNA_CAP program.
   Total program time is ~15 minutes and each well contains a volume of 50 µl.

Elute mRNA

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Place on the magnetic stand and wait 2 minutes.
3. Remove and discard all supernatant.
4. Remove from the magnetic stand.

5. Add 100 µl BWB to each well.
6. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Pipette 10 times.
7. Place on the magnetic stand and wait 2 minutes.
8. Remove and discard all supernatant.
9. With a 20 µl pipette, remove all residual BWB.
10. Remove from the magnetic stand.
11. Add 25 µl ELB to each well.
12. Mix using either method:
    - Seal and shake at 2200 rpm for 1 minute.
    - Slowly pipette until the beads are resuspended, and then seal.
13. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
14. Centrifuge at 280 × g for 10 seconds.
15. Place on the preprogrammed thermal cycler and run the mRNA_ELT program.
    Total program time is ~6 minutes and each well contains a volume of 25 µl.

Clean Up mRNA

1. In a 1.7 ml tube on ice, combine exactly the following volumes to prepare Fragmentation Master Mix. Multiply each volume by the number of samples.
   - Nuclease-free ultrapure water (10.5 µl)
   - EPH3 (10.5 µl)

   Volumes include reagent overage for accurate pipetting.
2. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
3. Add 25 µl BBB to each well.
4. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Pipette 10 times.
5. Incubate at room temperature for 5 minutes.
6. Place on the magnetic stand and wait 2 minutes.
7. Remove and discard 50 µl supernatant.
8. Remove from the magnetic stand.
9. Add 100 µl BWB to each well.
10. Mix using either method:

- Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
- Pipette 10 times.

11. Place on the magnetic stand and wait 2 minutes.
12. Remove and discard all supernatant.
13. With a 20 µl pipette, remove all residual BWB.
14. Remove from the magnetic stand.
15. Thoroughly pipette Fragmentation Master Mix to mix.
16. Add 19 µl Fragmentation Master Mix to each well.
17. Mix using either method:
    - Seal and shake at 2200 rpm for 1 minute.
    - Slowly pipette until the beads are resuspended, and then seal.
18. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
19. Incubate at room temperature for 2 minutes.
20. Centrifuge at 280 × g for 10 seconds.

Fragment and Denature mRNA

1. Place on the preprogrammed thermal cycler and run the DEN94_8 program.
   Total program time is ~10 minutes and each well contains a volume of 19 µl.
2. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
3. Place on the magnetic stand and wait 2 minutes.
4. Transfer 17 µl supernatant from each well to a new PCR plate.
5. Set aside the new PCR plate on ice.

Synthesize First Strand cDNA

This step reverse transcribes the hexamer-primed RNA fragments to produce first strand complementary DNA (cDNA). The First Strand Synthesis Mix includes Actinomycin D, which allows RNA-dependent synthesis and improves strand specificity while preventing spurious DNA-dependent synthesis.

Consumables

- FSA (First Strand Synthesis Mix)
- RVT (Reverse Transcriptase)
- 1.7 ml microcentrifuge tube, RNase-free

- Microseal 'B' adhesive film
- Prepare for a later procedure:
  - Agencourt AMPure XP
  - RSB (Resuspension Buffer)
  - SMM (Second Strand Marking Master Mix)

Warning

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.
RVT	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.
SMM	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly.

2. Save the following FSS program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 25 µl
   - 25°C for 10 minutes
   - 42°C for 15 minutes
   - 70°C for 15 minutes
   - Hold at 4°C

Procedure

1. In a 1.7 ml tube on ice, combine exactly the following volumes to prepare First Strand Synthesis Master Mix. Multiply each volume by the number of samples.

- FSA (9 µl)
- RVT (1 µl)

Volumes include reagent overage for accurate pipetting.

2. Thoroughly pipette First Strand Synthesis Master Mix to mix.
3. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
4. Add 8 µl First Strand Synthesis Master Mix to each well.
5. Pipette 10 times, and then seal.
6. Place on the preprogrammed thermal cycler and run the FSS program.
   Total program time is ~43 minutes and each well contains a volume of 25 µl.

Synthesize Second Strand cDNA

This step removes the RNA template and synthesizes a replacement strand to generate blunt-ended, double-stranded cDNA fragments. In place of deoxythymidine triphosphate (dTTP), deoxyuridine triphosphate (dUTP) is incorporated to quench the second strand during amplification and achieve strand specificity.

Consumables

- RSB (Resuspension Buffer)
- SMM (Second Strand Marking Master Mix)
- Agencourt AMPure XP
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

About Reagents

- Prepare 80% EtOH fresh and discard after one day. The protocol has three cleanup steps that require fresh 80% EtOH.

Preparation

1. Prepare 80% EtOH from absolute EtOH.
2. Save the following SSS program on the thermal cycler:
   - Choose the preheat lid option and set to 40°C
   - Reaction volume is 50 µl

- 16°C for 1 hour
- Hold at 4°C for ≤ 16 hours

Procedure

Generate cDNA

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Add 25 µl SMM to each well.
3. Pipette 10 times, and then seal.
4. Place on the preprogrammed thermal cycler and run the SSS program.
   Total program time is ~1 hour and each well contains a volume of 50 µl.

Clean Up cDNA

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Vortex AMPure XP to resuspend.
3. Add 90 µl AMPure XP to each well.
4. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Slowly pipette until the beads are resuspended.
5. Incubate at room temperature for 5 minutes.
6. Place on the magnetic stand and wait 5 minutes.
7. Remove and discard 130 µl supernatant.
8. Wash beads as follows.
   a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant.
9. Repeat wash a **second** time.
10. With a 20 µl pipette, remove all residual EtOH.
11. Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
12. Remove from the magnetic stand.
13. Add 19.5 µl RSB to each well.
14. Mix using either method:
    - Seal and shake at 2200 rpm for 1 minute.
    - Slowly pipette until the beads are resuspended, and then seal.

15. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
16. Incubate at room temperature for 2 minutes.
17. Centrifuge at 280 × g for 10 seconds.
18. Place on the magnetic stand and wait 2 minutes.
19. Transfer 17.5 µl supernatant from each well to a new PCR plate.
    Small amounts of bead carryover do not affect performance.

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

This step adds an adenine (A) nucleotide to the 3' ends of the blunt fragments to prevent them from ligating to each other during adapter ligation. A corresponding thymine (T) nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment.

Consumables

- ATL4 (A-Tailing Mix)
- Microseal 'B' adhesive film
- Prepare for a later procedure:
  - Anchor plate (RNA Index Anchors)
  - STL (Stop Ligation Buffer)

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
Anchor plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
ATL4	-25°C to -15°C	Thaw at room temperature. Flick to mix, and then centrifuge briefly.
STL	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.

2. Save the following ATAIL program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C

- Reaction volume is 30 µl
- 37°C for 30 minutes
- 70°C for 5 minutes
- Hold at 4°C

Procedure

1. If you are resuming the protocol after a safe stopping point, centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Add 12.5 µl ATL4 to each well.
3. Using a 200 µl pipette, pipette 10 times to mix, and then seal.
4. Place on the preprogrammed thermal cycler and run the ATAIL program.
   Total program time is ~38 minutes and each well contains a volume of 30 µl.

Ligate Anchors

This step ligates pre-index anchors to the ends of the double-stranded cDNA fragments to prepare them for dual indexing. A subsequent PCR amplification step adds the index adapter sequences.

Consumables

- Anchor plate (RNA Index Anchors) (green plate)
- LIGX (Ligation Mix)
- STL (Stop Ligation Buffer)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive film
- Prepare for a later procedure:
  - Agencourt AMPure XP

About Reagents

- Although stored at -25°C to -15°C, LIGX remains liquid and does not require thawing.
- Each well of the anchor plate is single-use and contains RNA Index Anchors. Anchor plate wells contain the same content and can be used in any order.
- The anchor plate is green and is distinct from the index adapter plate, which is colorless and used in a later step. Check the plate labels to make sure you are using the correct plate.

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
LIGX	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.

2. Save the following LIG program on the thermal cycler:
   - Choose the preheated lid option and set to 100°C
   - Reaction volume is 38 µl
   - 30°C for 10 minutes
   - Hold at 4°C

Procedure

Add Anchors

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Add the following volumes to each well *in the order listed*. Transfer RNA Index Anchors from the anchor plate to the PCR plate.

Order of Addition	Reagent	Volume for Sample Input ≤ 100 ng (µl)	Volume for Sample Input > 100 ng (µl)
1	RSB	2.5	0
2	RNA Index Anchors	2.5	5
3	LIGX	2.5	2.5

3. Using a 200 µl pipette, pipette 10 times to mix, and then seal.
4. Place on the preprogrammed thermal cycler and run the LIG program.
   Total program time is ~13 minutes and each well contains a volume of 38 µl.

Stop Ligation

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Add 5 µl STL to each well.

3. Pipette 15 times to mix.
   Each well contains a volume of 43 µl.

Clean Up Fragments

This step uses magnetic beads to purify the adapter-ligated fragments.

Consumables

- RSB (Resuspension Buffer)
- Agencourt AMPure XP
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

Procedure

1. Vortex AMPure XP to resuspend.
2. Add 34 µl AMPure XP to each well.
3. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Slowly pipette until the beads are resuspended.
4. Incubate at room temperature for 5 minutes.
5. Place on the magnetic stand and wait 5 minutes.
6. Remove and discard 67 µl supernatant.
7. Wash beads as follows.
   a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant.
8. Wash beads a **second** time.
9. With a 20 µl pipette, remove all residual EtOH.
10. Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
11. Remove from the magnetic stand.
12. Add 22 µl RSB to each well.
13. Mix using either method:

- Seal and shake at 2200 rpm for 1 minute.
- Slowly pipette until the beads are resuspended, and then seal.

14. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
15. Incubate at room temperature for 2 minutes.
16. Centrifuge at 280 × g for 10 seconds.
17. Place on the magnetic stand and wait 2 minutes.
18. Transfer 20 µl supernatant to the corresponding well of a new PCR plate. Amplification PCR cycles vary by input amount. When multiple samples are prepared, transfer to separate plates according to the number of PCR cycles specified in the *Amplify Library* on page 18 procedure.

SAFE STOPPING POINT

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

Amplify Library

This step uses PCR to selectively amplify the anchor-ligated DNA fragments and add indexes and primer sequences for cluster generation. The resulting product is a dual-indexed library: DNA fragments with adapters at each end.

For help with selecting index adapters, see *Pooling Preparation* on page 3.

Consumables

- EPM (Enhanced PCR Mix)
- Index adapter plate (UDP0XXX)

About Reagents

- Each well of the index adapter plate is single-use and contains > 10 µl UDP0XXX, which are premixed Index 1 (i7) and Index 2 (i5) adapters.
- The row and column labels are printed on the underside of the index adapter plate. Raise the plate overhead to check the labels.

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw at room temperature. Invert to mix, and then centrifuge briefly.
Index adapter plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge at 1000 × g for 1 minute.

2. Save the following PCR program on a thermal cycler using the appropriate number of PCR cycles, which are listed in the following table:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 50 µl
   - 98°C for 30 seconds
   - X cycles of:
     - 98°C for 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 4°C for ≤ 16 hours

   Adjust the program to optimize for different input amount.
   When multiple samples are amplified on one plate, make sure that the input for each sample is the same.

Input Amount (ng)	Number of PCR Cycles (X)
25	15
100	13
1000	10

   Total program time is ~44 minutes for 15 cycles, ~39 minutes for 13 cycles, and ~33 minutes for 10 cycles.

Procedure

1. If you are resuming the protocol after a safe stopping point, centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Using a new pipette tip for each well, pierce the foil covering the index adapter plate wells that you intend to use.

3. Add the following volumes to each well of the PCR plate ***in the order listed***. Transfer UDP0XXX from the index adapter plate to the PCR plate.
   - UDP0XXX (10 µl)
   - EPM (20 µl)
4. Pipette 10 times to mix, and then seal.
5. Place on the preprogrammed thermal cycler and run the PCR program.
   Each well contains a volume of 50 µl.

SAFE STOPPING POINT

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

Clean Up Library

This step uses magnetic beads to purify the dual-indexed libraries.

Consumables

- RSB (Resuspension Buffer)
- Agencourt AMPure XP
- Freshly prepared 80% EtOH
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.

Procedure

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Vortex AMPure XP to resuspend.
3. Add 50 µl AMPure XP to each well.

4. Mix using either method:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Slowly pipette until the beads are resuspended.
5. Incubate at room temperature for 5 minutes.
6. Place on the magnetic stand and wait 5 minutes.
7. Remove and discard 90 µl supernatant.
8. Wash beads as follows.
   a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant.
9. Wash beads a **second** time.
10. With a 20 µl pipette, remove all residual EtOH.
11. Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
12. Remove from the magnetic stand.
13. Add 17 µl RSB to each well.
14. Mix using either method:
    - Seal and shake at 2200 rpm for 1 minute.
    - Slowly pipette until the beads are resuspended, and then seal.
15. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
16. Incubate at room temperature for 2 minutes.
17. Centrifuge at 280 × g for 10 seconds.
18. Place on the magnetic stand and wait 2 minutes.
19. Transfer 15 µl supernatant from each well to the corresponding well of a new PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' or Microseal 'F' and store at -25°C to -15°C for up to 30 days.

Check Library

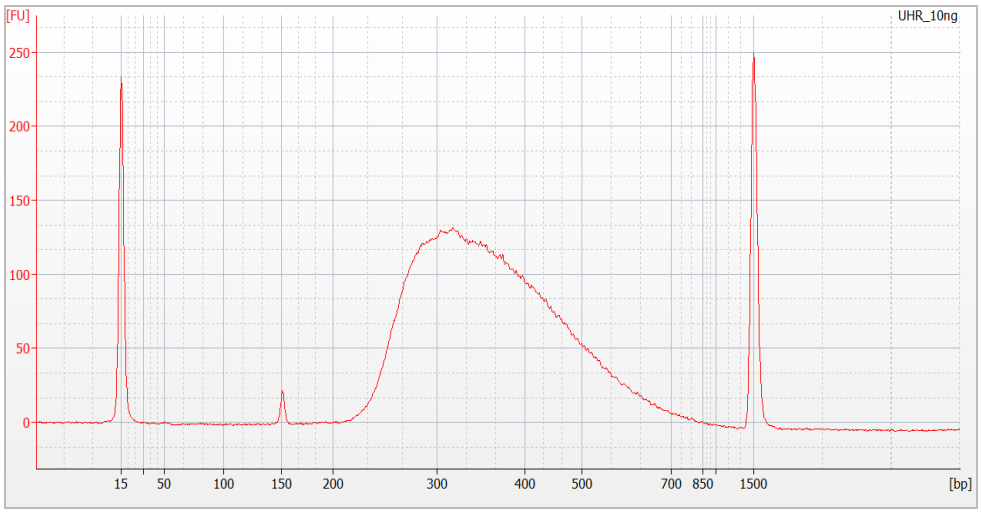
This step checks the concentration and quality of the final libraries.

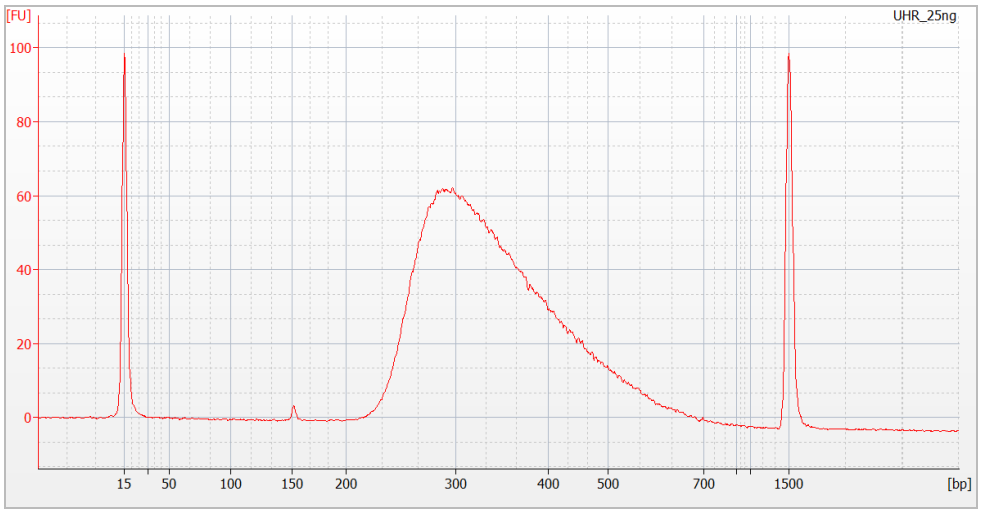
1. Analyze library using one of the following methods:
   - If using the NextSeq 1000/2000 Sequencing System, dilute libraries to 1:10000x dilution, and then analyze 2 µl library using KAPA qPCR library quantification kit.

- For all other sequencing systems, analyze 1 µl library using the Agilent 2100 Bioanalyzer and DNA 1000 Kit.

The following figure provides an example trace for a final library generated from 25 ng Universal Human Reference (UHR) RNA input.

Figure 2 Example Bioanalyzer Trace





2. [Optional] Analyze 2 µl library using the Qubit dsDNA BR Assay Kit for further quantification.
   For intact RNA samples, the average fragment length is ~300–400 bp. The expected insert size is ~160 bp.

Dilute Library to the Starting Concentration

This step dilutes libraries to the starting concentration for the NovaSeq 6000, NextSeq 500, NextSeq 550, NextSeq 1000, and NextSeq 2000 System. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration. Illumina recommends paired-end runs for sequencing. The number of cycles per index read is 10, and the number of cycles per read varies depending on the sequencing system.

1. Obtain the molarity value of the library or pooled libraries using the applicable method:
   - For libraries quantified with a Bioanalyzer only, use the molarity value obtained for the library.
   - For libraries quantified with a Bioanalyzer and Qubit, use the following formula to calculate the molarity value. Apply the average size from the Bioanalyzer and the concentration from the Qubit.
   - For libraries quantified with a KAPA qPCR, use the molarity value obtained for the library and 300–400 bp as the average library insert size.

$$\frac{ng/\mu l}{660\ g/mol \times average\ library\ size} \times 10^6 = Molarity\ (nM)$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
NextSeq 550 and NextSeq 500	1	1.1–1.4
NextSeq 1000 and NextSeq 2000	2	750
NovaSeq 6000	0.5	100

3. Dilute each library to the starting concentration for your system using RSB. Combine 10 µl each diluted library in a tube to pool libraries.
4. Follow the denature and dilute instructions for your system to dilute libraries to the final loading concentration.

If you are using the NextSeq 1000/2000 Control Software v1.2 and are not performing dark cycle sequencing, make sure to trim the T-overhang. See the *NextSeq 1000/2000 Sequencing System Guide (document # 1000000109376)* for more information on dark cycle sequencing.

Trim T-Overhang (Optional)

This step trims the first cycle nucleotide at FASTQ generation, removing the T overhang. See *NextSeq 1000/2000 Sequencing System Guide (document # 1000000109376* for more information on dark cycle sequencing.

Illumina Stranded mRNA adds a T nucleotide to cDNA inserts, which enables ligation of the insert to the adapter. The addition of the T nucleotide creates low diversity in the first cycle of each insert read, which can result in random base calls at the first sequencing cycle. To improve analysis quality, Illumina recommends trimming the first cycle nucleotide.

1. To trim using the FASTQ Toolkit app on BaseSpace Sequence Hub, perform the following actions.
   a. Expand the base trimming settings section.
   b. Enter 1 in the Trim reads at the 5'-end by n positions field.
2. To trim using a sample sheet, add the following settings to the sample sheet file.
   - Read1StartFromCycle,2
   - Read2StartFromCycle,2

   CAUTION

   FASTQ generation supports only one setting per run. To ensure correct settings for your libraries, use a separate sample sheet to demultiplex libraries without T-overhang.

Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this appendix, confirmed kit contents, and obtained all required consumables and equipment.

Acronyms

Acronym	Definition
A	Adenine
ATL4	A-Tailing Mix
BBB	Bead Binding Buffer
BWB	Bead Washing Buffer
cDNA	Complementary DNA
dsDNA	Double-stranded DNA
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
ELB	Elution Buffer
EPH3	Elute, Prime, Fragment 3HC Mix
EPM	Enhanced PCR Mix
EtOH	Ethanol
FSA	First Strand Synthesis Mix
LIGX	Ligation Mix
mRNA	Messenger RNA
PCR	Polymerase chain reaction
RIN	RNA Integrity Number
RPBX	RNA Purification Beads
RSB	Resuspension Buffer

Acronym	Definition
SMM	Second Strand Master Mix
STL	Stop Ligation Buffer
T	Thymine
UD	Unique dual
UHR	Universal Human Reference

Kit Contents and Storage

Make sure that you have all reagents identified in this section before starting library prep. The protocol requires one Illumina Stranded mRNA Prep, Ligation kit and at least one set of IDT for Illumina RNA UD Indexes. Combine all four sets to index up to 384 libraries.

Component	Kit Name	Illumina Catalog #
Library prep	Illumina Stranded mRNA Prep, Ligation (16 Samples)	20040532
	Illumina Stranded mRNA Prep, Ligation (96 Samples)	20040534
Indexes	IDT for Illumina RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples)	20040553
	IDT for Illumina RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples)	20040554
	IDT for Illumina RNA UD Indexes Set C, Ligation (96 Indexes, 96 Samples)	20040555
	IDT for Illumina RNA UD Indexes Set D, Ligation (96 Indexes, 96 Samples)	20040556

The prep kits provide reagents for mRNA selection, cDNA synthesis, and library prep in a 16- or 96-sample size. The indexes sets provide premixed Index 1 (i7) and Index 2 (i5) adapters with the adapters necessary for ligation.

The Illumina kits do not include Agencourt AMPure XP beads. For supplier information, see *Consumables and Equipment* on page 30.

Illumina Stranded mRNA Prep, Ligation (16 Samples) (20040532)

Illumina PolyA Capture

Quantity	Reagent	Description	Cap	Shipment	Storage
1	BBB	Bead Binding Buffer	Clear	2°C to 8°C	2°C to 8°C
3	BWB	Bead Washing Buffer	Yellow	2°C to 8°C	2°C to 8°C
1	ELB	Elution Buffer	Clear	2°C to 8°C	2°C to 8°C
1	RPBX	RNA Purification Beads	Clear	2°C to 8°C	2°C to 8°C

Illumina cDNA Synthesis

Quantity	Reagent	Description	Cap	Shipment	Storage
1	EPH3	Elute, Prime, Fragment 3HC Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	FSA	First Strand Synthesis Mix	Amber	-25°C to -15°C	-25°C to -15°C
1	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	RVT	Reverse Transcriptase	Clear	-25°C to -15°C	-25°C to -15°C
1	SMM	Second Strand Marking Master Mix	Clear	-25°C to -15°C	-25°C to -15°C

Illumina RNA Prep, Ligation

Quantity	Reagent	Description	Cap	Shipment	Storage
1	ATL4	A-Tailing Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	EPM	Enhanced PCR Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	LIGX	Ligation Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	STL	Stop Ligation Buffer	Red	-25°C to -15°C	-25°C to -15°C

Illumina Stranded mRNA Prep, Ligation (96 Samples) (20040534)

Illumina PolyA Capture

Quantity	Reagent	Description	Cap	Shipment	Storage
1	BBB	Bead Binding Buffer	Clear	2°C to 8°C	2°C to 8°C
1	BWB	Bead Washing Buffer	Clear	2°C to 8°C	2°C to 8°C
3	ELB	Elution Buffer	Clear	2°C to 8°C	2°C to 8°C
4	RPBX	RNA Purification Beads	Clear	2°C to 8°C	2°C to 8°C

Illumina cDNA Synthesis

Quantity	Reagent	Description	Cap	Shipment	Storage
4	EPH3	Elute, Prime, Fragment 3HC Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	FSA	First Strand Synthesis Mix	Amber	-25°C to -15°C	-25°C to -15°C
2	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	RVT	Reverse Transcriptase	Clear	-25°C to -15°C	-25°C to -15°C
4	SMM	Second Strand Marking Master Mix	Clear	-25°C to -15°C	-25°C to -15°C

Illumina RNA Prep, Ligation

Quantity	Reagent	Description	Cap Color	Shipment	Storage
4	ATL4	A-Tailing Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	EPM	Enhanced PCR Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	LIGX	Ligation Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
4	STL	Stop Ligation Buffer	Red	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples) (20040553)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	- 25°C to -15°C	- 25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set A (UDP0001–UDP0096)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples) (20040554)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set B (UDP0097–UDP0192)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set C, Ligation (96 Indexes, 96 Samples) (2004555)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set C (UDP0193–UDP0288)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set D, Ligation (96 Indexes, 96 Samples) (20040556)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set D (UDP0289–UDP0384)	-25°C to -15°C	-25°C to -15°C

Consumables and Equipment

The protocol has been optimized and validated using the items listed. When using alternate consumables and equipment, comparable performance is not guaranteed.

Ancillary Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes, RNase-free	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
1000 µl barrier pipette tips	General lab supplier
96-well twin.tec 250 µl PCR plates, semiskirted	One of the following suppliers: • Fisher Scientific, catalog # E951020303 • VWR, catalog # 47744-106
Agencourt AMPureXP, 60 ml	Beckman Coulter, catalog # A63881
Agilent DNA 1000 Kit	Agilent Technologies, catalog # 5067-1504
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Ethyl alcohol, pure (500 ml)	Sigma-Aldrich, catalog # E7023
Microseal 'B' PCR Plate Sealing Film	Bio-Rad, catalog # MSB-1001
Nuclease-free ultrapure water	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
RNaseZap[1]	General lab supplier
[Optional] Universal Human Reference RNA positive sample control	Agilent Technologies, catalog # 740000
[Optional] Agilent RNA 6000 Pico Kit	Agilent Technologies, catalog # Q32856
[Optional] Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
[Optional] Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, catalog # Q32850 or Q32853

[1] To decontaminate surfaces.

Ancillary Equipment

Equipment	Supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
2100 Bioanalyzer System	Agilent Technologies, catalog # G2939BA
[Shaking workflow] BioShake iQ high-speed thermoshaker	Q Instruments, catalog # 1808-0506
[Shaking workflow] BioShake PCR plate adapter	Q Instruments, catalog # 1808-1041
One of the following magnets: • [Shaking workflow] DynaMag-96 Side Magnet • [Pipette workflow] Magnetic Stand-96	The applicable supplier: • Thermo Fisher Scientific, catalog # 12331D • Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
One of the following 96-well thermal cyclers: • Bio-Rad C1000 Touch Thermal Cycler • T100 Thermal Cycler	The applicable supplier: • Bio-Rad, catalog # 1851196 • Bio-Rad, catalog # 1861096EDU
Vortexer	General lab supplier
[Optional] Qubit 2.0 Fluorometer	Thermo Fisher Scientific, catalog # Q32866

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
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Illumina Technical Support Telephone Numbers

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Singapore	1 800 5792 745	
South Korea	+82 80 234 5300	

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Thailand	+66 1800 011 304	
United Kingdom	+44 800 012 6019	+44 20 7305 7197
United States	+1 800 809 4566	+1 858 202 4566
Vietnam	+84 1206 5263	

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

Product documentation—Available for download from support.illumina.com.

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