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TruSeq® Small RNA Library Prep Reference Guide

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Document # 15004197 v02
July 2016

Customize a short end-to-end workflow guide with the Custom Protocol Selector
support.illumina.com/custom-protocol-selector.html

Revision History

Document	Date	Description of Change
Document # 15004197 v02	June 2016	• In Ligate Adapters About Reagents section, specified that T4 RNA Ligase 2, Deletion Mutant is a user-supplied consumable. • Corrected the volume of ethanol to 975 µl in the optional Concentrate Final Library step. • Added kit catalog numbers.
Document # 15004197 v01	January 2016	• Changed title of this document to Reference Guide. • Updated design of workflow diagram. • Renamed and combined some procedures as needed to improve continuity. • Simplified consumables information at the beginning of each section. • Revised step-by-step instructions to be more succinct. • Removed reference to obsolete Experienced User Cards and added references to Custom Protocol Selector and new protocol guide and checklist. • Removed box and tube part numbers from Kit Contents.
Part # 15004197 Rev. G	December 2014	• Kit name changed from sample prep to library prep. • Specified minimum amount if purified small RNA is used as input. • Replaced What's New section with Revision History. • Updated workflow diagram to include consumables. • Renamed Ligate 3' and 5' Adapters to *Ligate Adapters* with no change to the process. • Renamed DNA Template Storage to *Normalize Libraries* with no change to the process. • Modified name of Novex TBE consumables to match manufacturer name. • Updated *Run Gel Electrophoresis* procedures to indicate mixing vessel. • Updated *Additional Resources*. • Updated SDS link to support.illumina.com/sds.html.

Part # 15004197 Rev. F	February 2014	• Created new *Additional Resources* section.
		• Replaced *Tracking Tools* and *Best Practices* with a reference to content on the Illumina website.
		• Added reference to BaseSpace® to organize samples, libraries, pools, and runs.
		• Created new section of containing the following content:
		  • Acronyms
		  • Kit Contents
		  • Consumables and Equipment
		  • Indexed Adapter Sequences

Document	Date	Description of Change
Part # 15004197 Rev. E	February 2013	• Moved workflow diagram to start of protocol. • Reformatted the consumables list at the start of each procedure to include item quantity and storage temperature. • Corrected total volume of RNA and RTP mix in Perform Reverse Transcription procedure. • Corrected annotations on lower image of Small RNA Library from Total RNA Samples (Figure 12). • Changed final library storage concentration to 2 nM.

Part # 15004197 Rev. D	May 2012	• Added a What's New section.
		• Created a Getting Started section to contain the following sections:
		  • Added Acronyms
		  • Best Practices - contains Liquid Handling, Avoiding Cross-Contamination, TemperatureConsideration, and Equipment information
		  • RNA Input Recommendations
		  • Tracking Tools:
		  • Revised documentation download information.
		  • Removed sample sheet format guidelines and direct reader to sequencing analysis software user guide for detailed sample sheet guidelines
		  • Introduced Illumina Experiment Manager
		  • Indexed Adapter Sequences (renamed from Indexes)
		  • Pooling
		  • Kit Contents
		  • Consumables and Equipment:
		  • Revised user-supplied T4 RNA Ligase 2, Deletion Mutant consumable and supplier
		  • Removed text indicating that a Cluster Generation Kit is only required when not performing ethanol precipitation
		• Revised the Ligate 3' and 5' Adapters procedures to specify the use of T4 RNA Ligase 2, Deletion Mutant.
		• Specified using a preheated lid in thermal cycler preparation and procedures.
		• Removed the preparation section from each procedure in the Experienced User Card and incorporated preheating instructions as steps in the affected procedures.

Part # 15004197 Rev. C	April 2011	• Updated Sample Sheet section describing how to handle lanes with a single sample. • Updated protocol amplification and purification. • Updated kit contents and user supplied consumables.

Document	Date	Description of Change
Part # 15004197 Rev. B	February 2011	• Corrected index table. • Corrected RNA PCR Primer (RP1), part # 15013198. • Added Tracking Tools section. • Updated figure for Small RNA Library from Total RNA Samples. • Changed "microsRNAs" to "microRNA" in DNA chip trace figures. • Added instructions on how to use 0.5 ml tube as an alternative to gel breaker tubes. • Converted centrifuge rpm to × g.
Part # 15004197 Rev. A	November 2010	Initial release.

Table of Contents

Overview

Chapter 1

Introduction

This protocol explains how to prepare total RNA or purified small RNA using a Illumina® TruSeq® Small RNA Library Prep Kit. The goal of this protocol is to ligate adapters to each end of the RNA molecule, and then reverse transcribe and amplify to generate a cDNA library. A gel purification step prepares the library for clustering and sequencing.

The TruSeq Small RNA Library Prep Kit offers:

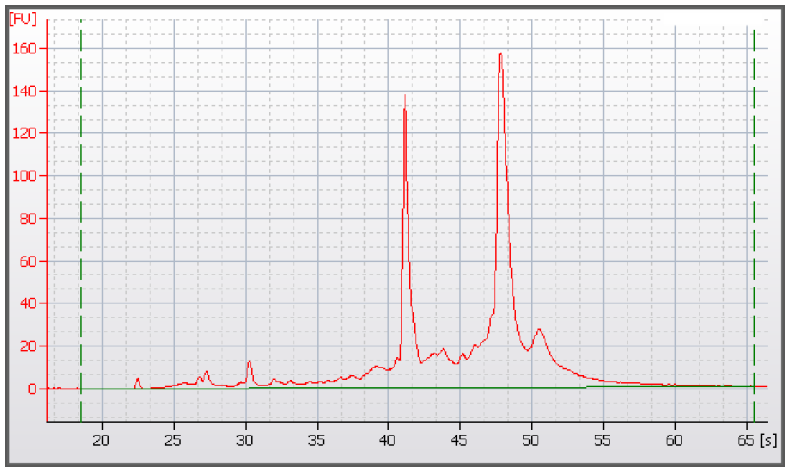
- Fast and easy sample preparation
  - Generate small RNA libraries directly from total RNA
  - Streamlined workflow for economical studies covering all small RNA transcripts in any species
- Multiplexed sequencing and analysis using 48 unique indexes
  - Allows microRNA (miRNA) and small RNA discovery and profiling
  - Six-base indexes distinguish samples in a flow cell lane
- RNA 3' adapters
  - Target small RNA and miRNA generated by Dicer or other RNA processing enzymes
- Indexes added during PCR
  - Significantly reduces ligation bias
  - Ensures accurate measurement of miRNA expression

RNA Input Recommendations

Total RNA Input

- The TruSeq Small RNA Library Prep Kit protocol is optimized for 1 µg of total RNA in 5 µl nuclease free water.
  - Lower amounts might result in inefficient ligation and low yield.
  - Quantify RNA using a fluorometric method.
- The TruSeq Small RNA Library Prep Kit protocol has been tested using 1 µg of high-quality universal human reference total RNA as input.
  - Small RNA populations can vary significantly between different tissue types and species.
  - Using RNA from other species, tissues, or qualities might require further optimization regarding the initial input amount and selection of desired bands during the final gel excision.
  - The types and coverage of small RNAs sequenced vary depending on which bands are selected during gel excision.
- Knowing the quality of the RNA starting material is important.
  - Degraded RNA can cause low yield, changes in observed expression patterns, or protocol failure.
  - RNA with DNA contamination results in underestimating the amount of RNA used.
  - For human or mammalian samples with an RNA Integrity Number (RIN) value ≥ 8, use an Agilent Technologies 2100 Bioanalyzer to check the total RNA integrity after isolation. Although this method does not directly measure small RNA, an RNA sample with degraded mRNA likely has degraded small RNA too.
  - Include a DNase step with the RNA isolation method.

Figure 1 Universal Human Reference (UHR) Starting RNA Bioanalyzer Trace



- Alternatively, you can run a formaldehyde 1% agarose gel and judge the integrity of RNA after staining with ethidium bromide.
- High-quality RNA shows a 28S rRNA band at 4.5 kb that is twice the intensity of an 18S rRNA band at 1.9 kb.
- Both kb determinations are relative to an RNA 6000 ladder.

Purified Small RNA Input

You can use previously isolated microRNA as starting material.

- A minimum of 10–50 ng of purified small RNA is required.
- This method results in fewer undesired bands during gel extraction.
- Purified small RNA ***must*** be in molecular grade water or 10 mM Tris-HC1, pH 8.5.

NOTE

Illumina has observed high sample variability using some commercial small RNA spin column purification methods. Validation of the purification method might be necessary.

Positive Control

Use Thermo Fisher Scientific Human Brain Total RNA (catalog # AM7962) as a positive control sample for this protocol. This preparation is certified to contain the small RNA fraction.

Additional Resources

Visit the TruSeq Small RNA Library Prep Kit support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

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

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html 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 Small RNA Library Prep Protocol Guide (document # 1000000005008)	Provides instructions for the experienced user.
TruSeq Small RNA Library Prep Checklist (document # 1000000005010)	Provides a checklist of steps for the experienced user.
TruSeq Small RNA Library Prep Consumables & Equipment (document # 1000000006959)	Provides an interactive checklist of user-provided consumables and equipment.
TruSeq Library Prep Pooling Guide (document # 15042173)	Provides pooling guidelines for preparing TruSeq libraries that require balanced index combinations. Review this guide before starting library prep.

Protocol

Introduction

This chapter describes the TruSeq Small RNA protocol.

- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Review Best Practices from the TruSeq Small RNA support page on the Illumina website.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before beginning library prep. Different methods are available depending on the sequencing instrument you are using. See the TruSeq Small RNA Library Prep Kit support page for more information.

Review the planning steps in the *TruSeq Library Prep Pooling Guide (document # 15042173)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

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***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- 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, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is effective for thermal cycling and easy to cut when using fewer than 96 wells.

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.
  - To pellet beads, centrifuge at 280 × g for 1 minute.

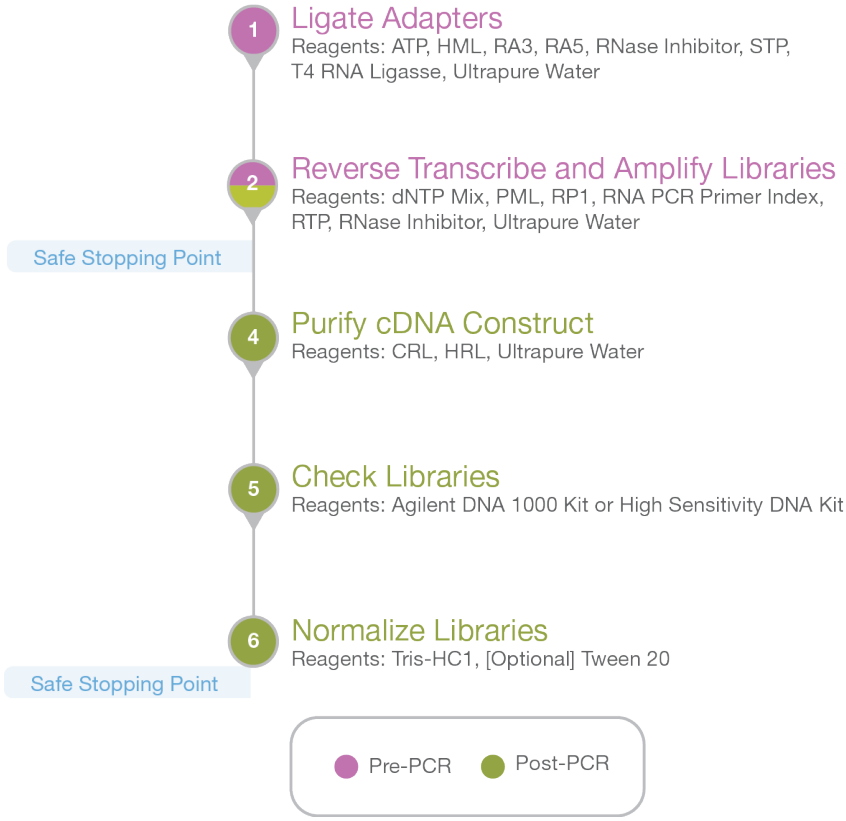
Handling Beads

- Pipette bead suspension slowly.
- When mixing, mix thoroughly.
- If beads are aspirated into the 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 appropriate magnet for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnet until the instructions specify to remove it.
  - Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

Library Prep Workflow

The following diagram illustrates the workflow using a TruSeq Small RNA Library Prep Kit. Safe stopping points are marked between steps.

Figure 2 TruSeq Small RNA Workflow



Ligate Adapters

This step ligates adapters to the 3' and 5' ends of the sample.

Consumables

- 10 mM ATP
- HML (Ligation Buffer)
- RA3 (RNA 3' Adapter)
- RA5 (RNA 5' Adapter)
- RNase Inhibitor
- STP (Stop Solution)
- T4 RNA Ligase
- Ultrapure water
- T4 RNA Ligase 2, Deletion Mutant (200 U/µl) (1 µl per sample)
- Nuclease-free 200 µl PCR tubes (3)

About Reagents

- Do not use the reaction buffer provided with T4 RNA Ligase 2, Deletion Mutant.
- T4 RNA Ligase 2, Deletion Mutant is a user-supplied consumable.
- In all reagent calculations, N is equal to the number of samples being prepared.
- Prepare PCR tubes on ice using a 96-well working rack.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
10 mM ATP	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
HML	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
RA3	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
RA5	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
RNase Inhibitor	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
STP	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
T4 RNA Ligase	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
Ultrapure water	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
T4 RNA Ligase 2, Deletion Mutant	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.

2 Preheat a thermal cycler to 70°C.

3 Choose the thermal cycler preheat lid option and set to 100°C.

Procedure

Ligate 3' Adapter

1. Combine the following volumes in a new 200 µl PCR tube on ice:
   - RA3 (1 µl)
   - 1 µg total RNA in nuclease-free water (5 µl)

   The total volume is 6 µl.
2. Pipette to mix, and then centrifuge briefly.
3. Place on the preheated thermal cycler.
4. Incubate at 70°C for 2 minutes.
5. Remove from the thermal cycler and place on ice.
6. Preheat the thermal cycler to 28°C.
7. Combine the following volumes in a new 200 µl PCR tube on ice. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
   - HML (2 µl)
   - RNase Inhibitor (1 µl)
   - T4 RNA Ligase 2, Deletion Mutant (1 µl)

   The total volume per sample is 4 µl.
8. Pipette to mix, and then centrifuge briefly.
9. Add 4 µl to the tube of RA3/total RNA mixture.
   The total volume is 10 µl.
10. Pipette to mix.
11. Place on the preheated thermal cycler.
12. Incubate at 28°C for 1 hour.
13. Add 1 µl STP and pipette to mix.
14. Continue incubating at 28°C for 15 minutes.
15. Remove from the thermal cycler and place on ice.

Ligate 5' Adapter

1. Preheat the thermal cycler to 70°C.
2. Add 1.1 × N µl RA5 to a new 200 µl PCR tube.
3. Place on the preheated thermal cycler.
4. Incubate at 70°C for 2 minutes.
5. Remove from the thermal cycler and place on ice.
6. Preheat the thermal cycler to 28°C.
7. Add 1.1 × N µl 10mM ATP to the tube of RA5.
8. Pipette to mix.
9. Add 1.1 × N µl T4 RNA Ligase to the RA5/ATP mixture.

10 Pipette to mix.

11 Add 3 µl to the tube of RA3 mixture.
The total volume is 14 µl.

12 Pipette to mix.

13 Place on the preheated thermal cycler.

14 Incubate at 28°C for 1 hour.

15 Remove from the thermal cycler and place on ice.

Reverse Transcribe and Amplify Libraries

Reverse transcription followed by amplification creates cDNA constructs based on the small RNA ligated with 3' and 5' adapters. This step selectively enriches RNA fragments with adapter molecules on both ends. The amplification is performed with 2 primers that anneal to the adapter ends.

Consumables

- 25 mM dNTP Mix
- PML (PCR Mix)
- RP1 (RNA PCR Primer)
- RNA PCR Primer Index (1 tube of each index being used)
- RTP (RNA RT Primer)
- RNase Inhibitor
- Ultrapure water
- 5X First Strand Buffer (2 µl per library)
- 100 mM DTT (1 µl per sample)
- High Sensitivity DNA chip (1 per library)
- Nuclease-free 200 µl PCR tubes (3 plus 1 per index used)
- SuperScript II Reverse Transcriptase (1 µl per library)

About Reagents

- For each reaction, only 1 of the 48 RNA PCR Primer indexes is used during amplification.
- Remaining 5' and 3' adapter-ligated RNA can be stored at -80°C for up to 7 days.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
5X First Strand Buffer	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
100 mM DTT	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.
SuperScript II Reverse Transcriptase	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds and then set aside on ice.

2 Preheat the thermal cycler to 70°C.

3 Choose the thermal cycler preheat lid option and set to 100°C.

4 Label a new 200 µl PCR tube 12.5 mM dNTP Mix.

Procedure

Dilute 25 mM dNTP Mix

1 Combine the following volumes in the 12.5 mM dNTP Mix tube to dilute to 12.5 mM. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent if you are preparing multiple libraries.

- 25 mM dNTP Mix (0.5 µl)
- Ultrapure water (0.5 µl)

The total volume per library is 1 µl.

2 Pipette to mix, and then centrifuge briefly.

3 Set aside on ice.

Perform Reverse Transcription

1 Add 6 µl each adapter-ligated RNA library to a new 200 µl PCR tube.

2 Add 1 µl RNA RT Primer to the tube of adapter-ligated RNA.

3 Pipette to mix, and then centrifuge briefly.

4 Place on the preheated thermal cycler.

5 Incubate at 70°C for 2 minutes.

6 Remove from the thermal cycler and place on ice.

7 Preheat the thermal cycler to 50°C.

8 Combine the following volumes in a new 200 µl PCR tube on ice. Multiply each volume by the number of libraries being prepared. Make 10% extra reagent if you are preparing multiple libraries.
- 5X First Strand Buffer (2 µl)
- 12.5 mM dNTP Mix (0.5 µl)
- 100 mM DTT (1 µl)
- RNase Inhibitor (1 µl)
- SuperScript II Reverse Transcriptase (1 µl)

The total volume per library is 5.5 µl.

9 Pipette to mix, and then centrifuge briefly.

10 Add 5.5 µl to the tube of adapter-ligated RNA/primer mix.

11 Pipette to mix, and then centrifuge briefly.
The total volume is 12.5 µl.

12 Place on the preheated thermal cycler.

13 Incubate at 50°C for 1 hour.

14 Remove from the thermal cycler and place on ice.

Amplify Libraries

1 Combine the following reagents in a new 200 µl PCR tube on ice to prepare the PCR master mix. Multiply each volume by the number of libraries being prepared. Make 10% extra reagent if you are preparing multiple libraries with the same index.
- Ultrapure water (8.5 µl)
- PML (25 µl)
- RP1 (2 µl)
- RPIX (2 µl)

The total volume per library is 37.5 µl.

2 Pipette to mix, and then centrifuge briefly.

3 Place on ice.

4 Add 37.5 µl PCR master mix to the adapter-ligated RNA mixture.
The total volume is 50 µl.

5 Pipette to mix, and then centrifuge briefly.

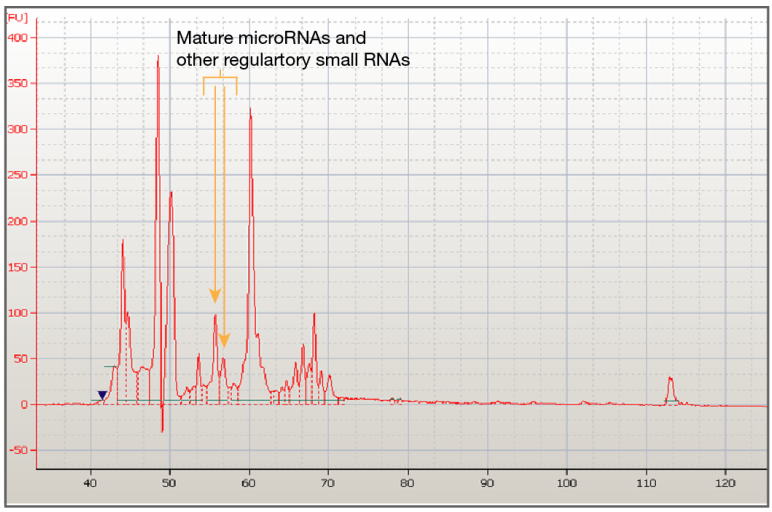
6 Place on ice.

7 Place on the preheated thermal cycler.

8 Incubate using the following program on the thermal cycler:
- Choose the preheat lid option and set to 100°C.
- 98°C for 30 seconds
- 11 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 15 seconds
- 72°C for 10 minutes
- 4°C hold

9 Run each library on a High Sensitivity DNA chip. The following figure shows typical results from human brain total RNA.

Figure 3 Sample Trace of Amplicons on a High Sensitivity DNA Chip



Amplification products can vary based on RNA input amount, tissue type, and species. This process was optimized using 1 µg of total RNA from mouse and human brain. If the gel image does not include clear and distinct bands, increase the number of PCR cycles (up to 15).

The amplification reaction can interfere with Bioanalyzer reagents. Diluting the library before running it on the High Sensitivity DNA chip might be necessary.

The bands of the high sensitivity chip can vary by library due to incorrect marker identification by the Bioanalyzer software.

SAFE STOPPING POINT

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

Purify cDNA Construct

This step gel purifies the amplified cDNA construct in preparation for subsequent cluster generation. Optional ethanol precipitation generates a more concentrated library in exchange for some yield.

NOTE
Libraries with unique indexes can be pooled and gel purified together. Purifying multiple libraries with the same index on a single gel is not recommended due to the risk of cross-contamination. If multiple libraries are run on a single gel, keep at least 4 empty wells between libraries. For more information, see *Pooling Guidelines* on page 34.

Consumables

- CRL (Custom RNA Ladder)
- HRL (High Resolution Ladder)
- Ultrapure water
- 5 µm filter tubes (2)
- Amplified cDNA construct (50 µl)
- DNA loading dye (13 µl)
- Gel breaker tubes (2)
- High Sensitivity DNA Kit (1 per library)
- Novex TBE gels, 6%, 10-well
- Novex TBE running buffer (5X)
- Nuclease-free 200 µl PCR tube
- Nuclease-free 1.5 ml microcentrifuge tubes (3)
- Razor blade
- Ultra pure ethidium bromide 10mg/ml (0.5 µl/ml in water)
- For optional ethanol precipitation:
  - 3 M NaOAc, pH 5.2 (30 µl)
  - 10 mM Tris-HC1, pH 8.5 (10 µl)
  - 70% ethanol (500 µl)
  - 100% ethanol (975 µl)
  - Glycogen (2 µl)
  - Pellet Paint NF Co-Precipitant (0.2 µl per library)

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CRL	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
HRL	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
Ultrapure water	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
Amplified cDNA Construct	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
Novex TBE running buffer (5X)	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.

Item	Storage	Instructions
Ultra pure ethidium bromide 10mg/ml	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
3 M NaOAc, pH 5.2	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
10 mM Tris-HC1, pH 8.5	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
100% Ethanol	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
Glycogen	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.
Pellet Paint NF Co-Precipitant	-25°C to -15°C	Thaw on ice. Centrifuge at 600 × g for 5 seconds, and then set aside on ice.

2 [Optional] Label a new 200 µl PCR tube 0.1X Pellet Paint.

3 Determine the volume of 1X TBE Buffer needed for gel electrophoresis. Dilute the 5X Novex TBE Buffer to 1X.

4 Place 6% Novex TBE gel into the gel electrophoresis unit per manufacturer instructions.

Procedure

Dilute Pellet Paint [Optional]

This step stains the DNA pellet with a removable dye so it is easier to see. To avoid pipetting small volumes, prepare sufficient pellet paint for at least 10 libraries.

1 Combine the following volumes in the 0.1X Pellet Paint tube. Multiply each volume by the number of libraries being prepared. Make 10% extra reagent if you are preparing multiple libraries.
- 1X Pellet Paint NF Co-Precipitant (0.2 µl)
- Ultrapure water (1.8 µl)

The total volume per sample is 2 µl.

2 Pipette to mix, and then centrifuge briefly.

Run Gel Electrophoresis

1 Combine 2 µl CRL and 2 µl DNA loading dye in a new 1.5 ml microcentrifuge tube.

2 Pipette to mix.

3 Combine 1 µl HRL and 1 µl DNA loading dye in a new 1.5 ml microcentrifuge tube.

4 Pipette to mix.

5 Combine all amplified cDNA construct (typically 48–50 µl) and 10 µl DNA Loading Dye in a new 1.5 ml microcentrifuge tube.

6 Pipette to mix.

7 Load 2 gel lanes with 2 µl CRL/loading dye mixture.

8 Load 1 gel lane with 2 µl HRL/loading dye mixture.

9 Load 2 gel lanes with 25 µl each of amplified cDNA construct/loading dye mixture. The total load volume is 50 µl.

10 Run the gel for 60 minutes at 145 V or until the blue front dye leaves the gel.

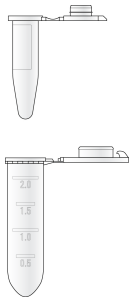
11 Remove the gel from the unit.

Recover Purified Construct

1 Open the cassette according to manufacturer instructions and stain the gel with ethidium bromide in a clean container for 2–3 minutes.

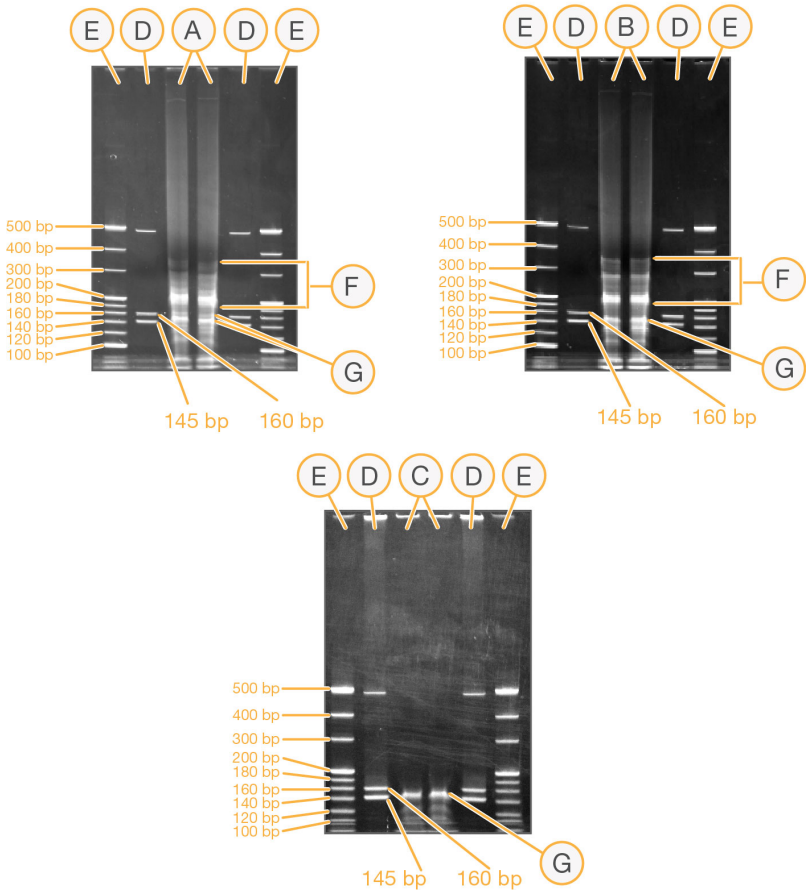
2 Place the gel breaker tube into a 2 ml microcentrifuge tube.

Figure 4 Insert Tube



3 View the gel on a Dark Reader transilluminator or a UV transilluminator.
Individual bands or pooled bands can be sequenced. The 147 nt band primarily contains mature miRNA generated from ~22 nt small RNA fragments. The 157 nt band contains piwi-interacting RNAs, some miRNAs, and other regulatory small RNA molecules. It is generated from ~30 nt RNA fragments.
The CRL in the following figure consists of 3 dsDNA fragments: 145 bp, 160 bp, and 500 bp.

Figure 5 Small RNA Library from Total RNA Libraries



A Human brain total RNA
B Mouse brain total RNA
C Prepurified miRNA
D CRL
E HRL
F Small noncoding RNAs
G miRNA

4 Using a razor blade, cut out the bands from the 2 lanes that correspond approximately to the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments as follows.

a Cut the gel along the top of the 160 bp CRL band and the bottom of the 145 bp CRL band.

b Connect the cuts on the sides to excise the gel. Both lanes can be combined into 1 slice.

The band containing the 22 nt small RNA fragment with both adapters is 147 nt long. The band containing the 30 nt RNA fragment with both adapters is 157 nt long.

5 Place the band of interest into the 0.5 ml gel breaker tube.

6 Centrifuge the nested tubes at 20,000 × g for 2 minutes to move the gel through the holes into the 2 ml tube.

7 If you are concentrating the final library, skip the remaining steps and proceed to *Concentrate Final Library [Optional]* on page 21.

8 Add 200 µl ultrapure water to the gel debris in the 2 ml tube.

9 Rotate or shake for at least 2 hours to elute the DNA.

NOTE
The tube can be rotated overnight.

10 Transfer the eluate and gel debris to the top of a 5 µm filter.

11 Centrifuge at 10 seconds at 600 × g.

Concentrate Final Library [Optional]

1 Add 300 µl ultrapure water to the gel debris in the 2 ml tube.

2 Rotate or shake for at least 2 hours to elute the DNA.

NOTE
The tube can be rotated overnight.

3 Transfer the eluate and gel debris to the top of a 5 µm filter.

4 Centrifuge at 600 × g for 10 seconds, and then discard the filter.

5 Add the following volumes to the eluate:
- Glycogen (2 µl)
- 3M NaOAc (30 µl)
- [Optional] 0.1X Pellet Paint (2 µl)
- 100% ethanol (975 µl)

6 Centrifuge at 20,000 × g on a benchtop microcentrifuge at 20 minutes at 4°C.

7 Remove and discard the supernatant. Leave the pellet intact.

8 If the pellet becomes loose, centrifuge at 20,000 × g for 2 minutes.

9 Wash the pellet with 500 µl 70% ethanol.

10 Centrifuge at 20,000 × g for 2 minutes.

11 Remove and discard the supernatant. Leave the pellet intact.

12 With the lid open, place the tube in a 37°C heat block until the pellet is dry (~7 minutes).

13 Resuspend the pellet in 10 µl 10 mM Tris-HC1, pH 8.5.

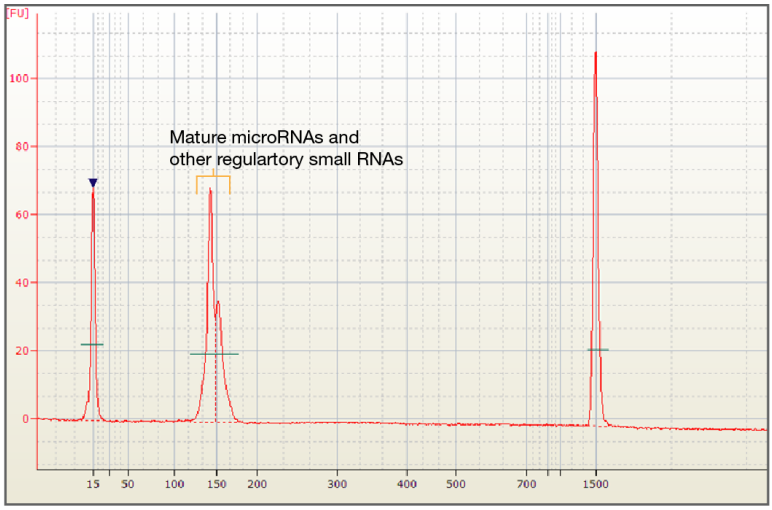
Check Libraries

Perform the following quality control analysis on your library.

1 Load 1 µl resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip, such as the DNA 1000 or High Sensitivity DNA.

NOTE
For libraries prepared without ethanol precipitation, use the High Sensitivity DNA chip.

Figure 6 Example DNA 100 Chip Trace of the Final Library from Human Brain Total RNA



2 Check the size, purity, and concentration of the library.
For clustering, use the total of all molarities from the Bioanalyzer. All peaks create clusters. For example, if there are 2 peaks, add the molarity of each peak. If there are 3 peaks, add the molarity of the 3 peaks together.

Normalize Libraries

This step normalizes DNA libraries to 2 nM.

Consumables

- Tris-HCl 10 mM, pH 8.5
- [Optional] Tween 20

About Reagents

- Tween 20 prevents adsorption of the library to plastic tubes upon repeated freeze-thaw cycles, which decreases cluster numbers over time.

Procedure

1. Normalize library concentration to 2 nM using Tris-HCl 10 mM, pH 8.5.
2. For storage, add Tween 20 to the library for a final concentration of 0.1% Tween 20.

SAFE STOPPING POINT

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

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 the required consumables and equipment.

Acronyms

Acronym	Definition
cDNA	Complementary DNA
CRL	Custom RNA Ladder
HML	Ligation Buffer
HRL	High Resolution Ladder
PCR	Polymerase Chain Reaction
PML	PCR Mix
RA3	RNA 3' Adapter
RA5	RNA 5' Adapter
RIN	RNA Integrity Number
RP1	RNA PCR Primer
RPI	RNA PCR Primer Index
RTP	RNA RT Primer
STP	Stop Solution
UHR	Universal Human Reference

Kit Contents

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

TruSeq Small RNA kits are configured for 24 reactions with 12 unique indexes per kit. The 48 indexes are divided into 4 kits. Each kit contains a core solutions box and an indexes box. Each MiniSeq kit contains 1 library prep kit and 2 MiniSeq reagent kits.

Kit Name	Catalog #
TruSeq Small RNA Library Prep Kit (Indexes 1–12)	RS-200-0012
TruSeq Small RNA Library Prep Kit (Indexes 13–24)	RS-200-0024
TruSeq Small RNA Library Prep Kit (Indexes 25–36)	RS-200-0036
TruSeq Small RNA Library Prep Kit (Indexes 37–48)	RS-200-0048
TruSeq Small RNA Set A MiniSeq Kit	20005613
TruSeq Small RNA Set B MiniSeq Kit	20005614
TruSeq Small RNA Set C MiniSeq Kit	20005615
TruSeq Small RNA Set D MiniSeq Kit	20005616

TruSeq Small RNA Core Solutions, Store at -25°C to -15°C

Slot	Reagent	Description
1	HML	Ligation Buffer
2	STP	Stop Solution
3–4	–	RNase Inhibitor
5	–	T4 RNA Ligase
6	–	10 mM ATP
7	–	25 mM dNTP Mix
8	PML	PCR Mix
9	HRL	High Resolution Ladder
10	CRL	Custom RNA Ladder
11–14	–	Ultrapure Water
15–20	–	Empty

TruSeq Small RNA Indices A, Store at -25°C to -15°C (RS-200-0012)

Slot	Reagent	Description
1	RTP	RNA RT Primer
2	RA3	RNA 3' Adapter
3	RA5	RNA 5' Adapter
4	RP1	RNA PCR Primer
5	RPI1	RNA PCR Primer Index 1
6	RPI2	RNA PCR Primer Index 2
7	RPI3	RNA PCR Primer Index 3
8	RPI4	RNA PCR Primer Index 4
9	RPI5	RNA PCR Primer Index 5

Slot	Reagent	Description
10	RPI6	RNA PCR Primer Index 6
11	RPI7	RNA PCR Primer Index 7
12	RPI8	RNA PCR Primer Index 8
13	RPI9	RNA PCR Primer Index 9
14	RPI10	RNA PCR Primer Index 10
15	RPI11	RNA PCR Primer Index 11
16	RPI12	RNA PCR Primer Index 12

TruSeq Small RNA Indices B, Store at -25°C to -15°C (RS-200-0024)

Slot	Reagent	Description
1	RTP	RNA RT Primer
2	RA3	RNA 3' Adapter
3	RA5	RNA 5' Adapter
4	RP1	RNA PCR Primer
5	RPI13	RNA PCR Primer Index 13
6	RPI14	RNA PCR Primer Index 14
7	RPI15	RNA PCR Primer Index 15
8	RPI16	RNA PCR Primer Index 16
9	RPI17	RNA PCR Primer Index 17
10	RPI18	RNA PCR Primer Index 18
11	RPI19	RNA PCR Primer Index 19
12	RPI20	RNA PCR Primer Index 20
13	RPI21	RNA PCR Primer Index 21
14	RPI22	RNA PCR Primer Index 22
15	RPI23	RNA PCR Primer Index 23
16	RPI24	RNA PCR Primer Index 24

TruSeq Small RNA Indices C, Store at -25°C to -15°C (RS-200-0036)

Slot	Reagent	Description
1	RTP	RNA RT Primer
2	RA3	RNA 3' Adapter
3	RA5	RNA 5' Adapter
4	RP1	RNA PCR Primer
5	RPI25	RNA PCR Primer Index 25
6	RPI26	RNA PCR Primer Index 26
7	RPI27	RNA PCR Primer Index 27
8	RPI28	RNA PCR Primer Index 28
9	RPI29	RNA PCR Primer Index 29
10	RPI30	RNA PCR Primer Index 30
11	RPI31	RNA PCR Primer Index 31
12	RPI32	RNA PCR Primer Index 32
13	RPI33	RNA PCR Primer Index 33
14	RPI34	RNA PCR Primer Index 34
15	RPI35	RNA PCR Primer Index 35
16	RPI36	RNA PCR Primer Index 36

Indices D, Store at -25°C to -15°C (RS-200-0048)

Slot	Reagent	Description
1	RTP	RNA RT Primer
2	RA3	RNA 3' Adapter
3	RA5	RNA 5' Adapter
4	RP1	RNA PCR Primer
5	RPI37	RNA PCR Primer Index 37
6	RPI38	RNA PCR Primer Index 38
7	RPI39	RNA PCR Primer Index 39
8	RPI40	RNA PCR Primer Index 40
9	RPI41	RNA PCR Primer Index 41
10	RPI42	RNA PCR Primer Index 42
11	RPI43	RNA PCR Primer Index 43
12	RPI44	RNA PCR Primer Index 44
13	RPI45	RNA PCR Primer Index 45
14	RPI46	RNA PCR Primer Index 46
15	RPI47	RNA PCR Primer Index 47
16	RPI48	RNA PCR Primer Index 48

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

Consumables

Consumable	Supplier
0.2 ml clean, nuclease-free microcentrifuge tubes	General lab supplier
1.5 ml clean, nuclease-free microcentrifuge tubes	General lab supplier
2 ml clean, nuclease-free microcentrifuge tubes	IST Engineering, part # 5488-100*
1 µg Total RNA in 5 µl nuclease-free water	User experimental samples
200 µl, clean, nuclease-free PCR tubes	General lab supplier
5 µm filter tube	IST Engineering, part # 5388-50
10 mM Tris-HC1, pH 8.5	General lab supplier
Cluster Generation Kit	Any current version of an Illumina Cluster Generation Kit
DNA 1000 Kit	Agilent Technologies, part # 5067-1504
DNA loading dye	Life Technologies, catalog # LC6678 or equivalent
Gel breaker tube	IST Engineering, part # 3388-100
High Sensitivity DNA Kit	Agilent, part # 5067-4626
Novex TBE gels, 6%, 10 well	Life Technologies, catalog # EC6265BOX
Novex TBE running buffer (5X)	Invitrogen, catalog # LC6675
Razor blade	General lab supplier
SuperScript II Reverse Transcriptase	Life Technologies, catalog # 18064-014
T4 RNA Ligase 2, Deletion Mutant • 2,000 (for up to 10 libraries) • 10,000 units (for up to 50 libraries)	Epicentre, catalog # LR2D1132K or LR2D11310K
Ultra pure ethidium bromide 10 mg/ml	General lab supplier
[Optional] Human brain total RNA	Life Technologies, catalog # AM7962
[Optional] Tween 20	Sigma-Aldrich, part # P7949
[Optional] 3 M NaOAc, pH 5.2	General lab supplier

Consumable	Supplier
[Optional] 70% Ethanol, room temperature	General lab supplier
[Optional] 100% Ethanol, -25°C to -15°C	General lab supplier
[Optional] Glycogen (2% w/v)	General lab supplier
[Optional] Pellet Paint NF Co-Precipitant	Novagen, part # 70748

* Or equivalent

Equipment

Equipment	Supplier
2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
Benchtop microcentrifuge	General lab supplier
Cooler block or 96-well working rack for 200 µl tubes	IST Engineering, part # 6388-001 or Stratagene, part # 410094*
Dark Reader transilluminator or UV transilluminator	Clare Chemical Research, part # D195M
Electrophoresis power supply	General lab supplier
Room temperature tube shaker or tube rotator	General lab supplier
Thermal cycler with heated lid	General lab supplier
XCell SureLock Mini-Cell electrophoresis unit	Life Technologies, catalog # EI0001
[Optional] 37°C heat block	General lab supplier
[Optional] 4°C microcentrifuge	General lab supplier

* Or equivalent

Index Sequences

The TruSeq Small RNA kits contain the following index adapter sequences.

Table 1 TruSeq Small RNA Indices A - Sequences 1–12

Index	Sequence	Index	Sequence
RPI1	ATCACG	RPI7	CAGATC
RPI2	CGATGT	RPI8	ACTTGA
RPI3	TTAGGC	RPI9	GATCAG
RPI4	TGACCA	RPI10	TAGCTT
RPI5	ACAGTG	RPI11	GGCTAC
RPI6	GCCAAT	RPI12	CTTGTA

Table 2 TruSeq Small RNA Indices B - Sequences 13–24

Index	Sequence	Index	Sequence
RPI13	AGTCAA	RPI19	GTGAAA
RPI14	AGTTCC	RPI20	GTGGCC
RPI15	ATGTCA	RPI21	GTTTCG
RPI16	CCGTCC	RPI22	CGTACG
RPI17	GTAGAG	RPI23	GAGTGG
RPI18	GTCCGC	RPI24	GGTAGC

Table 3 TruSeq Small RNA Indices C - Sequences 25–36

Index	Sequence	Index	Sequence
RPI25	ACTGAT	RPI31	CACGAT
RPI26	ATGAGC	RPI32	CACTCA
RPI27	ATTCCT	RPI33	CAGGCG
RPI28	CAAAAG	RPI34	CATGGC
RPI29	CAACTA	RPI35	CATTTT
RPI30	CACCGG	RPI36	CCAACA

Table 4 TruSeq Small RNA Indices D - Sequences 37–48

Index	Sequence	Index	Sequence
RPI37	CGGAAT	RPI43	TACAGC
RPI38	CTAGCT	RPI44	TATAAT
RPI39	CTATAC	RPI45	TCATTC
RPI40	CTCAGA	RPI46	TCCCGA
RPI41	GACGAC	RPI47	TCGAAG
RPI42	TAATCG	RPI48	TCGGCA

Pooling Guidelines

The TruSeq Small RNA Library Prep Kit can generate libraries compatible with Illumina multiplexed sequencing, with up to 48 libraries combined in 1 lane. When processing libraries in parallel, add the index at the amplification step following reverse transcription.

- Pool libraries immediately before gel purification or after gel purification and library validation. Pooling before gel purification can reduce the number of times gel purification is needed.
- Pool libraries in equimolar amounts. Determining library concentrations before gel purification can be challenging. If the peaks corresponding to the amplified small RNA species can be distinguished and quantified, the Agilent Bioanalyzer High Sensitivity DNA chip offers an estimate of library concentration.
- Libraries can also be pooled before gel purification and loaded into 2 gel lanes using equal small volumes. The volume of each pooled library depends on the number of libraries being pooled, with the total volume of a pool being 50 µl before gel purification. For example:
  - A pool of 12 libraries requires 4 µl per library.
  - A pool of 24 libraries requires 2 µl per library.
  - A pool of 48 libraries requires 1 µl per library.

Levels of small RNA vary widely by biological samples. Pooling by volume produces better results with libraries derived from similar species and tissues. For highly multiplexed runs, repool and resequence any libraries that do not provide sufficient coverage.

Review the *TruSeq Library Prep Pooling Guide (document # 15042173)* for guidance on choosing indexes to pool together.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 5 Illumina General Contact Information

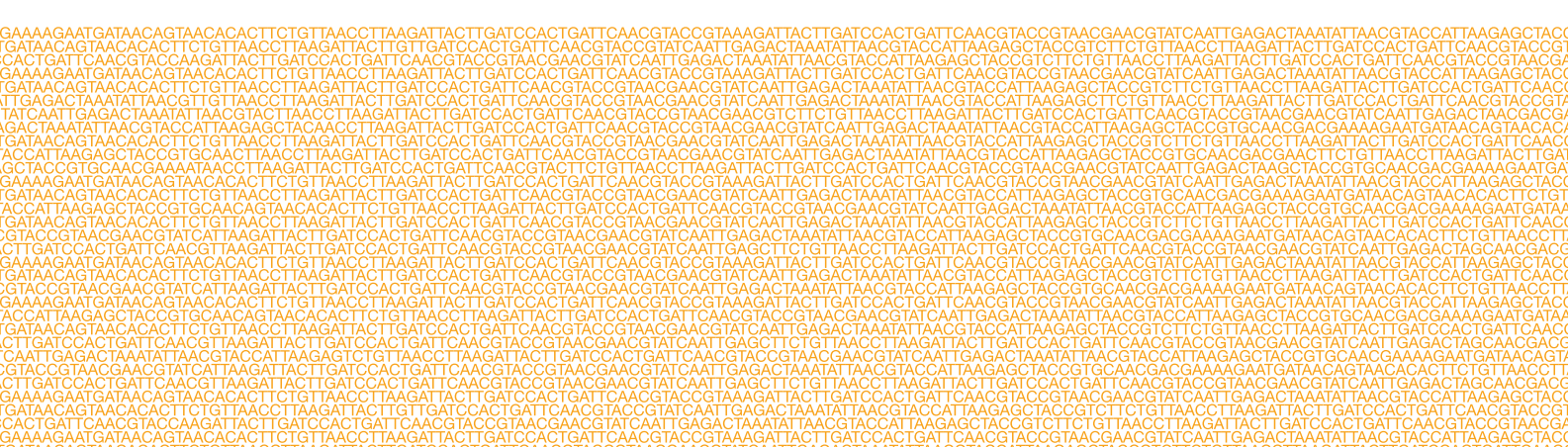
Website	www.illumina.com
Email	techsupport@illumina.com

Table 6 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Japan	0800.111.5011
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
China	400.635.9898	Singapore	1.800.579.2745
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	Taiwan	00806651752
Hong Kong	800960230	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000
Italy	800.874909		

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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