TruSeq® Small RNA
Sample Preparation Guide

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ILLUMINA PROPRIETARY
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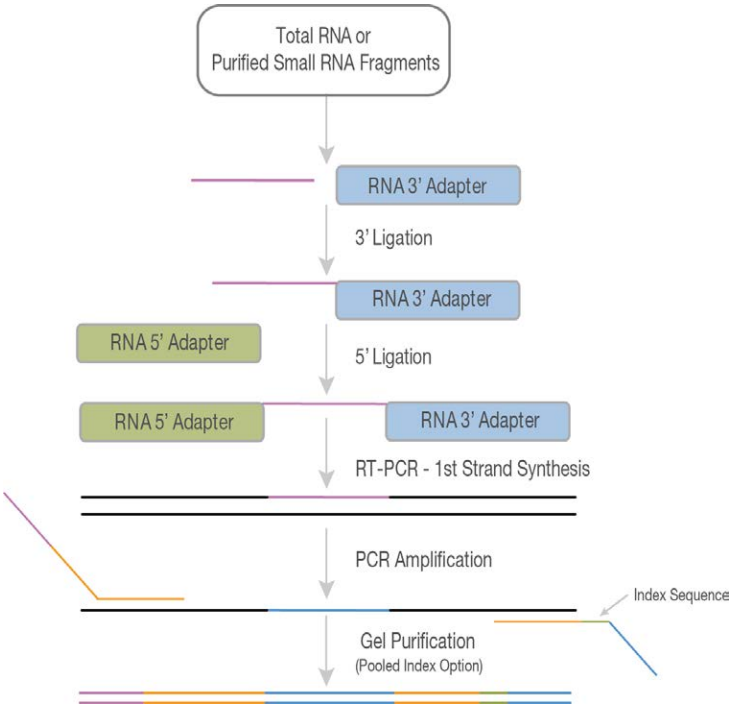
illumina®

Introduction

The Illumina® TruSeq® Small RNA Sample Preparation protocol is used to prepare various RNA species. The protocol takes advantage of the natural structure common to most known microRNA molecules. Most mature miRNAs have a 5'-phosphate and a 3'-hydroxyl group as a result of the cellular pathway used to create them. Because of this, the Illumina adapters in this kit are directly and specifically ligated to miRNAs.

This guide explains how to prepare libraries for subsequent cluster generation, using total RNA or purified small RNA as input. The protocol describes the steps for adapter ligation, reverse transcription, PCR amplification, and pooled gel purification to generate a library product.

Figure 1 Fragments after TruSeq Small RNA Sample Preparation



The RNA 3' adapter is specifically modified to target microRNAs and other small RNAs that have a 3' hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. The adapters are ligated to each end of the RNA molecule and

an RT reaction is used to create single stranded cDNA. The cDNA is then PCR amplified using a common primer and a primer containing one of 48 index sequences. The introduction of the index sequence at the PCR step separates the indexes from the RNA ligation reaction. This design allows for the indexes to be read using a second read and significantly reduces bias compared to designs that include the index within the first read.

The TruSeq Small RNA Sample Preparation allows for the use of 48 different index tags for multiplexing and analysis of directional and small RNA samples. For more information, see *Index Sequences* on page 37. Illumina multiplexed sequencing uses six-base indexes to distinguish different samples from one another in a single lane of a flow cell.

The kits are configured for 24 reactions with 12 different indexes per kit. The 48 indexes are divided into four different kits:

Table 1 TruSeq Small RNA Sample Prep Kits

Indexes	Catalog #	Contains a Core Solutions Box and the following Indices Box
1–12	RS-200-0012	A
13–24	RS-200-0024	B
25–36	RS-200-0036	C
37–48	RS-200-0048	D

What's New

The following changes were made in this guide revision:

- 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 *Supporting Information* containing the following content:
  - Acronyms
  - Kit Contents
  - Consumables and Equipment
  - Indexed Adapter Sequences

Additional Resources

The following resources are available for TruSeq Small RNA Sample Preparation protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSeq Small RNA Sample Prep Kit Support**.

Resource	Description
Training	Illustrates elements of the TruSeq Small RNA Sample Preparation process. Viewing these videos is recommended for new and less experienced users before starting sample preparation. Click **Training** on **TruSeq Small RNA Sample Prep Kit Support**
Best Practices	Provides best practices specific to this protocol. Review these best practices before starting sample preparation. Topics include: • Handling RNA • Handling Liquids • Avoiding Cross-Contamination • Temperature Considerations • Equipment Click **Best Practices** on **TruSeq Small RNA Sample Prep Kit Support**
TruSeq Small RNA Sample Preparation Experienced User Card and Lab Tracking Form (part # 15012191)	Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** Click **Documentation & Literature** on **TruSeq Small RNA Sample Prep Kit Support**
TruSeq Sample Preparation Pooling Guide (part # 15042173)	Provides TruSeq pooling guidelines for sample preparation. Review this guide before beginning library preparation. Click **Documentation & Literature** on **TruSeq Small RNA Sample Prep Kit Support**

Resource	Description
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate. To download the software, click **Downloads** on **TruSeq Small RNA Sample Prep Kit Support** To download the documentation, click **Documentation & Literature** on **TruSeq Small RNA Sample Prep Kit Support**
BaseSpace®	Sequencing data analysis tool that also enables you to organize samples, libraries, pools, and run in a single environment. For more information on BaseSpace see, support.illumina.com/sequencing/sequencing_software/basespace.ilmn

RNA Input Recommendations

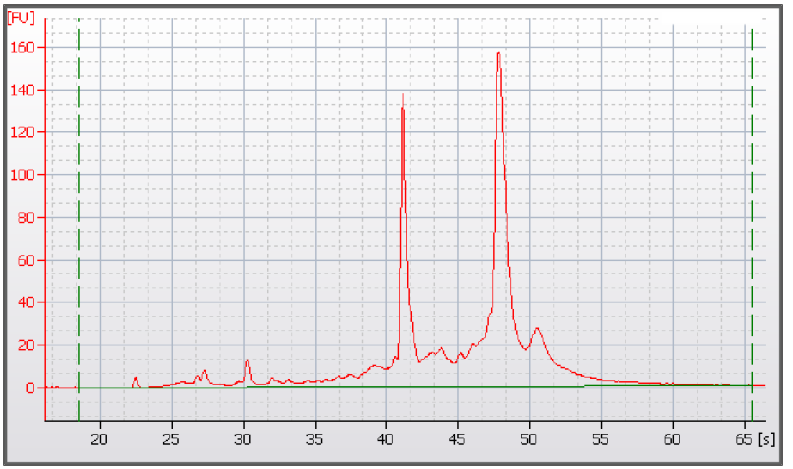
It is important to follow the TruSeq Small RNA Sample Preparation input recommendations.

Total RNA Input

- Optimization
  - The TruSeq Small RNA Sample Preparation protocol is optimized for 1 µg of total RNA in 5 µl nuclease free water, as quantified with a fluorometric method.
  - Lower amounts might result in inefficient ligation and low yield.
- Testing
  - The TruSeq Small RNA Sample Preparation 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.
  - Use of 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.
- It is important to know the quality of the RNA starting material.
  - Use of degraded RNA can result in low yield, changes in observed expression patterns, or failure of the protocol.
  - Illumina recommends that you check total RNA integrity following isolation, using an Agilent Technologies 2100 Bioanalyzer, for human (or mammalian) samples with an RNA Integrity Number (RIN) value ≥ 8. Although this does not directly measure small RNA, an RNA sample with degraded mRNA most likely has degraded small RNA as well.
  - RNA that has DNA contamination results in an underestimation of the amount of RNA used.
  - Illumina recommends including a DNase step with the RNA isolation method. However, contaminant DNA is removed during small RNA purification.

- The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 2 Starting RNA Bioanalyzer Trace



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

Purified Small RNA Input

You can also use previously isolated microRNA as starting material. Use the entire fraction of small RNA purified from 1–10 µg of total RNA. Fewer undesired bands are seen during the subsequent gel extraction using this method.

NOTE
Purified small RNAs ***must*** be in molecular grade water or 10 mM Tris-HCI, pH 8.5.

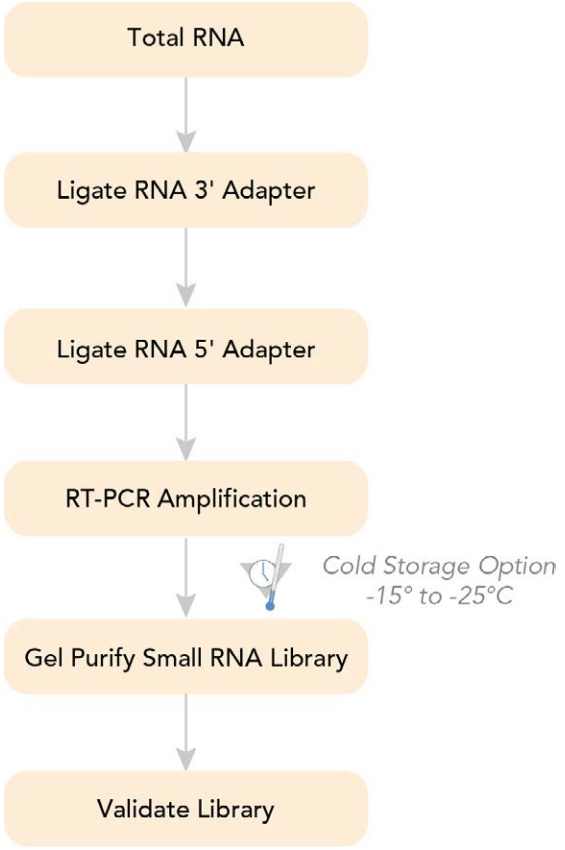
Positive Control

Illumina recommends using Ambion FirstChoice human brain total RNA (catalog # AM7962) as a positive control sample for this protocol. This preparation is certified to contain the small RNA fraction.

Sample Prep Workflow

The following diagram illustrates a single TruSeq Small RNA Sample Preparation workflow. When multiplexing libraries, all samples are processed in parallel through the RT-PCR Amplification process. After PCR amplification, libraries from separate samples can be pooled for a single gel isolation.

Figure 3 TruSeq Small RNA Sample Preparation Workflow



Prepare Adapter Setup

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library preparation.

- Do one of the following:
  - Use IEM to create and edit sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 5 for information on how to download IEM software and documentation from the Illumina website.
  - Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencers and analysis software. See *Additional Resources* on page 5 for information on how to access BaseSpace or download BaseSpace documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 5 for information on how to download the guide from the Illumina website.
- For more information on general TruSeq Small RNA Sample Prep pooling guidelines, see *Pooling Guidelines* on page 39.

Ligate 3' and 5' Adapters

This process describes the sequential ligation of the RNA 3' and RNA 5' RNA adapters to the sample.

Consumables

Item	Quantity	Storage	Supplied By
10 mM ATP	1 tube	-25°C to -15°C	Illumina
Ligation Buffer (HML)	1 tube	-25°C to -15°C	Illumina
RNA 3' Adapter (RA3)	1 tube	-25°C to -15°C	Illumina
RNA 5' Adapter (RA5)	1 tube	-25°C to -15°C	Illumina
RNase Inhibitor	1 tube	-25°C to -15°C	Illumina
Stop Solution (STP)	1 tube	-25°C to -15°C	Illumina
T4 RNA Ligase	1 tube	-25°C to -15°C	Illumina
Ultra Pure Water	1 tube	-25°C to -15°C	Illumina
Nuclease-free 200 µl PCR tubes	3	15°C to 30°C	User
T4 RNA Ligase 2, Deletion Mutant (200 U/µl)	1 µl per sample	-25°C to -15°C	User

NOTE
It is important to use a 96-well working rack when handling 200 µl PCR tubes on ice.

Preparation

- Remove the Illumina-supplied consumables and T4 RNA Ligase 2, Deletion Mutant from -25°C to -15°C storage and thaw on ice.
- Briefly centrifuge the thawed consumables at 600 × g for 5 seconds, then place them on ice.
- Pre-heat the thermal cycler to 70°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C.

Ligate 3' Adapter

1 Set up the ligation reaction in a sterile, nuclease-free 200 µl PCR tube on ice using the following:

Reagent	Volume (µl)
RNA 3' Adapter (RA3)	1
1 µg Total RNA in nuclease-free water	5
Total Volume	**6**

2 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly.

3 Place the tube on the pre-heated thermal cycler. Close the lid and incubate the tube at 70°C for 2 minutes and then immediately place the tube on ice.

NOTE
It is important to keep the RNA 3′ Adapter on ice after the 70°C incubation to prevent secondary structure formation.

4 Pre-heat the thermal cycler to 28°C.

5 Prepare the following mix in a separate, sterile, nuclease-free 200 µl PCR tube on ice. Multiply each reagent volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.

Reagent	Volume (µl)
Ligation Buffer (HML)	2
RNase Inhibitor	1
T4 RNA Ligase 2, **Deletion Mutant**	1
Total Volume per Sample	**4**

NOTE
Do not use the reaction buffer supplied with T4 RNA Ligase 2, Deletion Mutant. This enzyme retains activity in ligation buffer.

6 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly.

7 Add 4 µl of the mix to the reaction tube from step 1 and gently pipette the entire volume up and down 6–8 times to mix thoroughly. The total volume of the reaction is 10 µl.

8 Place the tube on the pre-heated thermal cycler. Close the lid and incubate the tube at 28°C for 1 hour.

9 With the reaction tube on the thermal cycler, add 1 µl Stop Solution (STP) and gently pipette the entire volume up and down 6–8 times to mix thoroughly. Continue to incubate the reaction tube on the thermal cycler at 28°C for 15 minutes and then place the tube on ice.

Ligate 5' Adapter

1 Pre-heat the thermal cycler to 70°C.

2 Prepare a master mix, by aliquoting 1.1 × N µl RNA 5' Adapter (RA5) into a separate, nuclease-free 200 µl PCR tube, with N equal to the number of samples being prepared.

3 Place the PCR tube on the pre-heated thermal cycler. Close the lid and incubate the tube at 70°C for 2 minutes and then immediately place the tube on ice.

NOTE
It is important to keep the RNA 5' Adapter on ice after the 70°C incubation to prevent secondary structure formation. When handling the RNA 5' RNA Adapter, pipette from one tube on ice to another tube on ice and pipette mix the reactions.

4 Pre-heat the thermal cycler to 28°C.

5 Add 1.1 × N µl 10mM ATP to the RNA 5' Adapter aliquot tube, with N equal to the number of samples being prepared. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

6 Add 1.1 × N µl T4 RNA Ligase to the RNA 5' Adapter aliquot tube, with N equal to the number of samples being prepared. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

7 Add 3 µl of the mix from the RNA 5' Adapter aliquot tube to the reaction from step 9 of *Ligate 3' Adapter* on page 12. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
The total volume of the reaction is 14 µl.

8 Place the tube on the pre-heated thermal cycler. Close the lid and incubate the reaction tube at 28°C for 1 hour and then place the tube on ice.

Reverse Transcribe and Amplify

Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 3' and 5' adapters. This process selectively enriches those fragments that have adapter molecules on both ends. PCR is performed with two primers that anneal to the ends of the adapters.

Consumables

Item	Quantity	Storage	Supplied By
25 mM dNTP Mix	1 tube	-25°C to -15°C	Illumina
PCR Mix (PML)	1 tube	-25°C to -15°C	Illumina
RNA PCR Primer (RP1)	1 tube	-25°C to -15°C	Illumina
RNA PCR Primer Index (1–48) (RPI1–RPI48)	1 tube of each index being used	-25°C to -15°C	Illumina
RNA RT Primer (RTP)	1 tube	-25°C to -15°C	Illumina
RNase Inhibitor	1 tube	-25°C to -15°C	Illumina
Ultra Pure Water	1 tube	-25°C to -15°C	Illumina
5X First Strand Buffer	2 µl per sample	-25°C to -15°C	User
100 mM DTT	1 µl per sample	-25°C to -15°C	User
High Sensitivity DNA Chip	1 per sample	15°C to 30°C	User
Nuclease-free 200 µl PCR tubes	3 + 1 per index used	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 µl per sample	-25°C to -15°C	User

NOTE

The TruSeq Small RNA Sample Prep Kits contain up to 48 different indexed PCR primers, each with a different index. During the PCR step, RNA PCR Primer is used with every library with only one of the 48 RNA PCR Primer indexes per library.

Preparation

- Remove the Illumina-supplied consumables, 5X First Strand Buffer, 100 mM DTT, and SuperScript II Reverse Transcriptase from -25°C to -15°C storage and thaw on ice.
- Briefly centrifuge the thawed consumables at 600 × g for 5 seconds, then place them on ice.
- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 5 for information on how to download the guide from the Illumina website.
- Pre-heat the thermal cycler to 70°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C.

Dilute 25 mM dNTP Mix

1 Dilute the 25 mM dNTPs by premixing the following reagents in a separate, sterile, nuclease-free, 200 µl PCR tube. Multiply each reagent volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.

Reagent	Volume (µl)
25 mM dNTP Mix	0.5
Ultra Pure Water	0.5
Total Volume per Sample	**1.0**

2 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly.

3 Label the tube "12.5 mM dNTP Mix" and then place it on ice.

Perform Reverse Transcription

1 Transfer 6 µl of each 5' and 3' adapter-ligated RNA to a separate, sterile, nuclease-free, 200 µl PCR tube.

NOTE
Store the remaining 5' and 3' adapter-ligated RNA at -80°C for up to 7 days, if desired.

2 Add 1 µl RNA RT Primer to each tube containing adapter-ligated RNA.

3 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly.

4 Place the tube on the pre-heated thermal cycler. Close the lid and incubate the tube at 70°C for 2 minutes and then immediately place the tube on ice.

5 Pre-heat the thermal cycler to 50°C.

6 Prepare the following mix in a separate, sterile, nuclease-free, 200 µl PCR tube placed on ice. Multiply each reagent volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.

Reagent	Volume (µl)
5X First Strand Buffer	2
12.5 mM dNTP mix	0.5
100 mM DTT	1
RNase Inhibitor	1
SuperScript II Reverse Transcriptase	1
Total Volume per Sample	**5.5**

7 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly.

8 Add 5.5 µl of the mix to the reaction tube from step 4. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, and then centrifuge briefly. The total volume is 12.5 µl.

9 Place the tube on the pre-heated thermal cycler. Close the lid and incubate the tube at 50°C for 1 hour and then place the tube on ice.

Perform PCR Amplification

1 Prepare a separate PCR tube for each index used. Combine the following reagents in a separate, sterile, nuclease-free, 200 µl PCR tube placed on ice. Multiply each reagent volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples with the same index.

Reagent	Volume (µl)
Ultra Pure Water	8.5
PCR Mix (PML)	25
RNA PCR Primer (RP1)	2
RNA PCR Primer Index (RPIX)	2
Total Volume per Sample	**37.5**

NOTE
For each reaction, only one of the 48 RNA PCR Primer indexes is used during the PCR step.

2 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, centrifuge briefly, and then place the tube on ice.

3 Add 37.5 µl of PCR master mix to the reaction tube from step 9 of *Perform Reverse Transcription* on page 15.

4 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, centrifuge briefly, and then place the tube on ice.
The total volume is 50 µl.

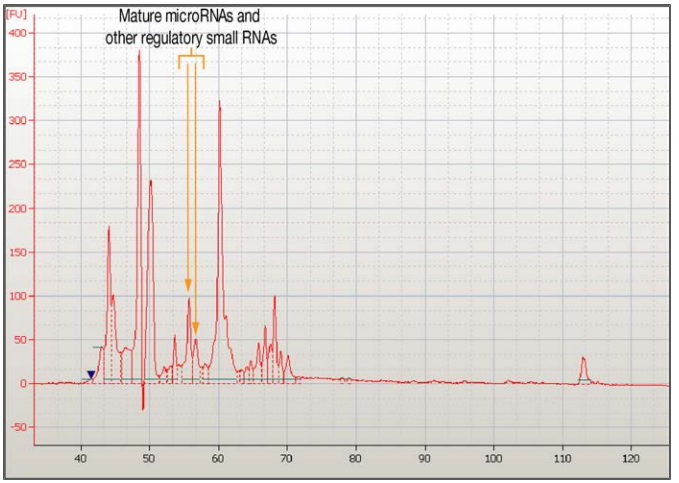
5 Place the tube on the thermal cycler. Close the lid and amplify the tube on the thermal cycler using the following PCR cycling conditions:
   a Choose the thermal cycler pre-heat lid option and set to 100°C.
   b 98°C for 30 seconds
   c 11 cycles of:
      — 98°C for 10 seconds
      — 60°C for 30 seconds
      — 72°C for 15 seconds
   d 72°C for 10 minutes
   e 4°C hold

6 Run each sample on a High Sensitivity DNA Chip according to the manufacturer's instructions. The following figure shows typical results from human brain total RNA.

NOTE

- 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 clear and distinct bands are not observed in the gel image, the number of PCR cycles can be adjusted to a maximum of 15 cycles.
- Components of the amplification reaction can interfere with the bioanalyzer reagents. It may be necessary to dilute the sample before running on the High Sensitivity DNA Chip.
- The bands of the high sensitivity chip can shift from sample to sample due to an incorrect identification of the marker by the bioanalyzer software.

Figure 4 Human Brain Total RNA Sample Trace of Amplicons on High Sensitivity DNA Chip



SAFE STOPPING POINT

If you do not plan to proceed immediately to *Purify cDNA Construct* on page 19, you can safely stop the protocol here. If you are stopping, store the samples at -25°C to -15°C for up to 7 days. When proceeding, thaw the samples on ice.

Purify cDNA Construct

This process gel purifies the amplified cDNA construct in preparation for subsequent cluster generation. After gel purification, the cDNA is eluted and can be concentrated by ethanol precipitation if desired. Ethanol precipitation results in a more concentrated final library, at the cost of some yield. Libraries produced without ethanol precipitation can require special handling during denaturation before loading onto a flow cell.

Individual libraries with unique indexes can be pooled and gel purified together. Combine equal volumes of the library or molar amounts and then load the samples on the gel according to the procedures. Do not load more than 30 µl of sample per well.

NOTE
It is important to follow this procedure exactly to ensure reproducibility.

NOTE
The workflow for pooling more than three samples becomes advantageous to pool by volume. In testing, using the same sample type, a 25% CV of reads per index was achieved.

CAUTION
Illumina does not recommend purifying multiple samples containing the same index on a single gel due to the risk of cross-contamination between libraries. If multiple samples are run on a single gel, keep at least four empty wells between samples.

Consumables

Item	Quantity	Storage	Supplied By
Custom RNA Ladder (CRL)	1 tube	-25°C to -15°C	Illumina
High Resolution Ladder (HRL)	1 tube	-25°C to -15°C	Illumina
Ultra Pure Water	1 tube	-25°C to -15°C	Illumina
5 µm filter tubes	2	15°C to 30°C	User
5X Novex TBE Buffer	as needed	-25°C to -15°C	User
6% Novex TBE PAGE Gel, 1.0 mm, 10 well	1	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Amplified cDNA Construct	50 µl	-25°C to -15°C	User
DNA Loading Dye	13 µl	15°C to 30°C	User
Gel breaker tubes	2	15°C to 30°C	User
High Sensitivity DNA Kit	1 per sample	15°C to 30°C	User
Nuclease-free 200 µl PCR tube	1	15°C to 30°C	User
Razor blade	1	15°C to 30°C	User
Ultra Pure Ethidium Bromide 10mg/ml	0.5 µg/ml in water	-25°C to -15°C	User

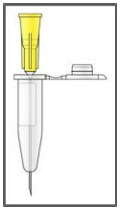
[Optional] Consumables for ethanol precipitation

Item	Quantity	Storage	Supplied By
3 M NaOAc, pH 5.2	30 µl	-25°C to -15°C	User
10 mM Tris-HCI, pH 8.5	10 µl	-25°C to -15°C	User
70% Ethanol	500 µl	15°C to 30°C	User
100% Ethanol	975 µl	-25°C to -15°C	User
Glycogen	2 µl	-25°C to -15°C	User
Pellet Paint NF Co-Precipitant	0.2 µl per sample	-25°C to -15°C	User

Preparation

- Remove the consumables from -25°C to -15°C storage and thaw on ice.
- Briefly centrifuge the thawed consumables at 600 × g for 5 seconds, then place them on ice.
- Review *Pooling Guidelines* on page 39.

- If you are unable to purchase gel breaker tubes, from the tube opening, puncture the bottom of a sterile, nuclease-free, 0.5 ml microcentrifuge tube 3–4 times with a 21-gauge needle.



[Optional] Dilute Pellet Paint NF Co-Precipitant

For ethanol precipitation only. This process marks the visualization of the DNA pellet with a removable dye, making it easier to see and track.

1 Dilute the Pellet Paint NF Co-Precipitant in a separate, sterile, nuclease-free, 200 µl PCR tube. Multiply each reagent volume by the number of samples being prepared, plus 10% extra reagent. Prepare enough pellet paint for a minimum of 10 samples to avoid pipetting small volumes.

Reagent	Volume (µl)
1X Pellet Paint NF Co-Precipitant	0.2
Ultra Pure Water	1.8
Total Volume per Sample	**2.0**

2 Gently pipette the entire volume up and down to mix thoroughly, and then centrifuge briefly.

3 Label the tube “0.1X Pellet Paint“.

Run Gel Electrophoresis

1 Determine the volume of 1X TBE Buffer needed. Dilute the 5X TBE Buffer to 1X for use in electrophoresis.

2 Assemble the gel electrophoresis apparatus per the manufacturer's instructions.

3 Mix 2 µl Custom RNA Ladder with 2 µl DNA Loading Dye.

4 Mix 1 µl High Resolution Ladder with 1 µl DNA Loading Dye.

5 Mix all of the amplified cDNA construct (typically 48–50 µl) with 10 µl DNA Loading Dye.

6 Load 2 µl of mixed Custom RNA Ladder and loading dye in two wells on the 6% PAGE Gel. See Figure 6.

7 Load 2 µl High Resolution Ladder and loading dye in a different well. See Figure 6.

8 Load two wells with 25 µl each of mixed Amplified cDNA Construct and loading dye on the 6% PAGE Gel. Load a total volume of 50 µl on the gel.

9 Run the gel for 60 minutes at 145 V or until the blue front dye exits the gel. Proceed immediately to step 10.

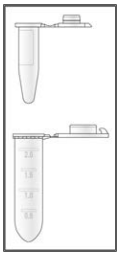
10 Remove the gel from the apparatus.

Recover Purified Construct

1 Open the cassette according to the manufacturer's instructions and stain the gel with Ethidium Bromide (0.5 µg/ml in water) in a clean container for 2–3 minutes.

2 Place the gel breaker tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube.

Figure 5 Place 0.5 ml Tube into 2 ml Tube



3 View the gel on a Dark Reader transilluminator or a UV transilluminator.

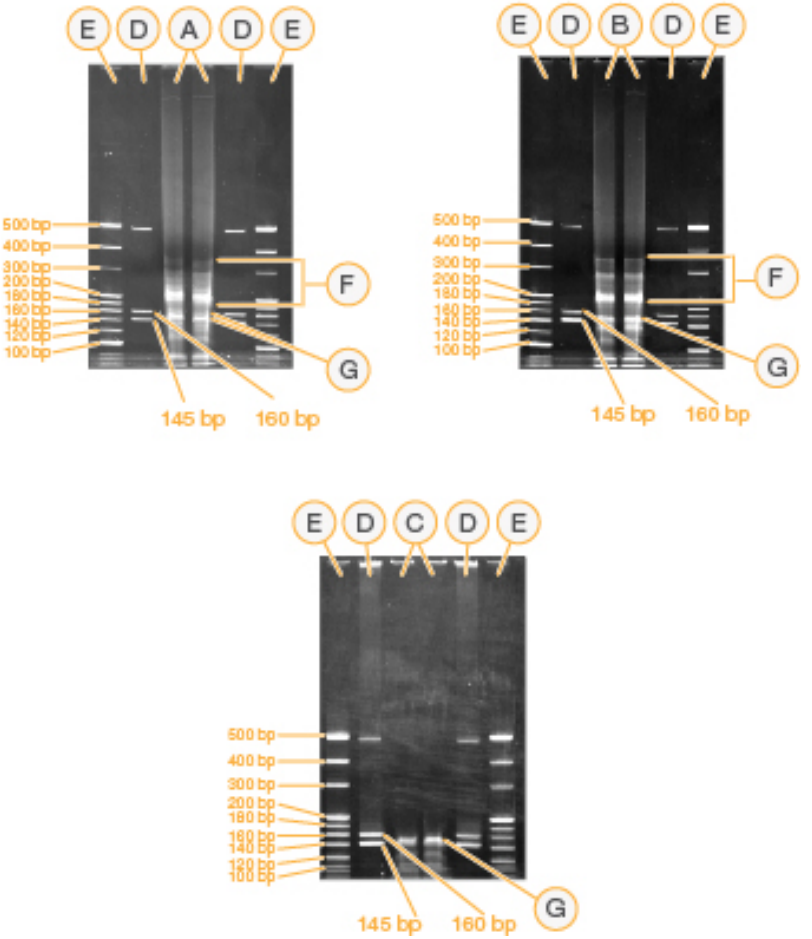
The following figure shows gel analysis of a human and a mouse brain small RNA library. The Custom RNA Ladder consists of three dsDNA fragments 145 bp, 160 bp, and 500 bp.

Sequencing can be conducted on individual bands or from pooled bands. The 147 nt band primarily contains mature microRNA generated from approximately 22 nt small RNA fragments. A second, 157 nt band containing piwi-interacting RNAs, as well as some microRNAs and other regulatory small RNA molecules, is generated from approximately 30 nt RNA fragments.

NOTE

Typically both bands are taken in a single slice and sequenced, but the gel can be parsed for finer resolution of sequencing results.

Figure 6 Small RNA Library from Total RNA Samples



A Human Brain Total RNA
B Mouse Brain Total RNA
C Pre-purified microRNA
D Custom RNA Ladder
E High Resolution Ladder
F Small Non-coding RNAs
G MicroRNA

NOTE

The voltage and run time can vary with different electrophoresis equipment. Optimize the running time so that the 145 bp band from the Custom RNA Ladder is close to the bottom of the gel.

4 Using a razor blade, cut out the bands from both lanes that correspond approximately to the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments. MicroRNAs often vary in length, often called isomiRs. The tighter the band selection, the tighter size distribution of the final microRNA representation.

Align the razor blade with the top of the 160 bp band of the Custom RNA Ladder, then with the bottom of the 145 bp band of the Custom RNA Ladder. Excise the gel fragment by connecting these cuts on the sides. Both lanes can be combined into one slice.

The band containing the 22 nt RNA fragment with both adapters are a total of 147 nt in length. The band containing the 30 nt RNA fragment with both adapters are 157 nt in length.

5 Place the band of interest into the 0.5 ml Gel Breaker tube from step 2.

6 Centrifuge the stacked tubes at 20,000 × g in a microcentrifuge for 2 minutes at room temperature to move the gel through the holes into the 2 ml tube. Make sure that the gel has all moved through the holes into the bottom tube.

7 If precipitating, proceed to *[Optional] Concentrate Final Library by Ethanol Precipitation* on page 25. If not precipitating, add 200 µl Ultra Pure Water to the gel debris in the 2 ml tube.

8 Elute the DNA by rotating or shaking the tube at room temperature for at least 2 hours. The tube can be rotated or shaken overnight, if desired.

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

10 Centrifuge the filter for 10 seconds at 600 × g.

11 Proceed to *Validate Library* on page 27.

[Optional] Concentrate Final Library by Ethanol Precipitation

For higher concentration.

1 Add 300 µl Ultra Pure Water to the gel debris in the 2 ml tube.

2 Elute the DNA by rotating or shaking the tube at room temperature for at least 2 hours. The tube can be rotated overnight, if desired.

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

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

5 Add 2 µl Glycogen, 30 µl 3M NaOAc, 2 µl 0.1X Pellet Paint (optional) and 975 µl of pre-chilled -25°C to -15°C 100% Ethanol to the eluate.

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

NOTE
The precipitation mix can be incubated at -80°C for 20–30 minutes, if desired.

7 Remove and discard the supernatant, leaving the pellet intact.

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

8 Wash the pellet with 500 µl of room temperature 70% Ethanol.

9 Centrifuge at 20,000 × g at room temperature for 2 minutes.

10 Remove and discard the supernatant, leaving the pellet intact.

11 Dry the pellet by placing the tube, lid open, in a 37°C heat block for 5–10 minutes or until dry.

12 Resuspend the pellet in 10 µl 10 mM Tris-HCI, pH 8.5.

13 Proceed to *Validate Library* on page 27.

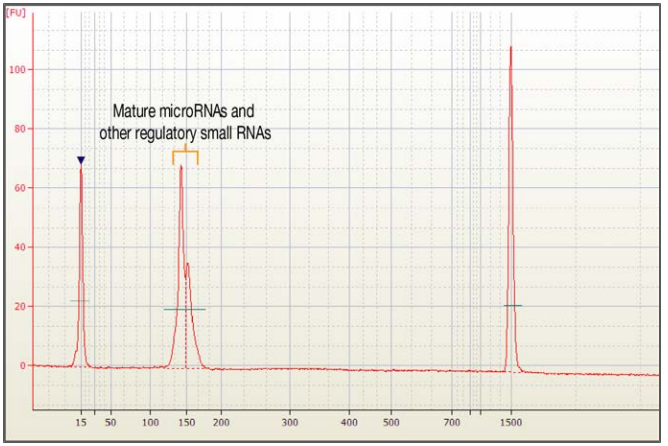
Validate Library

Illumina recommends performing the following quality control analysis on your sample library.

1 Load 1 µl of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the DNA-1000 or High Sensitivity DNA chip. Run samples prepared without ethanol precipitation on a High Sensitivity DNA chip due to their low concentration.

The following is an example of a library prepared using the TruSeq Small RNA Sample Preparation with typical results from human brain total RNA.

Figure 7 Example: DNA 1000 Chip Trace of the Final Library from a Human Brain Total RNA sample



2 Check the size, purity, and concentration of the sample.

NOTE

For clustering, use the total of all the molarities from the bioanalyzer. All peaks create clusters, even if they are adapter dimer, other small RNA, etc. For example, if there are two peaks, add the molarity of each peak. If there are three peaks, add the molarity of the three peaks together. For more information, see the user guide for your Illumina sequencing platform.

DNA Template Storage

This process describes how to prepare DNA templates for cluster generation.

1. Normalize the concentration of sample library to 2 nM using Tris-HCl 10 mM, pH 8.5.
2. For long-term storage, add Tween 20 to the sample library to a final concentration of 0.1% Tween 20. This helps to prevent adsorption of the template to plastic tubes upon repeated freeze-thaw cycles, which would decrease the cluster numbers from a sample over time.
3. Do one of the following:
   - Proceed to cluster generation. For more information, see the user guide for your Illumina sequencing platform.
   - Cap the tube that contains the sample library and store at -25°C to -15°C for up to 7 days before sequencing.

Supporting Information

The protocols described in this guide assume that you are familiar with the contents of this section and have obtained all of the requisite equipment and consumables.

Acronyms

Table 2 TruSeq Small RNA Sample Preparation Acronyms

Acronym	Definition
cDNA	Complementary DNA
CRL	Custom RNA Ladder
EUC	Experienced User Card
HML	Ligation Buffer
HRL	High Resolution Ladder
IEM	Illumina Experiment Manager
LTF	Lab Tracking Form
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

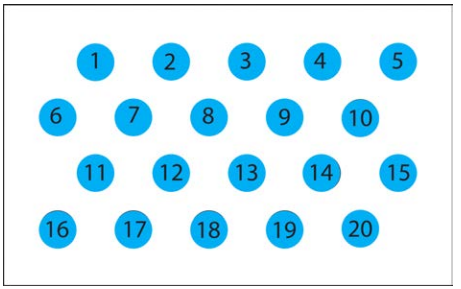
Check to make sure that you have all of the TruSeq Small RNA Sample Prep reagents identified in this section before starting the TruSeq Small RNA Sample Preparation protocol.

Core Solutions, Box Contents

Store at -25°C to -15°C

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

Figure 8 TruSeq Small RNA Sample Prep Kit, Core Solutions Box, part # 15016911



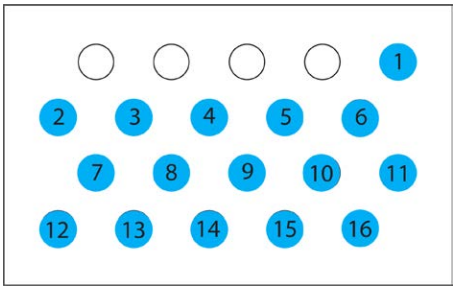
Slot	Reagent	Part #	Description
1	HML	15013206	Ligation Buffer
2	STP	15016304	Stop Solution
3–4	–	15003548	RNase Inhibitor
5	–	1000587	T4 RNA Ligase
6	–	15007432	10 mM ATP
7	–	11318102	25 mM dNTP Mix
8	PML	15022681	PCR Mix
9	HRL	15019401	High Resolution Ladder
10	CRL	15019413	Custom RNA Ladder
11–14	–	1001913	Ultra Pure Water
15–20	–	–	Empty

Indices A, Box Contents

Store at -25°C to -15°C

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

Figure 9 TruSeq Small RNA Sample Prep Kit, Indices A Box, part # 15016912



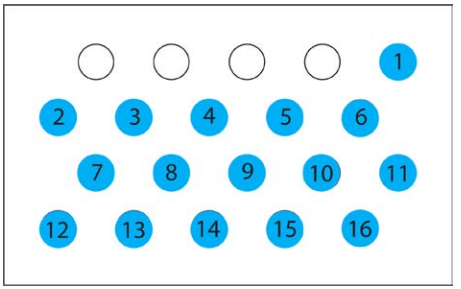
Slot	Reagent	Part #	Description
1	RTP	15013981	RNA RT Primer
2	RA3	15013207	RNA 3' Adapter
3	RA5	15013205	RNA 5' Adapter
4	RP1	15013198	RNA PCR Primer
5	RPI1	15013181	RNA PCR Primer Index 1
6	RPI2	15013185	RNA PCR Primer Index 2
7	RPI3	15013186	RNA PCR Primer Index 3
8	RPI4	15013187	RNA PCR Primer Index 4
9	RPI5	15013188	RNA PCR Primer Index 5
10	RPI6	15013189	RNA PCR Primer Index 6
11	RPI7	15013190	RNA PCR Primer Index 7
12	RPI8	15013191	RNA PCR Primer Index 8
13	RPI9	15013192	RNA PCR Primer Index 9
14	RPI10	15013193	RNA PCR Primer Index 10
15	RPI11	15013195	RNA PCR Primer Index 11
16	RPI12	15013196	RNA PCR Primer Index 12

Indices B, Box Contents

Store at -25°C to -15°C

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

Figure 10 TruSeq Small RNA Sample Prep Kit, Indices B Box part # 15016914



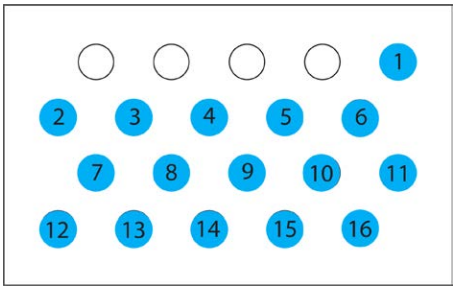
Slot	Reagent	Part #	Description
1	RTP	15013981	RNA RT Primer
2	RA3	15013207	RNA 3' Adapter
3	RA5	15013205	RNA 5' Adapter
4	RP1	15013198	RNA PCR Primer
5	RPI13	15016268	RNA PCR Primer Index 13
6	RPI14	15016269	RNA PCR Primer Index 14
7	RPI15	15016270	RNA PCR Primer Index 15
8	RPI16	15016271	RNA PCR Primer Index 16
9	RPI17	15016272	RNA PCR Primer Index 17
10	RPI18	15016273	RNA PCR Primer Index 18
11	RPI19	15016274	RNA PCR Primer Index 19
12	RPI20	15016275	RNA PCR Primer Index 20
13	RPI21	15016276	RNA PCR Primer Index 21
14	RPI22	15016277	RNA PCR Primer Index 22
15	RPI23	15016278	RNA PCR Primer Index 23
16	RPI24	15016279	RNA PCR Primer Index 24

Indices C, Box Contents

Store at -25°C to -15°C

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

Figure 11 TruSeq Small RNA Sample Prep Kit, Indices C Box, part # 15016916



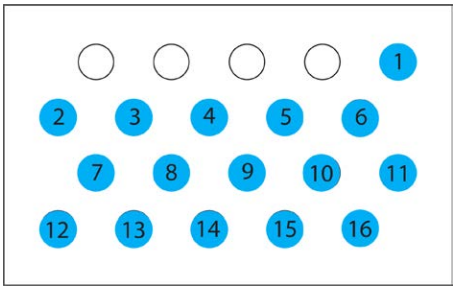
Slot	Reagent	Part #	Description
1	RTP	15013981	RNA RT Primer
2	RA3	15013207	RNA 3' Adapter
3	RA5	15013205	RNA 5' Adapter
4	RP1	15013198	RNA PCR Primer
5	RPI25	15016280	RNA PCR Primer Index 25
6	RPI26	15016281	RNA PCR Primer Index 26
7	RPI27	15016282	RNA PCR Primer Index 27
8	RPI28	15016283	RNA PCR Primer Index 28
9	RPI29	15016284	RNA PCR Primer Index 29
10	RPI30	15016285	RNA PCR Primer Index 30
11	RPI31	15016286	RNA PCR Primer Index 31
12	RPI32	15016287	RNA PCR Primer Index 32
13	RPI33	15016288	RNA PCR Primer Index 33
14	RPI34	15016289	RNA PCR Primer Index 34
15	RPI35	15016290	RNA PCR Primer Index 35
16	RPI36	15016291	RNA PCR Primer Index 36

Indices D, Box Contents

Store at -25°C to -15°C

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

Figure 12 TruSeq Small RNA Sample Prep Kit Indices D Box, part # 15016918



Slot	Reagent	Part #	Description
1	RTP	15013981	RNA RT Primer
2	RA3	15013207	RNA 3' Adapter
3	RA5	15013205	RNA 5' Adapter
4	RP1	15013198	RNA PCR Primer
5	RPI37	15016292	RNA PCR Primer Index 37
6	RPI38	15016293	RNA PCR Primer Index 38
7	RPI39	15016294	RNA PCR Primer Index 39
8	RPI40	15016295	RNA PCR Primer Index 40
9	RPI41	15016296	RNA PCR Primer Index 41
10	RPI42	15016297	RNA PCR Primer Index 42
11	RPI43	15016298	RNA PCR Primer Index 43
12	RPI44	15016299	RNA PCR Primer Index 44
13	RPI45	15016300	RNA PCR Primer Index 45
14	RPI46	15016301	RNA PCR Primer Index 46
15	RPI47	15016302	RNA PCR Primer Index 47
16	RPI48	15016303	RNA PCR Primer Index 48

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to TruSeq Small RNA Sample Preparation.

Table 3 User-Supplied Consumables

Consumable	Supplier
0.2 ml, 1.5 ml, and 2 ml clean, nuclease-free microcentrifuge tubes	General lab supplier (e.g., IST Engineering, VWR, Eppendorf, Fisher Scientific) IST Engineering, 2 ml tube part # 5488-100 www.isteng.com/biotech.asp
1 µg Total RNA in 5 µl nuclease-free water	User experimental samples
200 µl, clean, nuclease-free PCR tubes	General lab supplier
5X Novex TBE buffer	Invitrogen, part # LC6675
5 µm filter tube	IST Engineering, part # 5388-50
6% Novex TBE PAGE gel, 1.0 mm, 10 well	Invitrogen, part # EC6265BOX
Cluster Generation Kit	Any current version of an Illumina Cluster Generation Kit
DNA 1000 chip	Agilent, part # 5067-1504
DNA loading dye	Invitrogen, part # LC6678 or equivalent
Gel Breaker tube (Note: Alternatively, use a nuclease-free, 0.5 ml microcentrifuge tube)	IST Engineering, part # 3388-100
High Sensitivity DNA Kit	Agilent, part # 5067-4626
Razor blade	General lab supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014

Consumable	Supplier
T4 RNA Ligase 2, Deletion Mutant 2,000 (for up to 10 libraries) or 10,000 units (for up to 50 libraries)	Epicentre, catalog # LR2D1132K or LR2D11310K
Ultra pure ethidium bromide 10 mg/ml	General lab supplier

Table 4 User-Supplied Consumables - Optional for Ethanol Precipitation

Consumable	Supplier
10 mM Tris-HCI, pH 8.5	General lab supplier
3 M NaOAc, pH 5.2	General lab supplier
70% Ethanol, room temperature	General lab supplier
100% Ethanol, -25°C to -15°C	General lab supplier
Glycogen (2% w/v)	General lab supplier
Pellet Paint NF Co-Precipitant	Novagen, part # 70748

Table 5 User-Supplied 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 or equivalent
Dark Reader transilluminator or UV transilluminator	Clare Chemical Research, part # D195M
Electrophoresis power supply	General lab supplier

Equipment	Supplier
Room temperature tube shaker or tube rotator	General lab supplier
Thermal cycler	General lab supplier
XCell Sure Lock Mini-Cell electrophoresis unit	Invitrogen, part # EI0001

Table 6 User-Supplied Equipment- Optional for Ethanol Precipitation

Equipment	Supplier
37°C heat block	General lab supplier
4°C microcentrifuge	General lab supplier

Index Sequences

The TruSeq Small RNA Sample Prep Kit contains the following indexed adapter sequences:

Table 7 Indices A Box - Index 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 8 Indices B Box - Index 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 9 Indices C Box - Index 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 10 Indices D Box - Index 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 Sample Prep Kit can be used to construct libraries that are compatible with Illumina multiplexed sequencing, with up to 48 samples combined into a single lane. While processing samples in parallel, incorporate the index at the amplification step following reverse transcription. Pool samples immediately before gel purification or after gel purification and library validation. Pooling before gel purification can greatly reduce the number of necessary gel purifications.

Pool libraries in equimolar amounts. However, 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 two lanes of the PAGE gel, using equal small volumes. The volume of each sample that is pooled depends on the number of samples being pooled, with the total volume of a pool being 50 µl before gel purification. For example:

- A pool of 12 samples requires 4 µl per sample
- A pool of 24 samples requires 2 µl per sample
- A pool of 48 samples requires 1 µl per sample

Different biological samples can have widely varying levels of small RNA. Pooling by volume gives better results with samples derived from similar species and tissues. In the case of highly multiplexed runs, if necessary, repool and resequence any samples that do not provide sufficient coverage.

Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)* for guidance on choosing indexes to pool together. See *Additional Resources* on page 5 for information on how to download the guide from the Illumina website.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 11 Illumina General Contact Information

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

Table 12 Illumina Customer Support Telephone Numbers

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

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

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

Part # 15004197 Rev. F

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