TruSeq™ Small RNA Sample Preparation Guide

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Introduction 3
Sample Prep Workflow 5
Best Practices 6
RNA Input Recommendations 7
Pooling 8
Tracking Tools 9
Kit Contents 11
Consumables and Equipment 17
Ligate 3' and 5' Adapters 20
Reverse Transcribe and Amplify 23
Purify cDNA Construct 29
Validate the Library 36
DNA Template Storage 37
Indexes 39
Technical Assistance 43

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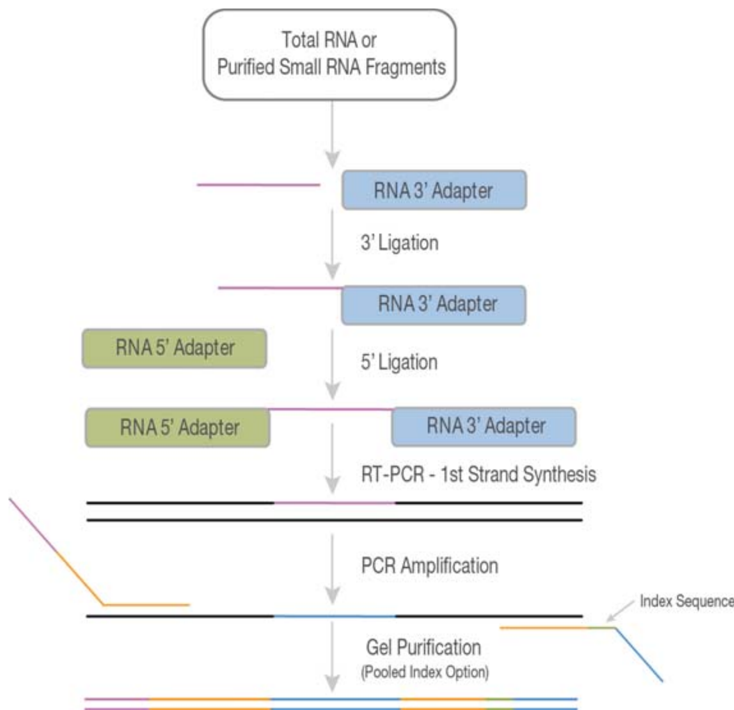
Introduction

The Illumina® TruSeq™ Small RNA Sample Preparation protocol is used to prepare a variety of 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 which include the index within the first read.

One feature of the TruSeq Small RNA Sample Preparation protocol is to allow use of 48 different index tags to make use of the Illumina multiplexing capability for analysis of directional and small RNA samples (see *Indexes* on page 39). The Illumina multiplexing system employs six-base indices to distinguish different samples from one another in a single lane of a flowcell. The kits are configured for 24 reactions with 12 different indexes per kit. The 48 indexes are divided into four different kits:

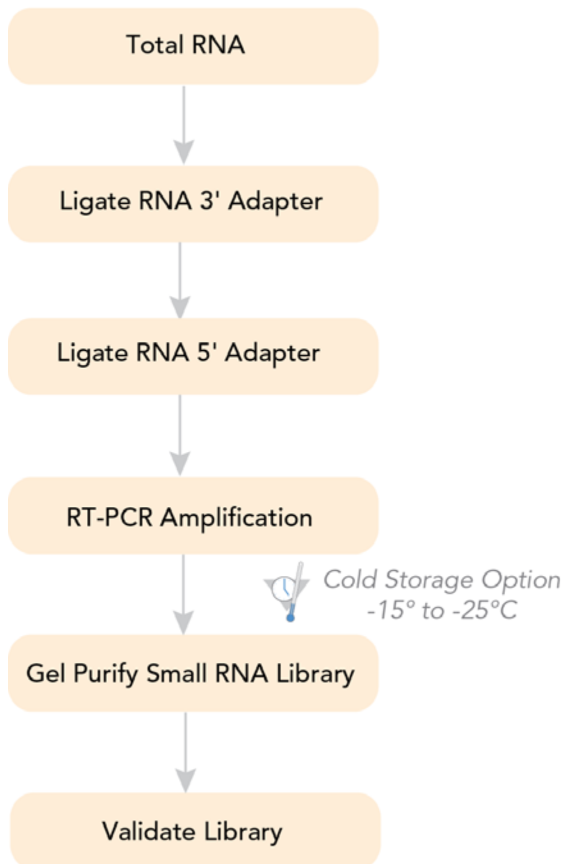
Table 1 TruSeq Small RNA Sample Prep Kits

Indices	Catalog #	Contains Core Solutions Box and 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

Sample Prep Workflow

The following diagram illustrates a single TruSeq Small RNA Sample Preparation workflow. For 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 2 TruSeq Small RNA Sample Preparation Workflow



Best Practices

RNA is highly susceptible to degradation by RNAse enzymes. RNAse enzymes are present in cells and tissues, and carried on hands, labware, and dust. They are very stable and difficult to inactivate. For these reasons, it is important to follow best laboratory practices while preparing and handling RNA samples.

- When harvesting total RNA, use a method that quickly disrupts tissue and isolates and stabilizes RNA.
- Wear gloves and use sterile technique at all times.
- Reserve a set of pipettes for RNA work. Use sterile RNAse-free filter pipette tips to prevent cross-contamination.
- Use disposable plasticware that is certified to be RNAse-free. Illumina recommends the use of non-sticky sterile RNAse-free microcentrifuge tubes. These should not be used for other lab work.
- All reagents should be prepared from RNAse-free components, including ultra pure water.
- Store RNA samples by freezing. Keep samples on ice at all times while working with them. Avoid extended pauses in the protocol until the RNA is in the form of double-stranded DNA.
- Use RNAse/DNAse decontamination solution to decontaminate work surfaces and equipment prior to starting this protocol.

RNA Input Recommendations

Total RNA Input

This protocol has been optimized using 1 µg of high-quality human or mouse brain 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 may require further optimization with regard to the initial input amount and selection of the desired bands during the final gel excision. The types and coverage of small RNAs sequenced will also vary depending on which bands are selected during gel excision.

It is very important to use high-quality RNA as the starting material. Use of degraded RNA can result in low yield or failure of the protocol. Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value greater than 8.

Alternatively, you can run a formaldehyde 1% agarose gel and judge the integrity of RNA upon staining with ethidium bromide. High quality human RNA will show a 28S rRNA band at 4.5 kb that should be twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to a RNA 6000 ladder. The mRNA will appear as a smear from 0.5–12 kb.

Purified Small RNA Input

You can also use previously isolated micro RNA as starting material. Use the entire fraction of small RNA purified from 1–10 µg of total RNA. Fewer undesired bands will be 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.

Pooling

The TruSeq Small RNA Sample Preparation kit can be used to construct libraries that are compatible with Illumina multiplexing, 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 prior to gel purification, or after gel purification and library validation. Pooling prior to 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. The Agilent Bioanalyzer High Sensitivity DNA chip offers an estimate of library concentration if the peaks corresponding to the amplified small RNA species can be distinguished and quantified.

Libraries can also be pooled prior to gel purification 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 would use 4 µl each, a pool of 24 samples would use 2 µl each, and a pool of 48 samples would use 1 µl each. These pools would be loaded into two lanes of the PAGE gel.

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.

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- **Experienced User Cards** to guide you through the protocol, but with less detail than provided in this user guide.
- **Lab Tracking Form** to record lab equipment start and stop times and record the barcode of each reagent and plate used in the protocol.
- **Sample Sheet Template** to record information about your samples for later use in data analysis.

NOTE

All of these documents can be downloaded via http://www.illumina.com/support/documentation.ilmn.

Lab Tracking Form

Create a copy of the lab tracking form for each run. Use it to track information about your sample preparation such as operator information, start and stop times, reagent lot numbers, and barcodes. This form can be filled out and saved online or printed and filled in by hand.

Sample Sheet

The sample sheet is a file that describes the samples in each lane, including the indexes used, and is required for demultiplexing following sequencing. For instructions on using the sample sheet to direct demultiplexing, see the analysis pipeline documentation.

The sample sheet is a comma-separated values (*.csv) file that contains the sample name and related information. Create the sample sheet using Excel or another text editing tool that supports .csv files. Fill in your sample sheet according to the guidelines provided in this section.

Include lanes with multiplexed samples in the sample sheet, listing the information below. Lanes with a single sample can be left out of the sample sheet. These single-sample lanes can then be aligned to a reference genome, as specified in the CASAVA config.template.txt file.

Figure 3 Example: Sample Sheet

	A	B	C	D	E	F	G	H	I
1	FCID	Lane	SampleID	SampleRef	Index	Description	Control	Recipe	Operator
2	FC612PV	1	sample1	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
3	FC612PV	1	sample2	E_coli	TAGCTT	desc1	N	R1	j. doe
4	FC612PV	2	sample3	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
5	FC612PV	2	sample4	CMV	CGATGT	desc1	N	R1	j. doe
6	FC612PV	2	sample5	E_coli	CTTGTA	desc1	N	R1	j. doe
7	FC612PV	3	sample6	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
8	FC612PV	3	sample7	CMV	CGATGT	desc1	N	R1	j. doe
9	FC612PV	3	sample8	E_coli	CTTGTA	desc1	N	R1	j. doe
10	FC612PV	4	sample9	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
11	FC612PV	4	sample10	CMV	CGATGT	desc1	N	R1	j. doe
12	FC612PV	4	sample11	E_coli	CTTGTA	desc1	N	R1	j. doe
13	FC612PV	5	sample12	phi	TTAGGC	desc1	N	R1	j. doe
14	FC612PV	6	sample13	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
15	FC612PV	6	sample14	CMV	CGATGT	desc1	N	R1	j. doe
16	FC612PV	6	sample15	E_coli	CTTGTA	desc1	Y	R1	j. doe
17	FC612PV	7	sample16	Potato	ATCACG	desc1	Y	R1	j. doe
18	FC612PV	7	sample17	CMV	CGATGT	desc1	Y	R1	j. doe
19	FC612PV	7	sample18	CMV	TGACCA	desc1	Y	R1	j. doe

SampleSheet (2)

The sample sheet has the following fields:

Table 2 Sample Sheet Fields

Column Header	Description
FCID	The flow cell ID
Lane	A positive integer indicating the lane number (1–8)
Sample ID	The sample ID. This can be used to specify samples in the CASAVA config.template.txt file.
Sample Ref	The reference sequence for the sample. This can be used to specify a reference genome in the CASAVA config.template.txt.
Index	The index sequence
Description	The sample description
Control	Y indicates the lane is a control lane N indicates a sample
Recipe	The recipe used during sequencing
Operator	The name or ID of the operator

CAUTION

To avoid misidentifying samples, ensure that the sample IDs entered in the sample sheet correctly correspond to the RNA samples used.

Kit Contents

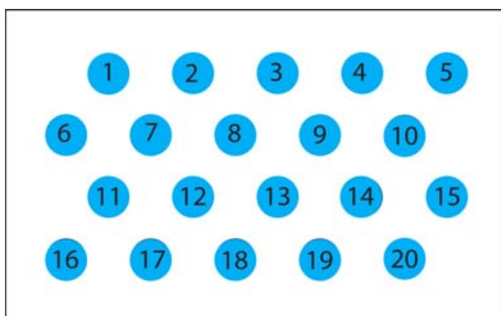
Check to ensure that you have all of the TruSeq Small RNA Sample Prep reagents identified in this section before proceeding. You will receive one TruSeq Small RNA Sample Prep Core Solutions box (24 samples) and one TruSeq Small RNA Sample Prep Indices box (A, B, C, or D) per TruSeq Small RNA Sample Prep Kit (see Table 1 on page 4).

Core Solutions, Box Contents

Store at -15° to -25°C

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

Figure 4 TruSeq Small RNA Sample Prep Core Solutions Box, part # 15016911



1 Ligation Buffer (HML), part # 15013206
2 Stop Solution (STP), part # 15016304
3 RNase Inhibitor, part # 15003548
4 RNase Inhibitor, part # 15003548
5 T4 RNA Ligase, part # 1000587
6 10 mM ATP, part # 15007432
7 25 mM dNTP Mix, part # 11318102
8 PCR Mix (PML), part # 15022681
9 High Resolution Ladder, part # 15019401
10 Custom RNA Ladder, part # 15019413

11 Ultra Pure Water, part # 1001913

12 Ultra Pure Water, part # 1001913

13 Ultra Pure Water, part # 1001913

14 Ultra Pure Water, part # 1001913

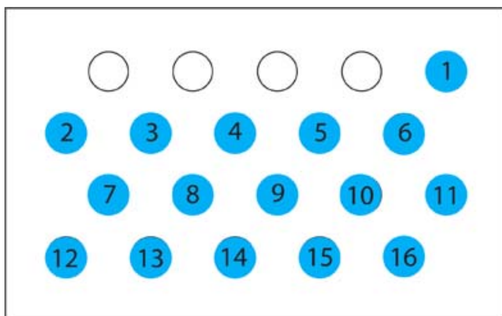
15 *Positions 15–20 are empty*

Indices A, Box Contents

Store at -15° to -25°C

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

Figure 5 TruSeq Small RNA Sample Prep Indices A Box, part # 15016912



1 RNA RT Primer (RTP), part # 15013981

2 RNA 3' Adapter (RA3), part # 15013207

3 RNA 5' Adapter (RA5), part # 15013205

4 RNA PCR Primer (RP1), part # 15013198

5 RNA PCR Primer Index 1 (RPI1), part # 15013181

6 RNA PCR Primer Index 2 (RPI2), part # 15013185

7 RNA PCR Primer Index 3 (RPI3), part # 15013186

8 RNA PCR Primer Index 4 (RPI4), part # 15013187

9 RNA PCR Primer Index 5 (RPI5), part # 15013188

10 RNA PCR Primer Index 6 (RPI6), part # 15013189

11 RNA PCR Primer Index 7 (RPI7), part # 15013190

12 RNA PCR Primer Index 8 (RPI8), part # 15013191

13 RNA PCR Primer Index 9 (RPI9), part # 15013192

14 RNA PCR Primer Index 10 (RPI10), part # 15013193

15 RNA PCR Primer Index 11 (RPI11), part # 15013195

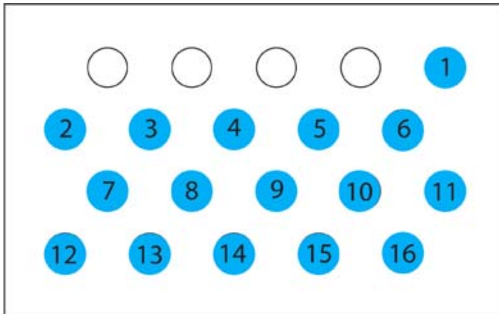
16 RNA PCR Primer Index 12 (RPI12), part # 15013196

Indices B, Box Contents

Store at -15° to -25°C

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

Figure 6 TruSeq Small RNA Sample Prep Indices B Box part # 15016914



1 RNA RT Primer (RTP), part # 15013981

2 RNA 3' Adapter (RA3), part # 15013207

3 RNA 5' Adapter (RA5), part # 15013205

4 RNA PCR Primer (RP1), part # 15013198

5 RNA PCR Primer Index 13 (RPI13), part # 15016268

6 RNA PCR Primer Index 14 (RPI14), part # 15016269

7 RNA PCR Primer Index 15 (RPI15), part # 15016270

8 RNA PCR Primer Index 16 (RPI16), part # 15016271

9 RNA PCR Primer Index 17 (RPI17), part # 15016272

10 RNA PCR Primer Index 18 (RPI18), part # 15016273

11 RNA PCR Primer Index 19 (RPI19), part # 15016274

12 RNA PCR Primer Index 20 (RPI20), part # 15016275

13 RNA PCR Primer Index 21 (RPI21), part # 15016276

14 RNA PCR Primer Index 22 (RPI22), part # 15016277

15 RNA PCR Primer Index 23 (RPI23), part # 15016278

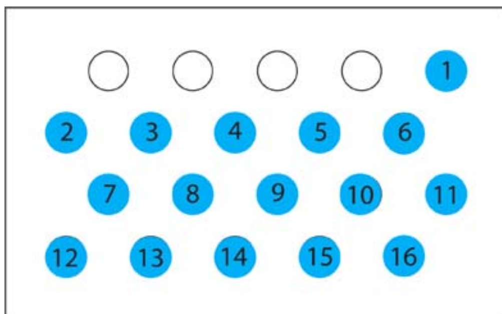
16 RNA PCR Primer Index 24 (RPI24), part # 15016279

Indices C, Box Contents

Store at -15° to -25°C

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

Figure 7 TruSeq Small RNA Sample Prep Indices C Box, part # 15016916



1 RNA RT Primer (RTP), part # 15013981

2 RNA 3' Adapter (RA3), part # 15013207

3 RNA 5' Adapter (RA5), part # 15013205

4 RNA PCR Primer (RP1), part # 15013198

5 RNA PCR Primer Index 25 (RPI25), part # 15016280

6 RNA PCR Primer Index 26 (RPI26), part # 15016281

7 RNA PCR Primer Index 27 (RPI27), part # 15016282

8 RNA PCR Primer Index 28 (RPI28), part # 15016283

9 RNA PCR Primer Index 29 (RPI29), part # 15016284

10 RNA PCR Primer Index 30 (RPI30), part # 15016285

11 RNA PCR Primer Index 31 (RPI31), part # 15016286

12 RNA PCR Primer Index 32 (RPI32), part # 15016287

13 RNA PCR Primer Index 33 (RPI33), part # 15016288

14 RNA PCR Primer Index 34 (RPI34), part # 15016289

15 RNA PCR Primer Index 35 (RPI35), part # 15016290

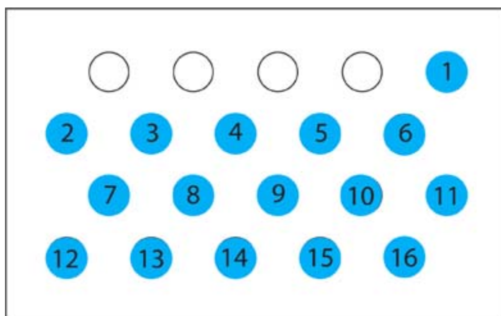
16 RNA PCR Primer Index 36 (RPI36), part # 15016291

Indices D, Box Contents

Store at -15° to -25°C

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

Figure 8 TruSeq Small RNA Sample Prep Indices D Box, part # 15016918



1 RNA RT Primer (RTP), part # 15013981

2 RNA 3' Adapter (RA3), part # 15013207

3 RNA 5' Adapter (RA5), part # 15013205

4 RNA PCR Primer (RP1), part # 15013198

5 RNA PCR Primer Index 37 (RPI37), part # 15016292

6 RNA PCR Primer Index 38 (RPI38, part # 15016293

7 RNA PCR Primer Index 39 (RPI39), part # 15016294

8 RNA PCR Primer Index 40 (RPI40), part # 15016295
9 RNA PCR Primer Index 41 (RPI41), part # 15016296
10 RNA PCR Primer Index 42 (RPI42), part # 15016298
11 RNA PCR Primer Index 43 (RPI43), part # 15016298
12 RNA PCR Primer Index 44 (RPI44), part # 15016299
13 RNA PCR Primer Index 45 (RPI45), part # 15016300
14 RNA PCR Primer Index 46 (RPI46), part # 15016301
15 RNA PCR Primer Index 47 (RPI47), part # 15016302
16 RNA PCR Primer Index 48 (RPI48), part # 15016303

Consumables and Equipment

Check to ensure 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
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 www.isteng.com/biotech.asp
6% Novex TBE PAGE gel, 1.0 mm, 10 well	Invitrogen, part # EC6265BOX
Cluster Generation Kit (required when not performing ethanol precipitation)	Any Illumina Cluster Generation Kit http://www.illumina.com
DNA 1000 chip	Agilent, part # 5067-1504
DNA loading dye	Invitrogen, part # LC6678 or equivalent
Gel Breaker tube (Note: If you are unable to purchase the gel breaker tube, use a nuclease-free, 0.5 ml microcentrifuge tube)	IST Engineering, part # 3388-100
High Sensitivity DNA chip	Agilent, part # 5067-4626
Razor blade	General lab supplier
SuperScript II reverse transcriptase with 100 mM DTT and 5X first strand buffer	Invitrogen, part # 18064-014

Table 3 User-Supplied Consumables (Continued)

Consumable	Supplier
T4 RNA Ligase 2, truncated	NEB, part # M0242S
Ultra pure ethidium bromide 10 mg/ml	General lab supplier

Table 4 User-Supplied Consumables - Optional for Ethanol Precipitation

Consumable	Supplier
10 mM Tris-HCl, pH 8.5	General lab supplier
3 M NaOAc, pH 5.2	General lab supplier
70% Ethanol, room temperature	General lab supplier
100% Ethanol, -15° to -25°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
Benchtop microcentrifuge	General lab supplier
Bioanalyzer	Agilent
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 transilllluminator	Clare Chemical Research, part # D195M
Electrophoresis power supply	General lab 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

Ligate 3' and 5' Adapters

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

Illumina-Supplied Consumables

- Ligation Buffer (HML)
- 10 mM ATP
- RNA 3' Adapter (RA3)
- RNA 5' Adapter (RA5)
- RNase Inhibitor
- Stop Solution (STP)
- T4 RNA Ligase
- Ultra Pure Water

User-Supplied Consumables

- T4 RNA Ligase 2, Truncated

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 from -15° to -25°C storage and thaw on ice.
- Briefly centrifuge the thawed Illumina-supplied consumables to 600 xg for 5 seconds, then place them on ice.
- Pre-heat the thermal cycler to 70°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, then centrifuge briefly.

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

NOTE
It is very 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, truncated	1
Total Volume	**4**

6 Gently pipette the entire volume up and down 6–8 times to mix thoroughly, 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 should be 10 µl.

8 Incubate the tube on the pre-heated thermal cycler at 28°C for 1 hour.

9 With the reaction tube remaining 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 Aliquot 1.1 x N µl of the RNA 5' Adapter (RA5) into a separate, nuclease-free 200 µl PCR tube, with N equal to the number of samples being processed for the current experiment.

3 Incubate the adapter on the pre-heated thermal cycler at 70°C for 2 minutes and then immediately place the tube on ice.

NOTE

It is very 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 X N µl of 10mM ATP to the aliquoted RNA 5' Adapter tube, with N equal to the number of samples being processed for the current experiment. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

6 Add 1.1 X N µl of T4 RNA Ligase to the aliquoted RNA 5' Adapter tube, with N equal to the number of samples being processed for the current experiment. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

7 Add 3 µl of the mix from the aliquoted RNA 5' Adapter tube to the reaction from step 9 of *Ligate 3′ Adapter*. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.

   The total volume of the reaction should now be 14 µl.

8 Incubate the reaction tube on the pre-heated thermal cycler 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.

Illumina-Supplied Consumables

- 25 mM dNTP Mix
- PCR Mix (PML)
- RNA PCR Primer (RP1)
- RNA PCR Primer Index (1–48) (RPI1–RPI48)
  (1 tube of each, depending on the RNA PCR Primer Indices being used)
- RNA RT Primer (RTP)
- RNase Inhibitor
- Ultra Pure Water

User-Supplied Consumables

- 5' and 3' Adapter-ligated RNA (6 µl)
- 5X First Strand Buffer
- 100 mM DTT
- High Sensitivity DNA Chip
- SuperScript II Reverse Transcriptase

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 Indices per library.

Preparation

- Remove the Illumina-supplied consumables from -15° to -25°C storage and thaw on ice.
- Briefly centrifuge the thawed Illumina-supplied consumables to 600 xg for 5 seconds, then place them on ice.
- Pre-heat the thermal cycler to 70°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, then centrifuge briefly.

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

Perform Reverse Transcription

1 Combine the following in a separate, sterile, nuclease-free, 200 µl PCR tube.

Reagent	Volume (µl)
5' and 3' Adapter-ligated RNA	6
RNA RT Primer (RTP)	1
Total Volume per Sample	7

NOTE
The remaining 5' and 3' adapter-ligated RNA may be stored at -80°C.

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

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

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

5 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**

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

7 Add 5.5 µl of the mix to the reaction tube from step 3. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.
The total volume should now be 12.5 µl.

8 Incubate the tube in the pre-heated thermal cycler 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.

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 Indices is used during the PCR step.

2. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly, then place the tube on ice.
3. Add 37.5 µl of PCR master mix to the reaction tube from step 8 of *Perform Reverse Transcription*.
4. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly and place the tube on ice.
   The total volume should now be 50 µl.

5 Amplify the tube in the thermal cycler using the following PCR cycling conditions:
   a 30 seconds at 98°C
   b 11 cycles of:
      — 10 seconds at 98°C
      — 30 seconds at 60°C
      — 15 seconds at 72°C
   c 10 minutes at 72°C
   d Hold at 4°C

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 may 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. The number of PCR cycles can be adjusted, to a maximum of 15 cycles, if clear and distinct bands are not observed in the gel image.

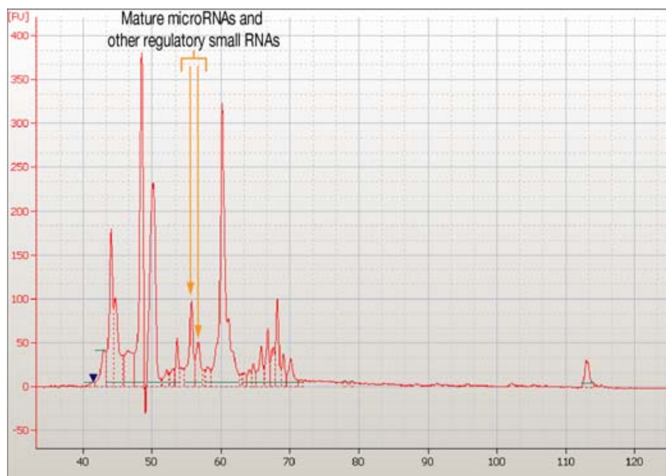
NOTE

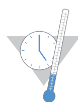
Components of the amplification reaction may interfere with the Bioanalyzer reagents. It may be necessary to dilute the sample before running on the high sensitivity DNA chip.

NOTE

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





SAFE STOPPING POINT

If you do not plan to proceed to Purify cDNA Construct immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. 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 will result in a more concentrated final library, at the cost of some yield. Libraries produced without ethanol precipitation may require special handling during denaturation before loading onto a flow cell.

At this point in the protocol, individual libraries with unique indices may 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 instructions below. 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.

Illumina-Supplied Consumables

- Custom Ladder
- High Resolution Ladder
- Ultra Pure Water

User-Supplied Consumables

- Gel Breaker Tubes
- 5X Novex TBE Buffer
- 5 µm Filter Tube
- 6% Novex TBE PAGE Gel, 1.0 mm, 10 well (1 per 2 sample run)

- Amplified cDNA Construct (50 µl)
- Razor Blade
- DNA Loading Dye
- High Sensitivity DNA Chip
- Ultra Pure Ethidium Bromide 10mg/ml
- Optional items for ethanol precipitation:
  - 10 mM Tris-HCI, pH 8.5
  - 3 M NaOAc, pH 5.2
  - 70% Ethanol, room temperature
  - 100% Ethanol, -15° to -25°C
  - Glycogen
  - Pellet Paint NF Co-Precipitant

NOTE

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, as shown below.

Figure 10 Puncture 0.5 ml Microcentrifuge Tube

Preparation

- Remove the Illumina-supplied consumables from -15° to -25°C storage and thaw on ice.
- Briefly centrifuge the thawed Illumina-supplied consumables to 600 xg for 5 seconds, then place them on ice.

Dilute Pellet Paint NF Co-Precipitant [Optional, 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, then centrifuge briefly.

3 Label the tube “0.1X Pellet Paint“.

Run the 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 of Custom Ladder with 2 µl of DNA Loading Dye.

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

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

6 Load 2 µl of mixed Custom Ladder and loading dye in two wells on the 6% PAGE Gel, as shown in Figure 12.

7 Load 2 µl of High Resolution Ladder and loading dye in a different well, as shown in Figure 12.

8. Load two wells with 25 µl each of mixed Amplified cDNA Construct and loading dye on the 6% PAGE Gel. A total volume of 50 µl should be loaded 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 the next step.
10. Remove the gel from the apparatus.

Recover the 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.

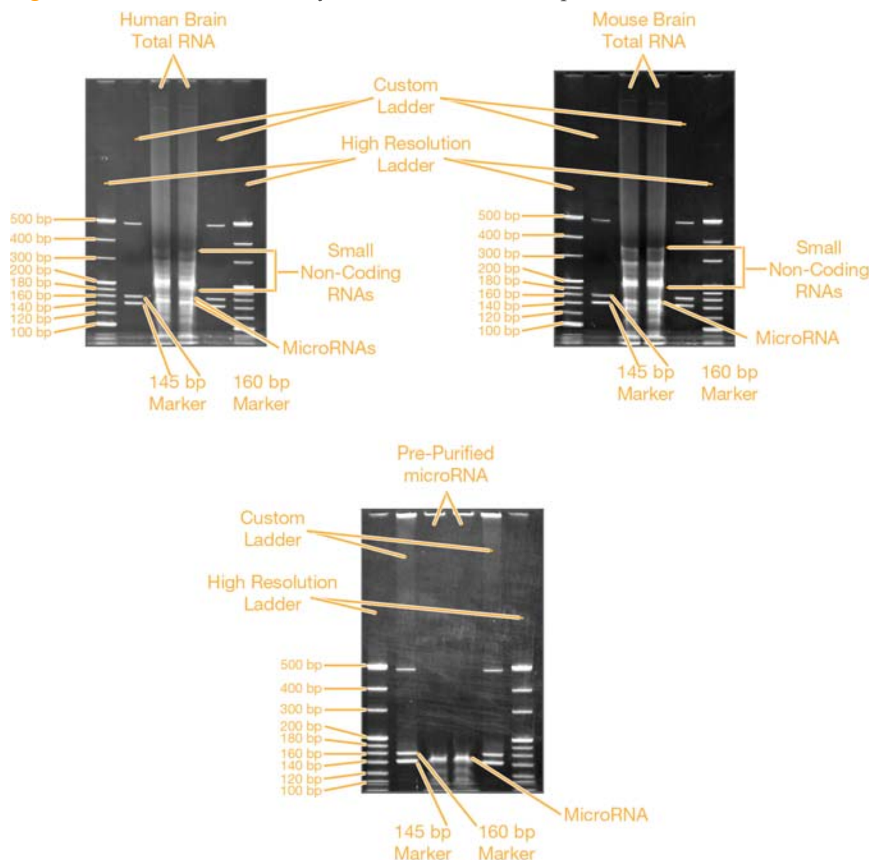
Figure 11 Place 0.5 ml Tube into 2 ml Tube

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 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.

Figure 12 Small RNA Library from Total RNA Samples



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 Ladder is close to the bottom of the gel.

4 Using a razor blade, cut out the bands corresponding to approximately the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments. MicroRNAs often vary in length, often called iso-mirs. 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 Ladder, then with the bottom of the 145 bp band of the Custom Ladder. Excise the gel fragment by connecting these cuts on the sides.

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 to 20,000 xg in a microcentrifuge for 2 minutes at room temperature to move the gel through the holes into the 2 ml tube. Ensure that the gel has all moved through the holes into the bottom tube.
7. If precipitating, proceed to *Concentrate the Final Library by Ethanol Precipitation [Optional, for higher concentration]*. If not precipitating, add 200 µl of 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 to 600 xg.
11. Proceed to *Validate the Library* on page 36. During cluster generation, this library may need to be denatured using the protocol in *DNA Template Storage* on page 37.

Concentrate the Final Library by Ethanol Precipitation [Optional, for higher concentration]

1. Add 300 µl of 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 to 600 xg.
5. Add 2 µl of Glycogen, 30 µl of 3M NaOAc, 2 µl of 0.1X Pellet Paint (optional) and 975 µl of pre-chilled -15° to -25°C 100% Ethanol.
6. Immediately centrifuge to 20,000 xg 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 to 20,000 xg at room temperature for 2 minutes.

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

9 Centrifuge to 20,000 xg 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 the Library* on page 36.

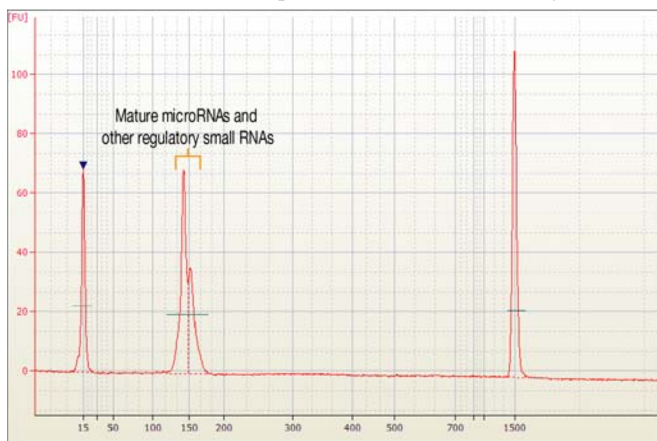
Validate the Library

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. Samples prepared without ethanol precipitation should be run on a High Sensitivity DNA chip due to their low concentration.

   An example of a library prepared using the TruSeq Small RNA Sample Preparation Protocol is shown below with typical results from human brain total RNA.

   Figure 13 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 *cBot User Guide*.

DNA Template Storage

The storage concentration of the prepared library will depend on whether the library is ethanol precipitated after gel purification. Samples that are not ethanol precipitated can be adjusted to 2 nM and denatured for clustering using the following procedure. Samples that are ethanol precipitated can be adjusted to 10 nM and denatured for clustering using the standard protocol (reference the *cBot User Guide* or *Cluster Station User Guide*).

Adjust the concentration for your prepared DNA samples (or pools of samples) to the desired concentration using Tris-HCI 10 mM, pH 8.5. For long-term storage of DNA samples, add Tween 20 to the sample to a final concentration of 0.1% Tween. 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.

Perform the following procedure to denature 2 nM libraries before loading on the flow cell. It uses more volume of the lower-concentration library to denature at the standard 1 nM concentration.

User-Supplied Consumables

- 2 N NaOH
- Hybridization Buffer (HT1) (provided in any Illumina Cluster Generation Kit)
- 10 mM Tris-HCI 10 mM, pH 8.5
- 0.2 ml eight-tube strip

Procedure

1 Combine the following in a 0.2 ml eight-tube strip:

Reagent	Volume (µl)
2 nM Template DNA	10
Tris-HCI 10 mM, pH 8.5	9
2 N NaOH	1
Total Volume	**20**

The template final concentration should be 1 nM.

2 Vortex briefly to mix the template solution.

3. Pulse centrifuge the solution.
4. Incubate for five minutes at room temperature to denature the template into single strands.
5. Place the denatured DNA template on ice until you are ready to proceed to final dilution.

Indexes

Table 7 TruSeq Small RNA Sample Prep Index Sequences

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
RPI1	ATCACG	RPI13	AGTCAA	RPI25	ACTGAT	RPI37	CGGAAT
RPI2	CGATGT	RPI14	AGTTCC	RPI26	ATGAGC	RPI38	CTAGCT
RPI3	TTAGGC	RPI15	ATGTCA	RPI27	ATTCCT	RPI39	CTATAC
RPI4	TGACCA	RPI16	CCGTCC	RPI28	CAAAAG	RPI40	CTCAGA
RPI5	ACAGTG	RPI17	GTAGAG	RPI29	CAACTA	RPI41	GACGAC
RPI6	GCCAAT	RPI18	GTCCGC	RPI30	CACCGG	RPI42	TAATCG
RPI7	CAGATC	RPI19	GTGAAA	RPI31	CACGAT	RPI43	TACAGC
RPI8	ACTTGA	RPI20	GTGGCC	RPI32	CACTCA	RPI44	TATAAT
RPI9	GATCAG	RPI21	GTTTCG	RPI33	CAGGCG	RPI45	TCATTC
RPI10	TAGCTT	RPI22	CGTACG	RPI34	CATGGC	RPI46	TCCCGA
RPI11	GGCTAC	RPI23	GAGTGG	RPI35	CATTTT	RPI47	TCGAAG
RPI12	CTTGTA	RPI24	GGTAGC	RPI36	CCAACA	RPI48	TCGGCA

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Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 8 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 9 Illumina Customer Support Telephone Numbers

Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850
Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at http://www.illumina.com/msds.

Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to http://www.illumina.com/support/documentation.ilmn. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.

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