Small RNA v1.5
Sample Preparation Guide

FOR RESEARCH USE ONLY

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Introduction

This protocol explains how to prepare small RNA libraries using the alternative v1.5 for subsequent sequencing during cluster generation. Libraries prepared by this method should be loaded only on single-read flowcells for cluster generation.

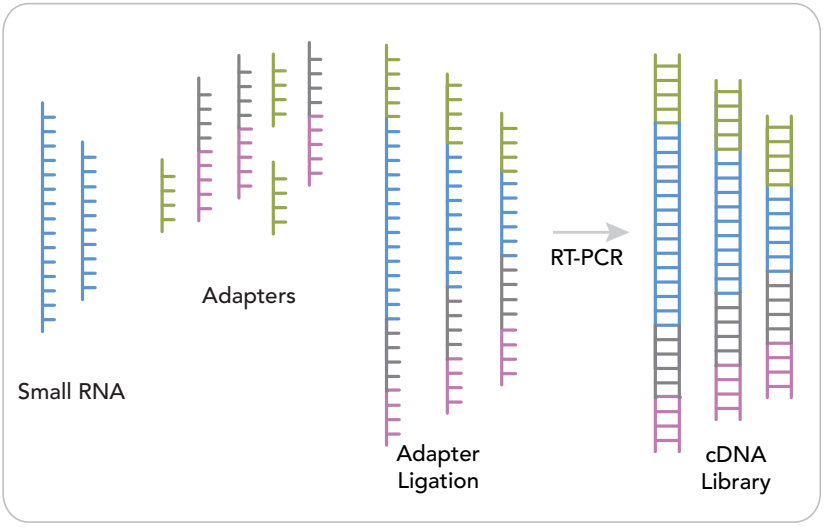
NOTE
The small RNA sequencing primer (part # 1001375) and single-read sequencing methods should be used for the sequencing reactions.

This protocol requires one of the following Illumina products:

- Small RNA Sample Prep Kit (8 samples) FC-102-1009
- Small RNA Sample Prep Kit (40 samples) FC-102-1010

This protocol is designed to use either total RNA or purified small RNAs as input. You ligate the adapters necessary for use during cluster creation, reverse-transcribe, and PCR amplify to generate the following template:

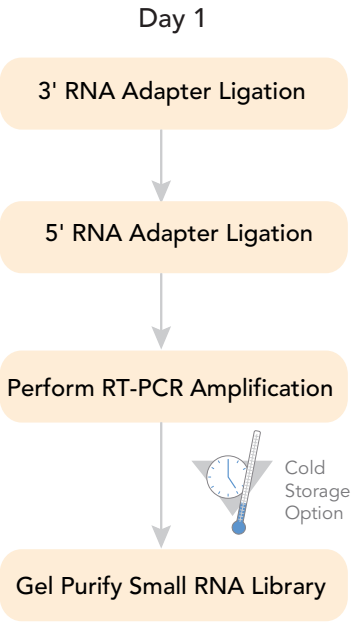
Figure 1 Fragments after Small RNA v1.5 Sample Preparation



The v1.5 small 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 3' adapter is required for reverse transcription and corresponds to the surface-bound amplification primer on the flow cell. The 5' small RNA adapter is necessary for amplification of the small RNA fragments.

Sample Prep Workflow

Figure 2 Small RNA v1.5 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

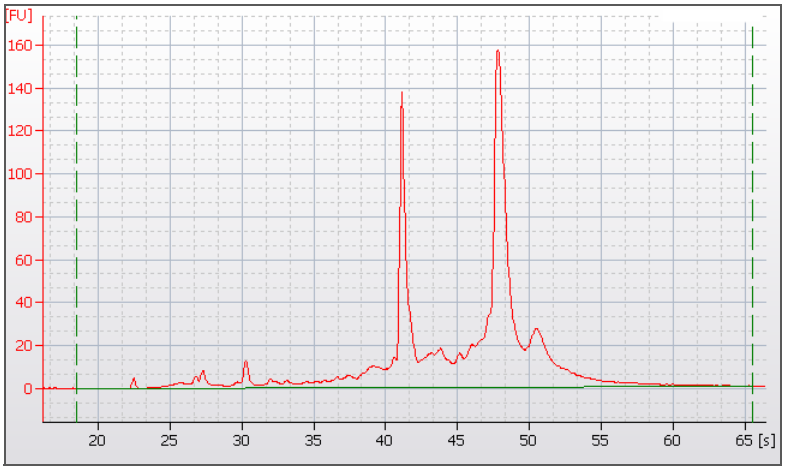
Total RNA Input

This protocol is suitable for 1–10 µg of total RNA. Lower amounts may result in inefficient ligation and low yield. The protocol has been optimized using 1 µg of high-quality human or mouse brain total RNA as input. 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 type 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.

Figure 3 is a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 3 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 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. Small RNA will not be specifically visible.

Purified Small RNA Input

You can also use previously isolated mRNA 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.

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.

Kit Contents

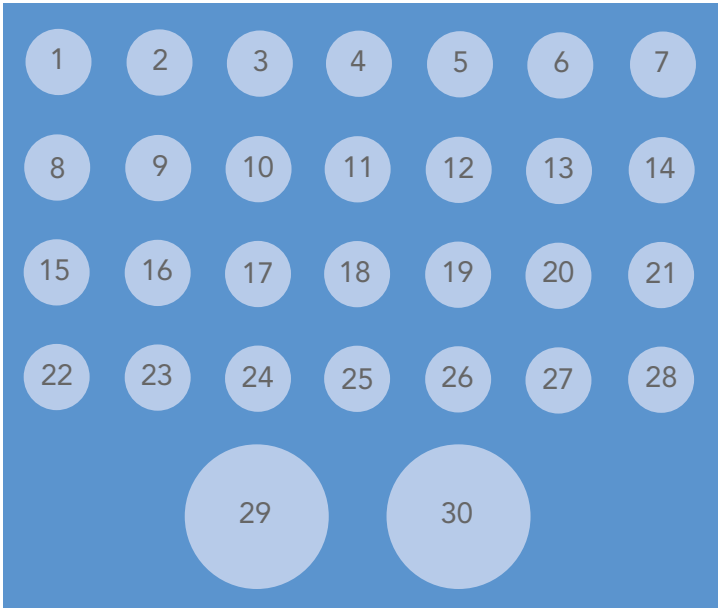
Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

Kit Contents, Box 1

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 Small RNA v1.5 Sample Prep Kit, Box 1



1 SRA Ladder, part # 1001665

2 SRA Gel Loading Dye, part # 1001661

3 Glycogen, part # 15009052

4 T4 RNA Ligase, part # 1000587

5 10X T4 RNA Ligase Buffer, part # 1000588

6 RNase Inhibitor, part # 15003548

7 SRA RT Primer, part # 1000597

8 25 mM dNTP Mix, part # 1001663

9 Phusion™ Polymerase (Finnzymes Oy), part # 1000584

10 5X Phusion HF Buffer (Finnzymes Oy), part # 1000585

11 Primer GX1, part # 1000591

12 Primer GX2, part # 1000592

13 25 bp Ladder, part # 1001662

14 10X Gel Elution Buffer, part # 1000571

15 Resuspension Buffer, part # 1001388

16 *Positions 16–28 are empty*

17 Ultra Pure Water (store at 2° to 8°C), part # 1000467

18 SRA 0.3 M NaCl, part # 1000573

Kit Contents, Box 2

Store at Room Temperature

These components are shipped at room temperature.

- Spin X Cellulose Acetate Filter

Kit Contents, Bag 1

Store at -15° to -25°C

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

- SRA 5' Adapter, part # 1000595
- SRA 3' Adapter, part # 1000596 (The SRA 3' adapter is not used in this v1.5 protocol.)

NOTE

The kit contents for the Small RNA standard and v1.5 protocols are the same. However, the SRA 3' Adapter is not used in the Small RNA v1.5 protocol described in this document. To perform the Small RNA standard protocol, which uses this adapter, see the *Small RNA Sample Preparation Guide*.

NOTE

Briefly centrifuge the tubes before use, as the contents may have settled on the sides.

Kit Contents, Bag 2

Store at -15° to -25°C

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

- 10X v1.5 sRNA 3' Adapter, part # 15000263

NOTE

All reagents are supplied in excess to guarantee you have the quantity necessary to perform eight small RNA sample preparations. It is normal to have leftover reagents following the preparation of eight samples.

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 1 User-Supplied Consumables

Consumable	Supplier
1–10 µg Total RNA in 5 µl nuclease-free water	User experimental samples
[Optional] Positive control RNA	Ambion, catalog # AM7962 or equivalent
3 M NaOAc, pH 5.2	General lab supplier
5X Novex TBE buffer	Invitrogen, part # LC6675
6% Novex TBE PAGE gel, 1.0 mm, 10 well	Invitrogen, part # EC6265BOX
10 mM ATP	Epicenter, part # R109AT or any molecular grade substitute
10X T4 RNL2 truncated reaction buffer	NEB-supplied
21-gauge needles	General lab supplier
100 mM $MgCl_2$	a 100 mM solution can be prepared from 1 M MgCl2 (USB, part # 78641) or any molecular grade substitute
70% Ethanol, room temperature	General lab supplier
75% Ethanol, room temperature	General lab supplier
100% Ethanol, -15° to -25°C	General lab supplier
100% Ethanol, room temperature	General lab supplier
Clean 0.2 ml, 0.5 ml, and 2.0 ml nuclease-free microcentrifuge tubes	General lab supplier
Clean scalpels	General lab supplier
DNA loading dye	Invitrogen, part # LC6678 or equivalent

Table 1 User-Supplied Consumables (Continued)

Consumable	Supplier
Nuclease-free water	General lab supplier
SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer	Invitrogen, part # 18064-014
T4 RNA Ligase 2	NEB, part # M0242S
Ultra Pure Ethidium Bromide 10 mg/ml	General lab supplier

Table 2 Equipment Checklist

Equipment	Supplier
4°C microcentrifuge (for ethanol precipitation)	General lab supplier
Benchtop microcentrifuge	General lab supplier
Dark Reader transilluminator or UV transillluminator	Clare Chemical Research, part # D195M
Electrophoresis power supply	General lab supplier
Heat block	General lab supplier
Room temperature tube rotator	General lab supplier
Savant speed vac	General lab supplier
Thermal cycler	General lab supplier
XCell Sure Lock Mini-Cell electrophoresis unit	Invitrogen, part # EI0001

Ligate the 3' and 5' Adapters

This process describes the first ligation reaction of the v1.5 small RNA 3' adapter, followed by the 5' adapter ligation. This process ligates adapters to the 3' and 5' ends of the isolated small RNA.

Illumina-Supplied Consumables

- 10X v1.5 sRNA 3' Adapter
- RNase Inhibitor
- SRA 5' Adapter
- SRA RT Primer
- T4 RNA Ligase
- Ultra Pure Water

NOTE
The SRA 3' adapter is not used in the v1.5 protocol. Use the v1.5 sRNA 3' adapter.

User-Supplied Consumables

- 1 M $MgCl_2$ Solution
- 1–10 µg Total RNA in 5 µl Nuclease-free Water
- 10 mM ATP
- 10X T4 RNL2 Truncated Reaction Buffer (NEB-supplied)
- Nuclease-free Water
- T4 RNA Ligase 2, Truncated (NEB-supplied)

NOTE
Prepare fresh dilutions of the adapter and primer with each use.

Procedure

1 Dilute the 10X v1.5 sRNA 3' (1:10) adapter by mixing 1 µl adapter with 9 µl of nuclease-free water.

2 Dilute the SRA RT primer (1:5) by mixing 1 µl primer with 4 µl nuclease-free water.

3 Dilute the 1 M $MgCl_2$ (1:10) solution by mixing 100 µl with 900 µl nuclease-free water.

4 Preheat the PCR thermal cycler to 22°C and the heat block to 70°C.

5 Set up the ligation reactions in a sterile, nuclease-free 200 µl microcentrifuge tube using the following:

Reagent	Volume (µl)
1–10 µg Total RNA in Nuclease-free Water	5
Diluted 1X v1.5 sRNA 3' Adapter	1.0

6 Incubate at 70° C for 2 minutes, then transfer immediately to ice.

7 Add the following reagents and mix well:

Reagent	Volume (µl)
10X T4 RNL2 Truncated Reaction Buffer	1.0
100 mM $MgCl_2$	0.8
T4 RNA Ligase 2, Truncated	1.5
RNase Inhibitor	0.5

8 Incubate on the preset thermal cycler at 22°C for 1 hour.

9 With 5 minutes remaining, prepare the 5' adapter for ligation by heating it at 70°C for 2 minutes, then transferring it to ice.

10 Add the following reagents to the ligation mixture from step 8 and mix well:

Reagent	Volume (µl)
10 mM ATP	1.0
SRA 5' Adapter	1.0
T4 RNA Ligase	1.0

11 Incubate on the preset thermal cycler at 20°C for 1 hour.

12 Do one of the following:

- It is the preferred method that you proceed to *Reverse Transcribe and Amplify* on page 16.
- If necessary, you can store the ligated adapters at 4°C on the thermal cycler overnight if you do not plan to proceed to the next step immediately.

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. The PCR is performed with two primers that anneal to the ends of the adapters.

Illumina-Supplied Consumables

- 25 mM dNTP Mix
- 5X Phusion HF Buffer (Finnzymes Oy)
- Phusion Polymerase (Finnzymes Oy)
- Primer GX1
- Primer GX2
- RNase Inhibitor
- SRA RT Primer
- Ultra Pure Water

User-Supplied Consumables

- 5' and 3' Adapter-ligated RNA (4.0 µl)
- SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer

Prepare Template

1 Preheat the PCR thermal cycler to 70°C.

2 Combine the following in a sterile, nuclease-free, 200 µl microcentrifuge tube:

Reagent	Volume (µl)
5' and 3' Ligated RNA	4
Diluted SRA RT Primer	1.0
Total Volume	**5**

3 Briefly centrifuge the mixture, then heat the mixture at 70°C on the preset thermal cycler for 2 minutes.

4 Place the tube on ice.

Dilute the 25 mM dNTP Mix

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

Reagent	Volume (µl)
Ultra Pure Water	0.5
25 mM dNTP Mix	0.5
Total Volume per Sample	**1**

2 Label the tube "12.5 mM dNTP Mix."

Reverse Transcription

1 Preheat the PCR thermal cycler to 48°C.

2 Premix the following reagents in the order listed in a separate tube. The following mix is for one sample. Multiply each 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	1.0
RNase Inhibitor	0.5
Total Volume per Sample	**4**

3 Add 4 µl of the mix to the iced tube containing the primer-annealed template material.

The total volume should now be 9 µl (5 µl of template preparation and 4 µl of reverse transcription).

4 Heat the sample on the preset thermal cycler to 48°C for 3 minutes.

5 Add 1 µl SuperScript II Reverse Transcriptase. The total volume should now be 10 µl.

6 Incubate on the preset thermal cycler at 44°C for 1 hour.

Prepare the PCR Master Mix

1 Premix the following reagents in the listed order in a separate tube. The following mix is for one sample. Multiply each volume by the number of samples being prepared.

Reagent	Volume (µl)
Ultra Pure Water	27
5X Phusion HF Buffer	10
Primer GX1	1.0
Primer GX2	1.0
25 mM dNTP Mix	0.5
Phusion DNA Polymerase	0.5
Total Volume per Sample	**40**

PCR Amplification

1 Add 40 µl of PCR master mix into a sterile, nuclease-free 200 µl PCR tube.

2 Add 10 µl of single strand reverse-transcribed cDNA.

3 Amplify the PCR on the thermal cycler using the following PCR cycling conditions:

NOTE

This process can be programed and saved as the 'Illumina Small RNA Library Amplification'.

a 30 seconds at 98°C

b 12 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

NOTE

Amplification conditions may vary based on RNA input amount, tissue type, and species. This protocol was optimized using 1 µg of total RNA from mouse and human brain. The number of PCR cycles can be adjusted if clear and distinct bands are not observed in the gel image. However, only run between 12 and 15 cycles.

SAFE STOPPING POINT

This is a safe stopping point. If you are stopping, store your sample at -15° to -25°C.

Purify cDNA Construct

This process gel purifies the amplified cDNA construct in preparation for subsequent cluster generation.

NOTE

It is important to follow this procedure exactly to ensure reproducibility. Illumina does not recommend purifying multiple samples 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

- 1X Resuspension Buffer
- 10X Gel Elution Buffer
- 25 bp Ladder
- Glycogen
- Spin-X Cellulose Acetate Filter
- Ultra Pure Water

User-Supplied Consumables

- 3 M NaOAc, pH 5.2
- 5X Novex TBE Buffer
- 6% Novex TBE PAGE Gel, 1.0 mm, 10 well
- 21-gauge Needles
- Amplified cDNA Construct (50 µl)
- Clean Scalpels
- DNA Loading Dye
- 70% Ethanol, room temperature
- 100% Ethanol -15° to -25°C
- Ultra Pure Ethidium Bromide

Prepare the Gel Electrophoresis Reagents and Apparatus

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.

Run the Gel Electrophoresis

1 Mix 1 µl of 25 bp Ladder with 1 µl of DNA Loading Dye.

2 Mix 50 µl of Amplified cDNA Construct with 10 µl of DNA Loading Dye.

3 Load 2 µl of mixed 25 bp Ladder and loading dye in one well on the 6% PAGE Gel.

4 Load two wells with 25 µl each of mixed Amplified cDNA Construct and loading dye on the 6% PAGE Gel.

5 Run the gel for 30–35 minutes at 200 V or until the front dye completely exits the gel.

NOTE
The voltage and run time can vary with different electrophoresis equipment. Optimize the running time so that the 100 bp band from the 25 bp Ladder is close to the bottom of the gel.

6 Remove the gel from the apparatus.

Dilute the 10X Gel Elution Buffer

1 Dilute the 10X Gel Elution Buffer into a fresh tube.

Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

Reagent	Volume (µl)
Ultra Pure Water	90
10X Gel Elution Buffer	10
Total Volume per Sample	**100**

Recover the Purified Construct

1 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 in Figure 5.

Figure 5 Puncture 0.5 ml Microcentrifuge Tube

2 Place the 0.5 ml microcentrifuge tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube, as shown in Figure 6.

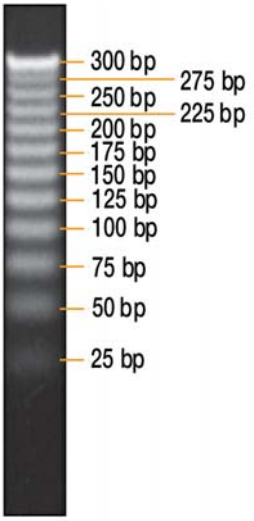
Figure 6 Place 0.5 ml Tube into 2 ml Tube

3 Using the supplied Novex wedge, pry apart the cassette and stain the gel with the Ethidium Bromide in a clean container for 2–3 minutes.

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

The 25 bp Ladder consists of 12 dsDNA fragments between 25 bp and 300 bp in 25 bp increments. The 300 bp band is approximately 2–3 times brighter than the other bands.

Figure 7 25 bp Ladder



5 Using a clean scalpel, cut out the bands corresponding to approximately the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments.

The band containing the 22 nt RNA fragment with both adapters are a total of 93 nt in length. The band containing the 30 nt RNA fragment with both adapters are 100 nt in length.

NOTE

Do not cut the 75 bp band out, as this is adapter dimers.

Figure 8 contains two gel images representing small RNA libraries generated from human and mouse brain total RNA and Figure 9 contains a third library made from small RNA fragments purified from 1 µg of human brain total RNA.

Sequencing can be conducted on individual bands or from pooled bands. The 93 nucleotide band primarily contains mature microRNA generated from approximately 22 nucleotide small RNA fragments.

A second band containing piwi-interacting RNAs, as well as some microRNAs and other regulatory small RNA molecules, corresponds to 100 nucleotides in length and is generated from approximately 30 nucleotide RNA fragments.

Figure 8 Small RNA Libraries from Total RNA

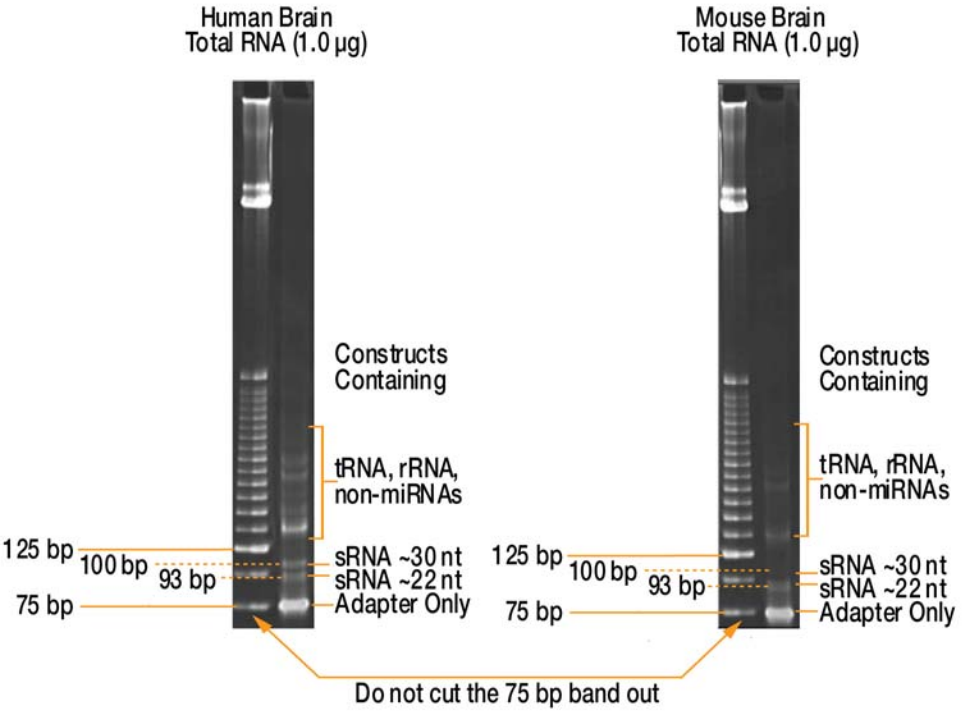
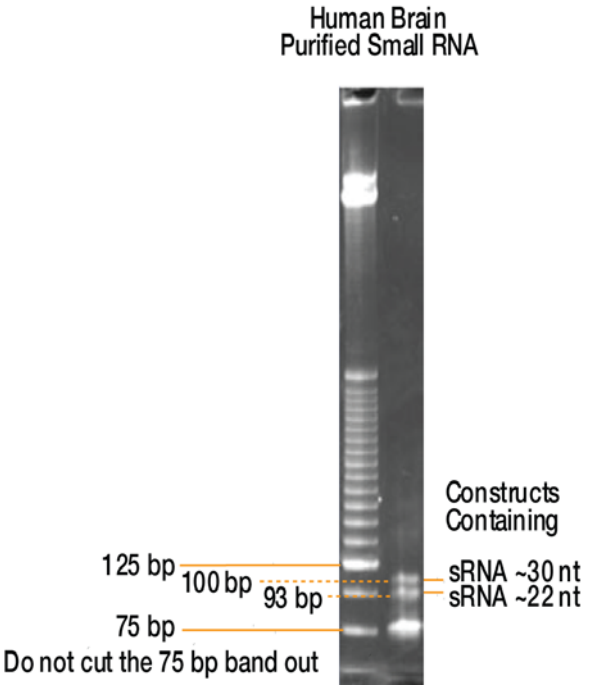


Figure 9 Purified Small RNA Libraries



6 Place the band of interest into the 0.5 ml microcentrifuge tube from step 1.

7 Centrifuge the stacked tubes on a benchtop microcentrifuge to 14,000 rpm (approximately 20,000 xg) 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.

8 Add 100 µl of 1X gel elution buffer to the gel debris in the 2 ml tube.

9 Elute the DNA by rotating the tube gently at room temperature for 2 hours or overnight if necessary.

10 Transfer the eluate and the gel debris to the top of a Spin-X cellulose acetate filter.

11 Centrifuge the filter a benchtop microcentrifuge to 14,000 rpm (approximately 20,000 xg) for 2 minutes at room temperature.

12 Add 1 µl of Glycogen, 10 µl of 3M NaOAc, and 325 µl of pre-chilled, -15° to -25°C 100% Ethanol to the Spin-X tube.

13 Immediately centrifuge the Spin-X tube on a benchtop microcentrifuge to 14,000 rpm (approximately 20,000 xg) for 20 minutes.

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

15 Wash the pellet with 500 µl of room temperature 70% ethanol.

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

17 Dry the pellet using the speed vac.

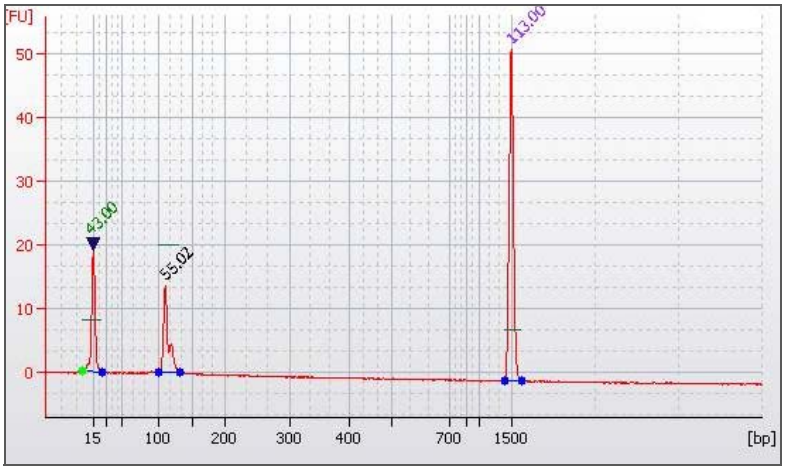
18 Resuspend the pellet in 10 µl Resuspension Buffer.

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.

Figure 10 Final miRNA Bioanalyzer Trace on a DNA-1000 Chip



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

You can confirm the final product by cloning 1 µl of the product into Invitrogen Zero Blunt TOPO vector, and sequence using conventional technology.

Notes

Notes

Notes

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 3 Illumina General Contact Information

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

Table 4 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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