Small RNA Sample Preparation Guide

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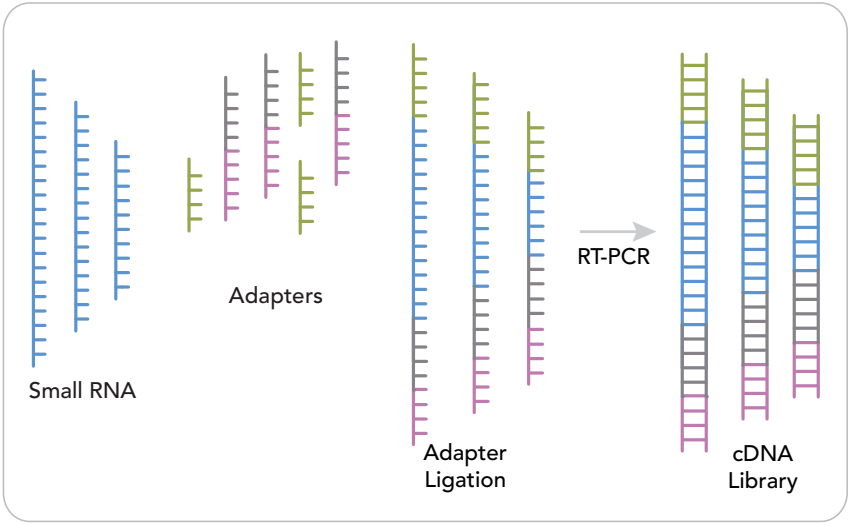
Introduction

This protocol explains how to prepare libraries of small RNA for subsequent cDNA sequencing during cluster generation.

You physically isolate small RNA, ligate the adapters necessary for use during cluster creation, and reverse-transcribe and PCR to generate the following template format:

Figure 1 Fragments after Small RNA Sample Preparation

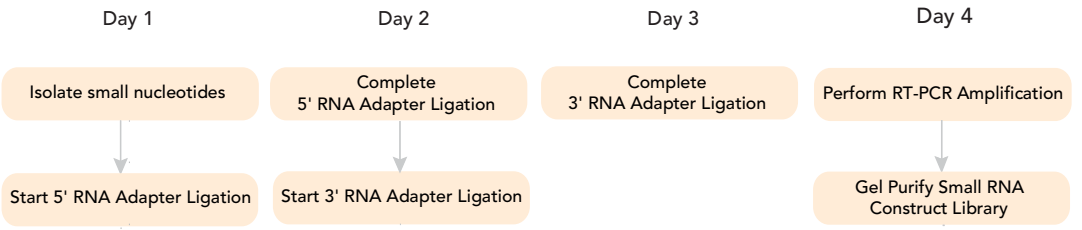


The 5' small RNA adapter is necessary for amplification of the small RNA fragment. This adapter also contains the DNA sequencing primer binding site. The 3' small RNA adapter is necessary for reverse transcription and corresponds to the surface bound amplification primer on the flow cell used during cluster generation.

Sample Prep Workflow

You need a minimum of 4 days to complete this protocol.

Figure 2 Small RNA Sample Preparation Workflow



NOTE
The sample may be stored in ethanol precipitation, if necessary.

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

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.

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. The mRNA will appear as a smear from 0.5–12 kb.

Kit Contents

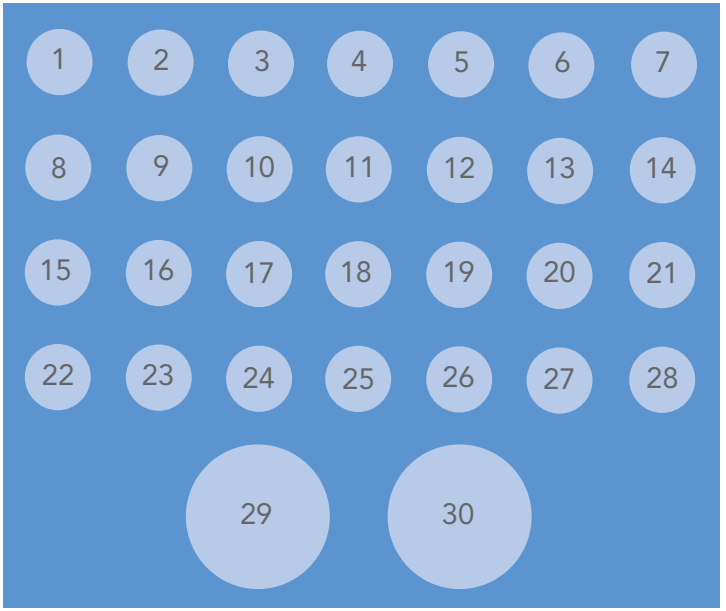
Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

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 3 Small RNA 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*

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

30 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

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

The kit contents for the Small RNA standard and v1.5 protocols are the same. However, the 10X v1.5 sRNA 3' Adapter is not used in the Small RNA standard protocol described in this document. To perform the Small RNA v1.5 protocol, which uses this adapter, see the *Small RNA v1.5 Sample Preparation Guide*.

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
3 M NaOAc, pH 5.2	General lab supplier
5' and 3' adapter-ligated RNA (4.5 µl)	General lab supplier
5X Novex TBE buffer	Invitrogen, part # LC6675
6X DNA loading dye	General lab supplier
6% Novex TBE PAGE gel, 1.0 mm, 10 well	Invitrogen, part # EC6265BOX
15% Novex TBE-urea PAGE gel, 1.0 mm, 10 well	General lab supplier
21-gauge needles	General lab supplier
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 scalpels	General lab supplier
Purified total RNA (10 µg) in 10 µl volume	General lab supplier
SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer	Invitrogen, part # 18064-014
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
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

Isolate Small RNA

This process purifies small RNA from total RNA by separating them based on nucleotide length and removing a band from the denaturing gel that corresponds to the nucleotide length of interest. This process discusses methods for studying small RNA in the range of 18–30 nucleotides. RNA of other lengths can be queried by altering the size range of the nucleotides isolated in this initial step.

The starting material, total RNA, can be isolated by a number of techniques. Ensure that the total RNA was purified using a method that retains small RNA. If you are not sure about the quality of your RNA, start with a commercial total RNA that is certified for micro RNA study.

Illumina-Supplied Consumables

- Glycogen
- Spin-X Cellulose Acetate Filters
- SRA Gel Loading Dye
- SRA Ladder
- SRA 0.3 M NaCl
- Ultra Pure Water, thawed and stored at 2° to 8°C

User-Supplied Consumables

- 5X Novex TBE Buffer
- 15% Novex TBE-urea PAGE Gel, 1.0 mm, 10 well
- 21-gauge Needles
- 75% Ethanol, room temperature
- 100% Ethanol, room temperature
- Clean Scalpels
- Purified Total RNA (10 µg) in 10 µl volume
- Ultra Pure Ethidium Bromide 10 mg/ml

Prepare Gel Electrophoresis Reagents and Apparatus

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 of libraries. This holds true for every gel purification step in this process.

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

NOTE
Use nuclease free water for all electrophoresis buffer dilutions.

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

NOTE
Clean the gel electrophoresis apparatus thoroughly between experiments.

3 Pre-run the 15% TBE-urea PAGE Gel for 15–30 minutes at 200 V and wash the wells using 1X TBE.

Sample Electrophoresis

1 While the gel is pre-running, mix 2 µl of SRA Ladder with 2 µl of SRA Gel Loading Dye in a sterile, RNase-free, 200 µl PCR tube.

2 Mix 10 µl (10 µg) of Total RNA with 10 µl of SRA Gel Loading Dye in a sterile, RNase-free 200 µl PCR tube.

3 Heat the sample and ladder tubes at 65°C for 5 minutes on a thermal cycler.

4 Place the tubes on ice.

5 Centrifuge the tubes at 13,000 rpm (approximately 18,000 xg) for 10 seconds at room temperature to collect the entire column of the tube.

6 Load both the entire SRA Ladder and sample RNA on the same gel with several lanes between them.

7 Run the gel at 200 V for 1 hour.

8 Remove the gel from the apparatus.

Recover the Isolated RNA

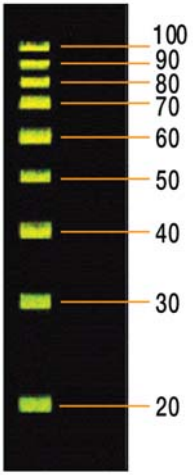
1 Puncture the bottom of a sterile, nuclease-free, 0.5 ml microcentrifuge tube 4–5 times with a 21-gauge needle.

2 Place the 0.5 ml microcentrifuge tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube.

3 Pry apart the cassette and stain the gel with TBE/Ethidium Bromide in a clean container for 2 minutes.

4 View the gel on a Dark Reader transilluminator or a UV transilluminator. The SRA Ladder ranges from 20–100 bases in 10 base increments.

Figure 4 SRA Ladder



5 Using a clean scalpel, cut out a band of gel corresponding to the 18–30 nucleotide bands in the marker lane.

6 Place the gel slice 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.

8 Add 300 µl of SRA 0.3 M NaCl to the gel debris in the 2 ml tube and elute the RNA by rotating the tube gently at room temperature for 4 hours.

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

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

11 Add 1 µl of Glycogen and 750 µl of room temperature 100% Ethanol to the Spin-X tube.

12 Incubate at -80°C for 30 minutes.

13 Immediately centrifuge the Spin-X tube on a 4°C microcentrifuge to 14,000 rpm (approximately 20,000 xg) for 25 minutes.

14 Remove the supernatant and discard it.

15 Wash the pellet with 750 µl of room temperature 75% Ethanol.

16 Remove the supernatant and discard it.

17 Allow the RNA pellet to air dry.

18 Resuspend the RNA pellet in 5.7 µl of Ultra Pure Water.

Ligate the 5' Adapter

This process ligates adapters to the 5' end of the isolated small RNA.

Illumina-Supplied Consumables

- 10X T4 RNA Ligase Buffer
- Glycogen
- RNase Inhibitor
- SRA 5' Adapter
- SRA Gel Loading Dye
- SRA Ladder
- SRA 0.3 M NaCl
- T4 RNA Ligase
- Ultra Pure Water

User-Supplied Consumables

- 5X Novex TBE Buffer
- 15% Novex TBE-urea PAGE gel, 1.0 mm, 10 well
- 21-gauge Needles
- 75% Ethanol, room temperature
- 100% Ethanol, room temperature
- Clean Scalpels
- Isolated Small RNA
- Ultra Pure Ethidium Bromide 10 mg/ml

Ligate the Adapter

1 Transfer the small RNA into a 200 µl PCR tube.

2 Add the following in the indicated order to each tube of PAGE gel isolated small RNA. Starting volume of small RNA is 5.7 µl.

Reagent	Volume (µl)
SRA 5' Adapter	1.3
10X T4 RNA Ligase Buffer	1.0
RNase Inhibitor	1.0
T4 RNA Ligase	1.0
Total Volume	**10**

3 Incubate at 20°C for 6 hours on a thermal cycler and hold overnight at 2° to 8°C.

4 Stop the reaction by adding 10 µl of SRA Gel Loading Dye.

Prepare 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.

3 Pre-run the 15% TBE-urea gel for 15–30 minutes at 200 V.

4 Wash the wells using 1X TBE.

Sample Electrophoresis

1 While the gel is pre-running, mix 2 µl of SRA Ladder with 2 µl of SRA Gel Loading Dye in a sterile, RNase-free, 200 µl PCR tube.

2 Heat the ligated sample and ladder tubes at 65°C for 5 minutes on a thermal cycler just before loading on the gel.

3 Place the tubes on ice.

4 Centrifuge the tubes at 13,000 rpm (approximately 18,000 xg) for 10 seconds at room temperature to collect the entire column of the tube.

5 Load both the entire SRA Ladder and sample RNA on the same gel with several lanes between them.

6 Run the gel at 200 V for 1 hour.

7 Remove the gel from the apparatus.

Recover the Isolated RNA

1 Puncture the bottom of a sterile, nuclease-free, 0.5 ml microcentrifuge tube 4–5 times with a 21-gauge needle.

2 Place the 0.5 ml microcentrifuge tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube.

3 Pry apart the cassette and stain the gel with TBE/ethidium bromide in a clean container for 2 minutes.

4 View the gel on a Dark Reader transilluminator or a UV transilluminator. The SRA Ladder ranges from 20–100 bases in 10 base increments. See Figure 4 on page 14.

5 Using a clean scalpel, cut out a band of gel corresponding to the 40–60 nucleotide bands in the marker lane.

6 Place the band 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.

8 Add 300 µl of SRA 0.3 M NaCl to the gel debris in the 2 ml tube.

9 Elute the RNA by rotating the tube gently at room temperature for 4 hours.

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

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

12 Add 1 µl of Glycogen and 750 µl of room temperature 100% Ethanol to the Spin-X tube.

13 Incubate at -80°C for 30 minutes.

14 Immediately centrifuge the Spin-X tube on a 4°C microcentrifuge to 14,000 rpm (approximately 20,000 xg) for 25 minutes.

15 Remove the supernatant and discard it.

16 Wash the pellet with 750 µl of room temperature 75% Ethanol.

17 Remove the supernatant and discard it.

18 Allow the RNA pellet to air dry.

19 Resuspend the RNA pellet in 6.4 µl of Ultra Pure Water.

Ligate the 3' Adapter

This process ligates a defined RNA adapter to the 3' end of the isolated small RNA.

Illumina-Supplied Consumables

- 10X T4 RNA Ligase Buffer
- Glycogen
- RNase Inhibitor
- SRA 3' Adapter
- SRA Gel Loading Dye
- SRA Ladder
- SRA 0.3 M NaCl
- T4 RNA Ligase
- Ultra Pure Water

User-Supplied Consumables

- 5' Adapter-ligated Small RNA in 4.5 µl Ultra Pure Water
- 5X Novex TBE Buffer
- 10% Novex TBE-urea PAGE gel, 1.0 mm, 10 well
- 21-gauge Needles
- 75% Ethanol, room temperature
- 100% Ethanol, room temperature
- Clean Scalpels
- Ultra Pure Ethidium Bromide 10 mg/ml

Ligate the Adapter

1 Transfer the ligated RNA into a 200 µl PCR tube.

2 Add the following in the indicated order to each tube of 5' RNA adapter-ligated small RNA. Starting volume of small RNA is 6.4 µl.

Reagent	Volume (µl)
SRA 3' Adapter	0.6
10X T4 RNA Ligase Buffer	1.0
RNase Inhibitor	1.0
T4 RNA Ligase	1.0
Total Volume	**10**

3 Incubate at 20°C for 6 hours on a thermal cycler and hold overnight at 2° to 8°C.

4 Stop the reaction by adding 10 µl of SRA Gel Loading Dye.

Prepare 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.

3 Pre-run the 10% TBE-urea gel for 15–30 minutes at 200 V.

4 Wash the wells using 1X TBE.

Sample Electrophoresis

1 While the gel is pre-running, mix 2 µl of SRA Ladder with 2 µl of SRA Gel Loading Dye in a sterile, RNase-free, 200 µl PCR tube.

2 Heat the ligated sample and ladder tubes at 65°C for 5 minutes on a thermal cycler just before loading on the gel.

3 Place the tubes on ice.

4 Centrifuge the tubes at 13,000 rpm (approximately 18,000 xg) for 10 seconds at room temperature to collect the entire column of the tube.

5 Load both the entire SRA Ladder and sample RNA on the same gel with several lanes between them.

6 Run the gel at 200 V for 1 hour.

7 Remove the gel from the apparatus.

Recover the Isolated RNA

1 Puncture the bottom of a sterile, nuclease-free, 0.5 ml microcentrifuge tube 4–5 times with a 21-gauge needle.

2 Place the 0.5 ml microcentrifuge tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube.

3 Pry apart the cassette and stain the gel with TBE/ethidium bromide in a clean container for 2 minutes.

4 View the gel on a Dark Reader transilluminator or a UV transilluminator. The SRA Ladder ranges from 20–100 bases in 10 base increments. See Figure 4 on page 14.

5 Using a clean scalpel, cut out a band of gel corresponding to the 70–90 nucleotide bands in the marker lane.

6 Place the band 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.

8 Add 300 µl of SRA 0.3 M NaCl to the gel debris in the 2 ml tube.

9 Elute the RNA by rotating the tube gently at room temperature for 4 hours.

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

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

12 Add 1 µl of Glycogen and 750 µl of room temperature 100% Ethanol to the Spin-X tube.

13 Incubate at -80°C for 30 minutes.

14 Immediately centrifuge the Spin-X tube on a 4°C microcentrifuge to 14,000 rpm (approximately 20,000 xg) for 25 minutes.

15 Remove the supernatant and discard it.

16 Wash the pellet with 750 µl of room temperature 75% Ethanol.

17 Remove the supernatant and discard it.

18 Allow the RNA pellet to air dry.

19 Resuspend the RNA pellet in 4.5 µl of Ultra Pure Water.

Reverse Transcribe and Amplify

Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 5' and 3' adapters. This process selectively enriches those RNA 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 DNA Polymerase (Finnzymes Oy)
- Primer GX1
- Primer GX2
- RNase Inhibitor
- SRA RT Primer
- Ultra Pure Water

User-Supplied Consumables

- 5' and 3' Adapter-ligated RNA (4.5 µl)
- SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer

Template Preparation

1 Combine the following in a sterile, RNase-free, 200 µl microcentrifuge tube:

Reagent	Volume (µl)
Purified 5' and 3' Ligated RNA	4.5
SRA RT Primer	0.5
Total Volume	**5**

2 Heat the mixture at 65°C on a thermal cycler for 10 minutes.

3 Place the tube on ice.

Dilute the 25 mM dNTP Mix

1 Premix the following reagents in a separate, sterile, RNase-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	**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	**4**

3 Add 4 µl of the mix to the cooled 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. 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	28
5X Phusion HF Buffer	10
Primer GX1	0.5
Primer GX2	0.5
25 mM dNTP mix	0.5
Phusion DNA Polymerase	0.5
Total Volume	**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 process:

- a 30 seconds at 98°C
- b 15 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

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
- 6X DNA Loading Dye
- 21-gauge Needles
- 70% Ethanol, room temperature
- 100% Ethanol, -15° to -25°C
- Ultra Pure Ethidium Bromide
- Amplified cDNA Construct (50 µl)
- Clean Scalpels

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 6X DNA Loading Dye.

2 Mix 50 µl of amplified cDNA construct with 10 µl of 6X 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.

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	**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.

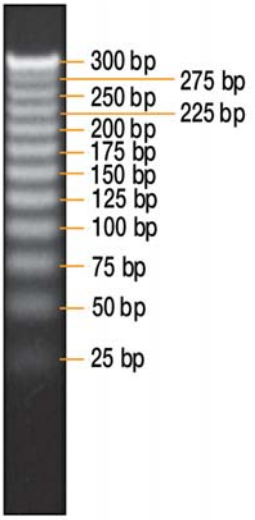
Figure 6 Place 0.5 ml Tube into 2 ml Tube

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 approximately a 92 bp band in the sample lanes.

6 Place the band 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.

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

11 Centrifuge the filter on 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 -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 at room temperature.

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.

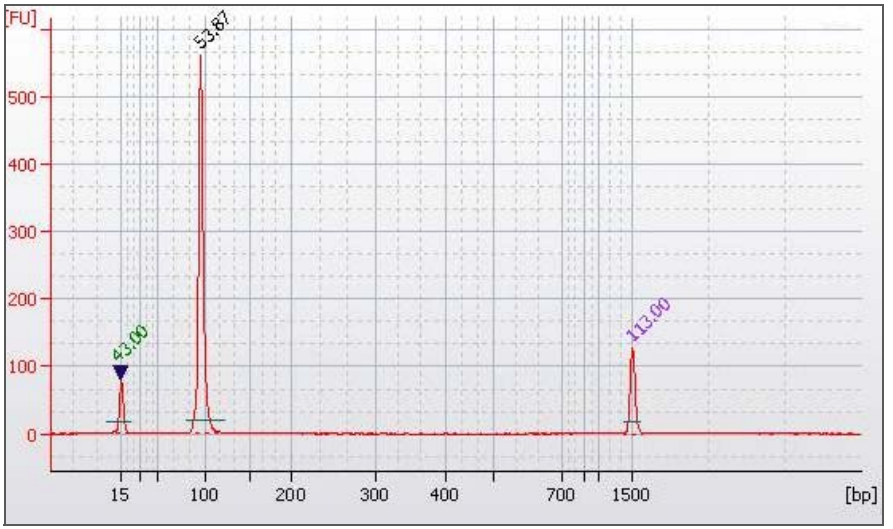
18 Resuspend the pellet in 10 µl resuspension buffer.

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 8 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

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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