Small RNA Sample Prep v1.5.0

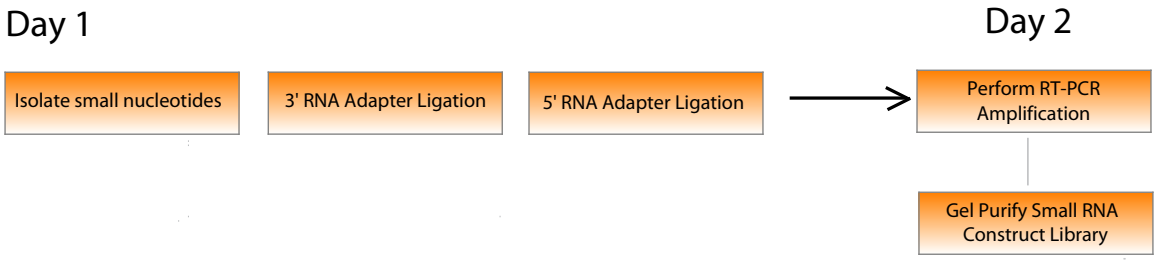
Pre-Release Protocol

Introduction

This protocol explains how to prepare libraries of small RNA for subsequent sequencing on the Illumina Cluster Station and Genome Analyzer.

This guide describes the Small RNA Sample Preparation v1.5 Pre-Release Protocol. This pre-release protocol requires supplementing commercially-released products with additional components. Support may be limited. The final released product may differ from pre-release protocols.

Workflow



Pre-Release Protocol Required Coponents

Illumina Supplied Components

This protocol requires the following components, which are contained in the Small RNA Sample Prep kits:

- -1 Small RNA Sample Prep Kit (8 samples) FC-102-1009
- -5 Small RNA Sample Prep Kit (40 samples) FC-102-1013

User Supplied Supplemental Components

SuperScript II Reverse Transcriptase with 100 mM DTT and 5X first strand buffer (Invitrogen, part # 18064-014)

v1.5 Component	User Supplied	Catalog Number
3' sRNA Ligase v1.5	T4 RNA Ligase2, truncated	M0242S NEB
3' sRNA Adaptor v1.5	See Illumina representative	

Purify Small RNA fraction

Purifying 20–30 nt Area from1 µg of Total RNA (1 µg/µl)

1. Remove the comb from the 15% TBU gel and rinse out the wells thoroughly with 1X TBE.
2. Pre-run the 15% TBU gel for 15–30 minutes at 200 V, and wash the wells using 1X TBE.
3. Mix 1 µl (1 µg) of total RNA with 1 µl of 2X formamide loading dye in a 200 µl PCR tube.
4. Mix the sample by vortex annd spin down.
5. Mix 2 µl of RNA ladder with 2 µl of 2X loading dye in another 200 µl PCR tube.
6. Heat the sample at 65°C for 5 minutes, put on ice, centrifuge, and load the sample and the ladder in two independent wells.
7. Run the gel at 200 V for 1 hour, and stain the gel with 1X TBE/EtBr for 2 minutes.
8. Cut out the corresponding gel band (20–30 nt) and transfer to a 0.5 ml tube with 4–5 pores punctured by a 21 gauge needle on the bottom.
9. Set this tube into a 2 ml round-bottom Eppendorf tube, and spin the gel through the hole into the 2 ml tube at 1400 RPM for 2 minutes.
10. Add 300 µl of 0.3 M NaCl to the tube, and elute the DNA by rotating the tube gently at room temperature for 4 hours.
11. Transfer the elute and the gel debris onto the top of a Spin-X filter, and spin at full speed for 2 minutes.
12. Add 750 µl of 100% EtOH and 3 µl of glycogen to the sample, and incubate at -80°C for 30 minutes.
13. Centrifuge at approximately 14,000 rpm for 25 minutes at 4°C in a microcentrifuge.
14. Carefully remove the supernatant and wash the pellet with 750 µl of room temperature 75% EtOH.
15. Allow the RNA pellet to air dry, and then dissolve the RNA in total of 6.0 µl of DEPC-treated water.

Ligate 3' and 5' RNA Adapters


NOTE The PR 3' Adapter is not used in the v1.5 protocol. Use the v1.5 sRNA 3' Adapter.

3' Adaptor Ligation and Purification

1. Set up the 3' adaptor ligation reaction:
   - Purified miRNA (6.0 µl)
   - 3' sRNA Adapter v1.5 (1 µl)
2. Incubate at 70°C for 2 minutes and put on ice.
3. Add the following reagents and mix well:
   - 10X ligation buffer (-ATP) (1 µl)
   - 3' sRNA ligase v1.5 (1.5 µl)
   - RNaseOut (0.5 µl)
4. Incubate at 22°C for 1 hour.
5. When there are 5 minutes left during the above incubation, prepare the 5' adapter for ligation by heating the 5' adapter at 70°C for 2 minutes, and put it on ice.
6. Add the following reagents to the ligation mixture and mix well:
   - 10 mM ATP (1 µl)
   - 5' adapter (0.5 µl)
   - T4 RNA ligase (1 µl)
7. Incubate at 20°C for 6 hours in a thermal cycler and keep at 4°C until the next day.

Reverse Transcribe and Amplify the Small RNA Ligated with Adapters

User-Supplied

- ⇦ 5' and 3' adapter-ligated RNA (4.5 µl)
- ⇦ SuperScript II Reverse Transcriptase with 100 mM DTT and 5X first strand buffer (Invitrogen, part # 18064-014)

Template Preparation

1. Combine the following in a sterile, RNase-free, 200 µl microcentrifuge tube:
   - 5' and 3' ligated RNA (4.5 µl)
   - SRA RT primer (0.5 µl)

   The total volume should be 5 µl.
2. Heat the mixture at 65°C in a thermal cycler for 10 minutes.
3. Place the tube on ice.

Reverse Transcribe and Amplify the Small RNA Ligated with Adapters (continued)

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.
   - Ultra pure water (0.5 µl)
   - 25 mM dNTP mix (0.5 µl)

   The total volume should be 1 µl.
2. Label the tube "12.5 mM dNTP Mix."

Reverse Transcription

1. Premix the following reagents in the order listed in a separate tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
   - 5X first strand buffer (2 µl)
   - 12.5 mM dNTP mix (0.5 µl)
   - 100 mM DTT (1 µl)
   - RNase OUT (0.5 µl)
2. 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).
3. Heat the sample to 48°C in a thermal cycler for 3 minutes.
4. Add 1 µl SuperScript II Reverse Transcriptase. The total volume should now be 10 µl.
5. Incubate in a thermal cycler at 44°C for 1 hour.

Prepare the PCR Master Mix

⇦ 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.
- Ultra pure water (28 µl)
- 5X Phusion HF buffer (10 µl)
- Primer GX1 (0.5 µl)
- Primer GX2 (0.5 µl)
- 25 mM dNTP mix (0.5 µl)
- Phusion DNA Polymerase (0.5 µl)

The total volume should be 40 µl.

Reverse Transcribe and Amplify the Small RNA Ligated with Adapters (continued)

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 in the thermal cycler using the following PCR protocol:
   a. 30 seconds at 98°C
   b. 9 to 12 cycles of:
      10 seconds at 98°C
      30 seconds at 60°C
      15 seconds at 72°C

Purify the Amplified cDNA Construct

This protocol gel purifies the amplified cDNA construct in preparation for loading on the Illumina Cluster Station.

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.
6. Remove the gel from the apparatus.

Dilute the 10X Gel Elution Buffer

⇦ 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.

- Ultra pure water (90 µl)
- 10X gel elution buffer (10 µl)

The total volume should be 100 µl.

Purify the Amplified cDNA Construct (continued)

Recover the Purified Construct

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 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 18 dsDNA fragments between 25 bp and 450 bp in 25 bp increments plus a fragment at 500 bp. An additional fragment at 2652 bp is provided above the ladder. The 125 bp is approximately 2–3 times brighter than all bands except the 500 bp and 2652 bp bands to provide internal orientation.
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 step1.
7. Centrifuge the stacked tubes at 14000 rpm in a microcentrifuge for 2 minutes at room temperature to move the gel through the holes into the 2 ml 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 filter.
11. Centrifuge the filter for 2 minutes at 14000 rpm.
12. Add 1 µl of glycogen, 10 µl of 3M NaOAc, and 325 µl of -20°C 100% ethanol.
13. Immediately centrifuge to 14000 rpm for 20 minutes in a benchtop microcentrifuge.
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.