Title

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Abstract

This protocol describes a library preparation method for sequencing small RNA. The method uses degenerate adapters to alleviate the problem of bias in the ligation steps during small RNA library preparation, and it optimizes several other parameters to make it appropriate for use specifically with plasma RNA.

Introduction

Extracellular RNAs (exRNAs) have been identified in every biofluid that has been tested. In biofluids, they have been found in extracellular vesicles, ribonucleoprotein complexes, and lipoprotein complexes. ExRNAs are of considerable interest because they can serve as signaling molecules between cells, they have the potential to serve as biomarkers for prediction and diagnosis of disease, and exRNAs or the extracellular particles that carry them might be used for therapeutic purposes.

The Extracellular RNA Communication Consortium (ERCC) is a group of laboratories funded by the U.S. National Institutes of Health. One goal of the ERCC is to develop robust and standardized methods for collecting and processing of biofluids, separating different types of exRNA-containing particles, and isolating and analyzing exRNAs. The "Reference Profiles group":http://exrna.org/referenceprofiles/ within the consortium is tasked with collecting reliable profiles of the spectrum of extracellular RNAs found in healthy individuals. These reference profiles will serve as benchmarks for comparison with the exRNA profiles from patients with disease. The present protocol for small RNA library preparation was developed by the ERCC Reference Profiles group for use with RNA isolated from human plasma.

Key elements of this small RNA library preparation method include the use of 4 random nucleotides on the end of the adapters ligated to the small RNAs of interest, the use of

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higher than usual adapter concentrations, and the use of increased amounts of polyethylene glycol (PEG) in the ligation steps. Small RNA library preparation methods that lack such degenerate adapters have been found to exhibit significant bias in the representation of different RNA sequences.[1-4] The use of random adapters is designed to alleviate that problem. The use of high adapter concentrations and macromolecular crowding agents such as PEG reduces bias by driving the ligation reactions toward completion. It should be noted that the use of improved ligation conditions and increased adapter concentrations also results in the formation of more adapter dimers, so purification of desired ligation products from such unwanted side products by size fractionation is essential in this protocol.

PCR amplification Small RNA libraries may require 10-20 cycles of PCR amplification depending on the type and amount of input. It has been shown that increased amplification does not significantly affect library bias.[1,5] Increased number of PCR cycles will, however, increase the amount of adapter dimers that must be separated from the library. Typically, a single PCR and gel purification step is sufficient to remove most of the adapter dimer products from insert-containing PCR products.

Size selection Because of the large excess of adapter dimers in low-input small RNA libraries, electrophoretic purification of PCR products is often necessary. This can be done using either acrylamide or agarose gels, as long as the gel can sufficiently resolve the insert-containing fragments ($==_{150bp}$) from the adapter dimer fragments ($==_{125bp}$).

A full list of the protocols developed by the ERCC is available at the "exRNA Portal":http://exrna.org/resources/protocols/, the ERCC's website.

This protocol is one of four protocols relating to a 2018 Nature Biotechnology paper.

Library Preparation for small RNA sequencing using 4N adapters: In house 4N Protocol A

"10.1038/protex.2018.049":http://dx.doi.org/10.1038/protex.2018.049

Library Preparation for small RNA sequencing using 4N adapters: In house 4N Protocol B

"10.1038/protex.2018.050":http://dx.doi.org/10.1038/protex.2018.050

Library Preparation for small RNA sequencing using 4N adapters: In house 4N Protocol C

"10.1038/protex.2018.081":http://dx.doi.org/10.1038/protex.2018.081/

Modified TruSeq Small RNA Library Prep using Randomized 4N Adapters: In house 4N

Protocol D

"10.1038/protex.2018.052":http://dx.doi.org/10.1038/protex.2018.052

Reagents

Oligonucleotide sequences[1]

5' adapter (desalted) – 5'
rGrUrUrCrArGrArArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCr(N:2525252525)r(N)r(N)r(N)r(N)-3'
3' adapter (HPLC purification) – 5'- /5rApp/(N1:25252525)(N1)(N1)
(N1)TGGAATTCTCGGGTGCCAAGG/3ddC/-3'

Reagents for 3' Ligation

TruSeq Small RNA Kit (Illumina RS-200-0012)
T4 RNA Ligase 2, Deletion Mutant (NEB M0242)
50% PEG 8000 (included with T4 RNA ligase)
Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Reagents for adapter depletion

E. coli single-stranded DNA binding protein (SSB) (Promega M3011)
5' deadenylase (NEB M0331)
RecJf (NEB M0264)

Reagents for 5' Ligation

10mM ATP and T4 RNA ligase 1 from TruSeq Small RNA Kit

Reagents for Reverse Transcription

RNA RT primer, 25 mM dNTP mix and RNase inhibitor from TruSeq Small RNA Kit
SuperScript II (Invitrogen 18064-014)
5x First strand buffer (included with Superscript II)
0.1 M DTT (included with Superscript II)
Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Reagents for PCR Amplification

TruSeq Small RNA Kit

Reagents for Gel Purification

3% agarose cassettes for Pippin Prep (Sage Science CDP3010)
QIAquick PCR purification kit (Qiagen 28104)

Reagents for Library Validation

High sensitivity DNA chip for Bioanalyzer (Agilent 5067-4626)

Equipment

Equipment for Ligations and Reverse Transcription

Vacuum concentrator
Thermal cycler

Equipment for PCR Amplifications

Thermal cycler

Equipment for Gel Purification

Pippin Prep (Sage Science, PIP0001) size selection instrument
Nanodrop spectrophotometer

Equipment for Library Validation

Agilent 2100 Bioanalyzer

Procedure

Thermocycler Setup

    70°C   2 minutes

    28°C   1 hour

    30°C 45 minutes

    37°C 45 minutes

    70°C   2 minutes

    28°C   1 hour

    70°C   2 minutes

    50°C   1 hour

    98°C 30 seconds

    98°C 10 seconds

60°C 30 seconds   20X

    72°C 15 seconds

    72°C 10 minutes

     4°C   Hold

Prepare Adapter Stocks

3' adapter: dilute to 4 µM in water

5' adapter: dilute to 10 µM in water

Prepare PEG Pellets

1.   Dilute 50% PEG to 15% PEG by adding water.

2.   Aliquot 10 µL of 15% PEG to PCR Tube.

3.   Dry PEG until it is a pellet.

4.   Store in dark conditions.

3' Ligation: Set Up on Ice.

1.   Add 1 µL of 4 µM 3'-4N adapter to each tube.

2.   Add 5 µL of each RNA sample.

3.   Gently mix, vortex, and centrifuge.

4.   Incubate at 70°C for 2 minutes.

5.   Immediately place on ice for 2 minutes.

6.   Mix, vortex, and centrifuge.

7.   Preheat thermocycler to 28°C.

8.   **Prepare Ligation Master Mix:**

Reagent	Volume (µL)
Ligation Buffer (HML)[2]	2
RNase Inhibitor	1

T4 RNA Ligase 2, Deletion Mutant   1

Total Volume per Sample   4

9.   Add 4 µL of Ligation Master Mix to each tube.

10.   Gently mix, Vortex, and Centrifuge.

11.   Incubate at 28°C for 1 hour.

Adapter Depletion

12.   Add 1 µg of *E. coli* SSBP to each tube (1 µg/µL – will need to make dilution from

stock.)

13.   Incubate on ice for 10 minutes.

14.   Add 1 µL of 5' deadenylase.

15.   Incubate at 30°C for 45 minutes.

16.   Add 1 µL of RecJf.

17.   Incubate at 37°C for 45 minutes.

5' Ligation

18.   Preheat thermocycler to 70°C.

19.   Prepare a master mix by adding 1.1 x N (N = number of samples) 10 µM 5'-4N

adapter to separate PCR tube.

20.   Incubate at 70°C for 2 minutes.

21.   Place on ice for 2 minutes.

22.   Preheat thermocycler to 28°C.

23.   Add 1.1 x N (N = number of samples) 10 mM ATP to the RNA 5' Adapter PCR Tube.

24.   Add 1.1 x N (N = number of samples) T4 RNA Ligase to the RNA 5' and ATP PCR Tube.

25.   Gently mix, Vortex, and Centrifuge.

26.   Add 3 µL of the master mix to each sample (reaction from step 17).

27. Incubate at 28°C for 1 hour.

28. Place on ice for 2 minutes.

Reverse Transcription and Amplification

29. Mix, Vortex, and Centrifuge.

30. Concentrate samples down from 14 μL to 6 μL.

31. Add 1 μL of RNA RT Primer to each tube.

32. Gently mix and centrifuge briefly.

33. Incubate at 70°C for 2 minutes.

34. Place on ice for 2 minutes.

35. Preheat thermocycler to 50°C.

36. **Prepare RT Master Mix:**

Reagent	Volume (μL)
5X First Strand Buffer	2
12.5 mM dNTP Mix[3]	0.5
100mM DTT	1
RNAse Inhibitor	1
SuperScript II RT	1
Total Volume per Sample	5.5

37. Add 5.5 μL of RT Master Mix to each sample. (Total volume is 12.5 μL.)

38. Gently mix, Vortex, and Centrifuge.

39. Incubate at 50°C for 1 hour.

40. **Prepare PCR Master Mix:**

Reagent	Volume (μL)
Ultra Pure Water	8.5

PCR Mix   25

RNA PCR Primer   2

Total Volume per Sample   35.5

41.   Add 35.5 µL of PCR Master Mix to each tube.

42.   Add 2 µL of unique PCR Primer Index to each tube. (Total volume is 50 µL.)

43.   Gently mix and centrifuge.

44.   PCR Program:

    98°C 30 sec.   1X

    98°C 10 sec.

    60°C 30 sec.   20X

    72°C 15 sec.

    72°C 10 min.   1X

    4°C   Hold

Quality Control

1.   Bioanalyzer High Sensitivity DNA Chip (Library peak should be 152bp.)

Size Selection – Pippin Prep:

1.   Can either pool libraries or run libraries independently.

2.   Purify libraries with QIAquick PCR Purification kit (5 µg). Elute in 10 µL water.

3.   Nanodrop purification – Add no more than 5 µg of PCR product, minimum in low

     nanograms.

4.   For each lane, bring total volume of library(s) + TE buffer to 30µL.

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5. Add 10 µL of supplied loading buffer (at RT).

6. Size select for 152 bp. Device-to-device variation likely. Make sure to save some PCR product for optimizing. Our program is set at 117-146 Range.

7. Bioanalyzer HS DNA Chip (Peak at 152bp ONLY – may see some adapter-dimer and ~160bp peaks.)

Troubleshooting

1) Oligonucleotide sequences © 2016 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.

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

3) Dilute 25 mM dNTP 1:1 to a final concentration of 12.5 mM.

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