Illumina Small RNA cloning protocol using Random Adapters

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Abstract

This protocol corrects small RNAs deep sequencing biases caused by RNA ligases. The key modification is the use of pooled adapters with the sequences below. See the cited publication for further details.

Citation: Ravi Sachidanandam Illumina Small RNA cloning protocol using Random Adapters. **protocols.io** dx.doi.org/10.17504/protocols.io.cccssv
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Before start

5' adapter-4N: 5'GUUCAGAGUUCUACAGUCCGACGAUCNNNN
3' adapter-4N: 5' rApp /NNNNTGGAATTCTCGGGTGCCAAGG/ 3ddC/
Reverse primer:
ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTGXXXXXXTAGAGCATACGGCAGAAGACGAAC
Forward primer: AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA

Protocol

Step 1.
Section I: Size selection and Gel purification of RNA sample

Step 2.
Prepare a 12% polyacrylamide/urea gel (thickness will depend on the amount of total RNA being run. As a guide, we use 1.0mm for <20µg,1.5mm for >20µg).

Step 3.
Spike RNA samples with 32P-labeled 19, 24, 28 and/or 33bp oligos (10,000 counts per oligo, per minute) – depending on the application.

Step 4.
Load the sample(s) and run the gel at constant 10W for 1-1.5 hrs (until the first dye front reaches the middle of the gel).

Step 5.
Add an equal amount of Gel Loading Buffer II (1X), heat samples at 95C for 5 minutes.

DURATION
00:05:00

Step 6.
Chill on ice for 1 minute.

DURATION
00:01:00

Step 7.

Carefully grind gel slices by hand using a pestle.

Step 8.
Expose a phosphor-screen over the gel for 10-15 minutes (time will vary depending on radioactivity intensity and imager sensitivity)

Step 9.
Add 420µL of 0.4M NaCl.

AMOUNT
420 µl Additional info:

Step 10.
Spin briefly in a centrifuge at maximum speed.

Step 11.
Flash freeze samples for 1 minute in a bath of EtOH and dry ice.

Step 12.
Transfer eluant to a fresh microcentrifuge tube.

Step 13.
Add 20µg GlycoBlue, mix, and add 2.5 volumes of 100% EtOH

Step 14.
Spin at 4C for 30 minutes.

DURATION
00:30:00

Step 15.
Incubate at -20C for 3-6+ hours.

Step 16.
Air dry for <5 min and resuspend pellet in 13µL DEPC-MilliQ H20

AMOUNT
13 µl Additional info:

Step 17.
Here, one can insert a gel slice into a dialyzer tube (Novagen, D-Tube Dialyzer Midi, MWCO 3.5 kDA – Cat. No. 71506-3) along with 450ul of water.

Step 18.
Reverse the poles and run for 2 minutes to pull the sample off the dialysis tubing wall

DURATION
00:02:00

Step 19.
Position the dialyzer into a gel box, submerged in 1xTAE, such that the dialysis tubing has the current running perpendicularly through it.

Step 20.
Pull out all of the water into a fresh microcentrifuge tube, mix in 20µg GlycoBlue and add 2.5 volumes of 100% EtOH

Step 21.
Wash with 70% EtOH and then remove ALL EtOH.

Step 22.
Spin at 4C for 30 minutes

DURATION
00:30:00

Step 23.
Wash with 70% EtOH and then remove ALL EtOH

Step 24.
Air dry for <5 min and resuspend pellet in 13µL DEPC-MilliQ H20

AMOUNT
13 µl Additional info:

Step 25.
Run at 100V for 18mins.

DURATION
00:18:00

Step 26.
Set up the ligation mix as described in the cited publication.

Step 27.
Incubate at 37C for 4 hours

DURATION
04:00:00

Step 28.
Add 20µl Gel Loading Buffer II

AMOUNT
20 µl Additional info:

Step 29.
Section II: 3’ linker ligation

Step 30.
Section III: Gel purification of 3’ ligated RNA product

Step 31.
Prepare a 1.0mm, 12% polyacrylamide/urea gel

Step 32.
Heat inactivate at 95C for 5 minutes

DURATION
00:05:00

Step 33.
Heat samples at 95C for 5 minutes

DURATION
00:05:00

Step 34.
Chill for 1 minute

Step 35.
Load the sample(s) and run the gel at constant 10W for 1.5-2 hrs (until the first dye front reaches the bottom of the gel)

Step 36.
Expose a phosphorimager screen over the gel for 30-45 minutes (time will vary) (Figure 2)

Step 37.
Excise the precise band corresponding to the desired size of small RNA (including the ligated radiolabeled oligo(s)) into a microcentrifuge tube

Step 38.

Spin briefly in a centrifuge at maximum speed

Step 39.
Carefully grind gel slices using a pestle

Step 40.
Load 5ng of 32P-labeled Decade Marker as a size marker

Step 41.
Add 420µL of 0.4M NaCl

AMOUNT
420 µl Additional info:

Step 42.
Quickly freeze samples for 1 minute in a bath of EtOH and dry ice

Step 43.
Incubate overnight at room temperature (RT) with agitation (secured on a vortex)

DURATION
15:00:00

Step 44.
Spin gel slice homogenate through micropore filter at full speed for 1 minute at RT

Step 45.
Transfer eluant to a microcentrifuge tube

Step 46.
Add 20µg GlycoBlue, mix, and add 2.5 volumes of 100% EtOH

Step 47.
Incubate at -20C for 3-6+ hours

Step 48.
Spin at 4C for 30 minutes

DURATION
00:30:00

Step 49.
Wash with 70% EtOH and then remove ALL EtOH

Step 50.
Air dry for <5 min and resuspend pellet in 13µL DEPC-MilliQ H20

AMOUNT
13 µl Additional info:

Step 51.
Section IV: 5’ linker ligation

Step 52.
Set up the linker ligation reaction mix as described in the publication

Step 53.
Incubate at 37C for 2 hours

DURATION
02:00:00

Step 54.
Add 20µl Gel Loading Buffer II

AMOUNT
20 µl Additional info:

Step 55.
Heat inactivate at 95C for 5 minutes

DURATION
00:05:00

Step 56.
Section V: Gel purification of 5’ and 3’ ligated RNA product

Step 57.
Prepare a 1.0mm, 12% polyacrylamide/urea gel

Step 58.
Load 2.5ng of 32P-labeled Decade Marker as a size marker

Step 59.
Heat samples at 95C for 5 minutes

Step 60.
Chill for 1 minute

Step 61.
Expose a phosphorimager screen over the gel for 1+ hours (time will vary) (Figure 3)

Step 62.
Load the sample(s) and run the gel at constant 10W for 2+ hrs (until first dye front passes the bottom of the gel)

Step 63.
Spin briefly in a centrifuge at maximum speed

Step 64.
Excise the precise band corresponding to the desired size of small RNAs (including the ligated radiolabeled oligo(s)) into a microcentrifuge tube

Step 65.
Spin briefly in a centrifuge at maximum speed

Step 66.
Add 420µL of 0.4M NaCl

AMOUNT
420 µl Additional info:

Step 67.
Quickly freeze samples for 1 minute in a bath of EtOH and dry ice

Step 68.
Spin gel slice homogenate through micropore filter at full speed for 1 minute at RT

Step 69.
Transfer eluant to a microcentrifuge tube

Step 70.
Add 20µg GlycoBlue, mix, and add 2.5 volumes of 100% EtOH

Step 71.
Incubate at -20oC for 3-6+ hours

Step 72.
Spin at 4C for 30 minutes

DURATION
00:30:00

Step 73.
Carefully grind gel slices using a pestle

Step 74.
Air dry for <5 min and resuspend pellet in 6.3µL DEPC-MilliQ H20

AMOUNT
6 µl Additional info:

Step 75.
Section VI: Reverse transcription

Step 76.
Incubate at 72C for 2 minutes

DURATION
00:02:00

Step 77.
Incubate overnight at room temperature (RT) with agitation (secured on a vortex)

Step 78.
Centrifuge at RT for 1 minute

Step 79.
Cool on ice for 2 minutes

DURATION
00:02:00

Step 80.
Add 8.4µL Reverse Transcriptase (RT) Mix (see publication for details)

Step 81.
Split into two tubes (9µL each)

Step 82.
Add either 1µL Superscript III RT (Invitrogen) (+RT) or 1µL DEPC-MilliQ H20 (-RT control)

Step 83.
Heat to 70C for 15 minutes

DURATION
00:15:00

Step 84.
Store at -20C until use

Step 85.
Incubate at 50C for 1 hour

DURATION
01:00:00

Step 86.
Section IX: Quantification of the purified PCR products using a high Sensitivity bioanalyzer

Step 87.
Section VIII: Ampure Cleanup of amplified cDNA -- Using a 1.8X ratio, clean up the PCR products following the manufacture’s instructions

Step 88.
Section VII: PCR amplification of cDNA - see details in publication