NEXTflex™ Small RNA Sequencing for Small RNA Starting Material

Bioo Scientific

Abstract

This protocol is for the NEXTflex™ Small RNA Sequencing Kit v2, designed to prepare small RNA libraries for sequencing using Illumina® sequencers. This kit utilizes adapters with randomized ends to greatly reduce sequence bias in small RNA sequencing library construction.

Please see the full manual for additional details.

(The protocol below describes preparation of small RNA sequencing libraries from **Small RNA** starting material; for total RNA samples, please see this protocol.)

Citation: Bioo Scientific NEXTflex™ Small RNA Sequencing for Small RNA Starting Material. **protocols.io** dx.doi.org/10.17504/protocols.io.dre53d
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Guidelines

Contents, Storage and Shelf Life

The NEXTflexÃ‚Â¢Ã‚Ã‚Â¢ Small RNA Sequencing Kit v2 contains enough material to prepare 24 RNA samples for IlluminaÃ‚Ã‚Â® compatible next-generation sequencing. The shelf life of all reagents is 6 months when stored properly. All components can safely be stored at -20Ã‚Ã‚Â°C, except: Adapter Depletion Solution, Resuspension Buffer, Nuclease-free Water, and Elution Buffer, which can be stored at room temperature.

Kit Contents	Amount
RED CAP	
3' NEXTflex™ 4N Adenylated Adapter	24 µL
AIR™ Ligase	24 µL
AIR™ Ligase Buffer	60 µL
50% PEG	144 µL
Adapter Depletion Solution	750 µL
RNase Inhibitor	26.5 µL
PURPLE CAP	
5' NEXTflex™ 4N Adapter	36 µL
T4 RNA Ligase 1	36 µL
ATP	36 µL

BLUE CAP	
NEXTflex™ RT Primer	24 µL
M-MuLV Reverse Transcriptase	48 µL
10X M-MuLV Buffer	96 µL
dNTPs	96 µL

GREEN CAP	
NEXTflex™ Universal Primer	24 µL
NEXTflex™ Barcode Primer 1	24 µL
5X DuroTaq Master Mix	120 µL

ORANGE CAP	
6X Loading Dye	150 µL
Ready to Load Low MW Ladder	300 µL

YELLOW CAP	
Resuspension Buffer	1000 µL

WHITE CAP	
Nuclease-free Water	1.5 mL

CLEAR CAP	
microRNA Control	10 µL

CLEAR CAP BOTTLE	
Elution Buffer (10X)*	2 mL

*see Reagent Preparation for dilution procedure

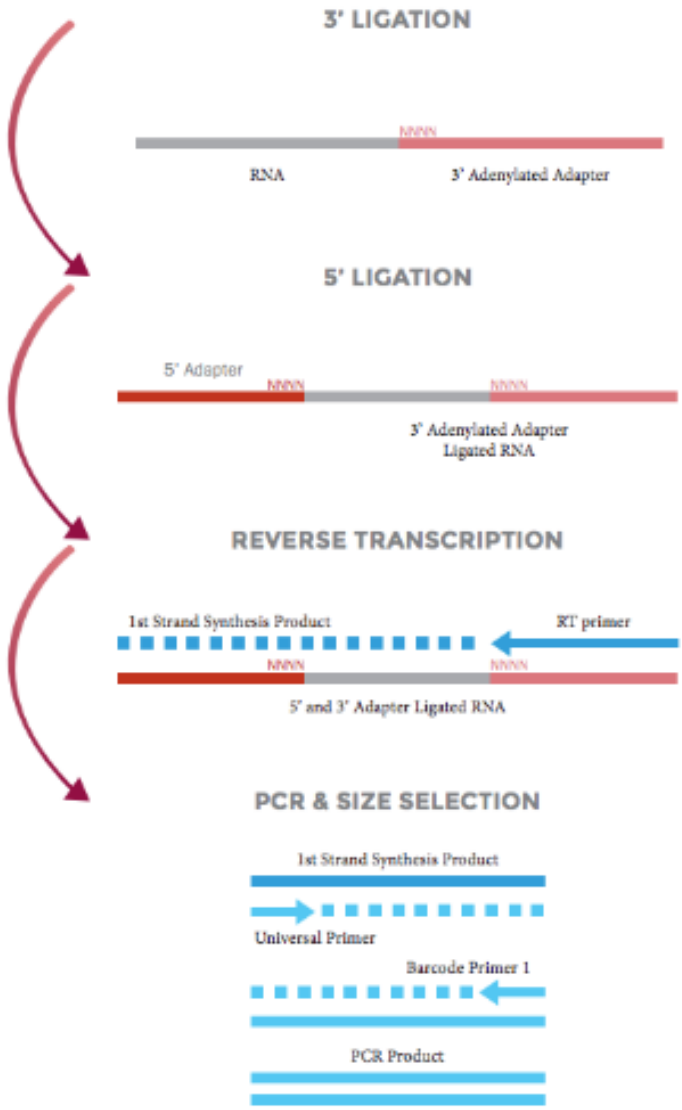
Required Materials Not Provided

ÃÂ¢ÃÂÃÂ¢ 1-10 ÃÂÃÂµg total RNA or purified small RNA from 1-10 ÃÂÃÂµg total RNA in up to 4 ÃÂÃÂµL Nucleasefree Water
ÃÂ¢ÃÂÃÂ¢ Isopropanol
ÃÂ¢ÃÂÃÂ¢ 80% Ethanol
ÃÂ¢ÃÂÃÂ¢ 2, 10, 20, 200 and 1000 ÃÂÃÂµL pipettes
ÃÂ¢ÃÂÃÂ¢ RNase-free pipette tips
ÃÂ¢ÃÂÃÂ¢ Microcentrifuge
ÃÂ¢ÃÂÃÂ¢Nuclease-free 1.7 mL microcentrifuge tubes
ÃÂ¢ÃÂÃÂ¢ 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
ÃÂ¢ÃÂÃÂ¢ Sterile disposable pestles (Fisher Cat # K749521-1500 or similar)
ÃÂ¢ÃÂÃÂ¢ Thin wall nuclease-free PCR tubes
ÃÂ¢ÃÂÃÂ¢ Thermocycler
ÃÂ¢ÃÂÃÂ¢ Heat block
ÃÂ¢ÃÂÃÂ¢ 6% TBE PAGE gels (1.0 mm) (Life Technologies Cat # EC6265BOX)
ÃÂ¢ÃÂÃÂ¢ 1X TBE buffer
ÃÂ¢ÃÂÃÂ¢ Clean razor or scalpel
ÃÂ¢ÃÂÃÂ¢ Nucleic acid stain such as SYBR Gold (Invitrogen)
ÃÂ¢ÃÂÃÂ¢ UV transilluminator or gel documentation instrument
ÃÂ¢ÃÂÃÂ¢ Gel Electrophoresis apparatus
ÃÂ¢ÃÂÃÂ¢ Electrophoresis power supply

Ã□Â¢Ã□Â□Ã□Â¢ Vortex
Ã□Â¢Ã□Â□Ã□Â¢ 0.45Ã□Â□Ã□Âµm, 2 mL Spin-X Centrifuge tube (Sigma Cat # CLS8162)
Ã□Â¢Ã□Â□Ã□Â¢ Agencourt AMPure XP 60 mL (Beckman Coulter Genomics, Cat # A63881)
Ã□Â¢Ã□Â□Ã□Â¢ Magnetic Stand -96 (Ambion, Cat # AM10027) or similar
Ã□Â¢Ã□Â□Ã□Â¢ Magnetic stand for micrcentrifuge tubes (Life Technologies DynaMagÃ□Â¢Ã□Â□Ã□Â¢-2 or similar)

Optional Materials Not Provided
Ã□Â¢Ã□Â□Ã□Â¢Ã□Â□Ã□Â If multiplexing: NEXTflexÃ□Â¢Ã□Â□Ã□Â¢ Small RNA Barcodes A Ã□Â¢Ã□Â□Ã□Â□ D (513305, 513306, 513307, 513308)

NEXTflexÃ□Â¢Ã□Â□Ã□Â¢ Small RNA Sample Preparation Flow Chart



Starting Material
The NEXTflexÃ□Â¢Ã□Â□Ã□Â¢ Small RNA Sequencing Kit v2 has been optimized and validated using total RNA (1 - 10 Ã□Â□Ã□Âµg), purified small RNA (from 1 - 10 Ã□Â□Ã□Âµg total RNA), and a synthetic miRNA pool (Ã□Â¢Ã□Â□Ã□Â¥100 pg). Best results are obtained with high quality starting material. The use of degraded RNA may result in poor yields or lack of sequencing output data. Bioo Scientific recommends running total RNA on a 1 - 2% agarose gel or examining its integrity using an Agilent Bioanalyzer. High quality total RNA preparations should have an 28S band that is twice as intense as

the 18S band of ribosomal RNA. At low concentrations, small RNA is difficult to detect on a gel; however it can be detected using an Agilent Bioanalyzer Small RNA assay (see Figure 2 in Appendix A). We recommend beginning with total RNA or a preparation of highly enriched small RNA by PAGE selecting small RNAs around 15-45 nt long or using a phenol-based small RNA isolation method such as BiooPureÃ□Â¢Ã□Â□Ã□Â¢ (Cat # 5301) for enrichment.

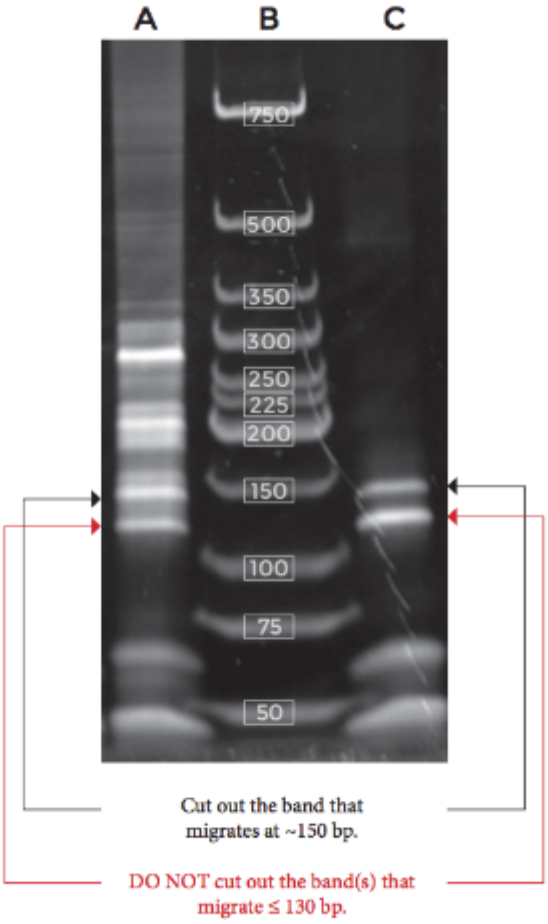
If the user is performing the procedure for the first time, we recommend using the microRNA Control included in the kit: 5Ã□Â¢Ã□Â□Ã□Â□Phos/CUCAGGAUGGCGGAGCGGUCU/3Ã□Â¢Ã□Â□Ã□Â□. This positive control sample consists of 21 RNA nucleotides and does not match any known sequence in miRBase. When running a positive control reaction, the user should add 1 Ã□Â□Ã□ÂµL of the microRNA Control in STEP A instead of their small RNA sample and expect to observe a 147 nt PCR product. The microRNA control may degrade with multiple freeze thaw cycles or exposure to nucleases. If you plan on using the control multiple times, we recommend aliquoting into several tubes and storing at -20Ã□Â□Ã□Â°C. For a total RNA positive control, human brain total RNA (Ambion catalog number AM7962 or similar) is recommended.

Reagent Preparation

1. Vortex and micro centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.
2. Add 18 mL of molecular biology-grade water to each bottle of Elution Buffer (10X) to make a 1X Elution Buffer. Check box on bottle to show water has been added.

Figure 2

6% TBE-PAGE gel
A. PCR product from library constructed from 1 Ã□Â□Ã□Âµg human brain total RNA
B. Ready to Load Low MW Ladder
C. PCR product from library constructed from 1 Ã□Â□Ã□ÂµL miRNA control
NOTE: 25 bp band not shown. 225 bp & 250 bp bands may run as a single band.



References

Jayaprakash et al. Identification and remediation of biases in the activity of RNA ligases in small-RNA deep sequencing. Nucl. Acids Res. (2011) 39 (21):e141

Materials

96 well PCR Plate Non-skirted MPS-499 by Phenix Research

Agencourt AMPure XP A63880 by Beckman Coulter

Magnetic Stand -96 AM10027 by Life Technologies

Sterile disposable pestles K749521-1500 by Fisher Scientific

6% TBE PAGE gels (1.0 mm) EC6265BOX by Life Technologies

0.45µm, 2 mL Spin-X Centrifuge tube CLS8162 by Sigma Aldrich

Magnetic stand for micrcentrifuge tubes 12321D by Life Technologies

NEXTflex Small RNA-Seq Kit v2 5132-03 by Bioo Scientific

Protocol

Reagent Preparation

Step 1.

Vortex and micro centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.

Reagent Preparation

Step 2.

Add 18 mL of molecular biology-grade water to each bottle of Elution Buffer (10X) to make a 1X Elution Buffer. Check box on bottle to show water has been added.

NEXTflex™ 4N Adenylated Adapter Ligation

Step 3.

Allow 50% PEG to come to room temperature before use.

NOTES

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Materials for "NEXTflex™ 4N Adenylated Adapter Ligation" section.

Bioo Scientific Supplied

RED CAP - 3’ NEXTflex™ 4N Adenylated Adapter, AIR™ Ligase, 50% PEG, NEXTflex™ RNase Inhibitor

WHITE CAP - Nuclease-free Water

User Supplied

RNA (1-10 µg total RNA or small RNA isolated from total RNA) in 4 µL Nuclease-free Water

96 well PCR plate

Thermocycler

Ice

NEXTflex™ 4N Adenylated Adapter Ligation

Step 4.

For each reaction combine the following in a 96 well PCR plate:

4 µL RNA (in Nuclease-free Water)
1 µL 3’ NEXTflex™ 4N Adenylated Adapter

NEXTflex™ 4N Adenylated Adapter Ligation

Step 5.

Heat at 70°C for 2 minutes then immediately place on ice.

DURATION

00:02:00

NEXTflex™ 4N Adenylated Adapter Ligation

Step 6.

Add the following components to each well and mix well:

1 µL	AIR™ Ligase
1 µL	AIR™ Ligase Buffer

2.5 µL	50% PEG (Note: 50% PEG is very viscous, please pipette carefully)
0.5 µL	RNase Inhibitor

NEXTflex™ 4N Adenylated Adapter Ligation

Step 7.
Incubate at 22°C for 2 hours in a thermocycler. For ligations to 2’ O-methylated small RNAs, such as those found in plants, incubate at 16°C overnight.

DURATION
02:00:00

Excess 3’ Adapter Removal

Step 8.
Add 10 µL of Nuclease-free Water to each sample and mix by pipetting.

NOTES
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Materials for 'Excess 3’ Adapter Removal' section

Bioo Scientific Supplied
RED CAP - Adapter Depletion Solution
YELLOW CAP - Reusupension Buffer
WHITE CAP - Nuclease-free Water

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
Isopropanol
80% Ethanol, freshly prepared
Magnetic Stand
***10 µL of 3’ NEXTflex™ 4N Adenylated Adapter Ligated RNA (from section 'NEXTflex™ 4N Adenylated Adapter Ligation')**

Excess 3’ Adapter Removal

Step 9.
Add 6 µL of Adapter Depletion Solution and mix well by pipetting.

AMOUNT
6 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

Excess 3’ Adapter Removal

Step 10.
Add 40 µL of AMPure XP beads and 60 µL of ispropanol and mix well by pipetting.

Excess 3’ Adapter Removal

Step 11.
Incubate for 5 minutes.

DURATION
00:05:00

Excess 3’ Adapter Removal

Step 12.

Magnetize beads until solution is clear.

Excess 3’ Adapter Removal

Step 13.

Remove and discard supernatant.

Excess 3’ Adapter Removal

Step 14.

Wash #1: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION

00:00:30

NOTES

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IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Excess 3’ Adapter Removal

Step 15.

Wash #2: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION

00:00:30

NOTES

Bioo Scientific 03 Sep 2015

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Excess 3’ Adapter Removal

Step 16.

Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.

DURATION

00:03:00

Excess 3’ Adapter Removal

Step 17.

Remove plate from magnetic stand and resuspend bead pellet in 22 µL of Resuspenion Buffer by pipetting volume up and down. Ensure that beads are completely resuspended.

Excess 3’ Adapter Removal

Step 18.

Incubate 2 minutes. During incubation, add 6 µL of Adapter Depletion Solution to a new, empty well.

DURATION

00:02:00

Excess 3’ Adapter Removal

Step 19.

Magnetize sample until solution appears clear.

Excess 3’ Adapter Removal

Step 20.

Transfer 20 µL of supernatant to the well containing 6 µL Adapter Depletion Solution and mix well by pipette.

Excess 3’ Adapter Removal

Step 21.

Add 40 µL of AMPure XP beads.

AMOUNT

40 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

Excess 3’ Adapter Removal

Step 22.

Add 60 µL of isopropanol and mix well by pipetting.

Excess 3’ Adapter Removal

Step 23.

Incubate for 5 minutes.

DURATION

00:05:00

Excess 3’ Adapter Removal

Step 24.

Magnetize sample until solution appears clear.

Excess 3’ Adapter Removal

Step 25.

Remove and discard supernatant.

Excess 3’ Adapter Removal

Step 26.

Wash #1: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION

00:00:30

NOTES

Bioo Scientific 03 Sep 2015

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Excess 3’ Adapter Removal

Step 27.

Wash #2: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION

00:00:30

NOTES

Bioo Scientific 03 Sep 2015

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Excess 3’ Adapter Removal

Step 28.

Incubate sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.

DURATION
00:03:00

Excess 3’ Adapter Removal

Step 29.
Remove plate from magnetic stand and resuspend bead pellet in 12 µL of Nuclease-free Water by pipetting volume up and down. Ensure that beads are completely resuspended.

Excess 3’ Adapter Removal

Step 30.
Incubate for 2 minutes.

DURATION
00:02:00

Excess 3’ Adapter Removal

Step 31.
Magnetize sample until solution appears clear.

Excess 3’ Adapter Removal

Step 32.
Transfer 11 µL of supernatant to a new well.

5’ NEXTflex™ 4N Adapter Ligation

Step 33.
For each reaction, add 1.5 µL of the 5’ NEXTflex™ 4N Adapter and heat at 70°C for 2 minutes, then immediately place on ice.

DURATION
00:02:00

NOTES
Bioo Scientific 18 Aug 2015
Materials for section "5’ NEXTflex™ 4N Adapter Ligation"

Bioo Scientific Supplied
PURPLE CAP - 5’ NEXTflex™ 4N Adapter, ATP, T4 RNA Ligase 1, RNase Inhibitor
RED CAP - AIR™ Ligase Buffer, 50% PEG (warm to room temperature before use)

User Supplied
Heatblock or thermocycler
96-well PCR plate or PCR tubes
Ice
***11 µL of 3’ NEXTflex™ 4N Adenylated Adapter Ligated RNA (from section "Excess 3’ Adapter Removal"**)

5’ NEXTflex™ 4N Adapter Ligation

Step 34.
For each reaction, combine the following components in a well of a 96-well PCR plate and mix thoroughly:

12.5 µL	3’ NEXTflex™ 4N Adapter Ligated RNA & 5’ NEXTflex™ 4N Adapter
1.5 µL	AIR™ Ligase Buffer
1.5 µL	ATP
1.5 µL	T4 RNA Ligase 1

3.5 µL	50% PEG (Note: 50% PEG is very viscous, please pipette carefully)
0.5 µL	RNase Inhibitor

5’ NEXTflex™ 4N Adapter Ligation

Step 35.
Incubate at 20°C for 1 hour in a thermocycler.

DURATION
01:00:00

Reverse Transcription-First Strand Synthesis

Step 36.
Add 1 µL NEXTflex™ RT primer to each sample. Heat at 70°C for 2 minutes then immediately place on ice.

DURATION
00:02:00

NOTES
Bioo Scientific 18 Aug 2015
Materials for section "Reverse Transcription-First Strand Synthesis"

Bioo Scientific Supplied
BLUE CAP - NEXTflex™ RT primer, 10X M-MuLV buffer, M-MuLV Reverse Transcriptase, dNTPs
WHITE CAP - Nuclease-free Water

User Supplied
96 well PCR Plate
Thermocycler
***5’ and 3’ NEXTflex™ Adapter Ligated RNA (21 µL) (from section "5’ NEXTflex™ 4N Adapter Ligation"**)

Reverse Transcription-First Strand Synthesis

Step 37.
For each sample, add the following components and mix well.

4 µL	10X M-MuLV Buffer
4 µL	dNTPs
8 µL	Nuclease-free Water
2 µL	M-MuLV Reverse Transcriptase

Reverse Transcription-First Strand Synthesis

Step 38.
Incubate in a thermocycler at 44°C for 1 hour followed by 90°C for 10 minutes. The procedure may be safely stopped at this step and samples stored at -20°C.

Bead Cleanup

Step 39.
Add 10 µL of Adapter Depletion Solution to each sample and mix well by pipette

NOTES
Bioo Scientific 18 Aug 2015
Materials for section "Bead Cleanup"

Bioo Scientific Supplied
RED CAP - Adapter Depletion Solution

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
Isopropanol
80% Ethanol, freshly prepared
Magnetic Stand
***40 µL of First Strand Synthesis product (from section "Reverse Transcription-First Strand Synthesis")**

Bead Cleanup

Step 40.
Add 40 µL of AMPure XP beads and 90 µL of ispropanol and mix well by pipette.

AMOUNT
40 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

Bead Cleanup

Step 41.
Incubate for 5 minutes.

DURATION
00:05:00

Bead Cleanup

Step 42.
Magnetize sample until solution is clear.

Bead Cleanup

Step 43.
Remove and discard supernatant.

Bead Cleanup

Step 44.
Wash #1: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION
00:00:30

NOTES
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IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Bead Cleanup

Step 45.
Wash #2: Add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove with a P200 or P300 set to 200 µL.

DURATION
00:00:30

NOTES
Bioo Scientific 03 Sep 2015

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

Bead Cleanup

Step 46.

Incubate sample for 3 minutes. After one minute, remove any residual liquid that may have collected at the bottom of the well.

DURATION

00:03:00

Bead Cleanup

Step 47.

Remove plate from magnetic stand and resuspend bead pellet in 19 µL of Nuclease-free Water by pipetting. Ensure that beads are completely resuspended.

Bead Cleanup

Step 48.

Incubate for 2 minutes.

DURATION

00:02:00

Bead Cleanup

Step 49.

Magnetize until solution is clear.

Bead Cleanup

Step 50.

Transfer 18 µL of supernatant to a new well.

PCR Amplification

Step 51.

For each PCR reaction add the following to the 18 µL purified First Strand Synthesis product (from section "Bead Cleanup"):

5 µL 5X DuroTaq Master Mix
1 µL NEXTflex™ Barcode Primer 1 or Barcoded Primer from NEXTflex™ Small RNA Barcodes Kit
1 µL NEXTflex™ Universal Primer

NOTES

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Materials

Bioo Scientific Supplied
GREEN CAP - NEXTflex™ Barcode Primer 1, NEXTflex™ Universal Primer, 5X DuroTaq Master Mix

User Supplied
(Optional) NEXTflex™ Barcode Primer (NEXTflex™ Small RNA Barcodes: 513305, 513306, 513307, or 513308)
Thermocycler
***18 µL Purified First Strand Synthesis product (from section "Bead Cleanup")**

PCR Amplification

Step 52.

Cycle as follows: (Make sure thermocycler is above 80°C before placing samples on block)

2 min	95°C	
20 sec	95°C	
30 sec	60°C	Repeat 12 -18 cycles
15 sec	72°C	
2 min	72°C	

Gel Electrophoresis

Step 53.
Add 5 µL of 6X Gel Loading Dye to each PCR product and mix well

NOTES
Bioo Scientific 17 Aug 2015
Materials

Bioo Scientific Supplied
ORANGE CAP - 6X Gel Loading Dye, Ready to Load Low MW Ladder

User Supplied
6% TBE-PAGE Gel
1X TBE Buffer
Nucleic acid stain such as SYBR Gold (Invitrogen)
UV transilluminator or other visualization tool
Clean razor or scalpel
Nuclease-free 1.7 mL microcentrifuge tube
***PCR Product (25 µL) (from section "PCR Amplification")**

Gel Electrophoresis

Step 54.
Load purified PCR products onto a 6% TBE-PAGE gel. We recommend leaving 1-2 lanes between samples prepared with the same barcode primer to avoid cross contamination. Samples prepared with different barcodes and that will be sequenced together may be run in adjacent lanes.

REAGENTS
6% TBE PAGE gels (1.0 mm) EC6265BOX by Life Technologies

Gel Electrophoresis

Step 55.
In an adjacent lane load 10 µL of Ready to Load Low MW Ladder.

Gel Electrophoresis

Step 56.
Run the gel with 1X TBE buffer at 200 V until the lower dye band is near the bottom of the gel (0.5-1 cm). The gel should run for approximately 30 minutes. Run times may vary depending on individual equipment.

DURATION
00:30:00

Gel Electrophoresis

Step 57.
Carefully remove the gel from the glass plates and stain with a nucleic acid stain such as SYBR Gold (Invitrogen) per manufacturer instructions.

Gel Electrophoresis

Step 58.

Visualize gel bands on a UV transilluminator or other gel documentation instrument.

Gel Electrophoresis

Step 59.

Using a clean razor cut out the 150 bp band and place into clean 1.7 mL tube. Do not cut out the 130 bp band; this is adapter dimer product. See Figure 2 in Guidelines for example. The ladder band at 200 bp is twice as intense as the other bands and can be used for orientation.

Nucleic Acid Elution and Purification

Step 60.

Briefly centrifuge the microcentrifuge tube containing the gel slice to collect the gel slice at the bottom of the tube.

NOTES

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Materials

Bioo Scientific Supplied

YELLOW CAP - Resuspension Buffer

CLEAR CAP BOTTLE - 1X Elution Buffer (Dilute prior to use as described in the Reagent Preparation section)

User Supplied

Agencourt AMPure XP Magnetic Beads (room temperature)

Isopropanol

80% Ethanol

Nuclease-free 1.7 mL microcentrifuge tubes

Spin-X Centrifuge tube (Sigma)

Sterile disposable pestles (Fisher Cat # K749521-1500 or similar)

Magnetic stand for micrcentrifuge tubes (Life Technologies DynaMag™-2 or similar)

***Gel Slice (in 1.7 mL tube) (from section "Gel Electrophoresis")**

Nucleic Acid Elution and Purification

Step 61.

Crush the gel slice thoroughly with a disposable pestle. Leave the pestle in the tube.

REAGENTS

Sterile disposable pestles K749521-1500 by Fisher Scientific

Nucleic Acid Elution and Purification

Step 62.

Add 300 µL of Elution Buffer to each tube and then remove the pestle, ensuring that as much gel as possible has been washed from the pestle.

Nucleic Acid Elution and Purification

Step 63.

Let gel pieces soak at least 2 hours or overnight at room temperature with agitation. DO NOT incubate longer than overnight.

Nucleic Acid Elution and Purification

Step 64.

Pulse spin tubes to collect all eluate from wall and lid.

Nucleic Acid Elution and Purification

Step 65.

Carefully transfer the eluate (including crushed gel) to the top of a Spin-X Centrifuge tube (Sigma). Cutting the end off of a P1000 tip can help in transfer of larger gel pieces. Centrifuge the Spin-X tube at 16,000 x g for 2 minutes. Dispose of the spin filter.

REAGENTS

0.45µm, 2 mL Spin-X Centrifuge tube CLS8162 by Sigma Aldrich

DURATION

00:02:00

Nucleic Acid Elution and Purification

Step 66.

Add to each tube and mix well*:

50 µL	AMPure XP Beads
350 µL	Isopropanol

AMOUNT

50 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

Nucleic Acid Elution and Purification

Step 67.

Incubate at room temperature for 10 minutes. Agitation during this incubation may increase efficiency of recovery.

DURATION

00:10:00

Nucleic Acid Elution and Purification

Step 68.

Magnetize sample until solution appears clear.

Nucleic Acid Elution and Purification

Step 69.

Wash #1: Carefully remove and discard the supernatant, add 950 µL 80% ethanol, incubate 30 seconds, and remove.

DURATION

00:00:30

Nucleic Acid Elution and Purification

Step 70.

Wash #2: Carefully remove and discard the supernatant, add 950 µL 80% ethanol, incubate 30 seconds, and remove.

DURATION

00:00:30

Nucleic Acid Elution and Purification

Step 71.

Dry sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the tube.

DURATION

00:03:00

Nucleic Acid Elution and Purification

Step 72.

Remove plate from magnetic rack and resuspend bead pellet in 13 µL of Resuspension Buffer by pipetting volume up and down. Ensure that beads are completely resuspended and rehydrated.

Nucleic Acid Elution and Purification

Step 73.

Incubate for 2 minutes.

DURATION

00:02:00

Nucleic Acid Elution and Purification

Step 74.

Magnetize for 5 minutes or until supernatant appears clear.

DURATION

00:05:00

Nucleic Acid Elution and Purification

Step 75.

Transfer 12 µL of supernatant to a clean 1.7 mL tube. This is your sequencing library.

Nucleic Acid Elution and Purification

Step 76.

Check the size distribution and concentration of the final library by Bioanalyzer High Sensitivity DNA Assay (Agilent).

NOTES

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If desired, ethanol precipiatation or other nucleic acid purification methods may be used for purification of supernatant after this step. For ethanol precipitation, it is recommended to transfer eluate from this step to a clean microcentrifuge tube, as pellets can be difficult to handle in the Spin-X tubes.

Warnings

Bioo Scientific strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or Bioo Scientific at nextgen@biooscientific.com.

• Do not use the kit past the expiration date.

• This kit contains a single Barcoded Primer. To enable multiplexing, please use the appopriate combination of NEXTflex™ Small RNA Barcodes during the PCR Amplification step.

• Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).

• RNA sample quality may vary between preparations. It is the user's responsibility to optimize the initial RNA input amount to obtain desired PCR bands for gel excision and sequencing. Refer to the Starting Material section for additional information.

• Vortex and micro centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.

• Do not remove AIR Ligase or T4 RNA Ligase 1 enzymes from -20°C until immediately before use and return to -20°C immediately after use.

• Some total RNA extraction and purification methods may not efficiently isolate small RNAs. Users should verify that their extraction and purification method also isolates small RNAs.