NEXTflex™ Rapid Directional qRNA-Seq™ Kit

Bioo Scientific

Abstract

The NEXTflex™ Rapid Directional qRNA-Seq Kit is designed to prepare directional, strandspecific RNA libraries for sequencing using Illumina® sequencers while enabling the high precision measurement of RNA molecules.

Please see the full manual for additional details.

Citation: Bioo Scientific NEXTflex™ Rapid Directional qRNA-Seq™ Kit. **protocols.io**
dx.doi.org/10.17504/protocols.io.dm849v
Published: 24 Aug 2015

Guidelines

OVERVIEW:
The NEXTflex™ Rapid Directional qRNA-Seq Kit is designed to prepare directional, strandspecific RNA libraries for sequencing using Illumina® sequencers while enabling the high precision measurement of RNA molecules. The NEXTflex™ Rapid Directional qRNA-Seq Kit efficiently generates libraries equivalent to conventional directional, strand-specific RNASeq libraries, but with the added feature of Molecular Indexing™, a DNA labeling technology developed by Cellular Research, Inc. Directionality is retained by adding dUTP during the second strand synthesis step and subsequent cleavage of the uridine-containing strand using Uracil DNA Glycosylase. The strand that's sequenced is the cDNA strand. This kit contains a set of 96 distinct molecular labels on the sequencing adapters. Each label consists of an 8 nucleotide barcode tag. During the ligation reaction, each cDNA fragment end independently and randomly ligates to a single label from this pool of 96 adapters to result in a total of 96 x 96 = 9,216 possible combinations across both ends. For every clone sequenced, paired-end reads reveal the chosen label on each end along with adjoining cDNA sequence. This allows for differentiation between re-sampling of the same molecule and sampling of a different molecule of identical sequence. Analysis using molecular indexing information provides an absolute, digital measurement of gene expression levels, irrespective of common amplification distortions observed in many RNA-Seq experiments. In addition to encoding cDNA fragments at the molecular level, the kit also allows for the application of sample-specific barcodes during the library preparation PCR step. A more detailed description of the use of molecular indexing for RNA-Seq is available in our product application note, Directional qRNA-Seq: Combining the Power of Stranded RNA-Seq with the Quantitative Precision of Molecular Labels, available for download here or contact us at BiooNGS@BiooScientific.com.

For deconvolution of unique read fragments, Bioo Scientific now offers a dqRNASeq script. Using read pairs aligned to transcripts and FASTQ files, this script will generate a table listing fragments, start/stop sites (USS) in transcripts, and molecular labels (STL). The script will also generate a table listing the total number of read pairs per transcript and the number of read pairs after STL, USS, and STL/USS correction. The script can be downloaded here or contact us at BiooNGS@BiooScientific.com.

Required Materials not Provided

•Total RNA Input: 10 ng – 1 µg total RNA for enrichment by NEXTflex™ Poly(A) Beads (Cat. # 512979, 512980, 512981). NEXTflex™ Poly(A) Beads are available separately.
• mRNA Input: ~1 ng - 100 ng isolated mRNA.
• rRNA-depleted RNA Input: ~1ng - 100 ng. Bioo Scientific recommends Ribo-Zero™ (Epicentre) or RiboMinus™ (Life Technologies) for rRNA depletion.
• 100% Ethanol (stored at room temperature)
• 80% Ethanol (stored at room temperature)
• 2, 10, 20, 200 and 1000 µL pipettes
• RNase-free pipette tips
• Nuclease-free 1.5 mL microcentrifuge tubes
• Thin wall nuclease-free 0.5 mL microcentrifuge tubes
• 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
• Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
• Agencourt AMPure XP 60 mL (Beckman Coulter Genomics, Cat # A63881)
• Magnetic Stand -96 (Ambion, Cat # AM10027) or similar for post PCR cleanup
• Microcentrifuge
• Thermocycler
• Heat block
• Vortex

Revision History

Version	Date	Description
V14.03	March 2014	Initial Product Launch
V14.07	July 2014	The Molecular Indices/Adapters, post-adapter ligation cleanup and PCR parameters have been optimized to improve efficiency. Please ensure PCR programs are updated.
V14.09	September 2014	The starting material necessary for RNA has been reduced to 10 ng total RNA when poly(A) enriched over NEXTflex™ Poly(A) Beads (Cat. # 512979, 512980, 512981).
V15.04	April 2014	The name of the NEXTflex™ Directional qRNA Molecular Labels (1 µM) has been changed to the NEXTflex™ Molecular Index Adapters (1 µM).

Contents, Storage and Shelf Life

The NEXTflex™ Rapid Directional qRNA-Seq™ Kit contains enough material to prepare 8 RNA samples for Illumina® compatible sequencing. The shelf life of all reagents is 12 months when stored properly. All components can safely be stored at -20°C.

Kit Contents	Amount
BROWN CAP	
NEXTflex™ RNA Fragmentation Buffer	40 µL
RED CAP	
NEXTflex™ First Strand Synthesis Primer	8 µL
NEXTflex™ Directional First Strand Synthesis Buffer Mix	32 µL
NEXTflex™ Rapid Reverse Transcriptase	8 µL
BLUE CAP	
NEXTflex™ Directional Second Strand Synthesis Mix	200 µL
PINK CAP	
NEXTflex™ Adenylation Mix	36 µL
YELLOW CAP	
NEXTflex™ Ligation Mix	220 µL
NEXTflex™ Molecular Index Adapters (1 µM)	16 µL
GREEN CAP	
NEXTflex™ Uracil DNA Glycosylase	8 µL
NEXTflex™ qRNA-Seq™ Universal Forward Primer (10 µM)	16 µL
NEXTflex™ PCR Master Mix	96 µL
ORANGE CAP	
NEXTflex™ qRNA-Seq™ Barcoded Primers 1-4 (10 µM)	10 µL
GRAY CAP	
Nuclease-free Water	1.5 mL
WHITE CAP	
Resuspension Buffer	(2) 1.5 mL

Required Materials not Provided

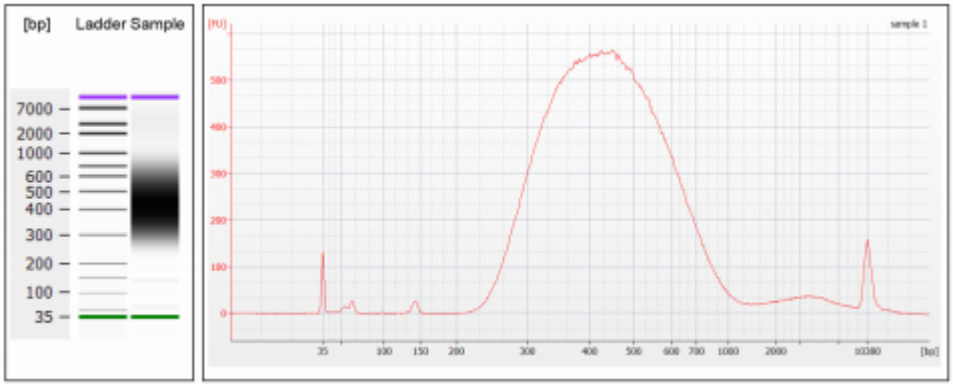
• Total RNA Input: 10 ng – 1 µg total RNA for enrichment by NEXTflex™ Poly(A) Beads (Cat. # 512979, 512980, 512981). NEXTflex™ Poly(A) Beads are available separately.
• mRNA Input: ~1 ng - 100 ng isolated mRNA.
• rRNA-depleted RNA Input: ~1ng - 100 ng. Bioo Scientific recommends Ribo-Zero™ (Epicentre) or RiboMinus™ (Life Technologies) for rRNA depletion.
• 100% Ethanol (stored at room temperature)
• 80% Ethanol (stored at room temperature)
• 2, 10, 20, 200 and 1000 µL pipettes
• RNase-free pipette tips
• Nuclease-free 1.5 mL microcentrifuge tubes
• Thin wall nuclease-free 0.5 mL microcentrifuge tubes
• 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
• Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
• Agencourt AMPure XP 60 mL (Beckman Coulter Genomics, Cat # A63881)
• Magnetic Stand -96 (Ambion, Cat # AM10027) or similar for post PCR cleanup
• Microcentrifuge
• Thermocycler
• Heat block
• Vortex

NEXTflex™ Rapid Directional qRNA-Seq™ Sample Preparation Flow Chart

Starting Material

The NEXTflex™ Rapid Directional qRNA-Seq™ Kit has been optimized and validated using poly(A) enriched or rRNA depleted RNA (~1 ng - 100 ng). Only 10 ng - 1 µg of total RNA are required if NEXTflex™ Poly(A) beads are used to enrich for mRNA*. Bioo Scientific recommends examining total RNA integrity using an Agilent Bioanalyzer. High quality total RNA preparations should have an RNA Integrity Number (RIN) greater than or equal to 8. Alternatively, total RNA may be run on a 1 - 2% agarose gel and integrity examined by staining with ethidium bromide. High quality RNA should have a 28S band that is twice as intense as the 18S band of ribosomal RNA. Lower amounts of starting material result in higher duplication rates and other changes in sequencing data quality.
*Low RNA inputs may reduce library complexity. Read our application note about constructing high quality RNA-Seq libraries from limited amounts of total RNA. Request a PDF copy by emailing BiooNGS@BiooScientific.com.

Library Validation



Materials

NEXTflex™ Poly(A) Beads 512979 by Bioo Scientific

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

Adhesive PCR Plate Seal MSB1001 by Bio-rad Laboratories

Agencourt AMPure XP A63880 by Beckman Coulter

Magnetic Stand -96 AM10027 by Life Technologies

Protocol

RNA Fragmentation

Step 1.

For each reaction combine the following in a nuclease-free microcentrifuge tube or plate:

14 µL	RNA (in Nuclease-free Water or Elution Buffer)
5 µL	NEXTflex™ RNA Fragmentation buffer
19 µL	TOTAL

REAGENTS

NEXTflex™ Poly(A) Beads 512979 by Bioo Scientific

NOTES

Bioo Scientific 13 Aug 2015

Materials

Bioo Scientific Supplied

BROWN CAP - NEXTflex™ RNA Fragmentation Buffer

GRAY CAP - Nuclease-free Water

User Supplied

mRNA enriched from 10 ng – 1 µg total RNA by NEXTflex™ Poly(A) Beads, or ~1 ng - 100 ng mRNA/rRNA-depleted RNA in up to 14 µL Nuclease-free Water or Elution Buffer

Nuclease-free microcentrifuge tube or plate

Thermocycler or heatblock set to 95°C

Ice

RNA Fragmentation

Step 2.

Mix thoroughly by pipetting.

RNA Fragmentation

Step 3.

Heat for 10 minutes at 95°C, immediately place on ice.

DURATION

00:10:00

First Strand Synthesis

Step 4.

For each reaction, add 1 µL NEXTflex™ First Strand Synthesis Primer to the fragmented RNA (from

Section "RNA Fragmentation").

NOTES

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Materials

Bioo Scientific Supplied

RED CAP - NEXTflex™ First Strand Synthesis Primer, NEXTflex™ Directional First Strand Synthesis Buffer Mix, NEXTflex™ Rapid Reverse Transcriptase

User Supplied

Thermocycler

Ice Fragmented

RNA (from Section "RNA Fragmentation")

First Strand Synthesis

Step 5.

Heat at 65°C for 5 minutes, immediately place on ice.

DURATION

00:05:00

First Strand Synthesis

Step 6.

For each reaction, combine the following in a nuclease-free microcentrifuge tube or plate:

20 µL	Fragmented RNA + NEXTflex™ First Strand Synthesis Primer
4 µL	NEXTflex™ Directional First Strand Synthesis Buffer Mix
1 µL	NEXTflex™ Rapid Reverse Transcriptase
25 µL	TOTAL

First Strand Synthesis

Step 7.

Mix thoroughly by pipetting.

First Strand Synthesis

Step 8.

Incubate at 25°C for 10min.

DURATION

00:10:00

First Strand Synthesis

Step 9.

Incubate at 50°C for 50min.

DURATION

00:50:00

First Strand Synthesis

Step 10.

Incubate at 70°C for 15min.

DURATION

00:15:00

Second Strand Synthesis

Step 11.

For each reaction, combine the following in a nuclease-free microcentrifuge tube or plate:

25 µL	First Strand Synthesis product (from Section "First Strand Synthesis")
25 µL	NEXTflex™ Directional Second Strand Synthesis Mix (contains dUTP)
50 µL	TOTAL

NOTES

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Materials

Bioo Scientific Supplied

BLUE CAP - NEXTflex™ Directional Second Strand Synthesis Mix

User Supplied

Thermocycler

Ice

First Strand Synthesis product (from Section "First Strand Synthesis")

Second Strand Synthesis

Step 12.

Mix thoroughly by pipetting.

Second Strand Synthesis

Step 13.

Incubate for 60 minutes at 16°C.

DURATION

01:00:00

Bead Cleanup

Step 14.

Add 90 µL of well mixed AMPure XP Beads to each well containing sample. Mix thoroughly by pipetting.

AMOUNT

90 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

NOTES

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Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

User Supplied

Adhesive PCR Plate Seal

Agencourt AMPure XP

Magnetic Beads (room temperature)

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

Second Strand Synthesis product (from Step "Second Strand Synthesis")

Bioo Scientific 14 Aug 2015

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

User Supplied

Adhesive PCR Plate Seal

Agencourt AMPure XP Magnetic Beads (room temperature)

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand Second Strand Synthesis product (from "Second Strand Synthesis" section)

Bead Cleanup

Step 15.

Incubate the plate for 5 minutes at room temperature.

DURATION

00:05:00

Bead Cleanup

Step 16.

Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION

00:05:00

Bead Cleanup

Step 17.

Remove and discard all of the supernatant from the plate taking care not to disturb the beads.

Bead Cleanup

Step 18.

With plate on stand, add 200 µL of freshly prepared 80% ethanol to each well without disturbing the beads and incubate the plate for at least 30 seconds at room temperature. Carefully, remove and discard the supernatant.

DURATION

00:00:30

Bead Cleanup

Step 19.

Repeat step 18, for a total of two ethanol washes. Ensure the ethanol has been removed.

Bead Cleanup

Step 20.

Remove the plate from the magnetic stand and let dry at room temperature for 2 minutes.

REAGENTS

Magnetic Stand -96 AM10027 by Life Technologies

DURATION

00:02:00

Bead Cleanup

Step 21.

Resuspend dried beads in 17 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure that the beads are completely rehydrated and re-suspended.

Bead Cleanup

Step 22.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup

Step 23.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

REAGENTS
Magnetic Stand -96 AM10027 by Life Technologies

DURATION
00:05:00

Bead Cleanup

Step 24.
Transfer 16 µL of the clear supernatant to a fresh well for the next step.

Bead Cleanup

Step 25.
The procedure may be stopped at this point and the reactions stored at -20°C.

Adenylation

Step 26.
For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

16 µL	Second Strand Synthesis product (from Step "Bead Cleanup")
4.5 µL	NEXTflex™ Adenylation Mix
20.5 µL	TOTAL

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

NOTES
Bioo Scientific 13 Aug 2015
Materials

Bioo Scientific Supplied
PINK CAP - NEXTflex™ Adenylation Mix
User Supplied
Thermocycler
Purified Second Strand Synthesis product (from Section "Bead Cleanup")

Adenylation

Step 27.
Mix thoroughly by pipetting.

Adenylation

Step 28.
Incubate at 37°C for 30min.

DURATION
00:30:00

Adenylation

Step 29.

Incubate at 70°C for 5min.

DURATION

00:05:00

Adapter Ligation

Step 30.

For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

20.5 µL	3' Adenylated DNA (from Section "Adenylation")
27.5 µL	NEXTflex™ Ligation Mix
2.0 µL	NEXTflex™ Molecular Index Adapters (1 µM)
50 µL	TOTAL

REAGENTS

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

NOTES

Bioo Scientific 13 Aug 2015

Materials

Bioo Scientific Supplied

YELLOW CAP - NEXTflex™ Ligation Mix (remove right before use and store immediately after use at -20°C), NEXTflex™ Molecular Index Adapters (1 µM)

User Supplied

Thermocycler

3' Adenylated DNA (from Section "Adenylation")

Adapter Ligation

Step 31.

Mix thoroughly by pipetting.

Adapter Ligation

Step 32.

Incubate on a thermocycler for 10 minutes at 30°C.

DURATION

00:10:00

Bead Cleanup 2

Step 33.

Add 40 µL of well mixed AMPure XP Beads to each well containing sample Mix thoroughly by pipetting.

AMOUNT

40 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

NOTES

Bioo Scientific 13 Aug 2015

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

User Supplied

Adhesive PCR Plate Seal
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
Adapter Ligated DNA (from Section "PCR Amplification")

Bead Cleanup 2

Step 34.
Incubate the plate for 5 minutes at room temperature.

DURATION
00:05:00

Bead Cleanup 2

Step 35.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 2

Step 36.
Remove and discard all of the supernatant from the plate taking care not to disturb the beads.

REAGENTS
Adhesive PCR Plate Seal MSB1001 by Bio-rad Laboratories

Bead Cleanup 2

Step 37.
With plate on stand, add 200 µL of freshly prepared 80% ethanol to each well without disturbing the beads and incubate the plate for at least 30 seconds at room temperature. Carefully, remove and discard the supernatant.

DURATION
00:30:00

Bead Cleanup 2

Step 38.
Repeat step 37, for a total of two ethanol washes. Ensure the ethanol has been removed.

Bead Cleanup 2

Step 39.
Remove the plate from the magnetic stand and let dry at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 2

Step 40.
Resuspend dried beads in 51 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure that the beads are completely rehydrated and re-suspended.

Bead Cleanup 2

Step 41.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 2

Step 42.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

REAGENTS
Magnetic Stand -96 AM10027 by Life Technologies

DURATION
00:05:00

Bead Cleanup 2

Step 43.
Transfer 50 µL of the clear supernatant to a fresh well.

Bead Cleanup 2

Step 44.
Add 40 µL of well mixed AMPure XP Beads to each well containing sample. Mix thoroughly by pipetting.

AMOUNT
40 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

Bead Cleanup 2

Step 45.
Incubate the plate for 5 minutes at room temperature.

DURATION
00:05:00

Bead Cleanup 2

Step 46.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 2

Step 47.
Remove and discard all of the supernatant from the plate taking care not to disturb the beads.

Bead Cleanup 2

Step 48.
With plate on stand, add 200 µL of freshly prepared 80% ethanol to each well without disturbing the beads and incubate the plate for at least 30 seconds at room temperature. Carefully, remove and discard the supernatant.

DURATION
00:00:30

Bead Cleanup 2

Step 49.
Repeat step 48, for a total of two ethanol washes. Ensure the ethanol has been removed.

Bead Cleanup 2

Step 50.
Remove the plate from the magnetic stand and let dry at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 2

Step 51.
Resuspend dried beads in 34 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure that the beads are completely rehydrated and re-suspended.

Bead Cleanup 2

Step 52.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 2

Step 53.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 2

Step 54.
Transfer 33 µL of the clear supernatant to a fresh well for the next step.

Bead Cleanup 2

Step 55.
The procedure may be stopped at this point and the reactions stored at -20°C.

PCR Amplification

Step 56.
For each sample (from Section "Bead Cleanup 2"), add 1 µL NEXTflex™ Uracil DNA Glycosylase and mix.

NOTES
Bioo Scientific 13 Aug 2015
Materials

Bioo Scientific Supplied
GREEN CAP - NEXTflex™ Uracil DNA Glycosylase, NEXTflex™ qRNA-Seq™ Universal Forward Primer, NEXTflex™ PCR Master Mix
ORANGE CAP - NEXTflex™ qRNA-Seq™ Barcoded Primer
User Supplied
Thermocycler
96 Well PCR Plate
Purified Adapter Ligated DNA (from Section "Bead Cleanup 2")

PCR Amplification

Step 57.
Incubate at 37°C for 30 minutes

DURATION
00:30:00

PCR Amplification

Step 58.

Incubate at 98°C for 2 minutes, then transfer to ice.

DURATION

00:02:00

PCR Amplification

Step 59.

For each sample, combine the following reagents on ice in a nuclease-free 96 well PCR Plate:

34 µL	Glycosylase-treated Adapter Ligated DNA
12 µL	NEXTflex™ PCR Master Mix
2 µL	NEXTflex™ qRNA-Seq™ Universal Forward Primer
2µL	NEXTflex™ qRNA-Seq™ Barcoded Primer
50 µL	TOTAL

REAGENTS

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

PCR Amplification

Step 60.

Mix thoroughly by pipetting.

PCR Amplification

Step 61.

PCR cycles:

2 min	98°C	
30 sec	98°C	
30 sec	65°C	Repeat 15 cycles*
60 sec	72°C	
4 min	72°C	

NOTES

Bioo Scientific 13 Aug 2015

*PCR cycles will vary depending on the amount of starting material and quality of your sample. Further optimization may be necessary. Always use the least number of cycles possible.

Bead Cleanup 3

Step 62.

Add 40 µL of well mixed AMPure XP Beads to each well containing sample. Mix thoroughly by pipetting.

AMOUNT

40 µl Additional info:

REAGENTS

Agencourt AMPure XP A63880 by Beckman Coulter

NOTES

Bioo Scientific 13 Aug 2015

Materials

Bioo Scientific Supplied

WHITE CAP - Resuspension Buffer

User Supplied

Adhesive PCR Plate Seal

Agencourt AMPure XP
Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand
PCR Amplified Libraries (from Section "PCR Amplification")

Bead Cleanup 3

Step 63.
Incubate the plate for 5 minutes at room temperature.

DURATION
00:05:00

Bead Cleanup 3

Step 64.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 3

Step 65.
Remove and discard all of the supernatant from the plate taking care not to disturb the beads.

Bead Cleanup 3

Step 66.
With plate on stand, add 200 µL of freshly prepared 80% ethanol to each well without disturbing the beads and incubate the plate for at least 30 seconds at room temperature. Carefully, remove and discard the supernatant.

DURATION
00:00:30

Bead Cleanup 3

Step 67.
Repeat step 66, for a total of two ethanol washes. Ensure the ethanol has been removed.

Bead Cleanup 3

Step 68.
Remove the plate from the magnetic stand and let dry at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 3

Step 69.
Resuspend dried beads in 51 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure that the beads are completely rehydrated and re-suspended.

Bead Cleanup 3

Step 70.
Incubate resuspended beads at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 3

Step 71.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 3

Step 72.
Transfer 50 µL of the clear supernatant to a fresh well.

Bead Cleanup 3

Step 73.
Add 40 µL of well mixed AMPure XP Beads to each well containing sample. Mix thoroughly by pipetting.

AMOUNT
40 µl Additional info:

REAGENTS
Agencourt AMPure XP A63880 by Beckman Coulter

Bead Cleanup 3

Step 74.
Incubate the plate for 5 minutes at room temperature.

DURATION
00:05:00

Bead Cleanup 3

Step 75.
Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION
00:05:00

Bead Cleanup 3

Step 76.
Remove and discard all of the supernatant from the plate taking care not to disturb the beads.

Bead Cleanup 3

Step 77.
With plate on stand, add 200 µL of freshly prepared 80% ethanol to each well without disturbing the beads and incubate the plate for at least 30 seconds at room temperature Carefully, remove and discard the supernatant.

DURATION
00:00:30

Bead Cleanup 3

Step 78.
Repeat step 77, for a total of two ethanol washes. Ensure the ethanol has been removed.

Bead Cleanup 3

Step 79.
Remove the plate from the magnetic stand and let dry at room temperature for 2 minutes.

DURATION
00:02:00

Bead Cleanup 3

Step 80.
Resuspend dried beads in 17 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure that the beads are completely rehydrated and re-suspended.

Bead Cleanup 3

Step 81.

Incubate resuspended beads at room temperature for 2 minutes.

DURATION

00:02:00

Bead Cleanup 3

Step 82.

Place the plate on the magnetic stand for 5 minutes at room temperature or until the supernatant appears completely clear.

DURATION

00:05:00

Bead Cleanup 3

Step 83.

Transfer 16 µL of the clear supernatant to a fresh well.

NOTES

Bioo Scientific 14 Aug 2015

Non-multiplexed libraries can be normalized using Tris-HCl (10 mM), pH 8.5 with 0.1% Tween 20. For multiplexed libraries, transfer 10 µL of each normalized library for pooling in the well of a new 96 Well PCR Plate. Mix thoroughly by pipetting.

Bioo Scientific 14 Aug 2015

qPCR is recommended to quantitate DNA library templates for optimal cluster density.

Bioo Scientific 14 Aug 2015

We recommend quantifying your library with a fluorometer and checking the size using an Agilent Bioanalyzer. If on the Bioanalyzer trace there are two bands, one of expected size and one of higher molecular weight, a portion of your adapter ligated inserts have annealed to each other forming a bubble product. This occurs due to the long adapter length and is more prevalent when there are too many PCR cycles. This type of double band will not affect your sequencing results as the double stranded product will be denatured prior to cluster generation. As an extra verification step, a portion of your product can be denatured manually by heating the sample to 95°C for 5 minutes and then placing it on ice.

Bead Cleanup 3

Step 84.

Proceed to cluster generation or store at -20°C.

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.

• The NEXTflex™ Directional First Strand Synthesis Buffer Mix is yellow in color.

• DTT in buffers may precipitate after freezing. If precipitate is seen, vortex buffer for 1-2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once precipitate is in solution.

• Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.

• Try to maintain a laboratory temperature of 20 – 25°C (68° – 77 °F).
• RNA sample quality may vary between preparations. High quality RNA should have a 28S band that is twice as intense as the 18S band of ribosomal RNA.
• Vortex and micro-centrifuge each component prior to use, to ensure material has not lodged in the cap or the side of the tube.