Small RNA Sequencing using NEXTflex™ Small RNA-Seq Kit v3 from Bioo Scientific

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Abstract

GENERAL INFORMATION

Product Overview

The NEXTflex™ Small RNA-Seq Kit v3 can be used to prepare small RNA libraries from total RNA for Illumina-compatible next-generation sequencing. The NEXTflex Small RNA-Seq Kit v3 is designed to greatly reduce formation of adapter-dimer product in small RNA-seq library preparation, allowing completely gel-free library preparation from typical input amounts, or allowing libraries to be created from low input amounts with a PAGE-based size selection of the final library. This kit utilizes patent-pending adapters with randomized ends to greatly reduce sequence bias in small RNA sequencing library construction (1), allowing more accurate identification and quantification of microRNAs, piRNAs, and other small RNAs.

This manual includes protocols for **size selection using a gel-free (Step H1)** or **PAGE-based (Step H2)** method. *The gel-free protocol described in this manual is recommended for library preparations where a sufficient amount of ~150 bp product and no ~130 bp adapter-dimer product is seen after 16 cycles of PCR*, which is typically achieved when using 1µg of total RNA starting material.

Note:

1) This protocol can be adapted for compatibility with sequencing platforms other than Illumina.

The sequences of the adapters (provided in Figure 1) and PCR primers must be modified for the specific sequencing platform.

2) This protocol is specific for **version 3** of the NEXTflex™ Small RNA-Seq Kit:

Bioo Scientific, Catalog # 5132-05 (8 reactions), or Catalog # 5132-06 (48 reactions).

Modifications to the protocol may be required for updated versions of the kit.

References
1. Jayaprakash, A.D., et al., Identification and remediation of biases in the activity of RNA ligases in small-RNA deep sequencing. Nucleic Acids Res, 2011. 39(21): p. e141.

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Guidelines

1) Start with 1ug of high quality (determined by Bioanalyzer) total RNA.

2) All steps with beads should be done carefully, pipetting up and down until the sample and beads are clearly mixed and the beads are evenly distributed throughout the sample.

3) Ensure that during bead cleanup steps, the ethanol washes are completely removed from the beads.

4) The NEXTflex Small RNA Sequencing Kit v3 protocol requires 1.5-2 days for completion. Approximate times to complete each step and safe Stopping Points are noted in the protocol; however, careful planning and time management are important for efficient and successful small RNA library preparation. If performing the protocol for the first time, we highly recommend preparing a library with the included microRNA control.

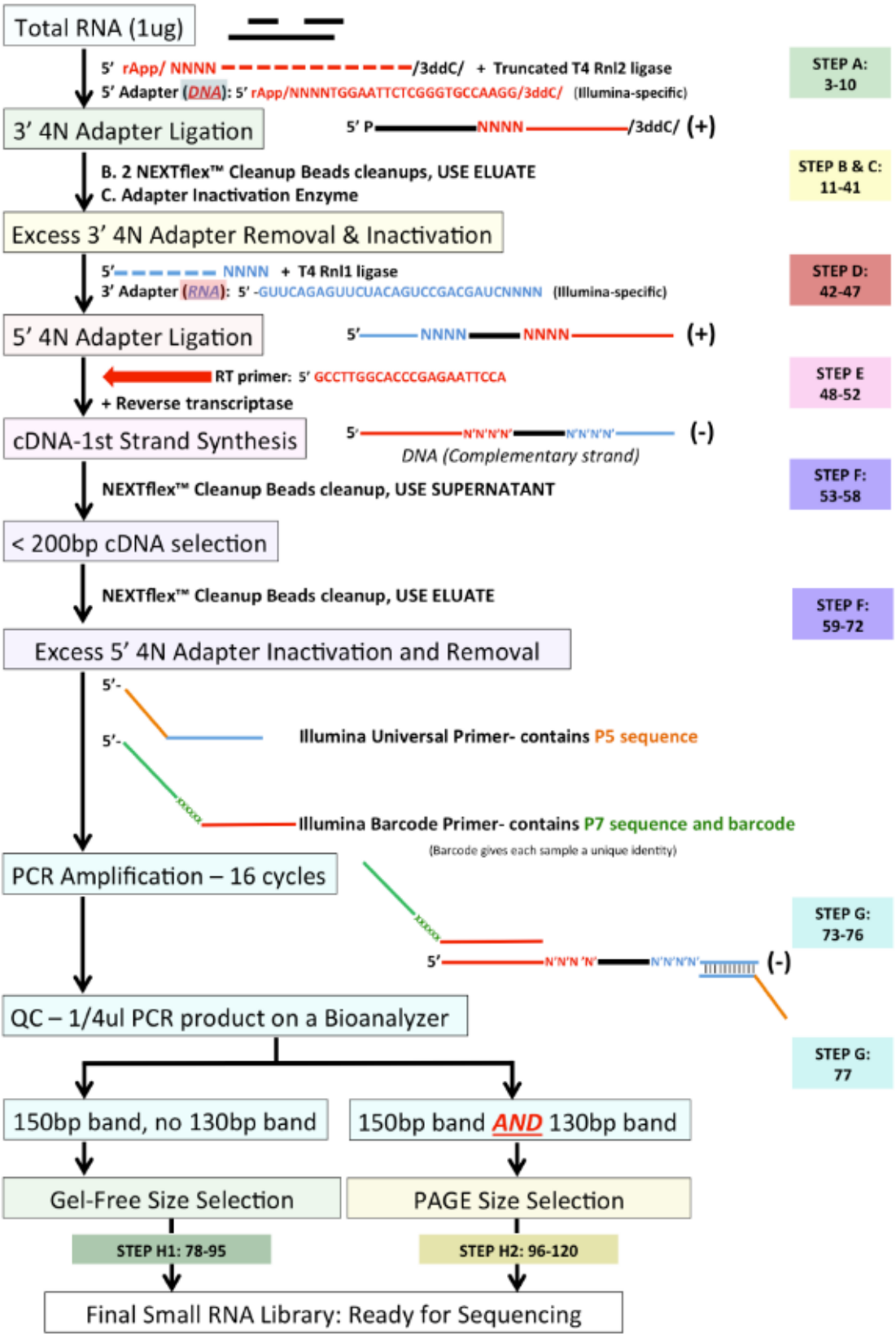
5) There are bead cleanup steps in this protocol in which supernatants must be either be carried over to downstream steps, or in which they are to be discarded. First time users are encouraged to retain all supernatants from bead cleanups in order to troubleshoot failed experiments.

Before start

Determine the concentration and quality of the total RNA sample on an Agilent Bioanalyzer, using Agilent RNA nano or pico kits.

Protocol

Step 1.



FINAL LIBRARY PRODUCT-Double-stranded DNA

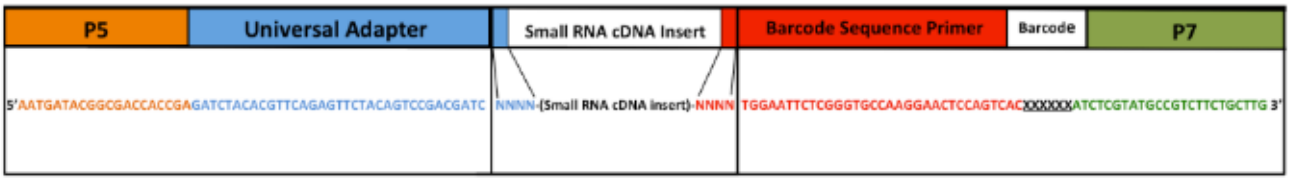


Figure 1: Overview of Small RNA Seq protocol.

Glossary

rAPP- 5' adenylation modification

3ddC- 3' dideoxycytosine

(+) - Sense strand

(-) - Antisense strand

Materials

Step 2.

NEXTflex™ Small RNA-Seq Kit v3 (Illumina® Compatible) (Bioo Scientific, Catalog # 5132-05 (8 reactions),

or Catalog # 5132-06 (48 reactions).

RNA (1µg total RNA) in up to 10.5 µL Nuclease-free Water (**IMPORTANT: *see Note below***).

Thin wall nuclease-free, low binding PCR tubes.

Nuclease-free, low binding microcentrifuge tubes.

Isopropanol, Molecular Biology Grade (Fisher Scientific, Catalog # BP2618-1)

Absolute ethanol (200 proof), Molecular Biology Grade (Fisher Scientific, Catalog # BP2818-4) for making 80% ethanol, freshly prepared each time.

2, 10, 20, 200 and 1000 µL pipettes.

RNase-free pipette tips.

Microcentrifuge.

Magnetic stand for microcentrifuge tubes (Life Technologies DynaMag™-2, Catalog # 12321D) or similar.

Thermocycler.

Heat block.

Vortex.

Ice.

If conducting PAGE Purification of the final library, these additional items will be needed:

Nuclease-free, low binding 1.7 mL microcentrifuge tubes

0.45µm, 2 mL Spin-X Centrifuge tube (Sigma, Catalog # CLS8162).

Sterile disposable pestles (Fisher Scientific, Catalog # K749521-1500) or similar.

6% TBE PAGE gels (1.0 mm) (Life Technologies Cat # EC6265BOX).

1X TBE Buffer.

Electrophoresis power supply.

Nucleic acid stain such as SYBR Gold (Invitrogen).

UV transilluminator or other visualization tool.

Clean razor or scalpel.

NOTES

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.

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 3.

Approximate time to complete: 2.5 hours

Background:

The 3' 4N adenylated adapter is a population of **DNA oligonucleotides** identical to each other but for the **4 nucleotides at the 5' terminal which are degenerate (4N)**. This design ameliorates the inherent base-specific biases of RNA ligase, thus allowing more accurate representation of the bonafide relative quatities of a sample's small RNA species in the final sequencing library.

Click on the following link to access the publication demonstrating the effectiveness of this pooling strategy:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3241666/

The ligase used in this reaction is a truncated form of T4 RNA ligase 2 (Rnl2), which lacks adenylation activity, but maintains its ligase functionality. Also, unlike its full length counterpart, this truncated

ligase cannot ligate the phosphorylated 5' end of DNA or RNA to the 3' end of RNA.

It will ligate only a 5'-end pre-adenylated substrate (DNA or RNA) to the 3' end of an RNA molecule. As such, RNA molecules from the input sample will not be adenylated and thus themselves become substrates for ligation. This prevents circularization or concatenation of input RNA, and specifies the ligation of the adenylated adapter to the 3' end of the input RNA molecules.

Furthermore, the 3' 4N adapters possess at their 3' end a dideoxy nucleotide, thus preventing its ligation to the 5' end of the input RNA, and also to itself.

Figure 2 below shows the final products of this step.

Materials:

Bioo Scientific Supplied

RED CAP - NEXTflex™ 3' 4N Adenylated Adapter, NEXTflex™ 3' Ligation Buffer, NEXTflex™ 3' Ligation Enzyme Mix

WHITE CAP - Nuclease-free Water

User Supplied

RNA (1µg total RNA) in up to 10.5 µL Nuclease-free Water

Nuclease-free, low binding PCR tubes

Thermocycler

Heat block (set to 70°C)

Ice

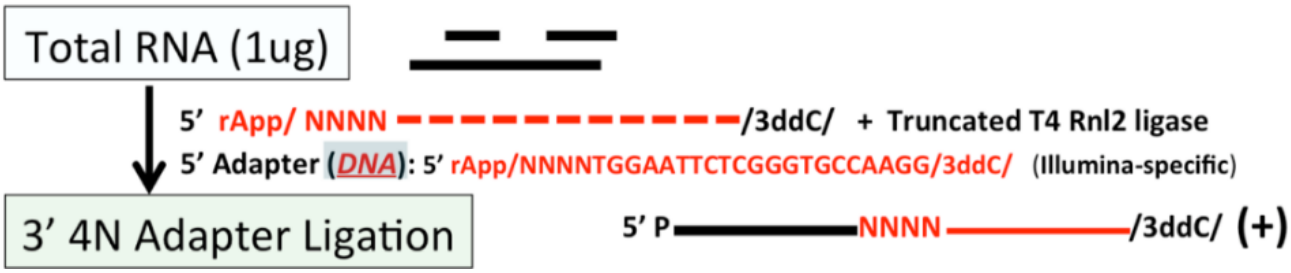


Figure 2: 3' 4N Adenylated Adapter Ligation overview.

rAPP- 5' adenylation modification

3ddC- 3' dideoxycytosine

(+) - Sense strand

NOTES

Do not remove NEXTflex™ 3' Ligation Enzyme Mix from -20°C until immediately before use and return to -20°C immediately after use.

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 4.

For each sample, combine the following reagents on ice in a nuclease-free PCR tube.

_ µL	RNA (1µg)
_ µL	Nuclease-free Water
10.5 µL	TOTAL

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 5.

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

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 6.

Incubate on ice for 5 minutes.

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 7.

Note: Be sure to mix the following reaction until visibly homogenous by pipetting. After mixing, briefly centrifuge to collect contents of tube at the bottom.

For each sample, combine the following reagents on ice in a nuclease-free, low binding PCR tube:

10.5 µL	Denatured RNA (from Step 1)
1 µL	NEXTflex™ 3' 4N Adenylated Adapter
7 µL	NEXTflex™ 3’ Ligation Buffer
1.5 µL	NEXTflex™ 3’ Ligation Enzyme Mix
20 µL	TOTAL

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 8.

Mix thoroughly by pipetting, ensuring that tube contents are homogenous.

Briefly centrifuge tubes to collect liquid at bottom of tube.

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 9.

Incubate at 25°C for 2 hours in a thermocycler with the lid left open.

30 minutes before the end of this incubation, place NEXTflex™ Cleanup Beads (BROWN CAP) at room temperature so that it will be ready for the step in STEP B.

STEP A: NEXTflex™ 3' 4N Adenylated Adapter Ligation

Step 10.

Proceed immediately to Step B: Excess 3' Adapter Removal.

STEP B: Excess 3’ Adapter Removal

Step 11.

Approximate time to complete: 1 - 2 hours

Background:

Unligated adapters that are carried downstream in small RNA workflows pose a particular problem, as they form side products known as adapter dimers that tend to dominate the final sequencing reaction.

Steps B and C in this protocol serve to effectively remove free, unligated adapters, thus eliminating the need for tedious PAGE-based isolation of the final desired library from adapter dimers after PCR amplification of the library.

Figure 3 below displays the workflow for this step.

Materials:

Bioo Scientific Supplied

RED CAP - NEXTflex™ Adapter Depletion Solution

YELLOW CAP - Resuspension Buffer

WHITE CAP - Nuclease-free Water

BROWN CAP - NEXTflex™ Cleanup Beads (equilibrate to room temperature for at least 30 minutes; resuspend just prior to use by vortexing for at least 30 seconds).

User Supplied

Isopropanol

80% Ethanol, freshly prepared

Nuclease-free, low binding microcentrifuge tubes

Magnetic Stand for microcentrifuge tubes

***20 µL of NEXTflex™ 3' 4N Adenylated Adapter Ligated RNA (from Step A)**

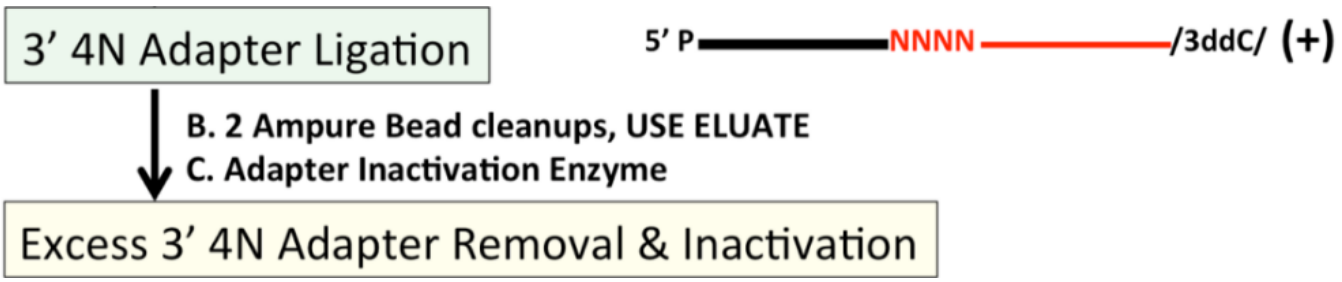


Figure 3: Excess 3’ Adapter Removal and Excess Adapter Inactivation.

3ddC- 3' dideoxycytosine

(+) - Sense strand

STEP B: Excess 3’ Adapter Removal

Step 12.

To each sample, add 25 µL of NEXTflex™ Adapter Depletion Solution and mix well by pipetting.

Ensure that tube contents are homogenous after mixing.

After mixing, transfer entire sample to a new nuclease-free, low binding microcentrifuge tube.

STEP B: Excess 3’ Adapter Removal

Step 13.

Add 40 µL of properly resuspended, room temperature equilibrated NEXTflex™ Cleanup Beads to the sample, and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

NOTES

Thoroughly resuspend NEXTflex™ Cleanup Beads by vortexing for at least 30 seconds, visually ensuring that beads are no longer settled at the bottom of the bottle.

STEP B: Excess 3’ Adapter Removal

Step 14.

Immediately add 60 µL of isopropanol and mix well by pipetting.

Pipette gently and thoroughly until beads are homogenously distributed.

STEP B: Excess 3’ Adapter Removal
Step 15.

Incubate for 5 minutes at room temperature.

STEP B: Excess 3’ Adapter Removal
Step 16.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP B: Excess 3’ Adapter Removal
Step 17.

Remove supernatant and save for recovery in a new tube (in case final small RNA library generation fails. See **'Note 17'** below).

Ensure that all of the supernatant is removed, visually inspecting the tubes.

CONTINUE ONTO STEP 18 OF PROTOCOL WITH THE BEADS IN THE ORIGINAL TUBE.

NOTE 17: Recovery in case of failure to obtain library at end of protocol*.*

Supernatants to be discarded should be saved in low binding tubes and frozen until the end of the protocol as a failsafe in case library generation fails. Potential pitfalls in the protocol include mistakenly taking the wrong fraction in the bead cleanup/size selection procedures to subsequent steps.

To recover sample, thaw saved supernatants on ice. Add 40ul (*for saved supernatants from other downstream steps, be certain to adjust the specifically required volume of beads for those steps*) of resuspended NEXTflex™ Cleanup Beads to a low binding tube, magnetize for 5 minutes, remove supernatant while beads are on the magnet.

Add completely thawed saved supernatants to the beads, resuspend well, and proceed with protocol from step 15 above.

NOTES

Always ensure at steps such as this when supernatants must be removed completely.

Begin aspirating the supernatant from just below its surface and steadily and slowly track the liquid level while aspirating all the way down, making sure to avoid touching the beads.

Additionally, aspirating slowly allows sufficient time for surface tension to drag all the supernatant to the bottom of the tube.

STEP B: Excess 3’ Adapter Removal

Step 18.

While the tube is on the magnet, add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant.

Repeat this step for a total of 2 ethanol washes.

Remove ethanol completely with samples still engaged on the magnet.

NOTES

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

STEP B: Excess 3’ Adapter Removal

Step 19.

Incubate samples for a total of 3 minutes on the magnet.

After the first minute, remove tubes from magnet, briefly centrifuge to collect residual ethanol at bottom of tube.

Replace tubes on the magnet, and incubate for the final 2 minutes.

Once the beads have visually moved to the side of the tube, remove all residual liquid that may have collected at the bottom of the well with a pipette.

STEP B: Excess 3’ Adapter Removal

Step 20.

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

Spin samples down briefly.

STEP B: Excess 3’ Adapter Removal
Step 21.

Incubate for 2 minutes at room temperature.

STEP B: Excess 3’ Adapter Removal
Step 22.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP B: Excess 3’ Adapter Removal
Step 23.

TRANSFER 20ul OF SUPERNATANT TO A NEW MICROCENTRIFUGE TUBE....

DO NOT DISCARD THIS SUPERNATANT!

Use this supernatant in the next step.

STEP B: Excess 3’ Adapter Removal
Step 24.

Add 25 µL of NEXTflex Adapter Depletion Solution to the 20ul of supernatant from previous step, and mix well by pipetting.

STEP B: Excess 3’ Adapter Removal
Step 25.

Add 40 µL of properly resuspended, room temperature equilibrated NEXTflex Cleanup Beads and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

STEP B: Excess 3’ Adapter Removal
Step 26.

Immediately add 60 µL of isopropanol and mix well by pipetting.

Pipette gently and thoroughly until beads are homogenously distributed.

STEP B: Excess 3’ Adapter Removal
Step 27.

Incubate for 5 minutes at room temperature.

STEP B: Excess 3’ Adapter Removal
Step 28.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP B: Excess 3’ Adapter Removal
Step 29.

Remove supernatant and save for recovery in a new tube (in case final small RNA library generation fails. See **'Note 17'** in STEP B-17 above). For recovery, restart procotol at Step 27 after adding thawed saved supernatants to beads.

Ensure that all of the supernatant is removed, visually inspecting the tubes.

CONTINUE ONTO STEP 30 OF PROTOCOL WITH THE BEADS IN THE ORIGINAL TUBE.

STEP B: Excess 3’ Adapter Removal
Step 30.

While the tube is on the magnet, add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant.

Repeat this step for a total of 2 ethanol washes.

Remove ethanol completely with samples still engaged on the magnet.

NOTES

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

STEP B: Excess 3’ Adapter Removal
Step 31.

Incubate samples for a total of 3 minutes on the magnet.

After the first minute, remove tubes from magnet, briefly centrifuge to collect residual ethanol at bottom of tube.

Replace tubes on the magnet, and incubate for the final 2 minutes.

Once the beads have visually moved to the side of the tube, remove all residual liquid that may have collected at the bottom of the well with a pipette.

STEP B: Excess 3’ Adapter Removal

Step 32.

Remove tubes from magnetic stand and resuspend bead pellet in 13 µL of Nuclease-free water by pipetting. Ensure that beads are completely resuspended.

STEP B: Excess 3’ Adapter Removal

Step 33.

Incubate for 2 minutes at room temperature.

STEP B: Excess 3’ Adapter Removal

Step 34.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP B: Excess 3’ Adapter Removal

Step 35.

Transfer 11.5ul of supernatant to a new low binding PCR tube.

STEP B: Excess 3’ Adapter Removal

Step 36.

Proceed immediately to Step C: Excess Adapter Inactivation.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step C: Excess Adapter Inactivation.

STEP C: Excess Adapter Inactivation

Step 37.

Approximate time to complete: 45 minutes

Materials:

Bioo Scientific Supplied

PINK CAP - NEXTflex™ Adapter Inactivation Buffer, NEXTflex™ Adapter Inactivation Enzyme

User Supplied

Nuclease-free, low binding PCR tubes

Thermocycler

Ice

***11.5 µL of Purified NEXTflex™ 3' 4N Adenylated Adapter Ligated RNA (from Step B)**

STEP C: Excess Adapter Inactivation

Step 38.

For each sample, combine the following reagents on ice in a Nuclease-free, low binding PCR tube.

11.5 µL	Purified NEXTflex™ 3' 4N Adenylated Adapter Ligated RNA (from Step B)
2.0 µL	NEXTflex™ Adapter Inactivation Buffer
0.5 µL	NEXTflex™ Adapter Inactivation Enzyme
14 µL	TOTAL

STEP C: Excess Adapter Inactivation

Step 39.

Mix thoroughly by pipetting, ensuring that tube contents are homogenous.

Briefly centrifuge tubes to collect liquid at bottom of tube.

STEP C: Excess Adapter Inactivation

Step 40.

Incubate as follows with lid set to 60°C, but left open on thermocycler (see note below):

15 min	12°C
20 min	50°C
hold	4°C

NOTES

- Set thermocycler lid to 60°C, but leave it in the open position during the 12°C incubation, and place it on for the 50°C incubation.

  Keep samples on ice until PCR block is at 12°C, then transfer the tubes to the thermocycler.

STEP C: Excess Adapter Inactivation

Step 41.

Proceed immediately to Step D: NEXTflex 5' 4N Adapter Ligation.

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 42.

Approximate time to complete: 1.5 hours

Background:

The 5' adapter is an RNA oligonucleotide whose complementary sequence will base pair to the NEXTflex Universal Primer (used in Step G).

As is the case for the 3' adenylated adapter, the 5' adapter also contains a 4 nucleotide region of degeneracy, but at the 3' end of the oligonucleotide, which will form the junction with the 5' end of the RNA species in the reaction.

T4 RNA ligase 1 (Rnl1) in a buffer containing ATP is used to ligate the adapter to the 5' end of the purified 3' 4N adenylated adapter ligation products from STEP A.

Figure 4 below displays the workflow and the final products of this step.

Materials:

Bioo Scientific Supplied

LIGHT PURPLE CAP - NEXTflex™ 5' 4N Adapter, NEXTflex™ 5' Ligation Buffer, NEXTflex™ Ligation Enzyme Mix

User Supplied

Nuclease-free, low binding PCR tubes

Thermocycler

Heat block (set to 70°C)

Ice

***14 µL of Purified NEXTflex™ 3' 4N Adenylated Adapter Ligated RNA (from Step C)**

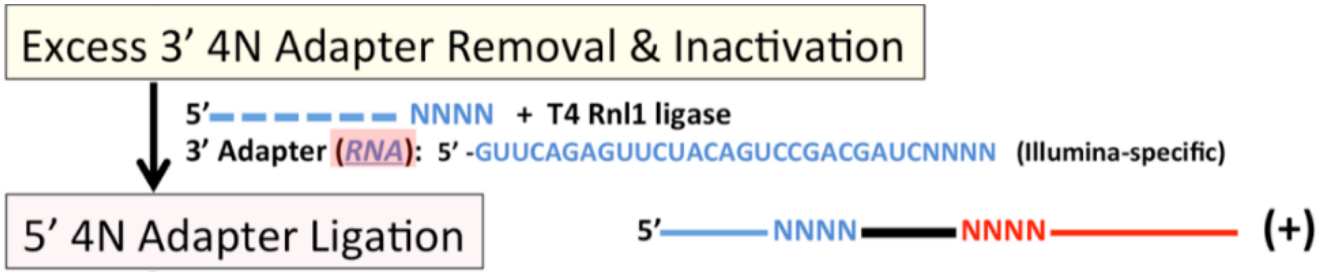


Figure 4: 5' 4N Adapter Ligation overview.

(+) - Sense strand

NOTES

Do not remove NEXTflex™ NEXTflex™ 5' Ligation Enzyme Mix from -20°C until immediately before use and return to -20°C immediately after use.

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 43.

Heat 1.5 uL of NEXTflex 5' 4N adapter per reaction at 70°C for 2 minutes in heat block, then immediately place on ice.

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 44.

Note: Be sure to mix the following reaction until visibly homogenous by pipetting or brief vortexing. For each sample, combine the following reagents on ice in a nuclease-free, low binding PCR tube:

14 µL Purified NEXTflex™ 3' 4N Adenylated Adapter Ligated RNA (from Step C)
1.5 µLNEXTflex 5' 4N Adapter
7.5 µLNEXTflex™ 5’ Ligation Buffer
2 µL NEXTflex™ 5’ Ligation Enzyme Mix
25 µL TOTAL

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 45.

Mix thoroughly by pipetting, ensuring that the tube contents are homogenous.

Briefly centrifuge tubes to collect liquid at bottom of the tube.

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 46.

Incubate at 20°C for 1 hour in a thermocycler with the lid left open.

STEP D: NEXTflex™ 5' 4N Adapter Ligation

Step 47.

Proceed immediately to Step E: Reverse Transcription - First Strand Synthesis.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step E: Reverse Transcription - First Strand Synthesis.

STEP E: Reverse Transcription-First Strand Synthesis

Step 48.

Approximate time to complete: 1 hour

Background:

The successfully ligated RNAs have (as shown in diagram below), the 5' and 3' adapters attached. The RT primer is complementary to the 3' adapter, and serves as a primer for reverse transcriptase to create the 1st strand cDNA.

Figure 5 below displays the details of the reaction in this step.

Materials:

Bioo Scientific Supplied

BLUE CAP - NEXTflex™ RT Buffer, M-MuLV Reverse Transcriptase

User Supplied

Nuclease-free, low binding PCR tubes

Thermocycler

Ice

***25 µL of 5’ and 3’ NEXTflex™ Adapter Ligated RNA (from Step D)**

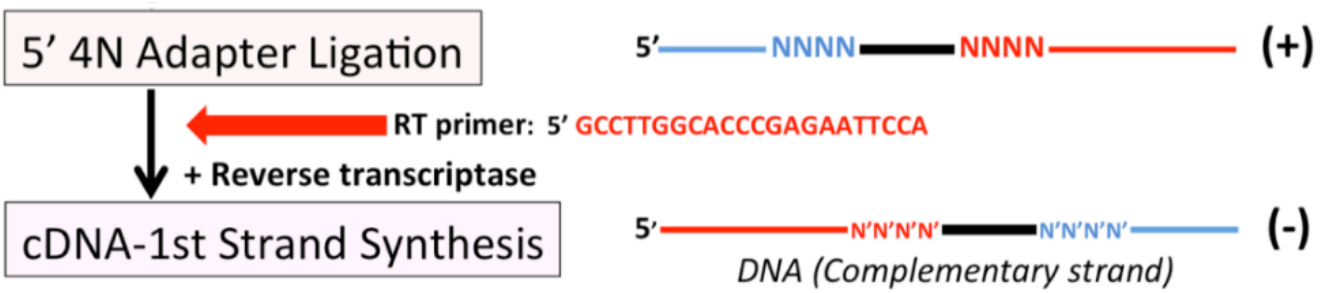


Figure 5: Reverse transcription of the successfully ligated RNA inserts by a primer complementary to the 3' 4N adapter.

(+) - Sense strand

(-) - Antisense strand

STEP E: Reverse Transcription-First Strand Synthesis

Step 49.

For each sample, combine the following reagents on ice in a Nuclease-free, low binding PCR tube:

25 µL5' and 3' NEXTflex™ Adapter Ligated RNA (from Step D)
13 µLNEXTflex™ RT Buffer
2 µL M-MuLV Reverse Transcriptase
40 µLTOTAL

STEP E: Reverse Transcription-First Strand Synthesis

Step 50.

Mix thoroughly by pipetting, ensuring that the tube contents are homogenous.

Briefly centrifuge tubes to collect liquid at bottom of the tube.

STEP E: Reverse Transcription-First Strand Synthesis

Step 51.

Incubate as follows on thermocycler with lid temperature of 100°C.

30 min 42°C

10 min 90°C

STEP E: Reverse Transcription-First Strand Synthesis

Step 52.

Spin tubes down to collect any droplets that may have condensed on lids, and proceed immediately to Step F: Bead Cleanup.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at 4°C or up to one week at -20°C. To restart, thaw frozen samples on ice before proceeding to Step F: Bead Cleanup.

STEP F: Bead Cleanup

Step 53.

Approximate time to complete: 30 - 45 minutes

Figure 6 below displays an overview of these steps.

Materials:

Bioo Scientific Supplied

RED CAP - NEXTflex™ Adapter Depletion Solution

BROWN CAP - NEXTflex™ Cleanup Beads (equilibrate to room temperature for at least 30 minutes; resuspend just prior to use by vortexing for at least 30 seconds).

WHITE CAP - Nuclease-Free Water

User Supplied

Isopropanol

80% Ethanol, freshly prepared

Magnetic Stand

Nuclease-free, low binding microcentrifuge tubes

***40 µL of First Strand Synthesis product (from Step E)**

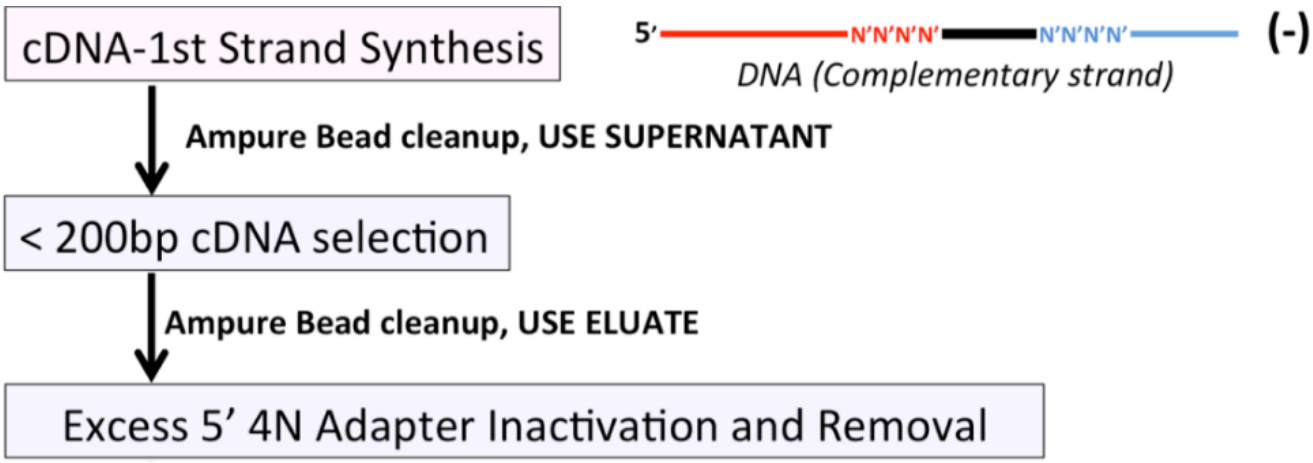


Figure 6: Excess 5' 4N Adapter Inactivation and Removal.

(-) - Antisense strand

STEP F: Bead Cleanup

Step 54.

To each sample, add 20 µL of NEXTflex™ Cleanup Beads and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

Transfer entire sample to a low binding microcentrifuge tube.

NOTES

Use beads that have been equilibrated to room temperature for at least 30 minutes; resuspend just prior to use by vortexing for at least 30 seconds).

STEP F: Bead Cleanup

Step 55.

Add 22 µL isopropanol and mix well by pipetting.

Pipette gently and thoroughly until beads are homogenously distributed.

STEP F: Bead Cleanup

Step 56.

Incubate for 5 minutes at room temperature.

STEP F: Bead Cleanup

Step 57.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP F: Bead Cleanup

Step 58.

Transfer 70 µL of supernatant to a new low binding microcentrifuge tube.

DO NOT DISCARD SUPERNATANT: this solution contains the cDNA product. Take care to not transfer beads along with clear supernatant.

STEP F: Bead Cleanup

Step 59.

Ensure that new tubes are removed from magnetic stand.

STEP F: Bead Cleanup

Step 60.

Add 10 µL NEXTflex™ Adapter Depletion Solution and mix well by pipetting.

Pipette gently and thoroughly, until solution appears homogenous.

STEP F: Bead Cleanup

Step 61.

Add 20 µL of NEXTflex Cleanup Beads and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

NOTES

Resuspend room temperature beads just prior to use by vortexing for at least 30 seconds.

STEP F: Bead Cleanup

Step 62.

Add 68 µL of isopropanol and mix well by pipetting.

Pipette gently and thoroughly until beads are homogenously distributed.

STEP F: Bead Cleanup

Step 63.

Incubate for 5 minutes at room temperature.

STEP F: Bead Cleanup

Step 64.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP F: Bead Cleanup

Step 65.

Remove supernatant and save for recovery in a new tube (in case final small RNA library generation fails. See **'Note 17'** in STEP B-17 above).

In case recovery of the saved supernatant from this step (65) is required, ensure that NEXTflex™ Cleanup Bead volume is 20ul (and not 40ul as mentioned in Note 17). Restart procotol at Step 63 after adding thawed saved supernatants to beads.

Ensure that all of the supernatant is removed, visually inspecting the tubes.

CONTINUE ONTO STEP 66 OF PROTOCOL WITH THE BEADS IN THE ORIGINAL TUBE.

STEP F: Bead Cleanup

Step 66.

While the tube is on the magnet, add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant.

Repeat this step for a total of 2 ethanol washes.

Remove ethanol completely with samples still engaged on the magnet.

NOTES

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

STEP F: Bead Cleanup

Step 67.

Incubate samples for a total of 3 minutes on the magnet.

After the first minute, remove tubes from magnet, briefly centrifuge to collect residual ethanol at bottom of tube.

Replace tubes on the magnet, and incubate for the final 2 minutes.

Once the beads have visually moved to the side of the tube, remove all residual liquid that may have collected at the bottom of the well with a pipette.

STEP F: Bead Cleanup

Step 68.

Remove sample from magnetic stand and resuspend bead pellet in 20 µL Nuclease-free Water by pipetting volume up and down. Ensure that beads are completely resuspended.

STEP F: Bead Cleanup

Step 69.

Incubate for 2 minutes at room temperature.

STEP F: Bead Cleanup

Step 70.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP F: Bead Cleanup

Step 71.

Transfer 18 µL of supernatant to a new low binding PCR tube.

STEP F: Bead Cleanup

Step 72.

Proceed immediately to Step G: PCR Amplification.

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored overnight at -20°C. To restart, thaw frozen samples on ice before proceeding to Step G: PCR Amplification.

STEP G: PCR Amplification

Step 73.

Approximate time to complete: 40 - 60 minutes

Figure 7 below displays the details of the reaction in this step.

Materials:

Bioo Scientific Supplied

GREEN CAP - NEXTflex™ Barcode Primers, NEXTflex™ Universal Primer, NEXTflex™ Small RNA PCR Master Mix

User Supplied

Low binding PCR tubes

Thermocycler

Ice

***18 µL Purified First Strand Synthesis Product (from Step F)**

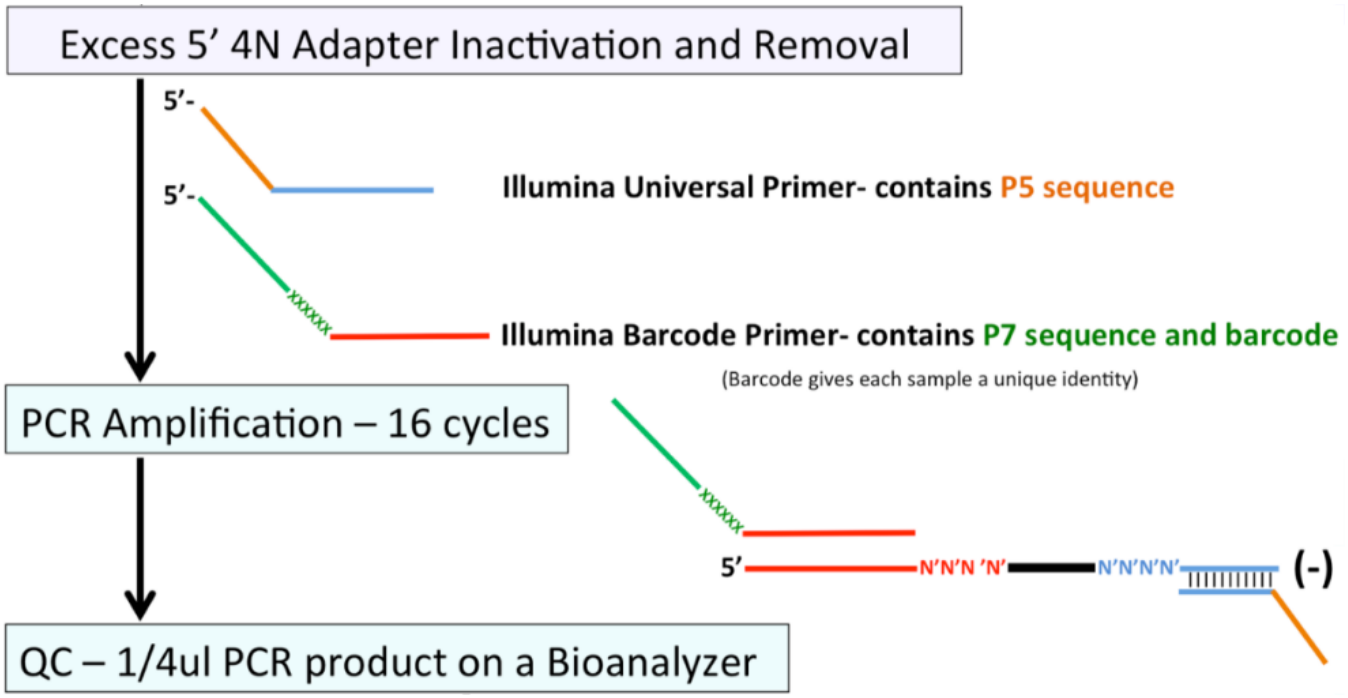


Figure 7: PCR amplification.

(-) - Antisense strand

STEP G: PCR Amplification

Step 74.

For each sample, combine the following reagents on ice in a nuclease-free low bind PCR tubes:

18 µL	Purified First Strand Synthesis Product (From Step F)
1 µL	NEXTflex™ Universal Primer
1 µL	NEXTflex™ Barcoded Primer (a different barcoded primer should be used for each sample that will be multiplexed for sequencing)
5 µL	NEXTflex™ Small RNA PCR Master Mix
25 µL	TOTAL

STEP G: PCR Amplification

Step 75.

Mix thoroughly by pipetting, ensuring that tube contents are homogenous.

Briefly centrifuge tubes to collect liquid at bottom of tube.

STEP G: PCR Amplification

Step 76.

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

2 min	95°C	
20 sec	95°C	16 cycles.
30 sec	60°C	
15 sec	72°C	
2 min	72°C	

STEP G: PCR Amplification

Step 77.

Following PCR, products may be analyzed by Agilent Bioanalyzer HS DNA Assay, TBE-PAGE gel, or similar.

For analysis by Bioanalyzer, we recommend running 1 µL of PCR product diluted 1/4 with nuclease-free water. The Bioanalyzer software may not correctly identify the peak sizes, so it is recommended to also run a library created with miRNA control to help identify the 150 bp peak. Presence of a strong 150 bp band indicates a successful library preparation, and absence of a band 130 bp indicates that gel-free size selection may be used. See Figure 8 below for examples of traces that indicate samples that are amenable to gel-free size selection (A), or that require PAGE-based size selection (B)

Note: A peak of 65 bp may be present. This peak represents excess PCR primer and will not negatively affect downstream sequencing or quantification by Qubit dsDNA HS Assay.

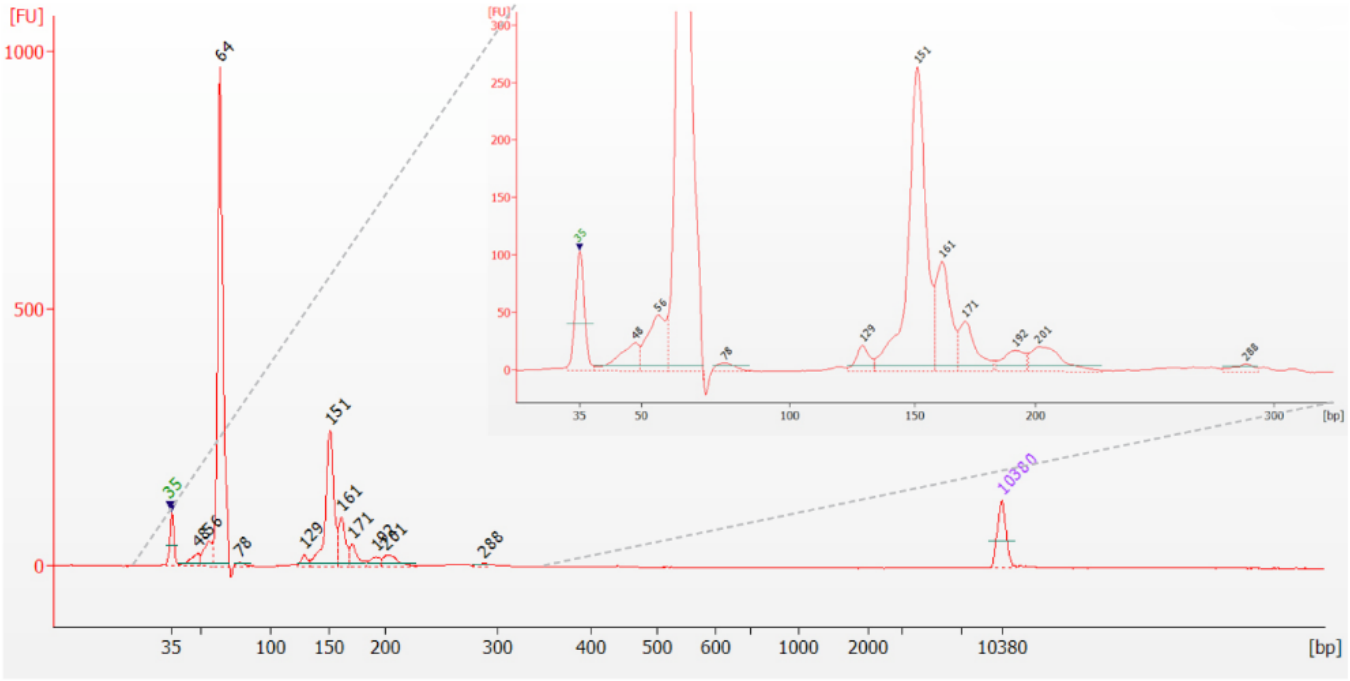


Figure 8(A): Sample Bioanalyzer HS DNA images of samples that could be size selected with option H1: Gel-Free Size Selection Cleanup

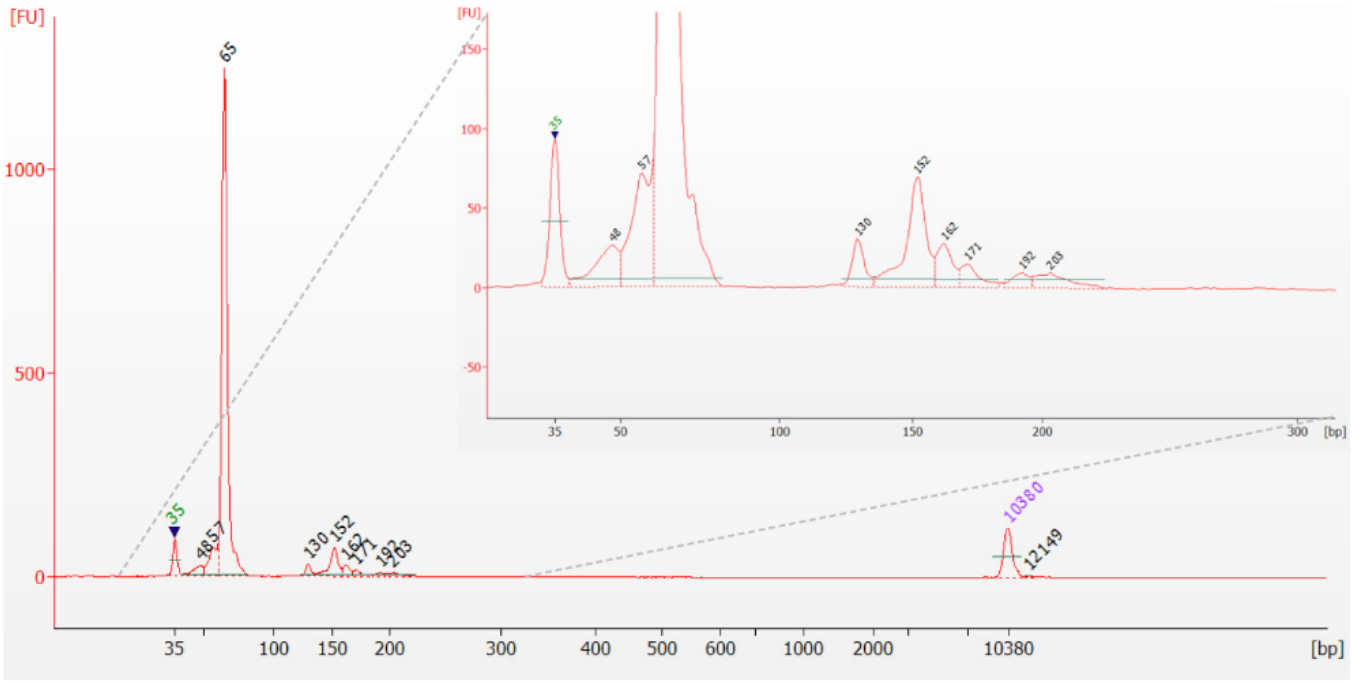


Figure 8(B): Sample Bioanalyzer HS DNA images of samples that could be size selected with H2:

PAGE Size Selection and Cleanup.

For analysis by PAGE gel, we recommend mixing 5 µL of PCR product with 1 µL of 6x Loading Dye and running on a 6% TBE-PAGE gel alongside 5 µL of Ready to Load Low Molecular Weight Ladder, and staining with SYBR Gold.

If an 150bp product is present, **and NO detectable 130bp product (adapter dimer)**, proceed immediately to Step H1: Gel-Free Size Selection Cleanup.

If an 150bp product is present, **and there is an 130bp product (adapter dimer)**, proceed immediately to Step H2: Gel Electrophoresis & Nucleic Acid Elution and Purification.

See Figure 9 below for examples of PAGE gel images that indicate samples that are amenable to gel-free size selection (A), or that require PAGE-based size selection (B).

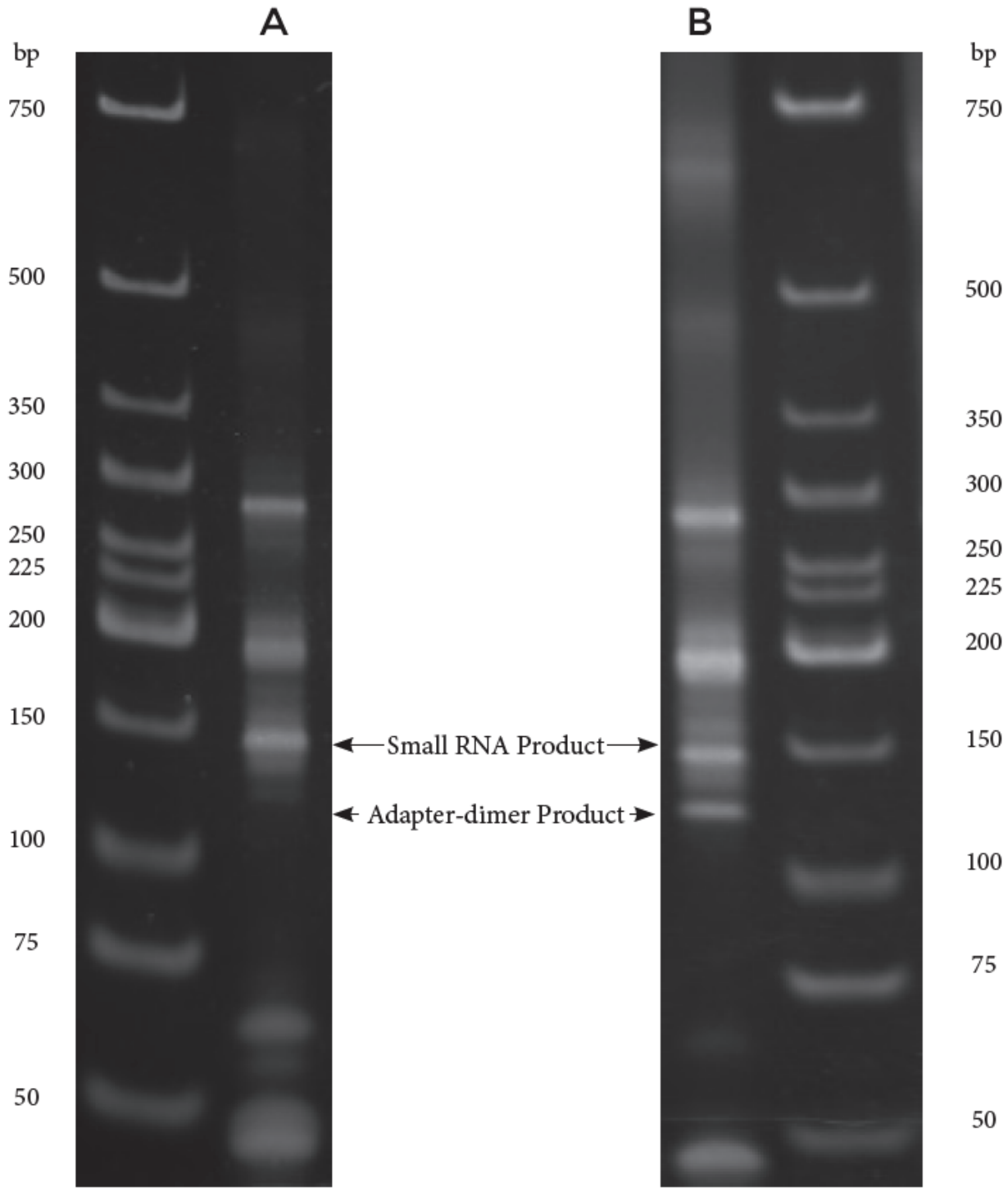


Figure 9: Sample PAGE images of samples that could be size selected with option H1: Gel-Free Size Selection Cleanup (A) or H2: PAGE Size Selection and Cleanup (B).

STOPPING POINT: Alternatively, the procedure may be stopped at this point with samples stored up to one week at -20°C. To restart, thaw frozen samples on ice before proceeding to Step H1: Gel-Free Size Selection Cleanup or Step H2: Gel Electrophoresis & Nucleic Acid Elution and Purification.

STEP H1: Gel-Free Size Selection & Cleanup

Step 78.

Approximate time to complete: 45 minutes

Figure 10 below displays an overview of this section.

Materials:

Bioo Scientific Supplied

WHITE CAP - Nuclease-Free Water

YELLOW CAP - Resuspension Buffer

BROWN CAP - NEXTflex Cleanup Beads (equilibrate to room temperature for at least 30 minutes; resuspend just prior to use by vortexing for at least 30 seconds)

User Supplied

80% Ethanol, freshly prepared

Nuclease-free, low binding microcentrifuge tube

Magnetic Stand

***25 µL of PCR Product (from Step G)**

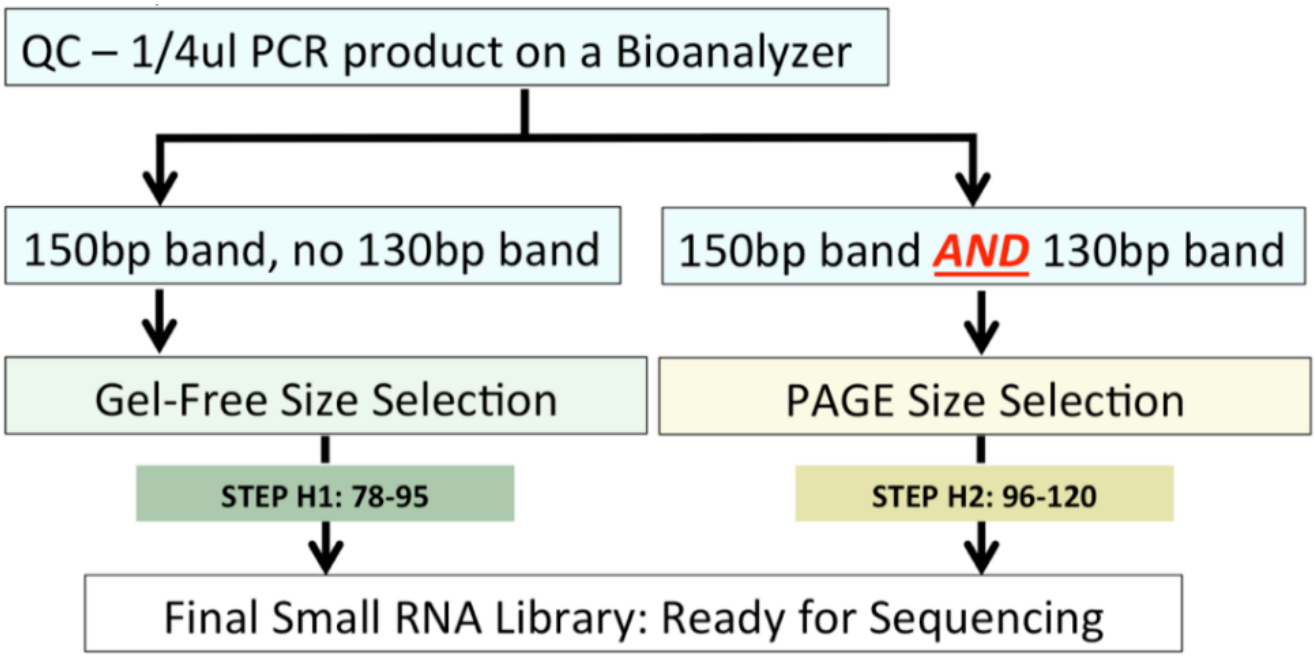


Figure 10: Overview of QC and size selection approaches.

STEP H1: Gel-Free Size Selection & Cleanup

Step 79.

Ensure the volume of all samples is 25 µL. If less, add Nuclease-free Water to bring the entire volume up to 25 uL.

NOTES

Ensuring that the volume of the PCR reaction is 25ul is critical, as bead-based size selection is dependent on the specific ratio of cleanup beads to sample.

STEP H1: Gel-Free Size Selection & Cleanup

Step 80.

Add 32.5 µL of properly resuspended, room temperature equilibrated NEXTflex™ Cleanup Beads and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

STEP H1: Gel-Free Size Selection & Cleanup

Step 81.

Incubate for 5 minutes at room temperature.

STEP H1: Gel-Free Size Selection & Cleanup

Step 82.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP H1: Gel-Free Size Selection & Cleanup

Step 83.

Transfer 52.5 µL of supernatant to a new nuclease-free, low binding microcentrifuge tube.

DO NOT DISCARD SUPERNATANT, this solution contains the amplified product. Take care to not transfer beads along with clear supernatant.

STEP H1: Gel-Free Size Selection & Cleanup

Step 84.

Ensure that new microcentrifuge tubes with supernatant from previous step are not on the magnet.

STEP H1: Gel-Free Size Selection & Cleanup

Step 85.

Add 30 µL of properly resuspended, room temperature equilibrated NEXTflex Cleanup Beads to each supernatant from previous step, and mix well by pipetting.

Pipette gently and thoroughly, until beads are homogenously distributed.

STEP H1: Gel-Free Size Selection & Cleanup

Step 86.

Incubate for 5 minutes at room temperature.

STEP H1: Gel-Free Size Selection & Cleanup

Step 87.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP H1: Gel-Free Size Selection & Cleanup

Step 88.

Remove supernatant and save for recovery in a new tube (in case final small RNA library generation fails. See **'Note 17'** in STEP B-17 above).

In case recovery of the saved supernatant from this step (88) is required, ensure that NEXTflex™ Cleanup Bead volume is 30ul (and not 40ul as mentioned in Note 17). Restart procotol at Step 86 after adding thawed saved supernatants to beads.

Be careful to dispose of all the liquid, without disturbing the beads.

CONTINUE ONTO STEP 89 OF PROTOCOL WITH THE BEADS IN THE ORIGINAL TUBE.

STEP H1: Gel-Free Size Selection & Cleanup

Step 89.

While the tube is on the magnet, add 180 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant.

Repeat this step for a total of 2 ethanol washes.

Remove ethanol completely with samples still engaged on the magnet.

NOTES

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

STEP H1: Gel-Free Size Selection & Cleanup

Step 90.

Incubate samples for a total of 3 minutes on the magnet.

After the first minute, remove tubes from magnet, briefly centrifuge to collect residual ethanol at bottom of tube.

Replace tubes on the magnet, and incubate for the final 2 minutes.

Once the beads have visually moved to the side of the tube, remove all residual liquid that may have collected at the bottom of the well with a pipette.

STEP H1: Gel-Free Size Selection & Cleanup

Step 91.

Remove samples from magnetic stand and resuspend bead pellet in 13.5 µL of Resuspension Buffer by pipetting volume up and down. Ensure that beads are completely resuspended.

STEP H1: Gel-Free Size Selection & Cleanup

Step 92.

Incubate for 2 minutes at room temperature.

STEP H1: Gel-Free Size Selection & Cleanup

Step 93.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP H1: Gel-Free Size Selection & Cleanup

Step 94.

Transfer 12 µL of supernatant to a new low binding microcentrifuge tube.

This is your sequencing library.

STEP H1: Gel-Free Size Selection & Cleanup

Step 95.

Check the size distribution of the final library by Bioanalyzer High Sensitivity DNA Assay (Agilent) and the concentration by Qubit dsDNA HS Assay (Life Technologies).

Figure 11(A) below shows an example of a successfully prepared small RNA library.

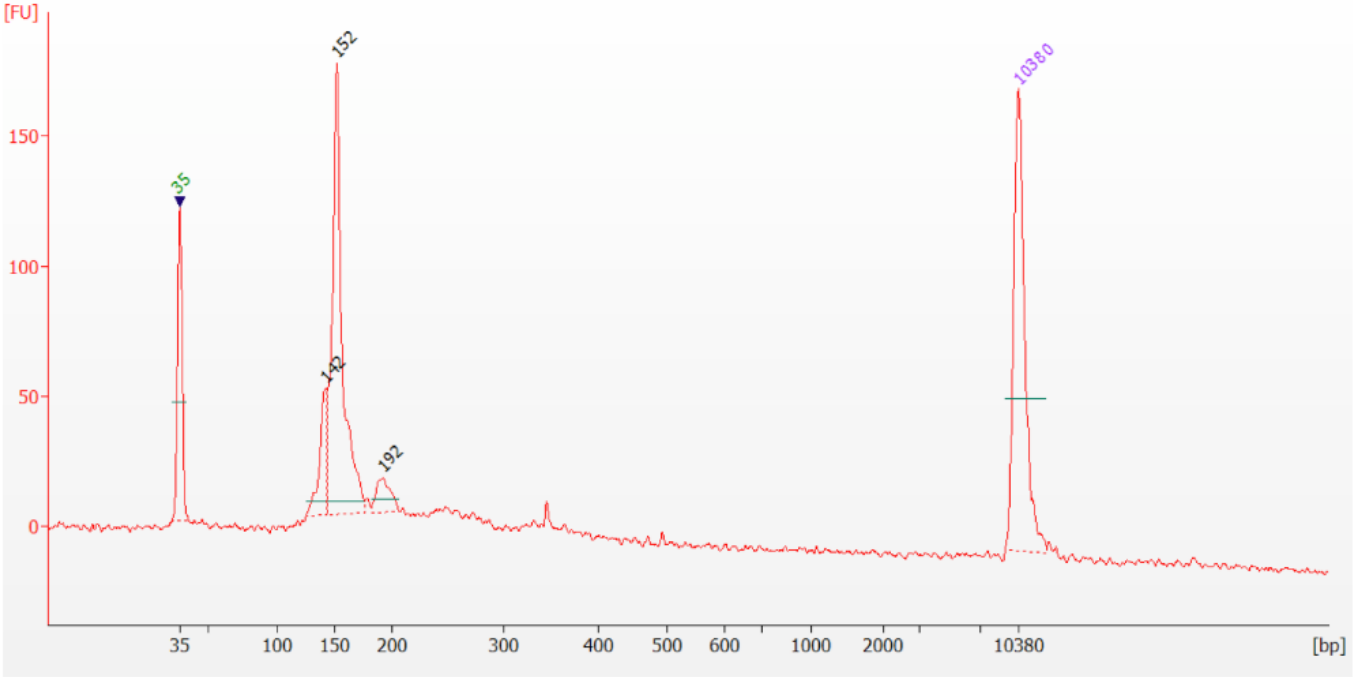


Figure 11(A): Sample Bioanalyzer HS DNA traces from libraries created from MCF-7 total RNA using gel-free protocol. *Occasionally, a peak of 65 bp will be seen. This peak represents excess PCR primer and will not negatively affect sequencing or quantification by Qubit dsDNA HS Assay.*

(If significant high molecular weight products remain (see Figure 11(B) below for an example trace of a library that would need to be subjected to a second round of size-selection), bring total sample volume to 25 µL with nuclease free water and repeat Step H1).

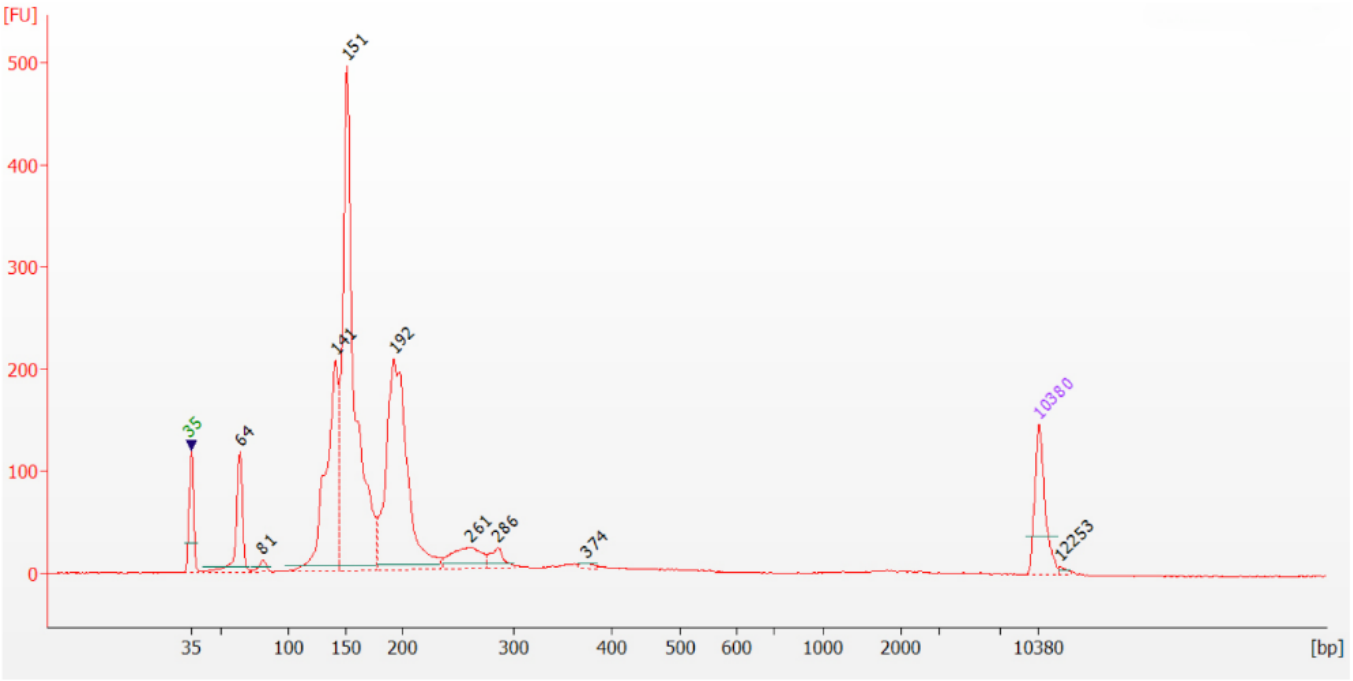


Figure 11(B): Sample Bioanalyzer HS DNA trace of a library that would benefit from repeating Step H1: Gel-Free Size Selection Cleanup. *Elimination of the products ≥180 bp will result in a greater proportion of reads representing small RNAs. Note that repeating step H1 will result in some overall loss.*

After second Gel-Free Size Selection, if library traces reflect successful removal of high molecular weight species, proceed to cluster generation for sequencing as per manufacturer's protocol.

STEP H2: PAGE Size Selection & Cleanup

Step 96.

Note: This option is recommended if an 150bp product is present, **and also** an 130bp adapter dimer after analyzing the PCR products from STEP G (see Figure 8(B) and 9(B).

Note: This step can be replaced with selection by Pippin Prep or Blue Pippin (See Resources tab at http://www.biooscientific.com/SmallRNA)

Approximate time to complete: 3 hours - overnight

Materials:

Bioo Scientific Supplied

YELLOW CAP - Resuspension Buffer

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

CLEAR CAP BOTTLE - NEXTflex™ Elution Buffer

BROWN CAP - NEXTflex™ Cleanup Beads

User Supplied

Isopropanol

80% Ethanol

Nuclease-free, low binding 1.7 mL microcentrifuge tubes

Spin-X Centrifuge tube (Sigma)

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

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

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

***25 µL of PCR Product (from Step G)**

STEP H2: PAGE Size Selection & Cleanup

Step 97.

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

STEP H2: PAGE Size Selection & Cleanup

Step 98.

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.

STEP H2: PAGE Size Selection & Cleanup

Step 99.

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

STEP H2: PAGE Size Selection & Cleanup

Step 100.

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.

STEP H2: PAGE Size Selection & Cleanup

Step 101.

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

STEP H2: PAGE Size Selection & Cleanup

Step 102.

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

STEP H2: PAGE Size Selection & Cleanup

Step 103.

Using a clean razor, cut out the 150 bp band and place into clean 1.7 mL nuclease-free, low binding microcentrifuge tube. Do not cut out the 130 bp band; this is adapter dimer product. The ladder band at 200 bp is twice as intense as the other bands and can be used for orientation.

STEP H2: PAGE Size Selection & Cleanup

Step 104.

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

STEP H2: PAGE Size Selection & Cleanup

Step 105.

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

STEP H2: PAGE Size Selection & Cleanup

Step 106.

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

STEP H2: PAGE Size Selection & Cleanup

Step 107.

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

Ensure that NEXTflex Cleanup Beads are placed at room temperature at least 30 minutes before the end of this incubation.

STEP H2: PAGE Size Selection & Cleanup

Step 108.

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

STEP H2: PAGE Size Selection & Cleanup

Step 109.

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 for transfers of larger gel pieces. Centrifuge the Spin-X tube at 16,000 x g for 2 minutes. Dispose of the spin filter.

STEP H2: PAGE Size Selection & Cleanup

Step 110.

Ensure that NEXTflex Cleanup Beads are equilibrated to room temperature for at least 30 minutes prior to this step, and that the beads are thoroughly resuspended by vortexing for 30 seconds, with visual confirmation of bead resuspension.

Add to each tube and mix well (*Pipette gently and thoroughly, until beads are homogenously distributed)*:

NEXTflex Cleanup Beads	50 µL
Isopropanol	350 µL

STEP H2: PAGE Size Selection & Cleanup

Step 111.

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

STEP H2: PAGE Size Selection & Cleanup

Step 112.

Pulse spin tubes to collect solution from walls and lid of tube and to pellet beads.

STEP H2: PAGE Size Selection & Cleanup
Step 113.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP H2: PAGE Size Selection & Cleanup
Step 114.

Remove supernatant and save for recovery in a new tube (in case final small RNA library generation fails. See **'Note 17'** in STEP B-17 above).

In case recovery of the saved supernatant from this step (114) is required, ensure that NEXTflex™ Cleanup Bead volume is 50ul (and not 40ul as mentioned in Note 17). Restart procotol at Step 111 after adding thawed saved supernatants to beads.

Be careful to dispose of all the liquid, without disturbing the beads.

CONTINUE ONTO STEP 115 OF PROTOCOL WITH THE BEADS IN THE ORIGINAL TUBE.

STEP H2: PAGE Size Selection & Cleanup
Step 115.

Add 950 µL 80% ethanol, incubate for 30 seconds, then remove all of the supernatant. Repeat this step for a total of two ethanol washes.

NOTES

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

STEP H2: PAGE Size Selection & Cleanup
Step 116.

Incubate samples for a total of 3 minutes on the magnet.

After the first minute, remove tubes from magnet, briefly centrifuge to collect residual ethanol at bottom of tube.

Replace tubes on the magnet, and incubate for the final 2 minutes.

Once the beads have visually moved to the side of the tube, remove all residual liquid that may have

collected at the bottom of the well with a pipette.

STEP H2: PAGE Size Selection & Cleanup
Step 117.

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

STEP H2: PAGE Size Selection & Cleanup
Step 118.

Incubate for 2 minutes at room temperature.

STEP H2: PAGE Size Selection & Cleanup
Step 119.

Magnetize samples until solution appears clear (times can range from 2-5 minutes for the clearing of the solution).

STEP H2: PAGE Size Selection & Cleanup
Step 120.

Transfer 12 µL of supernatant to a clean nuclease-free, low binding microcentrifuge tube. This is your sequencing library.

STEP H2: PAGE Size Selection & Cleanup
Step 121.

Check the size distribution of the final library by Bioanalyzer High Sensitivity DNA Assay (Agilent) and the concentration by Qubit dsDNA HS Assay (Life Technologies).

Proceed to cluster generation for sequencing as per manufacturer's protocol.