USER GUIDE

Trio RNA-Seq

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I. Introduction

A. Background

Trio RNA-Seq provides an end-to-end solution for whole transcriptome RNA-Seq library construction using as little as 500 pg of total RNA. Trio RNA-Seq is powered by three unique technologies: Ribo-SPIA for robust amplification of cDNA from low-input samples, highly efficient DimerFree library preparation for minimal adaptor dimers, and AnyDeplete (formerly InDA-C) for customizable, targeted depletion of abundant, unwanted sequences such as rRNA and globin. Together these technologies offer high-sensitivity whole-transcriptome library preparation with maximal sequencing efficiency.

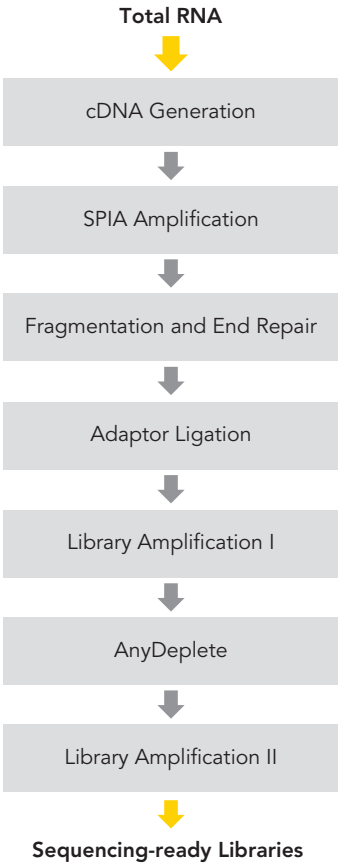
Trio RNA-Seq is available in 8, 32 or 96 reactions and includes 8, 32 or 96 barcodes, respectively, for multiplex sequencing. AnyDeplete probes are available off-the-shelf for human rRNA and mouse rRNA+globin, or can be customized to target any transcript.

B. Workflow

Beginning with total RNA, amplified cDNA is generated with priming at the 3' end as well as randomly throughout the transcriptome. Next, libraries are prepared by fragmentation of the ds-cDNA, end repair to generate blunt ends, and adaptor ligation. Finally, customizable AnyDeplete probes are added to the sample to facilitate degradation of unwanted transcripts. The remaining library molecules are then amplified to produce Illumina-compatible libraries ready for sequencing. A diagram of the workflow from total RNA to fully constructed library is provided in Figure 1.

I. Introduction

Figure 1. Trio RNA-Seq Workflow.



C. Performance Specifications

Trio RNA-Seq is designed to generate RNA-Seq libraries suitable for either single-read or paired-end sequencing on Illumina NGS platforms, starting with as little as 500 pg and up to 50 ng total RNA.

D. Quality Control

Every lot of Trio RNA-Seq undergoes functional testing to meet specifications for library generation performance.

I. Introduction

E. Storage and Stability

Trio RNA-Seq is shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product contains components with multiple storage temperatures.

The vials labeled First Strand Enzyme Mix (blue: A3) and K562 RNA (clear: RNA ver 1) should be removed from inside the kit box upon delivery and stored separately at –80 °C.

Bead binding buffer (clear: BB ver 1) should be stored at 4 °C.

All remaining components should be stored at –20 °C in a freezer without a defrost cycle.

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

F. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at www.nugen.com/products/trio-rna-seq

G. Before You Start

Please review this User Guide before using this kit for the first time, including the "Kit Components", "Planning the Experiment", "Overview", "Protocol" and "FAQ" sections. For more information, visit the Trio RNA-Seq page at www.nugen.com/products/trio-rna-seq.

New to NGS? Contact NuGEN Technical Support at techserv@nugen.com for tips and tricks on getting started.

II. Kit Components

A. Reagents Provided

Table 1. cDNA Generation and Library Preparation Reagents

COMPONENT	0357-08 PART NUMBER	0357-32 PART NUMBER	0357-96 PART NUMBER	VIAL LABEL	VIAL NUMBER
DNase Buffer Mix	S02280	S02294	S02397	Clear	DB VER 2
HL-dsDNase	S02281	S01491	S02398	Clear	—
First Strand Primer Mix	S01262	S02295	S02160	Blue	A1 VER 4
First Strand Buffer Mix	S02282	S02296	S02399	Blue	A2 VER 13
First Strand Enzyme Mix	S02250	S02297	S02252	Blue	A3 VER 7
Second Strand Buffer Mix	S01132	S01192	S02411	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	S01193	S02400	Yellow	B2 VER 2
SPIA Primer Mix	S02283	S02298	S02401	Red	SP1 VER 1
SPIA Buffer Mix	S02284	S02299	S02402	Red	SP2 VER 1
SPIA Enzyme Mix	S02285	S02300	S02403	Red	SP3 VER 1
End Repair Buffer Mix	S01464	S02301	S02404	Blue	ER1 VER 3
End Repair Enzyme Mix	S01510	S01533	S01909	Blue	ER2 VER 4
End Repair Enhancer	S01562	S01503	S02405	Blue	ER3
Ligation Buffer Mix	S01466	S01534	S01910	Yellow	L1 VER 4
Ligation Enzyme Mix	S01467	S01535	S01911	Yellow	L3 VER 4
Amplification Buffer Mix	S01903	S01914	S02406	Red	P1 VER 4

II. Kit Components

Table 1 cDNA Generation and Library Preparation Reagents, *continued*

COMPONENT	0357-08 PART NUMBER	0357-32 PART NUMBER	0357-96 PART NUMBER	VIAL LABEL	VIAL NUMBER
Amplification Primer Mix	S02286	S02302 (2)	S02408 (2)	Red	P2 VER 16
Amplification Enzyme Mix	S01644	S02064	S02407	Red	P3 VER 2
AnyDeplete Buffer Mix	S02291	S02306	S02390	Purple	AD1 VER 1
AnyDeplete Probe Mix	See Table 2 for available probe mixes				
AnyDeplete Enzyme Mix I	S02292	S02307	S02391	Purple	AD2 VER 1
AnyDeplete Enzyme Mix II	S02293	S02308	S02392	Purple	AD3 VER 1
DNA Resuspension Buffer Mix	S02287	S02303	S02303 (3)	Clear	DR1
Bead Binding Buffer	S02288	S02304 (2)	S02410 (5)	Clear	BB VER 1
K562 Control RNA	S02289	S02289	S02289	Clear	RNA VER 1
32-Plex Ligation Adaptor Plate	---	S02317	---	---	L2V23DR
96-Plex Ligation Adaptor Plate	---	---	S02366	---	L2V23DR
Ligation Adaptor Mix	S02309	—	—	Yellow	L2V23DR-BC1
	S02310	—	—	Yellow	L2V23DR-BC2
	S02311	—	—	Yellow	L2V23DR-BC3
	S02312	—	—	Yellow	L2V23DR-BC4
	S02313	—	—	Yellow	L2V23DR-BC5
	S02314	—	—	Yellow	L2V23DR-BC6
	S02315	—	—	Yellow	L2V23DR-BC7
	S02316	—	—	Yellow	L2V23DR-BC8

Table 2. AnyDeplete Probe Mix

COMPONENT	XXXX-08 PART NUMBER	XXXX-32 PART NUMBER	XXXX-96 PART NUMBER	VIAL LABEL	VIAL NUMBER
Trio RNA-Seq AnyDeplete Probe Mix- Human rRNA	S02290	S02305	S02394	Purple	AD10 VER 1
Trio RNA-Seq AnyDeplete Probe Mix - Mouse rRNA	S02353	S02355	S02395	Purple	AD11 VER 1
Trio RNA-Seq AnyDeplete Probe Mix- Custom	S0XXXX	S02369	S0XXXX	Purple	ICxxxx

Note: The reagents in Trio RNA-Seq are similar to reagents in our other kits; however, unless the component part numbers are identical, these reagents do not have exactly the same composition and therefore are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Agilent 2100 Bioanalyzer or 2200 TapeStation Instrument, or other equipment for electrophoretic analysis of nucleic acids
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL tube strips or plates
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - 2–20 µL or 5–50 µL multichannel pipette and 20–200 µL or 20–300 µL multichannel pipette for sample mixing
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Qubit® 2.0 or 3.0 Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries.

II. Kit Components

- **Reagents**
  - Agencourt RNAClean XP Beads or AMPure XP Beads (Beckman Coulter, Cat. #A63987 or A63881)
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793) optional; for diluting nucleic acids
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Nuclease-free water (Alfa Aesar, Cat. #J71786), for purification steps
  - Agilent High Sensitivity DNA Kit (Agilent, Cat. #5067-4626) or equivalent
  - EvaGreen®, 20X (Biotium, Cat. #31000)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL PCR strip tubes or 0.2 mL thin-wall PCR plates
  - Low-retention microcentrifuge tubes (DNA LoBind Tubes, Eppendorf Cat. #0030108035 or 0030108051)
  - Magnetic stand for 0.2 mL strip tubes or plates. A low-elution volume or "side pull"-style magnet is recommended for this system (Thermo Fisher Scientific Cat. #12027, or 12332D; Promega Cat. #V8351; others). Other magnetic stands may be used as well, although their performance has not been validated by NuGEN.
  - Cleaning solutions such as RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, Cat. #AM9780) and DNA OFF™ (MP Biomedicals, Cat. #11QD0500)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

To Order

- Alfa Aesar, www.alfa.com
- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Eppendorf, www.eppendorf.com
- MP Biomedicals, www.mpbio.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

III. Planning the Experiment

A. Input RNA Requirements

1. RNA Quantity

Total RNA input must be between 500 pg and 50 ng. Inputs outside of these ranges may affect reaction stoichiometry, resulting in sub-optimal libraries. Lower input amounts will potentially result in insufficient yields depending on the requirements of the analytical platform. We strongly recommend quantification of total RNA to ensure the minimum input requirement is met.

2. RNA Purity

RNA samples must be free of contaminating proteins and other cellular material, organic solvents (including phenol and ethanol) and salts used in many RNA isolation methods. When preparing small amounts of RNA, we recommend using a commercially available system that does not require organic solvents. If using an RNA isolation method based on organic solvents, such as TRIzol, we recommend column purification after isolation.

One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples should be in excess of 1.8.

3. RNA Integrity

RNA samples of high molecular weight with little or no evidence of degradation will perform very well with this product. When using degraded total RNA, we recommend using somewhat higher inputs in order to achieve yield and data quality similar to that of more intact RNA samples. Depending on available input, RNA integrity can be determined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip or RNA 6000 Pico LabChip. While it is impossible to guarantee satisfactory results with all degraded samples, this system may work with many samples that are moderately degraded.

4. DNase Treatment

Thorough DNase treatment of RNA is required with this system. The presence of genomic DNA in the RNA sample will have adverse effects on assay performance and data quality. An integrated DNase treatment workflow is included in section V.A. of this user guide.

B. Working with the 32- or 96-Plex Adaptor Plate

The Adaptor Plate included with the 32 and 96 reaction Trio RNA-Seq kits contain adaptor mixes, each with a unique eight-base barcode. Each well (first 32 wells, A01–H04, or all 96 wells, respectively) contains sufficient volume for preparation of a single

library. Trio RNA-Seq Adaptor Plates are sealed with a foil seal designed to provide airtight storage.

Thaw adaptor plate on ice and spin down. Do NOT warm above room temperature. Make sure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice after centrifuging. When pipetting the adaptor mixes, puncture the seal for each well you wish to use with a fresh pipet tip, and transfer the appropriate volume of adaptor into your sample. The remaining wells of the plate should remain sealed for use at a later date. Cover used wells with a new foil seal (e.g., AlumaSeal II) to prevent any remaining adaptor-containing liquid from contaminating future reactions.

For details regarding barcode color balancing for multiplex sequencing, please see Appendix A on page 31.

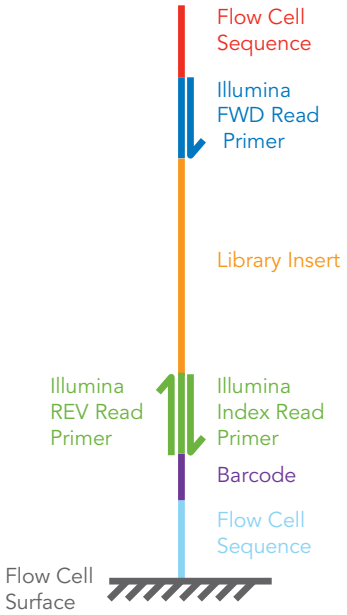
C. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

D. Sequencing Recommendations and Guidelines

Trio RNA-Seq produces RNA-Seq libraries compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer. These libraries contain an 8 bp barcode. These barcodes differ from the sequences used by Illumina and can be found in Appendix A.

Figure 2. Trio RNA-Seq Library Structure.



E. Data Analysis

Once the data have been parsed according to sample, additional sample-specific data analysis may be employed according to the requirements of the experiment.

Note: For data analysis, we recommend trimming the first 5 bp from the 5' end of the forward, and, if applicable, reverse read. If these bases are not trimmed, poor alignment may result. For experiments with short library inserts, such as with FFPE samples, additional trimming may be required. Please contact NuGEN Technical Support for additional recommendations.

IV. Overview

A. Overview

Trio RNA-Seq is performed in the following stages:

1. cDNA synthesis	
a. First strand cDNA synthesis	1.0 hour
b. Second strand cDNA synthesis and purification	2.0 hours
c. SPIA amplification	1.5 hours
2. Library construction	
a. Fragmentation and end repair	0.7 hours
b. Adaptor ligation and purification	1.5 hours
c. Library amplification and purification	1.5 hours
3. AnyDeplete	
a. Probe binding	0.25 hours
b. Targeted depletion	0.66 hours
c. Library amplification and purification	1.0 hour
Total time to prepare amplified library	**~10 hours**

B. Protocol Notes

Controls

- We recommend the routine use of a positive control RNA, using the K562 control RNA provided with the kit or other suitable RNA, especially the first time a reaction is set up. The use of a positive control RNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- Routine use of a low-template control (50 pg) is recommended to monitor the work environment for non-specific amplification issues. Avoid running negative controls (i.e., no RNA input reactions). The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular bioanalyzer traces in a low template control (LTC) reaction.
- Due to the high sensitivity inherent in the amplification stage of the protocol, we strongly recommend taking measures to minimize the potential for the carryover of previously amplified samples into new amplification reactions. The two steps to accomplish this are: 1) designating separate workspaces for "pre-amplification"and "post-amplification" steps and materials, and 2) implement-

ing routine clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in the Appendix.
- When working with picogram amounts of RNA we strongly recommend the use of low retention tubes for storage and dilution of the samples in order to reduce the loss of RNA due to adhesion to polypropylene surfaces.

General Workflow

- Set up no fewer than 4 reactions at a time (for 8-reaction kit) and 8 reactions at a time (for 32- and 96-reaction kits). This ensures sufficient reagent recoveries for the full nominal number of amplifications from the kit. Making master mixes for fewer samples at a time may affect reagent recovery volumes.
- Thaw components used in each step and immediately place them on ice. Keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing. Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme mixes nor warm any enzyme or primer mixes.
- When preparing master mixes, use the minimal amount of extra material to ensure all reactions in the kit. The Trio RNA-Seq Quick Protocol will automatically calculate an appropriate overfill volume which can be used as a guideline in setting up master mixes.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to mix via pipetting, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

Reagents

- Components and reagents from other NuGEN products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

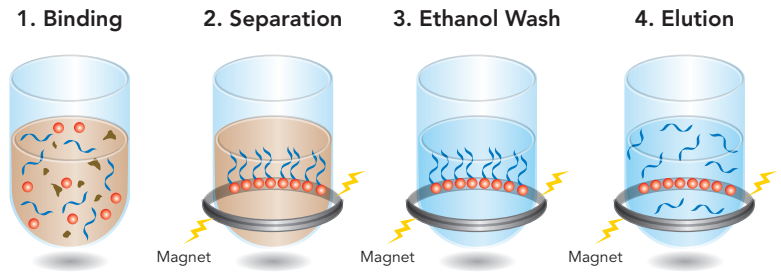
C. Agencourt® Beads

Agencourt RNAClean XP or Ampure XP Beads (Agencourt beads) are suitable for use with Trio RNA-Seq. There are modifications to the Agencourt beads standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads. The bead purification process used for cDNA purification before amplification consists of:

IV. Overview

1. Binding of DNA to Agencourt beads
2. Magnetic separation of beads from supernatant
3. Ethanol wash of bound beads to remove contaminants
4. Elution

Figure 3. Agencourt bead purification process.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

Tips and Notes

- Remove beads and Bead Binding Buffer from 4 °C and leave at room temperature for at least 30 minutes. Before use, ensure that they have completely reached room temperature. Cold beads and buffer will result in reduced recovery.
- Fully resuspend beads by inverting and tapping before adding to the sample.
- Note that we recommend specific sample to bead volume ratios in our Agencourt bead protocols. In many cases our recommendations differ from the standard Beckman Coulter protocol.
- Use a magnetic plate or stand for 0.2 mL strip tubes or plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- Take care to minimize bead loss throughout the procedure. Any significant loss of beads after the binding steps and ethanol washes will impact DNA yields.
- After the binding step has been completed, it is important to minimize bead loss when removing the binding buffer. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- During the ethanol washes, keep the samples on the magnet. The beads should not be allowed to disperse; the magnet will keep the beads on the walls of the sample wells or tubes.
- It is critical that all residual ethanol be removed prior to elution. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow

the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air-drying time.
- After drying the beads for 10 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.
- It is strongly recommended that strip tubes or partial plates are firmly placed when used with the magnetic plate. We don't advise the use of individual tubes, as they are not very stable on the magnetic plates.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 3 following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C. For thermal cyclers with a fixed-temperature heated lid use the default settings (typically 100–105 °C).

Table 3. Thermal Cycler Programming

cDNA SYNTHESIS AND AMPLIFICATION		VOLUME
Program 1 DNase Treatment	37 °C – 10 min, 60 °C – 5 min, hold at 4 °C	12.5 µL
Program 2 First Strand Synthesis	4 °C – 1 min, 25 °C – 10 min, 42 °C – 10 min, 70 °C – 15 min, hold at 4 °C	25 µL
Program 3 Second Strand Synthesis	4 °C – 1 min, 25 °C – 10 min, 50 °C – 30 min, 80 °C – 20 min, hold at 4 °C	50 µL
Program 4 SPIA Amplification	4 °C – 1 min, 47 °C – 60 min, 80 °C – 20 min, hold at 4 °C	10 µL
LIBRARY CONSTRUCTION		**VOLUME**
Program 5 Fragmentation and End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	15 µL
Program 6 Adaptor Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	30 µL
Program 7 Library Amplification I	72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 4–6x* (95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C	100 µL
ANYDEPLETE		**VOLUME**
Program 8 Probe Binding	95 °C – 2 min, 50 °C – 1 min, 65 °C – 10 min, hold at 4 °C	25 µL
Program 9 Targeted Depletion	60 °C – 30 min, 95 °C – 5 min, hold at 4 °C	50 µL
Program 10 Library Amplification II	95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 6x(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C	100 µL

Important Note: The number of cycles (*) used for Library Amplification I depends on the starting amount of RNA. Please refer to Table 12 for a general guide to choosing the appropriate number of cycles for the PCR amplification reaction. Alternatively, real-time PCR can be used to determine the appropriate number of PCR cycles. For more information, contact NuGEN Technical Support.

V. Protocol

For each section of the protocol, remove reagents from storage as listed. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol or store samples as directed. Return all reagents to their appropriate storage conditions promptly after use unless otherwise instructed.

Important Note: Carry out steps A (DNase Treatment) through E (SPIA Amplification) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA removal solution such as DNA-OFF (MP Biomedicals, Cat. #QD0500) to avoid the potential introduction of previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix F of this user guide. If you have any questions on this important topic, please contact NuGEN Technical Support (techserv@nugen.com, (888) 654-6544).

A. DNase Treatment

Table 4. DNase Master Mix

REAGENT	DNase BUFFER (CLEAR: DB VER 2)	HL-dsDNase (CLEAR)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	1 µL	1.5 µL

! *Do not vortex any enzyme mixes.*

1. Spin down the contents of HL-dsDNase and place on ice.
2. Thaw DB at room temperature. Mix by vortexing, spin and place on ice.
3. Prepare a master mix by combining DB and HL-dsDNase in a 0.5 mL capped tube according to the volumes shown in Table 4. Mix well by pipetting, spin and place on ice.
4. Add 2.5 µL of DNase Master Mix per sample to a 0.2 mL PCR tube or strip.
5. Add 10 µL of total RNA sample (500 pg to 50 ng) to each 0.2 mL tube containing DNase Master Mix for a total of 12.5 µL. If sample volume is less than 10 µL, add nuclease-free water up to 10 µL. Mix well by pipetting, spin and place on ice.
6. Place the tubes or strips in a pre-warmed thermal cycler programmed to run Program 1 (DNase Treatment; see Table 3):

   37 °C – 10 min, 60 °C – 5 min, hold at 4 °C

7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

B. First Strand cDNA Synthesis

Table 5. First Strand Master Mix

REAGENT	FIRST STRAND BUFFER (BLUE: A2 VER 13)	FIRST STRAND PRIMER MIX (BLUE: A1 VER 4)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 7)
STORAGE	–20 °C	–20 °C	–80 °C
1X REACTION VOLUME	6.25 µL	5 µL	1.25 µL

1. Spin down the contents of A3 and place on ice.
2. Thaw A1 and A2 at room temperature. Mix by vortexing, spin and place on ice.
3. Prepare a master mix by combining A1, A2 and A3 in a 0.5 mL capped tube according to the volumes shown in Table 5. Mix well by pipetting, spin and place on ice.
4. Add 12.5 µL of First Strand Master Mix to each sample tube for a total of 25 µL. Mix well by pipetting, spin and place on ice.
5. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 3):

   4 °C – 1 min, 25 °C – 10 min, 42 °C – 10 min, 70 °C – 15 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Second Strand cDNA Synthesis

Table 6. Second Strand Master Mix

REAGENT	SECOND STRAND BUFFER MIX (YELLOW: B1 VER 3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 2)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	24.25 µL	0.75 µL

1. Remove Agencourt beads and Bead Binding Buffer from 4 °C storage and place on the bench top to reach room temperature for use in the next step.
2. Spin down the contents of B2 and place on ice.
3. Thaw B1 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 6. Mix well by pipetting, spin and place on ice.
5. Add 25 µL of Second Strand Master Mix to each sample tube for a total of 50 µL. Mix well by pipetting, spin and place on ice.
6. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 3):

   4 °C – 1 min, 25 °C – 10 min, 50 °C – 30 min, 80 °C – 20 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation.

D. cDNA Purification

Table 7. Agencourt Bead Master Mix

REAGENT	AGENCOURT BEADS	BEAD BINDING BUFFER (BB VER 1)
STORAGE	4 °C	4 °C
1X REACTION VOLUME	30 µL	50 µL

1. Ensure the Agencourt beads and Bead Binding Buffer have completely reached room temperature before proceeding.

Prepare 70% ethanol daily using fresh ethanol stocks.

2. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container.

   Note: Measure both the ethanol and the water carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce amplification yield.
3. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
4. Prepare a master mix by combining the resuspended Agencourt beads and BB according to the volumes shown in Table 7. Mix well by vortexing.
5. At room temperature, add 80 µL (1.6 volumes) of Agencourt Bead Master Mix to each reaction and mix by pipetting 10 times.
6. Incubate at room temperature for 10 minutes.

7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Keeping the tubes on the magnet, carefully remove the binding buffer and discard it.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the washes.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat the wash two more times.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
12. Air-dry the beads on the magnet for 15 to 20 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with SPIA amplification.
13. Continue to SPIA Amplification with the cDNA still bound to the dry beads.

E. SPIA Amplification

Table 8. SPIA Master Mix

REAGENT	SPIA BUFFER MIX (RED: SP2 VER 1)	SPIA PRIMER MIX (RED: SP1 VER 1)	SPIA ENZYME MIX (RED: SP3 VER 1)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	2.5 µL	2.5 µL

! *Do not vortex SPIA enzyme mix.*

1. Thaw SP3 on ice and mix the contents by inverting gently 5 times. Ensure the enzymes are well mixed without introducing bubbles, spin and place on ice.
2. Thaw reagents SP1 and SP2 at room temperature, mix by vortexing, spin and place on ice.

! *Use SPIA Master Mix immediately after preparation*

3. Prepare a master mix by sequentially combining SP2, SP1 and SP3 in a 0.5 mL capped tube according to the volumes shown in Table 8. Mix well by pipetting, spin and place on ice.
4. Add 10 µL of SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 7 µL and mix thoroughly by pipet-

ting at least 8–10 times. Attempt to get the majority of the beads in suspension and remove most of the beads from the tube walls.

Note: The beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of SPIA master mix will elute the cDNA from the beads.

5. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (SPIA Amplification; see Table 3):

   4 °C – 1 min, 47 °C – 60 min, 80 °C – 20 min, hold at 4 °C

6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice. **Do not re-open the tubes in the pre-amplification workspace.**

Important Note: At this point the tubes should be removed from the pre-amplification workspace. Carry out all remaining steps in a postamplification workspace using dedicated post-amplification consumables and equipment. Take care to avoid the introduction of previously amplified cDNA into your pre-amplification workspace. For more information on our recommendations for workflow compartmentalization and routine lab cleanup, please refer to Appendix F of this user guide. If you have any questions on this important topic, please contact NuGEN Technical Support (techserv@nugen.com, (888) 654-6544).

7. Continue to End Repair with the Agencourt beads in solution.

Optional stopping point: Store samples at 4 °C overnight after SPIA Amplification.

F. Fragmentation and End Repair

Table 9. End Repair Master Mix

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 VER 3)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)	END REPAIR ENHANCER (BLUE: ER3)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	3.5 µL	0.5 µL	1 µL

1. Spin down the contents of ER2 and ER3 and place on ice.
2. Thaw ER1 at room temperature. Mix by vortexing, spin and place on ice.
3. Prepare a master mix by combining ER1, ER2 and ER3 in a 0.5 mL capped tube according to the volumes shown in Table 9. Mix well by pipetting, spin and place on ice.
4. Add 5 µL of End Repair Master Mix to each sample tube for a total of 15 µL. Mix by pipetting, spin and place on ice.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Fragmentation and End Repair; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
7. Continue to Adaptor Ligation with the Agencourt beads in solution.

G. Adaptor Ligation

Table 10. Ligation Master Mix

REAGENT	DNA RESUSPENSION BUFFER (CLEAR: DR1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	4.5 µL	6.0 µL	1.5 µL

1. Remove Bead Binding Buffer from 4 °C and DNA Resuspension Buffer Mix from –20 °C. Place on the bench top to reach room temperature for use in the next step.
2. Spin down L3 and place on ice.
3. Thaw Adaptor Mixes (L2V22DR-BC) or Adaptor Plate on ice, spin down and place on ice.
4. Thaw L1 and DR1 at room temperature. Mix L1 by vortexing. Spin L1 and DR1 and place on ice.
5. Add 3 µL of the appropriate barcoded Adaptor Mix to each sample. Mix thoroughly by pipetting, spin and place on ice. Make sure a unique barcode is used for each sample to be multiplexed together on the sequencer.

6. Prepare a master mix by combining DR1, L1 and L3 in a 0.5 mL capped tube according to the volumes shown in Table 10. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.

   Note: The L1 Ligation Buffer Mix is very viscous. Please be sure to pipet this reagent slowly.

7. Add 12 µL of the Ligation Master Mix to each reaction tube for a total of 30 µL. Mix thoroughly by pipetting slowly and gently, spin and place on ice. Proceed immediately with the incubation.

8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 6 (Adaptor Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C

9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

H. Adaptor Ligation Purification

1. Ensure the Bead Binding Buffer (BB VER 1) DNA Resuspension Buffer Mix (clear: DR1) have completely reached room temperature before proceeding.

2. Mix DR1 by vortexing, spin and place at room temperature.

3. Prepare a 70% ethanol wash solution.

4. Add 70 µL of nuclease-free water to each sample tube. Mix thoroughly by pipetting.

5. Add 80 µL of BB to each sample tube. Mix thoroughly by pipetting.

6. Incubate at room temperature for 10 minutes.

7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

8. Keeping the tubes on the magnet, carefully remove the binding buffer mix and discard it.

9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the washes.

10. Remove the 70% ethanol wash using a pipette.

11. Repeat the 70% ethanol wash one more time, for a total of two washes.

Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

12. Air-dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with the protocol.
13. Remove the tubes from the magnet.
14. Add 40 µL of DR1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
15. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
16. Carefully remove 40 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes and place on ice.

Optional stopping point: Store samples at –20 °C.

I. Library Amplification I

Table 11. Library Amplification I Master Mix

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 16)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	20 µL	39.5 µL	0.5 µL

1. Remove Agencourt beads from 4 °C and place on the bench top to reach room temperature for use in the next step.
2. Spin down P3 and place on ice.
3. Thaw P1 and P2 at room temperature. Mix by vortexing, spin and place on ice.
4. Make a master mix by combining P1, P2, and P3 in a 0.5 mL capped tube according to the volumes shown in Table 11. Mix thoroughly by pipetting. Spin and place on ice. Use immediately.
5. Add 60 µL of the Library Amplification I Master Mix to each sample tube for a total of 100 µL. Mix thoroughly by pipetting. Spin and place on ice.

6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (Library Amplification I; see Table 3):

   72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), N(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C

Important Note: The number of cycles used for PCR amplification depends on the starting amount of RNA. Whenever using a sample for the first time with the kit, or using a new amount of input, perform real-time PCR as described in Appendix C to determine the appropriate number of cycles for your sample. Please refer to Table 12 for a general guide to choosing the appropriate number of cycles for the PCR amplification reaction. Note that these guidelines are based on high-quality RNA inputs.

Table 12. Recommended PCR Cycles for Library Amplification I

STARTING INPUT	PCR CYCLES (N)
500 pg – 1 ng	6
1 ng – 50 ng	4

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Optional stopping point: Store samples at –20 °C.

J. Library Amplification I Purification

1. Remove the AnyDeplete Buffer Mix (purple: AD1 VER 1), AnyDeplete Probe Mix (purple: AD1x VER 1), AnyDeplete Enzyme Mix I (purple: AD2 VER 1) and DNA Resuspension Buffer Mix (clear: DR1) from –20 °C storage for use in the next step.
2. Spin down AD2 and place on ice.
3. Thaw AD1, AD1x and DR1 at room temperature. Spin down and place on ice.
4. Prepare a 70% ethanol wash solution.
5. Ensure the Agencourt beads have completely reached room temperature before proceeding.

6. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
7. Add 100 µL (1.0 volumes) of the bead suspension to each sample. Mix thoroughly by pipetting up and down.
8. Incubate at room temperature for 10 minutes.
9. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
10. Carefully remove 200 µL of the binding buffer and discard it.

    Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into AnyDeplete, so ensure beads are not removed with the binding buffer or the wash.
11. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
12. Remove the 70% ethanol wash using a pipette.
13. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the second wash, it is critical to remove as much of the ethanol as possible. Remove the ethanol wash with a pipet, allow excess ethanol to collect at the bottom of the tubes, and remove any remaining ethanol with a fresh pipet tip.
14. Air dry the beads on the magnet for 10 minutes. Continue to Section K, Probe Binding, while the beads are drying on the magnet.

K. Probe Binding

Table 13. Probe Binding Master Mix

REAGENT	ANYDEPLETE BUFFER MIX (PURPLE: AD1 VER 1)	ANYDEPLETE PROBE MIX (PURPLE: AD1x VER 1 or ICxxxx)	ANYDEPLETE ENZYME MIX I (PURPLE: AD2 VER 1)	DNA RESUSPENSION BUFFER (CLEAR: DR1)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	9 µL	1 µL	10 µL

1. Prepare a master mix by combining AD1, AD1x or ICxxxx, AD2 and DR1 in a 0.5 mL capped tube according to the volumes shown in Table 13. Mix thoroughly, spin and place on ice.
2. Inspect the tubes air drying on the magnet carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
3. Remove the tubes from the magnet.
4. Resuspend samples in 25 µL of Probe Binding Master Mix.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 8 (Probe Binding; see Table 3):

   95 °C – 2 min, 50 °C – 1 min, 65 °C – 10 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

L. Targeted Depletion

Table 14. Targeted Depletion Master Mix

REAGENT	ANYDEPLETE BUFFER MIX (PURPLE: AD1 VER 1)	ANYDEPLETE ENZYME MIX II (PURPLE: AD3 VER 1)	DNA RESUSPENSION BUFFER (CLEAR: DR1)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	4 µL	16 µL

1. Spin down AD3 and place on ice.
2. Thaw AD1 and DR1 at room temperature. Mix AD1 by vortexing. Spin down both tubes and place on ice.
3. Prepare a master mix by combining AD1, DR1 and AD3 in a 0.5 mL capped tube according to the volumes shown in Table 14. Mix thoroughly by pipetting, spin and place on ice.
4. Add 25 µL of Targeted Depletion Master Mix to each sample tube for a total of 50 µL. Mix thoroughly by pipetting, spin and place on ice.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Targeted Depletion; see Table 3):

   60 °C – 30 min, 95 °C – 5 min, hold at 4 °C

6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

M. Library Amplification II

Table 15. Library Amplification II Master Mix

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 16)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	10 µL	39.5 µL	0.5 µL

1. Remove Bead Binding Buffer (clear: BB VER 1) from 4 °C and DNA Resuspension Buffer Mix (clear: DR1) from –20 °C and place on the bench top to reach room temperature for use in the next step.
2. Spin down P3 and place on ice.
3. Thaw P1 and P2 at room temperature. Mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining P1, P2 and P3 in a 0.5 mL capped tube according to the volumes shown in Table 15. Mix thoroughly by pipetting, spin and place on ice.
5. Add 50 µL of the Library Amplification II Master Mix to each sample tube for a total of 100 µL. Mix thoroughly by pipetting, spin and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 10 (Library Amplification II; see Table 3):

   95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 6x(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Optional stopping point: Store samples at 4 °C.

N. Library Amplification II Purification

1. Ensure BB and DR1 have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution.
3. Add 100 µL (1.0 volumes) of BB to each sample. Mix thoroughly by pipetting up and down.
4. Incubate at room temperature for 10 minutes.
5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove 200 µL of the binding buffer and discard it.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the final yield, so ensure beads are not removed with the binding buffer or the wash.
7. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat the 70% ethanol wash one more time, for a total of two washes.

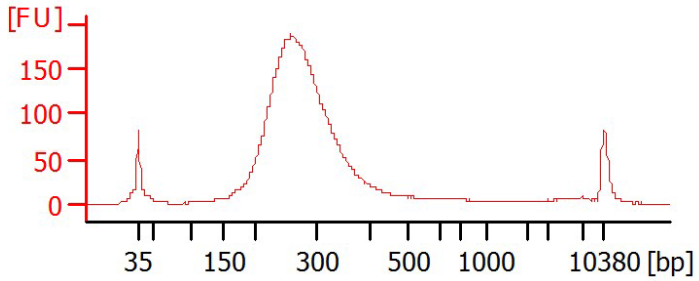
   Note: With the second wash, it is critical to remove as much of the ethanol as possible. Remove the ethanol wash with a pipet, allow excess ethanol to collect at the bottom of the tubes, and remove any remaining ethanol with a fresh pipet tip.
10. Air dry the beads on the magnet for 10 minutes.
11. Remove the tubes from the magnet.
12. Add 30 µL of DR1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
13. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
14. Carefully remove 30 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes and place on ice.

Optional stopping point: Store samples at –20 °C.

O. Quantitative and Qualitative Assessment of the Library

1. Assess the library by running 1 µL of 5 ng/µL library on the High Sensitivity DNA Chip (Agilent Technologies). A typical fragment distribution for high quality inputs is shown in Figure 4.

Figure 4. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA Chip from 1 ng K562 total RNA starting material.



2. Quantify the library using a qPCR-based method.
3. Validate the library as described in Illumina User Guides for DNA library construction, e.g., Genomic DNA Sample Prep Manual (Cat. #FC-102-1001).

VI. Technical Support

For help with any of our products, please contact NuGEN Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only). You may also send faxes to 888.296.6544 (toll-free) or email techserv@nugen.com.

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe@nugen.com.

In all other locations, contact your NuGEN distributor for technical support.

VII. Appendix

A. Barcode Sequences and Guidelines for Multiplex Experiments

Barcode sequences for the 32- and 96-plex Adaptor Plates are given below, with barcodes in 32 reaction kits found in wells A01–H04. Barcodes are color balanced in pairs (i.e. A01 + B01, C01 + D01, etc.) and in sets of 8 by column. Barcodes 1–8 in the 8-reaction kit correspond to plate positions A01–H01, respectively.

Table 16. Barcode sequences for Trio RNA-Seq adaptors

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CGCTACAT	A05	AGGTTCCT	A09	GCCTTAAC
B01	AATCCAGC	B05	GAACCTTC	B09	ATTCCGCT
C01	CGTCTAAC	C05	AAGTCCTC	C09	ATCGTGGT
D01	AACTCGGA	D05	CCACAACA	D09	GCTACAAC
E01	GTCGAGAA	E05	ATAACGCC	E09	TCTACGCA
F01	ACAACAGC	F05	CCGGAATA	F09	CTCCAATC
G01	ATGACAGG	G05	CCAAGTAG	G09	ACTCTCCA
H01	GCACACAA	H05	AAGGACCA	H09	GTCTCATC
A02	CTCCTAGT	A06	ACGCTTCT	A10	GCCAGAAT
B02	TCTTCGAC	B06	CTATCCAC	B10	AATGACGC
C02	GACTACGA	C06	TGACAACC	C10	GTACCACA
D02	ACTCCTAC	D06	CAGTGCTT	D10	ACGATCAG
E02	CTTCCTTC	E06	TCACTCGA	E10	TAACGTCG
F02	ACCATCCT	F06	CTGACTAC	F10	CGCAACTA
G02	CGTCCATT	G06	GTGATCCA	G10	AACACTGG
H02	AACTTGCC	H06	ACAGCAAG	H10	CCTGTCAA
A03	GTACACCT	A07	TGCTGTGA	A11	TCCTGGTA
B03	ACGAGAAC	B07	CAACACAG	B11	CATCAACC
C03	CGACCTAA	C07	CCACATTG	C11	AGCAGACA
D03	TACATCGG	D07	TAGTGCCA	D11	GAAGACTG
E03	ATCGTCTC	E07	TCGTGCAT	E11	TCTAGTCC
F03	CCAACACT	F07	CTACATCC	F11	CTCGACTT
G03	TCTAGGAG	G07	CATACGGA	G11	CTAGCTCA
H03	CTCGAACA	H07	TGCGTAAC	H11	TCCAACTG
A04	ACGGACTT	A08	CAGGTTCA	A12	GACATCTC
B04	CTAAGACC	B08	AGAACCAG	B12	ACTGCACT
C04	AACCGAAC	C08	GAATGGCA	C12	GTTCCATG
D04	CCTTAGGT	D08	AGGCAATG	D12	ACCAAGCA
E04	CCTATACC	E08	TAGGAGCT	E12	CTCTCAGA
F04	AACGCCTT	F08	CGAACAAC	F12	ACTCTGAG
G04	TCCATTGC	G08	CATTCGTC	G12	GCTCAGTT
H04	CAAGCCAA	H08	AGCCAACT	H12	ATCTGACC

B. Preventing Off-Target Amplification

Due to the high sensitivity inherent in the Trio RNA-Seq kit, we have developed a set of recommendations designed to minimize the potential generation of off-target amplification products. We strongly recommend implementing these procedures.

We have three general recommendations:

1. Designate separate workspaces for "pre-amplification" and "post-amplification" steps and materials. This provides the best work environment for processing RNA using this highly sensitive amplification protocol.

    - Pre-amplification includes all steps and materials related to RNA sample handling and dilution, DNase Treatment, First Strand cDNA Synthesis, Second Strand cDNA Synthesis and SPIA Amplification. Ideally the isolated pre-amplification workspace will be in a separate enclosed workspace.
    - We recommend the use of "PCR Workstation" enclosures with UV illumination for use as pre-amplification workspaces. After Adaptor Ligation, you may remove your material to the post-amplification area.
    - Post-amplification includes all steps and materials related to the handling of the library molecules including Adaptor Ligation Purification, Library Amplification I, Library Amplification I Purification, AnyDeplete, Library Amplification II and Library Amplification II Purification.

2. Implement routine clean-up protocols for workspaces as standard operating procedure.

    - Initially clean the entire lab thoroughly with DNA-OFF and RNaseZap.
    - In the pre-amplification area, remove all small equipment, then clean every surface. Before reintroducing any equipment, clean every piece of equipment thoroughly. Clean wells of thermal cyclers and magnetic plates with a cotton swab or by filling with cleaning solution.
    - Always wear gloves and don fresh gloves upon entry into the pre-amplification area. Frequently change gloves while working in this area, especially prior to handling stock reagents, cells, reactions and RNA samples.
    - Stock this area with clean (preferably new) equipment (pipettes, racks, consumables).
    - Make it a policy to carry out regular cleaning of all workspaces.
    - Do not open amplified product reaction vessels in the pre-amplification workspace.

3. Use low-template controls to detect and troubleshoot contamination. The clearest indication that an amplification reaction is contaminated is the appearance of significant amounts of amplified product in a low-template control (LTC).

    In the absence of contamination:

    - LTC yields for Trio RNA-Seq amplifications are typically at or below 10% of yield for actual samples.
    - Products generated from uncontaminated LTC reactions yield much lower alignment to the target genome.

In the presence of contamination:

- LTC yields are generally significantly higher than 10% of yield for actual samples.
- Contaminated LTC yields can be as high as or even higher than template containing reactions (i.e. your experimental samples or positive controls).
- LTC libraries may have much lower alignment to the target genome depending on the source of contamination.

C. Using qPCR to determine the Number of PCR Cycles in Library Amplification I

In the Trio RNA-Seq protocol, library amplification is performed by preparing an Amplification Master Mix and adding 60 µL of this master mix to 40 µL of library after adaptor ligation for a total PCR volume of 100 µL. If you wish to use qPCR to guide the number of cycles used in library amplification in order to ensure there is no excess amplification performed, you may perform a 1/10th scale qPCR reaction as follows.

1. Prepare a PCR master mix according to the volumes shown in Table 17.

Table 17. Library Amplification qPCR Master Mix

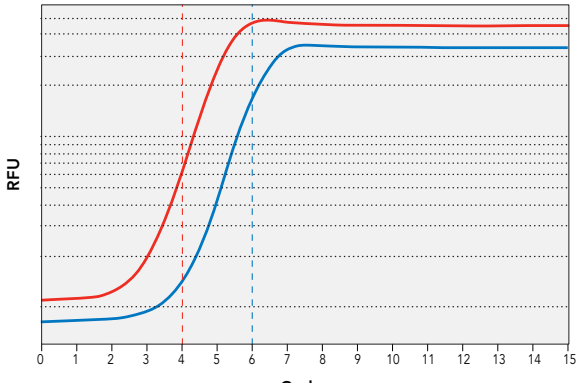
REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 16)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)	20X EVAGREEN
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2 µL	3.95 µL	0.05 µL	0.5 µL

2. Aliquot 6.5 µL of PCR master mix per well. Add 4.0 µL of purified library (after ligation but before PCR amplification) for a total qPCR volume of 10.5 µL.
3. Perform real-time qPCR with the following cycling conditions:

   72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 40x(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C

The cycle number used for subsequent library amplification should be within the exponential phase of the amplification plot (4 or 6 cycles in Figure 5 as an example).

Figure 5. Stylized qPCR amplification plot.



For PCR, choose a cycle number within the exponential phase of amplification.

4. To amplify the remaining 36 µL of library, prepare an amplification master mix according to the volumes shown in Table 18.

Table 18. Library Amplification I Master Mix

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 16)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	18 µL	35.55 µL	0.45 µL

5. Add 36 µL of library to 54 µL of amplification master mix for a total PCR volume of 90 µL.
6. PCR amplify with the following cycling conditions, where N is the number of cycles determined from the above real-time qPCR assay:

   72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), N(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C
7. Continue to Section J, Library Amplification I Purification.

VII. Appendix

D. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with Trio RNA-Seq?
Trio RNA-Seq provides all necessary buffers, primers, enzymes and depletion probes for cDNA synthesis, library construction, and targeted depletion. A control RNA (K562 total RNA) is provided to enable testing of the workflow. Nuclease-free water, Agencourt beads and EvaGreen for the optional PCR optimization step are not provided.

Q2. What is the concentration of the control RNA?
The control RNA (K562 total RNA) is provided at a concentration of 1 µg/µL.

Q3. What equipment is required or will be useful?
Required equipment includes a microcentrifuge; pipettes; vortexer; a thermal cycler; a magnetic plate for 0.2 mL tubes, strips, or plates; and a spectrophotometer or fluorometer. An Agilent Bioanalyzer or Tapestation may also be useful for optional analytical tests. A comprehensive list of required and recommended equipment can be found in Section II.B.

Q4. Can this system be used with other library preparation workflows?
Trio RNA-Seq is an end-to-end solution designed to generate libraries for Illumina sequencing starting from total RNA and has not been tested with alternative library preparation systems.

Q5. Can this system be used without AnyDeplete targeted depletion?
Yes. Please contact NuGEN technical support at techserv@nugen.com for information regarding the no-AnyDeplete workflow.

Input Recommendations

Q6. What methods do you recommend for RNA isolation?
We recommend a column-based method, including:

- Norgen Biotek Total RNA Purification Kit
- Zymo Research Quick-RNA™ Kits
- Arcturus PicoPure® RNA Isolation Kit
- Ambion PureLink® RNA Mini Kit
- Qiagen RNeasy Kits

For FFPE RNA isolation, we recommend a kit designed for FFPE samples, including:

- Norgen Biotek FFPE RNA Purification Kit
- Zymo Research Quick-RNA™ FFPE Kit
- Arcturus® Paradise® PLUS FFPE RNA Isolation Kit

- PureLink™ FFPE RNA Isolation Kit
- Qiagen RNeasy FFPE Kit

Organic methods such as TRIzol® Reagent should be subsequently followed with a column-based clean-up method.

Q7. Can I use TRIzol® or other phenol-chloroform based extractions for RNA isolation?
We do not recommend the use of TRIzol® or similar methods as any carry over of organic solvent may inhibit downstream enzyme activity. If using TRIzol extracted RNA, we recommend using a column-based purification of the RNA prior to input into the kit.

Q8. Can I use carrier RNA during RNA isolation?
We do not recommend the use of carriers during RNA isolation. If a carrier is required, please contact Technical Support for more information.

Q9. How much total RNA do I need for library generation?
Trio RNA-Seq can be used with purified total RNA in the range of 500 pg to 50 ng. Input amounts outside this range may produce unsatisfactory and variable results.

Q10. Do I need to perform an rRNA depletion or poly(A) enrichment step before processing samples with Trio RNA-Seq?
rRNA depletion or poly(A) enrichment is not required. The input range of 500 pg to 50 ng refers to total RNA.

Q11. Can I use poly(A) RNA as an alternative to total RNA?
Trio RNA-Seq has not been tested for use with poly(A) RNA.

Q12. Do I need to use high-quality total RNA?
Trio RNA-Seq is designed to work with purified total RNA. When using purified total RNA, samples should be of high molecular weight with little or no evidence of degradation. While it is impossible to guarantee the highest levels of performance when using RNA of lower quality, this system should allow the successful analysis of a wide range of samples. For FFPE or degraded samples, we recommend using total RNA inputs of 10–50 ng. With highly degraded samples, users may experience reduced sequencing metrics.

Q13. Do you recommend DNase treatment of purified total RNA samples?
Yes. When using purified total RNA samples, large amounts of contaminating genomic DNA may amplify during the process. For this reason DNase treatment is incorporated into the workflow.

Q14. Can I skip DNase treatment if I already incorporated DNase treatment during RNA isolation?
Residual amounts of genomic DNA can be amplified in final libraries and impact sequencing results. For this reason we recommend performing DNase treatment as a part of the Trio RNA-Seq workflow, even if samples have already been treated.

Q15. Can I use Trio RNA-Seq with RNA from any organism?
Trio RNA-Seq has been designed for use with total RNA isolated from a broad range of different organisms. The system requires obtaining organism-specific probes to target specific transcripts for depletion. See Table 2 in the User Guide for a list of available probe sets, or contact NuGEN Technical Support at techserv@nugen.com for more information on custom probe designs.

General Workflow

Q16. Can I perform fewer than 4 or 8 reactions at a time?
We recommend a minimum batch size of 4 reactions for an 8-reaction kit, and 8 reactions for a 32- or 96-reaction kit. Smaller batch sizes may result in difficulty pipetting small volumes and lead to poor performance. In addition, this ensures sufficient reagent recoveries for the full number of reactions in the kit. Making master mixes for fewer than 4 or 8, respectively, samples at a time may affect reagent recovery volumes.

Q17. Does Trio RNA-Seq deplete ribosomal RNA?
Yes. Trio RNA-Seq features NuGEN's targeted depletion technology, AnyDeplete, which can be customized to any transcript, any organism.

Q18. Can I modify the number of PCR amplification cycles recommended by Trio RNA-Seq when using different RNA input amounts?
Generally speaking, fewer PCR cycles will be needed when working with larger input amounts. See Table 12 of the User Guide for guidelines on the number of cycles to use.

Q19. Can I combine the barcoded libraries prior to the PCR amplification step?
This is not recommended. The stoichiometry of barcoded libraries may be adversely affected by this modification to the workflow. We suggest that the libraries be amplified and quantitated independently before being pooled for use on the sequencer.

Q20. Are Trio RNA-Seq libraries stranded?
No, libraries prepared with Trio RNA-Seq are not stranded.

Q21. Where can I safely stop in the protocol?
It is safe to stop after SPIA Amplification, Adaptor Ligation Purification, Library Amplification I, Library Amplification II and Library Amplification II Purification. After SPIA Amplification and Library Amplification II, store reaction products only at 4 °C overnight.

VII. Appendix

SPRI Bead Purifications

Q22. What is the difference between RNAClean XP and AMPure XP SPRI beads? Can both be used interchangeably?
RNAClean XP beads are certified to be RNase and DNase free. We have tested both RNAClean XP and AMPure XP beads in our kits and observe no difference in performance between products.

Q23. What magnetic separation devices do you recommend for the SPRI bead purifications?
Due to the large number of commercially available magnets, we do not have a comprehensive list of compatible products. However, many magnets are compatible. As long as the magnet is strong enough to clear the solution of magnetic beads, it can be applied to the system. We have the following guidelines for selecting a magnetic separation device:

a. Use a magnet designed for 0.2 mL tubes (PCR tubes), tube strips, or plates. Compared to magnets that are designed for 1.5 mL tubes, these minimize loss that can occur when samples are transferred from one tube to another.

b. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated.

c. Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the NuGEN workflow. This is also helpful to gain familiarity with the purification workflow.

Q24. How can I ensure maximum recovery of sample from the SPRI bead purification?

a. Allow the SPRI beads to reach room temperature before use; cold beads result in lower yields.

b. Ensure that the beads are fully resuspended in solution before adding to the sample.

c. Always use fresh ethanol during the washing steps. When preparing the ethanol, measure out the ethanol and water separately to ensure the desired ethanol concentration is obtained.

d. Mix the bead suspension and sample thoroughly to ensure maximum binding of the samples to the beads.

Library Quantification and Qualification

Q25. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?
Please refer to section V.II. of the User Guide for guidelines on quantitative and qualitative assessment. We recommend using a qPCR based-method in combination with the Agilent Bioanalyzer or Tapestation for the most accurate quantification.

VII. Appendix

Q26. How many bases do the Trio RNA-Seq adaptors add to the library?
The adaptors add 122 bp to the library.

Q27. What is the expected library size?
Trio RNA-Seq libraries generated with high-quality human total RNA are 300 bp on average.

Q28. What sequencers are compatible with your libraries?
Trio RNA-Seq libraries are compatible with Illumina sequencing platforms.

Q29. What kind of sequencing primers can I use with your library?
Trio RNA-Seq is designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications. Illumina sequencing primers are used for multiplex sequencing.

Q30. Can Trio RNA-Seq be used with paired-end sequencing?
Yes, Trio RNA-Seq can be used for both single end and paired-end sequencing. Special consideration should be given to the expected insert size in the paired-end assay. The expected distances between the 5'-most and 3'-most coordinates of paired-end reads will depend on the average fragment size of the insert pool.

Q31. How much material should I load into the sequencer?
Please follow manufacturer's recommendations for library QC, quantitation, balancing and loading of the amplified library on the sequencer.

Q32. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Each barcode is a minimum edit distance of 3 from any other barcode. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one barcode becomes a different barcode. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012), Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

Q33. What percentage of rRNA reads can I expect in my data?
The number of rRNA reads present in the sequencing results is dependent on the abundance of rRNA transcripts in the starting material. For a sample containing 10% mRNA and 90% rRNA, a 90% depletion of rRNA transcripts results in a sample containing 53% mRNA and 47% rRNA (i.e. 10% and 9% of the original pool of RNA, respectively).

Custom AnyDeplete

Custom depletion designs can be tailored to any transcript, any organism. Please contact techserv@nugen.com for more information.

VII. Appendix

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