USER GUIDE

Ovation® SoLo RNA-Seq Systems

HUMAN PART NO. 0500 (PART NO. 0500 INCLUDES PART NOS. 0407 AND S02218)

MOUSE PART NO. 0501 (PART NO. 0501 INCLUDES PART NOS. 0407 AND S02239)

DROSOPHILA PART NO. 0502 (PART NO. 0502 INCLUDES PART NOS. 0407 AND S02240)

AND CUSTOMS

M01406 v4

NuGEN®

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Contents

I. Introduction

A. Background

The Ovation SoLo RNA-Seq System provides an end-to-end solution for strand-specific RNA-Seq library construction using either cell lysates or isolated RNA. Starting with as little as 1–500 cells or 10 pg – 10 ng of total RNA from a range of sample types, including FFPE tissue, the Ovation SoLo RNA-Seq System provides a solution for a range of low input applications. The Ovation SoLo RNA-Seq System also integrates NuGEN's AnyDeplete technology (formerly InDA-C) to provide targeted depletion of unwanted transcripts, resulting in a significant reduction in sequencing reads derived from rRNA and any other targeted transcripts for more efficient use of sequencing resources. The system also includes an 8 bp barcode for a high degree of sample multiplexing followed by an 8 bp randomer for identification of unique molecules.

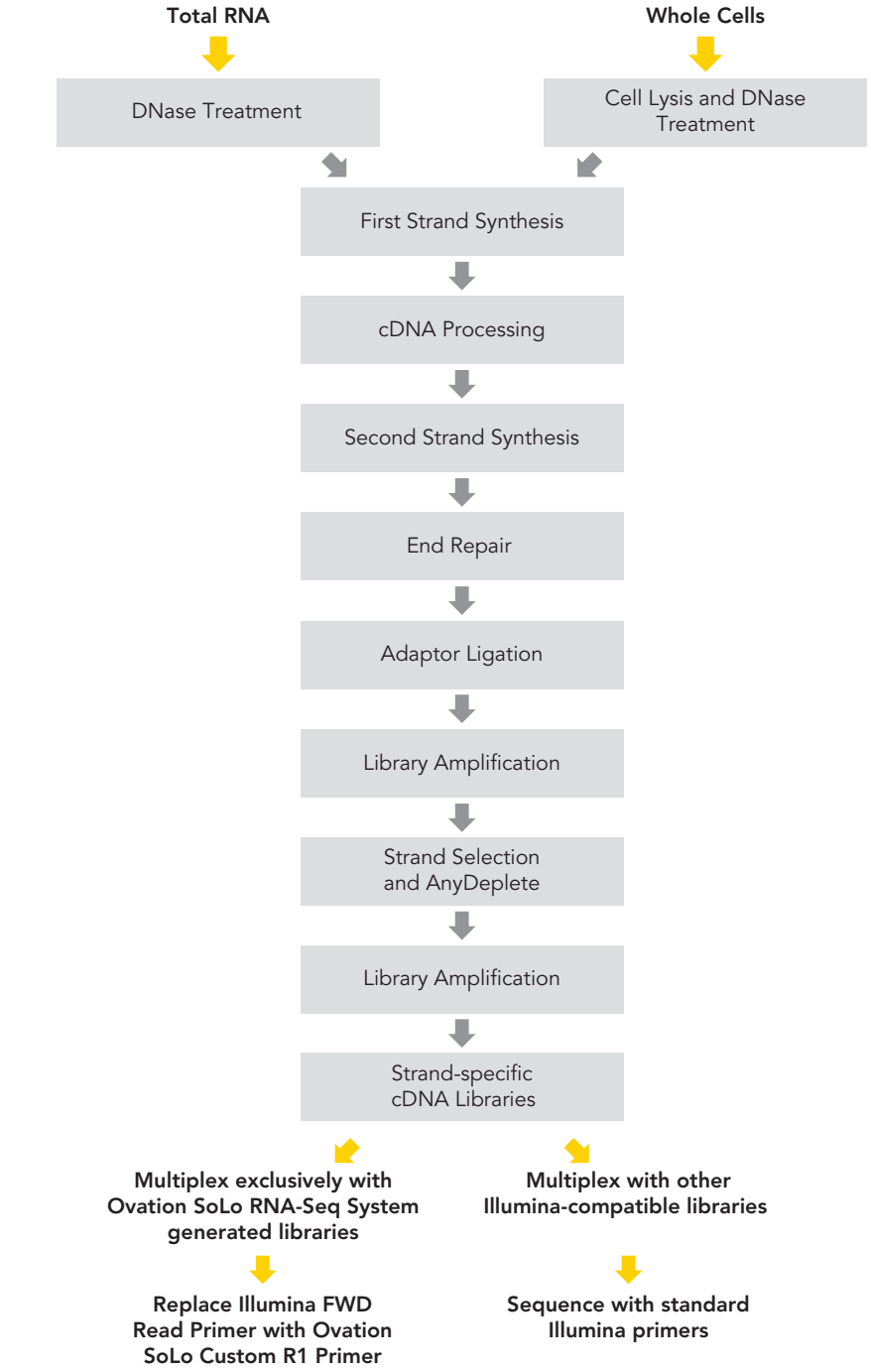
The Ovation SoLo RNA-Seq Systems, Part Nos. 0500, 0501 and 0502, each provide sufficient reagents for the construction of RNA-Seq libraries, with depletion of human, mouse or Drosophila rRNA, respectively. Also included are barcoded adaptors to enable flexible multiplexing for whole transcriptome sequencing.

B. Workflow

The Ovation SoLo RNA-Seq System begins with total RNA or whole cell inputs and generates sequencing ready libraries compatible with Illumina platforms (Figure 1).

I. Introduction

Figure 1. Ovation SoLo RNA-Seq System workflow.



I. Introduction

C. Performance Specifications

The Ovation SoLo RNA-Seq System is designed to generate RNA-Seq libraries suitable for either single read or paired-end sequencing on Illumina sequencing platforms. It is a robust system compatible with a range of whole cell or total RNA inputs. Starting with as little as 1 cell or 10 picograms of total RNA up to 500 cells or 10 ng of total RNA, the Ovation SoLo RNA-Seq System generates Illumina-compatible libraries in about 15 hours. Multiple optional stopping points are provided during the workflow to allow for flexibility.

D. Quality Control

Every lot of the Ovation SoLo RNA-Seq System undergoes functional testing to meet specifications for library generation performance.

E. Storage and Stability

The Ovation SoLo RNA-Seq System is shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product contains components with multiple storage temperature requirements.

- Lysis Buffer (purple cap) should be removed from the top of the shipping carton upon delivery and stored at room temperature.
- All other components should be stored at –20 °C on the internal shelves of a freezer without a defrost cycle.

The kit 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)

An SDS for this product is available on the NuGEN website at www.nugen.com/products/ovation-solo-rna-seq-system

II. Components

A. Reagents Provided

Table 1. Ovation SoLo RNA-Seq System Core Kit reagents (Part Nos. 0407-32, 0407-96 and 0407-A01)

COMPONENT	PART NUMBER 0407-32	PART NUMBER 0407-96	PART NUMBER 0407-A01	VIAL LABEL	VIAL NUMBER
Lysis Buffer	S02194	S02537	S02537	Purple	VER 2
DTT Solution	S02195	S02538	S02538	Purple	VER 1
RNase Inhibitor	S02196	S02539	S02539	Purple	—
DNase Buffer	S02197	S02540	S02540	Clear	VER 1
HL-dsDNase	S02198	S02458	S02458	Clear	—
First Strand Primer Mix	S02199	S02541	S02541	Blue	A1 VER 17
First Strand Buffer Mix	S02200	S02542	S02542	Blue	A2 VER 12
First Strand Enzyme Mix	S02201	S02378	S02378	Blue	A3 VER 6
cDNA Processing Enzyme I	S01884	S02543	S02543	Orange	DP1
cDNA Processing Enzyme II	S01885	S02544	S02544	Orange	DP2
cDNA Processing Reagent I	S02202	S02545	S02597	Orange	DP3 VER 2
cDNA Processing Reagent II	S02203	S02546	S02598	Orange	DP4 VER 2
cDNA Processing Reagent III	S02204	S02547	S02599	Orange	DP5
cDNA Processing Enzyme III	S02205	S02548	S02600	Orange	DP6
cDNA Processing Reagent IV	S02206	S02549	S02601	Orange	DP7
cDNA Processing Enzyme IV	S02207	S02550	S02602	Orange	DP8

II. Components

Ovation SoLo RNA-Seq System Core Kit reagents (Part Nos. 0407-32, 0407-96 and 0407-A01), *continued*					
COMPONENT	**PART NUMBER 0407-32**	**PART NUMBER 0407-96**	**PART NUMBER 0407-A01**	**VIAL LABEL**	**VIAL NUMBER**
Second Strand Buffer Mix	S02208	S02551	S02587	Yellow	B1 VER 10
Second Strand Primer Mix	S02209	S02552	S02588	Yellow	B4 VER 4
Second Strand Enzyme	S02210	S02553	S02589	Yellow	B2 VER 3
End Repair Enzyme Mix	S02211	S02554	S02603	Blue	ER2 VER 6
End Repair Enzyme Mix II	S02222	S02555	S02604	Blue	ER6 VER 1
Ligation Buffer Mix	S02212	S02556	S02605	Yellow	L1 VER 4
Ligation Enzyme Mix	S01848	S02557	S01690	Yellow	L3 VER 4
Amplification Buffer Mix	S01893	S02559	S02606	Red	P1 VER 4
Amplification Enzyme Mix	S01895	S02560	S02560	Red	P3 VER 2
Amplification Primer Mix I	S02216	S02561	S02608	Red	P2 VER 15
Amplification Primer Mix II	S02217	S02562	S02609	Red	P5 VER 2
Strand Selection Enzyme Mix II	S01738	S02563	S02563	Purple	SS4
AnyDeplete Enzyme Mix II	S02220	S02564	S02611	Purple	AD3 ver 2
32-Plex Adaptor Plate	S02221			—	L2V21DR-BC
96-Plex Adaptor Plate		S02238	S02574	—	L2V21DR-BC
Nuclease-free Water	S01001 (2)	S01001 (1) S01113 (2)		Green	D1
DNA Resuspension Buffer Mix	S01901 (2)	S02558		Clear	DR1
Ovation SoLo Custom R1 Primer	S02225	S02565	S02565	Clear	—

II. Components

Any of the AnyDeplete Probe Mixes listed below may be used in combination with the Ovation SoLo RNA-Seq System Core Kit. The contents of the kit will vary according to which AnyDeplete probes are purchased.

Table 2. Catalog AnyDeplete Probe Mixes

COMPONENT	PART NUMBER 0407-32	PART NUMBER 0407-96 and -A01	VIAL LABEL	VIAL NUMBER
SoLo AnyDeplete Probe Mix, Human	S02218	S02510	Purple	—
SoLo AnyDeplete Probe Mix, Mouse	S02239	S02519	Purple	—
SoLo AnyDeplete Probe Mix, Drosophila	S02240	S02574	Purple	—

II. Components

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.
- **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)
  - Tween® 20 (Sigma-Aldrich, Cat. #P9416)
  - 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
  - Magnetic stand for 0.2 mL strip tubes or plates (Beckman Coulter Cat. #A29164 or A32782; Thermo Fisher Scientific Cat. #12331D, 12027, or 12332D; Promega Cat. #V8351; others)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Cleaning solutions such as RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, Cat. #AM9780) and DNA OFF™ (MP Biomedicals, Cat. #11QD0500)

II. Components

To Order

- Alfa Aesar, www.alfa.com
- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, biotium.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 Requirements

The Ovation SoLo RNA-Seq System is designed to work with direct cell inputs from 1–500* cells, or with high quality total RNA from 10 pg – 10 ng.

1. RNA Quantity

Total RNA input must be between 10 pg and 10 ng. Inputs outside of this range may affect reaction stoichiometry, resulting in sub-optimal libraries. Inputs below 10 pg total RNA may result in insufficient yields depending on the requirements of the analytical platform.

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 a method 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 between 1.8–2.0.

3. RNA Integrity

RNA samples of high molecular weight with little or no evidence of degradation will perform very well with this product. 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. The instrument provides a sensitive and rapid way of confirming RNA integrity prior to processing. 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 is required to minimize adverse effects on assay performance and data quality. The protocol incorporates a DNase treatment for this purpose.

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

The Adaptor Plate included with the Ovation SoLo RNA-Seq System contains adaptor mixes, each with a unique eight-base barcode. Each well (first 32 wells, A01–H04, or all

* Direct cell inputs may be need to be optimized depending on cell type.

96 wells, respectively) contains sufficient volume for preparation of a single library. The Ovation SoLo RNA-Seq System 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 41.

C. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

D. Sequencing Recommendations and Guidelines

The Ovation SoLo RNA-Seq System produces RNA-Seq libraries compatible with Illumina NGS platforms. Ovation SoLo libraries contain an 8 bp barcode followed by 8 random bases to allow multiplexed sequencing and identification of unique molecules. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer. The barcode sequences must be entered into the Illumina software prior to parsing and analysis, as detailed in section E. These barcodes differ from the sequences used by Illumina and can be found in Appendix A.

Multiplexing Guidelines

The barcodes in the Ovation SoLo RNA-Seq System are color balanced by column. Libraries should be prepared with 8 or more samples at a time using at least one full column (8 samples) of barcodes. The Ovation SoLo RNA-Seq System offers two approaches to multiplex sequencing, as shown in Figure 2, to allow flexibility in experimental design.

Figure 2. Multiplexing strategy used by the Ovation SoLo RNA-Seq System

The Ovation SoLo RNA-Seq System libraries can be multiplexed and sequenced with other Illumina compatible libraries using the standard Illumina forward read primer (A) or multiplexed and sequenced using a custom read 1 primer supplied with the kit (B). Sequencing with custom primers may not be supported on all Illumina platforms. Please follow the custom primer recommendations for your specific sequencer. Sequence the forward read with either the standard Illumina sequencing primer OR the Ovation SoLo Custom R1 Primer as described below. Continue with standard Illumina primers for the index and reverse reads.

A. For Ovation SoLo RNA-Seq System libraries multiplexed with non-Ovation SoLo RNA-Seq System libraries, use the standard Illumina forward sequencing primer according to recommended protocols. Use no more than 60% of the flow cell for Ovation SoLo RNA-Seq System libraries.

B. For dedicated Ovation SoLo RNA-Seq System sequencing runs, use the Ovation SoLo Custom R1 primer, provided at 100 µM. Dilute 1:200 with hybridization buffer for use during the forward read (R1). Do not use the Illumina standard R1 sequencing primer nor a PhiX control.

Index Read Recommendations

The SoLo RNA-Seq System uses 8-base barcodes for sample multiplexing. In addition to the 8-base barcode, the index is immediately followed by 8 random bases, which can be used for duplicate read determination using the NuGEN Duplicate Marking Tool, NuDup (nugentechnologies.github.io/nudup/). Capturing this information is optional for customers interested in identifying duplicates that arise from PCR. The libraries should be sequenced using 16 cycles for the index read. The 8-base barcode sequences can be found in Appendix A.

E. Parsing and Generating an index.fastq File For De-duplication

Illumina does not provide a simple way to obtain the sequence information contained in the 16-base pair index read including the 8 random bases that are necessary for optional duplicate read determination. NuGEN's recommended methods to generate the necessary index fastq file are provided below.

MiSeq Instrument

During parsing, the built-in MiSeq software replaces the barcode sequence from each library with a numerical substitute, which removes the duplicate information provided by the N8 sequence present after the barcode. To retrieve this information using the MiSeq instrument, we recommend modification of the MiSeq config file to allow generation of an index fastq file during data analysis. This will generate a 16-base index file that is compatible with the NuDup script.

If you are unfamiliar with editing the config file, we recommend you request assistance from Illumina Technical Support to make this modification.

The steps are as follows:

1. Stop the MiSeq Reporter process.
2. Locate the "MiSeq Reporter.exe.config" file located in C:/Illumina/MiSeq Reporter
3. Open config file and search for a line that reads:

   `"<add key="CreateFastqForIndexReads" value="0"/>".`

   - If this line is present, change the value from "0" to "1".
   - If this line is not present, add the line to the config file using the add keys function under the appSettings tab with the value set as "1".
4. Restart the MiSeq reporter process.
5. Requeue the run for data analysis if required. The 8-base barcodes followed by NNNNNNNN should be entered into the sample sheet to enable proper multiplex library parsing.

HiSeq and NextSeq Systems

When setting up a HiSeq or NextSeq run, specify 16 bases of index sequencing (no sample sheet is required). However, if you wish to include a sample sheet, specify only the 8 bases of the actual barcode. Do not include the N8 in your sample sheet.

Use the method described below to parse and generate index fastq files for HiSeq and NextSeq sequencers. This workflow uses bcl2fastq2 version 2.17.

1. Browse to the location of the run folder (called "RunFolder" in this example).
2. Run bcl2fastq2. Use the "`--use-bases-mask Y*,I8Y*`" option to generate an index fastq file along with the forward read (for paired end reads use "`--use-bases-mask Y*,I8Y*,Y*`").

   For example:

```
/usr/local/bin/bcl2fastq --runfolder-dir . --output-dir ./
Data/Intensities/BaseCalls/ --no-lane-splitting --sample-
sheet SampleSheet.csv --use-bases-mask Y*,I8Y* --minimum-
trimmed-read-length 0 --mask-short-adapter-reads 0
```

This combines data for all lanes, creating one fastq file for the forward read, one fastq file for the index read, and one fastq file for the reverse read (if applicable) for each barcode.

Note: To generate the read and index fastq files without parsing, modify the --use-bases-mask option to "`--use-bases-mask Y*,Y*`". The generated fastq files can then be parsed using alternative software. In this case, the index fastq file will be labeled "R2" rather than "I1".

For parsing Ovation SoLo libraries with other Illumina sequencers, please contact NuGEN Technical Support.

F. 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. After parsing, quality trimming, and alignment of reads, the N8 sequence can be used to identify unique molecules. For more information on unique molecule identification with NuDup, see Appendix D.

Note: For data analysis, we recommend trimming the first 5 bp from the 5' end of the forward read. If these bases are not trimmed from the forward read, poor alignment may result.

IV. Overview

A. Overview

The library preparation process used in the Ovation SoLo RNA-Seq System is performed in the following stages. Review detailed protocol for optional stopping points.

1. DNase treatment and primer annealing	0.5 hours
2. First strand cDNA synthesis	1.0 hours
3. cDNA processing	3.0 hours
4. Second strand synthesis	1.0 hours
5. End repair	1.0 hours
6. Adaptor ligation and purification	2.0 hours
7. Library amplification I and purification	3.0 hours
8. AnyDeplete	1.0 hours
9. Library amplification II and purification	2.25 hours
Total time to prepare amplified library	**14.75 hours (5.5 hours hands-on)**

B. Protocol Notes

- We recommend the routine use of a positive control total RNA with input amounts of 100 pg, especially the first time you set up a reaction. The use of a positive control RNA will establish a baseline of performance and provide the opportunity to familiarize yourself with the bead purification steps. These steps 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 no-template control (NTC) is recommended to monitor the work environment for potential nucleic acid contamination or carryover of previous libraries. NTC samples may yield non-specific amplification products that are visible on a Bioanalyzer trace or gel. Contact NuGEN Technical Support for recommendations regarding library validation.
- Set up no fewer than eight reactions at a time. This ensures sufficient reagent recoveries for the full nominal number of amplifications from the kit. Making master mixes for fewer than eight samples at a time may affect reagent recovery volumes.
- When preparing master mixes, use a minimal amount of extra material to ensure 32 reactions in the kit.
- Thaw components used in each step and immediately place them on ice. It is best not to thaw all reagents at once.

- Use the water provided with the kit (green: **D1**) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Buffers, primer mixes and adaptor mixes may be thawed at room temperature prior to placing on ice. Once thawed, gently vortex and spin briefly to collect the contents. Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme mixes.
- 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. Do not warm any enzyme or primer mixes.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer unless otherwise instructed.
- Mix all solutions thoroughly by pipetting as instructed in the text. For best results, use a multichannel pipettor to mix the sample after the addition of each reagent or master mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.
- 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 cDNA purification protocols. Make the ethanol mixes fresh daily. 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

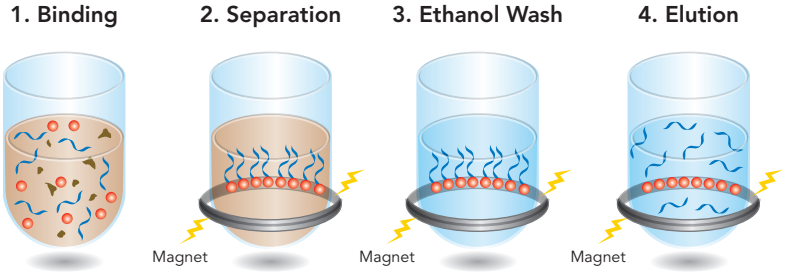
There are significant modifications to the Agencourt beads standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads.

The bead purification processes used in this kit consist of the following steps:

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 of bound DNA from beads

IV. Overview

Figure 3. Agencourt bead purification process overview.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes before use to ensure that they have completely reached room temperature. Cold beads reduce recovery.
- Fully resuspend the beads by inverting and tapping before adding to sample.
- Note that the ratio of bead volume to sample volume varies at each step of the protocol. The bead:sample ratios used differ from the standard Agencourt protocol.
- It is critical to let the beads separate on the magnet for a full 5 minutes. Removing binding buffer before the beads have completely separated will impact DNA yields.
- After completing the binding step, 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.
- Any significant loss of beads during the ethanol washes will impact DNA yields, so make certain to minimize bead loss throughout the procedure.
- Ensure that the ethanol wash is freshly prepared from fresh ethanol stocks at the indicated concentration. 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 sample wells or tubes in a small ring. It is critical that all residual ethanol be removed prior to continuing with the next step. 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 the time specified in the protocol, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding.
- We strongly recommend that strip tubes or partial plates be firmly placed when used with the magnetic plate. We do not advise the use of individual tubes as they are difficult to position stably 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 a 100 µL reaction volume capacity. 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 to 100 °C only when sample temperature reaches above 30 °C. For thermal cyclers with a fixed temperature heated lid (e.g., ABI GeneAmp® PCR 9600 and 9700 models), use the default settings (typically 100–105 °C).

Table 3. Thermal cycler programming

CELL LYSIS (OPTIONAL)		
Program 1 Cell Lysis	70 °C – 2 min, hold at 4 °C	15.6 µL/reaction
DNase TREATMENT AND PRIMER ANNEALING		
Program 2 DNase Treatment and Primer Annealing	37 °C – 10 min, 65 °C – 5 min, hold at 4 °C	18 µL/reaction
FIRST STRAND cDNA SYNTHESIS		
Program 3 First Strand cDNA Synthesis	25 °C – 5 min, 40 °C – 30 min, 70 °C – 10 min, hold at 4 °C	20 µL/reaction
cDNA PROCESSING		
Program 4 cDNA Processing I	37 °C – 30 min, hold at 4 °C	21 µL/reaction
Program 5 cDNA Processing II	90 °C – 20 min, hold at 4 °C	23 µL/reaction
Program 6 cDNA Processing III	37 °C – 30 min, 70 °C – 10 min, hold at 4 °C	27 µL/reaction

Thermal cycler programming, *continued*

Program 7 cDNA Processing IV	37 °C – 30 min, 75 °C – 20 min, hold at 4 °C	35 µL/reaction
SECOND STRAND SYNTHESIS		
Program 8 Second Strand Synthesis	25 °C – 15 min, 37 °C – 15 min, 70 °C – 10 min, hold at 4 °C	43 µL/reaction
END REPAIR		
Program 9 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	45 µL/reaction
ADAPTOR LIGATION		
Program 10 Adaptor Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	65 µL/reaction
LIBRARY AMPLIFICATION qPCR		
Program 11 Library Amplification qPCR	70 °C – 10 min, 35 cycles (94 °C – 30 s, 60 °C – 30 s, 72 °C – 1 min); 72 °C – 5 min, hold at 4 °C	10 µL/reaction
LIBRARY AMPLIFICATION I		
Program 12 Library Amplification I	70 °C – 10 min, n* cycles (94 °C – 30 s, 60 °C – 30 s, 72 °C – 1 min); 72 °C – 5 min, hold at 10 °C	50 µL/reaction
ANYDEPLETE		
Program 13 Probe Binding	37 °C – 10 min, 95 °C – 2 min, 50 °C – 1 min, 65 °C – 10 min, hold at 4 °C	25 µL/reaction
Program 14 Targeted Depletion	55 °C – 30 min, 95 °C – 5 min, hold at 4 °C	50 µL/reaction
LIBRARY AMPLIFICATION II		
Program 15 Library Amplification II	95 °C – 2 min, 2 cycles (95 °C – 30 s, 60 °C – 90 s); 6 cycles (95 °C – 30 s, 65 °C – 90 s); 65 °C – 5 min, hold at 4 °C	100 µL/reaction

*The precise number of PCR cycles required depends on a number of factors including sample type, quality and input, and should be determined empirically each type a new sample type or input is used.

V. Protocol

The Ovation SoLo RNA-Seq System is designed to work with whole cell inputs or total RNA. Process 8 samples or more at a time to ensure sufficient reagents for all reactions in the kit.

- **For cell input**, start with 1–500* cells. Sort cells directly into lysis buffer as described in the protocol, or resuspend cell pellets into lysis buffer.

  Begin the protocol at **Section A., DNase Treatment and Primer Annealing — Cell Input**.

- **For total RNA input**, start with 10 pg – 10 ng of total RNA in 10 µL of low-EDTA TE buffer (recommended) or nuclease-free water. For inputs lower than 100 pg, dilute RNA in low-EDTA TE + 0.1% Tween-20 to reduce sample loss due to binding microcentrifuge tubes.

  Begin the protocol at **Section C., DNase Treatment and Primer Annealing-Total RNA Input**.

Important: The Ovation SoLo RNA-Seq System generates sequencing-ready libraries from as little as a single cell or 10 pg total RNA. As such, this system is very sensitive to variations in the protocol as well as to environmental contaminants. Please read the user guide carefully prior to using this system for the first time. Follow all steps in the protocol as written to avoid introducing bias into the samples at any step in the workflow.

Carry out Sections A (DNase Treatment) through H (Adaptor Ligation) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with RNA and DNA decontamination solutions such as RNase Zap and DNA-OFF to avoid the potential introduction of RNases or off-target nucleic acids into sequencing libraries. For more information please refer to Appendix C of this user guide or contact NuGEN Technical Services at techserv@nugen.com, 888.654.6544 or 650.590.3674.

A. DNase Treatment and Primer Annealing — Cell Input

1. Remove DTT Solution (purple: **VER 1**), RNase Inhibitor (purple), DNase Buffer (clear: **VER 1**), HL-dsDNase (clear), First Strand Primer Mix (blue: **A1 VER 17**) and Nuclease-free Water (green: **D1**) from –20 °C storage.
2. Spin down the contents of RNase Inhibitor and HL-dsDNase and place on ice.
3. Thaw the other reagents at room temperature. Mix the DTT Solution, DNase Buffer and First Strand Primer Mix by vortexing, spin and place on ice.

* Direct cell inputs may need to be optimized depending on cell type.

V. Protocol

4. Spin down the Lysis Buffer (purple: VER 2) and place at room temperature.
5. Prepare Cell Lysis Master Mix by combining Lysis Buffer, DTT Solution and RNase Inhibitor according to the volumes shown in Table 4. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 4. Cell Lysis Master Mix (volumes listed are for a single reaction)

LYSIS BUFFER (PURPLE: VER 2)	DTT SOLUTION (PURPLE: VER 1)	RNase INHIBITOR (PURPLE)
2.8 µL	1.7 µL	0.5 µL

Thorough mixing of cells in Lysis Master Mix is crucial for efficient cell lysis

6. Resuspend pellets of up to 500* cells in 5 µL of Cell Lysis Master Mix or sort cells directly into 5 µL of Cell Lysis Master Mix. For cell sorting workflows do not exceed 1 µL of sheath fluid carryover per 5 µL of Cell Lysis Master Mix. Once sorting is complete, spin down plates for 1 minute at 1000 x g.
7. Mix cells thoroughly by pipetting up and down at least 10 times with a pipettor set to 4 µL. Continue immediately or freeze lysed cells at –80 °C.

Optional stopping point.

8. Add 7 µL of nuclease-free water to lysed cell pellets for a total volume of 12 µL. For sorted cells, add nuclease-free water to lysed cells up to 12 µL. Mix cells thoroughly by pipetting up and down at least 10 times with a pipettor set to 10 µL.
9. Prepare Primer Annealing Master Mix by combining First Strand Primer Mix and DNase Buffer according to the volumes shown in Table 5. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 5. Primer Annealing Master Mix (volumes listed are for a single reaction)

DNase BUFFER (CLEAR: VER 1)	FIRST STRAND PRIMER MIX (BLUE: A1 VER 17)
1 µL	2.6 µL

10. Add 3.6 µL of Primer Annealing Master Mix to each sample tube containing 12 µL of cell lysate for a total of 15.6 µL. Mix cells thoroughly by pipetting up and down at least 10 times with a pipettor set to 12 µL. Spin down and place on ice.

* Direct cell inputs may need to be optimized depending on cell type

11. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Cell Lysis; see Table 3):

    70 °C – 2 min, hold at 4 °C

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

13. Prepare DNase Master Mix by combining HL-dsDNase and RNase Inhibitor according to the volumes shown in Table 6. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 6. DNase Master Mix (volumes listed are for a single reaction)

HL-dsDNase (CLEAR)	RNase INHIBITOR (PURPLE)
2 µL	0.2 µL

14. Add 2.2 µL of DNase Master Mix to each sample tube for a total of 17.8 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 12 µL. Spin down and place on ice.

15. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (DNase Treatment and Primer Annealing; see Table 3):

    37 °C – 10 min, 65 °C – 5 min, hold at 4 °C

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

17. Continue immediately to the First Strand cDNA Synthesis protocol.

B. First Strand cDNA Synthesis — Cell Lysate

1. Remove First Strand Buffer Mix (blue: **A2 VER 12**), First Strand Enzyme Mix (blue: **A3 VER 6**) and RNase Inhibitor (purple) from –20 °C storage.

2. Spin down the contents of First Strand Enzyme Mix and RNase Inhibitor and place on ice.

3. Thaw the First Strand Buffer Mix at room temperature, mix by vortexing, spin and place on ice.

4. Prepare a master mix by combining First Strand Buffer Mix, First Strand Enzyme Mix and RNase Inhibitor in a 0.5 mL capped tube, according to the volumes shown in Table 7. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 7. First Strand Master Mix — Cell Lysate (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 12)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 6)	RNase INHIBITOR (PURPLE)
1 µL	1 µL	0.2 µL

5. Add 2.2 µL of First Strand Master Mix to each sample tube for a total of 20 µL. Mix thoroughly by pipetting up and down 10 times with a pipettor set to 16 µL. Spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (First Strand cDNA Synthesis; see Table 3):

   25 °C – 5 min, 40 °C – 30 min, 70 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately to **Section E., cDNA Processing**, or store at –20 °C.

Optional stopping point.

C. DNase Treatment and Primer Annealing — Total RNA Input

1. Remove DNase Buffer (clear), HL-dsDNase (clear), First Strand Primer Mix (blue: **A1 VER 17**), DTT Solution (purple: **VER 1**) and Nuclease-free Water (green: **D1**) from –20 °C storage.
2. Spin down the contents of HL-dsDNase and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, spin and place on ice.
4. Prepare DNase Treatment and Primer Annealing Master Mix by combining DNase Buffer, First Strand Primer Mix, DTT Solution, nuclease-free water and HL-dsDNase according to the volumes shown in Table 8. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 8. DNase Treatment and Primer Annealing Master Mix (volumes listed are for a single reaction)

DNase BUFFER (CLEAR: VER 1)	FIRST STRAND PRIMER MIX (BLUE: A1 VER 17)	DTT SOLUTION (PURPLE: VER 1)	NUCLEASE-FREE WATER (GREEN: D1)	HL-dsDNase (CLEAR)
1 µL	2.6 µL	1.7 µL	0.7 µL	2 µL

5. Add 8 µL of DNase Treatment and Primer Annealing Master Mix to each sample tube containing 10 µL of RNA for a total volume of 18 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 14 µL. Spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (DNase Treatment and Primer Annealing; see Table 3):

   37 °C – 10 min, 65 °C – 5 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately to the First Strand cDNA synthesis protocol.

D. First Strand cDNA Synthesis — Total RNA Input

1. Remove First Strand Buffer Mix (blue: **A2 VER 12**) and First Strand Enzyme Mix (blue: **A3 VER 6**) from –20 °C storage.
2. Spin down the contents of First Strand Enzyme Mix and place on ice.
3. Thaw the First Strand Buffer Mix at room temperature, mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining First Strand Buffer Mix and First Strand Enzyme Mix in a 0.5 mL capped tube according to the volumes shown in Table 9. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 9. First Strand Master Mix — Total RNA (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 12)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 6)
1 µL	1 µL

5. Add 2 µL of First Strand Master Mix to each sample tube for a total of 20 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 16 µL. Spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (First Strand cDNA Synthesis; see Table 3):

   25 °C – 5 min, 40 °C – 30 min, 70 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately to the cDNA Processing protocol or store at –20 °C.

Optional stopping point.

E. cDNA Processing

1. Remove cDNA Processing Enzyme I (orange: **DP1**), cDNA Processing Enzyme II (orange: **DP2**), cDNA Processing Reagent I (orange: **DP3 VER 2**) and cDNA Processing Reagent II (orange: **DP4 VER 2**) from –20 °C storage.
2. Spin down contents of cDNA Processing Enzyme I and cDNA Processing Enzyme II and place on ice.
3. Thaw cDNA Processing Reagent I and cDNA Processing Reagent II at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining cDNA Processing Enzyme I and cDNA Processing Enzyme II in a 0.5 mL capped tube, according to the volumes shown in Table 10. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 10. cDNA Processing Enzyme Master Mix (volumes listed are for a single reaction)

cDNA PROCESSING ENZYME I (ORANGE: DP1)	cDNA PROCESSING ENZYME II (ORANGE: DP2)
0.5 µL	0.5 µL

5. Add 1 µL of the cDNA Processing Enzyme Master Mix to each sample tube for a total of 21 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 17 µL. Spin down and place on ice.

The cDNA Processing Enzyme Master Mix is viscous. Pipet slowly and mix well.

6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (cDNA Processing I; see Table 3):

   37 °C – 30 min, hold at 4 °C

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

8. Add 2 µL of cDNA Processing Reagent I (orange: **DP3 VER 2**) to each sample tube for a total of 23 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 18 µL. Spin down and place on ice.

9. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (cDNA Processing II; see Table 3):

   90 °C – 20 min, hold at 4 °C

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

11. Add 2 µL of cDNA Processing Reagent II (orange: **DP4 VER 2**) to each sample tube for a total of 25 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 20 µL. Spin down and place on ice.

12. Remove cDNA Processing Reagent III (orange: **DP5**) and cDNA Processing Enzyme III (orange: **DP6**) from –20 °C storage.

13. Spin down contents of cDNA Processing Enzyme III and place on ice.

14. Thaw cDNA Processing Reagent III at room temperature. Mix by vortexing, spin down and place on ice.

15. Prepare a master mix by combining cDNA Processing Reagent III and cDNA Processing Enzyme III in a 0.5 mL capped tube according to the volumes shown in Table 11. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 11. cDNA Processing III Master Mix (volumes listed are for a single reaction)

cDNA PROCESSING REAGENT III (ORANGE: DP5)	cDNA PROCESSING ENZYME III (ORANGE: DP6)
1 µL	1 µL

16. Add 2 µL of the cDNA Processing III Master Mix to each sample tube for a total of 27 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 22 µL. Spin down and place on ice.

17. Place the tubes in a pre-warmed thermal cycler programmed to run Program 6 (cDNA Processing III; see Table 3):

    37 °C – 30 min, 70 °C – 10 min, hold at 4 °C

18. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
19. Remove cDNA Processing Reagent IV (orange: **DP7**) and cDNA Processing Enzyme IV (orange: **DP8**) from –20 °C storage.
20. Spin down contents of cDNA Processing Enzyme IV and place on ice.
21. Thaw cDNA Processing Reagent IV at room temperature. Mix by vortexing, spin down and place on ice.
22. Prepare a master mix by combining cDNA Processing Enzyme IV and cDNA Processing Reagent IV in a 0.5 mL capped tube according to the volumes shown in Table 12. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 12. cDNA Processing IV Master Mix (volumes listed are for a single reaction)

cDNA PROCESSING REAGENT IV (ORANGE: DP7)	cDNA PROCESSING ENZYME IV (ORANGE: DP8)
7 µL	1 µL

23. Add 8 µL of cDNA Processing Master Mix IV to sample tube for a total of 35 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 28 µL. Spin down and place on ice.
24. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (cDNA Processing IV; see Table 3):

    37 °C – 30 min, 75 °C – 20 min, hold at 4 °C
25. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
26. Continue immediately to the Second Strand Synthesis protocol.

F. Second Strand Synthesis

1. Remove Second Strand Buffer (yellow: **B1 VER 10**), Second Strand Primer Mix (yellow: **B4 VER 4**) and Second Strand Enzyme Mix (yellow: **B2 VER 3**) from –20 °C storage.
2. Spin down the contents of Second Strand Enzyme Mix and place on ice.
3. Thaw Second Strand Primer Mix and Second Strand Buffer at room temperature. Mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining Second Strand Buffer Mix, Second Strand Primer Mix and Second Strand Enzyme Mix in a 0.5 mL capped tube according

to the volumes shown in Table 13. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 13. Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 10)	SECOND STRAND PRIMER MIX (YELLOW: B4 VER 4)	SECOND STRAND ENZYME (YELLOW: B2 VER 3)
3.5 µL	3.5 µL	1 µL

5. Add 8 µL of the Second Strand Master Mix to each sample tube for a total of 43 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 35 µL. Spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 8 (Second Strand Synthesis; see Table 3):

   25 °C – 15 min, 37 °C – 15 min, 70 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice. Continue immediately to the End Repair protocol.

G. End Repair

1. Remove End Repair Enzyme Mix (blue: **ER2 VER 6**) and End Repair Enzyme Mix II (blue: **ER6 VER 1**) from –20 °C storage.
2. Spin down the contents of both enzyme mixes and place on ice.
3. Prepare a master mix by combining End Repair Enzyme Mix I and End Repair Enzyme Mix II in a 0.5 mL capped tube according to the volumes shown in Table 14. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 14. End Repair Master Mix (volumes listed are for a single reaction)

END REPAIR ENZYME MIX (BLUE: ER2 VER 6)	END REPAIR ENZYME MIX II (BLUE: ER6 VER 1)
1 µL	1 µL

4. Add 2 µL of End Repair Master Mix to each sample tube for a total of 45 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 36 µL. Spin down and place on ice.

5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (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. Continue immediately to the Adaptor Ligation protocol or store at –20 °C.

Optional stopping point.

H. Adaptor Ligation

1. Remove Ligation Buffer Mix (yellow: **L1 VER 4**), Ligation Enzyme Mix (yellow: **L3 VER 4**), the Adaptor Plate (**L2V21DR-BC**) and Nuclease-free Water (green: **D1**) from –20 °C storage. Also remove the Agencourt beads from 4 °C storage and DNA Resuspension Buffer Mix (clear: **DR1**) from –20 °C storage. Place at room temperature for use after Adaptor Ligation.
2. Spin down contents of Ligation Enzyme Mix and Adaptor plate and place on ice.
3. Thaw Ligation Buffer Mix and nuclease-free water at room temperature. Mix by vortexing, spin and place on ice.

Add barcoded adaptor mixes to samples and mix well prior to addition of Ligation Master Mix.

4. Add 3.25 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.
5. Prepare a master mix by combining Ligation Buffer Mix, Ligation Enzyme Mix and nuclease-free water in a 0.5 mL capped tube according to the volumes shown in Table 15. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 15. Ligation Master Mix (volumes listed are for a single reaction)

LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)	NUCLEASE-FREE WATER (GREEN: D1)
13 µL	2 µL	1.75 µL

The Ligation Master Mix is viscous. Pipet slowly and mix well.

6. Add 16.75 µL of the Ligation Master Mix to each sample tube for a total of 65 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 52 µL. Spin down and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 10 (Adaptor Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Adaptor Ligation Purification protocol or store at –20 °C.

Optional stopping point.

I. Adaptor Ligation Purification

Allow Agencourt beads 30 minutes to reach room temperature before proceeding with purification.

1. Ensure the Agencourt beads and DNA Resuspension Buffer Mix (clear: **DR1**) have completely reached room temperature before proceeding. Spin down contents of DNA Resuspension Buffer Mix and leave at room temperature.
2. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples.
3. Add 35 µL of nuclease-free water to each sample for a total of 100 µL.
4. Add 100 µL (1.0 volumes) of the bead suspension to each sample. Mix thoroughly by pipetting up and down.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. 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 Library Amplification I, so ensure beads are not removed with the binding buffer or the wash.

Prepare 70% ethanol daily using fresh ethanol stocks.

8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. 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.

11. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 30 µL of DNA Resuspension Buffer Mix to the dried beads. Mix thoroughly to ensure all beads are resuspended.
14. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
15. 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.
16. Continue immediately to the Library Amplification Optimization qPCR protocol or store at –20 °C.

Optional stopping point.

J. Library Amplification Optimization qPCR

Note: Due to the wide variety of sample type, quantity and quality used with the Ovation SoLo RNA-Seq System, the number of library amplification cycles required for a given sample must be determined empirically. While NuGEN recommends performing this step for every sample, users may proceed immediately from I. Adaptor Ligation Purification to K. Library Amplification I after this step has been optimized. For additional information, please contact NuGEN Technical Support.

1. Remove DNA Resuspension Buffer Mix (clear: **DR1**), Amplification Buffer Mix (red: **P1 VER 4**), Amplification Primer Mix I (red: **P2 VER 15**), Amplification Enzyme Mix (red: **P3 VER 2**), Nuclease-free Water (green: **D1**), and 20X EvaGreen from –20 °C storage.
2. Spin down Amplification Enzyme Mix and place on ice.
3. Thaw DNA Resuspension Buffer Mix, Amplification Buffer Mix, Amplification Primer Mix I, 20X EvaGreen and nuclease-free water at room temperature. Mix by vortexing, spin and place on ice.

4. Add 3 µL of DNA Resuspension Buffer Mix to each sample. Mix thoroughly by pipetting.
5. Prepare a Master Mix by combining Amplification Buffer Mix, Amplification Primer Mix I, Amplification Enzyme Mix, and EvaGreen in a 0.5 mL capped tube according to the volumes shown in Table 16. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the master mix volume. Spin down and place on ice. Use immediately.

Table 16. qPCR Master Mix (volumes listed are for a single reaction)

AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX I (RED: P2 VER 15)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)	20x EvaGreen	NUCLEASE-FREE WATER (GREEN: D1)
2 µL	1.9 µL	0.1 µL	0.5 µL	2.5 µL

6. Aliquot 3 µL of each sample into a fresh set of tubes. Store the remaining 30 µL of sample on ice or at –20 °C.
7. Add 7 µL of qPCR Master Mix to 3 µL of each sample. Mix thoroughly by pipetting, spin down, and place on ice.
8. Load each 10 µL sample into qPCR tubes or plates.
9. Run qPCR on the samples using Program 11 (Library Amplification qPCR, see Table 3):

   70 °C – 10 min, 35 cycles (94 °C – 30 s, 60 °C – 30 s, 72 °C – 1 min); 72 °C – 5 min, hold at 4 °C
10. Visualize the amplification curves as log fluorescence vs. cycle number (i.e. Log Rn vs. Cycle or Log RFU vs. Cycle). The cycle number used for subsequent library amplification should be within the exponential phase of the amplification. For guidance on selecting cycle number, please contact NuGEN Technical Support.
11. Proceed to Library Amplification I.

K. Library Amplification I

1. Remove Amplification Buffer Mix (red: **P1 VER 4**), Amplification Primer Mix I (red: **P2 VER 15**) and Amplification Enzyme Mix (red: **P3 VER 2**) from –20 °C storage. Also remove the Agencourt RNAClean XP Beads from 4 °C storage and DNA Resuspension Buffer Mix (clear: **DR1**) from –20 °C storage. Place at room temperature for use after Library Amplification I.
2. Spin down Amplification Enzyme Mix and place on ice.
3. Thaw Amplification Buffer Mix and Amplification Primer Mix I at room temperature. Mix by vortexing, spin and place on ice.

4. Prepare a master mix by combining Amplification Buffer Mix, Amplification Primer Mix I, Amplification Enzyme Mix and nuclease-free water in a 0.5 mL capped tube according to the volumes shown in Table 17. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 17. Library Amplification I Master Mix (volumes listed are for a single reaction)

AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX I (RED: P2 VER 15)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
10 µL	9.5 µL	0.5 µL

5. Add 20 µL of the Library Amplification I Master Mix to each sample tube for a total of 50 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 40 µL. Spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 12 (Library Amplification I; see Table 3):

   70 °C – 10 min, n* cycles (94 °C – 30 s, 60 °C – 30 s, 72 °C – 1 min); 72 °C – 5 min, hold at 10 °C

   ***Important:** The number of amplification cycles should be determined empirically by the user for each input and sample type.
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Proceed to the Library Amplification I Purification protocol or store at –20 °C.

Optional stopping point.

L. Library Amplification I Purification

Allow Agencourt beads 30 minutes to reach room temperature before proceeding with purification.

1. Ensure the Agencourt beads and DNA Resuspension Buffer Mix (clear: **DR1**) have completely reached room temperature before proceeding. Spin down contents of DNA Resuspension Buffer Mix and leave at room temperature.
2. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples.
3. Add 40 µL (0.8 volumes) of the bead suspension to the Library Amplification I reaction product. Mix thoroughly by pipetting up and down.
4. Incubate at room temperature for 10 minutes.

Prepare 70% ethanol daily using fresh ethanol stocks.

5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove 90 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 forward in the protocol, 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. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
11. Remove the tubes from the magnet.
12. Add 50 µL of DNA Resuspension Buffer Mix 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 50 µL of the eluate, ensuring as few beads as possible are carried over, then transfer to a fresh set of PCR tubes.
15. At room temperature, add 40 µL (0.8 volumes) of the freshly resuspended bead suspension to the eluate. Mix thoroughly by pipetting up and down.
16. Incubate at room temperature for 10 minutes.
17. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
18. Carefully remove 90 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 forward in the protocol, so ensure beads are not removed with the binding buffer or the wash.
19. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
20. Remove the 70% ethanol wash using a pipette.

21. 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. 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.

22. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

23. Remove the tubes from the magnet.

24. Add 25 µL of DNA Resuspension Buffer Mix to the dried beads. Mix thoroughly to ensure all beads are resuspended.

25. Transfer tubes to the magnet and let stand for 3 minutes to clear the solution of beads.

26. Carefully remove 25 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.

27. Continue immediately to Library Quantification or store at –20 °C.

Optional stopping point.

M. Library Quantification

Quantify the library using the Qubit dsDNA HS Assay. Alternatively, a NanoDrop or Bioanalyzer may also be used for library quantification.

N. AnyDeplete

1. Remove Amplification Buffer Mix (red: **P1 VER 4**), AnyDeplete Probe Mix (purple), Strand Selection Enzyme Mix II (purple: **SS4**), Amplification Enzyme Mix (red: **P3 VER 2**) and Nuclease-free Water (green: **D1**) from –20 °C storage.

2. Spin down the Strand Selection Enzyme Mix II and Amplification Enzyme Mix and place on ice.

3. Thaw Amplification Buffer Mix, AnyDeplete Probe Mix and nuclease-free water at room temperature. Mix Amplification Buffer Mix and AnyDeplete Probe Mix by vortexing, spin and place on ice.

4. Aliquot 10 ng of library into a new 0.2 mL capped tube. Store remaining library at –20 °C.

5. Use nuclease-free water to dilute each library to a total volume of 8.5 µL.

6. Prepare a master mix by combining Amplification Buffer Mix, AnyDeplete Probe Mix, Strand Selection Enzyme Mix II and Amplification Enzyme Mix in a 0.5 mL capped tube according to the volumes shown in Table 18. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 18. Probe Binding Master Mix (volumes listed are for a single reaction)

AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	ANYDEPLETE PROBE MIX (PURPLE)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
5 µL	10 µL	0.5 µL	1 µL

7. Add 16.5 µL of the Probe Binding Master Mix to each sample tube for a total of 25 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 20 µL. Spin down and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 13 (Probe Binding; see Table 3):

   37 °C – 10 min, 95 °C – 2 min, 50 °C – 1 min, 65 °C – 10 min, hold at 4 °C
9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Remove Amplification Buffer Mix (red: **P1 VER 4**), AnyDeplete Enzyme Mix II (purple: **AD3 VER 2**) and Nuclease-free Water (green: **D1**) from –20 °C storage.
11. Spin down AnyDeplete Enzyme Mix II and place on ice.
12. Thaw Amplification Buffer Mix and nuclease-free water at room temperature. Mix by vortexing, spin and place on ice.
13. Prepare a master mix by combining Amplification Buffer Mix, AnyDeplete Enzyme Mix II and nuclease-free water in a 0.5 mL capped tube according to the volumes shown in Table 19. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 19. Targeted Depletion Master Mix (volumes listed are for a single reaction)

AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	ANYDEPLETE ENZYME MIX II (PURPLE: AD3 VER 2)	NUCLEASE-FREE WATER (GREEN: D1)
5 µL	2.5 µL	17.5 µL

14. Add 25 µL of the Targetd Depletion Master Mix to each sample tube for a total of 50 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 40 µL. Spin down and place on ice.
15. Place the tubes in a pre-warmed thermal cycler programmed to run Program 14 (Targeted Depletion; see Table 3):

    55 °C – 30 min, 95 °C – 5 min, hold at 4 °C
16. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
17. Continue immediately to the Library Amplification II protocol or store at –20 °C.

Optional stopping point.

O. Library Amplification II

1. Remove Amplification Buffer Mix (red: **P1 VER 4**), Amplification Primer Mix II (red: **P5 VER 2**) and Amplification Enzyme Mix (red: **P3 VER 2**) from –20 °C storage. Also remove the Agencourt beads from 4 °C storage and DNA Resuspension Buffer Mix (clear: **DR1**) from –20 °C storage. Place at room temperature for use after Library Amplification II.
2. Spin down Amplification Enzyme Mix and place on ice.
3. Thaw Amplification Buffer Mix and Amplification Primer Mix II at room temperature. Mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining Amplification Buffer Mix, Amplification Primer Mix II and Amplification Enzyme Mix in a 0.5 mL capped tube according to the volumes shown in Table 20. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume. Spin down and place on ice. Use immediately.

Table 20. Library Amplification II Master Mix (volumes listed are for a single reaction)

AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX II (RED: P5 VER 2)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
10 µL	39.5 µL	0.5 µL

V. Protocol

5. Add 50 µL of the Library Amplification Master Mix I to each sample tube for a total of 100 µL. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 80 µL. Spin down and place on ice.
6. Mix by pipetting, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 15 (Library Amplification II; see Table 3):

   95 °C – 2 min, 2 cycles (95 °C – 30 s, 60 °C – 90 s); 6 cycles (95 °C – 30 s, 65 °C – 90 s); 65 °C – 5 min, hold at 4 °C
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Library Amplification II Purification protocol or store at –20 °C.

Optional stopping point.

P. Library Amplification II Purification

Allow Agencourt beads 30 minutes to reach room temperature before proceeding with purification.

1. Ensure the Agencourt beads and DNA Resuspension Buffer Mix (clear: **DR1**) have completely reached room temperature before proceeding. Spin down contents of DNA Resuspension Buffer Mix and leave at room temperature.
2. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples.
3. At room temperature, add 100 µL (1.0 volumes) of the bead suspension to the Library Amplification II reaction product. 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 yield of final library, so ensure beads are not removed with the binding buffer or the wash.

Prepare 70% ethanol daily using fresh ethanol stocks.

7. With 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. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
11. Remove the tubes from the magnet.
12. Add 50 µL of DNA Resuspension Buffer Mix 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 50 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes.
15. At room temperature, add 50 µL (1.0 volumes) of the freshly resuspended bead suspension to the eluate. Mix thoroughly by pipetting up and down.
16. Incubate at room temperature for 10 minutes.
17. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
18. Carefully remove 100 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 yield, so ensure beads are not removed with the binding buffer or the wash.
19. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
20. Remove the 70% ethanol wash using a pipette.
21. Repeat the 70% ethanol wash one more time, for a total of two washes.

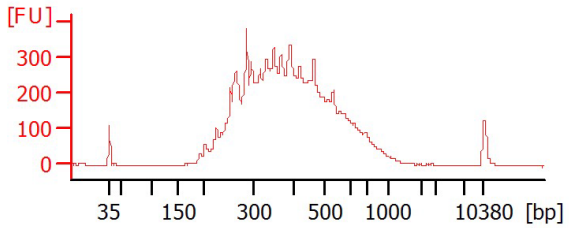
    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Remove the ethanol wash with a pipet, allow excess ethanol to collect at the bottom of the tubes, and remove any remaining ethanol with a fresh pipet tip.
22. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
23. Remove the tubes from the magnet.
24. Add 25 µL of DNA Resuspension Buffer Mix to the dried beads. Mix thoroughly to ensure all beads are resuspended.
25. Transfer tubes to the magnet and let stand for 3 minutes to clear the solution of beads.

26. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over, then transfer to a fresh set of PCR tubes and place on ice.
27. Proceed to Quantitative and Qualitative Assessment of the Library.

Q. 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 is as shown in Figure 4.
2. Quantify the library using a qPCR-based method, such as the KAPA Library Quantification Kit.
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).

Figure 4. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA Chip from 100 pg Universal Human Reference total RNA starting material.



Note: Ovation SoLo RNA-Seq libraries can be prepared for sequencing using the standard Illumina protocol. The forward read should be sequenced using either the standard Illumina sequencing primer OR the SoLo Custom R1 Primer as described in Section III. D., Sequencing Recommendations and Guidelines.

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 sets of 8 by column.

Table 21. Barcode sequences for dedicated read (DR) 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

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Barcode sequences for dedicated read (DR) adaptors, *continued*					
PLATE POSITION	**BARCODE SEQUENCE**	**PLATE POSITION**	**BARCODE SEQUENCE**	**PLATE POSITION**	**BARCODE SEQUENCE**
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 Ovation SoLo RNA-Seq System, 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, NuGEN's First Strand cDNA Synthesis, cDNA Processing, Second Strand cDNA Synthesis, End Repair and Adaptor Ligation. 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.

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 negative 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 'negative' control or No Template Control (NTC).

   In the absence of contamination:

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

   In the presence of contamination:

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

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C. Ovation Solo Lysis Buffer and Cell Lysis Master Mix Preparation

To prepare additional Lysis Buffer and Cell Lysis Master Mix for cell sorting and other workflows, the following reagents are required:

- Water, nuclease-free (Sigma Aldrich)
- Tris HCl, 1M, pH 7.5 or 8.0, RNase-free (ThermoFisher Scientific)
- SDS Solution, 10% w/v, Molecular biology grade (Promega)
- NP-40 Detergent Surfact-Amps™ Solution, 10% w/v NP-40 (ThermoFisher Scientific)
- Dithiothreitol (DTT) Solution, Ultrapure, 0.1M (ThermoFisher Scientific)
- RNase Inhibitor 40 µ/µL (1U=inhibition of 5 ng RNase by 50%) (New England Biolabs)

Prepare Lysis Buffer

1. Prepare 5 mL of Lysis Buffer by combining the reagents shown in Table 22:

Table 22. Ovation SoLo Lysis Buffer ((volumes listed are for 5 mL).

REAGENT	VOLUME	FINAL CONCENTRATION
Nuclease-free water	2.85 mL	—
1M Tris HCl	50 µL	10 mM
0.2 % w/v SDS	100 µL	10 % w/v SDS Solution
10% w/v NP-40 Solution	2 mL	4% w/v NP-40
Total volume	**5 mL**	

2. Mix thoroughly by vortexing. Store at room temperature for up to 6 months. Alternatively, store at –20 °C and minimize freeze-thaws.

Prepare Cell Lysis Master Mix

1. Remove 0.1 M DTT Solution and Lysis Buffer from storage and bring to room temperature.
2. Prepare 11.5 mM DTT by combining 11.5 µL of 0.1 M DTT Solution and 88.5 µL nuclease-free water. Mix well by vortexing and place on ice.
3. Remove RNase Inhibitor from –20 °C storage. Spin down the contents and place on ice.
4. Prepare Cell Lysis Master Mix by combining 2.8 µL of Lysis Buffer, 1.7 µL of 11.5 mM DTT, and 0.5 µL RNase inhibitor per sample. Use immediately.

5. After cell sorting is complete, spin down plates or tubes for 1 minute.
6. Mix cells thoroughly by pipetting up and down at least 10 times with a pipettor set to 4 µL.
7. Continue immediately to section V. Protocol Section A., DNase Treatment and Primer Annealing-Cell Input, step 8, or freeze lysed cells at –80 °C.

D. De-duplication of Ovation SoLo RNA-Seq System Libraries with NuDup

The N8 sequence contained in Ovation SoLo RNA-Seq libraries provides unambiguous identification of unique library molecules using the NuGEN Duplicate Marking tool, NuDup, to discriminate between true PCR duplicates and independent adaptor ligation events to fragments with the same start site. NuDup can be found at nugentechnologies.github.io/nudup/.

Run NuDup using the index.fq file generated in Section III. E. and a SAM or BAM alignment file as input.

For single end reads:

```
python nudup.py -f index.fq -o outputname aligneddata.sam -s 8 -l 8
```

For paired-end reads:

```
python nudup.py -2 -f index.fq -o outputname aligneddata.sam -s
8 -l 8
```

Continue with downstream data analysis.

E. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Ovation SoLo RNA-Seq System?
The Ovation SoLo RNA-Seq System provides all necessary buffers, primers and enzymes necessary for library construction from cell lysates or isolated RNA. The kit also provides DNA Resuspension Buffer for purification elution steps. This kit does not provide EvaGreen used for Library Amplification qPCR nor the Agencourt RNAClean XP or AMPure XP Beads required for the purification steps.

Q2. Can this system be used with other library preparation workflows?
There is no need to combine the workflow with other library preparation workflows. The Ovation SoLo RNA-Seq System is an end-to-end solution designed to generate Illumina-compatible libraries starting from cells or total RNA.

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Q3. Does this system contain a SPIA®-based amplification?
No SPIA-based amplification is used in the Ovation SoLo RNA-Seq System.

Q4. Can I use Ovation SoLo RNA-Seq System with RNA from any organism?
The Ovation SoLo RNA-Seq System should be suitable for total RNA input from any organism. For whole cell inputs, optimization may be required. Contact information NuGEN Technical Support for information on custom targeted transcript depletion.

Q5. Do I need to use high-quality total RNA?
When using with the Ovation SoLo RNA-Seq System, 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 somewhat degraded samples. With such samples, users may experience lower yields and may encounter affected sequencing metrics.

Q6. Do I need to perform an rRNA depletion or Poly(A) enrichment step before processing with the Ovation SoLo RNA-Seq System?
The system is designed to use total RNA as input and utilizes NuGEN's customizable AnyDeplete technology (formerly InDA-C) to deplete targeted transcripts. rRNA depletion or Poly(A) enrichment is not necessary.

Q7. Can I sort my cells into PBS or another buffer prior to beginning the cell lysis protocol?
For best results we recommend sorting cells directly into the Lysis Buffer Master Mix prepared with the kit, or resuspending cell pellets in the Lysis Buffer Master Mix. Other buffers may inhibit reactions in the kit and yield variable sequencing results. Contact NuGEN Technical Support for additional information.

Q8. What cell types are compatible with the lysis buffer provided with the kit?
The lysis buffer has been tested with cell inputs between 1–500 cells and should be compatible with most dissociated cells. Some direct cell inputs may need to be optimized depending on cell type. Decreased performance may be observed with cell types containing excessive lipids, minerals, collagen, etc. or a cell wall. Non-dissociated cells, tissue, or LCM samples may require additional optimization of the workflow.

Q9. Can contaminating genomic DNA interfere with Ovation SoLo RNA-Seq System performance?
Yes, contaminating genomic DNA may be incorporated into libraries. For this reason DNase treatment is incorporated into both the Cell Lysis and Total RNA Input workflows.

Q10. Does cDNA generated with the Ovation SoLo RNA-Seq System require fragmentation?
No, fragmentation of cDNA occurs during the cDNA Processing steps in the protocol.

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Q11. Can I combine the barcoded libraries prior to amplification?
The stoichiometry of barcoded libraries may be adversely affected by this modification to the Ovation SoLo RNA-Seq System workflow. We suggest that the libraries be amplified and quantitated independently by qPCR before being balanced and pooled for use on the sequencer.

Q12. Can I use alternative magnetic separation devices?
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:

1. Use of a magnet designed for 0.2 mL tubes (PCR tubes) can help improve performance. 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.
2. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated. For the Ovation SoLo RNA-Seq System, the minimum is 25 µL and the maximum is 200 µL.
3. 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.

Q13. Where can I safely stop in the protocol?
Samples may be stored at –80 °C after Cell Lysis. Samples may also be placed in short-term storage at –20 °C after First Strand cDNA Synthesis, End Repair, Adaptor Ligation, Library Amplification, or after any purification step.

Q14. Are irregular Bioanalyzer traces normal for libraries generated with the Ovation SoLo RNA-Seq System?
Yes, irregular Bioanalyzer traces are normal for libraries generated with the Ovation SoLo RNA-Seq System.

Q15. What is the average size of the library generated by the Ovation SoLo RNA-Seq System?
Ovation SoLo RNA-Seq libraries are 300–350 bp in length for high-quality human samples and may be shorter for FFPE and other degraded samples. Libraries generated from model organism samples may also be longer or shorter than human libraries. Take care not to overestimate library size for loading on the sequencer.

Q16. How many bases do Ovation SoLo RNA-Seq System adaptors add to the library?
The adaptors add 132 bp to the Ovation SoLo RNA-Seq System library.

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Q17. Is the Ovation SoLo RNA-Seq System compatible with all Illumina sequencing platforms?
Illumina may not support the use of a custom sequencing primer or low diversity libraries on all platforms. Please follow the custom primer and low diversity library recommendations for your specific sequencer.

Q18. Can the Ovation SoLo RNA-Seq System be used with paired-end sequencing?
Yes, it 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 workflow generates library fragments with an average size of 330 bases.

Q19. 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.

Q20. What kind of sequencing primers can I use with your library?
Ovation SoLo RNA-Seq System generated libraries are designed to use standard Illumina sequencing primers for both single end and paired-end sequencing applications when multiplexed with standard Illumina libraries (see Section III.D., Sequencing Recommendations and Guidelines for more information). For dedicated Ovation SoLo RNA-Seq Sequencing runs, the Ovation SoLo Custom R1 primer must be used in place of the Illumina forward read primer. It is provided at a concentration of 100 µM. Due to the use of the Ovation SoLo Custom R1 primer, a PhiX control should not be included in the sequencing run.

Q21. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Our balanced 8-base barcodes differ by at least three nucleotides, so no single base error will result in an incorrect barcode assignment. We recommend that only perfect match barcode reads be used for binning. The sequences of these NuGEN barcodes must be input prior to parsing.

Q22. Do you recommend trimming sequences before downstream analysis?
Yes. We recommend trimming the 5′ end of the sequence as recommended in Section III. F. Data Analysis. This portion of the read corresponds to the overhang of the forward adaptor and may not accurately reflect the biological sequence.

Q23. 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 10% mRNA and 9% rRNA (i.e., the total RNA pool will consist of 53% mRNA and 47% rRNA).

F. Update History

This document, the Ovation SoLo RNA-Seq System user guide (M01406 v4), is an update to address the following topics.

Description	Section	Page(s)
Reorganize and rewrite content for better clarity.	Throughout	Throughout
Changed Insert Dependent Adaptor Cleavage (InDA-C) to AnyDeplete technology	Throughout	Throughout
Added part numbers for A01 reaction kits.	II.A	4–5
Correct typos throughout	Throughout	Throughout

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