USER GUIDE

# Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16

PART NOS. 0326, 0327

NuGEN®

A Tecan Group company

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# I. Introduction

## A. Background

The Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16 provide an end-to-end solution for strand-specific RNA-Seq library construction using as little as 100 ng of prokaryotic total RNA. The core technology in this product enriches for non-rRNA in NGS libraries by selective priming during first strand cDNA synthesis, as well as during the final library construction steps, using a selective adaptor cleavage reaction based on NuGEN's Insert Dependent Adaptor Cleavage (InDA-C) technology. The Systems can be applied to transcriptomes extracted from a broad range of prokaryotes.

The cDNA synthesis is carried out using proprietary primers to create double-stranded cDNA, which retains RNA strand information. The resulting sequencing reads can be aligned to the strand from which the RNA originated, enabling detection of both sense and antisense expression. No dedicated steps are required to reduce rRNA levels. The resulting cDNA is converted to NGS libraries using reagents and adaptors provided in the same kit. The Ovation Complete Prokaryotic RNA-Seq Multiplex Systems provide barcoding to further optimize efficiencies and cost savings in transcriptome sequencing.

The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems have been designed for strand-specific expression analysis by incorporation of a nucleotide analog during the second strand cDNA synthesis and subsequent ligation to a pair of double-stranded adaptors also containing the same analog in one strand. After ligation the cDNA strand and adaptor containing the analog are selectively removed (Strand Selection), leaving only one cDNA strand, with both adaptor sequences attached. This product is then converted into a sequence-ready library by PCR amplification (see Figure 1).

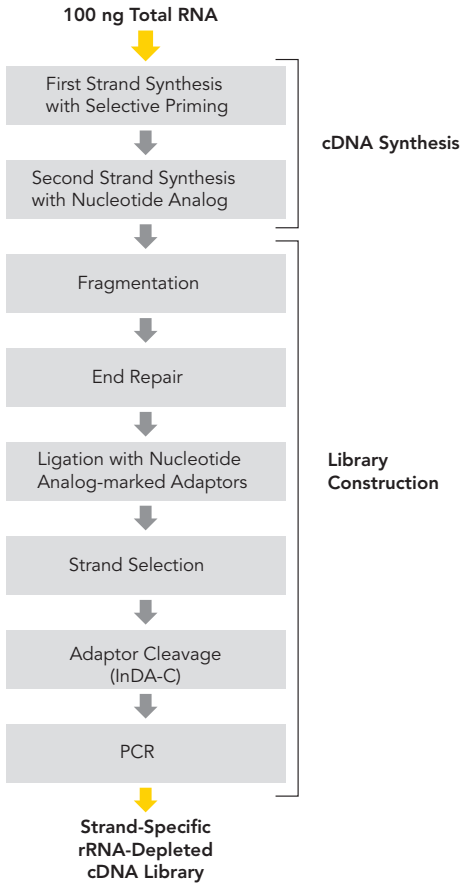
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 (Part No. 0326) and 9–16 (Part No. 0327) each provide eight unique dedicated read barcoded adaptors to prepare libraries for multiplex sequencing using a dedicated read design strategy with a second sequencing primer. In combination these kits enable up to 16-plex sequencing.

## B. Workflow

As shown in Figure 1, the streamlined workflow consists of double-stranded cDNA generation using selective priming, fragmentation of double-stranded cDNA, end repair to generate blunt ends, adaptor ligation, strand selection via nucleotide analog-targeted degradation and PCR amplification to produce the final library. The entire workflow, including fragmentation, can be completed in as few as seven hours, and yields DNA libraries ready for cluster formation for either single read or paired-end sequencing.

# I. Introduction

**Figure 1. Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems workflow.**




## C. Performance Specifications

The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are designed to generate DNA libraries suitable for either single read or paired-end sequencing on Illumina Genome Analyzer IIx/IIe (GAII), HiScan SQ or HiSeq 2000/2500 NGS platforms. They are fast, simple and robust systems capable of starting with 100 ng of total RNA to generate libraries suitable for use on the Illumina cBot Cluster Generation System in about seven hours.

## D. Quality Control

Every lot of the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems undergoes functional testing to meet specifications for library generation performance.

# I. Introduction

## E. Storage and Stability

The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are shipped on dry ice and should be unpacked immediately upon receipt.

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

- Vials labeled Agencourt® Beads (clear cap) should be removed from the top of the shipping carton upon delivery and stored at 4°C.
- All other components should be stored at –20°C on 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)

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

# II. Components

## A. Reagents Provided

**Table 1. Ovation Complete Prokaryotic RNA-Seq DR Multiplex System 1–8 and 9–16 Reagents (Part Nos. 0326-32 and 0327-32)**

| COMPONENT | PART NUMBER | VIAL CAP | VIAL NUMBER |
|---|---|---|---|
| First Strand Primer Mix | S01637 | Blue | A1 VER 10 |
| First Strand Buffer Mix | S01528 | Blue | A2 VER 8 |
| First Strand Enzyme Mix | S01529 | Blue | A3 VER 4 |
| Second Strand Buffer Mix | S01530 | Yellow | B1 VER 6 |
| Second Strand Enzyme Mix | S01531 | Yellow | B2 VER 4 |
| Second Strand Stop Buffer | S01554 | Yellow | B3 VER 2 |
| End Repair Buffer Mix | S01708 | Blue | ER1 VER 7 |
| End Repair Enzyme Mix | S01533 | Blue | ER2 VER 4 |
| End Repair Enhancer | S01709 | Blue | ER3 VER 2 |
| Ligation Buffer Mix | S01534 | Yellow | L1 VER 4 |
| Ligation Adaptor Mix | **0326-32**<br>S01645<br>S01646<br>S01647<br>S01648<br>S01649<br>S01650<br>S01651<br>S01652<br>**0327-32**<br>S01653<br>S01654<br>S01655<br>S01656<br>S01657<br>S01658<br>S01659<br>S01660 | Yellow | **0326-32**<br>L2V8DR-BC1<br>L2V8DR-BC2<br>L2V8DR-BC3<br>L2V8DR-BC4<br>L2V8DR-BC5<br>L2V8DR-BC6<br>L2V8DR-BC7<br>L2V8DR-BC8<br>**0327-32**<br>L2V8DR-BC9<br>L2V8DR-BC10<br>L2V8DR-BC11<br>L2V8DR-BC12<br>L2V8DR-BC13<br>L2V8DR-BC14<br>L2V8DR-BC15<br>L2V8DR-BC16 |
| Ligation Enzyme Mix | S01535 | Yellow | L3 VER 4 |

# II. Components

| **Ovation Complete Prokaryotic RNA-Seq DR Multiplex System 1–8 and 9–16 Reagents (Part Nos. 0326-32 and 0327-32),** *continued* | | | |
|---|---|---|---|
| **COMPONENT** | **PART NUMBER** | **VIAL CAP** | **VIAL NUMBER** |
| Strand Selection Buffer Mix I | S01710 | Clear | SS1 |
| Strand Selection Enzyme Mix I | S01537 | Purple | SS2 |
| Strand Selection Buffer Mix II | S01638 | Purple | SS3 VER 2 |
| Strand Selection Enzyme Mix II | S01738 | Purple | SS4 |
| Strand Selection Reagent | S01639 | Purple | SS5 |
| Strand Selection Enzyme Mix III | S01640 | Purple | SS6 |
| Ribosomal Depletion Enzyme Mix | S01641 | Purple | RD1 |
| Amplification Buffer Mix | S01642 | Red | P1 VER 4 |
| Amplification Primer Mix | S01643 | Red | P2 VER 7 |
| Amplification Enzyme Mix | S01644 | Red | P3 VER 2 |
| Nuclease-free Water | S01113 | Green | D1 |
| Agencourt Beads | S01502 | Clear | — |

# II. Components

## B. Additional Equipment, Reagents and Labware

**Required Materials**

- **Equipment**
  - Covaris S-series Sonication System
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of nucleic acids
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® UV-Vis Spectrophotometer for quantification of fragmented DNA
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL individual thin-wall PCR tubes or 8 X 0.2 mL strip PCR tubes or 0.2 mL thin-wall PCR plates
  - Magnetic separation device options:
    - Agencourt SPRIPlate Ring Super Magnet Plate (Beckman Coulter Genomics, Cat. #A32782)
    - Agencourt SPRIStand (Beckman Coulter Genomics, Cat. #A29182)
    - MagnaBot® II Magnetic Separation Device (Promega, Cat. #V8351)
    - DynaMag™-96 Bottom, Side, or Side Skirted (Invitrogen, Cat. #123-32D,123-31D, 120-27)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - RNeasy Mini Kit (QIAGEN, Cat. #74014) or RNase-Free DNase Set (QIAGEN, Cat. #79254)
  - RNA Clean and Concentrator™ Columns (Zymo Research Cat. #R1015) or RNeasy MinElute® Columns (QIAGEN, Cat. #74204)

# II. Components

**To Order**

- Affymetrix, Inc., www.affymetrix.com
- Beckman Coulter Genomics, www.beckmangenomics.com
- Covaris, www.covarisinc.com
- Invitrogen, www.invitrogen.com
- MP Biomedicals, www.mpbio.com
- Promega, www.promega.com
- QIAGEN, www.qiagen.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Zymo Research, www.zymoresearch.com

# III. Planning the Experiment

## A. Input RNA Requirements

### 1. RNA Quantity

Total RNA input must be between 100 ng and 500 ng. Inputs outside of this range 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 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 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. In many samples, 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 can work with many samples that are moderately degraded.

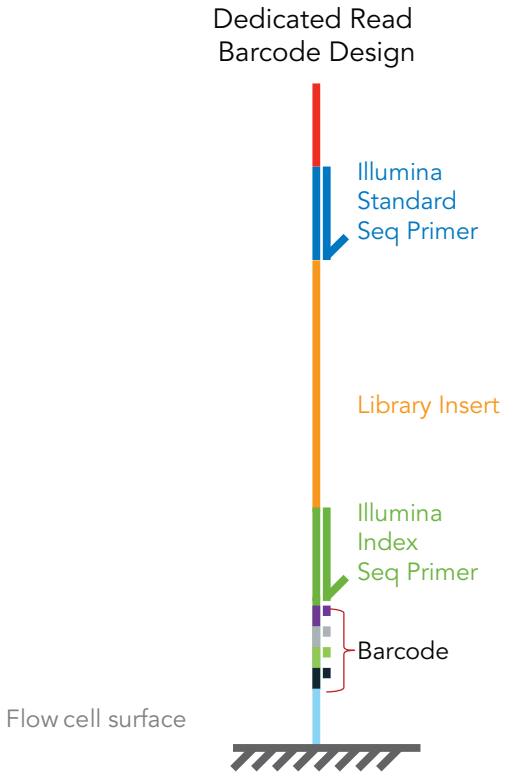
### 4. DNase Treatment

We highly recommend using DNase I-treated RNA with this system. The presence of genomic DNA in the RNA sample may have adverse effects on downstream analytical platforms. Also, if the total RNA sample contains a significant amount of genomic DNA, it may be difficult to accurately quantify the RNA concentration. The RNA input quantity may, therefore, be overestimated based on an absorbance measurement. Since it is important that RNA input be between 100 ng and 500 ng, we recommend using a DNase treatment that will remove genomic DNA during RNA purification.

## B. Using Ovation Complete Prokaryotic RNA-Seq DR Multiplex Libraries on Illumina NGS Systems

The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16 use a dedicated read (second sequencing primer) multiplex sequencing strategy, as shown in Figure 2.

**Figure 2. Multiplexing strategies used by the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16.**




The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16 use the same approach to multiplexing found in the standard Illumina method. These libraries should be sequenced using the Illumina protocol for multiplex sequencing. The DR barcode sequences are found in Appendix C of this user guide and must be entered into the Illumina software prior to the analysis.

## C. Amplified Library Storage

Amplified libraries may be stored at –20°C.

## D. Data Analysis and Parsing Multiplex Libraries

Data analysis for Next Generation Sequencing is an evolving field. The number of analysis strategies and software tools is growing rapidly. The specific analysis workflow for a given experiment will depend on many variables, including the type of experiment (DNA-Seq, Exome-Seq, RNA-Seq, etc.) and the goals of the particular study.

For the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequences of the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems barcodes, found in Appendix C, must be entered prior to parsing.

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

# IV. Overview

## A. Overview

**The library preparation process used in the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems is performed in the following stages:**

| | |
|---|---|
| 1. cDNA generation | 1.5 hours |
| 2. Fragmentation and purification | 1.25 hours |
| 3. End repair | 0.75 hours |
| 4. Adaptor ligation | 0.5 hours |
| 5. Strand selection and adaptor cleavage | 1.5 hours |
| 6. Library amplification and purification | 1.5 hours |
| **Total time to prepare amplified library** | **~7 hours** |

Components in the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are color coded, with each color linked to a specific stage of the process. Each stage requires making a master mix then adding it to the reaction, followed by incubation.

## B. Protocol Notes

- We recommend the routine use of a positive control RNA. 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. 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 no-template control (NTC) is recommended to monitor the work environment for potential carryover of previous libraries.
- Set up no fewer than four 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 four samples at a time may affect reagent recovery volumes.
- 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.
- 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.

- When instructed to pipet mix, 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.
- When preparing master mixes, use the minimal amount of extra material to ensure eight reactions in the kit.
- 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 as well. 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 Purification Beads

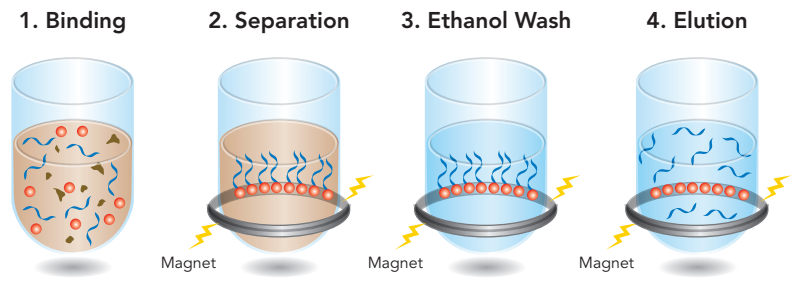
### Tips and Notes

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

**Figure 3. Agencourt Bead purification process overview.**




Reproduced from original picture from Agencourt/Beckman Coulter Genomics

### Additional Tips and Notes

- Remove beads from 4°C and leave at room temperature for at least 15 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 Agencourt 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 five 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 magnet, remove the specified quantity of binding buffer from each sample. Some liquid will remain at the bottom of the tube, but this will minimize bead loss.
- 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 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 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 to 105°C).

**Table 2. Thermal Cycler Programming**

| | |
|---|---|
| **PRIMER ANNEALING** | |
| **Program 1**<br>Primer Annealing | 65°C – 5 min |
| **cDNA SYNTHESIS** | |
| **Program 2**<br>cDNA Synthesis | 40°C – 30 min, hold at 4°C |
| **SECOND STRAND** | |
| **Program 3**<br>Second Strand Synthesis | 16°C – 60 min, hold at 4°C |
| **END REPAIR** | |
| **Program 4**<br>End Repair | 25°C – 30 min, 70°C – 10 min, hold at 4°C |
| **LIGATION** | |
| **Program 5**<br>Ligation | 25°C – 30 min, hold at 4°C |
| **STRAND SELECTION** | |
| **Program 6**<br>Strand Selection I | 72°C – 10 min, hold at 4°C |
| **Program 7**<br>Strand Selection II | 37°C – 10 min, 95°C – 2 min, 50°C – 30 sec, 65°C – 5 min, hold at 4°C |
| **ADAPTOR CLEAVAGE** | |
| **Program 8**<br>Adaptor Cleavage | 55°C – 5 min, 95°C – 5 min, hold at 4°C |
| **AMPLIFICATION** | |
| **Program 9**<br>Library Amplification | 95°C – 2 min, 2 cycles (95°C – 30 sec, 60°C – 90 sec); 18 cycles (95°C – 30 sec, 65°C – 90 sec); 65°C – 5 min, hold at 4°C |

# V. Protocol

## A. First Strand cDNA Synthesis

1. Remove First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and the nuclease-free water (green: D1) from –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice. Leave the nuclease-free water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 100 ng of total RNA (100 ng to 500 ng) to the primer, bringing the volume to 7 µL with nuclease-free water.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 2):

   65°C – 5 min
8. Immediately remove the tubes from the thermal cycler and snap chill on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 3.

**Table 3. First Strand Master Mix (volumes listed are for a single reaction)**

| FIRST STRAND BUFFER MIX (BLUE: A2 VER 8) | FIRST STRAND ENZYME MIX (BLUE: A3 VER 4) |
|---|---|
| 2.5 µL | 0.5 µL |

! *Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

10. Add 3 µL of the First Strand Master Mix to each sample tube.
11. Mix by pipetting, cap and spin the tubes and place on ice.
12. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 2):

    40°C – 30 min, hold at 4°C
13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately to the Second Strand cDNA Synthesis protocol.

## B. Second Strand cDNA Synthesis

1. Remove Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2), and Second Strand Stop Mix (yellow: B3) from –20°C storage.
2. Spin down the contents of B2 and place on ice.
3. Thaw reagents B1 and B3 at room temperature, mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 4.

**Table 4. Second Strand Master Mix (volumes listed are for a single reaction)**

| SECOND STRAND BUFFER MIX (YELLOW: B1 VER 6) | SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4) |
|---|---|
| 63.0 µL | 2.0 µL |

! *Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

5. Add 65 µL of the Second Strand Master Mix to each First Strand reaction tube.
6. Mix by pipetting, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand cDNA Synthesis; see Table 2):

   16°C – 60 min, hold at 4°C
8. Remove the tubes from the thermal cycler and spin to collect condensation.
9. Add 45 µL of the Second Strand Stop Buffer, B3. Mix by pipetting and spin.
10. Continue immediately to the cDNA Fragmentation protocol or store samples at –20°C.

## C. cDNA Fragmentation

1. Treat all DNA samples with the Covaris S-Series System according to the manufacturer's recommendations using the settings shown in Table 5 or other user-defined settings that produce fragmented DNA with a median size of 200 bp.

**Table 5. Covaris S-Series System Settings**

| PARAMETER | VALUE |
|---|---|
| Duty Cycle | 10% |
| Intensity | 5% |
| Cycles/Burst | 200 |
| Time(s) | 180 |
| Temperature (Water Bath) | 6–8°C |
| Power Mode Frequency | Sweeping |
| Degassing Mode | Continuous |
| Sample Volume | 120 µL |
| Water (FILL/RUN) | S2 – level 12<br>E210 – level 6 |
| AFA Intensifier | Yes |

2. Continue to the cDNA Purification protocol or store samples at –20°C.

## D. cDNA Purification

1. Ensure the Agencourt beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. 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. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce yield. (Sufficient wash solution should be prepared for all bead purification steps.)
4. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to 100 µL fragmented cDNA. Mix thoroughly by pipetting up and down.
5. Split each sample into two 140 µL aliquots.
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. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   **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 end repair, so ensure beads are not removed with the binding buffer or the wash.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
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 two 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 of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
13. Remove the tubes from the magnet.
14. Add 12 µL room temperature Nuclease-free Water (green: D1) to the first aliquot of dried beads. Mix thoroughly to ensure all beads are resuspended.
15. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
16. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
17. Carefully remove 10 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
18. Continue immediately to the End Repair protocol.

## E. End Repair

1. Remove End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2) and End Repair Enhancer (blue: ER3) from –20°C storage.
2. Thaw ER1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down contents of ER2 and ER3, and place on ice.

4. Prepare a master mix by combining ER1, ER2 and ER3 in a 0.5 mL capped tube, according to the volumes shown in Table 6.

**Table 6. End Repair Master Mix (volumes listed are for a single reaction)**

| END REPAIR BUFFER MIX (BLUE: ER1 VER 7) | END REPAIR ENZYME MIX (BLUE: ER2 VER 4) | END REPAIR ENHANCER (BLUE: ER3 VER 2) |
|---|---|---|
| 4.0 µL | 0.5 µL | 0.5 µL |

! *Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

5. Add 5 µL of the End Repair Master Mix to 10 µL of each sample.
6. Mix by pipetting, cap and spin the tubes and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (End Repair; see Table 2):

   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 Ligation protocol.

## F. Ligation

1. Remove Ligation Buffer Mix (yellow: L1), appropriate Ligation Adaptor Mixes (yellow: L2), Ligation Enzyme Mix (yellow: L3), and Nuclease-free Water (green: D1) from –20°C storage.
2. Thaw L1, L2 and D1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin L3 and place on ice.
4. Add 3 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting. Make sure a unique barcode is used for each sample.

   **Note:** For more information about running a multiplex experiment, see Appendix B.
5. Prepare a master mix by combining Nuclease-free Water (D1), L1 and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 7. Mix by pipetting slowly, without introducing bubbles, spin and place on ice.

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

**Table 7. Ligation Master Mix (volumes listed are for a single reaction)**

| NUCLEASE-FREE WATER (GREEN: D1) | LIGATION BUFFER MIX (YELLOW: L1 VER 4) | LIGATION ENZYME MIX (YELLOW: L3 VER 4) |
|---|---|---|
| 4.5 µL | 6.0 µL | 1.5 µL |

*Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

6. Add 12 µL of the Ligation Master Mix to each reaction tube. Mix thoroughly by pipetting slowly and gently, spin and place on ice. Proceed immediately with the incubation.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Ligation; see Table 2):

   25°C – 30 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 Strand Selection I protocol.

## G. Strand Selection I

1. Remove Strand Selection Buffer Mix I (clear: SS1) and Strand Selection Enzyme Mix I (purple: SS2) from –20°C storage.
2. Thaw SS1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down SS2 and place on ice.
4. Prepare a master mix by combining SS1 and SS2 in a 0.5 mL capped tube, according to the volumes shown in Table 8.

**Table 8. Strand Selection I Master Mix (volumes listed are for a single reaction)**

| STRAND SELECTION I BUFFER MIX (CLEAR: SS1) | STRAND SELECTION I ENZYME MIX (PURPLE: SS2) |
|---|---|
| 69 µL | 1 µL |

*Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

5. Add 70 µL of the Strand Selection I Master Mix to 30 µL of each sample.
6. Mix by pipetting, cap and spin the tubes and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 6 (Strand Selection I; see Table 2):

   72°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 Strand Selection I Purification protocol.

## H. Strand Selection I Purification

1. Ensure the Agencourt beads and Nuclease-free Water (green: D1) have completely reached room temperature before proceeding.
2. 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. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 80 µL (0.8 volumes) of the bead suspension to the Strand Selection I 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 165 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   **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 Strand Selection II, 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 final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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.
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 20 µL room temperature nuclease-free water (D1) 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 18 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
15. Continue immediately to the Strand Selection II protocol.

## I. Strand Selection II

1. Remove Strand Selection Buffer Mix II (purple: SS3), Strand Selection Enzyme Mix II (purple: SS4), Strand Selection Reagent (purple: SS5) and Strand Selection Enzyme Mix III (purple: SS6) from –20°C storage.
2. Thaw SS3 and SS5 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down SS4 and SS6 and place on ice.
4. Prepare a master mix by combining SS3, SS4, SS5, and SS6 in a 0.5 mL capped tube, according to the volumes shown in Table 9.

**Table 9. Strand Selection II Master Mix (volumes listed are for a single reaction)**

| STRAND SELECTION II BUFFER MIX (PURPLE: SS3 VER 2) | STRAND SELECTION II ENZYME MIX (PURPLE: SS4) | STRAND SELECTION REAGENT (PURPLE: SS5) | STRAND SELECTION ENZYME MIX III (PURPLE: SS6) |
|---|---|---|---|
| 5 µL | 0.5 µL | 1 µL | 0.5 µL |

! *Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

5. Add 7.0 µL of the Strand Selection II Master Mix to 18 µL of each sample.
6. Mix by pipetting, cap and spin the tubes and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (Strand Selection II; see Table 2):

   37°C – 10 min, 95°C – 2 min, 50°C – 30 sec, 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 Adaptor Cleavage protocol.

## J. Adaptor Cleavage

1. Prepare a master mix by combining SS3 and RD1 in a 0.5 mL capped tube according the volumes in Table 10.

*Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

**Table 10. Adaptor Cleavage Master Mix (volumes listed are for a single reaction)**

| NUCLEASE-FREE WATER (GREEN: D1) | STRAND SELECTION II BUFFER MIX (PURPLE: SS3 VER 2) | RIBOSOMAL DEPLETION ENZYME MIX (PURPLE: RD1) |
|---|---|---|
| 17.5 µL | 5 µL | 2.5 µL |

2. Add 25 µL of the Adaptor Cleavage Master Mix to 25 µL of each sample.
3. Mix by pipetting, cap and spin the tubes and place on ice.
4. Place the tubes in a pre-warmed thermal cycler programmed to run Program 8 (Adaptor Cleavage; see Table 2):

   55°C – 5 min, 95°C – 5 min, hold at 4°C
5. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue immediately to the Library Amplification protocol.

## K. Library Amplification

1. Remove Amplification Buffer Mix (red: P1 VER 4), Amplification Primer Mix (red: P2 VER 7), and Amplification Enzyme Mix (red: P3 VER 2) from –20°C storage.
2. Thaw P1 and P2 at room temperature. Mix each by vortexing, spin and place on ice.
3. Spin down P3 Amplification Enzyme Mix and place on ice.
4. Prepare a master mix by sequentially combining P1 and P2 in an appropriately sized capped tube, according to the volumes shown in Table 11. Add P3 Enzyme Mix at the last moment and mix well by pipetting taking care to avoid bubbles. Spin the tubes and place on ice.

*Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.*

**Table 11. Amplification Master Mix (volumes listed are for a single reaction)**

| NUCLEASE-FREE WATER (GREEN: D1) | AMP BUFFER MIX (RED: P1 VER 4) | AMP PRIMER MIX (RED: P2 VER 7) | AMP ENZYME MIX (RED: P3 VER 2) |
|---|---|---|---|
| 31.5 µL | 10 µL | 8 µL | 0.5 µL |

5. On ice, add 50 µL of the Amplification Master Mix to 50 µL of each sample.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Library Amplification; see Table 2):

95°C – 2 min, 2 cycles (95°C – 30 sec, 60°C – 90 sec); 18 cycles (95°C – 30 sec, 65°C – 90 sec); 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. Proceed to Bead Purification of the Amplified Material or store at –20°C.

## L. Bead Purification of the Amplified Material

1. Ensure the Agencourt beads and Nuclease-free Water (green: D1) have completely reached room temperature before proceeding.
2. 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. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 100 µL (1 volume) of the bead suspension to each reaction.
4. Mix thoroughly by pipetting up and down. It may be helpful to use a multichannel pipettor to ensure the incubation times are uniform.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet plate and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove only 185 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   **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.
8. Remove the tubes from the magnet, add 200 µL of freshly prepared 70% ethanol, mix thoroughly by pipetting up and down, transfer the tubes back to the magnet and let stand 3–5 minutes to completely clear the solution of beads.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash one more time, for a total of two washes.

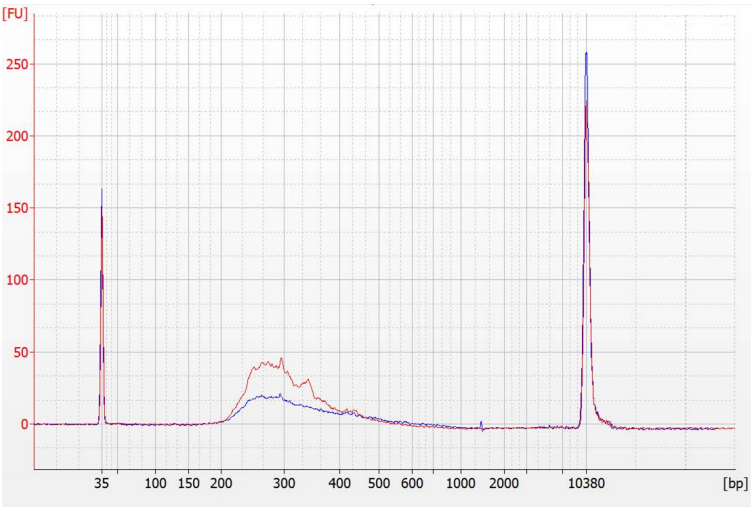
    **Note:** With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
11. Air dry the beads on the magnet for a minimum of 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 room temperature Nuclease-free Water (D1) to the dried beads. Mix thoroughly to ensure all beads are resuspended.
14. Incubate at room temperature for 5 minutes.
15. Transfer the tubes to the magnet and let stand for 2 minutes.
16. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to use a magnet stand to minimize bead carryover into any ensuing reactions.
17. Proceed to Quantitative and Qualitative Assessment of the Library.

## M. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer DNA Chip. Fragment distribution should be as shown in Figure 4.

**Figure 4. Fragment distribution on Bioanalyzer High Sensitivity DNA chip from 100 ng to 500 ng *E. coli* total RNA input.**




2. 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 Technical Support, please contact NuGEN at (U.S. only) 888.654.6544 (Toll-Free Phone) or 888.296.6544 (Toll-Free Fax) or email techserv-gn@tecan.com.

In Europe contact NuGEN at +31.13.5780215 (Phone) or +31.13.5780216 (Fax) or email at europe-gn@tecan.com.

In all other locations, contact your NuGEN distributors Technical Support team.

# VII. Appendix

## A. DNase Treatment of RNA

**DNase Treatment During Purification: Using the QIAGEN RNase-Free DNase Set and the RNeasy Mini RNA Purification Kit**

1. Homogenize sample in RLT buffer including ß-mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 µL), including any precipitate that may have formed, to the column.
5. Close the tube gently and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. For volumes greater than 700 µL, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 µL Buffer RW1 into the RNeasy mini column to wash, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
8. Add 10 µL DNase I to 70 µL Buffer RDD. Gently invert the tube to mix.

   **Note:** Other DNase I enzymes we recommend for use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 µL) or the DNase I (RNase-free) from New England BioLabs (use 10 µL).
9. Pipet the DNase I incubation mix (80 µL) directly onto the membrane inside the RNeasy mini column. Incubate at the bench top (~25°C) for 15 minutes.
10. Add 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer RPE to the RNeasy column.
14. Close the tube gently and centrifuge for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30–50 µL RNase-free water directly onto the RNeasy membrane.
17. Close the tube gently and centrifuge for 1 minute at ≥8000 X g (≥10,000 rpm) to elute.

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18. If yields of greater than 30 µg are expected, repeat elution step and collect in the same collection tube.

**DNase Treatment of RNA Post-purification: Using RNase-free DNase and either the RNA Clean and Concentrator™-5 Columns or the RNeasy MinElute® Columns**

**Note:** If you are unable to quantify your RNA because the sample is contaminated with DNA, we recommend DNase treatment followed by purification.

1. On ice, mix together 2.5 µL 10X DNase I Reaction buffer (Roche Cat. #04716728001 or USB Cat. #78316) with 1 µL rDNase (10 Units Roche Cat. #04716728001 or 2 Units USB Cat. #78311).
2. Add RNA sample (up to 500 ng) and add nuclease-free water (D1, green cap) to bring the final volume to 25 µL.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

**Purification with RNA Clean & Concentrator-5 (Zymo Research, Cat. #R1015)**

- Add 4 volumes (100 µL) of RNA binding buffer to the sample.
- Obtain one RNA Clean & Concentrator-5 Kit column and apply sample to column.
- Spin column for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- Add 200 µL wash buffer (with ethanol added as per vendor's specifications).
- After closing the column, spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- Add 200 µL fresh 80% ethanol, close cap and spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- Place the RNA Clean & Concentrator-5 Kit column in a fresh 1.5 mL collection tube.
- Add 10 µL nuclease-free water (green: D1) directly to the center of the filter in the tube and close the cap.

  **Important:** Do not use cold water!

- Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

**Purification with QIAGEN® RNeasy MinElute Cleanup Columns (QIAGEN, Cat. #74204)**

- Add 80 µL ice-cold nuclease-free water (D1, green cap) to the sample on ice.
- Add 350 µL Buffer RLT and mix by pipetting.

- Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
- Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.
- After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 µL Buffer RPE to the column and close the tube. Spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through, keeping the same collection tube.
- Add 500 µL 80% ethanol to the RNeasy MinElute Spin Column and close the tube.

  **Note:** Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
- Spin for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
- Add 14 µL nuclease-free water (D1, green cap) directly to the center of the filter in the tube and close the cap. Do not use cold water!
- Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

## B. Setting Up a Multiplex Experiment

Each Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems kit contains eight different barcodes that can be used for multiplex sequencing to interrogate several independently generated libraries on a single lane of the Illumina NGS platforms.

It is important when performing multiplex sequencing to produce each library independently, and not to mix adaptors during the actual library construction protocol. Multiplexing is achieved by mixing the amplified libraries prior to adding to the cBot or cluster station instrument.

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## C. Barcode Sequences for Multiplex Experiments

Barcode sequences for adaptors used in the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16 can be found in Table 12.

**Table 12. Barcode sequences for Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16.**

| LIGATION ADAPTOR MIX | BARCODE SEQUENCE |
|---|---|
| L2V8DR-BC1 | AAGGGA |
| L2V8DR-BC2 | CCTTCA |
| L2V8DR-BC3 | GGACCC |
| L2V8DR-BC4 | TTCAGC |
| L2V8DR-BC5 | AAGACG |
| L2V8DR-BC6 | CCTCGG |
| L2V8DR-BC7 | GGATGT |
| L2V8DR-BC8 | TTCGCT |
| L2V8DR-BC9 | ACACGA |
| L2V8DR-BC10 | CACACA |
| L2V8DR-BC11 | GTGTTA |
| L2V8DR-BC12 | TGTGAA |
| L2V8DR-BC13 | ACAAAC |
| L2V8DR-BC14 | CACCTC |
| L2V8DR-BC15 | GTGGCC |
| L2V8DR-BC16 | TGTTGC |

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## D. Frequently Asked Questions (FAQs)

**Q1. What materials are provided with the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems provide all necessary buffers, primers, enzymes and purification beads. The kit also provides nuclease-free water for purification elution steps.

**Q2. Does this system contain a SPIA®-based amplification?**
No. The cDNA is generated with selective primers, but no SPIA-based amplification is used.

**Q3. What equipment is required or will be useful?**
A comprehensive list of required and recommended equipment can be found in Section II.B of this user guide.

**Q4. Can I use the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems with RNA from any organism?**
This system has been designed specifically for prokaryotes. Performance with other organisms may vary.

**Q5. Do I need to use high-quality total RNA?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are 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 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 Complete Prokaryotic RNA-Seq DR Multiplex Systems?**
The system is designed to use total RNA as input. rRNA depletion or poly(A) enrichment are not necessary.

**Q7. How much total RNA do I need for amplification?**
The selective priming process is designed to deplete rRNA from 100–500 ng total RNA input.

**Q8. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems utilize optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the amount of adaptor dimer in the library.

**Q9. How does your protocol enable strand retention?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems utilize targeted degradation of an incorporated modified nucleotide to ensure library inserts all carry the same directionality.

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**Q10. Can contaminating genomic DNA interfere with the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems performance?**
When using purified total RNA samples, contaminating genomic DNA may be incorporated into libraries. For this reason we recommend DNase treatment during RNA purification. For an explanation of DNase requirements see section III.A.4. For DNase treatment of RNA samples, refer to Appendix A for guidelines.

**Q11. Can this system be used with other library preparation workflows?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are an end-to-end solution designed to generate libraries for Illumina sequencing starting from total RNA and have not been tested with alternative library preparation systems.

**Q12. How do I measure my amplified cDNA product yield? Can I use an Agilent Bioanalyzer to evaluate the product?**
Yes. Refer to section V.M of the user guide for guidelines on Quantitative and Qualitative Assessment.

**Q13. Where can I safely stop in the protocol?**
Samples can be placed in short-term storage at –20°C after B. Second Strand Synthesis, after C. cDNA Fragmentation or after any of the bead purification steps.

**Q14. Does NuGEN provide reagents for performing the fragmentation step of the protocol?**
We recommend using the Covaris instrument for cDNA fragmentation, as suggested in the "Materials" section of this user guide. NuGEN does not provide the reagents used in the fragmentation steps, but the user guide does specify suggested settings for the Covaris instrument.

**Q15. I don't have access to a Covaris instrument, can I use alternative fragmentation methods?**
We have evaluated only Covaris fragmented DNA during the development of these systems. Other mechanical means of fragmentation, such as sonication, may be suitable as long as the method generates a tight size distribution of DNA fragments with a median size of 200 bp.

**Q16. 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, and 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.

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2. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated. For the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems, the minimum is 12 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.

**Q17. How much material should I load into the cBot?**
Please follow manufacturer's recommendations for library QC, quantitation, balancing and loading of the amplified library on the cBot.

**Q18. Do the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems work with the Illumina Cluster Station (predecessor of the cBot instrument)?**
Yes, the Systems are also compatible with the Illumina Cluster Station.

**Q19. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?**
For experiments using the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems with dedicated read barcodes, please follow the Illumina recommendations on parsing barcodes. The NuGEN dedicated read barcodes are six-base unique barcode tags. The sequences of these NuGEN barcodes must be input prior to parsing.

**Q20. Can I combine the barcoded libraries prior to amplification?**
The stoichiometry of barcoded libraries may be adversely affected by this modification to the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems workflow. We suggest that the libraries be amplified and quantitated independently before being pooled for use on the cBot or Cluster Station.

**Q21. What kind of sequencing primers can I use with your library?**
The Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems are designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

**Q22. Can the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems be used with paired-end sequencing?**
Yes, they 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 libraries for fragments with an average size of 150 bases, corresponding to the expected distance between the 5´-most and 3´-most coordinates of paired-end reads.

**Q23. How many bases do the Ovation Complete Prokaryotic RNA-Seq DR Multiplex System adaptors add to the library?**
The adaptors add 125 bp to the library in Ovation Complete Prokaryotic RNA-Seq DR Multiplex System.

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**Q24. What is the expected yield of the amplified DNA library using the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems?**
The expected yield is 0.3–2 µg, depending on the quality and quantity of the input RNA. This amount is a large excess over the amount of DNA required for use on the cBot or Cluster Station.

**Q25. Can I use standard alignment algorithms to analyze strand-specific sequencing data?**
Yes. Strand-specific reads can be processed and mapped to reference sequences using the same methods used for other RNA-Seq libraries.

**Q26. Will the presence of extrachromosomal material in total RNA impact my data?**
It is possible to see a higher proportion of unmapped reads in the context of some bacterial strains with extrachromosomal content, such as plasmids.

**Q27. Will the use of RNA purification columns impact my data?**
We have observed changes in alignment metrics and expression profiles with the use of purification columns, such as the QIAGEN RNeasy column. We recommend consulting the manufacturer to ensure the RNAs of interest are retained after purification.

## E. Update History

This document, the Ovation Complete Prokaryotic RNA-Seq DR Multiplex Systems 1–8 and 9–16 user guide (M01288 v4.1) is an update to address the following topics.

| Description | Section | Page(s) |
|---|---|---|
| Updated legal information, emails, address, trademarks, and logo. | Througout | Througout |
| Updated Agencourt bead reagent information. | Throughout | Throughout |


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