USER GUIDE

# Ovation® Ultralow System V2

PART NO. 0344, 0344NB

NuGEN®
A Tecan Group company

## Patents, Licensing and Trademarks



The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to modify, reverse engineer, resell, repackage or further sublicense) under these patent applications and any patents issuing from these patent applications to use this product and methods, accompanying this user guide, for research and development purposes solely in accordance with the intended use described and the written instructions provided in this user guide. No license to make or sell products by use of this product is granted to the buyer whether expressly, by implication, by estoppels or otherwise. In particular, the purchase of this product does not include or carry any right or license to use, develop or otherwise exploit this product commercially and no rights are conveyed to the buyer to use the product or components of the product for purposes including commercial services or clinical diagnostics.

For information on purchasing a license to the Tecan Genomics patents for uses other than in conjunction with this product or to use this product for purposes other than research, please contact Tecan Genomics, Inc., 900 Chesapeake Drive, Redwood City, CA 94063. Phone 888-654-6544 or 650-590-3600; FAX 888-296-6544 or 650-590-3630.

### Warranty

Tecan Genomics warrants that this product meets the performance standards described in the Company's product and technical literature for a period of six months from the date of purchase, provided that the product is handled and stored according to published instructions, and that the product is not altered or misused. If the product fails to meet these performance standards, Tecan Genomics will replace the product free of charge or issue a credit for the purchase price. Tecan Genomics' liability under this warranty shall not exceed the purchase price of the product. Tecan Genomics shall assume no liability for direct, indirect, consequential or incidental damages arising from the use, results of use or inability to use its products. Tecan Genomics reserves the right to change, alter or modify any product to enhance its performance and design.

Tecan Genomics' products are developed, designed and sold FOR RESEARCH USE ONLY. This product is not to be used for diagnostic or therapeutic purposes, nor is it to be administered to humans or animals.

Except as expressly set forth herein, no right to modify, reverse engineer, distribute, offer to sell or sell Tecan Genomics' product is conveyed or implied by buyer's purchase of this Tecan Genomics product. The buyer agrees to use Tecan Genomics products accompanying the product insert in accordance with the intended use and the written instructions provided.

# Table of Contents

## Contents

# I. Introduction

## A. Background

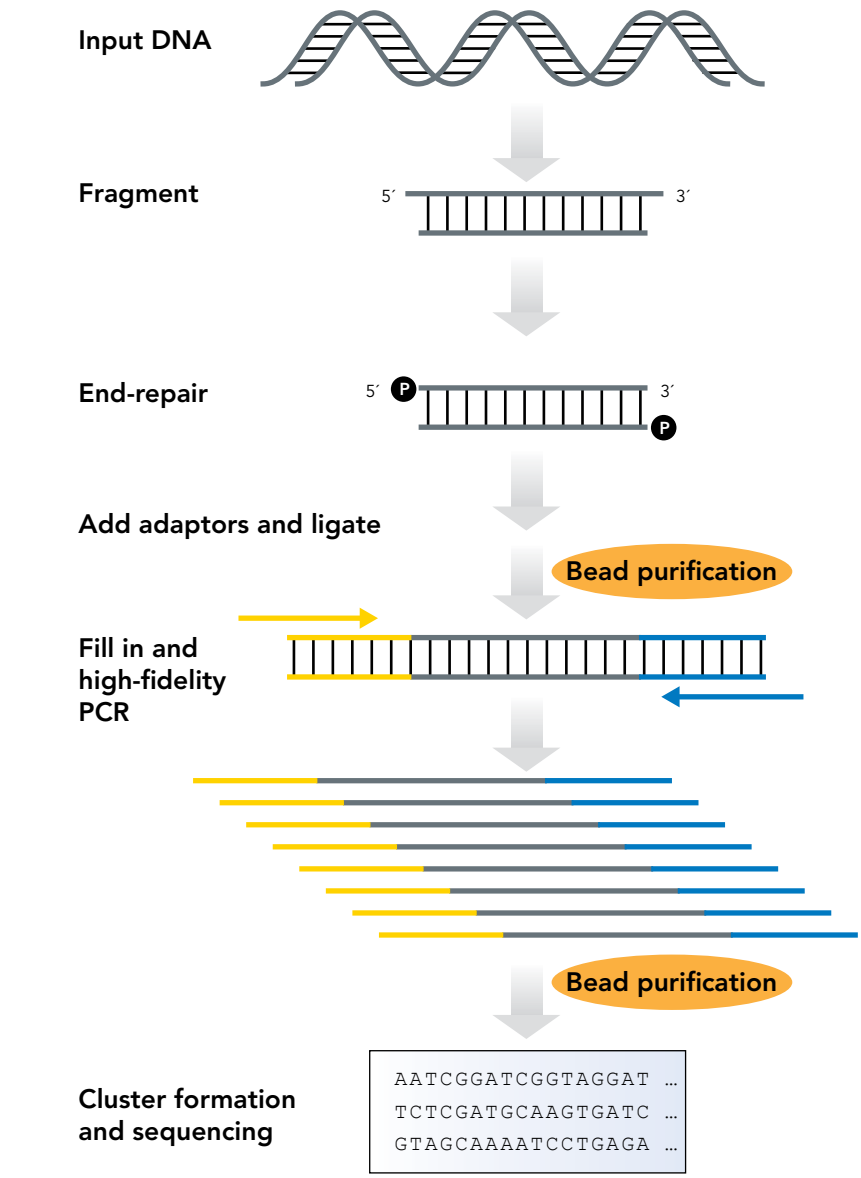
The Ovation® Ultralow System V2 provides a simple, fast and scalable solution for producing libraries used in next-generation sequencing starting with as little as 10 pg of double-stranded DNA. The library construction workflow is suitable for a wide range of sequencing applications including RNA-Seq, Digital Gene Expression (DGE), genomic DNA sequencing, target capture, amplicon sequencing, ChIP-Seq and more. These libraries are suitable for sequencing on Illumina sequencing platforms.

As shown in Figure 1, the streamlined workflow consists of four main steps: fragmentation of either genomic DNA or double-stranded cDNA, end repair to generate blunt ends, adaptor ligation with optional multiplexing and PCR amplification to produce the final library. The entire workflow including fragmentation can be completed in just over four hours, and yields DNA libraries ready for cluster formation and either single read or paired-end sequencing.

In addition to use with genomic and other double-stranded DNA sources, the Ovation Ultralow System V2 has been designed for seamless integration with NuGEN's Ovation RNA-Seq System V2 and Ovation RNA-Seq FFPE System (Part Nos. 7102 and 7150) to enable a complete end-to-end solution for library construction starting with total RNA. Importantly, for DNA sequencing applications, low abundance samples can be input directly to the library construction workflow without the need for pre-amplification.

# I. Introduction

Figure 1. Ovation Ultralow System V2 workflow.



# I. Introduction

## B. Performance Specifications

The Ovation Ultralow System V2 is designed to produce DNA libraries suitable for either single read or paired-end sequencing on Illumina sequencing platforms, without the need for gel-based size selection. Libraries suitable for cluster generation are produced in about four hours using as little as 10 pg input of double-stranded DNA.

## C. Quality Control

Every lot of the Ovation Ultralow System V2 undergoes functional testing to meet specifications for library generation performance.

## D. Storage and Stability

The Ovation Ultralow System V2 is shipped on dry ice and should be unpacked immediately upon receipt.

**Note:** This product contains components with multiple storage temperatures.

Vials labeled Agencourt® Beads (clear cap) should be removed from the top of the shipping carton upon delivery and stored at 4 °C (supplied with part no. 0344-32).

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 for at least six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

## E. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at www.nugen.com/products/ovation-ultralow-library-system-v2.

## F. Before You Start

Please review this User Guide before using this kit for the first time, including the "Kit Components", "Planning the Experiment", "Overview", "Protocol" and "FAQ" sections. For more information, visit the Ovation Ultralow System V2 page at www.nugen.com/products/ovation-ultralow-library-system-v2.

New to NGS? Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

# II. Components

## A. Reagents Provided

**Table 1. Ovation Ultralow System V2 Reagents (Part No. 0344)**

| COMPONENT | 0344NB-08 PART NUMBER | 0344-32/ 0344NB-32 PART NUMBER | 0344NB-A01 PART NUMBER | VIAL LABEL | VIAL NUMBER |
|---|---|---|---|---|---|
| End Repair Buffer Mix | S01464 | S01844 | S01686 | Blue | ER1 ver 3 |
| End Repair Enzyme Mix | S01510 | S01845 | S01687 | Blue | ER2 VER 4 |
| End Repair Enhancer | S01562 | S01846 | S01688 | Blue | ER3 |
| Ligation Buffer Mix | S01466 | S01847 | S01689 | Yellow | L1 VER 4 |
| Ligation Adaptor Mixes | S02309<br>S02310<br>S02311<br>S02312<br>S02313<br>S02314<br>S02315<br>S02316 | —<br>—<br>—<br>—<br>—<br>—<br>—<br>— | —<br>—<br>—<br>—<br>—<br>—<br>—<br>— | Yellow | L2V23DR-BC1<br>L2V23DR-BC2<br>L2V23DR-BC3<br>L2V23DR-BC4<br>L2V23DR-BC5<br>L2V23DR-BC6<br>L2V23DR-BC7<br>L2V23DR-BC8 |
| 32-Plex Adaptor Plate | — | S02317 | — | Yellow | L2V23DR-BC |
| 96-Plex Adaptor Plate | — | — | S02366 | Yellow | L2V23DR-BC |
| Ligation Enzyme Mix | S01467 | S01848 | S01690 | Yellow | L3 VER 4 |
| Amplification Buffer Mix | S02318 | S02066 | S02072 | Red | P1 VER 7 |
| Amplification Primer Mix | S02319 | S02068 | S02074 | Red | P2 VER 5 |
| Amplification Enzyme Mix | S02320 | S02067 | S01875 | Red | P3 VER 4 |

# II. Components

| COMPONENT | 0344NB-08 PART NUMBER | 0344-32/ 0344NB-32 PART NUMBER | 0344NB-A01 PART NUMBER | VIAL LABEL | VIAL NUMBER |
|---|---|---|---|---|---|
| Nuclease-free Water | S01001 | S01001 | S01113 | Green | D1 |
| Agencourt Beads | — | S01502 (Part No. 0344-32 only) | — | Clear | — |

**Ovation Ultralow System V2 Reagents (Part No. 0344NB-32)**

The 0344NB-32 version of the product includes all the same components as 0344-32 with the exception of Part Number S01502, Agencourt beads. This reagent can be sourced directly from Beckman Coulter, www.beckmancoulter.com.

The entire workflow requires a minimum of 3 mL of beads for 8 reactions, 12 mL of beads for 32 reactions, and 36 mL of beads for 96 reactions.

# II. Components

## B. Additional Equipment, Reagents and Labware

**Required Materials**

- **Equipment**
  - Covaris Ultrasonication 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
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793), for diluting nucleic acids
  - Agencourt® Ampure XP Beads for Part Nos. 0344NB (Beckman Coulter, Cat. #A63881)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 8 X 0.2 mL strip PCR 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). Other magnetic stands may be used as well, although their performance has not been validated by NuGEN.
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - OPTIONAL: MinElute® Reaction Cleanup Kit (QIAGEN®, Cat. #28204)
  - OPTIONAL: PhiX Control (Illumina, Cat. #FC-110-3001)

**To Order**

- Alfa Aesar, www.alfa.com
- Beckman Coulter, www.beckmancoulter.com
- Covaris, www.covarisinc.com
- Illumina, www.illumina.com
- MP Biomedicals, www.mpbio.com
- QIAGEN, www.qiagen.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

# III. Planning the Experiment

## A. Input DNA Requirements

The Ovation Ultralow System V2 is designed to work with inputs of 10 pg to 100 ng of fragmented genomic dsDNA or ds-cDNA. DNA samples must be free of contaminating proteins, RNA, organic solvents (including phenol and ethanol) and salts. Use of a commercially available system for DNA/cDNA isolation is recommended. The A260:A280 ratio for DNA samples should be in excess of 1.8. Use of DNA samples with lower ratios may result in low amplification yield.

## B. Working with the Adaptor Plates

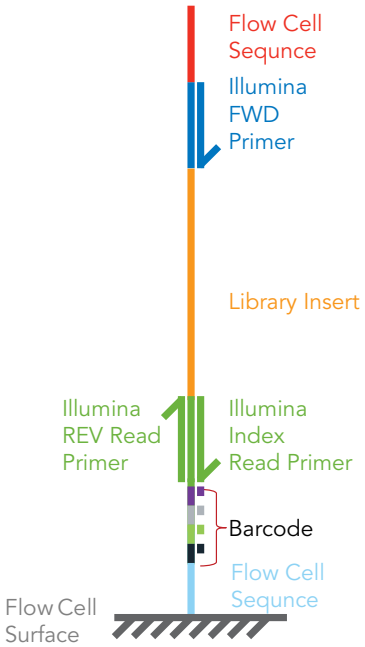
The Adaptor Plate included with the 32 and 96 reaction Ovation Ultralow System V2 kits contain adaptor mixes, each with a unique eight-base barcode. Each well (first 32 wells, A01– H04, or all 96 wells, respectively) contains sufficient volume for preparation of a single library. The Ovation Ultralow System V2 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 entire 15 µL of sample into each well. Mix well by pipetting, and transfer the reactions into PCR tubes. 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 21.

## C. Sequencing Recommendations and Guidelines

**Figure 2. Ovation Ultralow System V2 Library Structure.**




The Ovation Ultralow System V2 uses the same approach to multiplexing as the standard Illumina method and should be sequenced using the Illumina protocol for multiplex sequencing. The 8 bp barcode sequences are found in Appendix A of this user guide and must be entered into the Illumina software prior to data analysis.

## D. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

## E. Data Analysis and Parsing Multiplex Libraries

For the Ovation Ultralow System V2, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequences of the Ovation Ultralow System V2 barcodes will need to be entered prior to parsing. These sequences are found in Appendix A.

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. Protocol

## A. Overview

**The library preparation process used in the Ovation Ultralow System V2 is performed in three stages:**

| | |
|---|---|
| 1. End repair of sheared DNA | 0.75 hours |
| 2. Adaptor ligation and purification | 1.5 hours |
| 3. Amplification and purification | 1.75 hours |
| **Total time to prepare amplified library** | **~4 hours** |

Components in the Ovation Ultralow System V2 are color coded, with each color linked to a specific stage of the process. Performing each stage requires making a master mix then adding it to the reaction, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

## B. Protocol Notes

### Controls

- We recommend the routine use of a positive control DNA. Especially the first time you set up a reaction, the use of a positive control DNA will allow the establishment of a baseline of performance and provide the opportunity to become familiar with the bead purification steps.

### General Workflow

- Set up no fewer than four reactions at a time to ensure that you are not pipetting very small volumes.
- Thaw components used in each step and immediately place them on ice. Do not thaw all reagents at once.
- 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 it 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 that you are able to run the maximal number of reactions using the components provided in the kit.

### Reagents

- 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. Components and reagents from other NuGEN kits should not be used with the Ovation Ultralow System V2.
- Use only fresh ethanol stocks to make 70% ethanol used in the purification protocols.
- Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

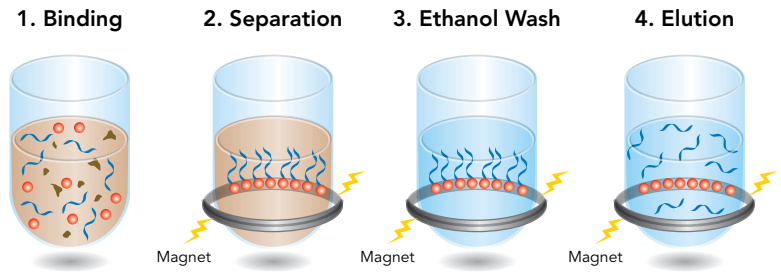
## C. Agencourt® Beads

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. However, you may review the Beckman Coulter user guide to become familiar with the manufacturer's recommendations.

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

### Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 15 minutes before use, and ensure that they have completely reached room temperature. Cold beads reduce recovery.
- Fully resuspend beads by inverting and tapping before adding to sample.

- Note that ratio of Agencourt bead volume to sample volume varies between the Ligation Purification protocol and the Amplified Library Purification 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. When the samples are on the magnet, the entire volume is often not removed. 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.
- It is strongly recommended that strip tubes or partial plates are 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 and equipped with a heated lid. Prepare the programs shown in Table 4, 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 2. Thermal Cycler Programming**

| END REPAIR | | VOLUME |
|---|---|---|
| **Program 1**<br>End Repair | 25 °C – 30 min, 70 °C – 10 min, hold at 4 °C | 15 µL |
| **LIGATION** | | **VOLUME** |
| **Program 2**<br>Ligation | 25 °C – 30 min, 70 °C – 10 min, hold at 4 °C | 30 µL |
| **AMPLIFICATION** | | **VOLUME** |
| **Program 3**<br>Amplification | 72 °C – 2 min, 95 °C – 3 min, 8–15* cycles (98 °C – 20 sec, 65 °C – 30 sec, 72 °C – 30 sec), 72 °C – 1 min, hold at 4 °C | 50 µL |

**Important Note:** The number of cycles (*) used for PCR amplification depends on the starting amount of genomic DNA. Please refer to Table 6 for a general guide to choosing the appropriate number of cycles for the PCR amplification reaction. Alternatively, real-time PCR can be used to determine the appropriate number of PCR cycles. For more information, contact NuGEN Technical Support.

## E. DNA Fragmentation

**Note:** Remove Agencourt beads from 4 °C and Nuclease-free Water (green: D1) from -20 °C and place on bench top.

1. Dilute appropriate amount of intact gDNA into 120 µL of 1X low-EDTA TE buffer.
2. Transfer 120 µL to Covaris snap cap microtube.
3. Fragment to desired insert size following Covaris recommended settings.

## F. DNA Purification

User may choose a nucleic acid column-based purification system that allows small volume elution, such as the MinElute® Reaction Cleanup Kit (QIAGEN®, Cat. #28204). The Agencourt bead-based purification protocol detailed below is provided for convenience.

# IV. Protocol

1. Ensure the Agencourt beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads.
3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. 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, ~1.5 mL per sample.)
4. Transfer entire 120 µL of fragmented DNA into two microcentrifuge tubes, 60 µL per tube.
5. At room temperature, add 120 µL (2 volumes) of the bead suspension to each tube and mix by pipetting 10 times.
6. Incubate at room temperature for 10 minutes.
7. Transfer the PCR tubes containing the bead-sample mixture to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove 160 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 5 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
13. Add 12 µL room temperature 1X low-EDTA TE buffer or Nuclease-free Water (green: D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. 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.

15. Transfer tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
16. 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.
17. Set aside the Agencourt beads and 70% ethanol at room temperature for use in the Ligation Purification and Amplified Library Purification protocols. Also set aside the Nuclease-free water (green:D1) for use throughout the protocol.
18. Continue immediately to the End Repair Protocol.

## G. End Repair

1. Obtain the End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2), End Repair Enhancer (blue: ER3) and Nuclease-free Water (green: D1) from –20 °C storage.

! *Do not vortex any enzyme mixes.*

2. Thaw ER1 and D1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down contents of ER2 and ER3 and place on ice.
4. Obtain the 10 µL fragmented DNA sample (10 pg – 100 ng) from the DNA Purification protocol. Alternatively, place 10 pg – 100 ng of DNA in 10 µL of low-EDTA TE buffer or Nuclease-free Water in a PCR tube.
5. Prepare a master mix by combining ER1, ER2 and ER3 in a 0.5 mL capped tube, according to the volumes shown in Table 3.

**Table 3. End Repair Master Mix (volumes listed are for a single reaction)**

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

| END REPAIR BUFFER MIX (BLUE: ER1 VER 3) | END REPAIR ENZYME MIX (BLUE: ER2 VER 4) | END REPAIR ENHANCER (BLUE: ER3) |
|---|---|---|
| 3.5 µL | 0.5 µL | 1.0 µL |

6. Add 5 µL of the End Repair Master Mix to each sample tube.
7. Mix by pipetting, cap and spin tubes and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (End Repair; see Table 2):

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

# IV. Protocol

## H. Ligation

1. Remove the Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mix (yellow: L2) and Ligation Enzyme Mix (yellow: L3) from –20 °C storage.
2. Thaw L1 and L2 on ice. Mix by vortexing, spin and place on ice.
3. Spin down L3 and place on ice.
4. Add L2 to each sample as follows:
   - If using adaptors from tubes (0344NB-08), add 6 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.
   - If using an adaptor plate (0344-32, 0344NB-32 or 0344NB-A01), add the entire 15 µL of sample to the appropriate adaptor well, mix well by pipetting, then transfer the entire sample to a PCR tube.

   **Note:** All samples intended to share the same sequencing flow cell lane should have unique ligation adaptors.
5. Make a master mix by combining D1, L1 and L3 in a 0.5 mL capped tube according to the volumes shown in Table 4. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.

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

*! For 0344-32, 0344NB-32 and 0344NB-A01, follow best practices for using adaptor plates described in section III.B above.*

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

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

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

6. Add 9 µL of the Ligation Master Mix to each 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 2 (Ligation; 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 with the Ligation Purification protocol.

# IV. Protocol

## I. Ligation Purification

1. Retrieve the Agencourt beads and 70% ethanol set aside previously and ensure they are at room temperature.
2. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads. (For 0344-32, an excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. Add 70 µL of room-temperature D1 to each ligation reaction.
4. At room temperature, add 80 µL (0.8 volumes) of the bead suspension to each reaction. Mix thoroughly by pipetting 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to a magnetic plate and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove only 160 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 PCR amplification, so ensure beads are not removed with the binding buffer or the wash.
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 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 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 40 µL 1X low-EDTA TE buffer to the dried beads. Mix thoroughly by pipetting to ensure all the beads are resuspended. Let stand on the bench top for 3 minutes.
14. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
15. Carefully remove 35 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.

16. Set aside the Agencourt beads and 70% ethanol at room temperature for use in the Amplified Library Purification protocol.
17. Continue immediately with the Library Amplification protocol.

## J. Library Amplification

1. Remove the Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2) and Amplification Enzyme Mix (red: P3) from –20 °C storage.
2. Thaw P1, P2 and P3 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin P3 and place on ice.
4. Make a master mix by sequentially combining P1 and P2 in an appropriately sized capped tube according to the volumes shown in Table 5. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles, spin and place on ice.

**Table 5. Amplification Master Mix (volumes listed are for a single reaction)**

| AMP BUFFER MIX (RED: P1 VER 7) | AMP PRIMER MIX (RED: P2 VER 5) | AMP ENZYME MIX (RED: P3 VER 4) |
|---|---|---|
| 12.75 µL | 1.25 µL | 1.0 µL |

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

5. On ice, add 15 µL of the Amplification Master Mix to each sample.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Library Amplification; see Table 2):

   72 °C – 2 min, 95 °C – 3 min, 8–15* cycles (98 °C – 20 s, 65 °C – 30 s, 72 °C – 30 s), 72 °C – 1 min, hold at 4 °C

**Important Note:** The number of cycles (*) used for PCR amplification depends on the starting amount of genomic DNA. Please refer to Table 6 for a general guide to choosing the appropriate number of cycles for the PCR amplification reaction. Alternatively, real-time PCR can be used to determine the appropriate number of PCR cycles. For more information, contact NuGEN Technical Support.

**Table 6. Recommended PCR Cycles for Library Amplification.**

| STARTING INPUT | PCR CYCLES |
|---|---|
| <1 ng | may require optimization |
| 1–10 ng | 13–15 |
| 10–50 ng | 10–12 |
| 50–100 ng | 7–9 |

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue with the Amplified Library Purification protocol.

## K. Amplified Library Purification

1. Retrieve the Agencourt beads and 70% ethanol set aside previously and ensure they are still at room temperature.
2. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to the sample. 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 50 µL (1 volume) of the bead suspension to each reaction.
4. Mix thoroughly by pipetting 10 times.
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 85 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 ligation, so ensure beads are not removed with the binding buffer or the wash.
8. With the plate 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 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 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 33 µL 1X low-EDTA TE buffer to the dried beads. Mix thoroughly to ensure all the beads are resuspended.

14. Transfer the tubes to the magnet and let stand for 2 minutes.

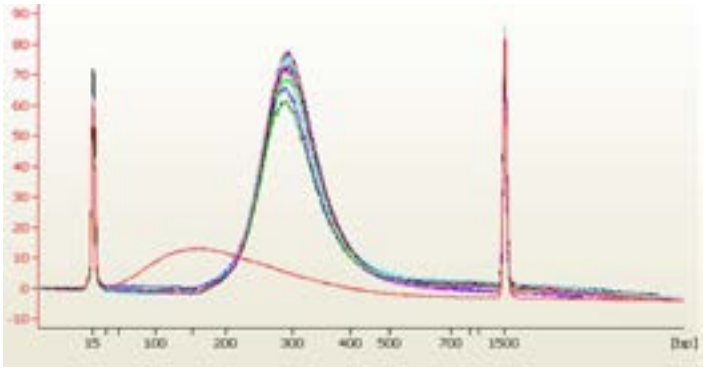
15. Carefully remove 30 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to let stand briefly on a magnet to minimize bead carryover.

16. Proceed to Quantitative and Qualitative Assessment of the Library.

## L. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer DNA 1000 Chip. If 150–200 base pair inserts were used, fragment distribution should be as shown in Figure 4. Larger insert sizes will shift the peak of the DNA trace.

**Figure 4. Fragment distribution on Bioanalyzer DNA 1000 Chip. Red = fragmented template DNA trace. Other traces from correctly constructed libraries.**




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).

# V. Technical Support

For help with any of our products, please contact Tecan NGS 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-gn@tecan.com.

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

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

# VI. Appendix

## A. Sequences of the Barcodes in the Multiplexed Reactions

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

**Important Note:** Part no. 0344-32 and 0344NB-32 barcode sequences were updated in May 2017. For more information, please go to the webpage at http://www.nugen.com/products/ovation-ultralow-library-system-v2 or contact Tech Support at techserv-gn@tecan.com.

All barcode sequences are separated by an edit distance of three. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012) Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

# VI. Appendix

**Table 7. Barcode sequences for barcoded adaptors (Part Nos. 0344NB-08, 0344-32, 0344NB-32 and 0344NB-A01).**

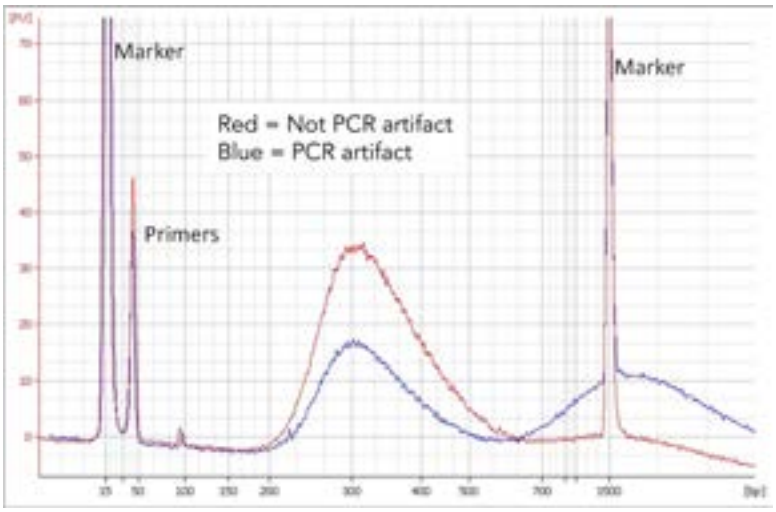
| PLATE POSITION | BARCODE SEQUENCE | PLATE POSITION | BARCODE SEQUENCE | PLATE POSITION | BARCODE SEQUENCE |
|---|---|---|---|---|---|
| A01 | CGCTACAT | A05 | AGGTTCCT | A09 | GCCTTAAC |
| B01 | AATCCAGC | B05 | GAACCTTC | B09 | ATTCCGCT |
| C01 | CGTCTAAC | C05 | AAGTCCTC | C09 | ATCGTGGT |
| D01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

# VI. Appendix

## B. PCR Enrichment Artifacts

In some instances, PCR enrichment may create artifacts in the downstream library size analysis which appear as high molecular weight species during Bioanalyzer or gel analysis (Figure 5). This phenomenon is due to the amplification of diverse library molecules that have the same adaptor sequences at their termini. As the concentration of library molecules increases during PCR, the adaptor ends begin to compete with the PCR primers for hybridization, resulting in partially hybridized species. Although this may impact PCR efficiency, it does not impact library quality for subsequent sequencing, nor does it affect quantitation by qPCR.

**Figure 5. Fragment distribution on Bioanalyzer 1000 Chip when PCR enrichment artifacts are present**




If desired, performing a single round of PCR in the presence of excess primer will resolve the material to a single peak of the correct library size.

When quantifying libraries that may have been subject to PCR enrichment artifacts, use the lower molecular weight peak to estimate library size and qPCR to determine concentration.

# VI. Appendix

## C. Frequently Asked Questions (FAQs)

### Input Recommendations

**Q1. Which NuGEN amplification system kits can be used to produce dsDNA for input to the Ovation Ultralow System V2?**
The Ovation RNA-Seq System V2 (Part No. 7102) and Ovation RNA-Seq FFPE System (Part No. 7150) have been validated to work with the Ovation Ultralow System V2 1–96.

**Q2. Can I use FFPE or other degraded DNA as input into NuGEN DNA-Seq Library Systems?**
We recommend using high quality DNA with the A260:A280 ratio in excess of 1.8. Use of DNA samples with lower ratios may result in low library yield.

### General Workflow

**Q3. 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 the Ovation Ultralow System V2. Other mechanical means of fragmentation, such as sonication, may be suitable.

**Q4. Does NuGEN provide reagents for performing the fragmentation step of the protocol?**
NuGEN does not provide the reagents used in the fragmentation steps. We suggest the Covaris instrument be utilized for DNA fragmentation, as suggested in the "materials" section of the User Guide.

**Q5. Can I modify the number of PCR amplification cycles recommended by the Ovation Ultralow System V2 workflow when using different DNA input amounts?**
Generally speaking, fewer PCR cycles will be needed when working with larger input amounts. See Table 6 of the User Guide for guidelines on the number of cycles to use.

**Q6. Can I combine the barcoded libraries prior to the PCR amplification step?**
This is not recommended. The stoichiometry of barcoded libraries may be adversely affected by this modification to the workflow. We suggest that the libraries be amplified and quantitated independently before being pooled for use on the sequencer.

### SPRI Bead Purification

**Q7. What is the difference between RNAClean XP and AMPure XP SPRI beads? Can both be used interchangeably?**
RNAClean XP beads are certified to be RNase and DNase free. We have tested both RNAClean XP and AMPure XP beads in our kits and observe no difference in performance between products.

# VI. Appendix

**Q8. What magnetic separation devices do you recommend for the SPRI bead purifications?**

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

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

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

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

**Q9. How can I ensure maximum recovery of sample from the SPRI bead purification?**

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

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

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

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

**Library Quantification and Qualification**

**Q10. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?**

Please refer to section IV. L. of the User Guide for guidelines on quantitative and qualitative assessment. We recommend using a qPCR based-method in combination with the Agilent Bioanalyzer or Tapestation for the most accurate quantification.

**Q11. How many bases do the Ovation Ultralow System V2 adaptors add to the library?**

The adaptors add 122 bp to the library.

# VI. Appendix

**Sequencing Recommendations**

**Q12. What sequencers are compatible with your libraries?**
Ovation Ultralow Systems V2 libraries are compatible with Illumina sequencing platforms.

**Q13. What kind of sequencing primers can I use with your library?**
The Ovation Ultralow System V2 is designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

**Q14. Can the Ovation Ultralow System V2 be used with paired-end sequencing?**
Yes, you can sequence Ovation Ultralow System V2 libraries as paired-end for subsequent paired-end data analysis.

**Q15. 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.

**Q16. What approach is used to minimize the impact of sequencing errors in the barcodes?**
Each barcode is a minimum edit distance of 3 from any other barcode. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one barcode becomes a different barcode. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012), Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

# VI. Appendix

## D. Update History

This document, the Ovation Ultralow System V2 user guide (M01379 v5.1), has been updated from the previous version to address the following topics:

| Description | Section | Page(s) |
|---|---|---|
| Updated NuGEN to Tecan Genomics legal entity. | Patents, Legal and Trademarks, VIII, Footer | Throughout |


NuGEN®
A Tecan Group company

**Tecan Genomics, Inc.**

**Headquarters USA**

900 Chesapeake Drive
Redwood City, CA 94063 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
cservice-gn@tecan.com
techserv-gn@tecan.com

**Europe**

P.O. Box 109
9350 AC Leek
The Netherlands
Tel: +31.13.5780215
Fax: +31.13.5780216
europe-gn@tecan.com

**Worldwide**

For our international distributors contact information, visit our website

**www.nugen.com**