USER GUIDE

Ovation® Ultralow System V2

REF 0344-32-FG, 0344NB-A01-FG, S02317-FG, S02366A-FG, and S02215-FG

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SYMBOL	MEANING
	Catalog Number
	Consult instructions for use
	Contains sufficient for <n> tests
	Warning
	Important
	Expiration Date
	Temperature limitation
	Optional stopping point
	Note
	Manufacturer

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I. Introduction

A. Overview

Intended Use

The Ovation Ultralow System V2 workflow provides a simple, fast and scalable solution for producing DNA-seq libraries starting with as little as 10 pg of double-stranded DNA. This workflow consists of the Ovation Ultralow V2 DNA-Seq Core kit as well as 8 nt single or unique dual index (UDI) Adaptor Plates. The UDI adaptors also contain UMI (Unique Molecular Identifier) sequences. Ovation Ultralow V2 DNA-seq workflow is intended for Research Use Only and not for use in diagnostic procedures.

Features

The Ovation Ultralow System V2 is compatible with dsDNA samples from a broad range of sample types. It comprises a streamlined add and incubate workflow for DNA library construction and provides single or UDI barcoding for scalability. This kit features Tecan's **DimerFree**® technology to minimize formation of adaptor dimers even at ultralow input amounts. Kit configurations include:

1. Ovation Ultralow System V2, Single Index, 32 reactions (Part No. 0344-32-FG and S02317-FG)
2. Ovation Ultralow System V2, Single Index, 96 reactions (Part No. 0344NB-A01-FG and S02366A-FG)
3. Ovation Ultralow System V2, UDI (with UMI), 96 reactions (Part No. 0344NB-A01-FG and S02215-FG)

Specifications

Input type:	dsDNA, including ds-cDNA
Input amount:	10 pg to 100 ng
# Reactions available:	32, and 96
Indexes available:	32 and 96 single index-8nt, 96 UDI (UMI)-8 nt
Sequencing platforms:	Illumina® NGS

B. Storage and Stability

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

All kits should be unpacked immediately upon receipt and stored as directed below.

All components of the kit should be stored at –20 °C in 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 are warranted to perform to specification through the labeled expiration date. Do not use kits that have passed the expiration date.

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide Tecan Genomics with the kit(s) and/or component(s) part number, and lot number information. Do not use damaged components.
2. Follow your institution's safety procedures for working with chemicals and handling of biological samples. Follow good laboratory practices and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary. Changing gloves between handling samples is recommended to avoid contamination of sample or reagents.
3. Consult your institution's environmental waste personnel on proper disposal of unused reagents. Check state and local regulations as they may differ from federal disposal regulations. This material may exhibit characteristics of hazardous waste requiring specific disposal requirements. Institutions should check their country hazardous waste disposal requirements.
4. An SDS for this product is available on the product webpage.

D. Before You Start

Please review this User Guide before using this kit for the first time, including the "Overview", "Kit Components", "Planning the Experiment", "Protocol" and "FAQ" sections.

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

A. Reagents Provided

Ovation Ultralow System V2 kit (Part No. 0344-32-FG and 0344NB-A01-FG) contains the reagents listed in Table 1 and is intended for 32 or 96 (A01) reactions. A01 fill sizes may be used in manual or automation workflows. A separate purchase of adaptor plates (Part No. S02317-FG, S02366A-FG and S02215-FG) is required for the library construction protocol.

Table 1. Ovation Ultralow System V2 Reagents (Part No. 0344-32-FG, 0344NB-A01-FG)

COMPONENT	0344-32-FG PART NUMBER	0344NB-A01-FG PART NUMBER	VIAL LABEL	VIAL NUMBER
End Repair Buffer Mix	S01844	S01686	Blue	ER1 VER 3
End Repair Enzyme Mix	S01845	S01687	Blue	ER2 VER 4
End Repair Enhancer	S01846	S01688	Blue	ER3
Ligation Buffer Mix	S01847	S01689	Yellow	L1 VER 4
Ligation Enzyme Mix	S01848	S01690	Yellow	L3 VER 4
Amplification Buffer Mix	S02066	S02072	Red	P1 VER 7
Amplification Primer Mix	S02068	S02074	Red	P2 VER 5
Amplification Enzyme Mix	S02067	S01875	Red	P3 VER 4
Nuclease-free Water	S01001	S01113	Green	D1

Table 2. Ovation Ultralow System V2 Adaptor Plates

COMPONENT	PART NUMBER	VIAL LABEL	VIAL NUMBER
32-plex Adaptor Plate	S02317-FG	Yellow	L2V23DR-BC
96-plex Adaptor Plate	S02366A-FG	Yellow	L2V23DR-BC
96-plex UDI Adaptor Plate*	S02215-FG	Yellow	L2V26

* The 96-plex UDI Adaptor plate is a custom product for research use only and has not undergone full development or validation. As a custom product, no performance claims are provided for this adaptor plate. Information about this UDI adaptor plate is provided in **Appendix A**.

Important: The reagents in the Ovation Ultralow System V2 DNA-seq workflow are similar to reagents in our other kits; however, unless the components' part numbers are identical, these reagents do not have exactly the same composition and therefore, are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - 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 spetrophotometer and cuvettes, or Nanodrop® UV-Vis Spectrophotometer
- **Reagents**
  - Ethanol, Absolute (200 Proof), Molecular Biology Grade (Sigma-Aldrich, Cat. #E7023; Fisher Scientific Cat. #BP2818), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Thermo Fisher Scientific, Cat. #J75793)
  - Agencourt® AMPure XP or RNAClean XP Beads (Beckman Coulter, Cat. #A63881 or A63987)
  - EvaGreen®, 20X (Biotium, Cat. #31000-T)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL nuclease-free microcentrifuge tubes
  - 8 X 0.2 mL thin-wall PCR strip tubes or 0.2 mL thin-wall 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 Tecan.
  - AlumaSeal II film (Thermo Fisher Scientific Cat. #6106W41)
  - 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)

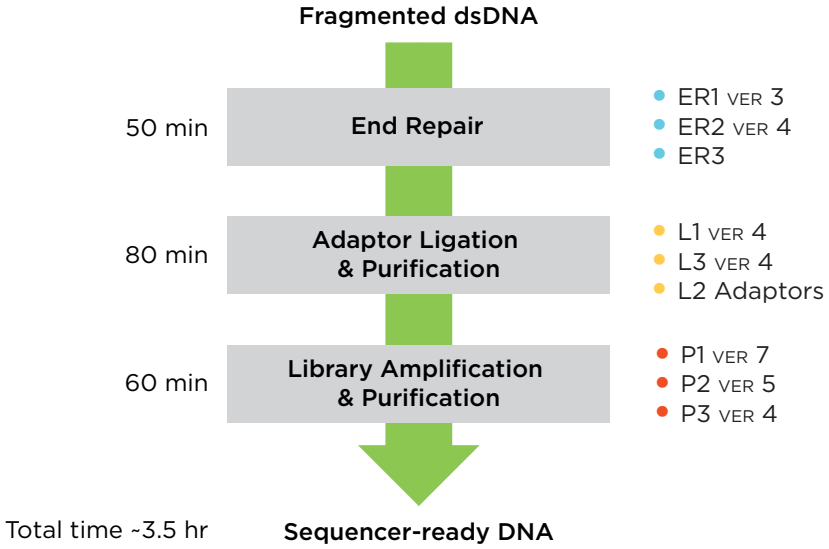
To Order:

- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Fisher Scientific, www.fishersci.com
- Illumina, www.illumina.com
- Qiagen, www.qiagen.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

A. Workflow and Time Required

The streamlined Ovation Ultralow System V2 workflow consists of four main steps: fragmentation of either genomic DNA or double-stranded cDNA, end repair, adaptor ligation and PCR amplification to produce the final library (Figure 1). The entire workflow can be completed in 3.5-4 hours, and yields DNA libraries ready for sequencing with Illumina NGS platforms.

Figure 1. Schematic of the Ovation Ultralow System V2 Workflow



B. Input DNA Requirements

DNA Quantity

DNA inputs must be between 10 pg and 100 ng of fragmented genomic dsDNA or ds-cDNA.

DNA Purity

DNA samples must be free of contaminating proteins, RNA and other cellular material, organic solvents (including phenol and ethanol), and salts used in many nucleic acid isolation methods. If using a DNA isolation method based on organic solvents, we recommend column purification after isolation.

One measure of DNA purity is the ratio of absorbance readings. The A260:A280 ratio for DNA samples should be more than 1.8 and A260:A230 should be in excess of 2.0. Use of DNA samples with lower ratios may results in poor performance.

DNA Integrity

Starting inputs should be fragmented to a size appropriate for inserts on Illumina sequencers. When using degraded DNA, we recommend using higher inputs in order to achieve yield and data quality similar to that of intact DNA samples. Depending on available input, DNA integrity can be determined using the Agilent 2100 Bioanalyzer or 5200 Fragment Analyzer. While it is impossible to guarantee satisfactory results with all degraded samples, this system may work with many samples that are degraded.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid and a 100 µL reaction volume capacity. Prepare thermocycler programs as 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 is above 30 °C. For thermal cyclers with a fixed temperature heated lid, use the default settings (typically 100-105 °C).

Table 3. Thermal Cycler Programming

END REPAIR		VOLUME
Program 1 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	15 µL
LIGATION		
Program 2 Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	30 µL
LIBRARY AMPLIFICATION qPCR OPTIMIZATION		
Program 3 qPCR Optimization	72 °C – 2 min, 95 °C – 3 min, 35 cycles (98 °C – 20 s, 65 °C – 30 s, 72 °C – 30 s), 72 °C – 1 min, hold at 4 °C	10.5 µL
AMPLIFICATION		
Program 4 Amplification	72 °C – 2 min, 95 °C – 3 min, N* cycles (98 °C – 20 s, 65 °C – 30 s, 72 °C – 30 s), 72 °C – 1 min, hold at 4 °C	100 µL

Important: The number of cycles (*) used for Library Amplification depends on the starting amount and quality of DNA and should be optimized by qPCR. For more information, contact Tecan NGS Technical Support.

D. Working with the 32- and 96-Plex Adaptor Plate

The Ovation Ultralow System V2 kits contain adaptor mixes with eight-base single- or unique dual-index indices. (Please review the part number corresponding to the single or UDI offering.) Each well 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 follow the protocol for mixing and transfer of the contents. 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 index sequencing, please see **Appendix A** on page 18.

Bead Purification

Agencourt Beads

Ampure XP or RNAClean XP Beads (Agencourt beads) are suitable for use with this kit. There are modifications to the Agencourt beads' standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads.

Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes prior to use. Cold beads and buffer will result in reduced recovery.
- Prior to use, ensure beads are fully resuspended by vortexing or inverting and tapping the tube.
- If using strip tubes or partial plates, ensure they are firmly placed on the magnetic plate. The use of individual tubes is not advised as they are not very stable on the magnetic plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- After the binding step has been completed, take care to minimize bead loss when removing the binding buffer. Loss of beads at this step may impact yields. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash is freshly prepared from fresh stocks. Lower percent mixes will reduce recovery.
- It is critical that all residual ethanol be removed prior to elution. Therefore, when removing the final wash, first remove most of the supernatant, then allow the excess to collect at the bottom of the tube before removing the remaining supernatant. This also reduces the required bead air-drying time.
- After drying the beads, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the next step.

Preparation of EtOH Wash Solution

Prepare a 70% EtOH wash solution by combining 100% EtOH and nuclease-free water. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes.

This protocol requires 0.8 mL of 70% EtOH solution per sample.

Table 4. EtOH Wash Recipe

1X REACTION VOLUME*	100% EtOH	NUCLEASE-FREE WATER
0.8 mL	0.56 mL	0.24 mL

*A minimum of 10% extra volume should be prepared for each sample.

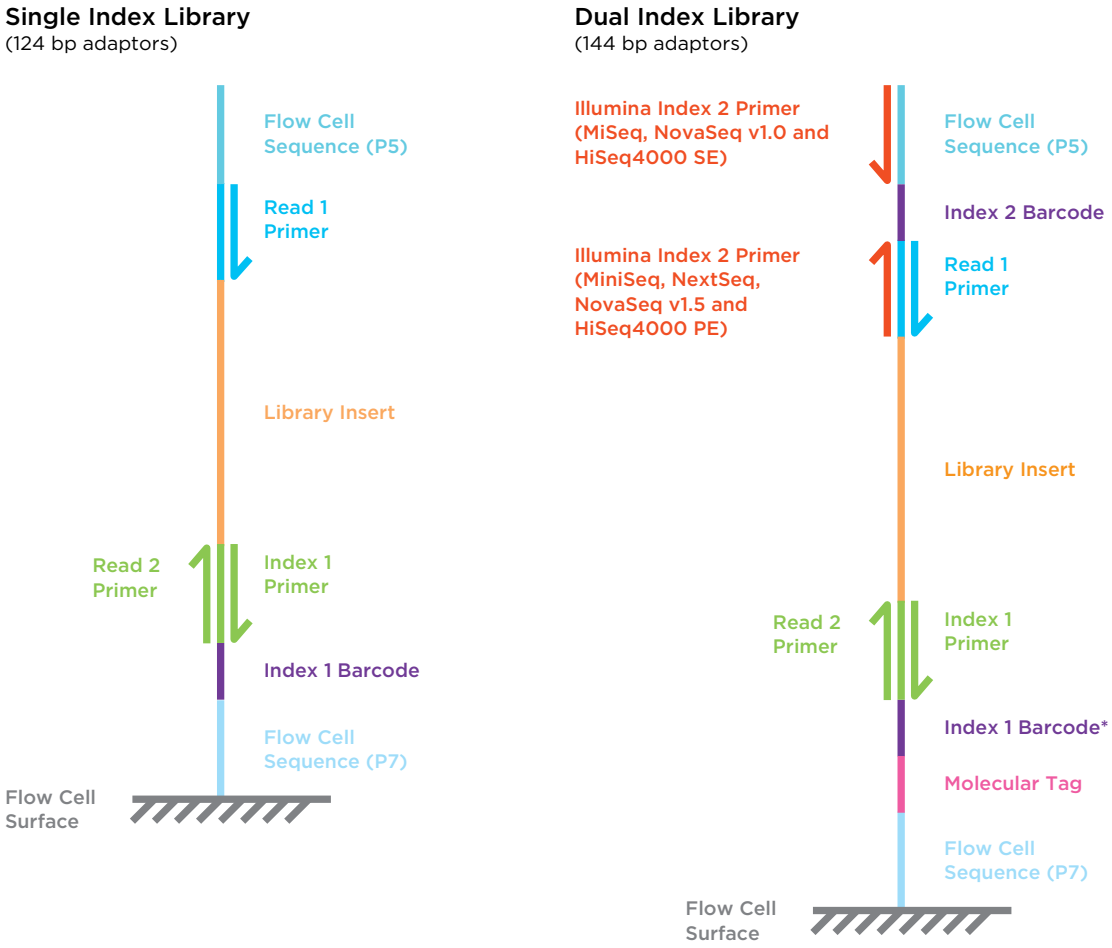
E. Sequencing Recommendations and Guidelines

Ovation Ultralow System V2 workflow produces libraries that are compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer.

Index Read Recommendations

UDI Adaptor Plates contain 8-base Unique Dual Indexes (UDI) for sample multiplexing (see Table 11). Both index 1 (i7) and index 2 (i5) should be sequenced for the detection of "index (barcode) hopping." Single index are also available with an index 1 (i7) index, 8 bp in length. Both these as well as the unique dual indexes differ from the sequences used by Illumina and can be found in **Appendix A**.

Figure 2. Ovation Ultralow System V2 DNA-Seq Library Structure



F. Data Analysis.

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

G. Amplified Library Storage

-20 °C

Amplified libraries may be stored at –20 °C.

Protocol Notes

Controls

- We recommend the routine use of a positive control DNA, especially the first time a reaction is set up. The use of a positive control DNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.

General Workflow

- Reagent fill volumes are sufficient for 10% overage consumption over six kit usages.
- Thaw components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Do not thaw all reagents at once.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing.
- Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme mixes nor warm any enzyme, adaptor or primer mixes.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip into the reaction mix.
- When instructed to mix via pipetting, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

Reagents

- 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 Tecan 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.

For each section of the protocol, recommended storage conditions listed in **Section I. B. Storage and Stability**. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

Return all reagents to their appropriate storage conditions promptly after use unless otherwise instructed.

A. Sample Preparation

If starting with intact DNA, shear DNA to 200–500 bp fragments using a Covaris Ultrasonicator and purify according to the protocol in **Appendix B**.

If beginning with already fragmented DNA, prepare 10 pg – 100 ng input DNA in 10 µL of low-EDTA TE Buffer (low TE) or Nuclease-free Water in a 0.2 mL PCR strip tube or plate. Continue to the next section.

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

B. 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.
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.

Note: Do not vortex any enzyme mixes.

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 5. Mix well by pipetting, spin down and place on ice.

Table 5. End Repair Master Mix

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 VER 3)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)	END REPAIR ENHANCER (BLUE: ER3)
1X REACTION VOLUME	3.5 µL	0.5 µL	1.0 µL

5. Add 5 µL of the End Repair Master Mix to each sample tube for a total volume of 15 µL. Mix well by pipetting, spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (End Repair, see Table 3):

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

7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately with the Ligation protocol.

C. 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 the entire 15 µL of sample to the appropriate L2 adaptor well, mix well by pipetting, then transfer the entire 18 µL of sample to a PCR tube.
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 6. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.

Important: The L1 Ligation Buffer Mix is very viscous. It is critical to pipet this reagent slowly and mix thoroughly.

Table 6. Ligation Master Mix

REAGENT	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)	NUCLEASE-FREE WATER (GREEN: D1)
1X REACTION VOLUME	6.0 µL	1.5 µL	1.5 µL

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 3):

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

D. Post-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.
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 47 µL (40 µL if not doing qPCR) 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 42 µL (35 µL if not doing qPCR) of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.

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

E. Library Amplification Optimization with qPCR (Optional)

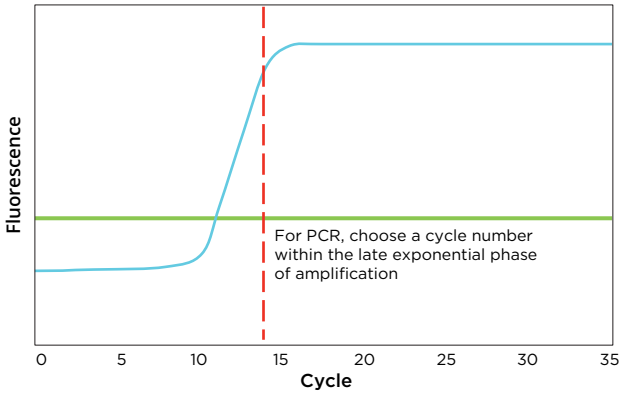
Note: qPCR optimization should be performed when running the kit for the first time or when using a new sample type or input or samples with unquantifiable input amounts or low quality.

Table 7. Amplification Master Mix for qPCR

REAGENT	AMPLIFICATION BUFFER MIX (P1)	AMPLIFICATION PRIMER MIX P2	AMPLIFICATION ENZYME MIX P3	20X EVAGREEN
1X REACTION VOLUME	2.6 µL	0.3 µL	0.2 µL	0.5 µL

1. Thaw P1, P2 and EvaGreen at room temperature. Mix by vortexing, spin and place on ice.
2. Spin down P3 and place on ice.
3. Prepare a master mix by sequentially combining P1, P2 and EvaGreen according to the volumes show in Table 7. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin and place on ice.
4. On ice, add 3.5 µL of the Amplification Master Mix to a qPCR strip tube or plate.
5. Add 7 µL of library sample to the qPCR strip tube or plate for a total of 10.5 µL. Mix well by pipetting, spin down and place on ice.
6. Place the tubes in a qPCR instrument programmed to run Program 3.
7. Select a cycle number for subsequent library amplification. The cycle number should be at the top of the exponential phase of the amplification plot (see Figure 3 below).

Figure 3. Stylized qPCR amplification plot.



Note: The cycle number used for subsequent library amplification should be determined empirically. Choose a cycle number within the mid to late exponential phase of the amplification plot (14 cycles in Figure 3).

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

F. Library Amplification

Note: Whenever using a sample for the first time with the kit, or using a new amount of input, perform real-time PCR as described above to determine the appropriate number of library amplification cycles for your sample.

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 8. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles, spin and place on ice.

Table 8. Amplification Master Mix

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 7)	AMPLIFICATION PRIMER MIX (RED: P2 VER 5)	AMPLIFICATION ENZYME MIX (RED: P3 VER 4)
1X REACTION VOLUME	12.75 µL	1.25 µL	1.0 µL

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 3):

   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

Note: The number of cycles used for PCR amplification depends on the starting amount of DNA. Note that the guidelines in Table 9 are based on high-quality human DNA inputs.

Table 9. 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

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

G. 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.

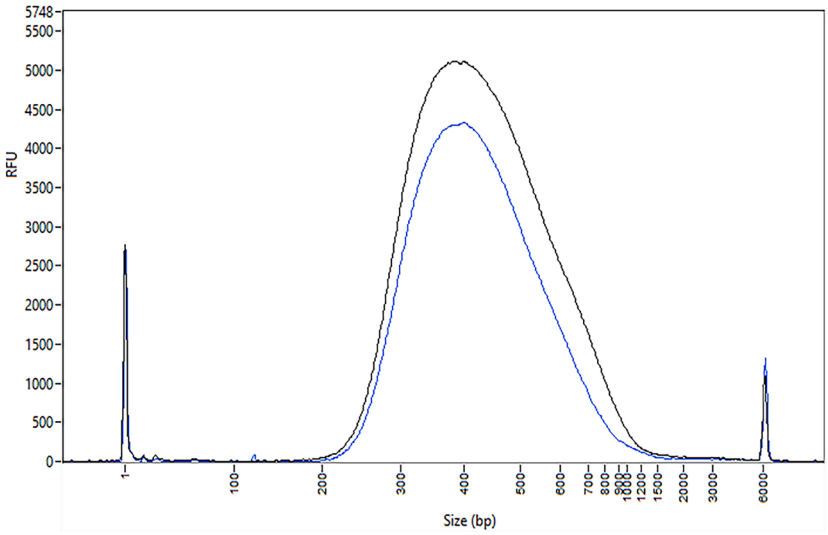
Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

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.

H. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer, Fragment Analyzer or equivalent to determine size distribution as shown in Figure 4.

Figure 4. Fragment distribution when 2 µL of final library is loaded onto a HS NGS Fragment Analyzer (1-6000 bp) from 10 ng (black) or 1 ng (blue) total DNA starting material.



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) or email Genomics.Support@tecan.com.

In Europe contact Tecan NGS Technical Support at Genomics.Support@tecan.com.

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

A. Index Sequences

Barcode sequences for the 32- and 96-plex Adaptor Plates are given below, with indices in 32 reaction kits found in wells A01–H04. Indices are color balanced in pairs (e.g. A01 + B01, C01 + D01, etc.) and in sets of 8 by column.

All index sequences are separated by an edit distance of three. For further details on the index 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.

Table 10. Index sequences for single index adaptors (Part Nos. S02317-FG and S02366A-FG)

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

Table 11. Index sequences for UDI adaptors (Part No. S02215-FG)

PLATE LOCATION	INDEX 1 SEQUENCE	INDEX 2 SEQUENCE	PLATE LOCATION	INDEX 1 SEQUENCE	INDEX 2 SEQUENCE	PLATE LOCATION	INDEX 1 SEQUENCE	INDEX 2 SEQUENCE
A01	CGCTACAT	AACCTACG	A05	AGGTTCCT	TCGAACCT	A09	GCCTTAAC	CCGTTATG
B01	AATCCAGC	GCATCCTA	B05	GAACCTTC	CAAGGTAC	B09	ATTCCGCT	TGTCGACT
C01	CGTCTAAC	CAACGAGT	C05	AAGTCCTC	AGCTACCA	C09	ATCGTGGT	CTCTATCG
D01	AACTCGGA	TGCAAGAC	D05	CCACAACA	CATCCAAG	D09	GCTACAAC	ACTGCTTG
E01	GTCGAGAA	CTTACAGC	E05	ATAACGCC	CTCACCAA	E09	TCTACGCA	CGCCTTAT
F01	ACAACAGC	ACCGACAA	F05	CCGGAATA	TCAGTAGG	F09	CTCCAATC	ATAGGTCC
G01	ATGACAGG	ACATGCCA	G05	CCAAGTAG	GAACGTGA	G09	ACTCTCCA	TGATCACG
H01	GCACACAA	GAGCAATC	H05	AAGGACCA	AGGAACAC	H09	GTCTCATC	CGGATCAA
A02	CTCCTAGT	CCTCATCT	A06	ACGCTTCT	CCTAAGTC	A10	GCCAGAAT	TACTAGCG
B02	TCTTCGAC	TACTGCTC	B06	CTATCCAC	AACGCACA	B10	AATGACGC	TGGACCAT
C02	GACTACGA	TTACCGAC	C06	TGACAACC	GTCAACAG	C10	GTACCACA	GCGCATAT
D02	ACTCCTAC	CCGTAACT	D06	CAGTGCTT	ACACCTCA	D10	ACGATCAG	ATCGCAAC
E02	CTTCCTTC	TTCCAGGT	E06	TCACTCGA	TATGGCAC	E10	TAACGTCG	TCAGCCTT
F02	ACCATCCT	CCATGAAC	F06	CTGACTAC	CGCAATGT	F10	CGCAACTA	CATTGACG
G02	CGTCCATT	TTCCTCCT	G06	GTGATCCA	ACTCAACG	G10	AACACTGG	ACAGGCAT
H02	AACTTGCC	CCAACTTC	H06	ACAGCAAG	GTCTGCAA	H10	CCTGTCAA	AGGTCTGT
A03	GTACACCT	GAGACCAA	A07	TGCTGTGA	CACGATTC	A11	TCCTGGTA	CAGATCCT
B03	ACGAGAAC	ACAGTTCG	B07	CAACACAG	AGAAGCCT	B11	CATCAACC	CTCCTGAA
C03	CGACCTAA	CTAACCTG	C07	CCACATTG	TACTCCAG	C11	AGCAGACA	AGAGGATG
D03	TACATCGG	TCCGATCA	D07	TAGTGCCA	CGTCAAGA	D11	GAAGACTG	CACCATGA
E03	ATCGTCTC	AGAAGGAC	E07	TCGTGCAT	CTGTACCA	E11	TCTAGTCC	CGGTAATC
F03	CCAACACT	GACGAACT	F07	CTACATCC	TCACCTAG	F11	CTCGACTT	GAGTGTGT
G03	TCTAGGAG	TTGCAACG	G07	CATACGGA	AACACCAC	G11	CTAGCTCA	AACTGAGG
H03	CTCGAACA	CCAACGAA	H07	TGCGTAAC	CGTCTTCA	H11	TCCAACTG	TGTGTCAG
A04	ACGGACTT	ATCGGAGA	A08	CAGGTTCA	AACGTAGC	A12	GACATCTC	TGTCACAC
B04	CTAAGACC	CCTAACAG	B08	AGAACCAG	GCAACCAT	B12	ACTGCACT	AGATCGTC
C04	AACCGAAC	CATACTCG	C08	GAATGGCA	GATCCACT	C12	GTTCCATG	CAATGCGA
D04	CCTTAGGT	TGCCTCAA	D08	AGGCAATG	ACCTAGAC	D12	ACCAAGCA	TGCTTGCT
E04	CCTATACC	TACAGAGC	E08	TAGGAGCT	CTAGCAGT	E12	CTCTCAGA	AATGGTCG
F04	AACGCCTT	CGAGAGAA	F08	CGAACAAC	TCGATGAC	F12	ACTCTGAG	AGTTGTGC
G04	TCCATTGC	AGGTAGGA	G08	CATTCGTC	TTGGTGCA	G12	GCTCAGTT	GTATCGAG
H04	CAAGCCAA	GAACGAAG	H08	AGCCAACT	AGTGCATC	H12	ATCTGACC	GTACGATC

The 96-plex UDI Adaptor plate is a custom product for research use only and has not undergone full development or validation. This adaptor plate contains 8-base Unique Dual Indexes (UDI) for sample multiplexing. Both index 1 (i7) and index 2 (i5) of UDI adaptors should be sequenced for the detection of "index (barcode) hopping."

B. Fragmented 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.

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.

C. Notes and Frequently Asked Questions (FAQs)

Input Recommendations and Kit Information

Note: The UDI+UMI adaptor plate (PN S02215-FG) is available to enable the detection and removal of index hopping and PCR duplicate reads. No performance claims are made and limited validation has been performed with the Ovation Ultralow System V2 and this adaptor plate.

Q1. Can I use FFPE or other degraded DNA as input into Tecan 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

Q2. 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 means of DNA fragmentation may be suitable.

Q3. Does Tecan provide reagents for performing the fragmentation step of the protocol?
Tecan 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.

Q4. 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 9 of the User Guide for guidelines on the number of cycles to use.

Q5. Can I combine the indexed libraries prior to the PCR amplification step?
This is not recommended. The stoichiometry of indexed libraries may be adversely affected by this modification to the workflow. We suggest that the libraries be amplified and quantified independently before being pooled for use on the sequencer.

SPRI Bead Purification

Q6. 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.

Q7. 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 Tecan workflow. This is also helpful to gain familiarity with the purification workflow.

Q8. 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

Q9. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?
Please refer to **Section IV. H**. 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.

Q10. How many bases do the Ovation Ultralow System V2 adaptors add to the library?
The single-indexed adaptors add 124 bp to the library and the Unique Dual Index adaptors add 144 bp to the library.

Q11. Why do my libraries have a wide insert size distribution?
Libraries may range in size and distribution depending on the method of fragmentation used. To achieve tighter distributions a double-sided bead clean up is recommended as well as optimization with fragmentation method utilized.

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

Q16. What approach is used to minimize the impact of sequencing errors in the indices?
Each index is a minimum edit distance of 3 from any other index. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one index becomes a different index. For further details on the index 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.

D. Update History

This document, the Ovation Ultralow System V2 user guide (M01379 v6.1), has been updated from the previous version to address the following topics:

DESCRIPTION	SECTION	PAGE(S)
Changed NuGEN/NuGEN Technologies to Tecan/Tecan Genomics.	Throughout	Throughout
Minor changes and corrections made throughout.	Throughout	Throughout
Content reorganized to streamline User Guide.	Throughout	Throughout
Tecan branding updated throughout.	Throughout	Throughout

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