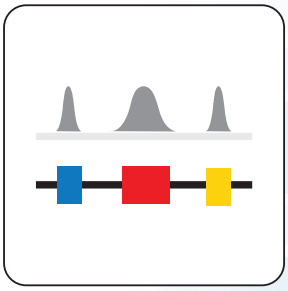
USER GUIDE



Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete™

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NuGEN®

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I. Introduction

A. Background

The Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit provides an end-to-end solution for strand-specific RNA-Seq library construction using as little as 100 ng of total RNA from microbiome samples. The core technology in this product enriches for non-rRNA in NGS libraries by selective priming during first strand cDNA synthesis as well as using AnyDeplete (formerly InDA-C) for depletion of unwanted transcript sequences during the final library construction steps. This kit is suitable for metatranscriptome analysis from microbiome samples.

The cDNA synthesis is carried out using proprietary primers to create double-stranded cDNA that 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. The cDNA is subsequently converted to NGS libraries using DimerFree™ library construction and unwanted reads are eliminated with AnyDeplete.

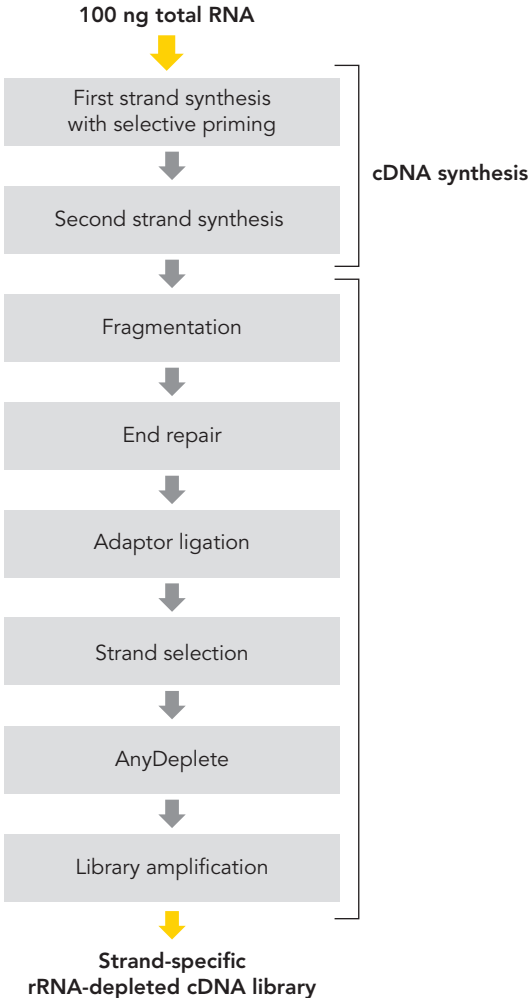
The Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit provides 32 unique barcoded adaptors to prepare libraries for multiplex sequencing.

B. Workflow

As shown in Figure 1, the Universal Prokaryotic RNA-Seq kit consists of double-stranded cDNA generation using selective priming, fragmentation of double-stranded cDNA, followed by end repair, adaptor ligation, AnyDeplete and PCR amplification to produce the final library. The entire workflow, including fragmentation, can be completed in as few as seven hours and yields cDNA libraries ready for either single read or paired-end sequencing on Illumina sequencing platforms.

I. Introduction

Figure 1. Universal Prokaryotic RNA-Seq workflow.



C. Perfomance Specifications

The Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit is designed to generate DNA libraries suitable for either single read or paired-end sequencing on Illumina NGS platforms. It is a simple and robust system capable of starting with 100 ng of total RNA to generate libraries suitable for use on the Illumina NGS systems in about seven hours.

D. Quality Control

Every lot of the Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit undergoes functional testing to meet specifications for library generation performance.

I. Introduction

E. Storage and Stability

The Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit is 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.
- The Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit 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 our website at www.nugen.com/products/universal-prokaryotic-rna-seq-library-prep

G. Before You Start

Please review this User Guide before using this kit for the first time, including the "Kit components", "Planning the experiment", "Overview", "Protocol" and "FAQ" sections. For more information, visit the Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete kit page at NuGEN.com (www.nugen.com/products/universal-prokaryotic-rna-seq-library-prep).

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

II. Components

A. Reagents Provided

Table 1. Universal Prokaryotic RNA-Seq, Prokaryotic AnyDeplete Reagents (Part Nos. 0363-32)

COMPONENT	PART NUMBER	VIAL LABEL	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
32-Plex Adaptor Plate	S02511	Yellow	L2V12DR-BC
Ligation Enzyme Mix	S01535	Yellow	L3 VER 4
Strand Selection Buffer Mix I	S01710	Purple	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

II. Components

Table 1 Ovation Universal RNA-Seq System (Part No. 0363), *continued*

COMPONENT	PART NUMBER	VIAL LABEL	VIAL NUMBER
Amplification Primer Mix	S01767	Red	P2 VER 10
Amplification Enzyme Mix	S01644	Red	P3 VER 2
Nuclease-free Water	S01113	Green	D1
Agencourt Beads	S01502	Clear	—

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

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Covaris S-series Sonication System
  - Agilent 2100 Bioanalyzer or 2200 TapeStation Instrument, or other equipment for electrophoretic analysis of nucleic acids
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL tube strips or plates
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - 2–20 µL or 5–50 µL multichannel pipette and 20–200 µL or 20–300 µL multichannel pipette for sample mixing
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid and 100 µL reaction capacity
  - Qubit® 2.0, 3.0 or 4 Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries.
- **Reagents**
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793) optional; for diluting nucleic acids
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Nuclease-free water (Alfa Aesar, Cat. #J71786), for purification steps
  - Agilent High Sensitivity DNA Kit (Agilent, Cat. #5067-4626) or equivalent
  - EvaGreen®, 20X (Biotium, Cat. #31000) optional; for optimizing Library Amplification with qPCR
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL PCR strip tubes or 0.2 mL thin-wall PCR plates
  - Low-retention microcentrifuge tubes (DNA LoBind Tubes, Eppendorf Cat. #0030108035 or 0030108051)
  - Magnetic stand for 0.2 mL strip tubes or plates. (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.

II. Components

- 96-well plate sealing foil (Thermo Fisher Scientific, Cat. #AB1720)
- 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)
- Disposable gloves
- Kimwipes
- Ice bucket

To Order:

- Agilent, www.agilent.com
- Alfa Aesar, www.alfa.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Covaris, www.covarisinc.com
- Eppendorf, www.eppendorf.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
- Zymo Research, www.zymoresearch.com

III. Planning the Experiment

A. Input RNA Requirements

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.

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.

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.

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. Amplified Library Storage

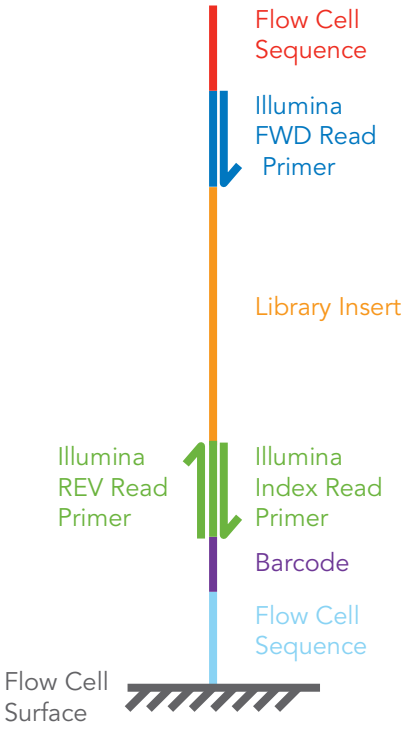
Amplified libraries may be stored at –20 °C.

C. Sequencing Recommendations and Guidelines

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

III. Planning the Experiment

Figure 2. Universal Prokaryotic RNA-Seq library structure.



D. Data Analysis

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

Note: The forward read from the Universal Prokaryotic RNA-Seq kit libraries represents the sense strand. This may be opposite to stranded data from other library preparation kits and may require slight modification to the data analysis workflow. Contact Tecan NGS Technical Support (techserv-gn@tecan.com) for more information.

IV. Overview

A. Overview

The library preparation process used in the Universal Prokaryotic RNA-Seq kit 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 Universal Prokaryotic RNA-Seq kit 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

Controls

- We recommend the routine use of a positive control RNA, especially the first time a reaction is set up. The use of a positive control RNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- Routine use of a no template control (NTC) is recommended to monitor the work environment for potential carryover contamination of previous libraries.

General Workflow

- Set up no fewer than 4 reactions at a time to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of samples from the kit. Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes.
- 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 preparing master mixes, use the minimal amount of extra material to ensure you are able to run the maximum number of reactions using the components provided in the kit.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip to 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

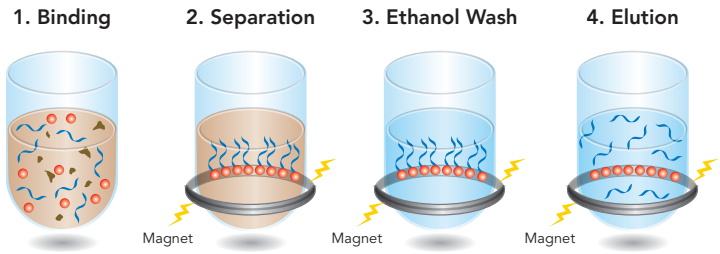
- Use the water provided with the kit (Green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Components and reagents from other Tecan NGS products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols.
- Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

C. Agencourt® Beads

There are modifications to the Agencourt beads' standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads. The bead purification process consists of:

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

Figure 3. Agencourt bead purification.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes 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.
- Note that our recommendations in the bead protocols may differ from the standard Beckman Coulter protocols. Please follow the protocol as written in this guide.
- 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.

IV. Overview

- Ensure that the 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- During the ethanol washes, do not allow the beads to disperse. Keep the samples on the magnet in order to keep the beads on the walls of the plate wells or tubes.
- It is critical that all residual ethanol be removed prior to elution. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This 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 amplification step.

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 2, 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, use the default settings (typically 100 to 105 °C).

Table 2. Thermal Cycler Programming

PRIMER ANNEALING		VOLUME
Program 1 Primer Annealing	65 °C – 5 min	7 µL
cDNA SYNTHESIS		**VOLUME**
Program 2 First Strand Synthesis	40 °C – 30 min, hold at 4 °C	10 µL
Program 3 Second Strand Synthesis	16 °C – 60 min, hold at 4 °C	75 µL
END REPAIR		**VOLUME**
Program 4 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	15 µL
LIGATION		**VOLUME**
Program 5 Ligation	25 °C – 30 min, hold at 4 °C	30 µL
STRAND SELECTION		**VOLUME**
Program 6 Strand Selection	72 °C – 10 min, hold at 4 °C	100 µL
ANYDEPLETE		**VOLUME**
Program 7 Probe Binding	37 °C – 10 min, 95 °C – 2 min, 50 °C – 30 s, 65 °C – 5 min, hold at 4 °C	25 µL

Table 2. Thermal Cycler Programming, *continued*

Program 8 Targeted Depletion	55 °C – 15 min, 95 °C – 5 min, hold at 4 °C	50 µL
AMPLIFICATION		**VOLUME**
Program 9 Library Amplification	95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), 18x*(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C	100 µL

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

V. Protocol

For each section of the protocol, remove reagents from storage as listed. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

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

A. Sample Preparation

1. Remove Nuclease-free Water (Green: D1) from –20 °C and place at room temperature.
2. Aliquot each total RNA input sample (100–500 ng) into a 0.2 mL strip tube or 96-well plate.
3. Dilute the RNA with D1 to a final volume of 5 µL and place on ice.

B. First Strand Synthesis

1. Remove A1 from –20 °C storage and thaw at room temperature. Mix by vortexing, spin down and place on ice.

Table 3. First Strand Master Mix

REAGENT	FIRST STRAND BUFFER MIX (BLUE: A2 VER 8)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 4)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	2.5 µL	0.5 µL

2. Spin down the contents of A3 and place on ice.
3. Thaw A2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Add 2 µL of A1 to each sample tube for a total of 7 µL. Mix well by pipetting, spin down and place on ice.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 2):

   65 °C – 5 min
6. Immediately remove the tubes from the thermal cycler and snap chill on ice.
7. Once Primer Annealing (Step 4) 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. Mix well by pipetting, spin down and place on ice.
8. Add 3 µL of First Strand Master Mix to each sample tube for a total of 10 µL. Mix well by pipetting, spin down and place on ice.
9. 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
10. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Second Strand cDNA Synthesis

1. Remove Agencourt beads from 4 °C storage and Nuclease-free Water (Green: D1) from –20 °C storage and place on the bench top to reach room temperature for use in the next section.
2. Remove B3 from –20 °C storage and thaw at room temperature. Mix by vortexing, spin down and place on ice.

Table 4. Second Strand Master Mix

REAGENT	SECOND STRAND BUFFER MIX (YELLOW: B1 VER 6)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	63.0 µL	2.0 µL

3. Spin down the contents of B2 and place on ice.
4. Thaw B1 at room temperature. Mix by vortexing, spin down and place on ice.
5. Prepare a master mix by combining B1 and B2 in a 0.5 mL capped tube according to the volumes shown in Table 4. Mix well by pipetting, spin down and place on ice.
6. Add 65 µL of Second Strand Master Mix to each sample tube for a total of 75 µL. Mix well by pipetting, spin down 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 B3 to each sample tube. Mix well by pipetting and spin down.

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

V. Protocol

D. 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 E210 – level 6
AFA Intensifier	Yes

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

E. cDNA Purification

1. Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution. (Sufficient wash solution should be prepared for all bead purification steps.)

Important:
- It is critical that the ethanol solution in the purification steps be prepared fresh on the day of the experiment from a recently opened stock container.
- Measure both the ethanol and the water carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce sample recovery.

3. 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.)

V. Protocol

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

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

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 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 to completely clear the solution of beads.
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.

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

V. Protocol

F. End Repair

Table 6. End Repair Master Mix

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 VER 7)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)	END REPAIR ENHANCER (BLUE: ER3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	4 µL	0.5 µL	0.5 µL

1. Spin down the contents of ER2 and ER3 and place on ice.
2. Thaw ER1 at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining ER1, ER2 and ER3 in a 0.5 mL capped tube according to the volumes shown in Table 6. Mix well by pipetting, spin down and place on ice.
4. Add 5 µL of End Repair Master Mix to each sample tube for a total of 15 µL. Mix by pipetting, spin down and place on ice.
5. Place the tubes in a thermal cycler programmed to run Program 4 (End Repair; see Table 2):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

G. Adaptor Ligation

Table 7. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	4.5 µL	6.0 µL	1.5 µL

1. Spin down L3 and place on ice.
2. Thaw Adaptor Plate (L2V12DR) on ice, spin down and return to ice.
3. Thaw L1 at room temperature. Mix by vortexing, spin down and place on ice.
4. Add 3 µL of the appropriate barcoded Adaptor Mix to each sample. Mix thoroughly by pipetting, spin down and place on ice. Make sure a unique barcode is used for each sample to be multiplexed together on the sequencer.

5. Prepare a master mix by combining 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 down 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.

6. Add 12 µL of Ligation Master Mix to each reaction tube for a total of 30 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
7. Place the tubes in a thermal cycler programmed to run Program 5 (Adaptor 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.

H. Strand Selection

1. Remove Agencourt beads from 4 °C storage and Nuclease-free Water (Green: D1) from –20 °C storage and place on the bench top to reach room temperature for use in the next step.

Table 8. Strand Selection Master Mix

REAGENT	STRAND SELECTION BUFFER MIX I (PURPLE: SS1)	STRAND SELECTION ENZYME MIX I (PURPLE: SS2)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	69 µL	1 µL

2. Thaw SS1 at room temperature. Mix by vortexing, spin down 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.
5. Add 70 µL of Strand Selection Master Mix to 30 µL of each sample for a total of 100 µL. Mix by pipetting, spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 6 (Strand Selection; see Table 2):

   72 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation.

V. Protocol

I. Strand Selection Purification

1. Ensure the Agencourt beads and 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. Add 80 µL (0.8 volumes) of the bead suspension to the Strand Selection 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 the binding buffer and discard it.

Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into subsequent steps of the protocol, so ensure beads are not removed with the binding buffer or the wash.

7. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat the 70% ethanol wash one more time, for a total of two washes.

Note: 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 (Green: 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 and, 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.

J. Probe Binding

Table 9. Probe Binding Master Mix

REAGENT	STRAND SELECTION II BUFFER MIX (PURPLE: SS3 VER 2)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	STRAND SELECTION REAGENT (PURPLE: SS5)	STRAND SELECTION ENZYME MIX III (PURPLE: SS6)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	0.5 µL	1 µL	0.5 µL

1. Thaw SS3 and SS5 at room temperature. Mix by pipetting, spin down and place on ice.
2. Spin down SS6 and SS4 and place on ice.
3. Prepare a master mix by combining SS3, SS5, SS6 and SS4 in a 0.5 mL capped tube, according to the volumes shown in Table 9.
4. Add 7 µL of Probe Binding Master Mix to each sample for a total of 25 µL. Mix by pipetting, spin down and place on ice.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (Probe Binding; see Table 2):

   37 °C – 10 min, 95 °C – 2 min, 50 °C – 30 s, 65 °C – 5 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

K. Targeted Depletion

Table 10. Targeted Depletion Master Mix

REAGENT	STRAND SELECTION II BUFFER MIX (PURPLE: SS3 VER 2)	RIBOSOMAL DEPLETION ENZYME MIX (PURPLE: RD1)	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	2.5 µL	17.5 µL

1. Thaw D1 and SS3 at room temperature. Mix SS3 by vortexing, spin down and place on ice.
2. Spin down RD1 and place on ice.
3. Prepare a master mix by combining SS3, RD1 and D1 in a 0.5 mL capped tube according the volumes in Table 10. Mix thoroughly by pipetting, spin down and place on ice.
4. Add 25 µL of Targeted Depletion Master Mix to each sample for a total of 50 µL. Mix by pipetting, spin down and place on ice.

V. Protocol

5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 8 (Targeted Depletion; see Table 2):

   55 °C – 15 min, 95 °C – 5 min, hold at 4 °C

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

L. Library Amplification

Note: The number of cycles used for PCR amplification should be optimized via real-time PCR whenever using a sample for the first time with the kit, or using a new amount of input. See **Appendix C** for a protocol to determine the appropriate number of cycles for your sample.

1. Remove Agencourt beads from 4 °C storage and Nuclease-free Water (Green: D1) from –20 °C storage and place on the bench top to reach room temperature for use in the next section.

Table 11. Library Amplification Master Mix

REAGENT	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 10)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	10 µL	8 µL	0.5 µL	31.5 µL

2. Thaw P1 and P2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Spin down P3 and place on ice.
4. Prepare a master mix by sequentially combining D1, P1 and P2 in an appropriately sized capped tube according to the volumes shown in Table 11. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
5. On ice, add 50 µL of Library Amplification Master Mix to each sample for a total of 100 µL. Mix by pipetting, spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Library Amplification; see Table 2):

   95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), 18x*(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C

   *The precise number of PCR cycles required depends on a number of factors including sample type, quality and input amount. The number of PCR cycles may be decreased or increased based on the requirements for a given sample and should be optimized via qPCR. Contact Tecan NGS Technical Support at techserv-gn@tecan.com for more information.

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

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

V. Protocol

M. Amplified Library Purification

1. Ensure the Agencourt beads and 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. Mix thoroughly by pipetting up and down.

Note: At this step, the reaction will be 200 µL. Pipet carefully to avoid spilling the sample.

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 the binding buffer and discard it.

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

7. Remove the tubes from the magnet, add 200 µL of freshly prepared 70% ethanol and mix thoroughly by pipetting up and down.
8. 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 27 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 and, ensuring as few beads as possible are carried over, transfer to a fresh set of tubes and place on ice.

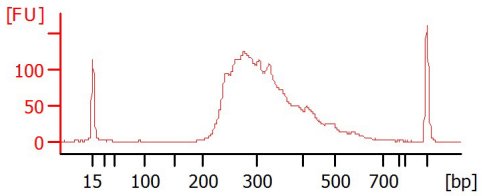
Optional stopping point: Store samples at –20 °C.

V. Protocol

N. Quantitative and Qualitative Assessment of the Library

1. Assess the library by performing a fragment distribution analysis according to manufacturer recommendations. A typical fragment distribution on the Bioanalyzer for *E. coli* libraries starting with 100 ng high quality RNA input, 200 bp Covaris fragmentation and 18 cycles is shown in Figure 4.

Figure 4. Fragment distribution analysis on Bioanalyzer DNA 1000 chip starting with 50 ng of the final library.



2. Quantify the library using a qPCR-based method.
3. Prepare libraries for sequencing following the Illumina "Denature and Dilute Libraries Guide" for your specific sequencer.

VI. Technical Support

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

In Europe contact Tecan at +31(0)135780215 (Phone) or email at europe-gn@tecan.com.

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

VII. Appendix

A. Barcode Sequences and Guidelines for Multiplex Experiments

Barcode sequences for the 32 barcode Adaptor Plate are given below. Barcodes are color balanced in sets of 8 by column.

Table 12. Barcode sequences for Universal Prokaryotic RNA-Seq adaptors.

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CACGTCTA	A03	TCAGCCTT
B01	AGCTAGTG	B03	AAGGCTCT
C01	ACTATCGC	C03	TGTTCCGT
D01	GCGTATCA	D03	GGAATGTC
E01	ACTCTCCA	E03	CATCCAAG
F01	CGTCCATT	F03	GTCAACAG
G01	AGCCGTAA	G03	TCGCTATC
H01	GAGTAGAG	H03	AGCCTATC
A02	ACGTCGTT	A04	TCGGATTC
B02	GTCCTGTT	B04	CGGAGTAT
C02	AGAAGCCT	C04	GAACCTTC
D02	GAAGATCC	D04	AGAGGATG
E02	TAGCTGAG	E04	ACGCTTCT
F02	ACGTCCAA	F04	CACAGGAA
G02	CACACATC	G04	ACGAATCC
H02	CGGATCAA	H04	CCTTCCAT

B. 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.
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.
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) with 1 µL rDNase (10 Units Roche Cat. #04716728001).
2. Add RNA sample (up to 500 ng) and add Nuclease-free Water (Green: D1) 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:

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

1. Add 4 volumes (100 µL) of RNA binding buffer to the sample.
2. Obtain one RNA Clean & Concentrator-5 Kit column and apply sample to column.
3. Spin column for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
4. Add 200 µL wash buffer (with ethanol added as per vendor's specifications).
5. After closing the column, spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. Add 200 µL fresh 80% ethanol, close cap and spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.

Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.

7. Place the RNA Clean & Concentrator-5 Kit column in a fresh 1.5 mL collection tube.
8. 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!

9. 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)

1. Add 80 µL ice-cold Nuclease-free Water (Green: D1) to the sample on ice.
2. Add 350 µL Buffer RLT and mix by pipetting.
3. Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
4. 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.
5. After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. 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.
7. 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.

8. Spin for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.

9. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the micro-centrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
10. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
11. Add 14 µL D1 directly to the center of the filter in the tube and close the cap. Do not use cold water!
12. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

C. Library Amplification Optimization with qPCR

In the Universal Prokaryotic RNA-Seq protocol, library amplification is performed by preparing an Amplification Master Mix and adding 50 µL of this master mix to 50 µL of library after target depletion for a total PCR volume of 100 µL. If you wish to use qPCR to guide the number of cycles used in library amplification in order to ensure there is no excess amplification performed, you may perform a 1/10th scale qPCR reaction as follows.

1. Prepare a PCR master mix according to the volumes shown in Table 13.

Table 13. PCR Master Mix (volumes listed are for a 10 µL qPCR reaction per sample)

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 10)	20X EvaGreen	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2.65 µL	1.0 µL	0.8 µL	0.5 µL	0.05 µL

2. Aliquot 5.0 µL of PCR master mix per well. Add 5.0 µL of purified library (after ligation but before PCR amplification) for a total qPCR volume of 10 µL.
3. Perform real-time qPCR with the following cycling conditions:

   95 °C – 2 min; 2x(95 °C – 30 s, 60°C – 90 s); 35x(95 °C – 30 s, 65 °C – 90 s)

The cycle number used for subsequent library amplification should be within the exponential phase of the amplification plot (17 or 18 cycles in Figure 5 as an example).

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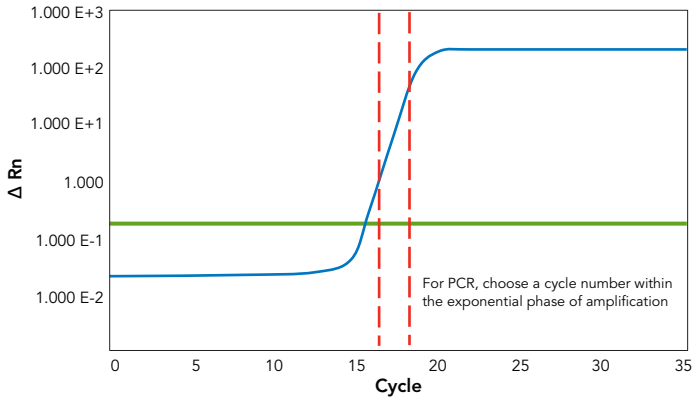
Figure 5. Stylized qPCR amplification plot.



4. To amplify the remaining 45 µL of library, prepare an amplification master mix according to the volumes shown in Table 14.

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

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION BUFFER MIX (RED: P1 VER 4)	AMPLIFICATION PRIMER MIX (RED: P2 VER 10)	AMPLIFICATION ENZYME MIX (RED: P3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	28.35 µL	9.0 µL	7.2 µL	0.45 µL

5. Add 45 µL of library to 45 µL of amplification master mix for a total PCR volume of 90 µL.
6. PCR amplify with the following cycling conditions, where N is the number of cycles determined from the above real-time qPCR assay:

   95 °C – 2 min; 2x(95 °C – 30 s, 60 °C – 90 s); N(95°C – 30 s, 65 °C – 90 s); 65 °C – 5 min; hold at 4 °C
7. Proceed with amplified library purification.

VII. Appendix

D. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with the Universal Prokaryotic RNA-Seq kit?
The Universal Prokaryotic RNA-Seq kit provides all necessary buffers, primers, enzymes and purification beads. The kit also provides nuclease-free water for purification and elution steps. EvaGreen for the optional Library Amplification Optimization step is not included.

Q2. What equipment is required or will be useful?
Required equipment includes a microcentrifuge; pipettes; vortexer; a thermal cycler; a magnetic plate for 0.2 mL tubes, strips, or plates; and a spectrophotometer or fluorometer. An Agilent Bioanalyzer or Tapestation may also be useful for optional analytical tests. A comprehensive list of required and recommended equipment can be found in **Section II.B**.

Input Recommendations

Q3. 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.

Q4. Can I use TRIzol® or other phenol-chloroform based extractions for RNA isolation?
We do not recommend the use of TRIzol® or similar methods as any carry over of organic solvents may inhibit downstream enzyme activity. If using TRIzol extracted RNA, we recommend using a column-based purification of the RNA prior to input into the kit.

Q5. Can I use the Universal Prokaryotic RNA-Seq kit with RNA from any organism?
This system has been designed specifically for prokaryotes. Performance with other organisms may vary.

Q6. Can I use carrier RNA during RNA isolation?
We do not recommend the use of carriers during RNA isolation. If a carrier is required, please contact Technical Support for more information.

Q7. Do I need to use high-quality total RNA?
The Universal Prokaryotic RNA-Seq kit is designed to work with purified total RNA. When using purified total RNA, samples should be of high molecular weight with little or no evidence of degradation. While it is impossible to guarantee the highest levels of performance when using RNA of lower quality, this system should allow the successful analysis of somewhat degraded samples. With such samples, users may experience lower yields and may encounter affected sequencing metrics.

Q8. Do I need to perform an rRNA depletion or poly(A) enrichment step before processing with the Universal Prokaryotic RNA-Seq kit?
The system is designed to use total RNA as input. rRNA depletion or poly(A) enrichment are not necessary.

Q9. How much total RNA do I need for this kit?
The Universal Prokaryotic RNA-Seq kit is designed to work from 100–500 ng total RNA input.

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Q10. Can contaminating genomic DNA interfere with the Universal Prokaryotic RNA-Seq kit 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 B** for guidelines.

General Workflow

Q11. Does this system contain a SPIA®-based amplification?
No. The cDNA is generated with selective primers, but no SPIA-based amplification is used.

Q12. Does Tecan 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. Tecan does not provide the reagents used in the fragmentation steps, but the user guide does specify suggested settings for the Covaris instrument.

Q13. 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.

Q14. Can this system be used with other library preparation workflows?
The Universal Prokaryotic RNA-Seq kit is 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.

Q15. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Universal Prokaryotic RNA-Seq kit utilizes optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the amount of adaptor dimer in the library.

Q16. How does your protocol enable strand retention?
The Universal Prokaryotic RNA-Seq kit utilizes targeted degradation of an incorporated modified nucleotide to ensure library inserts all carry the same directionality.

Q17. Can I combine the barcoded libraries prior to amplification?
The stoichiometry of barcoded libraries may be adversely affected by this modification to the Universal Prokaryotic RNA-Seq kit workflow. We suggest that the libraries be amplified and quantitated independently before being used for sequencing.

Q18. Where can I safely stop in the protocol?
Samples can be placed in short-term storage at –20 °C after second strand synthesis, cDNA purification, end repair, strand selection or after any of the bead purification steps.

SPRI Bead Purifications

Q19. 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.

Q20. 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.

Q21. 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

Q22. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?
Yes. Refer to S**ection V.N** of the user guide for guidelines on Quantitative and Qualitative Assessment.

Q23. How many bases do the Universal Prokaryotic RNA-Seq kit adaptors add to the library?
The adaptors add 125 bp to the size of the final library.

Sequencing Recommendations

Q24. 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.

Q25. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Each barcode is a minimum edit distance of 3 from any other barcode. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one barcode becomes a different barcode. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012), Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

VII. Appendix

Q26. What kind of sequencing primers can I use with your library?
The Universal Prokaryotic RNA-Seq kit is designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

Q27. Can the Universal Prokaryotic RNA-Seq libraries 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 with an average insert size of 200 bases. Contact Tecan NGS Technical Support (techserv-gn@tecan.com) for additional information.

Data Analysis

Q28. 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. Note that in libraries generated by the Universal Prokaryotic RNA-Seq kit, the forward read corresponds to the sense strand.

Q29. 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.

Custom AnyDeplete

Custom depletion designs can be tailored to any transcript, any organism. Please contact Tecan NGS Technical Support at techserv-gn@tecan.com for more information.

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