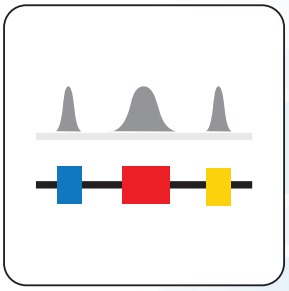
USER GUIDE



Universal RNA-Seq with NuQuant®

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I. Introduction

A. Background

The Universal RNA-Seq with NuQuant kit is an end-to-end solution for strand-specific RNA-Seq library construction using nanogram amounts of total RNA obtained from a broad range of tissues or cell lines. The workflow is compatible with both high quality and degraded RNA derived from FFPE samples.

This kit includes AnyDeplete technology for depletion of unwanted transcript sequences. AnyDeplete can be applied to a wide variety of transcripts to reduce uninformative sequencing reads for more efficient use of sequencing resources. This kit also features NuQuant, a novel fluorescent-based method of quantifying library molarity in minutes. NuQuant reduces the time and error associated with standard library QC.

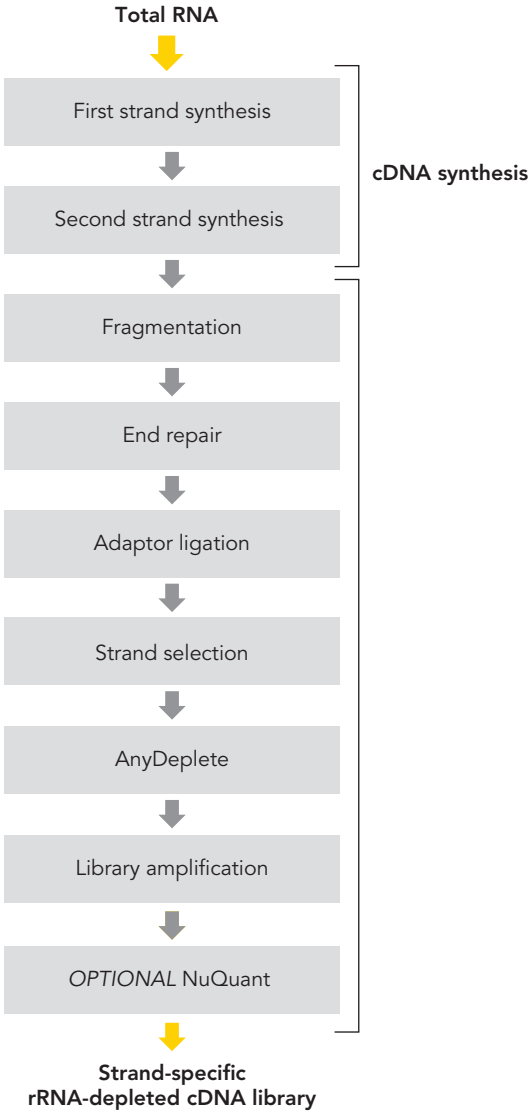
The Universal RNA-Seq with NuQuant kit provides 32 or 96 unique single-index adaptors to enable multiplex sequencing in order to further optimize efficiencies and cost savings during transcriptome analysis.

I. Introduction

B. Workflow

As shown in Figure 1, the streamlined workflow consists of double-stranded cDNA generation using a mixture of random and poly(T) priming, optional fragmentation of double-stranded cDNA, end repair, adaptor ligation, strand selection, targeted transcript depletion with AnyDeplete, and PCR amplification to produce the final library. The entire workflow, including fragmentation, can be completed in as few as 7 hours, and yields DNA libraries ready for either single read or paired-end sequencing on Illumina sequencing platforms.

Figure 1. Universal RNA-Seq with NuQuant workflow.



I. Introduction

C. Perfomance Specifications

The Universal RNA-Seq with NuQuant kit is designed to generate RNA-Seq libraries suitable for either single read or paired-end sequencing on Illumina NGS platforms. The Universal RNA-Seq with NuQuant kit provides a fast, simple and robust workflow for generation of Illumina compatible libraries from inputs of as little as 10 ng of high quality total RNA or 100 ng degraded RNA.

D. Quality Control

Every lot of the reagents in Universal RNA-Seq with NuQuant kit undergoes functional testing to meet specifications for library generation performance.

E. Storage and Stability

The Universal RNA-Seq with NuQuant kit is shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product may contain components with multiple storage temperature requirements.

- Vials labeled Agencourt® Beads (clear cap; provided only with 0364-32) should be removed from the top of the shipping carton upon delivery and stored at 4 °C.
- The Universal RNA-Seq with NuQuant 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)

An SDS for this product is available on the NuGEN website at www.nugen.com/products/universal-rna-seq-library-prep-nuquant.

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 RNA-Seq with NuQuant page at www.nugen.com/products/universal-rna-seq-library-prep-nuquant.

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 RNA-Seq with NuQuant (Part No. 0364)

COMPONENT	0364-32 PART NUMBER	0364-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
First Strand Primer Mix†	S01858	S02680	Blue	A1 ver 13
First Strand Buffer Mix	S01528	S02052	Blue	A2 ver 8
First Strand Enzyme Mix	S01529	S02053	Blue	A3 ver 4
Second Strand Buffer Mix	S01530 (2)	S02054 (2)	Yellow	B1 ver 6
Second Strand Enzyme Mix	S01531	S02055	Yellow	B2 ver 4
Second Strand Stop Buffer	S01554	S02266 (2)	Yellow	B3 ver 2
End Repair Buffer Mix	S01708	S02056	Blue	ER1 ver 7
End Repair Enzyme Mix	S01533	S01687	Blue	ER2 ver 4
End Repair Enhancer	S01709	S02057	Blue	ER3 ver 2
Ligation Buffer Mix	S01534	S01689	Yellow	L1 ver 4
32-plex Adaptor Plate	S02511	—	Yellow	L2V12DR-BC
96-plex Adaptor Plate	—	S01989	Yellow	L2V12DR-BC
Ligation Enzyme Mix	S01535	S01690	Yellow	L3 ver 4
Strand Selection Buffer Mix I	S01710	S01710 (3)	Purple	SS1
Strand Selection Enzyme Mix I	S01537	S02058	Purple	SS2
AnyDeplete Buffer Mix	S02306	S02427	Purple	AD1 ver 1
Strand Selection Enzyme Mix II	S01738	S02060	Purple	SS4
AnyDeplete Enzyme Mix I	S02307	S02428	Purple	AD2 ver 1
AnyDeplete Enzyme Mix II	S02308	S02471	Purple	AD3 ver 1
Amplification Reagent I	S02620	S02627	Red	AR1 ver 1
Amplification Reagent II	S02676	S02686	Red	AR2 ver 2
Amplification Enzyme Mix	S02679	S02629	Red	AR3 ver 1
Nuclease-free Water	S01113	S01113 (2)	Green	D1
NuQuant Buffer	S02517	S02517	Clear	—

† First strand random hexamers and oligo(dT) primers are available as separate components. For information, contact Tecan NGS Technical Support at techserv-gn@tecan.com.

II. Components

Table 1 Universal RNA-Seq with NuQuant (Part No. 0364), *continued*

COMPONENT	0364-32 PART NUMBER	0364-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
NuQuant Standard	S02512	S02512	Clear	—
Agencourt Beads	S01502 (4)	—	Clear	—

Table 2. AnyDeplete Probe Mixes

COMPONENT	PART NUMBER	VIAL LABEL	VIAL NUMBER
Human rRNA	ADU001	Purple	Human ADU001
Mouse rRNA + Globin	ADU002	Purple	Mouse ADU002
Drosophila	ADU003	Purple	Drosophila ADU003
Custom AnyDeplete Probe Mixes	Contact your local sales representative or Tecan NGS Technical Support for a list of available AnyDeplete designs or for a new custom AnyDeplete design.		

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Covaris Sonication System
  - Agilent 2100 Bioanalyzer or other 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
  - 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**
  - Agencourt RNAClean XP Beads or AMPure XP Beads (Beckman Coulter, Cat. #A63987 or A63881)
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793) optional; for diluting nucleic acids
  - For DNase treatment:
    - 1 M Tris-HCl (Invitrogen, Cat. #15567-027)
    - 0.1 M DTT (dithiothreitol) (Affymetrix, Cat. #70726 150 UL)
    - HL-dsDNase (ArcticZymes, Cat. #70800-201)
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Nuclease-free water (Alfa Aesar, Cat. #71786), for purification steps

II. Components

- 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; others). Other magnetic stands may be used as well, although their performance has not been validated by Tecan.
  - Cleaning solutions such as RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, Cat. #AM9780) and DNA OFF™ (MP Biomedicals, Cat. #11QD0500)
  - 96-well plate sealing foil (Thermo Fisher Scientific, Cat. #AB1720)
  - 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.covaris.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

III. Planning the Experiment

A. Input RNA Requirements

RNA Quantity

Total RNA input must be between 10–100 ng for high quality RNA, and 100–250 ng for degraded RNA. Inputs outside of these ranges 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. If using an RNA isolation method based on organic solvents, 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

When using degraded total RNA, we recommend using somewhat higher inputs in order to achieve yield and data quality similar to that of more intact RNA samples. RNA integrity can be determined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip®. The instrument provides a sensitive and rapid way of confirming RNA integrity prior to processing.

DNase Treatment

Thorough DNase treatment of RNA is required with this system. The presence of genomic DNA in the RNA sample will have adverse effects on assay performance and data quality. For samples that have NOT been DNase-treated, an integrated DNase treatment workflow is included in section **V.A.** of this user guide. For samples that have already been DNase-treated, follow the workflow in section **V.B.** of this user guide.

B. Working with the 32- and 96-Plex Adaptor Plates

The Adaptor Plate included with the 32 and 96 reaction Universal RNA-Seq with NuQuant kits contain adaptor mixes, each with a unique eight-base barcode. Each well contains sufficient volume for preparation of a single library. The Adaptor Plates are sealed with a foil seal designed to provide airtight storage.

Thaw adaptor plate on ice and spin down. Do NOT warm above room temperature. Make sure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice after centrifuging. When pipetting the adaptor mixes, puncture the seal for each well you wish to use with a fresh pipet tip, and transfer the appropriate volume of adaptor into your sample. The remaining wells of the plate should remain sealed for use at a later date. Cover used wells with a new foil seal (e.g., AlumaSeal II) to prevent any remaining adaptor-containing liquid from contaminating future reactions.

For details regarding barcode color balancing for multiplex sequencing, please see **Appendix A** on page 29.

III. Planning the Experiment

C. NuQuant

NuQuant® is a novel method to accurately measure molar concentrations of NGS libraries without the need for separate fragment size analysis. NuQuant is a proprietary method by which a specific number of fluorescent labels are incorporated into the library molecules during library preparation. Consequently, each library molecule has an equivalent number of labels incorporated, regardless of the size of the library fragment. The library molar concentration can be directly measured using fluorometers or standard plate readers.

NuQuant is compatible with Qubit 2.0, 3.0 or 4 as well as a wide variety of fluorescent plate readers.

For Qubit-based quantification, an app is required. The apps and installation instructions are available on GitHub: https://nugentechnologies.github.io/NuQuant/

D. Preparation and Storage of NuQuant Standard

Universal RNA-Seq with NuQuant includes a 50X NuQuant Standard stock solution. The fluorescence of this stock corresponds to a 32.3 µM Universal RNA-Seq with NuQuant library. A protocol for preparing the diluted standard for Qubit is provided below. For other fluorometers, a standard curve must be prepared. The diluted NuQuant Standard corresponds to a 644 nM library.

Preparation of Diluted NuQuant Standard for Qubit Fluorometers

Note:
- Concentrated and diluted NuQuant Standards should always be protected from light.
- Diluted NuQuant Standard may be stored at 4 °C for up to two months. Do not freeze diluted NuQuant Standard.

1. Remove concentrated 50X NuQuant Standard stock solution and NuQuant Buffer from storage.
2. Thaw concentrated standard on ice. Mix by vortexing, spin down and place on ice.
3. Prepare diluted NuQuant Standard in a DNA LoBind tube by combining 2 µL of 50X NuQuant. Standard stock solution and 98 µL of NuQuant Buffer. Mix thoroughly by vortexing, spin down and store at 4 °C.

E. Amplified Library Storage

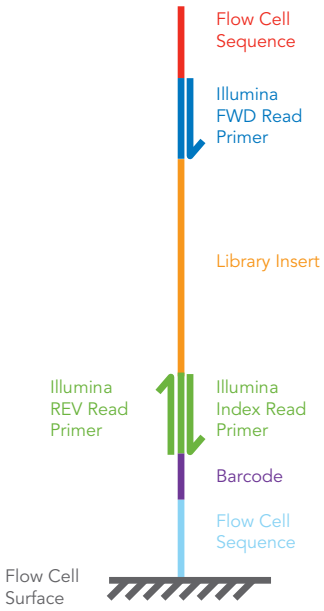
Amplified libraries may be stored at –20 °C.

F. Sequencing Recommendations and Guidelines

The Universal RNA-Seq with NuQuant 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. Universal RNA-Seq with NuQuant libraries contain an 8 bp barcode. These barcodes differ from the sequences used by Illumina and can be found in **Appendix A**.

III. Planning the Experiment

Figure 2. Universal RNA-Seq with NuQuant Library Structure.



G. 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 Universal RNA-Seq with NuQuant libraries represents the sense strand. This may be opposite to stranded data from other library preparation kits and may require slight modification of 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 RNA-Seq with NuQuant kit is performed in the following stages:

1.	DNase treatment	0.25 hours
2.	cDNA generation	1.5 hours
3.	Fragmentation and concentration	1.25 hours
4.	End repair	0.75 hours
5.	Adaptor ligation	0.5 hours
6.	Strand selection and AnyDeplete	1.5 hours
7.	Library amplification and purification	1.5 hours
	Total time to prepare amplified library	**7.25 hours**

Components in the Universal RNA-Seq with NuQuant 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 (32-reaction kit) or 8 reactions at a time (96-reaction kit) 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.

IV. Overview

- 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

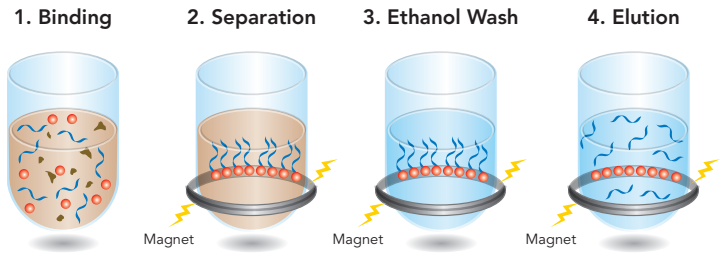
- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Components and reagents from other Tecan products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol 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.
- Ensure that all NuQuant reagents are at room temperature before use. Mix diluted libraries and standards thoroughly before performing NuQuant assay.

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:

- Binding of DNA to Agencourt beads
- Magnetic separation of beads from supernatant
- Ethanol wash of bound beads to remove contaminants
- 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 mag-

netic 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 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 3 following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C. For thermal cyclers with a fixed-temperature heated lid use the default settings (typically 100 to 105 °C).

Table 3. Thermal Cycler Programming

cDNA SYNTHESIS		**VOLUME**
Program 1 Primer Annealing	65 °C – 1 min	4 µL or 7 µL
Program 2 DNase Treatment	37 °C – 10 min, 55 °C – 10 min, hold at 4 °C	7 µL
Program 3 First Strand Synthesis	25 °C – 5 min, 40 °C – 5 min, 70 °C – 15 min, hold at 4 °C	10 µL
Program 4 Second Strand Synthesis	16 °C – 60 min, hold at 4 °C	75 µL
END REPAIR		**VOLUME**
Program 5 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	15 µL
LIGATION		**VOLUME**
Program 6 Adaptor Ligation	25 °C – 30 min, hold at 4 °C	30 µL
STRAND SELECTION		**VOLUME**
Program 7 Strand Selection I	72 °C – 10 min, hold at 4 °C	100 µL

IV. Overview

Table 3 Thermal Cycler Programming, *continued*

ANYDEPLETE		VOLUME
Program 8 Probe Binding	37 °C – 10 min, 95 °C – 2 min, 50 °C – 30 s, 65 °C – 5 min, hold at 4 °C	25 µL
Program 9 Targeted Depletion	60 °C – 30 min, 95 °C – 5 min, hold at 4 °C	50 µL
AMPLIFICATION		**VOLUME**
Program 10 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

*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. See **Appendix B** for a method to determine the appropriate number of cycles.

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.

Important: Several reagents in this kit have similar names. Please verify reagent names before use.

Note: For samples in which RNA has not been DNase treated, start with First Strand cDNA Synthesis with Integrated DNase Treatment (section **V.A.**); for samples in which the RNA has been DNase treated, proceed to First Strand cDNA Synthesis Using DNase-treated RNA (section **V.B.**).

A. First Strand cDNA Synthesis with Integrated DNase Treatment

Important: Thorough DNase treatment of RNA is required with this system. The presence of genomic DNA in the RNA sample will have adverse effects on assay performance and data quality. This workflow is for samples that have NOT been DNase treated. For samples that have already been DNase treated, proceed directly to section **V.B.**, First Strand cDNA Synthesis Using DNase-treated RNA.

For this protocol you will need the following additional reagents not included with the kit:

- 1 M Tris-HCl (pH 7.5)
- 0.1 M DTT
- HL-dsDNase
- 20 mM Nuclease-free $MgCl_2$

Table 4. HL-dsDNase Reaction Buffer

REAGENT	1 M TRIS-HCL	0.1 M DTT	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	—	–20 °C	—
1X REACTION VOLUME	7 µL	7 µL	86 µL

1. Remove Nuclease-free Water (green: D1) from –20 °C storage and place at room temperature.
2. Prepare the HL-dsDNase Reaction Buffer by combining 1 M Tris-HCl, 0.1 M DTT and D1 in a 0.5 mL capped tube according to the volumes shown in Table 4. Mix well by pipetting, spin down, and set aside for use in the DNase Master Mix (Table 5).
3. Remove First Strand Primer Mix (blue: A1 VER 13), First Strand Buffer Mix (blue: A2 VER 8), First Strand Enzyme Mix (blue: A3 VER 4) and HL-dsDNase from –20 °C storage for use in Tables 6 and 7.
4. Spin down A3 and HL-dsDNase and place on ice.
5. Thaw A1 and A2 at room temperature. Mix by vortexing, spin down and place on ice.

V. Protocol

6. Add 2 µL of total RNA (10–100 ng high quality total RNA or 100–250 ng degraded total RNA) to a 0.2 mL PCR tube and place on ice.
7. Add 2 µL of First Strand Primer Mix (A1) to each tube for a total of 4 µL. Mix by pipetting, spin, and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed run Program 1 (Primer Annealing; see Table 3):

   65 °C – 1 min
9. Immediately remove the tubes from the thermal cycler and snap chill on ice.

Table 5. DNase Master Mix

REAGENT	$MGCL_2$ SOLUTION	HL-dsDNASE REACTION BUFFER (TABLE 4)	HL-dsDNASE
STORAGE	—	–20 °C	–20 °C
1X REACTION VOLUME	1 µL	1 µL	1 µL

10. Once primer annealing is complete, prepare DNase Master Mix by combining HL-dsDNase Reaction Buffer (Table 4), $MgCl_2$ Solution and HL-dsDNase according to the volumes shown in Table 5.
11. Add 3 µL of DNase Treatment Master Mix to each sample tube for a total of 7 µL.
12. Mix by pipetting, spin down and place in a pre-warmed thermal cycler programmed to run Program 2 (DNase Treatment; see Table 3):

    37 °C – 10 min, 55 °C – 10 min, hold at 4 °C

Table 6. First Strand Master Mix

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

13. Prepare First Strand Master Mix by combining A2 and A3 in a 0.5 mL capped tube according to the volumes shown in Table 6.
14. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (First Strand cDNA Synthesis; see Table 3):

    25 °C – 5 min, 40 °C – 5 min, 70 °C – 15 min, hold at 4 °C
15. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
16. Continue immediately to section **V.C. Second Strand cDNA Synthesis**.

V. Protocol

B. First Strand cDNA Synthesis Using DNase-treated RNA

Important: Thorough DNase treatment of RNA is required with this system. The presence of genomic DNA in the RNA sample will have adverse effects on assay performance and data quality. This workflow is for samples that have been DNase treated prior to the Universal RNA-Seq with NuQuant protocol.

1. Remove Nuclease-free Water (green: D1) from –20 °C storage and place at room temperature.
2. Remove First Strand Primer Mix (blue: A1 VER 13), First Strand Buffer Mix (blue: A2 VER 8) and First Strand Enzyme Mix (blue: A3 VER 4) from –20 °C storage for use in Table 7.
3. Spin down A3 and place on ice.
4. Thaw A1 and A2 at room temperature. Mix by vortexing, spin down and place on ice.
5. Add 5 µL of total RNA (10–100 ng high quality total RNA or 100–250 ng degraded total RNA) to a 0.2 mL PCR tube.
6. Add 2 µL of First Strand Primer Mix (A1) to each tube for a total of 7 µL. Mix by pipetting, spin, and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed run Program 1 (Primer Annealing; see Table 3):

   65 °C – 1 min
8. Immediately remove the tubes from the thermal cycler and snap chill on ice.

Table 7. First Strand Master Mix

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

9. Once Primer Annealing is complete, prepare First Strand Master Mix by combining A2 and A3 in a 0.5 mL capped tube according to the volumes shown in Table 7.
10. 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.
11. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (First Strand cDNA Synthesis; see Table 3):

    25 °C – 5 min, 40 °C – 5 min, 70 °C – 15 min, hold at 4 °C
12. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
13. Continue immediately to section **V.C. Second Strand cDNA Synthesis**.

V. Protocol

C. Second Strand cDNA Synthesis

1. Remove Second Strand Stop Buffer (Yellow: B3 VER 2) from –20 °C for use after Program 4.

Table 8. 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 µL	2 µL

2. Spin down the contents of B2 and place on ice.
3. Thaw B1 and B3 at room temperature, mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining B1 and B2 in a 0.5 mL capped tube according to the volumes shown in Table 8.
5. 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.
6. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (Second Strand cDNA Synthesis; see Table 3):

   16 °C – 60 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation.
8. Add 45 µL of B3 to each sample tube for a total of 120 µL. Mix well by pipetting and spin down.
9. Continue immediately to section **D. cDNA Fragmentation** or store samples at –20 °C.

Note: cDNA fragmentation may be omitted from the protocol†. To skip cDNA fragmentation, add 25 µl Nuclease-free Water (Green: D1) and continue to section **E. cDNA Concentration**.

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

† Note: Contact Tecan NGS Technical Support for additional information.

V. Protocol

D. cDNA Fragmentation

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.
2. Fragment all DNA samples with Covaris Ultrasonication according to the manufacturer's recommendations.

Note:
- When processing cDNA derived from intact total RNA, a median fragment size of 200–500 bp is recommended.
- Alternative fragmentation methods may be suitable for use with the Universal RNA-Seq kit, but have not been validated by Tecan.

3. After fragmentation, collect 100 µL fragmented cDNA from the Covaris tube. If the collected volume is lower than 100 µL, add nuclease-free water to bring the total volume to 100 µL.

E. cDNA Concentration

1. Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution.

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 amplification yield.

3. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
4. At room temperature, add 180 µL (1.8 volumes) of bead suspension to 100 µL fragmented cDNA†. Mix thoroughly by pipetting.

Note: At this step, the reaction will be 280 µL. Pipet carefully to avoid spilling the sample.

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.

† If tubes have become noticably warped or plastic integrity compromised, transfer all of the sample to fresh tubes.

V. Protocol

9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

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.

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: It is critical to remove as much of the ethanol as possible. Use at least 2 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.
13. Remove the tubes from the magnet.
14. Add 11 µL room temperature D1 to the first aliquot of dried beads. Mix thoroughly to ensure all beads are resuspended.
15. Transfer the first aliquot of resuspended beads to the second aliquot of dried beads. Mix thoroughly to ensure all of the beads are resuspended.
16. Incubate at room temperature for 3 minutes.
17. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
18. Transfer 10 µL of eluate to a fresh 0.2 mL tube, ensuring as few beads as possible are carried over, and place on ice.

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

F. End Repair

Table 9. 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.

V. Protocol

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 9. 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 well by pipetting, spin down and place on ice.
5. Place the tubes in a thermal cycler programmed to run Program 5 (End Repair; see Table 3):

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

G. Adaptor Ligation

Table 10. 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.

Note: 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.

2. Thaw Adaptor Plate on ice, spin down, and return to ice.
3. Thaw L1 and D1 at room temperature. Mix by vortexing, spin down and place on ice.
4. Add 3 µL of the appropriate adaptor mix to each sample. Mix thoroughly by pipetting. 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 10. 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. Pipet this reagent slowly and mix thoroughly.

6. Add 12 µL of Ligation Master Mix to each sample 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 6 (Adaptor Ligation; see Table 3):

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

V. Protocol

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 11. 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.

Note: For 32 reaction kits, SS1 is provided in a 5 mL vial.

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 11.
5. Add 70 µL of the Strand Selection I 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 7 (Strand Selection I; see Table 3):

   72 °C – 10 min, hold at 4 °C

7. Remove the tubes from the thermal cycler and spin to collect condensation.

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.
3. Add 80 µL (0.8 volumes) of the bead suspension to the Strand Selection reaction product. Mix thoroughly by pipetting.
4. Incubate at room temperature for 10 minutes.
5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove 165 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

V. Protocol

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. 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 16 µL 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 15 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.

J. Probe Binding

Table 12. Probe Binding Master Mix

REAGENT	ANYDEPLETE BUFFER MIX (PURPLE: AD1 VER 1)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	ANYDEPLETE PROBE MIX (PURPLE: ADXX)	ANYDEPLETE ENZYME MIX I (PURPLE: AD2 VER 1)
STORAGE	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	5 µL	0.5 µL	4 µL	0.5 µL

1. Spin down SS4 and AD2 and place on ice.
2. Thaw AD1 and AnyDeplete Probe Mix at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining AD1, AnyDeplete probe mix, SS4 and AD2 in a 0.5 mL capped tube, according to the volumes shown in Table 12.
4. Add 10 µL of Probe Binding Master Mix to each sample for a total of 25 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 (Probe Binding; see Table 3):

   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 13. Targeted Depletion Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	ANYDEPLETE BUFFER MIX (PURPLE: AD1 VER 1)	ANYDEPLETE ENZYME MIX II (PURPLE: AD3 VER 1)
STORAGE	—	–20 °C	–20 °C
1X REACTION VOLUME	16 µL	5 µL	4 µL

1. Spin down AD3 and place on ice.
2. Thaw D1 and AD1 at room temperature. Mix AD1 by vortexing, spin down and place on ice.
3. Prepare a master mix by combining AD1, AD3 and D1 in a 0.5 mL capped tube according the volumes in Table 13. 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.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Targeted Depletion; see Table 3):

   60 °C – 30 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.

V. Protocol

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 B** 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 14. Library Amplification Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 2)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)
STORAGE	—	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	31.5 µL	10 µL	8 µL	0.5 µL

2. Spin down AR3 and place on ice.
3. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by sequentially combining D1, AR1 and AR2 in an appropriately sized capped tube, according to the volumes shown in Table 14. Add AR3 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.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 10 (Library Amplification; see Table 3):

   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 number of PCR cycles may be decreased or increased based on the requirements for a given sample.
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

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

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.

V. Protocol

3. At room temperature, add 100 µL of the Library Amplification reaction product to 100 µL (1.0 volumes) of bead suspension. Mix thoroughly by pipetting†.

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. While the tubes are still on the magnet, carefully remove 200 µL of the binding buffer and discard it.

Note: This is a modifiction to the standard protocol. Remove tubes from the magnet and resuspend beads in 70% ethanol during the wash steps rather than leaving the tubes on the magnet.

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

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

10. Air dry the beads on the magnet for 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.
11. Remove the tubes from the magnet.
12. Add 26 µL D1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
13. Incubate at room temperature for 5 minutes.
14. Transfer the tubes to the magnet and let stand for 2 minutes for the beads to completely clear the solution.
15. Carefully remove 25 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.

† If tubes have become noticably warped or plastic integrity compromised, transfer all 200 µL of sample to fresh tubes.

V. Protocol

N. Quantitative and Qualitative Assessment of the Library

Library Quantification with NuQuant†

Note: A detailed protocol for the Tecan NuQuant Qubit assay is provided below. For information on using NuQuant with other fluorometers and fluorescent plate readers, please contact Tecan NGS Technical Support.

1. Ensure NuQuant Buffer and diluted NuQuant Standard (prepared in section **III.D.**) have reached room temperature before proceeding.
2. Aliquot 200 µL NuQuant Buffer into a new thin-wall, clear, 0.5 mL tube. Label this tube S1.
3. Aliquot 195 µL NuQuant Buffer into thin-wall, clear, 0.5-mL tubes. Prepare one tube for the diluted standard and one tube for each library. Label the diluted standard tube S2.

Note: The S2 Standard represents a Universal RNA-Seq with NuQuant library with a concentration of 16.1 nM.

4. Add 5 µL of diluted NuQuant Standard or library to each tube containing NuQuant Buffer from Step 2. Close lids securely, mix thoroughly by vortexing, and spin briefly to collect all liquid into the bottom of the tube.

Important
- The diluted NuQuant Standard may settle over time. Mix thoroughly before aliquoting.
- If standards and libraries are not thoroughly mixed, inaccurate results may occur.

5. Measure samples as directed for your specific quantification platform:
   a. Qubit 2.0: Select "Univ. Plus" from the Qubit home screen. Follow the on screen prompts to read S1, S2, and samples.

Note: For Qubit 2.0, the "Assay Concentration" is displayed after reading each sample. To calculate the stock concentration of each library, select the "Calculate Stock Conc." button, and change the "Volume of Sample Used" to 5 µL. The correct stock concentration of your library will be displayed.

   b. Qubit 3.0 and 4: Navigate to the saved location of Tecan NuQuant app and select "Univ. Plus". Follow the on screen prompts to read S1, S2, and samples.

Note: For Qubit 3.0 and 4, enter the original sample volume (5 µL) after reading standard 2. The result for each sample will be displayed as the "Original Calculated Sample Concentration" and the "Qubit Tube Concentration."

† For quantification of libraries without use of NuQuant, a qPCR-based method is recommended. Contact Tecan NGS Technical Support for more information.

V. Protocol

Library Pooling and Assessment

1. Pool the libraries according to NuQuant concentration. An example using the calculation $V_1=(C_2/N*V_2)/C_1$, where C= concentration, N = number of libraries in the pool and V=volume, is provided in Table 15 below.

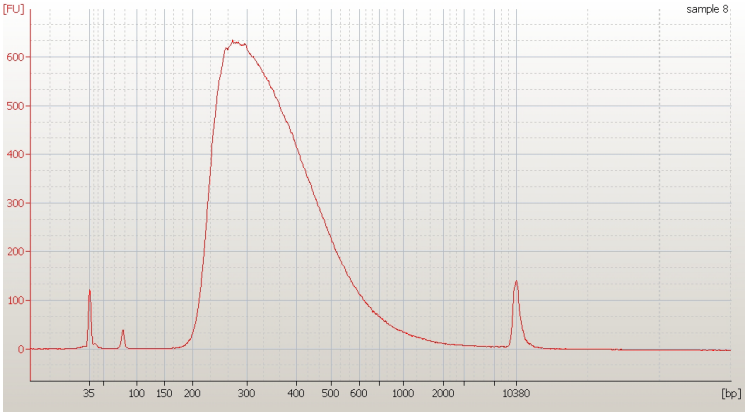
Table 15. Example Library Pooling Calculation using NuQuant Concentration

LIBRARY	NUQUANT CONCENTRATION (C_1)	TARGET CONCENTRATION OF POOL (C_2)	TARGET VOLUME OF POOL (V_2)	VOLUME OF LIBRARY TO ADD TO POOL (V_1)
1	250 nM	10 nM	200 µL	2.0 µL
2	220 nM			2.3 µL
3	280 nM			1.8 µL
4	240 nM			2.1 µL
Volume of low-EDTA or nuclease-free water to add				191.8 µL
Total volume of library pool				200 µL

2. Validate the library pool for sequencer loading following the Illumina guidelines, "Best practices for manually normalizing library concentrations" for your specific sequencer. See **Appendix A**. of this guide for guidelines on color balancing and multiplexing of Tecan libraries. A typical fragment distribution for Universal RNA-Seq with NuQuant libraries prepared with high quality input RNA is shown in Figure 4.

Important: As with any library quantitation method, individual operators, lab equipment and instruments will influence the optimal loading quantitation. Validation of the library pool should be determined empirically for each new library type and sequencer platform.

Figure 4. Library size distribution from total RNA sample. 100 ng of Universal Human Reference total RNA was processed using a 200 bp fragmentation target size. The resulting library was run on an Agilent Bioanalyzer using the High Sensitivity DNA assay. 15 cycles of PCR were performed.



3. Prepare libraries for sequencing following the Illumina "Denature and Dilute Libraries Guide" for your specific sequencer.

VI. 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 techserv-gn@tecan.com.

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

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

VII. Appendix

A. Barcode Sequences and Guidelines for Multiplex Experiments

Barcode sequences for the 32- and 96-plex Adaptor Plates are given below, with barcodes in the 32-plex Adaptor Plate found in wells A01–H04. Barcodes are color balanced in sets of 8 by column.

Table 16. Barcode sequences

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CACGTCTA	A05	ATGGCGAT	A09	CAAGAAGC
B01	AGCTAGTG	B05	AACGCCTT	B09	CTGATGAG
C01	ACTATCGC	C05	GTAAGGTG	C09	CTCGAACA
D01	GCGTATCA	D05	TGTCGACT	D09	TCGACAAG
E01	ACTCTCCA	E05	ACTCTGAG	E09	AGTGCATC
F01	CGTCCATT	F05	GATGGAGT	F09	TGGCTACA
G01	AGCCGTAA	G05	CTAGCTCA	G09	GCACACAA
H01	GAGTAGAG	H05	CTGTACCA	H09	GCATAGTC
A02	ACGTCGTT	A06	CCTGTCAA	A10	AACACGCT
B02	GTCCTGTT	B06	GGTCGTAT	B10	TTCACGGA
C02	AGAAGCCT	C06	CGCTGATA	C10	TGCTGTGA
D02	GAAGATCC	D06	TAGCTTCC	D10	CCTCGAAT
E02	TAGCTGAG	E06	CAAGTCGT	E10	TGTGGCTT
F02	ACGTCCAA	F06	GTCTCATC	F10	CTGTGGTA
G02	CACACATC	G06	ACCAAGCA	G10	TCACTCGA
H02	CGGATCAA	H06	AGTCAGGT	H10	ACTCCTAC
A03	TCAGCCTT	A07	TATCGCGA	A11	CCACAACA
B03	AAGGCTCT	B07	TAGCAGGA	B11	CCGCTTAA
C03	TGTTCCGT	C07	AGAAGGAC	C11	GTGGTATG
D03	GGAATGTC	D07	TGAGCTGT	D11	GGTGTACA
E03	CATCCAAG	E07	CAGAGTGA	E11	TCTAGGAG
F03	GTCAACAG	F07	AGGTTCCT	F11	TGGAAGCA
G03	TCGCTATC	G07	AGACCTTG	G11	AACACCAC
H03	AGCCTATC	H07	CTTCCTTC	H11	CATACGGA
A04	TCGGATTC	A08	CAGGTTCA	A12	CTCTCAGA
B04	CGGAGTAT	B08	ACTGGTGT	B12	AAGCTGGT
C04	GAACCTTC	C08	GGATTCAC	C12	GTGTCCTT
D04	AGAGGATG	D08	CACGATTC	D12	AGGTCTGT
E04	ACGCTTCT	E08	AGACATGC	E12	CATTCGTC
F04	CACAGGAA	F08	GACACAGT	F12	CTCACCAA
G04	ACGAATCC	G08	CCAGTTGA	G12	GACTACGA
H04	CCTTCCAT	H08	CATGGATC	H12	ATACGCAG

VII. Appendix

B. Library Amplification Optimization with qPCR

When using the kit for the first time, or working with a new sample type or input amount, we recommend performing a qPCR step prior to Library Amplification to determine the optimum number of cycles needed and ensure there is no excess amplification.

Perform a 1/10th scale qPCR reaction as follows:

Table 17. Library Amplification qPCR Master Mix

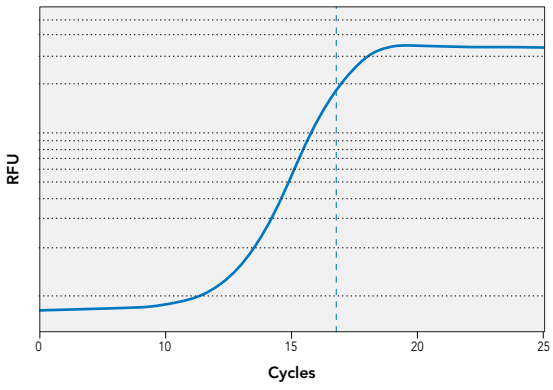
REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 2)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	20X EvaGreen
STORAGE	—	–20 °C	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2.65 µL	1 µL	0.8 µL	0.05 µL	0.5 µL

1. Prepare a PCR master mix according to the volumes shown in Table 17.
2. Aliquot 5.0 µL of PCR master mix per sample into a 0.2 mL qPCR strip or plate.
3. Add 5.0 µL of library (after Targeted Depletion) for a total qPCR volume of 10 µL. Mix well by pipetting, spin and place on ice.
4. 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 near the top of the exponential phase of the amplification plot (17 cycles in Figure 5 as an example).

Figure 5. Stylized qPCR Amplification Plot



VII. Appendix

For PCR, choose a cycle number within the exponential phase of amplification. Follow the steps below to amplify 45 µL of library.

Table 18. Library Amplification Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 2)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)
STORAGE	—	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	28.35 µL	9 µL	7.2 µL	0.45 µL

5. Spin down AR3 and place on ice.
6. Thaw D1, AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
7. Prepare a master mix by sequentially combining D1, AR1 and AR2 in an appropriately sized capped tube, according to the volumes shown in Table 18. Add AR3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
8. On ice, add 45 µL of Library Amplification Master Mix to each sample for a total of 90 µL.
9. Place the tubes in a pre-warmed thermal cycler programmed to run Program 11 (Library Amplification; see Table 3), 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

   *The number of PCR cycles may be decreased or increased based on the requirements for a given sample.
10. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
11. Proceed with section **M. Amplified Library Purification**.

C. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with the Universal RNA-Seq with NuQuant kit?
The Universal RNA-Seq with NuQuant kit provides all necessary buffers, primers and enzymes for library construction. The kit also provides nuclease-free water for purification elution steps. For custom AnyDeplete probes, please contact Tecan NGS Technical Support. Agencourt beads (provided with 32 reaction kit), HL-dsDNase for optional integrated DNase treatment and EvaGreen for optional qPCR must be purchased separately.

Q2. What equipment is required or will be useful?
A list of required and recommended equipment can be found in **section II.B.** of this user guide.

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Input Recommendations

Q3. 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.

Q4. Can I use the Universal RNA-Seq with NuQuant kit with RNA from any organism?
This system has been designed for use with a broad range of different organisms using organism-specific primers to target specific transcripts for depletion. AnyDeplete probe mixes for human (rRNA), mouse (rRNA plus globin) and Drosophila (rRNA) are available. Please contact Tecan NGS Technical Support for a list of available custom AnyDeplete designs or to design a new custom AnyDeplete probe mix.

Q5. 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 Tecan NGS Technical Support for more information.

Q6. Do I need to use high-quality total RNA?
The Universal RNA-Seq with NuQuant 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.

Q7. Do I need to perform an rRNA depletion or poly(A) enrichment step before processing with the Universal RNA-Seq with NuQuant kit?
The system is designed to use total RNA as input. rRNA depletion or poly(A) enrichment are not necessary. rRNA, as well as other unwanted transcript types, can be targeted for depletion using AnyDeplete.

Q8. How much total RNA do I need as input?
Refer to section **III.A.** for a discussion of RNA input requirements.

Q9. Can contaminating genomic DNA interfere with the Universal RNA-Seq with NuQuant kit performance?
Contaminating genomic DNA can interfere with accurate RNA quantification and may be incorporated into libraries. For samples that have not undergone a thorough DNase treatment during RNA purification or is suspected of containing contaminating genomic DNA, we recommend starting the protocol at section **V.A. First Strand cDNA Synthesis with Integrated DNase Treatment**.

General Workflow

Q10. Does this system contain a SPIA®-based amplification?
No. SPIA-based amplification is not used in this kit.

Q11. 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.

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Q12. 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 or enzymatic fragmentation, may be suitable as long as the method generates a tight size distribution of DNA fragments with the required median size.

Q13. Can I skip mechanical fragmentation before cDNA concentration?
Yes, fragmentation can be omitted from the protocol. Note that with high quality input samples, RNA depletion may be less efficient and transcript coverage may be biased towards the 5′ end. Contact Tecan NGS Technical Support for additional information.

Q14. Can this system be used with other library preparation workflows?
The Universal RNA-Seq with NuQuant 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 the protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Universal RNA-Seq with NuQuant kit utilizes optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the formation of adaptor dimer in the library.

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

Q17. 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

Q18. 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.

Q19. 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 of a magnet designed for 0.2 mL tubes (PCR tubes) can help improve performance. Compared to magnets that are designed for 1.5 mL tubes, these minimize loss that can occur when samples are transferred from one tube to another.

b. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated. For the Ovation Universal RNA-Seq System, the minimum volume is 12 µL and the maximum is 200 µL.

c. Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the NuGEN workflow. Performing this mock purification will also help you to gain familiarity with the purification workflow.

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Q20. 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

Q21. How do I measure my final library yield?

We recommend using NuQuant to accurately quantify the final libraries for multiplex pooling using a Qubit instrument. The final library pool concentration should be determined using a qPCR-based method before loading onto an Illumina sequencer. Alternatively, use standard NGS library QC methods to quantify your library or refer to the Illumina "Denature and Dilute Libraries Guide" for your specific sequencer.

Q22. How many bases do the Universal RNA-Seq with NuQuant adaptors add to the library?

The adaptors add 124 bp to the size of the final library.

Sequencing Recommendations

Q23. 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.

Q24. 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 three 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.

Q25. What kind of sequencing primers can I use with your libraries?

The Universal RNA-Seq with NuQuant kit is designed for use with the standard Illumina sequencing primers for both single-end and paired-end sequencing applications.

Q26. Can the Universal RNA-Seq with NuQuant 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.

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Data Analysis

Q27. 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 RNA-Seq with NuQuant kit, the forward read corresponds to the sense strand.

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 on available existing designs or to develop a new custom design.

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