USER GUIDE

Universal Plus™ Total RNA-Seq with NuQuant®

REF 9156, 9156B, 9156C, 9156D, 9157, 9157B, 9157C, 9157D, 9158, 9158B, 9158C, 9158D

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

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I. Introduction

A. Overview

Intended Use

Universal Plus Total RNA-Seq with NuQuant is an end-to-end solution for generation of RNA-Seq libraries derived from total RNA. Universal Plus Total RNA-Seq with NuQuant is intended for Research Use Only and not for use in diagnostic procedures.

Features

Universal Plus Total RNA-Seq with NuQuant is a streamlined solution for construction of RNA-Seq libraries from total RNA. This kit is compatible with high quality total RNA obtained from a broad range of tissues or cell lines. Degraded RNA samples may also be compatible, please contact Tecan NGS Technical Support for guidance. The Universal Plus Total RNA-Seq with NuQuant workflow consists of fragmentation of total RNA, followed by cDNA synthesis, **DimerFree**® library construction with Unique Dual Index (UDI) adaptors and **AnyDeplete**® for depletion of unwanted transcript sequences to provide more efficient use of sequencing resources. Included in the kit are reagents for Tecan's novel quantitation method, **NuQuant**®, for time- and cost-savings in measuring molarity for library pooling prior to multiplex sequencing. 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 library size.

Specifications

Input type:	Total RNA
Input amount:	10 ng – 500 ng
# Reactions available:	24, 96
Barcodes available:	Up to 384 UDI
Sequencing platforms:	Illumina NGS

B. Storage and Stability

Universal Plus Total RNA-Seq is provided as a single shipment. The core library preparation kit and UPlus Total RNA AnyDeplete Module are shipped on dry ice and should be unpacked immediately upon receipt.

- K562 Control RNA (RNA VER 1) should be stored at –80 °C (provided only in 24 reaction kits).

- All remaining components should be stored at –20 °C in a freezer without a defrost cycle.

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines are warrantied to perform to specifications for 6 months from the date of shipment. Expiration dates listed on the kit label may be longer than the warranty period. Do not use kits that have passed the expiration date.

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide Tecan Genomics with the kit(s) and/or component(s) part number, and lot number. Do not use damaged components.

2. Follow your institution's safety procedures for working with chemicals and handling of biological samples. Follow good laboratory practices and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary. Changing gloves between handling samples is recommended to avoid contamination of sample or reagents.
3. Consult your institution's environmental waste personnel on proper disposal of unused reagents. Check state and local regulations as they may differ from federal disposal regulations. This material may exhibit characteristics of hazardous waste requiring specific disposal requirements. Institutions should check their country hazardous waste disposal requirements.
4. If appropriate, an SDS for this product is available on the Tecan Genomics website at www.nugen.com/products/universal-plus-total-rna-seq-library-preparation-kit-nuquant

D. Before You Start

Please review this User Guide before using this kit for the first time, including the "Components", "Planning the Experiment", "Protocol" and "FAQ" sections.
For more information, visit the Universal Plus Total RNA-Seq page at www.nugen.com/products/universal-plus-total-rna-seq-library-preparation-kit-nuquant

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

A. Reagents Provided

This kit is intended for 24 (-24) or 96 (-A01) reactions. A01 fill sizes may be used in manual or automation workflows. For more information on automation, contact automation-gn@tecan.com.

Universal Plus Total RNA-Seq with NuQuant (Part No. 9156, 9156B, 9156C, 9156D, 9157, 9157B, 9157C, 9157D, 9158, 9158B, 9158C, 9158D) includes:

- 0361 (Universal Plus RNA-Seq with NuQuant)
- 0370 (UPlus Total RNA AnyDeplete Module)
- AnyDeplete Probe Mix.

Table 1. Universal Plus RNA-Seq with NuQuant (Part No. 0361)

COMPONENT	0361-24 PART NUMBER	0361-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
2X Fragmentation Buffer	S02621	S02423	Clear	FB1 VER 1
Actinomycin D	S02350	S02379	Brown	—
First Strand Buffer Mix	S02612	S02415	Blue	A2 VER 14
First Strand Enzyme Mix	S02613	S02416	Blue	A3 VER 6
Second Strand Buffer Mix	S02333	S02380 (2)	Yellow	B1 VER 11
Second Strand Enzyme Mix	S02614	S02417	Yellow	B2 VER 4
End Repair Buffer Mix	S01708	S02418	Blue	ER1 VER 7
End Repair Enzyme Mix	S01706	S02419	Blue	ER2 VER 4
End Repair Enhancer	S02615	S02420	Blue	ER3 VER 2
Ligation Buffer Mix	S01847	S01689	Yellow	L1 VER 4
Ligation Enzyme Mix	S02616	S02421	Yellow	L3 VER 4
Strand Selection Buffer Mix I	S02617	S02393 (2)	Purple	SS1
Strand Selection Enzyme Mix I	S02618	S02385	Purple	SS2
Strand Selection Enzyme Mix II	S02619	S02386	Purple	SS4
Amplification Reagent I	S02620	S02627	Red	AR1 VER 1
Amplification Reagent II	S02607	S02610	Red	AR2 VER 1
Amplification Enzyme Mix	S02628	S02629	Red	AR3 VER 1
K562 Control RNA	S02289	—	Clear	RNA VER 1
Nuclease-free Water	S01113	—	Green	D1

Table 1. Universal Plus RNA-Seq With NuQuant (Part No. 0361), *CONTINUED*

COMPONENT	0361-24 PART NUMBER	0361-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
Universal Plus UDI 24-Plex Adaptor Plate	S02622	—	—	—
Universal Plus UDI 96-Plex Adaptor Plate (Part No. 9156, 9157, 9158 for A01-fill only)	—	S02480	—	—
Universal Plus UDI-B 96-Plex Adaptor Plate (Part No. 9156B, 9157B, 9158B for A01-fill only)	—	S02690	—	—
Universal Plus UDI-C 96-Plex Adaptor Plate (Part No. 9156C, 9157C, 9158C for A01-fill only)	—	30185200	—	—
Universal Plus UDI-D 96-Plex Adaptor Plate (Part No. 9156D, 9157D, 9158D for A01-fill only)	—	30185201	—	—
NuQuant Buffer	S02516	S02517	Clear	—
NuQuant Standard	S02512	S02512	Clear	—

Table 2. UPlus Total RNA AnyDeplete Module (Part No. 0370)

COMPONENT	0370-24 PART NUMBER	0370-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
AnyDeplete Buffer Mix	S02594	S02427	Purple	AD1 VER 1
AnyDeplete Enzyme Mix I	S02595	S02428	Purple	AD2 VER 1
AnyDeplete Enzyme Mix II	S02705	S02706	Purple	AD3 VER 3

Table 3. AnyDeplete Probe Mix

COMPONENT	24 REACTIONS	96 REACTIONS	VIAL LABEL	VIAL NUMBER
UPlus Total RNA AnyDeplete Probe Mix — Human AnyDeplete	S02707	S02708	Purple	AD16 VER 1
UPlus Total RNA AnyDeplete Probe Mix — Mouse AnyDeplete	S02711	S02712	Purple	AD17 VER 1
UPlus Total RNA AnyDeplete Probe Mix — Custom AnyDeplete	—	—	Purple	—

To use Universal Plus with custom AnyDeplete probe mixes, contact your local Account Executive or Tecan NGS Technical Support (techserv-gn@tecan.com).

Note: The reagents in Universal Plus Total RNA-Seq with NuQuant 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**
  - 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**
  - Agencourt RNAClean XP Beads or AMPure XP Beads (Beckman Coulter, Cat. #A63987 or A63881)
  - Ethanol, Absolute (200 Proof), Molecular Biology Grade (Fisher Scientific Cat. #BP2818), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Fisher Scientific, Cat. #75793), for diluting nucleic acids
  - Nuclease-free water (Fisher Scientific, Cat. #BP2484), for diluting nucleic acids
  - Agilent High Sensitivity DNA Kit (Agilent, Cat. #5067-4626) or equivalent
  - EvaGreen® Dye, 20X in water (Biotium, Cat. #31000) optional; for optimizing Library Amplification with qPCR
- **Supplies and Labware**
  - Barrier (filter) pipette tips, nuclease-free
  - 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.
  - Cleaning solutions such as RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, Cat. #AM9780) and DNA OFF™ (MP Biomedicals, Cat. #11QD0500)
  - AlumaSeal II film (Sigma-Aldrich Cat. #Z721530)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

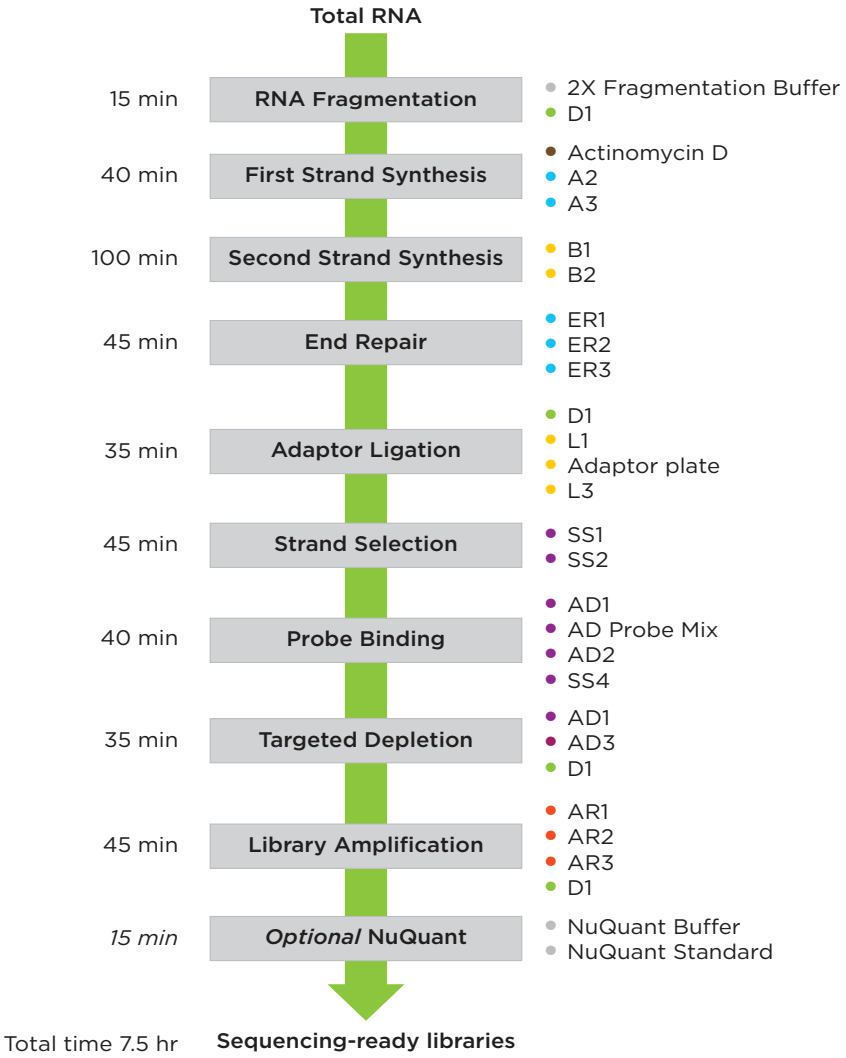
To Order:

- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Eppendorf, www.eppendorf.com
- Fisher Scientific, www.fishersci.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

A. Workflow and Time Required

The Universal Plus Total RNA-Seq with NuQuant consists of RNA fragmentation and double-stranded cDNA generation using a mixture of random and oligo(dT) priming, followed by end repair to generate blunt ends, ligation of UDI adaptors, strand selection, AnyDeplete, and PCR amplification to produce the final library. (Figure 1). The entire workflow can be completed in one day, and yields libraries ready for either single read or paired-end sequencing on Illumina sequencing platforms.

Figure 1. Universal Plus Total RNA-Seq Workflow.



B. Input RNA Requirements

RNA Quantity

Total RNA input must be between 10 ng – 500 ng. 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, such as TRIzol, we recommend column purification after isolation.

One measure of RNA purity is the ratio of absorbance readings. The A260:A280 ratio for RNA samples should be greater than 1.8 and A260:A230 should be greater than 2.0.

RNA Integrity

The Universal Plus Total RNA-Seq with NuQuant kit is designed for use with RNA samples of high molecular weight with little or no evidence of degradation. Moderately degraded RNA may also be compatible with this kit. RNA integrity can be determined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip or RNA 6000 Pico LabChip.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 4 following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C. For thermal cyclers with a fixed-temperature heated lid use the default settings (typically 100–105 °C).

Table 4. Thermal Cycler Programming

RNA FRAGMENTATION		VOLUME
Program 1 RNA Fragmentation	86 °C – 8 min, hold at 4 °C	20 µL
cDNA SYNTHESIS		**VOLUME**
Program 2 First Strand Synthesis	25 °C – 5 min, 42 °C – 15 min, 70 °C – 15 min, hold at 4 °C	25 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

TABLE 4. THERMAL CYCLER PROGRAMMING, *CONTINUED*

LIGATION		VOLUME
Program 5 Adaptor 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, 5x(95 °C – 2 min, 50 °C – 30 s), 65 °C – 5 min, hold at 4 °C	25 µL
Program 8 Targeted Depletion	65 °C – 30 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), 13x*(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.

D. Working with the 24- and 96-Plex Adaptor Plates

The Adaptor Plate included with the 24 and 96 reaction Universal Plus Total RNA-Seq with NuQuant kit contains Unique Dual Index (UDI) adaptor mixes with unique eight-base barcodes. Each well contains sufficient volume for preparation of a single library. The Universal Plus Total RNA-Seq 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 return the adaptor plate to 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 to prevent any remaining adaptor-containing liquid from contaminating future reactions.

For details regarding barcode sequences, please see **Appendix A** on page 27.

E. Bead Purifications

Agencourt® Beads

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

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

Preparation of EtOH Wash Solution

Prepare a 70% EtOH wash solution by combining 100% EtOH and nuclease-free water. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. This protocol requires 0.8 mL of 70% EtOH solution per sample.

Important:

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

Table 5. 70% EtOH Wash Recipe

EtOH SOLUTION	1X REACTION VOLUME*	100% EtOH	NUCLEASE-FREE WATER
70% EtOH	0.8mL	0.56 mL	0.24 mL

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

F. NuQuant

NuQuant® is a novel method to accurately measure molar concentrations of NGS libraries without the need for separate fragment size analysis. The library molar concentration can be directly measured using fluorometers or standard plate readers, then pooled and quantified for sequencing.

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/

Preparation of Diluted NuQuant Standard

Universal Plus Total RNA-Seq with NuQuant includes a 50X NuQuant Standard stock solution. The fluorescence of this stock corresponds to a 32.2 µM library generated with the kit. This stock solution should be diluted prior to use on fluorometers or fluorescent plate readers following the protocol below. The 1X NuQuant Standard corresponds to a 644 nM Universal Plus Total RNA-Seq library.

Note:

- NuQuant Standards should be protected from light.
- NuQuant Standard diluted to 1X for Qubit may be stored at 2–8 °C for up to two months. Do not freeze the 1X NuQuant Standard.

1. Remove concentrated 50X NuQuant Standard stock solution and thaw on ice. Mix by vortexing, spin down and place on ice.
2. Prepare diluted NuQuant Standard in a DNA LoBind tube.
   a. For use with Qubit, combine 2 µL of 50X NuQuant Standard stock solution and 98 µL of NuQuant Buffer. Mix thoroughly by vortexing, spin down and store at 2–8 °C.
   b. For use with fluorescent plate readers, make a fresh dilution of the 50X NuQuant Standard to 1X with Low-EDTA TE Buffer, 1X, pH 8.0. Mix thoroughly by vortexing and spin down.

NuQuant Application

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/

For other fluorometers, a standard curve must be prepared. For using NuQuant with a fluorescent plate reader, contact Tecan NGS Technical Support at techserv-gn@tecan.com.

Important: NuQuant quantitation must be performed prior to storage at –20 °C.

G. Sequencing Recommendations and Guidelines

Universal Plus Total 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. For sequencing recommendations and questions contact Tecan NGS Technical Support at techserv-gn@tecan.com.

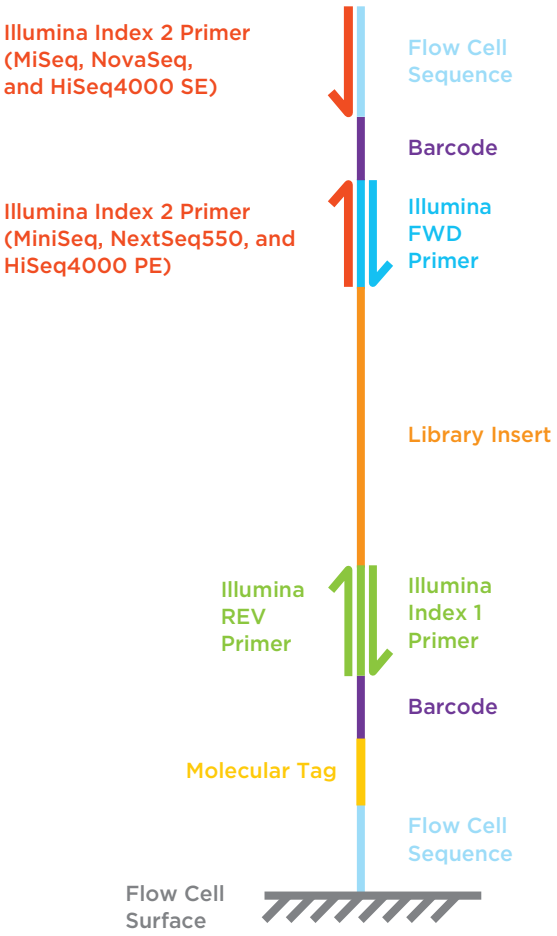
Index Read Recommendations

Universal Plus Total RNA-Seq libraries contain 8 base Unique Dual Indexes (UDI) for sample multiplexing. Both index 1 (i7) and index 2 (i5) should be sequenced for the detection of "index (barcode) hopping". These barcodes differ from the sequences used by Illumina and can be found in **Appendix A**.

The 24-reaction kit (9156-24, 9157-24, 9158-24) contains barcodes A01–H03 from the Universal Plus UDI 96-Plex Adaptor Plate (S02480).

The Universal Plus Total RNA-Seq with NuQuant kit includes an optional 8 nt molecular tag which can be used in conjunction with genomic sequence for duplicate read determination. To capture the molecular tag information, use 16 cycles for the index 1 (i7) read.

Figure 2. Universal Plus Total RNA-Seq Library Structure.



H. 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 Plus Total RNA-Seq 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 for more information.

I. Amplified Library Storage

-20 °C

Amplified libraries may be stored at –20 °C.

Protocol Notes

Controls

- We recommend the routine use of the K562 positive control RNA, provided at 1 µg/µL in the 24 reaction kits, 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 8 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 8 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; 24 reaction kit only) 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.

This workflow requires the following components:

- Part no. 0361 — Universal Plus RNA-Seq with NuQuant
- Part no. 0370 — UPlus Total RNA AnyDeplete Module
- AnyDeplete Probe Mix

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 2X Fragmentation Buffer (Clear: FB1) from –20 °C for use in the next section. Thaw at room temperature, mix by vortexing, spin down and place on ice.
2. Remove Nuclease-free water (Green: D1; 24 reaction kit only) from –20 °C and place at room temperature.
3. Aliquot each total RNA input sample (10 ng – 500 ng) into a 0.2 mL strip tube or 96-well plate.
4. Dilute the RNA with D1 to a final volume of 10 µL and place on ice.

B. RNA Fragmentation

1. Remove Actinomycin D (Brown) from –20 °C for use in the next section. Thaw at room temperature, mix by vortexing, spin down and place at room temperature.
2. Add 10 µL of 2X Fragmentation Buffer to each sample. Mix thoroughly by pipetting up and down at least 10 times.
3. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (RNA Fragmentation; see Table 4):

   86 °C – 8 min, hold at 4 °C
4. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

C. First Strand cDNA Synthesis

Table 6. First Strand Master Mix

REAGENT	ACTINOMYCIN D (BROWN)	FIRST STRAND BUFFER MIX (BLUE: A2 VER 14)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 6)
1X REACTION VOLUME	1.25 µL	2.75 µL	1 µL

1. Spin down the contents of A3 and place on ice.
2. Thaw A2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining Actinomycin D, A2 and A3 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 First Strand Master Mix to each sample tube for a total of 25 µL. Mix well by pipetting, spin down and place on ice.
5. Place the tubes in a thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 4):

   25 °C – 5 min, 42 °C – 15 min, 70 °C – 15 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

D. 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.

Table 7. Second Strand Master Mix

REAGENT	SECOND STRAND BUFFER MIX (YELLOW: B1 VER 11)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4)
1X REACTION VOLUME	48 µL	2 µL

2. Spin down the contents of B2 and place on ice.
3. Thaw B1 at room temperature. Mix by vortexing, spin down 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 7. Mix well by pipetting, spin down, and place on ice.
5. Add 50 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 3 (Second Strand Synthesis; see Table 4):

   16 °C – 60 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation.

E. cDNA Purification

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 sample recovery.

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 135 µL (1.8 volumes) of Agencourt beads to each reaction and mix by pipetting 10 times.

Note: At this step, the reaction will be 210 µL. Pipet carefully to avoid spilling the sample.

5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Keeping the tubes on the magnet, carefully remove the binding buffer and discard it.
8. 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.

9. Remove the 70% ethanol wash using a pipette.

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.

10. Air-dry the beads on the magnet for 5 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
11. Remove the tubes from the magnet.
12. Add 11 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 at least 5 minutes to completely clear the solution of beads.
15. Transfer 10 µL of sample to a fresh 0.2 mL tube.

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

F. End Repair

Table 8. 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)
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 8. 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 4 (End Repair; see Table 4):

   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

1. Thaw Adaptor Plate on ice, spin down, and return to ice.

Table 9. Ligation Master Mix

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

2. Spin down L3 and place on ice.
3. Thaw L1 at room temperature. Mix by vortexing, spin down and place on ice.
4. Add the entire 15 µL of sample to the appropriate adaptor well, mix well by pipetting, then transfer the entire 18 µL of sample to a PCR tube.

   Note: All samples intended to share the same sequencing flow cell lane should have unique barcoded adaptors.
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 9. 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 5 (Adaptor Ligation; see Table 4):

   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 section.

Table 10. Strand Selection Master Mix

REAGENT	STRAND SELECTION BUFFER MIX I (PURPLE: SS1)	STRAND SELECTION ENZYME MIX I (PURPLE: SS2)
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 10.
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 4):

   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 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. 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 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.

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.

9. Air-dry the beads on the magnet for 5 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.
10. Remove the tubes from the magnet.
11. Add 16 µL D1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
12. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
13. 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 11. Probe Binding Master Mix

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

1. Thaw AD1 and AnyDeplete Probe Mix at room temperature. Mix by pipetting, spin down and place on ice.
2. Spin down AD2 and SS4 and place on ice.
3. Prepare a master mix by combining AD1, AnyDeplete Probe Mix, AD2 and SS4 in a 0.5 mL capped tube, according to the volumes shown in Table 11.
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.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (Probe Binding; see Table 4):

   37 °C – 10 min, 5x(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 12. Targeted Depletion Master Mix

REAGENT	ANYDEPLETE BUFFER MIX (PURPLE: AD1 VER 1)	ANYDEPLETE ENZYME MIX II (PURPLE: AD3 VER 3)	NUCLEASE-FREE WATER (GREEN: D1)
1X REACTION VOLUME	5 µL	4 µL	16 µL

1. Thaw D1 and AD1 at room temperature. Mix AD1 by vortexing, spin down and place on ice.
2. Spin down AD3 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 12. 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 8 (Targeted Depletion; see Table 4):

   65 °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.

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; 24 reaction kit only) from –20 °C storage and place on the bench top to reach room temperature for use in the next section.

Table 13. Library Amplification Master Mix

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

2. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Spin down AR3 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 13. 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. 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 4):

   95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), 13x*(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C

   *The recommended number of PCR cycles given in the example above is based on starting with 10 ng of K562 total RNA. The number of PCR cycles may be increased or decreased as needed based on the requirements for a given sample.
7. Remove the tubes from the thermal cycler and spin to collect condensation.

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

M. Amplified Library Purification

1. Remove NuQuant reagents from storage for use in the next section:
   - Remove diluted NuQuant Standard from storage. Mix thoroughly by vortexing, spin and place at room temperature. Protect from light.
   - Remove NuQuant Buffer from storage and thaw at room temperature. Mix thoroughly by vortexing, spin and place on benchtop.

2. Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.
3. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
4. At room temperature, add 70 µL of bead suspension (0.7 volumes) to 100 µL of library amplification product. Mix thoroughly by pipetting.†
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove and discard the supernatant, taking care not to disturb the beads.
8. Remove the tubes from the magnet.
9. Add 50 µL of D1 to the bead pellet. Mix thoroughly to ensure all beads are resuspended.
10. Incubate at room temperature for 2 minutes.
11. At room temperature, add 40 µL of bead suspension (0.8 volumes) to 50 µL of library amplification product. Mix thoroughly by pipetting.
12. Incubate at room temperature for 10 minutes.
13. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
14. Carefully remove and discard the supernatant, taking care not to disturb the beads.
15. Remove the tubes from the magnet.
16. Add 200 µL of freshly prepared 70% ethanol and mix thoroughly by pipetting to resuspend the beads.
17. Transfer the tubes back to the magnet and let stand 5 minutes to completely clear the solution of beads.
18. Remove the 70% ethanol wash using a pipette.
19. Repeat the 70% ethanol wash one more time, for a total of two washes.

> **Important:** It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.

20. Air dry the beads on the magnet for a minimum of 5 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.
21. Remove the tubes from the magnet.
22. Add 26 µL D1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
23. Incubate at room temperature for 5 minutes.
24. Transfer the tubes to the magnet and let stand for at least 2 minutes for the beads to completely clear the solution.
25. 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.

† If tubes have become noticeably warped or plastic integrity compromised, transfer all 170 µL of the sample to fresh tubes.

N. Quantitative and Qualitative Assessment of the Library

Library Quantification with NuQuant†

Note: A detailed protocol for the 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. F.) 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 Plus Total 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 3. 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 NuGEN 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."

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 14 below.

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

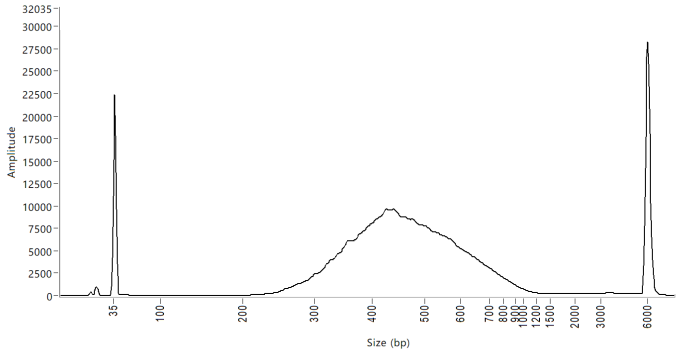
Table 14. Example Library Pooling Calculation using NuQuant Concentration

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 Plus Total RNA-Seq with NuQuant libraries prepared with high quality input RNA is shown in Figure 3.

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 3. Fragment distribution when 2 µL of 4.5 ng/µL library is loaded into a Fragment Analyzer hsDNA assay from 10 ng K562 total RNA starting material.



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

V. Technical Support

For help with any of our products, please contact Tecan NGS Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only) or email techserv-gn@tecan.com.

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (Phone) or email europe-gn@tecan.com.

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

A. Barcode Sequences and Guidelines for Multiplex Experiments

Unique Dual Index (UDI) barcode sequences for the Universal Plus UDI 96-Plex Adaptor Plate (S02480) are given below. Barcodes in the 24 reaction kits are found in wells A01–H03.

Text versions of all barcode sequences for the Universal Plus UDI 24-Plex Adaptor Plate (S02622) or Universal Plus UDI (S02480), UDI-B (S02690), UDI-C (30185200) and UDI-D (30185201) 96-Plex Adaptor plates can be found at https://tecangenomics.github.io/, or contact Tecan NGS Technical Support.

Table 15. Barcode sequences for Universal Plus UDI 96-Plex Adaptor Plate (Indexes 1-96; S02480)

PLATE LOCATION	INDEX 1 (I7) SEQUENCE	INDEX 2 (I5) SEQUENCE	PLATE LOCATION	INDEX 1 (I7) SEQUENCE	INDEX 2 (I5) SEQUENCE	PLATE LOCATION	INDEX 1 (I7) SEQUENCE	INDEX 2 (I5) SEQUENCE
A01	CGCTACAT	AACCTACG	A05	AGGTTCCT	TCGAACCT	A09	GCCTTAAC	CCGTTATG
B01	AATCCAGC	GCATCCTA	B05	GAACCTTC	CAAGGTAC	B09	ATTCCGCT	TGTCGACT
C01	CGTCTAAC	CAACGAGT	C05	AAGTCCTC	AGCTACCA	C09	ATCGTGGT	CTCTATCG
D01	AACTCGGA	TGCAAGAC	D05	CCACAACA	CATCCAAG	D09	GCTACAAC	ACTGCTTG
E01	GTCGAGAA	CTTACAGC	E05	ATAACGCC	CTCACCAA	E09	TCTACGCA	CGCCTTAT
F01	ACAACAGC	ACCGACAA	F05	CCGGAATA	TCAGTAGG	F09	CTCCAATC	ATAGGTCC
G01	ATGACAGG	ACATGCCA	G05	CCAAGTAG	GAACGTGA	G09	ACTCTCCA	TGATCACG
H01	GCACACAA	GAGCAATC	H05	AAGGACCA	AGGAACAC	H09	GTCTCATC	CGGATCAA
A02	CTCCTAGT	CCTCATCT	A06	ACGCTTCT	CCTAAGTC	A10	GCCAGAAT	TACTAGCG
B02	TCTTCGAC	TACTGCTC	B06	CTATCCAC	AACGCACA	B10	AATGACGC	TGGACCAT
C02	GACTACGA	TTACCGAC	C06	TGACAACC	GTCAACAG	C10	GTACCACA	GCGCATAT
D02	ACTCCTAC	CCGTAACT	D06	CAGTGCTT	ACACCTCA	D10	ACGATCAG	ATCGCAAC
E02	CTTCCTTC	TTCCAGGT	E06	TCACTCGA	TATGGCAC	E10	TAACGTCG	TCAGCCTT
F02	ACCATCCT	CCATGAAC	F06	CTGACTAC	CGCAATGT	F10	CGCAACTA	CATTGACG
G02	CGTCCATT	TTCCTCCT	G06	GTGATCCA	ACTCAACG	G10	AACACTGG	ACAGGCAT
H02	AACTTGCC	CCAACTTC	H06	ACAGCAAG	GTCTGCAA	H10	CCTGTCAA	AGGTCTGT
A03	GTACACCT	GAGACCAA	A07	TGCTGTGA	CACGATTC	A11	TCCTGGTA	CAGATCCT
B03	ACGAGAAC	ACAGTTCG	B07	CAACACAG	AGAAGCCT	B11	CATCAACC	CTCCTGAA
C03	CGACCTAA	CTAACCTG	C07	CCACATTG	TACTCCAG	C11	AGCAGACA	AGAGGATG
D03	TACATCGG	TCCGATCA	D07	TAGTGCCA	CGTCAAGA	D11	GAAGACTG	CACCATGA
E03	ATCGTCTC	AGAAGGAC	E07	TCGTGCAT	CTGTACCA	E11	TCTAGTCC	CGGTAATC
F03	CCAACACT	GACGAACT	F07	CTACATCC	TCACCTAG	F11	CTCGACTT	GAGTGTGT
G03	TCTAGGAG	TTGCAACG	G07	CATACGGA	AACACCAC	G11	CTAGCTCA	AACTGAGG
H03	CTCGAACA	CCAACGAA	H07	TGCGTAAC	CGTCTTCA	H11	TCCAACTG	TGTGTCAG
A04	ACGGACTT	ATCGGAGA	A08	CAGGTTCA	AACGTAGC	A12	GACATCTC	TGTCACAC
B04	CTAAGACC	CCTAACAG	B08	AGAACCAG	GCAACCAT	B12	ACTGCACT	AGATCGTC
C04	AACCGAAC	CATACTCG	C08	GAATGGCA	GATCCACT	C12	GTTCCATG	CAATGCGA
D04	CCTTAGGT	TGCCTCAA	D08	AGGCAATG	ACCTAGAC	D12	ACCAAGCA	TGCTTGCT
E04	CCTATACC	TACAGAGC	E08	TAGGAGCT	CTAGCAGT	E12	CTCTCAGA	AATGGTCG
F04	AACGCCTT	CGAGAGAA	F08	CGAACAAC	TCGATGAC	F12	ACTCTGAG	AGTTGTGC
G04	TCCATTGC	AGGTAGGA	G08	CATTCGTC	TTGGTGCA	G12	GCTCAGTT	GTATCGAG
H04	CAAGCCAA	GAACGAAG	H08	AGCCAACT	AGTGCATC	H12	ATCTGACC	GTACGATC

For UDI barcodes B-D and text versions of all barcode sequences visit https://tecangenomics.github.io/

Note: Manual demultiplexing of sequencing data generated on the Illumina MiniSeq, NextSeq, NovaSeq or HiSeq 4000 (PE runs only) instruments may require inputting the reverse complement of the Index 2 (i5) sequences.

B. Liibrary Amplification qPCR Optimization

When using the Universal Plus Total RNA-Seq kit for the first time, or when 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 16. Library Amplification qPCR Master Mix

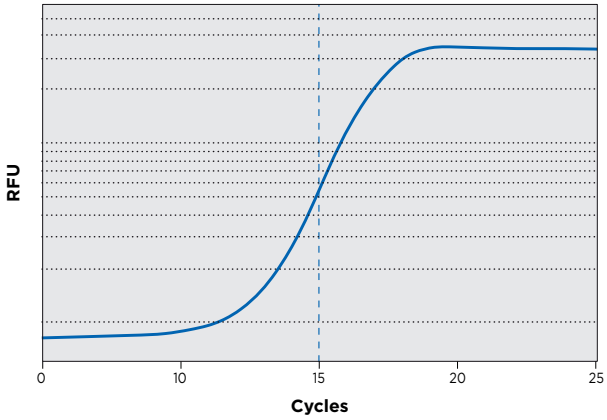
REAGENT	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	20X EVAGREEN	NUCLEASE-FREE WATER (GREEN: D1)
1X REACTION VOLUME	1 µL	0.8 µL	0.05 µL	0.5 µL	2.65 µL

1. Prepare a PCR master mix according to the volumes shown in Table 16.
2. Aliquot 5 µL of PCR master mix per sample into a 0.2 mL qPCR strip or plate.
3. Add 5 µL of purified library (after **K. Targeted Depletion** but before **L. Library Amplification**) 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), 30x(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C

   The cycle number used for subsequent library amplification should be within the exponential phase of the amplification plot (Figure 4).

Figure 4. Stylized qPCR Amplification Plot



To amplify the remaining 45 µL of library, prepare an amplification master mix according to the volumes shown below (Table 17) for standard workflows.

Table 17. Library Amplification Master Mix

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

1. Remove Agencourt beads from storage and place on the bench top to reach room temperature for use in the next step.
2. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down AR3 and place on ice.
4. Make a master mix by sequentially combining D1, AR1 and AR2 in an appropriately sized capped tube according to the volumes shown in Table 17. Add AR3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin the tubes and place on ice.
5. On ice, add 45 µL of Library Amplification Master Mix to 45 µL of sample for a total of 90 µL. Mix by pipetting, spin and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run the following program, using the number of cycles (N) determined by qPCR:

   95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), Nx(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately to section **M. Amplified Library Purification**.

C. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with Universal Plus Total RNA-Seq with NuQuant?
The Universal Plus Total RNA-Seq with NuQuant kit bundle (Part No. 9156, 9157, 9158) provides all necessary buffers, primers and enzymes for fragmentation, cDNA synthesis, library construction, AnyDeplete and NuQuant. SPRI purification beads and EvaGreen are not included.

Q2. What is the concentration of the control RNA?
The control RNA (K562 total RNA), included only with the 24 reaction kit, is provided at a concentration of 1 µg/µL.

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

Q4. Can this system be used with other library preparation workflows?
Universal Plus Total RNA-Seq is an end-to-end solution designed to generate libraries for Illumina sequencing starting from total RNA and has not been tested with alternative library preparation systems.

Input Recommendations

Q5. What methods do you recommend for RNA isolation?
We recommend a column-based method, including:

- Norgen Biotek Total RNA Purification Kit
- Zymo Research Quick-RNA™ Kits
- Arcturus PicoPure® RNA Isolation Kit
- Ambion PureLink® RNA Mini Kit
- Qiagen RNeasy Kits

Organic methods such as TRIzol® Reagent should be subsequently followed with a column-based clean-up method.

Q6. 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.

Q7. 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.

Q8. Can I use Universal Plus Total RNA-Seq with RNA from any organism?
Universal Plus Total RNA-Seq has been designed for use with total RNA inputs from any organism. Custom depletion of rRNA or other transcripts can be designed for any organism. Please contact Tecan NGS Technical Support (techserv-gn@tecan.com) for more information.

Q9. Do I need to use high-quality total RNA?
The Universal Plus Total RNA-Seq with NuQuant kit is designed for whole transcriptome RNA-Seq and will work well with high-quality total RNA. The kit has also been shown to be compatible with degraded samples such as FFPE. Contact Tecan NGS Technical Support for more information.

Q10. Can contaminating genomic DNA interfere with Universal Plus Total RNA-Seq with NuQuant?
Contaminating genomic DNA can be incorporated into libraries and inclusion of a DNase treatment during RNA isolation is recommended.

General Workflow

Q11. Does this system contain a SPIA®-based amplification?
No. The cDNA is generated with random and poly(T) primers, but no SPIA-based amplification is used.

Q12. Is it necessary to fragment my cDNA prior to End Repair and Adaptor Ligation?
No. Chemical fragmentation of the RNA is incorporated into the workflow.

Q13. Can I combine the barcoded libraries prior to the PCR amplification step?
This is not recommended. The stoichiometry of barcoded libraries may be adversely affected by this modification to the workflow. We suggest that the libraries be amplified and quantitated independently before being pooled for use on the sequencer.

Q14. Where can I safely stop in the protocol?
Samples can be placed in short-term storage at –20 °C after second strand synthesis, end repair, strand selection or after any of the bead purification steps.

SPRI Bead Purifications

Q15. 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 during the purification steps in this protocol.

Q16. 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.

Q17. 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

Q18. 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. Please refer to section **IV.N.** for guidelines on alternative library quantitative and qualitative assessments.

Q19. How many bases do Universal Plus Total RNA-Seq adaptors add to the library?

The adaptors add 144 bp to the library.

Sequencing Recommendations

Q20. What sequencers are compatible with your libraries?

Universal Plus Total RNA-Seq libraries are compatible with Illumina sequencing platforms.

Q21. 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.

Q22. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?

The barcodes provided in the Universal Plus UDI 96-Plex Adaptor Plate (S02480) are a minimum edit distance of 3 from other barcodes in the adaptor plate. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one barcode becomes a different barcode. The barcodes provided in the Universal Plus UDI-B 96-Plex Adaptor Plate (S02690), Universal Plus UDI-C 96-Plex Adaptor Plate (30185200) or Universal Plus UDI-D 96-Plex Adaptor Plate (30185201) are a minimum edit distance of 2 from other barcodes in the adaptor plate. 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.

Q23. What kind of sequencing primers can I use with your libraries?

Universal Plus Total RNA-Seq libraries are designed for use with the standard Illumina sequencing primers for both single-end and paired-end sequencing applications.

Q24. Can Universal Plus Total RNA-Seq libraries be used with paired-end sequencing?

Yes. The libraries produced using this kit 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 approximately 300 bases. Contact Tecan NGS Technical Support for additional information.

Data Analysis

Q25. Can I use standard alignment algorithms to analyze strand-specific sequencing data?

Yes. Strand-specific reads can be processed and mapped to reference sequences using the same methods used for other RNA-Seq libraries. Note that in libraries generated by Universal Plus Total RNA-Seq, the forward read corresponds to the sense strand.

Custom AnyDeplete

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

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