USER GUIDE

Universal Plus™ mRNA-Seq with NuQuant®

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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

Contents

I. Introduction

A. Overview

Intended Use

Universal Plus mRNA-Seq with NuQuant is an end-to-end solution for generation of stranded RNA-Seq libraries derived from poly(A)-selected RNA. Universal Plus mRNA-Seq with NuQuant is intended for Research Use Only and not for use in diagnostic procedures.

Features

Universal Plus mRNA-Seq with NuQuant offers a streamlined process for constructing mRNA-Seq libraries from total RNA. This kit is compatible with high quality total RNA obtained from a broad range of tissues or cell lines. This product includes all the reagents necessary for library construction, including pre-plated Unique Dual Index (UDI) adaptors with **DimerFree**® technology for efficient library construction without adaptor dimers. Tecan's novel library quantification method, **NuQuant**®, is integrated providing time- and cost-savings when measuring molarity for library pooling prior to multiplex sequencing. Finally, optional integration of **AnyDeplete**® is available with this kit, enabling an integrated solution for depletion of unwanted transcript sequences, providing more efficient use of sequencing resources.

Specifications

Input type:	High-quality total RNA
Input amount:	10 ng - 1 µg
# Reactions available:	96
Barcodes available:	Up to 384 UDI
Sequencing platforms:	Illumina NGS

B. Storage and Stability

Universal Plus mRNA-Seq is provided as two shipments. The core library preparation kit and optional AnyDeplete module are shipped on dry ice while the mRNA Selection Module is shipped at 4 °C. Both packages should be unpacked and inspected immediately upon receipt.

Important: This product contains components with multiple storage temperature requirements. All shipments should be unpacked immediately upon receipt and stored as directed below.

- The mRNA Selection Module (Part No. 0408) containing the Oligo(dT) Beads, mRNA Elution Buffer, mRNA Wash Buffer, and mRNA Binding Buffer should be stored at 2–8 °C.

- 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 through the labeled expiration date. Do not use kits that have passed the expiration date.

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide Tecan Genomics with the kit(s) and/or component(s) part number, and lot number. 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 lifesciences.tecan.com/universal-plus-mrna-seq-library-preparation-nuquant

D. 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 Plus mRNA-Seq page at lifesciences.tecan.com/universal-plus-mrna-seq-library-preparation-nuquant.

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

A. Reagents Provided

This kit is provided in a 96 (-A01) reaction format. A01 fill sizes may be used in manual or automation workflows.

Universal Plus mRNA-Seq with NuQuant (Part Nos. 0520, 0520B, 0520C and 0520D) is a bundle of:

- Part No. 0361, Universal Plus RNA-Seq with NuQuant
- Part No. 0408, mRNA Selection Module
- Part No. S02480 (0520, 0521, 0522), S02690 (0520B, 0521B, 0522B), 30185200 (0520C, 0521C, 0522C), or 30185201 (0520D, 0521D, 0522D) Unique Dual Index adaptor plate

Universal Plus mRNA-Seq with NuQuant and AnyDeplete (Part Nos. 0521, 0521B, 0521C, 0521D) also includes:

- Part No. 0359, AnyDeplete Module
- AnyDeplete Probe Mix

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

COMPONENT	0361-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
2X Fragmentation Buffer	S02423	Clear	FB1 VER 1
Actinomycin D	S02379	Brown	—
First Strand Buffer Mix	S02415	Blue	A2 VER 14
First Strand Enzyme Mix	S02416	Blue	A3 VER 6
Second Strand Buffer Mix	S02380 (2)	Yellow	B1 VER 11
Second Strand Enzyme Mix	S02417	Yellow	B2 VER 4
End Repair Buffer Mix	S02418	Blue	ER1 VER 7
End Repair Enzyme Mix	S02419	Blue	ER2 VER 4
End Repair Enhancer	S02420	Blue	ER3 VER 2
Ligation Buffer Mix	S01689	Yellow	L1 VER 4
Ligation Enzyme Mix	S02421	Yellow	L3 VER 4
Strand Selection Buffer Mix I	S02393 (2)	Purple	SS1
Strand Selection Enzyme Mix I	S02385	Purple	SS2
Strand Selection Enzyme Mix II	S02386	Purple	SS4
Amplification Reagent I	S02627	Red	AR1 VER 1
Amplification Reagent II	S02610	Red	AR2 VER 1

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

COMPONENT	0361-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
Amplification Enzyme Mix	S02629	Red	AR3 VER 1
Universal Plus UDI 96-Plex Adaptor Plate (Part No. 0520, 0521, 0522 for A01- fill only)	S02480	—	—
Universal Plus UDI-B 96-Plex Adaptor Plate (Part No. 0520B, 0521B and 0522B for A01-fill only)	S02690	—	—
Universal Plus UDI-C 96-Plex Adaptor Plate (Part No. 0520C, 0521C and 0522C for A01-fill only)	30185200	—	—
Universal Plus UDI-D 96-Plex Adaptor Plate (Part No. 0520D, 0521D and 0522D for A01-fill only)	30185201	—	—
NuQuant Buffer	S02517	Clear	—
NuQuant Standard	S02512	Clear	—

Table 2. mRNA Selection Module (Part No. 0408)

COMPONENT	0408-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
Oligo dT Beads	S02248	Clear	—
mRNA Elution Buffer	S02424	Clear	—
mRNA Wash Buffer	S02425	Clear	—
mRNA Binding Buffer	S02426	Clear	—

Table 3. AnyDeplete Module (Part No. 0359)

COMPONENT	0359-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
AnyDeplete Buffer Mix	S02427	Purple	AD1 VER 1
AnyDeplete Enzyme Mix I	S02428	Purple	AD2 VER 1
AnyDeplete Enzyme Mix II	S02392	Purple	AD3 VER 1

Table 4. AnyDeplete Probe Mix

COMPONENT	96 REACTIONS	VIAL LABEL	VIAL NUMBER
Universal Plus AnyDeplete Probe Mix — Human Globin	S02431	Purple	AD14 VER 1

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

Note: The reagents in Universal Plus mRNA-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

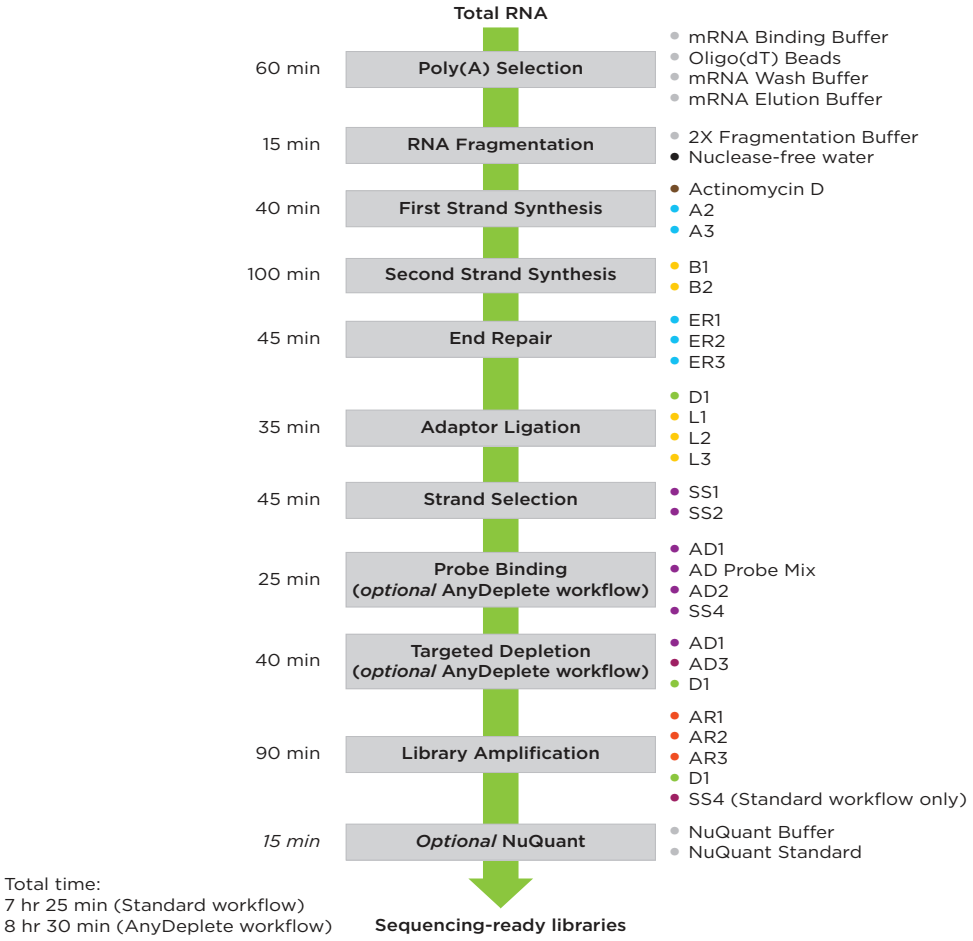
To Order:

- 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 mRNA-Seq with NuQuant workflow includes poly(A) RNA selection, RNA fragmentation and double-stranded cDNA generation using random and oligo(dT) priming. Library construction continues with end-repair of the cDNA, ligation of UDI adaptors, strand selection, and PCR amplification (Figure 1). An optional AnyDeplete step may be performed prior to library amplification to remove unwanted high-abundance transcripts (e.g. globin). The entire workflow can be completed in one day, and produces stranded cDNA libraries ready for either single read or paired-end sequencing on Illumina platforms.

Figure 1. Universal Plus mRNA-Seq with NuQuant Workflow.



B. Input RNA Requirements

RNA Quantity

Total RNA input must be between 10 ng – 1 µg. We strongly recommend accurate quantification of total RNA to ensure the minimum input requirement is met.

RNA Purity

RNA samples must be free from contaminating proteins, cellular debris, organic solvents (e.g., phenol, ethanol), and salts (e.g., GuHCl). For RNA isolated using organic solvents like TRIzol, column purification is recommended.

RNA purity may be assessed using 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

Universal Plus mRNA-Seq with NuQuant is designed for use with RNA samples of high molecular weight with little or no evidence of degradation. RNA integrity can be determined using the Agilent 5200 Fragment Analyzer System or Agilent 2100 Bioanalyzer.

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 5 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 5. Thermal Cycler Programming

mRNA SELECTION		**VOLUME**
Program 1 Poly(A) RNA Binding	65 °C – 5 min, hold at 4 °C	110 µL
Program 2 RNA Elution	80 °C – 2 min, hold at 25 °C	50 µL
RNA FRAGMENTATION		**VOLUME**
Program 3 RNA Fragmentation (300 bp insert size)	86 °C – 8 min, hold at 4 °C	20 µL
cDNA SYNTHESIS		**VOLUME**
Program 4 First Strand Synthesis	25 °C – 5 min, 42 °C – 15 min, 70 °C – 15 min, hold at 4 °C	25 µL
Program 5 Second Strand Synthesis	16 °C – 60 min, hold at 4 °C	75 µL

Table 5. Thermal Cycler Programming, *continued*

END REPAIR		VOLUME
Program 6 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	15 µL
LIGATION		**VOLUME**
Program 7 Adaptor Ligation	25 °C – 30 min, hold at 4 °C	30 µL
STRAND SELECTION		**VOLUME**
Program 8 Strand Selection	72 °C – 10 min, hold at 4 °C	100 µL
AMPLIFICATION (FOR STANDARD WORKFLOW)		**VOLUME**
Program 9 Library Amplification A	37 °C – 10 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), X*(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C	100 µL
ANYDEPLETE (OPTIONAL)		**VOLUME**
Program 10 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 11 Targeted Depletion	60 °C – 30 min, 95 °C – 5 min, hold at 4 °C	50 µL
AMPLIFICATION (FOR OPTIONAL ANYDEPLETE WORKFLOW)		**VOLUME**
Program 12 Library Amplification B	95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), X*(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 Adaptor Plates

Adaptor Plates included with Universal Plus mRNA-Seq contain Unique Dual Index (UDI) adaptor mixes with unique eight-base barcodes. Each well contains sufficient volume for preparation of a single library. Universal Plus mRNA-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. Centrifuge the adaptor plate briefly (<1000 rpm) to ensure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice. During the adaptor ligation step (Protocol **I. Adaptor Ligation**), puncture the seal for each well you wish to use with a fresh pipet tip, transfer the sample into the appropriate wells of the adaptor plate, and mix well by pipetting. Transfer the entire volume of the sample with adaptors to a new PCR tube. 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 33.

E. Bead Purifications

Agencourt® Beads

Ampure XP or RNA Clean XP Beads are compatible with this kit. Follow the specific protocols in this user guide, as they differ from standard Agencourt bead procedures.

Guidelines

- **Temperature**: Allow beads to sit at room temperature for at least 30 minutes after removing them from 4 °C. Cold beads and buffer can reduce recovery.
- **Resuspension**: Fully resuspend beads by vortexing or inverting and tapping the tube.
- **Magnet**: Use an appropriate magnet for the strip tubes or plate type to ensure optimal results.
- **Separation Time**: Let beads separate on the magnet for the full specified time. Premature removal of the binding buffer can affect yields.
- **Buffer Removal**: Minimize bead loss when removing the binding buffer. Carefully remove the specified amount without disturbing the beads.
- **Ethanol Wash**: Use freshly prepared 70% ethanol from fresh stocks. Lower concentrations can reduce recovery.
- **Residual Ethanol**: Ensure all residual ethanol is removed before elution. Remove most of the ethanol, let excess collect at the bottom, then remove the remainder. This reduces bead air-drying time.
- **Inspection**: After drying, inspect each tube to ensure all ethanol has evaporated before proceeding with amplification.

Preparation of EtOH Wash Solution

Prepare a 70% EtOH wash solution by combining 100% EtOH and nuclease-free water (Table 6). Make the ethanol mix 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 library recovery.

Table 6. 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 that accurately measures the molar concentration 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 with a compatible filter set. Libraries are then pooled and quantified for sequencing. See also "**Library Pooling and Validation**" on page 27.

Preparation of 1X NuQuant Standard

Universal Plus mRNA-Seq 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 diluted 1X NuQuant Standard corresponds to a 644 nM library.

The 50X and 1X 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. Thaw 50X NuQuant Standard stock solution on ice. Mix by vortexing, spin down and keep on ice.
2. Prepare 1X NuQuant Standard in a clearly labeled, DNA LoBind tube. 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.

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 at https://tecangenomics.github.io/NuQuant

For other fluorometers, a standard curve must be prepared. For using NuQuant with a fluorescent plate reader see **Section IV. Q. Quantitative and Qualitative Assessment of the Library** or contact Tecan NGS Technical Support at Genomics.Support@tecan.com.

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

G. Sequencing Recommendations and Guidelines

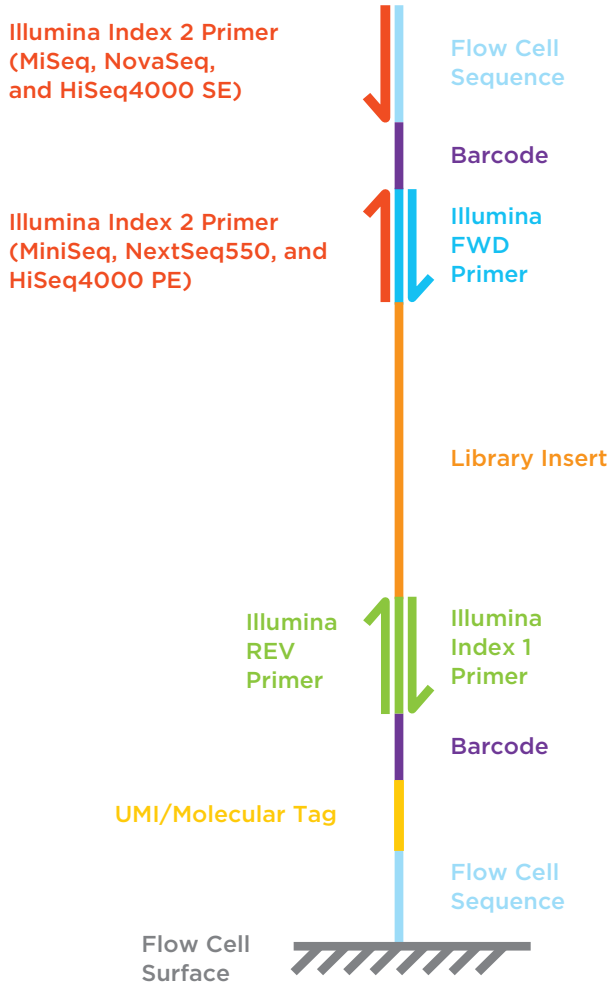
Universal Plus mRNA-Seq with NuQuant produces RNA-Seq libraries compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer (Figure 2). For sequencing recommendations and questions contact Tecan NGS Technical Support at Genomics.Support@tecan.com.

Index Read Recommendations

Universal Plus mRNA-Seq with NuQuant 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 Universal Plus mRNA-Seq with NuQuant kit includes an optional 8 nucleotide (nt) Index-based UMI (Unique Molecular Index or Molecular Tag) which can be used in conjunction with genomic sequence for duplicate read determination. To capture the UMI information, use 16 cycles for the index 1 (i7) read.

Figure 2. Universal Plus mRNA-Seq with NuQuant Library Structure.



H. Data Analysis

Universal Plus mRNA-Seq libraries should be demultiplexed with 0 or 1 mismatch(es) for Universal Plus UDI 96-Plex Adaptor Plate (S02480) and 0 mismatches for Universal Plus UDI Adaptor Plates B, C and D (S02690, 30185200, 30185201). Once the data have been parsed according to sample, additional sample-specific data analysis may be employed according to the requirements of the experiment. For more information on using index-based UMI sequences for deduplication, please contact Tecan Genomics Technical Support at Genomics.Support@tecan.com.

Note: The forward read from Universal Plus mRNA-Seq with NuQuant libraries represents the sense strand. This may differ from stranded data from other library preparation kits and could require adjustments to your data analysis workflow. For more information, contact Tecan NGS Technical Support.

Protocol Notes

Controls

- **Positive Control RNA**: Use routinely to monitor performance and establish a baseline. Multiple controls can highlight variability due to handling. New users may benefit from a practice run using only positive control input to familiarize themselves with the protocol.
- **No Template Control (NTC)**: Use routinely to check for potential carryover contamination.
- **No AnyDeplete Control**: Recommended for first-time AnyDeplete use to establish a baseline. Replace the AnyDeplete probe mix with nuclease-free water in the Probe Binding step.

General Workflow

- **Batch Setup**: Set up at least 8 reactions to avoid small volume pipetting and ensure sufficient reagent recovery.
- **Thawing**: Thaw components for each step and place them on ice immediately. Avoid thawing all reagents at once.
- **Precipitate Resolution**: If precipitate appears in buffer mixes, gently warm at room temperature for 2 minutes and vortex briefly to dissolve.
- **Enzyme Handling**: Keep enzyme mixes on ice after spinning. Do not vortex or warm enzyme, adaptor, or primer mixes.
- **Master Mixes**: Use minimal extra volume, typically 10% overage, to maximize the number of reactions.
- **Pipetting**: Ensure complete transfer by pipetting up and down several times. Mix by gently aspirating and dispensing at least half the total reaction volume.
- **Incubation Steps**: Allow the thermal cycler to reach the initial incubation temperature before placing tubes or plates in the block.

Reagents

- **Water**: Use nuclease-free water; avoid DEPC-treated water.
- **Component Compatibility**: Do not use components from other Tecan products.
- **Ethanol**: Use fresh stocks for washes. Prepare ethanol mixes fresh, measuring ethanol and water carefully. Lower ethanol concentrations can decrease yield during purification.

This protocol includes workflows for RNA-Seq library construction with and without AnyDeplete.

Standard workflow

Follow sections **A. Sample Preparation** through **L. Library Amplification A**, then continue to **P. Amplified Library Purification**.

Required components:

Part No. 0408 – mRNA Selection Module

Part No. 0361 – Universal Plus RNA-Seq with NuQuant

AnyDeplete workflows

Follow sections **A. Sample Preparation** through **K. Strand Selection Purification**, then continue to **M. Probe Binding (optional AnyDeplete workflow)**.

Required components:

Part No. 0408 – mRNA Selection Module

Part No. 0361 – Universal Plus RNA-Seq with NuQuant

Part No. 0359 – AnyDeplete Module

AnyDeplete Probe Mix

For each section of the protocol, remove reagents from the recommended storage conditions listed in "**B. Storage and Stability**" on page 1. Thaw and place reagents at room temperature or on ice as instructed. Continue immediately to the next section unless otherwise directed. Return all reagents to their appropriate storage conditions promptly after use unless otherwise instructed.

A. Sample Preparation

1. Place nuclease-free water at room temperature.
2. Aliquot each total RNA input sample (10 ng – 1 µg) into a 0.2 mL strip tube or 96-well plate.
3. Dilute the RNA with nuclease-free water to a final volume of 50 µL and place on ice.

Note: Keep nuclease-free water at room temperature for use in subsequent sections.

B. Poly(A) Selection

1. Remove mRNA Wash Buffer (Clear) and mRNA Elution Buffer (Clear) from storage and place at room temperature for use in the next section.
2. Remove mRNA Binding Buffer (Clear) and Oligo(dT) Beads (Clear) from storage and place at room temperature at least 10 minutes prior to use. Ensure these reagents have completely reached room temperature before proceeding.

Table 7. Oligo(dT) Bead Master Mix

REAGENT	OLIGO(dT) BEADS (CLEAR)	mRNA BINDING BUFFER (CLEAR)
1X REACTION VOLUME	10 µL	50 µL

3. Prepare Oligo(dT) Bead Master Mix by combining mRNA Binding Buffer and Oligo(dT) Beads in a 0.5 mL capped tube according to the volumes shown in Table 7.
4. Aliquot 60 µL of Oligo(dT) Bead Master Mix into a tube for each sample. Add 50 µL of prepared total RNA to the Oligo(dT) Bead Master Mix for a total of 110 µL. Mix thoroughly by pipetting up and down at least 10 times, taking care when pipetting to minimize foaming.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Poly(A) RNA Binding; see Table 5):

   65 °C – 5 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. RNA Elution

1. Remove 2X Fragmentation Buffer (Clear: FB1) from storage for use in the next section. Thaw at room temperature, mix by vortexing, spin down and place on ice.
2. Mix samples thoroughly by pipetting slowly up and down at least 10 times.
3. Incubate at room temperature for 5 minutes.
4. Repeat steps 2 and 3 once for a total of two room temperature incubations.
5. Transfer the tubes to the magnet and let stand for 2 minutes to completely clear the solution of beads.
6. Carefully remove and discard the binding buffer, ensuring that the beads are not disturbed.
7. Remove samples from the magnet.
8. Add 200 µL of mRNA Wash Buffer to the beads. Mix thoroughly by slowly pipetting up and down at least 10 times.
9. Transfer the tubes to the magnet and let stand for 2 minutes to completely clear the solution of beads.
10. Carefully remove and discard the binding buffer, ensuring that the beads are not disturbed.
11. Remove tubes from the magnet.
12. Add 50 µL of mRNA Elution Buffer to each sample. Resuspend beads by slowly pipetting up and down at least 10 times.
13. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (RNA Elution; see Table 5):

    80 °C – 2 min, hold at 25 °C
14. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

15. Add 50 µL of mRNA Binding Buffer to bead/elution buffer mix. Mix thoroughly by slowly pipetting up and down at least 10 times.
16. Incubate at room temperature 5 minutes.
17. Transfer the tubes to the magnet and let stand for 2 minutes to completely clear the solution of beads.
18. Carefully remove and discard the binding buffer, ensuring that the beads are not disturbed.
19. Remove the tubes from the magnet.
20. Add 200 µL of mRNA Wash Buffer to the beads. Mix thoroughly by slowly pipetting up and down at least 10 times.
21. Transfer the tubes to the magnet and let stand for 2 minutes to completely clear the solution of beads.
22. Continue to **D. RNA Fragmentation** with the tubes incubating on the magnet.

D. RNA Fragmentation

1. Remove Actinomycin D (Brown) from storage for use in the next section. Thaw at room temperature, mix by vortexing, spin down and place at room temperature.

Table 8. 1X Fragmentation Buffer

REAGENT	2X FRAGMENTATION BUFFER (CLEAR: FB1 VER 1)	NUCLEASE-FREE WATER
1X REACTION VOLUME	10 µL	10 µL

2. Prepare 1X Fragmentation Buffer by combining FB1 and nuclease-free water in a 0.5 mL capped tube according to the volumes shown in Table 8. Resuspend beads by slowly pipetting up and down at least 10 times.
3. Carefully remove and discard the binding buffer, ensuring that the beads are not disturbed.
4. Remove the tubes from the magnet.
5. Add 20 µL of 1X Fragmentation Buffer to each sample. Resuspend beads by slowly pipetting up and down at least 10 times.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (RNA Fragmentation; see Table 5).

   86 °C – 8 min, hold at 4 °C
7. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Transfer the tubes to the magnet and let stand for at least 2 minutes to completely clear the solution of beads.
9. Transfer 20 µL of fragmented mRNA to a new 0.2 mL tube and place on ice.

E. First Strand cDNA Synthesis

Table 9. 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 9. 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 4 (First Strand Synthesis; see Table 5):

   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.

F. Second Strand cDNA Synthesis

1. Remove Agencourt beads from storage and allow them to reach room temperature for use in the next section.

Table 10. 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 10. 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 5 (Second Strand Synthesis; see Table 5):

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

G. cDNA Purification

1. Ensure the Agencourt beads and nuclease-free water have reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution (Table 6).

Important
- Prepare the ethanol solution fresh on the day of the experiment using a recently opened stock container.
- Carefully measure both ethanol and water before mixing to avoid higher 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: The reaction volume will be 210 µL. Pipet carefully to avoid spills.

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

Note: Beads should remain on the tube walls. Ensure beads are not dispersed or removed during the binding buffer or wash steps. Loss of beads may result in diminished library yield.

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 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
11. Remove the tubes from the magnet.
12. Add 11 µL nuclease-free water 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.

H. End Repair

Table 11. 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 11. 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 6 (End Repair; see Table 5):

   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.

I. Adaptor Ligation

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

Table 12. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER	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 (15 µL sample + 3 µL adaptor mix) to a PCR tube.

Note: The L1 Ligation Buffer Mix is very viscous. Pipet this reagent slowly and mix thoroughly.

5. Prepare a master mix by combining nuclease-free water, L1 and L3 in a 0.5 mL capped tube according to the volumes shown in Table 12 Mix by pipetting slowly, without introducing bubbles, spin down and place on ice. Use the master mix immediately.

   Note: The Ligation Master Mix is very viscous. Pipet 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 7 (Adaptor Ligation; see Table 5):

   25 °C – 30 min, hold at 4 °C

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

J. Strand Selection

1. Remove Agencourt beads from storage and allow them to reach room temperature for use in the next section.

Table 13. 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 13.
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 8 (Strand Selection; see Table 5):

   72 °C – 10 min, hold at 4 °C

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

K. Strand Selection Purification

1. Ensure the Agencourt beads have 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 and discard the binding buffer.

Note: Beads should remain on the tube walls. Ensure beads are not dispersed or removed during the binding buffer or wash steps. Loss of beads may result in diminished library yield.

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 the beads on the magnet for 5 minutes. Inspect each tube carefully to ensure 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 nuclease-free water 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 to clear the solution of beads.
13. Carefully remove 15 µL of the eluate, minimizing bead carryover, and transfer to a fresh set of PCR tubes. Place on ice.
14. Continue to **L. Library Amplification A** (for standard workflow) or to **M. Probe Binding** (for optional AnyDeplete workflow).

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

L. Library Amplification A

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

1. Remove Agencourt beads from storage and place on the bench top to reach room temperature for use in the next section.

Table 14. Library Amplification A Master Mix

REAGENT	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	NUCLEASE-FREE WATER
1X REACTION VOLUME	10 µL	8 µL	0.5 µL	1 µL	65.5 µL

2. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Spin down AR3 and SS4 and place on ice.
4. Prepare a master mix by sequentially combining nuclease-free water, AR1 and AR2 in an appropriately sized capped tube according to the volumes shown in Table 14. Add AR3 and SS4 last and mix well by pipetting, taking care to avoid creating bubbles. Spin down and place on ice.
5. On ice, add 85 µL of Library Amplification A 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 A; see Table 5):

   37 °C – 10 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), **X***(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C

   * see Table 15. Library Amplification Cycling Guidelines. These are approximate values based on the use of high-quality human RNA inputs (RIN >9.5). Degraded RNA or RNA samples with low mRNA content may require additional cycles. Cycle numbers for all sample types should be determined empirically by qPCR (see **Appendix B**).

Table 15. Library Amplification Cycling Guidelines (mRNA-Seq without AnyDeplete)

INPUT MASS (NG RNA)	CYCLE RANGE
10	16 - 18
100	13 - 15
1000	10 - 12

7. Remove the tubes from the thermal cycler and spin to collect condensation and continue to **P. Amplified Library Purification**.

M. Probe Binding (optional AnyDeplete workflow)

Table 16. 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 an appropriately sized capped tube, according to the volumes shown in Table 166.
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 10 (Probe Binding; see Table 5):

   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.

N. Targeted Depletion (optional AnyDeplete workflow)

Table 17. Targeted Depletion Master Mix

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

1. Thaw 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 nuclease-free water in an appropriately sized capped tube according the volumes in Table 177. 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 11 (Targeted Depletion; see Table 5):

   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.

O. Library Amplification B (optional AnyDeplete workflow)

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

1. Remove Agencourt beads from storage and place on the bench top to reach room temperature for use in the next section.

Table 18. Library Amplification B 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
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 Nuclease-free water, AR1 and AR2 in an appropriately sized capped tube according to the volumes shown in Table 188. 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 B 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 12 (Library Amplification B; see Table 5):

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

   * see Table 19. Library Amplification Cycling Guidelines. These are approximate values based on the use of high-quality human RNA inputs (RIN >9.5). Degraded RNA or RNA samples with low mRNA content may require additional cycles. Cycle numbers for all sample types should be determined empirically by qPCR (see **Appendix C**).

Table 19. Library Amplification Cycling Guidelines (mRNA-Seq with AnyDeplete)

INPUT MASS (NG RNA)	CYCLE RANGE
10	18 - 20
100	15 - 17
1000	12 - 14

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

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

P. Amplified Library Purification

1. Remove NuQuant reagents from storage for use in the next section:
   - Mix 1X NuQuant Standard thoroughly by vortexing, spin, and place at room temperature. Protect from light.
   - Thaw NuQuant Buffer at room temperature, mix thoroughly by vortexing, spin, and place on the benchtop.
2. Ensure the Agencourt beads and nuclease-free water have 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.

   Important: If tubes are noticeably warped or compromised, transfer samples to fresh tubes before addition of Agencourt beads..

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, ensuring that the beads are not disturbed.
8. Remove the tubes from the magnet.
9. Add 50 µL of nuclease-free water to the bead pellet. Mix thoroughly to ensure all beads are resuspended.
10. Incubate at room temperature for 2 minutes.
11. Add 40 µL of bead suspension (0.8 volumes) to 50 µL of library amplification product (mixture of water and beads from step 9). 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, ensuring that the beads are not disturbed.
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 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 31 µL nuclease-free water 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 to completely clear the solution of beads.
25. Carefully remove 30 µL of the eluate, minimizing bead carryover, and transfer to a fresh set of PCR tubes. Place on ice..

Q. Quantitative and Qualitative Assessment of the Library

NuQuant Library Quantification with Qubit

> **Note:** A detailed protocol for library quantification with NuQuant assay on plate readers is provided in the . 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. NuQuant**) 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 1X NuQuant standard and one tube for each library. Label the 1X standard tube S2.

> **Note:** The S1 Standard represents a blank measurement (0 nM). The S2 Standard represents a library with a concentration of 16.1 nM.

4. Add 5 µL of 1X NuQuant Standard to tube S2. Add 5 µL library to each remaining 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 1X 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 "NuQuant 644" 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 "NuQuant 644". 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."

NuQuant Library Quantification with Plate Readers

I. Prepare Standard Curve

1. Ensure 1X NuQuant Standard (prepared in **Section III. F. NuQuant**) and low TE buffer have reached room temperature before proceeding.

Note: The 1X NuQuant standard will be the highest concentration in the standard curve.

2. Create serial dilutions of the 1X standard following the dilution series in Table 2020. Mix thoroughly by vortexing after creating each dilution.

Table 20. NuQuant serial dilution*

DILUTION #	DILUTION RATIO (Relative to 1X NuQuant Standard)	VOLUME	STANDARD CONC. (nM)
1	1	1X NuQuant Standard	644
2	1:2	40 µL Dilution #1 + 40 µL Low TE	322
3	1:4	40 µL Dilution #2 + 40 µL Low TE	161
4	1:8	40 µL Dilution #3 + 40 µL Low TE	80.5
5	1:16	40 µL Dilution #4 + 40 µL Low TE	40.3
6	1:32	40 µL Dilution #5 + 40 µL Low TE	20.1
7	—	40 µL Low TE	0

* It is recommended to load duplicates of the standards at each dilution.

II. Load Assay Plate

1. Load 20 µL of each standard dilution, in duplicate, into a plate.
2. Load 20 µL of each sample into the assay plate.

III. Run NuQuant Assay on Plate Reader

1. Load assay plate onto plate reader. Optimize gain and Z-position settings on the plate reader. Recommended settings for the Infinite M Plex and Infinite F Nano+ are provided below. Consult the Operating Manual for your specific plate reader instrument for more information on adjustment and application of individual settings.

Table 21. Measurement settings for the Infinite M Plex

PARAMETER	SETTING
Plate used for test	TEC384fb
Measurement mode	Fluorescence Intensity Top
Excitation	620 (9) nm
Emission	680 (20) nm
Lag time	0 µs
Integration time	20 µs (default)
Flash number	25
Settle time	0 ms
Gain	Calculated from well containing highest concentration standard
Z position	Calculated from well

Table 22. Measurement settings for the Infinite F Nano+

PARAMETER	SETTING
Plate used for test	BioRad96_PCR_Hardshell
Measurement mode	Fluorescence Intensity Top
Excitation	620 (20) nm
Emission	680 (30) nm
Lag time	0 µs
Integration time	20 µs (default)
Flash number	25
Settle time	0 ms
Gain	Calculated from well containing highest concentration standard
Mirror	Automatic (50%)

Note: Other compatible plates may be used, although their performance has not been validated by Tecan.

Library Pooling and Assessment

1. Pool the libraries according to NuQuant concentration. An example is provided in Table 23 below.

$$V_1 = \frac{(C_2 * V_2)}{(C_1 * N)}$$

C = concentration (nM), N = number of libraries in the pool, V = volume (µL).

Table 23. Example library pooling calculation using NuQuant concentration

LIBRARY (N=4)	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	240 nM	10 nM	200 µL	2.1 µL
2	75 nM			6.7 µL
3	102 nM			4.9 µL
4	197 nM			2.5 µL
		Volume of low-EDTA or nuclease-free water to add		183.8 µL
			Total volume of libarary 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. Tecan recommends a qPCR based method to validate and optimize NuQuant quantification. See **Appendix A** of this guide for guidelines on color balancing and multiplexing of Tecan libraries. A typical fragment distribution for Universal Plus mRNA-Seq with NuQuant libraries prepared with high quality input RNA is shown in Figure 3.

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

V. Technical Support

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

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (phone) or email Genomics.Support@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 (Table 24). UDI barcodes for plates B-D are available at https://tecangenomics.github.io/Barcodes.

Text versions of all barcode sequences for the 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/Barcodes, or contact Tecan NGS Technical Support.

Table 24. 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

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. Library Amplification qPCR Optimization for Standard Workflow

When using the Universal Plus kit for the first time, or when working with a new sample type or input amount, a qPCR step prior to Library Amplification is recommended to determine the optimal number of PCR cycles and prevent excess amplification.

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

Table 25. Library Amplification qPCR Master Mix

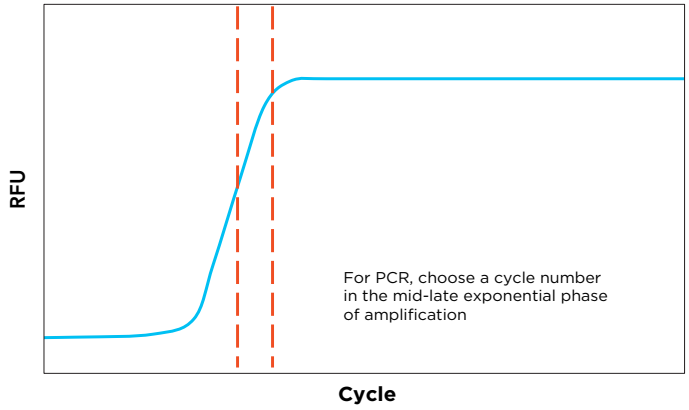
REAGENT	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	20X EvaGreen	NUCLEASE-FREE WATER
1X REACTION VOLUME	1 µL	0.8 µL	0.05 µL	0.1 µL	0.5 µL	6.05 µL

1. Prepare a PCR master mix according to the volumes shown in Table 255.
2. Aliquot 8.5 µL of PCR master mix per sample into a 0.2 mL qPCR strip or plate.
3. Add 1.5 µL of purified library (after **K. Strand Selection Purification** but before **L. Library Amplification A**) for a total qPCR volume of 10 µL. Mix well by pipetting, spin and place on ice.
4. Perform qPCR with the following cycling conditions:

   37 °C – 10 min, 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

   Choose a cycle number within the mid- to late-exponential phase of the amplification plot (Figure 4).

Figure 4. Stylized qPCR Amplification Plot



To amplify the remaining 13.5 µL of library, prepare an amplification master mix according to the volumes according to Table 266 for standard workflows.

Table 26. 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)	STRAND SELECTION ENZYME MIX II (PURPLE: SS4)	NUCLEASE-FREE WATER
1X REACTION VOLUME	9 µL	7.2 µL	0.45 µL	0.9 µL	58.95 µL

5. Remove Agencourt beads from storage and allow them to reach room temperature for use in the next step.
6. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin and place on ice.
7. Spin down AR3 and SS4 and place on ice.
8. Prepare a master mix by sequentially combining nuclease-free water, AR1 and AR2 in an appropriately sized capped tube according to Table 26. Add AR3 and SS4 last and mix well by pipetting, taking care to avoid creating bubbles. Spin and place on ice.
9. On ice, add 76.5 µL of Library Amplification Master Mix to 13.5 µL of sample for a total of 90 µL. Mix by pipetting, spin and place on ice.
10. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Library Amplification; see Table 5), using the number of cycles (N) determined by qPCR:

    37 °C – 10 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), **N**x(95 °C – 30 s, 65 °C – 90 s), 65 °C – 5 min, hold at 4 °C
11. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
12. Add 10 µL of nuclease-free water to each library. Mix well by pipetting and continue immediately to section **P. Amplified Library Purification**.

C. Library Amplification qPCR Optimization for AnyDeplete Workflow

When using the Universal Plus kit for the first time, or when working with a new sample type or input amount, a qPCR step prior to Library Amplification is recommended to determine the optimal number of PCR cycles and prevent excess amplification.

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

Table 27. Library Amplification qPCR Master Mix

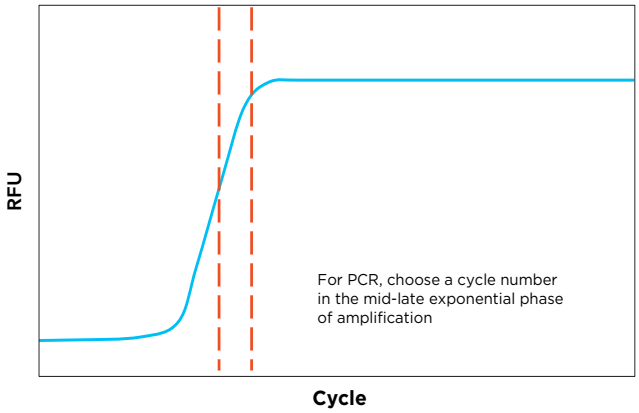
REAGENT	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (RED: AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	20X EvaGreen	NUCLEASE-FREE WATER
1X REACTION VOLUME	1 µL	0.8 µL	0.05 µL	0.5 µL	2.65 µL

1. Prepare a PCR master mix according to the volumes shown in Table 27.
2. Aliquot 5 µL of PCR master mix per sample into a 0.2 mL qPCR strip or plate.
3. Add 5 µL of library (after **N. Targeted Depletion (optional AnyDeplete workflow)** but before **O. Library Amplification B (optional AnyDeplete workflow)**) for a total qPCR volume of 10 µL. Mix well by pipetting, spin and place on ice.
4. Perform 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 5).

Figure 5. Stylized qPCR Amplification Plot



To amplify the remaining 45 µL of library, prepare an amplification master mix according to Table 288 for AnyDeplete workflows.

Table 28. Library Amplification B 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
1X REACTION VOLUME	9 µL	7.2 µL	0.45 µL	28.35 µL

1. Remove Agencourt beads from storage and allow them 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 nuclease-free water, AR1 and AR2 in an appropriately sized capped tube according to Table 28. Add AR3 last 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 B 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 Program 12 (Library Amplification B; see Table 5), using the number of cycles (N) determined by qPCR:

   95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 90 s), **N**x(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. Add 10 µL of nuclease-free water to each library. Mix well by pipetting and continue immediately to section **P. Amplified Library Purification**.

D. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with Universal Plus mRNA-Seq with NuQuant?
This kit includes all necessary buffers, primers and enzymes for poly(A) selection, library construction, AnyDeplete (Part No. 0521, 0521B, 0521C, 0521D, 0522, 0522B, 0522C, 0522D only) and NuQuant. SPRI purification beads and EvaGreen are not included.

Q2. What equipment is required or will be useful?
A comprehensive list of required and recommended equipment can be found in Section **II.B.**

Q3. Can this system be used with other library preparation workflows?
Universal Plus mRNA-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

Q4. What methods do you recommend for RNA isolation?
We recommend column-based methods, 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 followed with a column-based clean-up method.

Q5. Can I use TRIzol or other phenol-chloroform based extractions for RNA isolation?
We do not recommend TRIzol or similar methods due to potential organic solvent carryover, which may inhibit enzyme activity. If using TRIzol extracted RNA, perform a column-based purification of the RNA prior to using Universal Plus RNA-Seq.

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

Q7. Can I use Universal Plus mRNA-Seq with RNA from any organism?
Yes, it is compatible with polyadenylated RNA from any organism.

Q8. Do I need to use high-quality total RNA?
Yes, high quality RNA is required for efficient poly(A) selection.

Q9. What input amounts can be used with the Universal Plus mRNA-Seq with NuQuant kit?
The kit is designed for inputs between 10 ng – 1 µg of total RNA. Inputs outside this range may affect reaction stoichiometry and library quality.

Q10. Can contaminating genomic DNA interfere with Universal Plus mRNA Seq?
Yes, genomic DNA can be incorporated into libraries and may result in decreased library strandedness. Include DNase treatment during RNA isolation to minimize gDNA contamination.

General Workflow

Q11. Does this system contain a SPIA®-based amplification?
No. The cDNA is generated with random and poly(T) primers, without SPIA-based amplification.

Q12. Is it necessary to fragment my cDNA prior to End Repair and Adaptor Ligation?
No. Chemical fragmentation is incorporated as part of the workflow after poly(A) RNA selection.

Q13. Can I combine the barcoded libraries prior to the PCR amplification step?
No, this may affect stoichiometry. Amplify and quantify libraries independently before pooling.

Q14. Where can I safely stop in the protocol?
Samples can be stored at –20 °C after second strand synthesis, end repair, strand selection or after any of the bead purification steps. These safe stopping points allow for brief (overnight) storage. Please plan to continue the protocol as soon as possible.

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?
Use a magnet designed for 0.2 mL tubes, tube strips, or plates. Ensure the magnet is strong enough to clear the solution of beads. Test with a mock purification to ensure effectiveness.

Q17. How can I ensure maximum recovery of sample from the SPRI bead purification?
- Allow beads to reach room temperature before use.
- Fully resuspend beads before adding to samples.
- Use fresh ethanol for washes, measuring ethanol and water separately.
- Mix bead suspension and sample thoroughly for maximum binding.

Library Quantification and Qualification

Q18. How do I measure my final library yield?
Use NuQuant for accurate quantification of final libraries using either a Qubit instrument or a fluorescence plate reader. Use the NuQuant-determined concentration for multiplex library pooling. Determine final library pool concentration using a qPCR-based method before loading onto an Illumina sequencer. Refer to section **IV.Q** for alternative library assessments.

Q19. How many bases do the UDI 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 mRNA-Seq libraries are compatible with Illumina sequencing platforms.

Q21. How much material should I load into the sequencer?
Follow the manufacturer's recommendations for library QC, quantification, 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 UDI adaptors?
UDI adaptors in the Universal Plus UDI-A 96-Plex Adaptor Plate (S02480) are a minimum edit distance of 3, allowing for a maximum difference of 1 during demultiplexing. Indexes in UDI-B (S02690), UDI-C (30185200) and UDI-D (30185201) plates have a minimum edit distance of 2, allowing for a maximum difference of 0 during demultiplexing. For further details on the index sequence 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?
Use standard Illumina sequencing primers for both single-end and paired-end sequencing applications.

Q24. Can Universal Plus mRNA-Seq libraries be used with paired-end sequencing?
Yes, they can be used for both single-end and paired-end sequencing. Consider the expected insert size and planned sequencing read length when planning paired-end assays. 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 using standard methods. Note that in Universal Plus mRNA-Seq libraries, the forward read corresponds to the sense strand.

Q26. Will amplification introduce bias to my libraries?
In library preparation, input quantity and complexity determine the rate of PCR duplication, rather than the number of PCR cycles used. For more information see Ebbert *et. al.,* (2016). Evaluating the necessity of PCR duplicate removal from next-generation sequencing data and a comparison of approaches. *BMC Bioinformatics*, 17; Fu *et. al.,* (2018). Elimination of PCR duplicates in RNA-seq and small RNA-seq using unique molecular identifiers. *BMC Genomics*, 19.

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