USER GUIDE

Revelo™ RNA-Seq

REF 30184147, 30184149, 30184151, 30184204

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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

Revelo RNA-Seq is an end-to-end solution for generation of RNA-Seq libraries derived from total RNA. This kit is intended for Research Use Only and not for use in diagnostic procedures.

Features

Revelo RNA-Seq is a streamlined solution for whole transcriptome RNA-Seq with an optimized workflow for detection of rare transcripts (e.g. viral genomes). This kit is compatible with high-quality total RNA obtained from a broad range of tissues or cell lines. Degraded RNA samples may be compatible; please contact Tecan NGS Technical Support for guidance.

The Revelo RNA-Seq workflow consists of cDNA synthesis with integrated human rRNA depletion, Single Primer Isothermal Amplification (**SPIA**®) and cDNA fragmentation followed by **DimerFree**® library construction. 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 insert size.

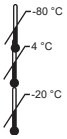
Specifications

Input type:	Total RNA
Input amount:	250 pg – 10 ng
# Reactions available:	8, 32, 96
Barcodes available:	8, 32, 96 single index; and 96 UDI
Sequencing platforms:	Illumina NGS

B. Storage and Stability

Revelo RNA-Seq is provided as two shipments. The core library preparation kit is shipped on dry ice while the bead binding buffer and NuQuant buffer are shipped on ice packs. 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.



- A3 VER 7 should be removed from the core kit box and stored at –80 °C.
- Bead binding buffer and NuQuant buffer should be stored at 4 °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 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/revelo-rna-seq-library-preparation-kit

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 Revelo RNA-Seq page at www.nugen.com/products/revelo-rna-seq-library-preparation-kit

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 8, 32 or 96 (A01) reactions. A01 fill sizes may be used in manual or automation workflows.[†]

Table 1. Revelo RNA-Seq, Human rRNA kit reagents

COMPONENT	30184147 (8 RXN)	30184149 (32 RXN)	30184151 (96/A01 RXN)	30184204 (96/A01 UDI)	VIAL LABEL	VIAL NUMBER
DNase Buffer Mix	S02280	S02294	S02457	S02457	Clear	DB VER 2
HL-dsDNase	30184155	30184156	30184157	30184157	Clear	-
Strand Selection Reagent	30184158	30184160	30184161	30184161	Purple	SS7 VER 1
First Strand Primer Mix	S01262	S02295	S02460	S02460	Blue	A1 VER 4
First Strand Buffer Mix	S02282	S02296	S02459	S02459	Blue	A2 VER 13
First Strand Enzyme Mix	S02250	S02297	S02270	S02270	Blue	A3 VER 7
Second Strand Buffer Mix	S01132	S01192	S02461	S02461	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	S01193	S02462	S02462	Yellow	B2 VER 2
SPIA Primer Mix	30184176	30184178	S02401 (2)	S02401 (2)	Red	SP1 VER 1
SPIA Buffer Mix	30184179	30184180	30184181	30184181	Red	SP2 VER 1
SPIA Enzyme Mix	30184182	30184183	S02403 (2)	S02403 (2)	Red	SP3 VER 1
End Repair Buffer Mix	30184184	30184185	S02404 (2)	S02404 (2)	Blue	ER1 VER 3
End Repair Enzyme Mix	S01510	30184186	S01909 (2)	S01909 (2)	Blue	ER2 VER 4
End Repair Enhancer	30184187	30184188	30184189	30184189	Blue	ER3 VER 3
Ligation Buffer Mix	S01466 (2)	30184190	S01689	S01689	Yellow	L1 VER 4
Ligation Enzyme Mix	30184191	S01467 (2)	S01535 (2)	S01535 (2)	Yellow	L3 VER 4
Amplification Reagent I	30184194	30184195	30184196	30184196	Red	AR1 VER 1
Amplification Reagent II	30184200	30184201	30184202	30184202	Red	AR2 VER 1

[†] Our automation size kits are designed to process 96 reactions in a maximum of two batches of samples on the standard platforms listed. If more than two batches are processed from a single kit, there may be insufficient reagent for 96 reactions due to overages required in automation workflows.

TABLE 1. REVELO RNA-SEQ, HUMAN rRNA KIT REAGENTS, *CONTINUED*

COMPONENT	30184147 (8 RXN)	30184149 (32 RXN)	30184151 (96/A01 RXN)	30184204 (96/A01 UDI)	VIAL LABEL	VIAL NUMBER
Amplification Enzyme Mix	30184197	30184198	30184199	30184199	Red	AR3 VER 1
DNA Resuspension Buffer Mix	S02287 (2)	S02303	S01901 (2)	S01901 (2)	Clear	DR1
Bead Binding Buffer Mix	S02288	S02288	S02410 (4)	S02410 (4)	Clear	BB VER 1
NuQuant Buffer	S02515	S02516	S02517	S02517	Clear	—
NuQuant Standard	S02512	S02512	S02512	S02512	Clear	—

Table 2. Revelo RNA-Seq Adaptor Mixes

COMPONENT	KIT NUMBER	PART NUMBER	VIAL LABEL	VIAL NUMBER
Ligation Adaptor Mix	30184147	S02309 S02310 S02311 S02312 S02313 S02314 S02315 S02316	Yellow	L2V23DR-BC1 L2V23DR-BC2 L2V23DR-BC3 L2V23DR-BC4 L2V23DR-BC5 L2V23DR-BC6 L2V23DR-BC7 L2V23DR-BC8
32-Plex Adaptor Plate	30184149	S02317	Yellow	L2V23DR-BC
96-Plex Adaptor Plate	30184151	S02477	Yellow	L2V23DR-BC
96-Plex Unique Dual Index Adaptor Plate	30184204	30184203	Yellow	L2V28

Note: The reagents in Revelo RNA-Seq are similar to reagents in our other kits; however, unless the component part numbers are identical (including version (VER) numbers), 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 50 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®, 20X (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. A low-elution volume or “side pull” style magnet is recommended for this system (Thermo Fisher Scientific Cat. #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)
- Disposable gloves
- Kimwipes
- Ice bucket

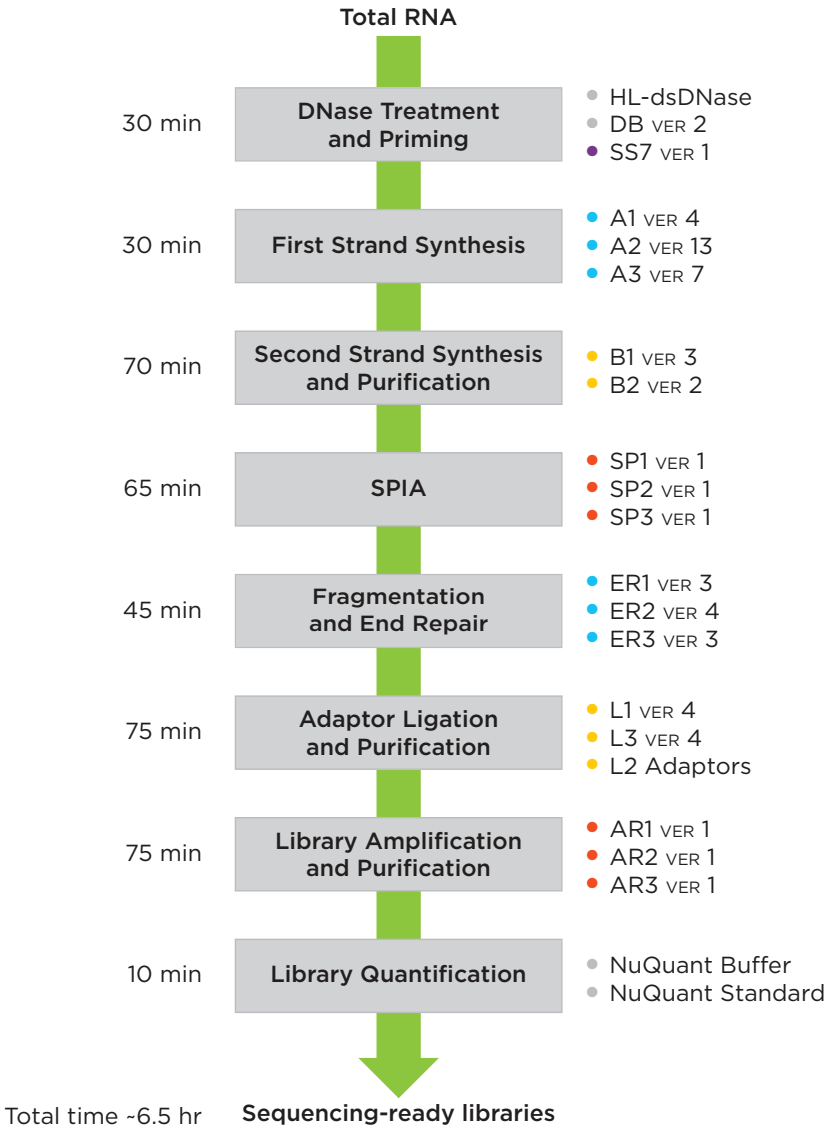
To Order:

- 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
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

A. Workflow and Time Required

The Revelo RNA-Seq workflow begins with first strand cDNA synthesis via priming at the 3' end of RNA as well as randomly throughout the transcriptome. Next, second strand cDNA is synthesized and amplified with SPIA, followed by end repair to generate blunt ends, ligation of barcoded adaptors, and PCR amplification to produce the final library (Figure 1). The entire workflow can be completed in one day, and yields cDNA libraries ready for either single read or paired-end sequencing on Illumina sequencing platforms.

Figure 1. Revelo RNA-Seq Workflow.



B. Input RNA Requirements

RNA Quantity

Total RNA input must be between 250 pg – 10 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 in excess of 1.8 and A260:A230 should be in excess of 2.0.

RNA Integrity

RNA samples of high molecular weight with little or no evidence of degradation will perform very well with this product. When using degraded total RNA, we recommend using somewhat higher inputs in order to achieve yield and data quality similar to that of more intact RNA samples. Depending on available input, RNA integrity can be determined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip or RNA 6000 Pico LabChip. While it is impossible to guarantee satisfactory results with all degraded samples, this system may work with many samples that are moderately degraded.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 50 µL reaction volume. Prepare the programs shown in Table 3 following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C. For thermal cyclers with a fixed-temperature heated lid use the default settings (typically 100–105 °C).

Table 3. Thermal Cycler Programming

cDNA SYNTHESIS		VOLUME
Program 1 DNase Treatment	37 °C – 10 min, 60 °C – 5 min, hold at 4 °C	10 µL
Program 2 Selective Priming	60 °C – 5 min, hold at 4 °C	12.5 µL
Program 3 First Strand Synthesis	25 °C – 10 min, 42 °C – 10 min, 70 °C – 5 min, hold at 4 °C	25 µL
Program 4 Second Strand Synthesis	25 °C – 10 min, 50 °C – 30 min, hold at 4 °C	50 µL
Program 5 SPIA	47 °C – 40 min, 80 °C – 20 min, hold at 4 °C	15 µL

TABLE 3. THERMAL CYCLER PROGRAMMING, *CONTINUED*

LIBRARY CONSTRUCTION		VOLUME
Program 6 Fragmentation and End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	22.5 µL
Program 7 Adaptor Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	45 µL
AMPLIFICATION		**VOLUME**
Program 8 Library Amplification Optimization with qPCR	72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 25x(95 °C – 30 s, 65 °C – 60 s)	10 µL
Program 9 Library Amplification	72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), N*(95 °C – 30 s, 65 °C – 60 s), 65 °C – 5 min, hold at 4 °C	50 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 32- and 96-Plex Adaptor Plates

The Adaptor Plate included with the 32 and 96 reaction Revelo RNA-Seq kits contain adaptor mixes with eight-base single- or dual-index barcodes. Each well contains sufficient volume for preparation of a single library. The Revelo 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. 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 29.

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 1.2 mL of 70% EtOH solution per sample.

Table 4. 70% EtOH Wash Recipe

1X REACTION VOLUME*	100% ETOH	NUCLEASE-FREE WATER
1.2 mL	0.84 mL	0.36 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

Revelo RNA-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 1X NuQuant Standard corresponds to a 644 nM Revelo 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.

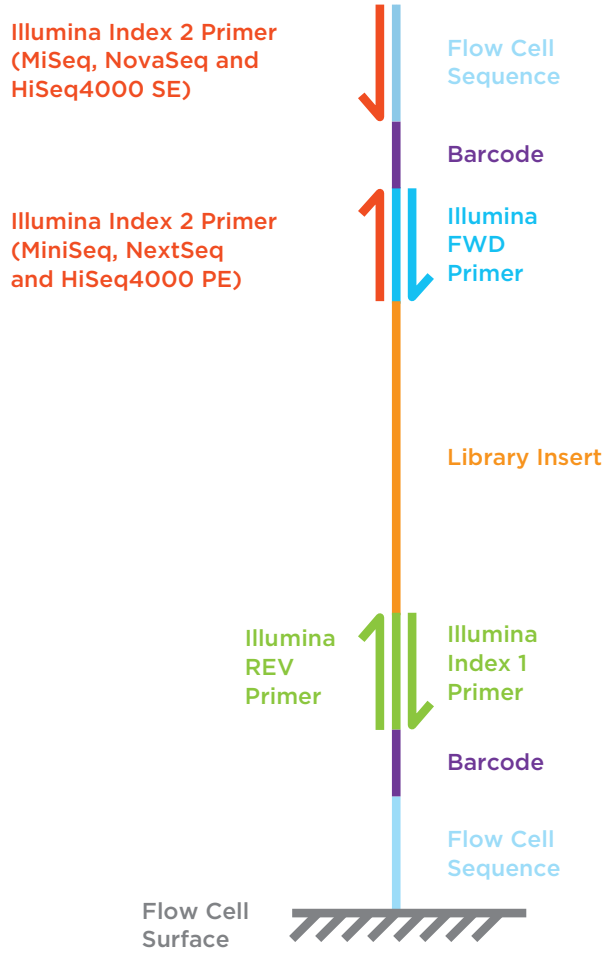
G. Sequencing Recommendations and Guidelines

Revelo 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

Revelo RNA-Seq libraries contain 8 nucleotide single or Unique Dual Indexes (UDI) for sample multiplexing. These barcodes differ from the sequences used by Illumina and can be found in Appendix A. When using the UDI kit, both index 1 (i7) and index 2 (i5) should be sequenced.

Figure 2. Revelo RNA-Seq Library Structure.



H. Data Analysis

Once the data have been parsed according to sample, a specialized adaptor trimming command is required at the 5' end of the reads. To do this we recommend using `cutadapt` (https://cutadapt.readthedocs.io/en/stable/) with `-g ACTTTGTGTTTGA` for single end (or `-g ACTTTGTGTTTGA -G ACTTTGTGTTTGA` for paired end). For data sequenced on an Illumina instrument using two-color chemistry (NextSeq, NovaSeq, MiniSeq), we recommend also adding `--nextseq-trim=20` option in `cutadapt`.

For experiments with short library inserts, such as degraded samples, additional trimming may be required. Contact Tecan NGS Technical Support for more information.

I. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

Protocol Notes

Controls

- We recommend the routine use of a positive control RNA, especially the first time a reaction is set up. The use of a positive control RNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- Do not use no-template controls (i.e. NTC; no nucleic acid/RNA controls) due to the high sensitivity inherent in the Revelo RNA-Seq kit. Host genome controls (e.g. no pathogen) or low-template controls (LTC, 50 pg) are recommended. For more information see **Appendix B**.
- Due to the high sensitivity inherent in this protocol a clean work environment is essential. We strongly recommend taking measures to minimize the potential for the carryover of previously amplified samples into new amplification reactions. The two steps to accomplish this are: 1) designating separate workspaces for "pre-amplification" and "post-amplification" steps and materials, and 2) implementing routine clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in **Appendix B**.
- When working with picogram amounts of RNA we strongly recommend the use of low retention tubes for storage and dilution of the samples in order to reduce the loss of RNA due to adhesion to polypropylene surfaces.

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

- Only use nuclease-free water with this kit. 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.

For each section of the protocol, remove reagents from storage as listed in "Storage and Stability" on page 1. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

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

Important: Carry out steps A (Sample Preparation) through G (SPIA Amplification) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA removal solution such as DNA-OFF (MP Biomedicals, Cat. #QD0500) to avoid the potential introduction of previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix B of this user guide. If you have any questions on this important topic, please contact Tecan NGS Technical Support (techserv-gn@tecan.com).

A. Sample Preparation

1. Thaw DNA Resuspension Buffer (DR1) at room temperature. Mix by vortexing, spin down and place on ice.
2. Aliquot each total RNA input sample (250 pg – 10 ng) into a 0.2 mL strip tube or 96-well plate.
3. Dilute the RNA with DR1 to a final volume of 8 µL and place on ice.
4. Leave DR1 at room temperature for use in the next step.

B. DNase Treatment

Table 5. DNase Master Mix

REAGENT	DNase BUFFER (CLEAR: DB VER 2)	HL-dsDNase (CLEAR)	DNA RESUSPENSION BUFFER (CLEAR: DR1)
1X REACTION VOLUME	1 µL	0.5 µL	0.5 µL

1. Spin down the contents of HL-dsDNase and place on ice.
2. Thaw DB at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining DB, HL-dsDNase and DR1 in a 0.5 mL capped tube according to the volumes shown in Table 5. Mix well by pipetting, spin down and place on ice.
4. Add 2 µL of DNase Master Mix to each sample for a total of 10 µL. Mix well by pipetting, spin down and place on ice.

5. Place the tubes or strips in a pre-warmed thermal cycler programmed to run Program 1 (DNase Treatment; see Table 3):

   37 °C – 10 min, 60 °C – 5 min, hold at 4 °C

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

C. Selective Priming

1. Thaw Strand Selection Reagent (SS7) on ice. Mix by vortexing, spin down and return to ice.
2. Add 2.5 µL of SS7 to each sample for a total of 12.5 µL. Mix well by pipetting, spin down and place on ice.
3. Place the tubes or strips in a pre-warmed thermal cycler programmed to run Program 2 (Selective Priming; see Table 3):

   60 °C – 5 min, hold at 4 °C

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

D. First Strand cDNA Synthesis

Table 6. First Strand Master Mix

REAGENT	FIRST STRAND PRIMER MIX (BLUE: A1 VER 4)	FIRST STRAND BUFFER MIX (BLUE: A2 VER 13)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 7)
1X REACTION VOLUME	5 µL	6.25 µL	1.25 µL

1. Spin down the contents of A3 and place on ice.
2. Thaw A1 and A2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining A1, 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 12.5 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 3 (First Strand Synthesis; see Table 3):

   25 °C – 10 min, 42 °C – 10 min, 70 °C – 5 min, hold at 4 °C

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

E. Second Strand cDNA Synthesis

1. Remove Agencourt beads from 4 °C storage and DR1 from from –20 °C storage. 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 3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 2)
1X REACTION VOLUME	24.25 µL	0.75 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 (1.5 mL capped tube for more than 16 samples) according to the volumes shown in Table 7. Mix well by pipetting, spin down, and place on ice.
5. Add 25 µL of Second Strand Master Mix to each sample tube for a total of 50 µL. Mix well by pipetting, spin down and place on ice.
6. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (Second Strand Synthesis; see Table 3):

   25 °C – 10 min, 50 °C – 30 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation.

F. cDNA Purification

Table 8. Agencourt Bead Master Mix

REAGENT	AGENCOURT BEADS	BEAD BINDING BUFFER (BB VER 1)
1X REACTION VOLUME	30 µL	50 µL

1. Ensure the Agencourt beads and BB 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. Recommended volumes are provided in Table 4.
- 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 vortexing. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
4. Prepare a master mix by combining the resuspended Agencourt beads and BB according to the volumes shown in Table 8. Mix well by vortexing.
5. At room temperature, add 80 µL (1.6 volumes) of Agencourt bead master mix to each reaction and mix thoroughly by pipetting at least 10 times or until beads are well mixed in solution.

Note: It is critical for the beads to be well dispersed in solution. Please ensure samples are mixed thoroughly.

6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Keeping the tubes on the magnet, carefully remove the binding buffer and discard it.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

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

10. Remove the 70% ethanol wash using a pipette.
11. Repeat the wash one time for a total of two washes.

Note: It is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

12. Air-dry the beads on the magnet for 3 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with SPIA amplification.
13. Continue to SPIA Amplification with the cDNA still bound to the dry beads.

G. SPIA Amplification

Table 9. SPIA Master Mix

REAGENT	SPIA PRIMER MIX (RED: SP1 VER 1)	SPIA BUFFER MIX (RED: SP2 VER 1)	SPIA ENZYME MIX (RED: SP3 VER 1)
1X REACTION VOLUME	3.75 µL	7.5 µL	3.75 µL

1. Thaw SP3 on ice and mix the contents by inverting gently 5 times. Ensure the enzymes are well mixed without introducing bubbles, spin down and place on ice.

2. Thaw reagents SP1 and SP2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by sequentially combining SP1, SP2 and SP3 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 15 µL of SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 10 µL and mix thoroughly by pipetting. Attempt to get the majority of the beads in suspension and remove most of the beads from the tube walls.

Note: The beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of SPIA master mix will elute the cDNA from the beads.

5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (SPIA Amplification; see Table 3):

   47 °C – 40 min, 80 °C – 20 min, hold at 4 °C

6. Remove the tubes from the thermal cycler, spin to collect condensation and place at room temperature. **Do not re-open the tubes in the pre-amplification workspace.**

Important: At this point the tubes should be removed from the pre-amplification workspace. Carry out all remaining steps in a post-amplification workspace using dedicated post-amplification consumables and equipment. Take care to avoid the introduction of previously amplified cDNA into your pre-amplification workspace. For more information on our recommendations for workflow compartmentalization and routine lab cleanup, please refer to Appendix B of this user guide. If you have any questions on this important topic, please contact Tecan NGS Technical Support (techserv-gn@tecan.com).

7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. 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.

H. Fragmentation and End Repair

Table 10. End Repair Master Mix

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 VER 3)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)	END REPAIR ENHANCER (BLUE: ER3 VER 3)
1X REACTION VOLUME	5.25 µL	0.75 µL	1.5 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 10. Mix well by pipetting, spin down and place on ice.
4. Add 7.5 µL of End Repair Master Mix to each sample tube for a total of 22.5 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 3):

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

I. Adaptor Ligation

1. Remove Agencourt beads from 4 °C and place on the bench top to reach room temperature for use in the next step.
2. Thaw adaptors (L2) on ice, spin down, and return to ice.

Table 11. Ligation Master Mix

REAGENT	DNA RESUSPENSION BUFFER (CLEAR: DR1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
1X REACTION VOLUME	9.5 µL	9 µL	1 µL

3. Spin down L3 and place on ice.
4. Thaw L1 and DR1 at room temperature. Mix by vortexing, spin down and place on ice.
5. Add barcoded adaptor mixes to each sample as follows:
   - If using adaptors from the 8 or 32 reaction kits, add 3 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.
   - If using an adaptor plate from the 96 reaction kits, add the entire 22.5 µL of sample to the appropriate adaptor well, mix well by pipetting, then transfer the entire sample back to a PCR tube and spin down.

6. Prepare a master mix by combining DR1, L1 and L3 according to the volumes shown in Table 11. 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.

7. Add 19.5 µL of Ligation Master Mix to each sample tube for a total of 45 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
8. Place the tubes in a thermal cycler programmed to run Program 7 (Adaptor Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Leave DR1 at room temperature for use in the next step.

J. Adaptor Ligation Purification

1. Ensure the Agencourt beads and DR1 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. Recommended volumes are provided in Table 4.
- 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 vortexing. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
4. Add 30 µL of nuclease-free water to each sample for a total of 75 µL. Mix thoroughly by pipetting.
5. At room temperature, add 60 µL (0.8 volumes) of Agencourt beads to each reaction for a total of 135 µL. Mix thoroughly by pipetting at least 10 times or until beads are well mixed in solution.

Note: It is critical for the beads to be well dispersed in solution. Please ensure samples are mixed thoroughly.

6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 3 minutes to completely clear the solution of beads.

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

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

10. Remove the 70% ethanol wash using a pipette.
11. Repeat the wash one time for a total of two washes.

Note: It is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step. Briefly spin down the tubes before placing on the magnet again.

12. Air-dry the beads on the magnet for 3 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
13. Add 38 µL (32 µL if not doing qPCR) of DR1 or low-EDTA TE to the bead pellet. Mix thoroughly to ensure all beads are resuspended.
14. Incubate at room temperature for 2 minutes.
15. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
16. Carefully remove 36 µL (30 µL if not doing qPCR) of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.

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

K. Library Amplification Optimization with qPCR

Note: qPCR optimization should be performed when running the kit for the first time or when using a new sample type or input.

Table 12. Library Amplification qPCR Master Mix

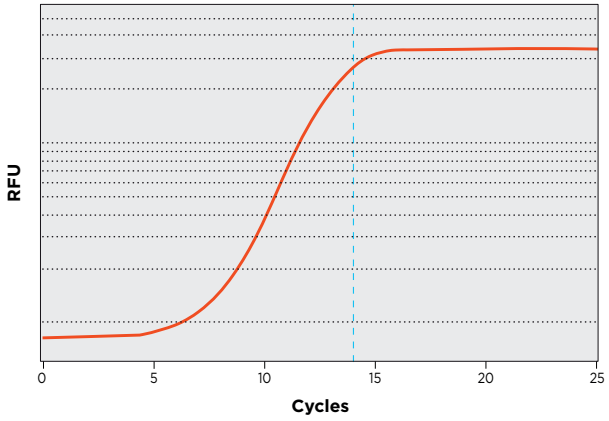
REAGENT	AMPLIFICATION REAGENT 1 (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	20X EVAGREEN	DNA RESUSPENSION BUFFER (DR1)
1X REACTION VOLUME	2 µL	1 µL	0.1 µL	0.5 µL	0.4 µL

1. Spin down AR3 and place on ice.

2. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Prepare a master mix by combining AR1, AR2, AR3, 20x EvaGreen, and DR1 in a 0.5 mL capped tube according to the volumes shown in Table 12. Mix well by pipetting, spin down and place on ice.
4. Add 4 µL of Library Amplification qPCR Master Mix to qPCR strip tube or plate for a total of 10 µL.
5. Transfer 6 µL of purified library to the qPCR strip tube or plate. Mix well by pipetting, spin down and place on ice.
6. Place the tubes in a real-time PCR instrument programmed to run Program 8 (Library Amplification qPCR; see Table 3):

   72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), 25x(95 °C – 30 s, 65 °C – 60s)
7. Select a cycle number for subsequent library amplification. The cycle number should be at the top of the exponential phase of the amplification plot (14 cycles in Figure 3 as an example).
8. Leave DR1 at room temperature for use in the next step.

Figure 3. Stylized qPCR amplification plot.



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

L. Library Amplification

1. Remove Agencourt beads from 4 °C and place on the bench top to reach room temperature for use in the next step.

Table 13. Library Amplification Master Mix

REAGENT	AMPLIFICATION REAGENT I (RED: AR1 VER 1)	AMPLIFICATION REAGENT II (AR2 VER 1)	AMPLIFICATION ENZYME MIX (RED: AR3 VER 1)	DNA RESUSPENSION BUFFER (DR1)
1X REACTION VOLUME	10 µL	5 µL	0.5 µL	4.5 µL

2. Spin down AR3 and place on ice.
3. Thaw AR1 and AR2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining AR1, AR2, AR3 and DR1 in a 0.5 mL capped tube according to the volumes shown in Table 13. Mix well by pipetting, spin down and place on ice.
5. Add 20 µL of Library Amplification Master Mix to each sample for a total of 50 µL. Mix well by pipetting, spin down and place on ice.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Library Amplification; see Table 3):

   72 °C – 2 min, 95 °C – 2 min, 2x(95 °C – 30 s, 60 °C – 60 s), N(95 °C – 30 s, 65 °C – 60s) , 65 °C – 5 min, hold at 4 °C

   Note: The number of cycles used for PCR amplification depends on the starting amount of RNA. Note that the guidelines in Table 14 are based on high-quality human RNA inputs.

7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Leave DR1 at room temperature for use in the next step.

Table 14. Recommended PCR Cycles for Library Amplification

STARTING INPUT	PCR CYCLES (N)
250 pg	10 cycles
500 pg	9 cycles
5 ng	7 cycles
10 ng	6 cycles

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

M. Library Amplification 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 DR1 have completely reached room temperature before proceeding.
3. Prepare a 70% ethanol wash solution.
4. 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.
5. Add 50 µL (1.0 volumes) of the bead suspension to each sample. Mix thoroughly by pipetting up and down at least 10 times or until the beads are well mixed in solution.

   Note: It is critical for the beads to be well dispersed in solution. Please ensure samples are mixed thoroughly.

6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
8. Keeping the tubes on the magnet, carefully remove the bead 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 library yield, so ensure beads are not removed with the binding buffer or the wash.

9. Add 50 µL of DR1 or low-EDTA TE to the bead pellet. Mix thoroughly to ensure all beads are resuspended.
10. Add 50 µL (1.0 volumes) of the bead suspension to each sample. Mix thoroughly by pipetting up and down.

    Note: It is critical for the beads to be well dispersed in solution. Please ensure samples are mixed thoroughly.

11. Incubate at room temperature for 10 minutes.
12. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
13. Keeping the tubes on the magnet, carefully remove the bead binding buffer and discard it.
14. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
15. Remove the 70% ethanol wash using a pipette.

16. Repeat the 70% ethanol wash one more time, for a total of two washes.

Note: With the second wash, it is critical to remove as much of the ethanol as possible. Remove the ethanol wash with a pipette, allow excess ethanol to collect at the bottom of the tubes, and remove any remaining ethanol with a fresh pipet tip. Briefly spin down the tubes before placing them back on the magnet.

17. Air dry the beads on the magnet for 3 minutes.
18. Add 32 µL of DR1 or low-EDTA TE to the bead pellet. Mix thoroughly to ensure all beads are resuspended.
19. Incubate at room temperature for 2 minutes.
20. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
21. Carefully remove 30 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.

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 Revelo RNA-Seq 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.

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

5. Measure samples as directed for your specific quantification platform:

   a. Qubit 2.0: Select “Revelo” 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 NuQuant app and select “Revelo”. 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 15 below.

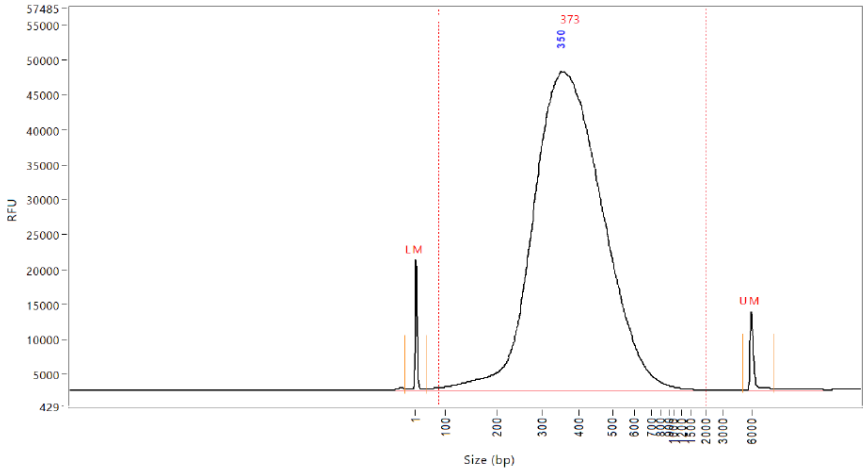
Table 15. Example Library Pooling Calculation using NuQuant Concentration

LIBRARY	NUQUANT CONCENTRATION (C_1)	TARGET CONCENTRATION OF POOL (C_2)	TARGET VOLUME OF POOL (V_2)	VOLUME OF LIBRARY TO ADD TO POOL (V_1)
1	220 nM	10 nM	200 µL	2.3 µL
2	160 nM			3.1 µL
3	140 nM			3.6 µL
4	120 nM			4.2 µL
			Volume of low-EDTA or nuclease-free water to add	186.9 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 Revelo RNA-Seq libraries prepared with high quality input RNA is shown in Figure 4.

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

Figure 4. Fragment distribution when 2 µL of final library is loaded onto a HS NGS Fragment Analyzer (1-6000 bp) 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.

† For libraries measured with NuQuant, load 2 µL of 10 nM final library onto a high-sensitivity fragment analysis assay. For mass-based library measurements, load 2 µL of 5 ng/µL final library.

VI. Technical Support

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

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (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

Barcode sequences for the 8-, 32- and 96-plex kits are given below.

Text versions of all barcode sequences can be found at https://tecangenomics.github.io/ or contact Tecan NGS Technical Support.

Barcodes shown in **Table 16** are color balanced in pairs and sets of 8 by plate column. Barcodes for the 32-Plex Adaptor Plate are given in plate positions A01–H04. Barcodes for the 8-reaction kit are given in plate positions A01–H01.

Unique dual indexes shown in **Table 17** are color balanced in sets of 8 by plate column.

Table 16. Barcode sequences for Revelo RNA-Seq Single Index (i7) kits

PLATE LOCATION	INDEX 1 (i7) SEQUENCE	PLATE LOCATION	INDEX 1 (i7) SEQUENCE	PLATE LOCATION	INDEX 1 (i7) SEQUENCE
A01	CGCTACAT	A05	AGGTTCCT	A09	GCCTTAAC
B01	AATCCAGC	B05	GAACCTTC	B09	ATTCCGCT
C01	CGTCTAAC	C05	AAGTCCTC	C09	ATCGTGGT
D01	AACTCGGA	D05	CCACAACA	D09	GCTACAAC
E01	GTCGAGAA	E05	ATAACGCC	E09	TCTACGCA
F01	ACAACAGC	F05	CCGGAATA	F09	CTCCAATC
G01	ATGACAGG	G05	CCAAGTAG	G09	ACTCTCCA
H01	GCACACAA	H05	AAGGACCA	H09	GTCTCATC
A02	CTCCTAGT	A06	ACGCTTCT	A10	GCCAGAAT
B02	TCTTCGAC	B06	CTATCCAC	B10	AATGACGC
C02	GACTACGA	C06	TGACAACC	C10	GTACCACA
D02	ACTCCTAC	D06	CAGTGCTT	D10	ACGATCAG
E02	CTTCCTTC	E06	TCACTCGA	E10	TAACGTCG
F02	ACCATCCT	F06	CTGACTAC	F10	CGCAACTA
G02	CGTCCATT	G06	GTGATCCA	G10	AACACTGG
H02	AACTTGCC	H06	ACAGCAAG	H10	CCTGTCAA
A03	GTACACCT	A07	TGCTGTGA	A11	TCCTGGTA
B03	ACGAGAAC	B07	CAACACAG	B11	CATCAACC
C03	CGACCTAA	C07	CCACATTG	C11	AGCAGACA
D03	TACATCGG	D07	TAGTGCCA	D11	GAAGACTG
E03	ATCGTCTC	E07	TCGTGCAT	E11	TCTAGTCC
F03	CCAACACT	F07	CTACATCC	F11	CTCGACTT
G03	TCTAGGAG	G07	CATACGGA	G11	CTAGCTCA
H03	CTCGAACA	H07	TGCGTAAC	H11	TCCAACTG
A04	ACGGACTT	A08	CAGGTTCA	A12	GACATCTC
B04	CTAAGACC	B08	AGAACCAG	B12	ACTGCACT
C04	AACCGAAC	C08	GAATGGCA	C12	GTTCCATG
D04	CCTTAGGT	D08	AGGCAATG	D12	ACCAAGCA
E04	CCTATACC	E08	TAGGAGCT	E12	CTCTCAGA
F04	AACGCCTT	F08	CGAACAAC	F12	ACTCTGAG
G04	TCCATTGC	G08	CATTCGTC	G12	GCTCAGTT
H04	CAAGCCAA	H08	AGCCAACT	H12	ATCTGACC

Table 17. Index 1 (i7) and Index 2 (i5) sequences for Revelo RNA-Seq UDI 96-Plex Adaptor Plate

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

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. Preventing Non-specific Amplification

The SPIA technology was developed to work with limiting amounts of starting material, hence it can detect environmental contamination. Due to the high sensitivity inherent in the Revelo RNA-Seq kit, it is important to maintain a clean work environment. We have developed a set of recommendations designed to minimize the potential generation of non-specific amplification products. We strongly recommend implementing these procedures.

We have three general recommendations:

1. Designate separate workspaces for "pre-amplification" and "post-amplification" steps and materials. This provides the best work environment for processing RNA using this highly sensitive amplification protocol.
    - Pre-amplification includes all steps and materials related to RNA Sample Preparation, DNase Treatment, Selective Priming, First Strand cDNA Synthesis, Second Strand cDNA Synthesis and SPIA Amplification. Ideally the isolated pre-amplification workspace will be in a separate enclosed workspace.
    - We recommend the use of "PCR Workstation" enclosures with UV illumination for use as pre-amplification workspaces. After Adaptor Ligation, you may remove your material to the post-amplification area.
    - Post-amplification includes all steps and materials related to the handling of the library molecules including Adaptor Ligation Purification, Library Amplification and Library Purification.
2. Implement routine clean-up protocols for workspaces as standard operating procedure.
    - Initially clean the entire lab thoroughly with DNA-OFF and RNaseZap.
    - In the pre-amplification area, remove all small equipment, then clean every surface. Before reintroducing any equipment, clean every piece of equipment thoroughly. Clean wells of thermal cyclers and magnetic plates with a cotton swab or by filling with cleaning solution.
    - Always wear gloves and don fresh gloves upon entry into the pre-amplification area. Frequently change gloves while working in this area, especially prior to handling stock reagents, cells, reactions and RNA samples.
    - Stock this area with clean (preferably new) equipment (pipettes, racks, consumables).
    - Make it a policy to carry out regular cleaning of all workspaces.
    - Do not open amplified product reaction vessels in the pre-amplification workspace.
3. Use low-template controls (LTC) instead of no-template controls to detect and troubleshoot contamination. The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular bioanalyzer traces in an LTC reaction.

    If environmental contamination is suspected, contact Tecan NGS Technical Support (techserv-gn@tecan.com).

C. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with Revelo RNA-Seq?
The Revelo RNA-Seq kit provides all necessary buffers, primers and enzymes for cDNA synthesis, human rRNA depletion, SPIA amplification, library construction 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?
Revelo 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

Q4. 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

For FFPE RNA isolation, we recommend a kit designed for FFPE samples, including:

- Norgen Biotek FFPE RNA Purification Kit
- Zymo Research Quick-RNA™ FFPE Kit
- Arcturus® Paradise® PLUS FFPE RNA Isolation Kit
- PureLink™ FFPE RNA Isolation Kit
- Qiagen RNeasy FFPE Kit

Organic methods such as TRIzol® Reagent should be subsequently 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 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.

Q6. Can I use carrier RNA during RNA isolation?
Do not use carrier RNA during RNA isolation. If a carrier is required, please contact Tecan NGS Technical Support for more information.

Q7. Can I use Revelo RNA-Seq with RNA from any organism?
Revelo RNA-Seq has been designed for use with total RNA inputs from human samples.

Q8. Do I need to use high-quality total RNA?
The Revelo RNA-Seq 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 RNA extracted from nasal swabs. Contact Tecan NGS Technical Support for more information.

Q9. Can contaminating genomic DNA interfere with Revelo RNA-Seq?
Contaminating genomic DNA can be incorporated into libraries. Inclusion of a DNase treatment during RNA isolation is recommended.

General Workflow

Q10. How much extra reagent is recommended when preparing the master mixes at each step?
A minimum amount of overage should be used in master mixes to ensure the full nominal number of reactions in the kit. The amount of overage needed depends on sample batch size, pipetting accuracy, and viscosity of reagents. We have found that 10% extra volume is sufficient at most steps. When preparing master mixes with particularly viscous components, including Fragmentation and End Repair, and Adaptor Ligation, 12-15% extra volume is recommended.

Q11. My input RNA samples are already fragmented (e.g. RNA derived from plasma, FFPE, nasal swabs, etc.). Can I skip the fragmentation and end repair step?
The fragmentation and end repair step is required in the Revelo RNA-Seq workflow. This kit has been demonstrated to work with moderately degraded samples.

Q12. 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.

Q13. Where can I safely stop in the protocol?
Samples can be placed in short-term storage at –20 °C after any of the bead purification steps.

Q14. How long can I store Revelo RNA-Seq libraries at –20 °C?
Libraries are stable at –20 °C for at least 4 months. Libraries must be protected from light.

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 or plate reader. The final library pool concentration should be determined using a qPCR-based method before loading onto an Illumina sequencer. Please refer to **Section V. N.** for guidelines on alternative library quantitative and qualitative assessments.

Q19. How many bases do Revelo RNA-Seq adaptors add to the library?

The adaptors add 136 bp to the library.

Sequencing Recommendations

Q20. What sequencers are compatible with your libraries?

Revelo 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 this kit 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. 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?

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

Q24. Can Revelo RNA-Seq libraries be used with paired-end sequencing?

Yes. The libraries produced using this kit can be used for both single- 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. Do Revelo RNA-Seq libraries have special data analysis requirements?

Once the data have been parsed according to sample, a specialized adaptor trimming command is required at the 5' end of the reads. To do this we recommend using `cutadapt` (https://cutadapt.readthedocs.io/en/stable/) with `-g ACTTTGTGTTTGA` for single end (or `-g ACTTTGTGTTTGA -G ACTTTGTGTTTGA` for paired end). For data sequenced on an Illumina instrument using two-color chemistry (NextSeq, NovaSeq, MiniSeq), we recommend also adding `--nextseq-trim=20` option in `cutadapt`. Contact Tecan NGS Technical Support for more information.

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