WTS Library Preparation - Illumina TruSeq

Purpose

To define the Whole Transcriptome Sequencing (WTS) library preparation procedure, using the Illumina TruSeq Stranded Total RNA Library Prep Gold kit.

Scope

Following extraction and initial Quality Control (QC), samples in the WTS workflow must undergo library preparation. A library consists of DNA fragments with ligated adapters and indices that are suitable for sequencing. This standard operating procedure (SOP) outlines the preparation of Illumina TruSeq Stranded Total RNA Library Prep Gold (48 or 96 samples) libraries from formalin-fixed paraffin-embedded (FFPE) and fresh frozen (FF) samples, for use on Illumina sequencers. The procedure uses the Illumina TruSeq Stranded Total RNA Library Prep Gold Sample Preparation Kits and includes the following steps: removal of ribosomal RNA (rRNA), first and second strand cDNA synthesis, adenylation of 3' ends, ligation of adapters, PCR amplification, and library validation. This SOP follows instructions provided by the manufacturer with minor modifications, and applies to all staff who perform WTS library preparation. The related document can be found in the Equipment User Manuals folder on the Genomics Quality SharePoint.

Related Document
Manufacturer-supplied protocol: *TruSeq® Stranded Total RNA Reference Guide* (Document 1000000040499 v00, October 2017)

Responsibilities

Management:
Review and update procedure as required

QA Manager:
Monitor quality output from this procedure

Laboratory Staff:
Follow this procedure and report any non-conformances that may occur

Reagents and Consumables

Item Description	Vendor	Catalogue #
Cervical Adenocarcinoma (HeLa-S3) Total RNA (positive control); GLCS_0029	ThermoFisher	AM7852
RNaseZap	Invitrogen	AM9780, AM9782
High Sensitivity RNA ScreenTape (7 tapes,112 samples)	Agilent	5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	Agilent	5067-5580
High Sensitivity RNA ScreenTape Ladder	Agilent	5067-5581
High Sensitivity D1000 Screen Tape (7 tapes,112 samples)	Agilent	5067-5584
High Sensitivity D1000 Reagents	Agilent	5067-5585
Plate Foil Seal	Agilent	5067-5154
96 Well plates, 150ul, conical, 25/pk	Agilent	5042-8502
Mx3000P Optical Strip Caps	Agilent	401425
Mx3000P Strip Tubes	Agilent	401428
PCR STRIP(8), 0.2ml NEUTRAL, ATT FLAT CAP,120/pk	Medstore	72.991.002
Sigma Nuclease-free water	Medstore	W4502-1L
Axygen Snaplock Centrifuge Microtubes (clear), 1.5mL, 250/pk (10 pks/case)	Medstore	MCT-150-L-C
Ethanol anhydrous 100% (brown bottle), case of 12X500ml	Medstore, Greenfield Specialty Alcohols	P006EAAN
Ethanol anhydrous (4X4L white jug, for cleaning only)	Medstore/Greenfield Specialty Alcohols	P016EAAN
TruSeq Stranded Total RNA Library Prep Gold (96 Samples)	Illumina	20020599
TruSeq RNA CD Index Plate (96 Indexes, 96 Samples)	Illumina	20019792 (order separately)

FOR 48-plex: TruSeq Stranded Total RNA Library Prep Gold (TruSeq Strnd Ttl RNA LPGold 48 Smpl, Component 15032619)	Illumina	20020598
TruSeq Strnd Ttl RNA LP(48 Smpl) (Component 15032615)	Illumina	
TruSeq Strnd RNA Core LPBox 2 (Component 15032611)	Illumina	
SuperScript II Reverse Transcriptase (10000units, 200units/µL)	Invitrogen	18064014
NucleoMag® NGS Clean-up and Size Select beads or AMPure XP Beads	Macherey-Nagel Beckman/Cedarlane	744970 A36881 (60 ml)
Agencourt RNA Clean XP	Beckman/Cedarlane	A63987 (40 mls)
Qubit dsDNA HS Assay Kit	ThermoFisher	Q32854
Qubit Assay Tubes	ThermoFisher	Q32856
Pipette Tips	Any Vendor	Various

Note: Plastic consumables may be substituted for an approved equivalent product

Equipment

Item Description	Vendor	Catalogue #
TapeStation 2200	Agilent	G2964AA
Vacufuge Plus OR CentriVap Benchtop Vacuum Concentrator	Eppendorf OR VWR	022820001 89004-916
Centrifuge	Eppendorf	Various
c1000 Thermocycler	Bio-Rad	1841100
Qubit 4.0 Fluorometer	Thermofisher Scientific	Q33226
Qubit Flex Fluorometer	Thermofisher Scientific	Q33327
Mini-centrifuge	VWR/Fisher	C1413-VWR230/05-090-100

Pipettes (mechanical)	Any Vendor	Various
Vortex	Fisher	02215365
Dynamag (magnetic rack)	ThermoFisher	12321D
Dynamag-96 (96 well magnetic rack)	ThermoFisher	123331D

Variables and Observations to Record

Record
Quality of Input RNA (ng, DV200)
Qubit Quantification (ng/µL) (final library)
% Adapter Contaminant (final library)
Library Size (bp) (Tapestation or Fragment Analyzer)
TruSeq Stranded Total RNA Library Prep Gold Lot Number
TruSeq RNA CD Index Plate Lot Number

Batch Controls

A no-template control (NTC) and positive RNA control must be included in each library synthesis batch. RNA positive controls are purchased from Thermofisher (Cat# AM7852, MISO alias: GLCS_0029). Control libraries are not sequenced but are included in each library synthesis batch and recorded in a MISO batch within the respective MISO CAP project(s). Control libraries must be synthesized at the same time as a production batch of libraries. The following library information is recorded and logged in MISO.

Sample Name	IDT Dual Indices	Insert Size (bp)	Qubit (ng/ul)
1) NTC negative control			
2) RNA positive control (GLCS_0029)			

Important Considerations

Reagent Lots and Expiration

- Prior to starting an assay, inspect reagents to ensure that they have not expired

    - Expired reagents may not be used for validated clinical assays, however, they may be used for RUO assays at the discretion of the Production Manager
- Lot numbers for critical reagents must be recorded on MISO.

RNA Handling

- Purified Total RNA must always be stored at -80°C.
- Record DV200 score, quants, dilutions, thermal cycler used, and all other QC information. DV200 is obtained using the TapeStation or Fragment Analyzer prior to starting library preparation, following either the TM. High Sensitivity TapeStation Assay or TM. Fragment Analyzer Assays SOP, as required.
- **DV200 CUT OFF IS 20%. Any RNA sample less than 20% DV200 will not be accepted for library preparation.**
- Enter the DV200 value into MISO as described in the LIMS Section below
- When kits are received, kit component boxes must be inspected for proper shipping temperature. rRNA Removal Beads (RRB) should never be frozen, or arrive frozen. If these reagents are clumpy, or frozen, isolate the reagent and contact Illumina customer support for a replacement. Discontinue use if these beads freeze or become clumpy at any time.
- Before beginning work each day, wipe down all pipettes, bench surfaces and centrifuge interiors with bleach, then wipe with 70% ethanol (made from 4L white jug ethanol).
- Before working with RNA and up to step "4 Adenylate 3' ends", wipe down surfaces with RNaseZap, followed by 70% ethanol.
- Do not mix and match reagents from multiple kits. Aliquot reagents where appropriate to minimize freeze thaw cycles, and indicate each thaw with a dot on the top of the respective tube, especially First Strand Synthesis Act D Mix (FSA) + SSII mix (section 2, first strand synthesis). Add 50µl of superscript II (SSII) to 450ul of FSA, gently vortex, spin and aliquot into 24 rxn aliquots (200µl), and freeze. Note that the volume of FSA provided by Illumina may not exactly equal 450 ul - measure with a pipette to ensure proper volume before addition of SSII.
- Reagents/enzymes may be thawed and then placed on ice or in 4°C fridge until ready for use, except for RBB, RRB, and ELP during ribosomal depletion steps.
- rRNA Removal Beads (RBB), Agencourt RNA Clean XP and AMPure XP (or NucleoMag) beads must be allowed to reach room temperature before use; 30 min at room temperature is sufficient. If these beads become frozen or clumpy, discard; they will no longer deplete ribosomal material efficiently. Keep these reagents at room temp at all times and do not place on ice.
- 70% and 80% ethanol wash solutions should be made fresh every day using molecular grade water and anhydrous ethanol (brown bottle only). Always use fresh ethanol and water aliquots to minimize risk of contamination between technicians. Always use aliquoted reagents; max 50ml aliquots. If you think you have touched the side of a pipette to the side of an aliquot tube (ie the shaft of the pipette), discard aliquot.
- Be sure beads are thoroughly bound to the side of the tube when washing, as loss of beads will reduce library diversity.

- During bead-based cleanups, ensure beads are dry before reconstituting in Resuspension Buffer (RSB). Residual ethanol may interfere with enzymatic reactions, or lead to adapter carry over. Beads should appear to be cracking from dryness. 15 min of dry time is an estimate. Depending on humidity, dry time may be as short as 7 min.
- Be sure not to carry over beads after elution. If beads do carryover, bind solution to magnetic rack again and transfer again to fresh strip tube.
- Use a 10µL pipette tip to remove residual liquid before adding 80% ethanol wash to AMPure XP (or NucleoMag) beads. This will significantly reduce contaminating adapter in FFPE samples (ligation and PCR cleanup steps), and ensure optimal enzyme reactions occur.
- Never remove a tube/strip tube of dried beads from a magnetic rack prior to addition of RSB in resuspension steps. "Soaking" the dried beads, prior to removing from a magnetic rack will prevent dried beads from dispersing into air, or "jumping" from the tube electrostatically.
- Record DV200 score, quants, dilutions, thermal cycler used, and all other QC information.
- Enzyme solutions should be 'flick' mixed and briefly spun down prior to use, buffers should be vortexed and spun down.
- Reactions in strip tubes should be briefly spun to collect material at bottom of reaction well, especially before and after thermocycler incubations, and/or before AMPure XP (or NucleoMag) additions (minifuge).
- Record all QC steps, including master lot tracking references, protocol version, tech performing prep/QC step, thermocycler(s) used in project running sheet.
- Use RNA Clean XP with 70% ethanol and 80% ethanol with AMPure XP (or NucleoMag) beads.
- Record a "hash mark" on kit box to indicate # of reactions used, and which index was used (limiting adapter in kit)
- Record all unusual observations, errors, or other issues.

Safety

- First Strand Synthesis Act D Mix (FSA) contains actinomycin D. Avoid inhalation, ingestion, skin and eye contact. Wear appropriate safety protection.

Freeze-Thaw

- Illumina does not recommend freeze-thawing indexes more than 4 times. Thaw the adapter plate at room temperature for 10 min and visually inspect that all wells are thawed, centrifuge at 1000RPM for 1 min. Wells may be punctured using a clean strip tube. With each new 96 plex adapter plate, thaw once and aliquot each adapter to strip tubes. Thaw only the strip tubes that are needed. Track the number of freeze-thaws on the individual adapter with a dot on the top of the strip tube lid. Follow Illumina recommendations for selection of TruSeq CD Indexes (Dual Indexing) to ensure optimal base diversity. NovaSeq S4 lanes may accommodate up to 20 TruSeq Total RNA libraries. Follow the index plate map below to select an appropriate combination of adapters. If multiplexing 20 libraries on a run, select 4 additional indexes from rows A-

D, column 9-12 (upper right block) (Figure A); For Research Use Only (RUO) NextSeq550 HighOutput flow cells a maximum of 5 libraries may be pooled, choose an indexing strategy as depicted in Figure B.

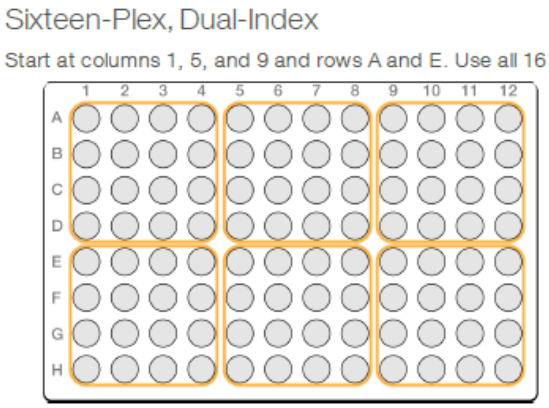

Figure A: NovaSeq Indexing Strategies

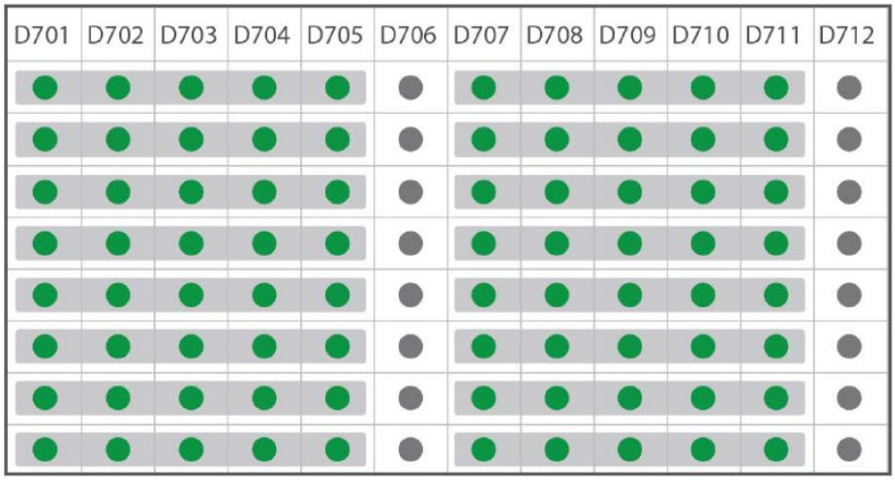

Figure B: NextSeq550 Indexing Strategies (RUO)

Procedure

1. Ribo-Zero Deplete and Fragment RNA

1. Thaw the following reagents and keep on ice/ 4°C:
    a. Elute, Prime, Fragment High Mix (EPH)
    b. Resuspension Buffer (RSB) (use an aliquot)
2. Bring the following reagents to room temperature before use (allow 30 min):
    a. Elution Buffer (ELB)
    b. rRNA Binding Buffer (RBB)
    c. rRNA Removal Beads (RRB)
    d. rRNA Removal Mix-Gold (RRM G)

e. Agencourt RNA Clean XP beads (Ribo-Zero Deplete and Fragment RNA stage only, use 70% EtOH)
f. AMPure XP beads or NucleoMag beads (Required for purifications after second strand cDNA synthesis, use 80% EtOH)

3. Prepare fresh 70% and 80% EtOH using nuclease-free water and anhydrous ethanol.
4. Dilute 200 ng of total RNA (FFPE) or 50ng of fresh frozen total RNA to a final volume of 10 µL with RSB in PCR plate or strip. Include an NTC (H20) and positive RNA control (200ng, GLCS_0029) in each batch. If required, reduce volume using a Vacufuge with a max temperature setting of 30°C. Note: the validated input range is 50-200 ng for fresh frozen samples. The default input is listed above, however, Management may specify a different input within this range as required.
    a. Do not completely dry the RNA aliquot.
    b. Add RSB to bring the total volume to 10 µL prior to next step.
5. Add 5 µL of room temperature, vortexed rRNA Binding Buffer (RBB) to each well with sample.
6. Add 5 µL of room temperature, vortexed rRNA Removal Mix- Gold (RRM G).
    a. Pipette up and down minimum 6 times to mix.
    b. Quickly spin down.
    c. Avoid excessive foaming, as this may reduce depletion efficiency.
7. Place sealed PCR plate/strip in a thermal cycler and incubate with program "RNA-denature" as follows:
    a. 68°C for 5 min
    b. Heated lid: 100°C.
8. Quick spin, then incubate the plate/strip with the samples at room temperature for a minimum of 1 min.
9. Vortex room temperature rRNA Removal Beads (RRB) to completely re-suspend the beads.
10. In a new 1.5 ml microfuge tube, add 35 µL of rRNA Removal Beads (RRB) for each sample.
11. Add entire volume of the sample (20 µL) to the 1.5ml microfuge tube containing rRNA Removal Beads.
    a. Pipette up and down quickly 20 times to mix the contents (set pipette to 45 µL), tap down any droplets on side of tube.
    b. Add sample to beads (**not** beads to sample).
12. Incubate the samples at room temperature for 1 min.
13. Place the microfuge tube on the magnetic stand and incubate for 1 min.
14. Transfer supernatant from each microfuge tube into a new 1.5ml microfuge tube.
15. Place the 1.5 ml microfuge tube on the magnetic stand for 1 min and verify that no beads remain.
16. Repeat step 13 if there are any beads remaining in the 1.5ml microfuge tube. Residual beads are a source of ribosomal contamination.

<u>Clean up</u>

17. Thaw the following reagents and keep on ice/ 4°C:

a. Elute, Prime, Fragment High Mix (EPF)
b. First Strand Synthesis Act D Mix (FSA)

18. Vortex Agencourt RNA Clean XP beads until they are well dispersed and add to each sample (depleted RNA should be in a 1.5ml microfuge tube):
    a. 193 µL of beads (for FFPE samples DV200<65) **OR;**
    b. 99 µL of beads (for non-degraded total RNA, DV200>65).
    c. Gently pipette up and down 10 times to mix.
19. Incubate samples at room temperature for 15 min.
20. Place the samples on the magnetic stand for ~5 min then remove the supernatant.
    a. CRITICAL: Use a 10 µL pipette tip to remove all residual supernatant before adding ethanol wash.
21. With the samples on the magnetic stand, add 200 µL of 70% EtOH to the samples without disturbing the beads.
22. Incubate samples for 30 seconds and then remove and discard supernatant.
23. Dry the beads at room temperature for 15 min, or until ethanol has evaporated and the beads have a matte appearance.
24. Add 11 µL of Elution Buffer (ELB) to each sample, soaking the dried beads. Mix by pipetting the entire volume up and down 10 times.
    a. Visually confirm bead resuspension.
25. Incubate samples for 2 min at room temperature.
26. Place tubes on magnetic stand at room temperature for 5 min. Transfer 8.5 µL of supernatant from each tube to a new PCR plate/strip.
27. Add 8.5 µL of Elute, Prime, Fragment High Mix (EPF) to each sample. Mix by pipetting up and down the entire volume 10 times, and briefly centrifuge.
28. Place the PCR plate/strip in a thermal cycler and incubate with program "Frag-Prime." Incubation times are variable, based on the quality of the RNA. Consult the table below to determine the length of fragmentation required:

Frag-Prime Program (with heated lid: 100 °C)

94°C	**min (see table below)
4°C	Hold

Sample Quality Measure (DV200)	**Fragmentation Time
DV200<55	No Fragmentation (0 min)
55<DV200<65	4 min
DV200>65	8 min

29. Record fragmentation time on sample tracking sheet.
30. Remove the PCR plate/strip with the samples and centrifuge briefly.

2. First Strand cDNA Synthesis

1. Thaw First Strand Synthesis Act D Mix (FSA) and bring to room temperature.
2. When opening a new box, make a master mix of SuperScript II and First Strand Synthesis Act D (FSA):
    a. 90 µL First Strand Synthesis Act D Mix (FSA) + 10 µL SuperScript II
    b. This is sufficient for 12.5 reactions – DO NOT make smaller batches.
    c. Minimize freeze/thaws of mix, and track by indicating with a dot on the top of the master mix tube.
3. Add 8 µL from the FSA + SSII master mix to each well of the PCR plate/strip containing the samples (17 µL from previous step, as shown below).
4. Gently pipette the entire volume (25 µL) up and down 6 times, then centrifuge briefly.

First Strand Synthesis PCR Mix, **1x**	
rRNA-depleted RNA sample	17 µL
FSA + SSII mix	8 µL
Total Reaction	**25 µL**

5. Place the PCR plate/strip in a thermal cycler and incubate with program "1st strand" as follows:
   1st Strand Program (with heated lid: 100 °C)

25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

6. Proceed immediately to next step.

3. Second Strand cDNA Synthesis

1. Thaw and keep on ice/4°C fridge:
    a. Second Strand Marking Master Mix (SMM)
    b. Resuspension Buffer (RSB)
2. Preheat thermal cycler to 16°C (pre-heat lid set to 30°C), program "2nd Strand"
3. Add 5 µL of Resuspension Buffer (RSB) and 20 µL of Second Strand Marking Mix (SMM) to each sample (25 µL from previous step, as shown below).
4. Gently pipette the entire volume (50 µL) up and down 6 times, centrifuge briefly.

Second Strand Synthesis PCR Mix, 1x	
Resuspension Buffer (RSB)	5 µL

Second Strand Marking Master Mix (SMM)	20 µL
First strand cDNA sample	25 µL
Total Reaction	**50 µL**

5. Place the PCR plate/strip on the preheated thermal cycler and incubate at **16°C for 1 hour**.
6. Remove the PCR plate/strip from the thermal cycler and bring to room temperature for 5 min.

Clean up

7. Vortex AMPure XP (or NucleoMag) beads until re-suspended and then add 90 µL to each sample.
8. Gently pipette the entire volume (140 µL) up and down 10 times.
9. Incubate at room temperature for 15 min.
10. Place the PCR plate/strip on a magnetic stand for 5 min then remove and discard 135 µL of the supernatant.
    a. CRITICAL: Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash.
11. Keep the microfuge tubes on the stand. Add 200 µL of fresh 80% EtOH without disturbing the beads.
12. Incubate for 30 seconds at room temperature. Remove and discard supernatant from each well.
13. Repeat steps 11 and 12 for a total of two 80% EtOH washes.
14. Keeping the PCR plate/strip on the magnetic stand, dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
15. Add 17.5 µL RSB to each sample well before removing from magnetic stand.
16. Remove strip from stand and gently pipette the entire volume up and down 10 times.
    a. Visually confirm that beads are fully re-suspended after drying.
17. Incubate samples at room temperature for 2 min.
18. Place PCR plate/strips on the magnetic stand for 5 min.
19. Transfer 15 µL of supernatant from each well to a corresponding well in a new PCR plate/strip.
20. SAFE STOP POINT. May store at -20 °C for up to 7 days.

4. Adenylate 3' Ends

1. Thaw the following and keep on ice/4°C fridge:
    a. A Tailing Mix (ATL)
    b. Resuspension Buffer (RSB)
    c. TruSeq RNA CD Index Plate (96 plex kit)
    d. Stop Ligation Buffer (STL)

e. Ligation Mix (LIG)

2. Remove AMPure XP (or NucleoMag) Beads from storage and keep them at room temperature for 30 min before use.
3. Add 2.5 µL of RSB and 12.5 µL of A-Tailing Mix to each the 15 µL of cDNA sample, and briefly centrifuge.

3' Adenylation Reaction, 1x	
Resuspension Buffer (RSB)	2.5 µL
A-tailing Mix (ATL)	12.5 µL
cDNA	15 µL
Total Reaction	**30 µL**

4. Place the PCR plate/strip in a thermal cycler using program "ATAIL70"and incubate as follows:

ATAIL70 Program (with heated lid: 100 °C)

37°C	30 min
70°C	5 min
4°C	Hold

5. Proceed immediately to next step.

5. Ligate Adapters

NOTE: **Ensure Library indices assigned are coordinated to avoid index collisions with other batches**

1. Select indices based on pooling guidelines.
2. Timing and order of reagent additions is important.
   a. Work quickly after addition of adapter.
   b. Accurate incubation timing and prompt addition of stop ligation buffer (STL) is critical to ensure low adapter contamination of final product library.
3. Preheat thermal cycler to 30 °C using program "LIG"

LIG Program (with heated lid: 100 °C)

30°C	10 min

4. Add 2.5 µL of RSB and 2.5 µL of Ligation Mix (LIG) to each 30 µL sample from the last step.

5. Add 2.5 µL of the appropriate RNA Adapter Index to each well of the PCR plate/strip with samples.
6. Gently pipette the entire volume (37.5 µL) up and down 10 times, and briefly centrifuge.

Ligation Reaction, 1x	
Resuspension Buffer (RSB)	2.5 µL
Ligation Mix (LIG)	2.5 µL
RNA Adapter Index	2.5 µL
3'-adenylated cDNA	30 µL
Total Reaction	**37.5 µL**

7. Place the PCR plate/strips in the preheated thermal cycler. Incubate at 30°C for 10 min using the LIG program (listed above).
8. After 10 min, add 5 µL of Stop Ligation Buffer (STL) to each sample.
9. Gently pipette the entire volume (42.5 µL) up and down 10 times, and briefly centrifuge.

Clean Up

10. Vortex AMPure XP (or NucleoMag) beads until re-suspended and then add 42 µL of beads to each sample.
11. Gently pipette the entire volume (84.5 µL) up and down 10 times.
12. Incubate at room temperature for 15 min.
13. Place the PCR plate/strip on a magnetic stand at room temperature for 5 min, then discard 79.5 µL of supernatant per well.
    a. CRITICAL: Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash.
14. Keeping the PCR plate/strip on the magnetic stand, add 200 µL of fresh 80% EtOH without disturbing the beads.
15. Incubate at room temperature for 30 seconds then discard the supernatant.
16. Repeat Steps 14 and 15 for a total of two 80% EtOH washes.
17. Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
18. Add 52.5 µL RSB to each well prior to removing from magnetic stand, then gently pipette the entire volume up and down 10 times.
    a. Visually confirm bead resuspension.
19. Incubate at room temperature for 2 min.
20. Place the PCR plate/strips on a magnetic stand for 5 min.
21. Transfer 50 µL supernatant to a new PCR plate/tube.

22. Add 50 µL of mixed AMPure XP (or NucleoMag) beads to each sample. Gently pipette the entire volume (100 µL) up and down 10 times.
23. Incubate at room temperature for 15 min.
24. Place the PCR plate/strip on a magnetic stand at room temperature for 5 min.
25. Remove and discard 95 µL supernatant from each well.
    a. CRITICAL: Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash.
26. Keeping the PCR plate/strip on the magnetic stand, add 200 µL of fresh 80% EtOH without disturbing the beads.
27. Incubate at room temperature for 30 seconds then discard the supernatant.
28. Repeat Steps 26 and 27 for a total of two 80% EtOH washes.
29. Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
30. Add 22.5 µL RSB to each well before removing from magnetic stand. Remove strip/plate from magnetic rack and gently pipette the entire volume up and down 10 times.
    a. Visually confirm bead resuspension.
31. Incubate at room temperature for 2 min.
32. Place the PCR plate/strips on a magnetic stand for 5 min or until liquid is clear.
33. Transfer 20 µL of supernatant from each well to a new PCR plate/strip tube.
34. SAFE STOP POINT. Place in -20 °C.

6. Library Amplification

1. Thaw the following and keep on ice/4°C fridge:
    a. PCR Master Mix (PMM)
    b. PCR Primer Cocktail (PPC)
2. Bring the following to room temperature:
    a. Resuspension Buffer (RSB)
    b. AMPure XP or NucleoMag beads (keep at room temperature for 30 min before use)
3. Add 5 µL of PCR Primer Cocktail (PPC) and 25 µL of PCR Master Mix (PMM) to each 20 µL sample from the last step.
4. Pipette up and down to mix, and briefly centrifuge.

Adapter-ligated cDNA PCR reaction, 1x	
PCR Primer Cocktail (PPC)	5 µL
PCR Master Mix (PMM)	25 µL
Adapter-ligated cDNA	20 µL
Total Reaction	**50 µL**

5. Place the PCR plate/strip in a thermal cycler using program "PCR" and incubate as follows:

PCR Program (with heated lid: 100 °C)

98°C	30 sec	1 Cycle
98°C	10 sec	15 Cycles
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1 Cycle
4°C	Hold	

Clean up

6. Note that Ampure XP (or NucleoMag) volumes vary between dual index and single index adapters.
7. Vortex beads until well dispersed.
8. Add 47.5 µL of beads to each sample.
9. Gently pipette the entire volume (100 µL) up and down 10 times.
10. Incubate at room temperature for 15 min.
11. Place the PCR plate/strip on a magnetic stand for 5 min then discard 95 µL of supernatant.
    a. CRITICAL: Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash.
12. Add 200 µL of freshly prepared 80% EtOH to each well, incubate for 30sec, then remove and discard supernatant.
13. Repeat step 12 for a total of two 80% EtOH washes.
14. Dry the beads at room temperature for 15 min, or until ethanol has evaporated (beads almost cracking).
15. Add 32.5 µL of RSB to each sample prior to removing from magnetic rack.
16. Remove samples from rack and gently pipette up and down 10 times to mix.
    a. Visually confirm resuspension.
17. Incubate at room temperature for 2 min.
18. Place the PCR plate/strip on a magnetic stand for 5 min.
19. Transfer 30 µL of supernatant to a labelled Matrix tube.

7. Assess Quality and Quantity of Library

1. Use Qubit HS dsDNA assay to quantify the cDNA library, following the TM. Genomics Qubit Fluorometric Quantitation SOP, located on the Genomics Quality SharePoint.

2. Use High Sensitivity D1000 screen tape and record average library size distribution, setting the region to ~190bp- 1000bp, following the TM. High Sensitivity TapeStation Assay SOP, located on the Genomics Quality SharePoint. The average library size will be used to adjust library concentration and is integrated into MISO library identities. If RT-qPCR quantification is utilized for RUO NextSeq pooled runs, RT-qPCR values are corrected by average library insert size. Record TapeStation file ID in sample tracking sheet.
3. Look for adapter contamination peaks at a size of approximately 130- 140 bp and record % adapter contaminant in sample tracking sheet (see section 9 Appendix, (2) adapter contamination for example). **Do not** proceed to RT-qPCR, normalization or library pooling if the samples are heavily contaminated by adapters (>10%). This may indicate a poor-quality library.

Important note: Please record QC observations in the Qubit/TapeStation verification logs located in each instrument's lab binder

8. LIMS Entries

1. For general MISO instruction and training, see TM. LIMS Usage - MISO.
2. Prior to library preparation, the DV200 % must be determined and recorded for total RNA samples aliquots.
    a. **DV200 CUT OFF IS 20%. Any RNA sample less than 20% DV200 will not be accepted for library preparation.**
    b. This value is also used to select fragmentation time.
    c. The TapeStation or Fragment Analyzer may be used to determine the DV200 value.
    d. DV200 values are added at the sample aliquot level in MISO, following the steps outlined below:
        i. Select all sample aliquots in the same batch
        ii. Select Add QCs: In the pop up window, enter 1 for QCs per Sample (DV200%) and 0 for Controls per QC
        iii. Fill in the Date and for the type select %DV200 in the results section
        iv. Please note: DV200 must be entered in every new sample aliquot received
        v. When all QC metrics have been entered, review to ensure all QC values are paired to the correct sample aliquot, and then Save
3. Propagate one or more MISO Libraries from their respective whole RNA Aliquots. If using the MISO bulk edit function, ensure that the order of samples has not been changed in MISO when propagating samples. Enter the following information for each Library:
    - Matrix Barcode: Use barcode scanner while column is selected
    - Box Alias and Position: Use Box Search column to find correct Box Alias
    - Creation Date: Defaults to current date, but set to date of library creation
    - SOP: Set to WT Library Prep – Illumina TruSeq v.X (see version at the footer of SOP)

- Thermal Cycler
- Design: Set to WT
    - Design tells analysis pipeline to do alignment against a whole transcriptome
- Platform: Set to Illumina
- Type: Set to Paired End
    - Type tells analysis pipeline to demultiplex one or two reads
- Index Kit: Set to TruSeq CD Indexes
- Index 1: Set to the index used for the library. Will autofill Index 2
    - Index is used by the analysis pipeline to demultiplex reads
- Kit: Set to TruSeq_Stranded_TotalRNA
- Kit Lot: The lot number of the TruSeq_Stranded_TotalRNA Kit
- QC Passed: See Library Quality Assessment for criteria
- Size (bp): Determined during Library Quality Assessment
- Volume: Set to 28 uL, unless elution volume was less than expected
    - Elution volume is 30 uL, but 2 uL are used for QC
- Concentration Determined during Library Quality Assessment
- GroupID: Do not enter a groupID unless you are certain that it is required (e.g. for re-validation). Group ID is inherited and has been set during sample accessioning.
    - GroupID is used by the analysis pipeline to determine which samples/libraries belong together, e.g. to determine which entries are top-ups: https://wiki.oicr.on.ca/x/6ARdBw
    - WGS and WT GroupIDs must match for analysis to proceed
- Prior to saving Libraries check that all information is correct and that the MISO entry matches LIMs tracking sheet

4. Propagate Library Aliquot. Prepare a 20uL aliquot at 5 ng/ul of the stock library and propagate a matching MISO Library Aliquot from the MISO Library:
    - Matrix Barcode: Use barcode scanner while column is selected
    - Box Alias and Position: Use Box Search column to find correct Box Alias
    - QC Passed: Set to True
    - Conc. 5 ng/uL
    - Volume: 20uL
    - Parent ng Used: Record the amount of stock library used to create the dilution
    - Parent Vol. Used: Record the volume (uL) of stock library used to create the dilution
    - GroupID: Do not enter a groupID unless you are certain that it is required (e.g. for re-validation). Group ID is inherited and has been set during sample accessioning.
        - GroupID is used by the analysis pipeline to determine which samples/libraries belong together, e.g. to determine which entries are top-ups: https://wiki.oicr.on.ca/x/6ARdBw
        - WGS and WT GroupIDs must match for analysis to proceed

- Creation Date: Defaults to current date, but set to date of dilution/aliquot creation
- Prior to saving Libraries, check that all information is correct and that the MISO entry matches LIMs tracking sheet

5. Add Library QC to all stock libraries that were prepared during the same library synthesis batch:
    a. Select all stock libraries prepared together in the same batch
    b. Select Add QCs: In the pop-up window,
        i. Enter 2 QCs per Library: Average Library Size (TapeStation or Fragment Analyzer) and Concentration (Qubit)
        ii. Select the name of the instrument used for each QC
        iii. Add 2 Controls per QC (Positive control, No Template Control)
    c. Fill in the two QC areas per Library ensuring each individual Library has both QC values entered
    d. Control 1: Assign as positive control with the lot number of the control used and indicate if the control passes
    e. Control 2: Assign as negative control, enter the lot number of nuclease-free water used and indicate if control passes
    f. When all QC metrics have been entered, review to ensure all QC values are paired to the correct Library, and then Save
6. Attach batch QC files to all stock libraries that were prepared during the same library synthesis batch:
    a. Select all stock libraries prepared together in the same batch
    b. Select Attach Files: Upload New Files
    c. Category:
        i. LIMS tracking sheet for scanned batch worksheet
        ii. Misc for Qubit csv file
        iii. TapeStation for run report
    d. Choose files: Attach the appropriate file corresponding to the category previously selected. Only one file can be uploaded at a time
    e. Upload
    f. Repeat previous steps until all three Batch QC files have been uploaded
7. After all QC has been entered and the appropriate files have been uploaded, Library aliquots can now be passed to GRP sequencing by following these steps:
    a. Move the Library aliquot tubes into the GRP "CAP Miseq Inbox" BOX1580 and update the locations in MISO
    b. Add the LDI for every sample into the Workset- GRP MiSeq QC Queue (For CAP-stream NovaSeq 2x101) in MISO.
    c. Send an email to GenomicsLibrarySubmissions@oicr.on.ca with the LDI's that were added to the Workset to alert the sequencing team that there are CAP libraries pending sequencing.

Version History

Version	Description of Changes
7.2	• Change log introduced to document 2025-03-05 • Updated section 8 “LIMS Entries” subpoint 5 (adding QC) to include entering 2 QCs per library (Average Library Size (TapeStation or FA) and Concentration(Qubit)) as well as selecting the name of the instrument used • Added a note under section 7 “Assess Quality and Quantity of Library” reminding users to record observations in the appropriate lab binder