SOP Title:

Illumina TruSeq Stranded Total RNA Library Prep Gold

1.0 Purpose and Scope

This standard operating procedure (SOP) is for the preparation of Illumina TruSeq Stranded Total RNA Library Prep Gold (48 or 96 samples) libraries from FFPE and fresh frozen samples at PM-OICR TGL 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: the removal of ribosomal RNA (rRNA), first and second strand cDNA synthesis, adenylation of 3' ends, ligation of adapters, PCR amplification, and library validation. The procedure requires 200ng of FFPE or fresh frozen total RNA.

Related document:

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

This SOP follows instructions provided by the manufacturer with minor modifications.

Related TGL documents:

- YYYY_MM_DD_SAMPLE SUBMISSION FORM_PI_Lastname_Firstname_TGL
- SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.doc
- 17_06_16_TruSeq_Stranded_Total_RNA_Lot_Tracking_Sheet
- SOP_TapeStation_4200_V1.0
- SOP_KAPA_Library_Quantification_Illumina_Platforms_V1.0.2_production
- truseq-library-prep-pooling-guide-15042173-01
- truseq-stranded-total-rna-sample-prep-ls-euc-ltf-15031060-e (lot control sheet)

2.0 Materials

Workspace		
Library prep room for all the steps prior to enrichment PCR amplification		
Post-PCR area for enrichment PCR amplification and all the subsequent steps		
Equipment and associated consumables		
Agilent	Agilent TapeStation 4200	
	High Sensitivity RNA ScreenTape (7 tapes,112 samples)	5067-5579
	High Sensitivity RNA ScreenTape Sample Buffer	5067-5580
	High Sensitivity RNA ScreenTape Ladder	5067-5581
	High Sensitivity D1000 Screen Tape (7 tapes,112 samples)	5067-5584
	High Sensitivity D1000 Reagents	5067-5585
	Plate Foil Seal	5067-5154
	96 Well plates, 150ul, conical, 25/pk	5042-8502
	Mx3000P Optical Strip Caps	401425
	Mx3000P Strip Tubes	401428
Eppendorf	Vacufuge Plus	022820001
Various	Thermal cycler (i.e. Applied Biosystems Veriti, BioRad T100, etc)	
Medstore	PCR STRIP(8), 0.2ml NEUTRAL, ATT FLAT CAP,120/pk	72.991.002
ThermoFisher	Dynamag (magnetic rack)	12321D
	Dynamag-96 (96 well magnetic rack)	123331D

Reagent kits		
Illumina (96 plex)	TruSeq Stranded Total RNA Library Prep Gold (96 Samples)	20020599
	TruSeq RNA CD Index Plate (96 Indexes, 96 Samples)	20019792 (order separately)
Illumina (48 plex)	**OR:** TruSeq Stranded Total RNA Library Prep Gold (TruSeq Strnd Ttl RNA LPGold 48 Smpl, Component 15032619)	20020598
	TruSeq Strnd Ttl RNA LP(48 Smpl) (Component 15032615)	
	TruSeq Strnd RNA Core LPBox 2 (Component 15032611)	
	TruSeq Stranded RNA LT Kit- 48 Samples Index Set A (Component 15032612), 6bp index	20020492 (order separately)

	TruSeq Stranded RNA LT Kit- 48 Samples Index Set B (Component 15032613), 6bp index	20020493 (order separately)
Medstore	SuperScript II Reverse Transcriptase (10000units, 200units/µL)	18064014
Beckman/Cedarlane	AMPure XP beads	A36881 (60 ml)
	Agencourt RNA Clean XP	A63987 (40 mls)
ThermoFisher	Qubit dsDNA HS Assay Kit	Q32854
	Qubit Assay Tubes	Q32856
Commonly used reagents		
Medstore	Sigma Nuclease-free water	W4502-1L
	Axygen Snaplock Centrifuge Microtubes (clear), 1.5mL, 250/pk (10 pks/case)	MCT-150-L-C
Medstore/Greenfield Specialty Alcohols	Ethanol anhydrous 100% (brown bottle), case of 12X500ml	P006EAAN
Medstore/Greenfield Specialty Alcohols	Ethanol anhydrous (4X4L white jug, cleaning only!)	P016EAAN

2.0 General Guidance:

- First Strand Synthesis Act D Mix (FSA) contains actinomycin D. Avoid inhalation, ingestion, skin and eye contact. Wear appropriate safety protection.
- Purified Total RNA must always be stored at -80°C.
- When kits are received, inspect kit component boxes 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. If at any time these beads become clumpy, or are frozen, discontinue use.
- When opening a new kit, record all lot numbers in TGL master lot tracking sheet (R:\Lab_tracking_forms\truseq-stranded-total-rna-sample-prep-ls-euc-ltf-15031060-e, associate all samples to the master lot tracking sheet using a letter key (“A”, “B”) in sample tracking sheet. Visit Illumina’s website to download and record all lot information using kit specific RGT#s. An illumina.com account is required. On illumina.com, under the customer number in the website menu, click on Order Management> My Tools> Product Lot Tracker, select box serial #, and enter RGTXXXXXXX. Upon kit receipt, label each kit subcomponent box with a letter (ie “A>Z) and store in respective fridge/freezers in a Ziplock bag to prevent mixing and matching of lots/shipments.
- All RNA seq libraries will undergo multiplex MiSeq Nano library validation using dual index sequencing. Follow Illumina’s TruSeq Sample Preparation Pooling Guide. Illumina does not recommend freeze-thawing the adapters more than 4X. Thaw the adapter plate at room temperature for 10 minutes and visually inspect that all wells are thawed, centrifuge at

1000RPM for 1 minute. 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. Use the next available sequence of adapters, thawing 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 the indexing strategy outlined in the figures below. Each row is a unique 500 series (I5) adapter from 501-508, each column is a unique 700 series (I7) adapter (figure 1). When building libraries, combine multiple rows of indexes to ensure each library within a pool will have a unique dual index. For example, each NextSeq550 run will be comprised of 5 libraries. In figure 2, select a block of 5 indexes for 5 libraries (highlighted in grey with green wells). Do not use indicies in columns 706, or 712, as they are suboptimal for 5-plex pools. Each 5-plex dual index pool will be combined into a maximum 80 plex pool for validation on the MiSeq.

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

Figure 1: TruSeq RNA CD Index Plate

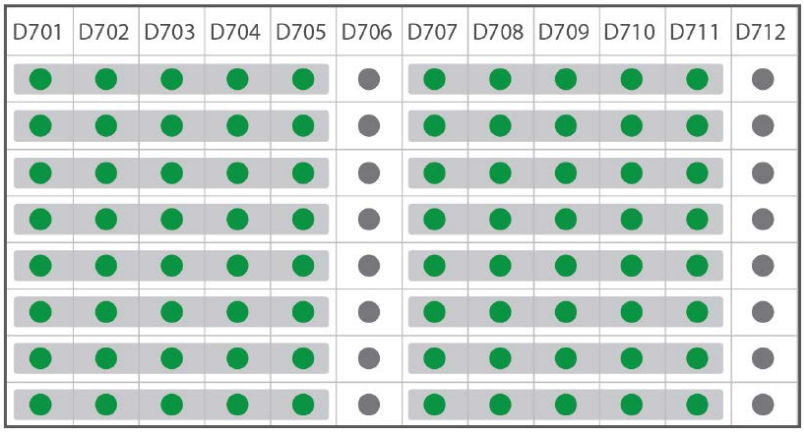


Figure 2: TruSeq RNA CD Index Plate Optimized 5-plex NextSeq550 Pools

- Before beginning work every day, wipe down all pipettes, bench surfaces and centrifuge interiors with peroxide wipes, then wipe with 70% ethanol (made from 4L white jug 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. Please note 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 beads must be allowed to reach room temperature before use; 30 mins 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, do not place on ice!
- 70% and 80% ethanol wash solutions should be made fresh every day using molecular grade H_20 and anhydrous ethanol (brown bottle only!). Always use personal ethanol and H_20 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 magnetic rack when washing, 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 minutes of dry time is an estimate. Depending on humidity, dry time may be as short as 7 minutes.
- 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 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, of "jumping" from the tube electrostatically.
- Record RINS, DV200 scores, quants, dilutions, thermal cycler used, and other QC information in individual sample sheets.

- 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 additions (minifuge).
- Record all QC steps in original sample sheet, including MISO LIMS IDs, LIB and LDI #s, master lot tracking references, protocol version, tech performing prep/QC step in sample sheet, thermocycler(s) used for reaction(s)
- Note: Use RNA Clean XP with 70% ethanol and 80% ethanol with AMPure XP 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 in sample sheet

4.0 Procedure

Qubit material to verify concentration using Qubit HS RNA Assay kit and following SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx. If sample concentration is excessively high, dilute stock solution and re-qubit to a reasonable concentration.

Run Agilent High Sensitivity RNA Tapestation and record DV200 and RIN. Use appropriate concentration. Do not go outside of recommended tape sample concentration (0.5-10 ng/µl), or RIN/DV200 will not be accurate. Use these results to determine fragmentation times for RNA samples (see 9.0 Appendix).

PRE-PCR AREA

1. Ribo-Zero Deplete and Fragment RNA

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

Elute, Prime, Fragment High Mix (EPH)
Resuspension Buffer (RSB) (use an aliquot)

Bring the following reagents to room temperature before use (allow 30 minutes):

Elution Buffer (ELB)
rRNA Binding Buffer (RBB)
rRNA Removal Beads (RRB)
rRNA Removal Mix-Gold (RRM G)
Agencourt RNA Clean XP beads (Ribo-Zero Deplete and Fragment RNA stage only, use 70%EtOH)
AMPure XP beads (Required for purifications after second strand cDNA synthesis, use 80% EtOH)

Prepare fresh 70% and 80% EtOH using nuclease-free water and anhydrous ethanol.

1) Dilute 200 ng of total RNA to a final volume of 10 µL with nuclease-free water in PCR plate or strip. If required, reduce volume using a Vacufuge with a max temperature setting of 30°C; do not completely dry RNA aliquot. Add RSB to bring the total volume to 10 µL prior to next step.
2) Add 5 µL of room temperature, vortexed rRNA Binding Buffer (RBB) to each well with sample.
3) Add 5 µL of room temperature, vortexed rRNA Removal Mix- Gold (RRM G). Pipette up and down 6 times to mix. Quick spin. Avoid excessive foaming, as this may reduce depletion efficiency.
4) Place sealed PCR plate/strip in a thermal cycler and incubate with program "RNA-denature" as follows:
   68°C for 5 min
   Heated lid: 100°C.
5) Quick spin, then incubate the plate/strip with the samples at room temperature for 1 minute.

6) Vortex room temperature rRNA Removal Beads (RRB) to completely re-suspend the beads.
7) In a new 1.5 ml microfuge tube, add 35 µL of rRNA Removal Beads (RRB) for each sample.
8) Add entire volume of the sample (20 µL) to the 1.5ml microfuge tube containing rRNA Removal Beads. Pipette up and down quickly 20 times to mix the contents (set pipette to 45 µL), tap down any droplets on side of tube.
   NB: add sample to beads (not beads to sample) order is important!
9) Incubate the samples at room temperature for 1 minute.
10) Place the microfuge tube on the magnetic stand and incubate for 1 minute.
11) Transfer supernatant from each microfuge tube into a new 1.5ml microfuge tube.
12) Place the 1.5 ml microfuge tube on the magnetic stand for 1 minute and verify no beads remain.
13) Repeat step 10 if there are any beads remaining in the 1.5ml microfuge tube. Residual beads are a source of ribosomal contamination.

Clean up

Thaw the following reagents and keep on ice/ 4°C:
Elute, Prime, Fragment High Mix (EPF)
First Strand Synthesis Act D Mix (FSA)

14) 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):
    - 193 µL of beads (for FFPE samples DV200<65), **or**
    - 99 µL of beads (for non-degraded total RNA, DV200>65).

    Gently pipette up and down 10 times to mix.
15) Incubate samples at room temperature for 15 minutes.
16) Place the samples on the magnetic stand for ~5 minutes then remove the supernatant. Use a 10 µL pipette tip to remove all residual supernatant before adding ethanol wash (critical step)!
17) With the samples on the magnetic stand, add 200 µL of 70% EtOH to the samples without disturbing the beads.
18) Incubate samples for 30 seconds and then remove and discard supernatant.
19) Dry the beads at room temperature for 15 minutes, or until ethanol has evaporated (beads almost cracking).
20) 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. Visually confirm bead resuspension!
21) Incubate samples for 2 minutes at room temperature.
22) Place tubes on magnetic stand at room temperature for 5 minutes. Transfer 8.5 µL of supernatant from each tube to a new PCR plate/strip.
23) 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, briefly centrifuge.

Skip steps 24 if RNA is heavily degraded.

24) Place the PCR plate/strip in a thermal cycler and incubate with program "Frag-Prime" as follows:
94°C for 0-8 min; 4°C HOLD**
Heated lid: 100 °C.
** variable time: DV200<55, 0 or no fragmentation, 55<DV200<65 4 minute fragmentation, DV200>65, 8 minute fragmentation. Refer to 9.0 appendix (1) RNA Fragmentation Times of this document for reference traces. Record fragmentation times in sample tracking sheets!
25) Remove the PCR plate/strip with the samples and centrifuge briefly.

2. First Strand cDNA Synthesis

Thaw First Strand Synthesis Act D Mix (FSA) and bring to room temperature.

1) When opening a new box, make a master mix of SuperScript II and First Strand Synthesis Act D (FSA)
   - 90 µL First Strand Synthesis Act D Mix (FSA) + 10 µL SuperScript II
   - This is sufficient for 12.5 reactions, never make smaller batches! Minimize freeze/thaws of mix, and track by indicating with a dot on the top of the master mix tube.

   Add 8 µL from the FSA + SSII master mix to each well of the PCR plate/strip containing the samples. 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
FSA + SSII mix	8
Total Reaction→	25

2) Place the PCR plate/strip in a thermal cycler and incubate with program "1st strand" as follows:

   25°C for 10 min; 42°C for 15 min; 70°C for 15 min; 4°C HOLD.
   Heated lid: 100°C; **proceed immediately to next step.**

3. Second Strand cDNA Synthesis

Thaw and keep on ice/4°C fridge:
Second Strand Marking Master Mix (SMM)
Resuspension Buffer (RSB)

Preheat thermal cycler to 16°C (pre-heat lid set to 30°C), program "2nd Strand"

1) Add 5 µL of Resuspension Buffer (RSB) and 20 µL of Second Strand Marking Mix (SMM) to each sample. Gently pipette the entire volume (50 µL) up and down 6 times, centrifuge briefly.
2) Place the PCR plate/strip on the preheated thermal cycler and incubate at **16ºC for 1 hour**.
3) Remove the PCR plate/strip from the thermal cycler and bring to room temperature for 5 minutes.

Second Strand Synthesis PCR Mix	**1x**
Resuspension Buffer (RSB)	5
Second Strand Marking Master Mix (SMM)	20
First strand cDNA sample	25
Total Reaction→	50

Clean up

4) Vortex AMPure XP beads until re-suspended and then add 90 µL to each sample. Gently pipette the entire volume (140 µL) up and down 10 times, and transfer to new 1.5mL microfuge tubes.
5) Incubate at room temperature for 15 minutes.
6) Place the PCR plate/strip on a magnetic stand for 5 minutes then remove and discard 135 µL of the supernatant. Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash (critical step)!
7) Keep the microfuge tubes on the stand. Add 200 µL of fresh 80% EtOH without disturbing the beads.
8) Incubate for 30 seconds at room temperature. Remove and discard supernatant from each well.
9) Repeat steps 7 and 8 for a total of two 80% EtOH washes.
10) 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).
11) Add 17.5 µL RSB to each sample well before removing from magnetic stand. Remove strip from stand and gently pipette the entire volume up and down 10 times (visually confirm beads fully re-suspended after drying!).
12) Incubate samples at room temperature for 2 mins.
13) Place PCR plate/strips on the magnetic stand for 5 mins. Transfer 15 µL of supernatant from each well to a corresponding well in a new PCR plate/strip.

SAFE STOP POINT. May store at -20 ºC for up to 7 days, preferable to continue protocol.

4. Adenylate 3' Ends

Thaw the following and keep on ice/4°C fridge:

A Tailing Mix (ATL)
Resuspension Buffer (RSB)
RNA Adapter Indices (48 plex kit), or TruSeq HT adapter plate (96 plex kit)
Stop Ligation Buffer (STL)
Ligation Mix (LIG)

Remove AMPure XP Beads from storage and allow 30 minutes to come to room temperature.

1) Add 2.5 µL of RSB and 12.5 µL of A-Tailing Mix to each sample, briefly centrifuge.
2) Place the PCR plate/strip in a thermal cycler using program "ATAIL70"and incubate as follows:
   37 °C for 30 min; 70 °C for 5 min 4 °C HOLD
   Heated lid: 100 °C. **Proceed immediately to next step.**

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

5. <u>Ligate Adapters</u>

Select indices based on pooling guidelines in general guidance section of this protocol when using the 96 plex kit and TruSeq RNA CD Index Plate. If using the 48 plex kit follow Illumina recommended combinations (truseq-library-prep-pooling-guide). Timing and order of reagent additions is important. Work quickly after addition of adapter, accurate incubation timing and prompt addition of stop ligation buffer (STL) is critical to low adapter contamination of final product library.

Preheat thermal cycler to 30 °C using program "LIG"

Heated lid: 100 °C

1) Add 2.5 µL of RSB and 2.5 µL of Ligation Mix (LIG) to each sample.
2) Add 2.5 µL of the appropriate RNA Adapter Index to each well of the PCR plate/strip with samples. Gently pipette the entire volume (37.5 µL) up and down 10 times, briefly centrifuge.
3) Place the PCR plate/strips in the preheated thermal cycler. Incubate at:
   30 °C for 10 minutes
   Heated lid: 100 °C.
4) Add 5 µL of Stop Ligation Buffer (STL) to each sample. Gently pipette the entire volume (42.5 µL) up and down 10 times, briefly centrifuge.

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

<u>Clean up</u>

5) Vortex AMPure XP beads until re-suspended and then add 42 µL of AMPure XP beads to each sample. Gently pipette the entire volume (84.5 µL) up and down 10 times.
6) Incubate at room temperature for 15 mins.
7) Place the PCR plate/strip on a magnetic stand at room temperature for 5 mins then discard 79.5 µL of supernatant per well. Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash (critical step)!
8) Keeping the PCR plate/strip on the magnetic stand, add 200 µL of fresh 80% EtOH without disturbing the beads.
9) Incubate at room temperature for 30 seconds then discard the supernatant.
10) Repeat Steps 8 and 9 for a total of two 80% EtOH washes.
11) Dry the beads at room temperature for 15 mins, or until ethanol has evaporated (beads almost cracking).
12) 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. Visually confirm bead resuspension.
13) Incubate at room temperature for 2 mins.
14) Place the PCR plate/strips on a magnetic stand for 5 mins.
15) Transfer 50 µL supernatant to a new PCR plate/tube.
16) Add 50 µL of mixed AMPure XP beads to each sample. Gently pipette the entire volume (100 µL) up and down 10 times.
17) Incubate at room temperature for 15 minutes.
18) Place the PCR plate/strip on a magnetic stand at room temperature for 5 mins.
19) Remove and discard 95 µL supernatant from each well. Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash (critical step)!
20) Keeping the PCR plate/strip on the magnetic stand, add 200 µL of fresh 80% EtOH without disturbing the beads.
21) Incubate at room temperature for 30 seconds then discard the supernatant.
22) Repeat Steps 20 and 21 for a total of two 80% EtOH washes.
23) Dry the beads at room temperature for 15 mins, or until ethanol has evaporated (beads almost cracking).
24) 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. Visually confirm bead resuspension.
25) Incubate at room temperature for 2 minutes.
26) Place the PCR plate/strips on a magnetic stand for 5 minutes or until liquid is clear.
27) Transfer 20 µL of supernatant from each well to a new PCR plate/strip tube.

SAFE STOP POINT. Place in -20 °C.

6. Library Amplification

Move to Post PCR Work Area (step 2)

Thaw the following and keep on ice/4°C fridge:

PCR Master Mix (PMM)
PCR Primer Cocktail (PPC)

Bring the following to room temperature:
Resuspension Buffer (RSB)
AMPure XP beads (allow 30 minutes to reach room temperature)

1) Add 5 µL of PCR Primer Cocktail (PPC) and 25 µL of PCR Master Mix (PMM) to each sample, pipette up and down to mix, and briefly centrifuge.
2) Place the PCR plate/strip in a thermal cycler using program "PCR" and incubate as follows:

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

98 °C for 30 sec; 15 cycles {98 °C for 10 sec;60 °C for 30 sec;72 °C for 30 sec}72 °C for 5 mins; 4 °C HOLD.
Heated lid: 100 °C.

Clean up (Note Ampure XP volumes vary between dual index and single index adapters!)

3) Vortex AMPure XP beads until well dispersed. Add 47.5 ul of AMPure XP beads if using the TruSeq RNA CD Index Plate (dual indexing, PN20019792) or 50 µL of AMPure XP beads (if using the single index adapters, PN 20020492 or PN20020493) to each sample. Gently pipette the entire volume (100 µL) up and down 10 times.
4) Incubate at room temperature for 15 minutes.
5) Place the PCR plate/strip on a magnetic stand for 5 mins then discard 95 µL of supernatant. Use 10µL pipette tip to remove all residual supernatant before adding ethanol wash (critical step)!
6) Add 200 µL of freshly prepared 80% EtOH to each well, incubate for 30sec, then remove and discard supernatant.
7) Repeat step 6 for a total of two 80% EtOH washes.
8) Dry the beads at room temperature for 15 mins, or until ethanol has evaporated (beads almost cracking).
9) Add 32.5 µL of RSB to each sample prior to removing from magnetic rack. Remove samples from rack and gently pipette up and down 10 times to mix. Visually confirm resuspension.
10) Incubate at room temperature for 2 mins.
11) Place the PCR plate/strip on a magnetic stand for 5 mins.

12) Transfer 30 µL of supernatant to a labelled 1.5 mL tube.

7. Assess Quality and Quantity of Library

1) Use Qubit HS dsDNA assay using TGL SOP to quantify the cDNA library (SOP_Qubit_Assay_for_Nucleic_Acid_Quantification.docx.).
2) Use High Sensitivity D1000 screen tape and record average library size distribution, setting the region to ~190bp- 1000bp. Average library size will be used to size correct the library after RT-qPCR quantification, and is used in LIMS sample IDs. Record Tapestation file ID in sample tracking sheet.

   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.

8. Sequencing

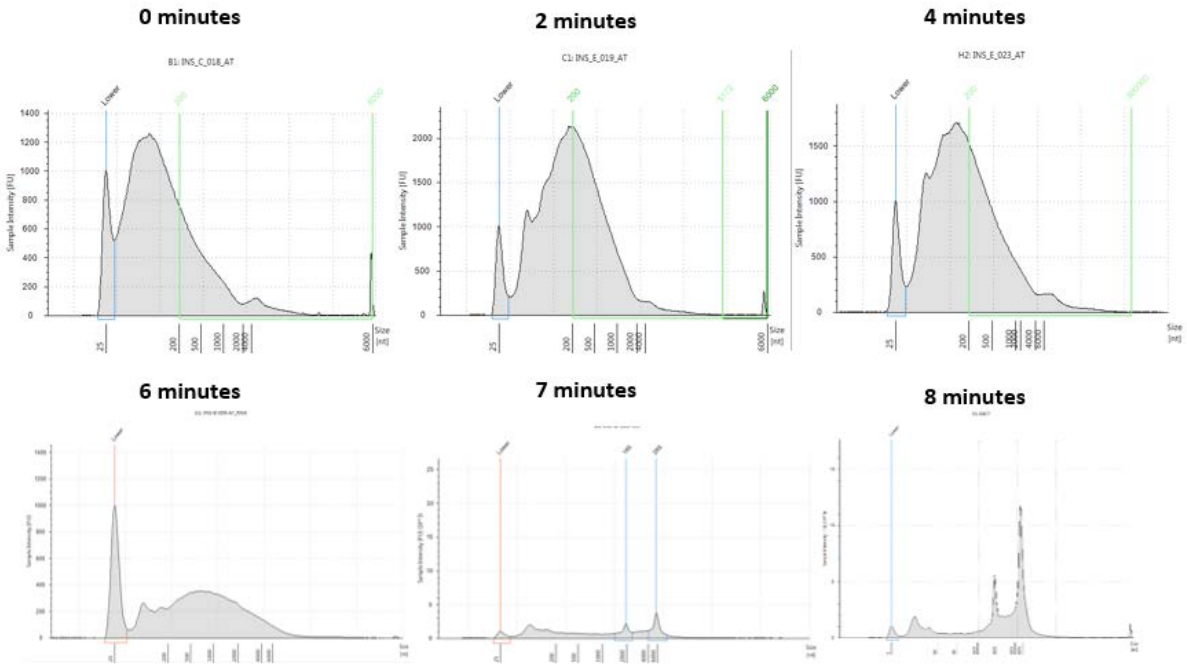
Pre-analytic quality control is performed on all libraries. Up to 96 dual barcoded libraries may be combined in one pool and sequenced on the MiSeq Nano flow cell, 150bpX8bpX8bpX150bp. After creating a multiplex pool, repeat RT-qPCR to ensure optimal cluster density when sequencing. Libraries with <35% ribosomal contaminant as determined by informatics, may be deep sequenced on the NextSeq550. If ribosomal contaminant is >35%, repeat library synthesis.

Deep sequencing of libraries is performed on a NextSeq550 High Output kit and each library is sequenced at a depth of 80 million clusters per library, 79bpX8bpX79bp. Ideally five RNA samples are pooled per NextSeq550 High Output Kit and a pool is composed of the recommended 5-plex single index pooling strategy for single or dual index barcoding.

9. Appendix

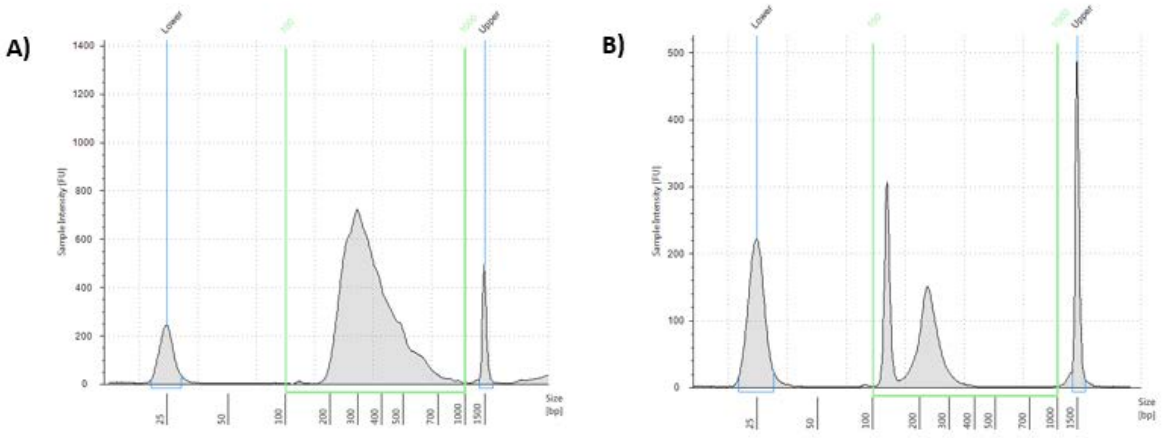
1) RNA Fragmentation Times

Below are examples of total RNA (from FFPE samples) analyzed on the Tapestation and their associated fragmentation time. Samples with a DV200<55 are not fragmented. Samples with 55<DV200<65 should be fragmented for 4 minutes. Samples with DV200>65 are fragmented for 8 minutes.



2) Adapter Contamination

The following snapshots depict the difference seen between those samples with and without adapter contamination, as identified by High Sensitivity D1000 Tapestation trace. Record adapter contamination in sample tracking sheet including % adapter contaminant (Tapestation analysis region selection).



A) High Sensitivity D1000 Tapestation trace of a sample with no adapter contamination.
B) Trace of a sample with heavy adapter contamination at ~130 bp.

6.0 Revision History

Version Number	Date *(yyyy-mm-dd)*	History of Change
1.1	2017-06-13	First draft by Shihab Sarwar
1.2	2017-06-22	Update and Edits by Kayla Marsh
1.3	2017-06-29	Edits by Dax Torti
1.4	2018-04-12	Edits by Kayla Marsh, minor symbol changes, reference to lot tracking on illumina website, update to dual indexing primers, new illumina product numbers

1.4.1	2018-04-24	Dax Torti, added dual indexing strategy, and pre-analytic QC sequencing guidance.
1.4.2	2018-07-03	Updated step 14 cleanup after ribosomal depletion to occur in 1.5ml tubes instead of PCR strip tubes to improve recovery of depleted Total RNA. Corrected final PCR bead cleanup volume options for dual index library preps.
1.4.3	2019-01-11	Update section 7 – library sizing from 190-1000 to exclude size of adapter
1.4.4	2019-02-27	Changed protocol to use Agencourt RNA Clean XP beads with 70% EtOH washes for RiboZero Deplete and Frament RNA. Changed recommended microfuge tubes to low retention. Added missing acronyms, updated bead handling recommendations.