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3' TagSeq Library Preparation Protocol

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ABSTRACT

Purpose: This protocol is used to construct libraries directed at the 3' ends of mRna for gene expression profiling studies and provides an alternative to standard RNA-Seq.

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PROTOCOL CITATION

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MATERIALS TEXT

A	B	C
Reagent	Vendor/Catalog #	Storage
10mM dNTPs	NEB/N0447L	freezer
0.1M DTT (aliquots)	ThermoFisher/707265ML	freezer
5X First Strand (FS) buffer	Takara(Clontech)/639537	freezer
SMARTScribe Reverse Transcriptase	Takara(Clontech)/639537	freezer
Klentaq DNA polymerase	DNA polymerase tech/SKU: 100	freezer
10X Klentaq 1 buffer	DNA polymerase tech/SKU: 100	freezer
3iLL-30TV primer	IDT	freezer
5iLL primer	IDT	freezer
Template Switching Oligos (TSO) 4 oligos	IDT	-80
Agencourt AMPure Beads XP	Beckman Coulter A63881	fridge
80% ETOH	Prepared fresh from 100% ETOH (UT Mkt; AC615090020)	flammable cabinet
Eppendorf twin-tec 96-well PCR plate	Eppendorf (VWR:95041-440)	shelf
Adhesive PCR plate foil seal	Fisher	shelf
96-well plate magnet	ThermoFisher AM10027	shelf
Bioanalyzer kits (HS and Pico)	Aligent: HS DNA: 5067-4626; RNA 6000 Pico: 5067-1513	fridge
Index Primers	IDT	freezer
Pippin Prep Cassettes/Reagents	BDF1510	shelf and fridge
MinElute Column	Qiagen #28006	fridge
PB Binding Buffer	Qiagen #28006	shelf
PE Wash Buffer	Qiagen #28006	shelf

Template Switching Oligos (4) specifications

1. Order on IDT
   1a. on IDT, under products and services, go to "DNA&RNA", click on "Custom RNA oligos", order RNA oligos by the tube and customize each oligo in their template.

   General specifications:
   Product: 100nmole RNA Oligo
   Guarantee: 0.3 nmol
   Purification: RNase-Free HPLC
   Additional Services: Level II Setup Fee

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A	B
swMWWM	/5Me-isodC/iisodG/iMe-isodC/CCATGCGGCTACACGACGCTCTTCCGATCTNNMWWMWHrGrGrG
swGC	/5Me-isodC/iisodG/iMe-isodC/CCATGCGGCTACACGACGCTCTTCCGATCTNNGCWTCHMWrGrGrG
swTG	/5Me-isodC/iisodG/iMe-isodC/CCATGCGGCTACACGACGCTCTTCCGATCTNNTGCMWrGrGrG
swMW	/5Me-isodC/iisodG/iMe-isodC/CCATGCGGCTACACGACGCTCTTCCGATCTNNMWrGrGrG

Index Primer 96 Well Adapter Plate
General Specifications:
Guarantee: 1.38 nmol
Final Concentration: 10 uM
Quantity: 1.38 nmol
Buffer: IDTE Buffer pH 8.0 (10mM Tris-HCl/0.1 mM EDTA)
Purification: Standard Desalting
Plate Product: Tru-Seq - Compatible Indexing Primer, 16 rxn
Plate Type: Matrix- Screw Top

IDT Upload Index Plate 1 UDI_Bolnick.xlsx

RNA Fragmentation and RT Primer Annealing

1

RNA Fragmentation and RT Primer Annealing

*Input material from customer should be AT LEAST 25ul of high quality DNAse-treated RNA in water ideally normalized to 50ng/ul. (acceptable range is 5ng/ul to 100ng/ul).

1.1 Clean bench, pipets, ice bucket, and all other equipment first with RNaseZAP and second with 70% EtOH. Prepare a bucket of ice and transfer RNA and reagents to thaw on ice.

1.2. Turn on a thermocycler and allow lid to preheat 10-15 minutes.

1.3. Label a 96-well plate with appropriate labels ie. plate#, date, initials, and SOP step (i.e. RNA fragmentation).

1.4. Pipet **RNA and nuclease-free H2O to a total volume of 10ul**(50ng-1000ng) into the proper well. This can be done with the Liquidator or a 20ul multichannel pipette.**If transferring RNA from tubes, have a lab member watch you pipet samples into 96-wellplate. (calculate RNA and water input in excel)**

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1.5. Make up the RNA fragmentation/RT master mix:

Number of samples ie 40*(extra 10% of total samples)=44 total samples to make mastermix

	A	B	C
1	Reagent	Volume per sample	Volume in master mix
2	dNTPs (10mM)	1ul	44 ul
3	0.1 M DTT	2ul	88ul
4	5x FS buffer	4ul	176ul
5	3iLL-30TV (10uM)	1ul	44ul
6	Total RNA (+H2O if needed)	10ul	
7	Total volume	18ul	352 ul

1.6. Mix by gentle inversion or pipetting and briefly spin master mix in minifuge.

1.7. Aliquot master mix into strip tubes to aid pipetting. With a multi-channel pipette, add **8ul of master mix**to each RNA sample. Using the same tips set at 8ul, pipet to mix 4-5 times.

1.8. Cover plate with foil seal, creating a secure seal around each well using a kimwipe or plate cover paddle/roller. Briefly spin plate in mini plate spinner centrifuge.

1.9. At the thermocycler, open and start the fragmentation program (User: TagSeq; Program: frag). When the block temperature approaches 95°C, put plate on the block, close the lid, and incubate for 2.5 minutes. Immediately move samples to ice and incubate for 2 minutes.

1.10. **Optional**: If needed, briefly spin plate and remove 1.5ul of a subset of samples for BA RNA Pico analysis to confirm fragmentation in the event of troubleshooting. Replace 1.5ul**1x FS buffer**back into each used well (Note: Using undiluted 5X FS buffer will cause prep to fail).

First-Strand cDNA Synthesis

2 First-Strand cDNA Synthesis

2.1. Briefly centrifuge the plate containing the fragmented RNA in the mini plate spinner and carefully remove the foil seal. Return the plate to ice.

2.2. Make the following master mix:

Number of samples ie 40 x (10%)= 44 total

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A	B	C	D
	A	B	C
1	Reagent	Volume per sample	Volume in master mix
2	Template switch oligo pool (10uM) (4 total)	0.1 ul	4.4
3	SmartScribe RT	1ul	44

2.3. To each sample, using a multi-channel pipette, add 2ul of the oligo/RT master mix. Reaction volume total will be 20ul for each sample. Set pipette to 15ul and mix 4-5 times. Cover with a foil seal as in 1.8. Briefly centrifuge plate in mini plate spinner.

2.4. At the thermocycler, open and start the first strand cDNA synthesis program (User: TagSeq; program: first or RT). As the block temperature approaches 42°C, place the plate in the thermocycler and run at 42°C for 1hr, 65°C for 15 min.

2.5. While the program is running, make sure AMPure beads are set out at room temperature for at least 30 minutes for preparation of step 5.

AMPure Bead Purification

3 **AMPure Bead Purification**

3.1. Vortex AMPure beads well and dispense enough beads for 45ul/sample in a 50mL reservoir. Prepare a second reservoir with nuclease-free H2O (30ul/sample).

3.2. Briefly centrifuge the First-Strand cDNA plate in the mini plate spinner and remove the foil seal. Use a multi-channel pipette to add**30ul of H2O**to each sample to bring the volume to 50ul.

3.3. Perform a 0.9x AMPure bead purification of the cDNA by adding**45ul of AMPure beads**to each sample. With the same tips and with the pipette still set to 45ul, mix sample 10 times until the beads are uniformly mixed with the reaction.

3.4.Cover plate with a foil seal. Incubate at room temperature for 15 minutes.Prepare 80% EtOH during this incubation step (200ul 80% EtOH needed/sample).

3.5.**Can use Liquidator for remaining steps depending on sample number. If using a multi-channel pipette, prepare a 3rdreservoir with 80% EtOH.**

3.6.Place plate on the Alpaqua magnet for 5 minutes to collect the beads. Carefully remove the foil seal. Then, remove the supernatant without disturbing the beads (approx. 90ul/sample).

3.7.Wash the beads with 100ul 80% EtOH. Incubate for 30s. Discard the ethanol wash.

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3.8.Repeat 3.7.

3.9.Using 20ul tips, remove any remaining EtOH from the bottom of the wells and air dry the samples for 3-5 minutes. Do Not Overdry!

3.10.Resuspend the samples in**15ul of water**by uniformly mixing by pipetting up and down 10 times. Incubate 2 min at room temperature. Place the plate on the MagnaBot II side magnet for approx. 5 min. to collect the beads.

3.11.**Transfer 10ul**of AMPure purified cDNA to a new 96-well plate using the Liquidator or multichannel pipette. Label the plate with the HT#, plate#, date, initials, and SOP step (i.e. cDNA amp).**Note: Save the remaining 5ul cDNA in the #6 freezer until picogreen of final libraries is completed (step 10).**

3.12.**This is a safe stopping point. Cover plate with foil seal and put in freezer #6 or proceed to cDNA Amplification.**

cDNA Amplification

4 cDNA Amplification

4.1. 1.1.Prepare the master mix for cDNA amplification.

Number of samples x 10%

A	B	C
Reagent	**Volume**	**Volume in Master Mix**
RNA/DNA/Nuclease free H2O	6ul	
dNTPs (10mM)	0.5ul	
10x Klentaq 1 buffer	2ul	
10uM 5iLL oligo	0.5ul	
10uM 3iLL-30TV	0.5ul	
Klentaq	0.5ul	
Purified cDNA	10ul	
Total Volume	20ul	

4.2. Mix by gentle inversion or pipetting and briefly spin master mix in minifuge.

4.3. Aliquot master mix into strip tubes. Using a multi-channel pipette, add**10ul of master mix**to each well with cDNA. Keep pipette at 10ul and mix 4-5 times before ejecting tips. Cover plate with a foil seal (step 3.8). Briefly centrifuge plate in mini plate spinner.
Place the plate in the thermocycler and select your labeled program.

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Enter the number of PCR cycles based on the RNA input (table below).

A	B
RNA Input	**PCR Cycle #**
<150ng	18
150-400ng	14
400-1000ng	10

A	B	C	D
Cycle Step	**Temp**	**Time**	**Cycles**
Inital Denaturation	94	5 min	1
Denaturation	94	1 min	10,14, or 18 cycles
Annealing	63	2 min	
Extension	72	2 min	
Hold	4	hold/infinity	1

4.4. Plate can be left at 4C overnight or proceed to bead cleanup.

AMPure Cleanup

5 **AMPure Cleanup**

5.1. Vortex room-temperature AMPure beads well and dispense enough beads for 45ul/sample in a 50mL reservoir. Prepare a second reservoir with nuclease-free H2O (30ul/sample).

5.2.Briefly centrifuge the cDNA plate in the mini plate spinner and remove the foil seal. Use a multi-channel pipette to add **30ul of H2O** to each sample to bring the volume to 50ul.

5.3.Perform a 0.9x AMPure bead purification of the cDNA by adding **45ul of AMPure beads** to each sample. With the same tips and with the pipette still set to 45ul, mix sample 10 times until the beads are uniformly mixed with the reaction.

5.4.Cover plate with a foil seal and incubate at room temperature for 15 minutes.Prepare 80% EtOH during this incubation step (200ul 80% EtOH needed/sample).

5.5.**Can use Liquidator for remaining steps depending on sample number. If using a multi-channel pipette, prepare a 3rdreservoir with 80% EtOH.**

5.6.Place plate on the Alpaqua magnet for 5 minutes to collect the beads. Carefully remove the foil seal. Then, remove the supernatant without disturbing the beads (approx. 90ul/sample).

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5.7.Wash the beads with 100ul 80% EtOH. Incubate for 30s. Discard the ethanol wash.

5.8.Repeat 5.7.

5.9.Using 20ul tips, remove any remaining EtOH from the bottom of the wells and air dry the samples for 5 minutes. Do Not Overdry!

5.10.Resuspend the samples in **22ul of water** by uniformly mixing by pipetting up and down 10 times.

5.11.Place the plate on the MagnaBot side magnet for approximately 5 min. to collect the beads.

5.12.**Transfer 10ul** of the amplified cDNAinto **two** new 96-well plates, one for Index PCR (step 8) and one to store the remaining cDNA (freezer #6). Label the plates with the HT#, plate#, date, initials, and SOP step (i.e. cDNA or index PCR).

5.13.**This is a safe stopping point. Index PCR Plate can be stored in freezer #6 or proceed to indexing PCR.**

Index Addition via PCR

6 Index Addition via PCR

6.1. Based on your own samples and index plate, document which index primer correlates to each sample.

6.2. Index plate: add 3ul of index (3.9uM) to each sample well. Next prepare the following mastermix.

A	B	C
Reagent	Volume	Volume in master mix
RNA/DNA/Nuclease free H2O	12.65ul	
dNTPS	.75ul	
10x PCR buffer	3 ul	
Klentaq	0.6ul	
3.9 uM index	3ul	
Amplified cDNA	10ul	
Total volume	30ul	

*****add index primer to each individual sample first and not into the mastermix

6.3. Mix by gentle inversion or pipetting and briefly spin master mix in minifuge.

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6.4. If using **index plate 1/2 :** Add **13.4ul master mix** to each sample
If using **index plate 3/4:** Add **17ul of master mix** to each sample well.
Pipet to mix 4-5 times.

6.5. Cover the plate with a foil seal and briefly centrifuge in mini plate spinner.

6.6. Place the plate in the thermocycler and run the following program:

A	B	C	D
Cycle Step	**Temp**	**Time**	**Cycles**
Initial Denaturation	95	5 min	1
Denaturation	95	40 sec	4 cycles
Annealing	63	2 min	
Extension	72	2 min	
Hold	4	hold/infinity	1

AMPure Cleanup

7 **AMPure Cleanup**

7.1. Vortex room-temperature AMPure beads well and dispense enough beads for 27ul/sample in a 50mL reservoir.

7.2. Perform a 0.9X AMPure bead purification of the cDNA by adding **27ul of AMPure beads** using a multichannel pipette to each sample.

7.3. Mix by pipetting sample 10 times until the beads are uniformly mixed with the reaction.

7.4. Cover plate with a foil seal and incubate at room temperature for 15 minutes. Prepare 80% EtOH during this incubation step (200ul 80% EtOH needed/sample).

7.5. **Can use Liquidator for remaining steps depending on sample number. If using a multi-channel pipette, prepare 2 more reservoirs with 80% EtOH and H2O.**

7.6. Place plate on the Alpaqua magnet for 5 minutes to collect the beads. Carefully remove the supernatant without disturbing the beads (approx. 52ul/sample).

7.7. Wash the beads 2X with 100ul 80% EtOH. Incubate for 30s. Discard the ethanol washes.

7.8. Using 20ul tips, remove any remaining EtOH from the bottom of the wells and air dry the samples for 5 minutes. Do Not Overdry!

7.9. Resuspend the samples in **28ul of water** by uniformly mixing by pipetting up and down 10 times.

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7.10.Place the plate on the MagnaBot side magnet for approximately 5 min. to collect the beads.

7.11.Remove **25ul to a new 96-well plate** labeled with plate name, date, initials, and “final libraries” or label of choice.

Picogreen and Library QC

8 Picogreen and Library QC

*Steps using bioanalyzer can be supplemented with a tapestation protocol

8.1. Picogreen the plate. **Note:** Use standards starting at 50ng/ul (18 or 14 cDNA PCR cycles) or at 25ng/ul (10 cDNA PCR cycles). If multiple plates, only analyze standards on one plate.

8.2. Run picogreen plate and save values.

8.3. **Note: If all samples have expected concentrations on picogreen, continue to 10.5.However, if a sample looks low on PicoGreen STOP and perform the following**:

a. Run the final library on Qubit to verify the PicoGreen results.

b. If concentration is good, replace the concentration in the picogreen Excel file and continue to step 10.5.

c. If concentration is low, determine if the RNA was previously run on a BA. If the RNA quality is good, proceed to d.
If the RNA is poor, **stop** here and notify sequencing facility or troubleshoot before continuing.

d. Obtain the amplified cDNA and run a Qubit with this sample as well as the UHR control and 1-2 other random samples that had good quality picogreen results. Continue to e.

e. Determine if the cDNA levels indicate a successful library was produced.

f. If the cDNA Qubit results are similar to other samples, this indicates a library was generated.Verify this by running the cDNA on a high-sensitivity BA chip. If a good library is seen on the BA, repeat steps 8-10.4 on this sample(s).

g. If the cDNA Qubit results are poor, indicating no library was produced, continue to h.

h. Obtain the original RNA if no previous BA has been run on this sample.Run sample on an appropriate RNA BA chip and inspect RNA quality.

i. If RNA quality is poor indicating degradation, **stop** here and troubleshoot before continuing.

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j. If RNA quality is good, repeat library prep for this sample(s).**Note: If samples require a reprep, STOP and wait until other TagSeq jobs for upcoming NovaSeq run are completed.Highlight problem samples in NovaSeq Google Doc. Perform repreps from all jobs in a single batch. Once repreps have acceptable picogreen results, continue to step 10.5, and pool samples with appropriate job.**
1.2.Dilute a subset of the samples to approximately 2-3ng/ul each (if <2ng/ul it may be hard to see clearly on BA) and run a single High Sensitivity Bioanalyzer chip to check library quality and concentration.

Pooling

9 Pooling

9.1. Designate pools to include 24-32 samples (sample number based on final volume of pool and keeping in mind step 10.1. you don't want a huge volume (i.e., >120ul)). **Only pool samples from the same job**.

9.2. Enter the ng amount for sample pooling for each pool. **If possible pool a minimum of 50ng for each sample.** Sample volumes will be automatically calculated. Adjust ng amount until each sample is in the range of 2-11ul. (2ul is the lower range for pipetting on the Tecan and 11ul is half the sample).

9.3. Briefly centrifuge final library plate and label 1.5ml tubes for each pool.

9.4. When finished, remove plate, seal with foil cover, and store in freezer. Continue to step 10 with pools.

Size Selection

10 Size Selection

10.1. Adjust volume of pool to 60ul either by drying down on the Speedvac (medium heat, manual setting) or adding nuclease-free H2O to increase the volume of the sample.

10.2. Run a 2% dye-free gel with the V1 marker on the Blue Pippin to size-select **350-550bp** fragments.
(**Note:**Do not use more than 30ul of each pool.Pre-Pippin pools can be stored in Freezer #4 job box in case of Pippin error. Use the Dymo Printer to label tubes; select a job box, record location of tubes in job folder.)

10.3. Label tubes as final lib, apply red dots, and run a High-Sensitivity Bioanalyzer chip to confirm proper size selection of the libraries. Store final libraries. (Can use a tapestation instead of bioanalyzer)

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