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Stranded Whole Transcriptome RNA-Seq for All RNA Types

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Abstract

Stranded whole transcriptome RNA-Seq described in this unit captures quantitative expression data for all types of RNA including, but not limited to miRNA (microRNA), piRNA (Piwi-interacting RNA), snoRNA (small nucleolar RNA), lincRNA (large non-coding intergenic RNA), SRP RNA (signal recognition particle RNA), tRNA (transfer RNA), mtRNA (mitochondrial RNA) and mRNA (messenger RNA). The size and nature of these types of RNA are irrelevant to the approach described here. Barcoded libraries for multiplexing on the Illumina platform are generated with this approach but it can be applied to other platforms with a few modifications.

Keywords

RNA-Seq; transcriptome; gene expression; Duplex-specific nuclease

INTRODUCTION

Few RNA-Seq protocols truly capture stranded whole transcriptomes for which RNA sizes range from 20–20,000 nt (Yoffe, et al., 2008). The loss of some RNA types with other protocols is typically due to the size of the RNA and either gel purification, poly(A)

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Internet Resources

<http://www.idtdna.com/analyzer/applications/oligoanalyzer/> IDT (Integrated DNA Technology) OligoAnalyzer 3.1.

http://support.illumina.com/downloads/multiplexing_sample_prep_guide_1005361.ilmn Illumina multiplexing guide.

selection or the method used to attach the adapters. The method described in this unit allows the quantification of all RNA species by RNA-seq regardless of size and without gel electrophoresis. Due to size constraints for Next Generation Sequencing (NGS), total RNA is first fractionated by size. Basic Protocol 1 (Miller, D., et al., 2013) begins with size separation into small RNA (smRNA <200 nt) and large RNA (LgRNA >200 nt) followed by fragmentation of LgRNA (FLgRNA), end repair and adapter ligations of both size fractions to retain strandedness (Basic Protocol 2) (Figure 1). The RNA is reverse transcribed into cDNA, amplified with PCR1 (Basic Protocol 3) and normalized with duplex-specific nuclease (DSN) to remove the abundant rRNA sequences (Basic Protocol 4). Barcodes are added by PCR2 for multiplexing on the Illumina HiSeq platform using four samples per lane to capture about 50 million reads per sample (Basic Protocols 5 and 6). By retaining strandedness while capturing the entire transcriptome the investigator can generate quantitative expression data for all types of RNA.

Basic Protocol 1

RNA PURIFICATION AND FRAGMENTATION

Optimally, all RNA should be isolated without degradation so that smaller sizes can be separated prior to fragmentation of the larger RNA for library generation. The smaller native RNA can be sequenced intact while the fragmented larger RNA is later sequenced to provide coverage across the entire transcript. Alternatively, total RNA of lesser quality can be fragmented and sequenced as a single pool. Total RNA is isolated from tissue culture cells using the Qias shredder to reduce genomic DNA size and viscosity. Then DNA and RNA are isolated with the AllPrep kit (Qiagen) in conjunction with the alternative RWT wash buffer in order to capture all sizes of RNA including miRNA. Size fractionation is then accomplished with the RNeasy MinElute kit (Qiagen) by manipulating ethanol concentrations. The LgRNA is chemically fragmented using a fragmentation reagent (Ambion). Always wear gloves and use aerosol resistant tips when pipetting samples and solutions. Determine RNA concentrations with the NanoDrop for accuracy.

Materials

Tissue culture cells or other suitable source for RNA

Microcentrifuge

1.7mL tubes (Avant)

RNeasy Mini AllPrep kit (Qiagen)

Ethanol (100%)

2-Mercaptoethanol (BME)

Qias shredder (Qiagen)

RWT buffer (Qiagen)

Nuclease-free water (Sigma)

RNeasy MinElute kit (Qiagen)

NanoDrop 2000 (ThermoFisher)

10X fragmentation reagent and stop solution (Ambion)

2100 Bioanalyzer (Agilent)

RNA 6000 Pico assay kit (Agilent)

Total RNA purification

1. Add 600µL RLT buffer containing 1% BME (vol/vol) to the pelleted cells ($\sim 10^7$) from one 60 cm² culture dish and transfer to a 1.7 ml tube.
2. Rake the tube across a rack approximately 20 times or vortex the sample until the cells are completely lysed.
3. Aliquot 300µL of the lysed sample and store at -80°C indefinitely.
4. Centrifuge 300µL of lysed sample through a Qias shredder column for 2 min at 17,000 g.
5. Apply 300µL of Qias shredder treated sample to the AllPrep AP DNA column and centrifuge for 20 s at 10,000 g. The column can be stored at -80°C for DNA collection according to manufacturer's recommendation.
6. (Optional) Re-apply the sample to an additional AP DNA column to ensure removal of residual DNA for accurate quantification. This protocol will not generate library inserts from source DNA contamination in the RNA fraction.
7. Add 450µL 100% ethanol (60% final vol/vol) to the AP DNA column flow-through and bind RNA to RNeasy Mini column by centrifugation for 15 sec at 10,000 g. Flow-through can be stored at -80°C for protein collection according to the manufacturer's recommendations.
8. Wash the RNeasy column (centrifuge at 10,000 g for 15 sec) with 500µL RWT buffer containing ethanol according to the manufacturer.
9. Wash the RNeasy column (centrifuge at 10,000 g for 15 sec) with 500µL RPE buffer containing ethanol according to the AllPrep kit instructions.
10. Wash the RNeasy column (centrifuge at 17,000 g for 2 min) with 500µL freshly made 80% ethanol (vol/vol) diluted with nuclease-free water.
11. In a clean 1.7mL tube, centrifuge the RNeasy column at 17,000 g for an additional 5 min to dry.
12. Transfer RNeasy column to a clean collection tube and allow it to dry with the lid open for at least 10 min.
13. Add 50–100µL nuclease-free water to the RNeasy column, incubate at room temperature for 1 min and collect RNA by centrifugation at 17,000 g for 1 min. Add a second elution for high concentrations of expected RNA yields over 30µg.
14. Determine the RNA concentration with the NanoDrop.

Size fractionation of total RNA—Total RNA is separated into two size-fractions using the RNeasy Minelute kit. Fractionate the sample by manipulating ethanol concentrations to determine the size of the RNA that will bind to or pass over the column. This will generate two separate RNA-Seq libraries for any given sample. You may also use total RNA that has not been fractionated and follow the protocol for fragmented large RNA (FLgRNA) but there will be some loss of reads for the smallest RNAs.

1. Transfer 1–5 µg RNA to a new 1.7mL tube and add nuclease-free water for a final volume of 100µL. Store additional RNA at –80°C.
2. Add 350µL RLT with 1% BME and 302µL ethanol (final 40% vol/vol) to the RNA sample.
3. Centrifuge over an RNeasy Minelute column for 15 sec at 10,000 g to bind the large RNA fraction >200 nt (LgRNA). The small RNA (smRNA) will flow through the column.
4. Add 375µL ethanol to the smRNA fraction (final 60% vol/vol) and bind to a clean RNeasy Minelute column by centrifugation for 15 sec at 10,000 g. Discard the flow-through.
5. Wash the RNeasy columns (centrifuge at 10,000 g for 15 sec) with 500µL RPE buffer containing ethanol according to AllPrep kit instructions.
6. Wash the RNeasy columns (centrifuge at 17,000 g for 2 min) with 500µL 80% ethanol (vol/vol) diluted with nuclease-free water.
7. Transfer the columns to clean 1.7mL centrifuge tubes and spin at 17,000 g for an additional 5 min to dry.
8. Transfer RNeasy columns to clean collection tubes and allow them to dry with the lid open for at least 10 min.
9. Add 40µL nuclease-free water to the smRNA and FLgRNA columns. For low concentration samples use less water.
10. Incubate the columns at room temperature for 1 min and centrifuge at 17,000 g for 1 min.

Fragmentation of LgRNA—RNA-Seq library inserts should be around 200bp or less. The size fractionated large RNA (LgRNA >200 nt) must be fragmented by some means. The chemical fragmentation method in this protocol is reproducible for small quantities of RNA.

1. Starting with 400ng of LgRNA or more in 36µL add 4µL 10X fragmentation reagent. This should provide adequate quantities of fragmented LgRNA (FLgRNA); however, lesser starting quantities can be used.
2. Incubate the mix at 70°C for 4 min.
3. Stop the reaction with the addition of 4µL 10X stop buffer to the fragmentation reaction. This should generate an average size of 150 nt.

Purify RNA with RNeasy Minelute—The ethanol concentration should be 60% (vol/vol) for the smRNA binding step following end repair (below). Ethanol concentrations should be 40% (vol/vol) for FLgRNA or LgRNA purifications.

1. Prepare aqueous RNA for RNeasy MinElute binding by adding the following components.
44μL RNA
56μL nuclease-free water
350μL RLT containing 1% BME
675μL ethanol (smRNA: final 60% ethanol vol/vol)
or
302μL ethanol (LgRNA or FLgRNA; final 40% ethanol vol/vol)
2. Bind the RNA to an RNeasy Minelute column by sequential centrifugations for 15 sec at 10,000 g. Discard the flow-through.
3. To the column add 500μL RPE buffer containing ethanol according to the manufacturer and wash by centrifugation for 15 sec at 10,000 g.
4. Add 500μL 80% ethanol and wash the RNeasy Minelute column by centrifugation for 2 min at 17,000 g.
5. Transfer the column to a clean 1.7mL centrifuge tube and spin at 17,000 g for an additional 2 min to dry.
6. Transfer RNeasy column to a clean 1.7mL collection tube and allow it to dry with the lid open for 5–10 min.
7. Add 19μL RNase-free water to the smRNA and FLgRNA columns.
8. Incubate the columns at room temperature for 1 min.
9. Centrifuge the column at 17,000 g for 1 min to elute the RNA.
10. Quantify the RNA fractions with the NanoDrop and run 5ng of each on the Bioanalyzer using the RNA 6000 Pico kit according to the manufacturer's recommendations. Typical RNA profiles are shown in Figure 2. Check the Total RNA, smRNA, FLgRNA and LgRNA fractions with the Bioanalyzer RNA Pico kit for incomplete size fractionation.

BASIC PROTOCOL 2: END REPAIR AND ADAPTER LIGATIONS

The enzymes used in this protocol allow the use of DNA adapters to be ligated only to RNA inserts. Low-level DNA contamination will not be efficiently ligated to the 3' adapters by the T4 RNA ligase 2 truncated KQ enzyme. It is necessary to prepare the ends of the RNA before the adapter ligations. The formation of adapter dimers is reduced with a hybridization step where the reverse transcription primer (RS-TS-PCR1A) is bound to the 3' adapter before the 5' adapter ligation. Otherwise, T4 RNA ligase 1 used for the 5' adapter ligation will form adapter dimers with single stranded 5' and 3' adapters. Hybridization of RS-TS-

PCR1A to the 3' adapter will block the formation of unwanted adapter dimers (Vigneault, et al., 2012).

Materials

smRNA or FLgRNA in 17 μ L RNase-free water
0.2mL PCR tubes
Thermocycler with a heated lid
Antarctic phosphatase (New England Biolabs)
RNasin Plus (Promega)
T4 polynucleotide kinase
10mM ATP (Epicentre)
RNase-free water (Sigma)
RNeasy Minelute kit (Qiagen)
T4 RNA Ligase 2, truncated KQ (New England Biolabs)
RS-TS 3' adapter (5 μ M)
RS-TS-PCR1A (5 μ M)
RT-TS 5' adapter (10 μ M)
T4 RNA Ligase 1 (New England Biolabs)

End repair

1. Combine the following reagents in a 0.2mL PCR tube for phosphatase treatment of smRNA or FLgRNA.
 - 17 μ L RNA in RNase-free water
 - 2 μ L 10X Antarctic phosphatase reaction buffer
 - 1 μ L Antarctic phosphatase
 - 0.5 μ L RNasin Plus
2. Incubate the reaction in a thermocycler under the following conditions.
 - 37°C for 30 min
 - 65°C for 5 min (inactivates the phosphatase)
 - 4°C hold
3. Mix the following reagents on ice for the kinase reaction.
 - 5 μ L 10X T4 polynucleotide kinase buffer
 - 5 μ L 10mM ATP
 - 2 μ L T4 polynucleotide kinase

0.5 μ L RNasin Plus

17 μ L nuclease-free water

4. Add the T4 kinase reaction mix to the phosphatase-treated RNA in the thermocycler.
5. Incubate the reaction for 60 min at 37°C.
6. Bring the sample volume to 100 μ L by adding 50 μ L nuclease-free water.
7. Purify the RNA with the RNeasy Minelute protocol and elute in 16 μ L nuclease-free water.
8. Quantify the RNA with the NanoDrop.

Adapter ligations

Block the excess 3' adapter from forming adapter dimers after the first ligation step by hybridizing an equal molar amount of the reverse transcription primer RS-TS-PCR1A to the 3' adapters. Then ligate the 5' adapter with T4 RNA ligase (Vigneault, et al., 2012).

1. Combine these components in a 0.2 μ L PCR tube to denature and ligate the 3' adapter with end-repaired smRNA or FLgRNA. Starting with 5 pmoles of end-repaired RNA is optimal. Based on the average sizes, this would require 210ng smRNA or 240ng FLgRNA. If the starting quantities are lower, adjust the amount of RS-TS 3' adapter proportionally.

14 μ L end-repaired RNA (5 pmoles)

1 μ L of RS-TS 3' adapter (5 μ M)

2. Heat denature the sample for 2 min at 70°C and hold at 4°C.
3. Ligate the 3' adapter in the following reaction mixture.

2 μ L 10X T4 RNA ligase 2 truncated KQ buffer

1 μ L RNasin Plus

2 μ L T4 RNA ligase 2 truncated KQ (200u/ μ L)

Incubate the reaction overnight at 16°C in the thermocycler.

4. Add 1 μ L of 5 μ M RS-TS-PCR1A primer (or equal molar amounts of the 3' adapter in the above ligation step) and incubate at 90°C for 30 sec, then hybridize at 55°C for 30 min and then hold at 4°C.
 5. Heat denature the RS-TS 5' adapter (10uM) for 2 min at 70°C and hold at 4°C. This can be denatured just once if it is kept cold or frozen at -20°C.
 6. Ligate the 5' adapter in the following reaction mixture.
- 21 μ L 3' adapter ligation/RS-TS-PCR1A hybrid
- 1 μ L 10X T4 RNA ligase 1 reaction buffer
- 3 μ L 10mM ATP

1μL RS-TS 5' adapter (10μM)

2μL RNase-free water

2μL T4 RNA ligase 1 (20u/μL)

7. Incubate the reaction in the thermocycler for 2 hr at 20°C.
8. Purify the ligated RNA with an RNeasy MinElute column containing the appropriate concentration of ethanol in the binding buffer as described in the Basic Protocol 1 (60% ethanol vol/vol for smRNA or 40% ethanol vol/vol for FLgRNA ligation reactions).
9. Elute the RNA in 11μL RNase-free water.

BASIC PROTOCOL 3: REVERSE TRANSCRIPTION AND PCR1

It is necessary to convert the adapter-ligated RNA libraries to cDNA using reverse transcription before the PCR1 amplification reaction. EDTA is added prior to PCR1 to remove excess MgCl₂ from the reverse transcription reaction.

Materials

0.2mL PCR tube

Adapter ligated RNA in 10μL RNase-free water

RS-TS PCR 1A primer (25μM)

Superscript RT III (Life Technologies)

RNase H (Life Technologies)

RS-TS PCR 2D primer (25μM)

40mM EDTA, pH8

100mM dNTP mix (Bioline)

Phusion DNA polymerase (New England Biolabs)

Thermocycler with a heated lid

Reverse transcription

1. Combine the following components in a 0.2mL PCR tube.
 - 10μL adapter-ligated RNA
 - 2.5μL RS-TS PCR1A primer (25μM)
2. Heat denature the mixture for 2 min at 70°C and hold at 4°C.
3. Add the following Superscript RTIII components to the denatured adapter-ligated RNA and RS-TS PCR1A mixture.
 - 15μL Superscript RTIII 2X reaction buffer
 - 2.5μL Superscript RTIII/RNaseOut enzyme mixture

4. Incubate the reverse transcription reaction for 60 min at 50°C and then hold the reaction at 4°C.
5. Add 1µL RNaseH (10u) and incubate for 20 min at 37°C and hold at 4°C or store at -20°C.

PCR1 amplification

1. Add the following reagents to the reverse-transcribed cDNA reaction.

20µL Phusion reaction buffer (5X)

2µL RS-TS-PCR2D (25µM)

1µL 100mM dNTP mix

2µL 40mM EDTA pH8

1µL Phusion DNA polymerase (2u)

43µL RNase-free water

2. Amplify the reaction using the following PCR conditions.

1 cycle:	98°C	30 sec
20 cycles:	98°C	10 sec
	55°C	30 sec
	72°C	15 sec
1 cycle:	72°C	10 min
	4°C	hold

SUPPORT PROTOCOL 1: AMPURE XP CALIBRATION AND CLEAN-UP FOR PCR1 REACTION

AMPure XP magnetic beads are used to remove enzymes, buffer components primers and adapter dimers from the PCR reactions. The volume ratio of the beads to the reaction will determine the size selection of the binding reaction. Calibrate AMPure XP beads for each lot to ensure accurate size selection. Use different size selection for PCR1 reactions and for PCR2 reactions because the size of the adapter dimers (PCR1-82bp, PCR2-113bp) are increased in PCR2 by the addition of the barcodes and flow cell binding sequences (Table 1, Figure 3).

Materials

AMPure XP magnetic beads (Beckman Coulter)

Magna-Sep magnetic 1.7ml tube stand (Life Technologies)

1.7mL tubes

100% ethanol

RNase-free water (Sigma)
EB elution buffer (Qiagen)
Tween 20 (Promega)
20 bp Ext Range DNA Ladder (Lonza)
2100 Bioanalyzer (Agilent)
Bioanalyzer HS DNA kit (Agilent)
Qubit dsDNA HS assay (Life Technologies)

AMPure XP bead calibration

1. Dilute 10 μ L (1 μ g) 20 bp Ext Range DNA Ladder with 900 μ L EBT (EB buffer containing 0.1% Tween 20).
2. In a 1.7ml tube, combine 80 μ L diluted 20 bp ladder with the appropriate volume of AMPure XP beads have been equilibrated to room temperature. The volume ratios and approximate cutoff sizes (bp) are listed below as demonstrated in Figure 3 and Table 1.
3. Vortex briefly and incubate the XP bead:PCR mixture at room temperature for 5 min.
4. Place the binding reaction on the magnetic stand for 5 min and remove the liquid while the tube is on the stand.
5. Add 700 μ L 80% ethanol (freshly made with nuclease-free water), invert the tube and rotate the tube 180° on the stand to force the beads to transfer to the opposite side of the tube.
6. Rotate the tube 180° again and allow the beads to transfer back.
7. Remove the tube from the stand and vortex sample for 3–5 sec until well mixed.
8. Place the tube back on the magnetic stand for 5 min and discard the wash.
9. Add an additional 700 μ L 80% ethanol and repeat the wash procedure without vortexing.
10. Discard the wash and briefly spin the tube oriented with the bead cluster facing outwards.
11. Place the tube back on the stand with the original orientation and remove any remaining wash solution.
12. Dry the beads completely with the lid open for about 6 min at 40°C or at room temperature for about 30 min.
13. Place the tube back on the magnetic stand and add 62 μ L EB elution buffer.
14. Remove the tube from the stand and mix well by pipetting up and down about 10 times.

15. Incubate at room temperature for at least 2 min.
16. Return the tube to the magnetic stand for 5 min and carefully transfer 60 μ L to a clean tube containing 1.2 μ L of 5% Tween 20.
17. Allow the samples to degas for 5 min and then without mixing run 1 μ L of each calibration reaction on a Bioanalyzer HS DNA chip.

AMPure XP bead clean-up for PCR reactions

1. Vortex AMPure XP beads well and transfer 160 μ L to a 1.7ml tube to obtain a ratio of 1.6:1 XP beads:PCR1 vol/vol ratio based on the AMPure XP bead calibration. Use 1.2:1 XP beads (120 μ L) to PCR2 volume (100 μ L). It is necessary to change the size selection for PCR2 since the adapter dimer size increases from 82bp (PCR1) to 113bp (PCR2).
2. Allow XP beads to equilibrate to room temperature; add the 100 μ L PCR reaction and vortex the mixture.
3. Incubate the XP:PCR binding reaction at room temperature for 5 min.
4. Place the binding reaction on the magnetic stand for 5 min and remove the liquid while the tube is on the stand.
5. Add 700 μ L 80% ethanol (freshly made with RNase-free water); invert the tube then rotate the tube 180° on the stand to force the beads to transfer to the opposite side of the tube.
6. Rotate the tube 180° again and allow the beads to transfer back.
7. Remove the tube from the stand and vortex sample for 3–5 sec until well mixed.
8. Place the tube back on the magnetic stand for about 5 min and discard the wash.
9. Add an additional 700 μ L 80% ethanol and repeat the wash procedure without vortexing.
10. Discard the wash and briefly spin the tube oriented with the bead cluster facing outwards.
11. Place the tube back on the stand with the original orientation and remove any remaining wash solution.
12. Dry the beads completely with the lid open for about 6 min at 40°C or at room temperature for about 30 min.
13. Carefully place the tube back on the magnetic stand and add 32 μ L EB elution buffer.
14. Remove the tube from the stand and mix well by pipetting up and down about 10 times and then gently mixing by hand.
15. Incubate at room temperature for at 2 min.
16. Return the tube to the magnetic stand for 5 min and carefully transfer 30 μ L to a clean tube containing 0.6 μ L of 5% Tween 20.

17. Repeat the AMPure XP bead clean-up with 1.6:1 ratio of beads again if there is significant adapter dimer contamination or it will result in low DSN and PCR2 efficiencies.

BASIC PROTOCOL 4: DUPLEX-SPECIFIC NUCLEASE (DSN) REDUCTION OF rRNA SEQUENCES

The reduction of rRNA sequences is important for effective RNA-Seq. Hybridizing PCR1 for five hours allows only high copy number sequences enough time to become double-stranded once denatured. DSN is specific only to dsDNA and will effectively cut these sequences and prevent further PCR amplification. Significant primer dimer contamination will interfere with this process (Figure 4). Salt concentrations are critical for proper melting temperature predictions so all DSN buffers are custom-made for pH adjustments by combining Tris-HCl and Tris Base (see Reagent and Solutions). It is also important that all buffer and enzyme additions to the hybridization be mixed well in the thermocycler chamber for proper temperature regulation. Salt and temperatures have been selected to prevent adapters that reside on the ends of the library at high concentration from forming stable hybrids (Table 2). Under these conditions rRNA sequences (as DNA) can form stable hybrids to become effective DSN targets. It is possible to do several samples in parallel at one- or two-min intervals but should be limited to eight or less to avoid temperature fluctuations.

Materials

PCR1 library DNA
Custom 10X DSN hybridization buffer (see recipe)
Tween 20 (Promega)
50% PEG 8000 (Life Technologies)
Nuclease-free water (Sigma)
Duplex-specific nuclease (Wako Chemicals)
Thermocycler (2 chamber or additional thermocycler)
2X custom DSN reaction buffer (see recipe)
2X custom DSN stop buffer (see recipe)
AMPure XP beads (Beckman Coulter)
EB elution buffer (Qiagen)

DSN reduction of rRNA sequences

1. Combine the following components in a 0.2μL PCR tube
 - 100ng PCR1 library DNA
 - 2μL custom 10X DSN hybridization buffer

Bring final volume to 20 μ L with nuclease-free water.

To generate libraries made from fragmented total RNA that has not been size fractionated, use 200ng of PCR1 product for the DSN hybridization reaction.

2. Mix the reaction with a pipette and centrifuge briefly.
3. Denature the hybridization mix at 98°C for 2 min.
4. Incubate the hybridization for 5 hr at 68°C.
5. Preheat a 20 μ L aliquot of custom 2X DSN reaction buffer in a separate thermocycler chamber at 68°C.
6. Add 20 μ L of preheated (68°C) 2X DSN reaction buffer to the hybridization reaction. The reaction tube must remain in the thermocycler. Due to the 68°C temperature, the pipette tip will tend to draw less volume when transferring the 20 μ L DSN enzyme reaction buffer and should be set around 25 μ L for transfer and mixing. Samples should be mixed well (10X) in the PCR chamber without allowing droplets to get on the sides of the tube or some of the reaction may not be exposed to the DSN enzyme treatment.
7. Pre-incubate the DSN reaction mix for 10 min at 68°C and then add 1 μ L (1U) DSN enzyme and mix well with the reaction tube in the thermocycler.
8. Incubate the DSN enzyme reaction for 10 min at 68°C.
9. Stop the DSN reaction by adding 41 μ L of cold custom DSN 2X stop buffer and store on ice until the clean-up with AMPure XP beads or store at -20°C.

DSN reaction clean-up with AMPure XP beads

1. Add the 82 μ L DSN reaction to 131 μ L AMPure XP beads pre-equilibrated to room temperature in a 1.7mL tube (1.6:1 vol/vol XP:DSN).
2. Follow the PCR1 AMPure XP clean-up.
3. Elute the DSN normalized library in 30 μ L and transfer to a clean 0.2mL PCR tube.

BASIC PROTOCOL 5: PCR2 AMPLIFICATION AND BARCODE ADDITION OF DSN NORMALIZED LIBRARIES

Illumina HiSeq platforms generate around 200 million reads per flow cell lane while only 50 million reads is sufficient per sample for total RNA fractions (smRNA and FLgRNA each). Adding different barcodes (or index sequences) to each separate library allows the user to combine samples into one lane and the data are later sorted out with bioinformatics. It is typically necessary to combine four standard eukaryotic RNA-Seq libraries per lane for sufficient numbers of reads on the Illumina HiSeq platform. Choose the barcodes before PCR2 since not all barcode sequences are compatible in a single lane. Follow the Illumina guidelines for barcode selection when multiplexing fewer than 12 samples per flow cell lane (Illumina support web site). Adjust the number of PCR cycles to produce around 15–20 ng/ μ L concentrations (450–600ng total).

Materials

0.2mL PCR tube
 DSN normalized library
 RS-TS PCRI-# barcoded primer (25uM)
 RS-TS PCR2D primer (25uM)
 100mM dNTP mix (Bioline)
 Phusion DNA polymerase (New England Biolabs)
 Thermocycler with a heated lid
 AMPure XP beads (Beckman Coulter)
 EB elution buffer (Qiagen)
 2100 Bioanalyzer (Agilent)
 Qubit dsDNA HS assay (Life Technologies)
 Bioanalyzer HS DNA kit (Agilent)

1. Add the following reagents to the DSN normalized library.

30μL DSN normalized library
 20μL Phusion reaction buffer (5X)
 2μL RS-TS I-# barcoded primer (25μM)
 2μL RS-TS PCR2D (25μM)
 1μL 100mM dNTP mix
 1μL Phusion DNA polymerase (2u)
 44μL RNase-free water

2. Amplify the reaction using the following PCR conditions.

1 cycle:	98°C	30 sec
20 cycles:	98°C	10 sec
	55°C	30 sec
	72°C	15 sec
1 cycle:	72°C	10 min
	4°C	hold

- 3.** Clean-up PCR2 barcoded libraries with 120μL AMPure XP beads equilibrated to room temperature using 1.2:1 ratio of AMPure XP beads to PCR2 reaction volumes based on the bead calibration (Support Protocol 1).
- 4.** Elute finished PCR2 RNA-Seq library in 30μL EB buffer and then add 0.6μL 5% Tween 20. To elute the DNA from the beads, add 32μL EB and remove only 30μL.

5. Determine the concentration of the PCR2 DNA using the Qubit dsDNA HS assay kit.
6. Dilute the PCR2 DNA to 0.5 ng/ μ L with EBT and determine the average fragment size using the Bioanalyzer HS DNA kit.
7. Repeat the AMPure XP bead clean-up again with a 1.2:1 ratio of beads again if there are significant adapter dimers present.

SUPPORT PROTOCOL 2: qRT-PCR VALIDATION OF DSN rRNA SEQUENCE REDUCTION

The DSN normalization must be validated by qRT-PCR to ensure adequate reduction of the rRNA sequences. Test with 5S rRNA primers for smRNA and 28S rRNA primers for FLgRNA libraries (Table 3). The 5.8S rRNA is not a valid indicator for smRNA libraries in most cell lines but 18S rRNA can also be used for FLgRNA. Suitable rRNA reductions are 50-, 100- and 200-fold for 5S, 18S and 28S rRNAs in these RNA-Seq libraries.

Materials

PCR1 library
PCR2 library
480 LightCycler (Roche)
LightCycler 480 SYBR Green I Master (Roche)
EB buffer (Qiagen)
Tween 20 (Promega)
5S rRNA PCR primers (ThermoFisher)
28S rRNA PCR primers (ThermoFisher)
EEF1A1 PCR primers (ThermoFisher)
U6 snRNA PCR primers (ThermoFisher)
Nuclease-free water
480 LightCycler 96 well plate (Roche)

qRT-PCR assay for rRNA reduction

1. Dilute PCR1 and PCR2 DNA 1:30 (vol/vol) to approximately 0.5ng/ μ L with EB buffer containing 0.1% Tween 20 (EBT).
2. Combine the following reagents in a 96 well 480 LightCycler plate for qRT-PCR in triplicate.
 - 5 μ L diluted PCR1 or PCR2
 - 1 μ L PCR primer mix (5 μ M forward and reverse)
 - 4 μ L RNase-free water

15µL LightCycler 480 SYBR Green I Master mix

3. Mix each reaction (in triplicate) 5X with a pipette.
4. Use the manufacturer's recommended qRT-PCR experimental design with a 60°C annealing temperature.
5. Determine the C_T values using the 480 LightCycler analysis program "Absolute Quantification Analysis Using Fit Points Method". Set the upper cycle count appropriately to eliminate secondary inflection curves from interfering with the C_T calculation (Figure 5).
6. Analyze the rRNA reduction using the C_T method (Livak, K. and Schmittgen, T., 2001). Test for the DSN reduction of 5S rRNA and 28S rRNA by comparing PCR2 to PCR1 with reference genes U6 snRNA and EEF1A1 for smRNA and FLgRNA libraries respectively. PCR primers should be designed for RNA-Seq validation since the library inserts are smaller than standard cDNA preparations. The qRT-PCR product sequence should be 80–100bp. PCR1 (or PCR2) can also be used for standard qRT-PCR analysis but require less input RNA (Figure 6).

BASIC PROTOCOL 6: QUANTIFICATION AND POOLING BARCODED LIBRARIES FOR SEQUENCING ON THE ILLUMINA PLATFORM

It is critical to accurately determine the concentrations before sequencing the libraries. Cluster identification on the flow cell is very sensitive to concentration and size of the library elements. Determine dsDNA concentrations with the Qubit HS DNA kit for more accuracy. Use the average size of the library elements obtained from the Bioanalyzer HS DNA chip to calculate the molecular weight. Then use the Qubit concentration with the Bioanalyzer average size (Region table) to calculate the nanomolar concentration (see below).

Materials

Qubit dsDNA HS assay (Life Technologies)
 2100 Bioanalyzer (Agilent)
 Bioanalyzer HS DNA kit (Agilent)
 EB buffer (Qiagen)
 Tween 20 (Promega)
 Nuclease-free water (Sigma)

Library concentration determination and multiplexing with barcodes

1. Determine the concentration of the libraries to be pooled using the Qubit HS DNA assay kit.
2. Dilute the libraries to 0.5–1.0 ng/µL with EB buffer containing 0.1% Tween 20 (EBT), mix and wait 5 min to degas.

3. Run 1 μ L of each diluted library on a Bioanalyzer HS DNA chip.
4. Determine the average fragment size for each library in the Bioanalyzer Region table.
5. Calculate the library concentration using the equation, $nM = \{[\text{conc (ng}/\mu\text{L})] \times (10^6 \mu\text{L/L})\} / \text{MW (ng/nmole)}$ such that $\text{MW dsDNA} = [(\text{size bp}) \times 607] + 158 = \text{ng/nmole}$.
6. Dilute each library with EBT to a concentration of 17.5nM, mix, wait 5 min to degas, and combine equal volumes for multiplexing.
7. Determine the final concentration of the pooled libraries using the Qubit HS DNA assay kit and Bioanalyzer HS DNA chip as before.
8. Check with the Illumina instrument operator for instructions for library submission. Typically, for running on the Illumina HiSeq2000, add 35 fmoles of the pooled libraries (2 μ L of 17.5nM) to 16.5 μ L EBT with a final volume of 18.5 μ L to be run in a single flow cell lane.

ADAPTING THIS APPROACH TO OTHER ADAPTERS

This original protocol is designed for the Illumina small RNA v1.5 single read flow cell but can be adapted to other platforms by changing the adapters and PCR1 primer lengths. Give careful consideration to the adapter lengths for proper thermal stability. Optimal adapter T_m should be more than 5°C below 68°C for the DSN hybridization and step yet high enough for the PCR primers to anneal. Additional sequences can be added during PCR2 along with the barcodes following the DSN normalization step. This strategy is demonstrated for the Illumina small RNA v1.5 sequences seen in Tables 2 and 3.

REAGENTS AND SOLUTIONS

Prepare all solutions with nuclease-free water (Sigma). Use aerosol resistant tips when pipetting and transferring samples and solutions. Wear gloves when handling all reagents. All primary stock solutions (*e.g.*, 1M Tris-HCl) were filter-sterilized and stored at room temperature. Mixed buffers (*e.g.*, DSN hybridization buffer) were prepared and stored frozen.

Materials

Tris-HCl (Sigma)
Tris Base (Sigma)
Nuclease-free water (Sigma)
Tween 20 (Promega)
50% PEG8000 (Life Technologies)
NaCl (Sigma)
Glycerol (Sigma)

1M MgCl₂ (Ambion)

EDTA (Sigma)

Custom DSN buffers

All duplex-specific nuclease (DSN) buffers are custom-made by mixing 1M stocks of Tris-HCl and Tris Base in order to adjust the pH without additional salts with the exception of the custom DSN stop buffer. Store all mixed buffer aliquots at −20°C. Reconstitute DSN in 50μL of custom DSN storage buffer (50mM Tris pH8) and 50μL glycerol according to the Evrogen directions.

DSN storage buffer (pH 8)

28μL 1M Tris-HCl

22μL 1M Tris Base

950μL nuclease-free water

10X DSN hybridization buffer (pH 8)

200μL 1M NaCl

280μL 1M Tris-HCl

220μL 1M Tris Base

4μL Tween 20

296μL 50% PEG8000

2X DSN reaction buffer (pH 8)

56μL 1M Tris-HCl

44μL 1M Tris Base

40μL 1M MgCl₂

860μL nuclease-free water

2X DSN stop buffer (pH 8)

500μL 1M NaCl

200μL 0.5M EDTA pH8

300μL nuclease-free water

COMMENTARY

Background

RNA-Seq using high-throughput sequencing is a relatively new method for analyzing the transcriptome (Nagalakshmi et al., 2008). Numerous approaches have been implemented, each with unique advantages and disadvantages. In general, libraries can be generated with

PCR amplification using random primers with attached adapters, directed adapter ligations after cDNA synthesis or before cDNA synthesis in order to retain strand information of the source transcripts (McGettigan, P., 2012). One of the challenges facing RNA-Seq experiments is the removal of rRNA sequences that otherwise monopolize most of the sequencing data due to abundance. There are several techniques available for removing rRNA sequences from the libraries: hybridization to complementary sequences that are immobilized to magnetic beads or degraded with RNaseH, or poly(A⁺) selection (Morlan et al., 2012), . Here we have updated our optimized degradation of rRNA sequences using duplex-specific nuclease (DSN) following hybridization of the PCR1-amplified library precursor (Miller, D., et al., 2013). Many protocols utilize size selection by gel electrophoresis, which fails to capture the whole transcriptome (Peng et al., 2012). Our method allows stranded whole transcriptome coverage of all RNA types and is executed entirely in the microcentrifuge tube.

This protocol was described previously (Miller, D., et al., 2013) but has been improved. RNA-Seq libraries for a single sample are generated in two parts in order to capture the entire transcriptome (Figure 1). Total RNA is size-fractionated and the large RNA (LgRNA) is fragmented to accommodate sequencing on the flow cell. Following end-polishing of small RNA (smRNA) and fragmented large RNA (FLgRNA), adapters are attached to preserve strandedness, RNA is converted to DNA and the samples are amplified using PCR. rRNA sequences are removed from both smRNA and FLgRNA using limited hybridization followed by digestion with duplex-specific nuclease (DSN). Another round of PCR is used to add the remaining adapter sequences containing the barcode sequences to allow multiplex sequencing on the Illumina platform.

Critical Parameters

RNA quality is not essential, but starting with total RNA with RIN values (Bioanalyzer RNA pico) greater than 8 will improve results. Starting with as much as 5µg or more of good quality RNA will improve results but less can be used (Figure 6). We recommend using unfractionated total RNA for library generation when starting with low quantities or degraded RNA (follow protocols for LgRNA). The DSN reaction is the most critical step of the protocol. It may be necessary to design your own adapters and primers to allow adequate T_m ranges for other library adapters (Tables 2 and 3). Avoiding excessive adapter dimers is important for successful DSN reduction of rRNA sequences. This requires AMPure XP bead calibrations for accurate size selection following PCR1 amplification. Adapter dimers can be reduced by hybridizing the 3' reverse transcription primer (RS-TS PCR1A) to the free 3' adapters not attached to the RNA before ligating the 5' adapter, which has been incorporated into this protocol (Vigneault, F., et al., 2012). The DSN reaction requires that the sample be mixed well in a thermocycler with two separate chambers or a second thermocycler for preheating the DSN reaction buffer before mixing. Care should be taken not to leave sample droplets on the sides of the incubation tube to ensure adequate hybridization. Accurate salts concentrations are another critical part of the DSN reaction. This is accomplished by making custom buffers that mix Tris-HCl and Tris Base for pH control without additional salts. Some consideration for qRT-PCR primer design and product size (<100 bp) is necessary for qRT-PCR validation of the DSN reaction (see Miller, D., et al. 2013). In spite of these

considerations, the DSN reaction will occasionally need to be repeated, likely due to the mechanics of mixing the reagents in the thermocycler. Adding the barcode in the PCR2 reaction allows the investigator to keep the adapter sequences short for the DSN hybridization reaction so that only high copy rRNA sequences will be targeted for degradation. It is important to utilize the Illumina multiplexing strategy and accurate library quantification for highly efficient clustering on the flow cell. DNA quantification with the Qubit fluorometer in combination with the Bioanalyzer works best while the NanoDrop is more accurate for RNA concentrations. Samples should be allowed to degas for about five minutes after mixing, before running them on the Bioanalyzer.

Troubleshooting

See Table 4.

Anticipated Results

It is reasonable to expect high quality results by multiplexing four libraries per lane on the Illumina HiSeq with around 50 million reads per library. Single reads of 50 bases (SR50) are adequate but SR100 runs will generate better splicing results. Using SR50 flow cell runs on cell line total RNA, it would be reasonable to identify: 12,000 known transcripts, 200 unknown transcripts, 140 snoRNAs, 100 miRNAs, 27 mitochondrial RNAs, 100 alternately spliced transcripts, 1,300 repeat element RNAs, and 500 significant SNPs (Miller, D., et al., 2013, Strong, et al., 2014). This protocol also works well for qRT-PCR from small quantities of RNA since the samples can be amplified as needed (Figure 6). We have not explored the limits of this approach for qRT-PCR but starting with as little as 100ng RNA and as many as 30 cycles of PCR amplification (in two steps) does not appear to compromise the data.

Time Considerations

This protocol can be completed in one week allowing an additional day for quantification and pooling. Sample groups for parallel processing should be limited to around eight during the DSN step to avoid significant temperature fluctuations. Typically, it is possible to have two sets of eight samples processed in parallel. The RNA samples can be frozen at -80°C for storage after any given step while the cDNA can be stored at -20°C after any given step. The DSN step should be finished on the same day to keep the hybridization times constant.

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Figure 1. General workflow for RNA-Seq libraries. Abbreviations; DSN (Duplex-specific nuclease), smRNA (small RNA <200nt), FLgRNA (fragmented large RNA >200nt), RPKM (Reads Per Kb per Million reads).

Figure 2.

RNA profiles. RNA fractions were run on the 2100 Agilent Bioanalyzer RNA pico chip to demonstrate successful size fractionation and fragmentation profiles. Abbreviations; small RNA (smRNA), large RNA (LgRNA), fragmented large RNA (FLgRNA).

AMPure XP Bead Calibration Data

Figure 3. AMPure XP bead calibration. The AMPure XP beads are combined with the 20bp ladder (vol/vol) at the indicated volume ratio for the beads. Following the clean-up, each sample was run on a Bioanalyzer HS chip.

Figure 4.

Effect of adapter dimers on DSN efficiency. A-D. Bioanalyzer HS profiles for PCR1 and PCR2 reactions with low and high amounts of adapter dimers (AD). E. qRT-PCR validation for DSN reaction of PCR2 libraries with low and high adapter dimers.

Figure 5. qRT-PCR for library with a secondary inflection in the C_T curve. Absolute Quantification Analysis Using Fit Points Method curve for qRT-PCR data analysis with upper cycle count adjusted below the secondary inflection.

Figure 6.

Comparison of standard cDNA synthesis and RNA-Seq PCR1 for qRT-PCR with various input quantities of total RNA for PCR1. LRP6 mRNA fold change from tumor samples before and after treatment.

Table 1**AMPure XP bead calibration**

Size selection with the AMPure XP beads are calibrated for each new lot by mixing different ratios of beads with the 20 bp ladder. The products are then run the Bioanalyzer HS DNA chip to determine the cut-off sizes for different ratios.

XP:sample vol/vol ratio	XP bead volume	Approx. size cut-off - bp
none	none	None
0.6:1	48µL	400
0.7:1	56µL	300
0.8:1	64µL	200
1.0:1	80µL	175
1.2:1	96µL	150
1.4:1	112µL	125
1.6:1	128µL	100
1.8:1	144µL	100
2.0:1	160µL	100

Table 2

Customized Illumina Oligos for Small RNA v1.5 Libraries. T_m calculations for ave size 200bp (lib before index) and average %GC in 20 mM NaCl. $T_m = 81.5 + 16.6 \times \log_{10}(\text{cation conc}) + 0.41 \times \text{pctGC} - (675.0 / \text{length})$. T_m calculations for PCR reactions were done using the IDT OligoAnalyzer 3.1 with the following Phusion PCR salts; 50mM KCl, 2mM MgCl₂.

Custom smRNA v1.5 Oligos	T_m -DSN HYB	T_m -PCR	%GC
RS-TS-5' adapter	60.4		50.0
RS-TS-5'/PCR2D overlap		61.9	50.0
RS-TS-5' end (final)	61.3		53.1
RS-TS-3' adapter	55.2		54.5
RS-TS-3' PCR1A	55.2		54.5
RS-TS-3'/PCR1A overlap		72.3	54.5
RS-TS-3' PCR-I-1	64.5		53.1
RS-TS-3'/PCR-I-1 overlap		72.3	54.5
RS-TS-3' end (final)	64.5		53.1
Targets	T_m -DSN HYB		%GC
5.8S rRNA	72.4		57.0
5S rRNA	76.4		59.5
18S rRNA	71.9		56.1
28S rRNA	77.2		69.4

Table 3

Custom Small RNA-Seq v1.5 Primers, Adapters and qRT-PCR Primer Sequences.

Small RNA-seq Version	Primer	Sequence
Custom Illumina v1.5	RS-TS-5' adapter	G*TTCAGAGTTCTACAGTCCGACGATC
	RS-TS-3' adapter ^a	/5rApp/GATCGGAAGAGCACACGTCTGAACTCCAGTCAC*/3ddC/
	RS-PCR2D ^a	A*ATGATACGGCGACCACCGACAGGTTTCAGAGTTCTACAGTCCGAC
	RS-TS-PCR1A ^a	G*TGACTGGAGTTCAGACGTGTGCTCTTCCGATC
	RS-TS-PCR-I-1 ^b	G*CAAGCAGAAGACGGCATACGAGAT CGTGAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
qRT-PCR Primers	5S rRNA Forward	CGATCTCGTCTGATCTCGGAAG
	5S rRNA Reverse	AGGCGGTCTCCCATCCAAG
	5.8S rRNA Forward	GGCTCGTGCGTCGATGAAG
	5.8S rRNA Reverse	CGCTCAGACAGGCGTAG
	18S rRNA Forward	CCAGTAAGTGCGGGTCATAAGC
	18S rRNA Reverse	CCATCCAATCGGTAGTAGCGAC
	28S rRNA Forward	TCAGACCCAGAAAAGGTGTTG
	28S rRNA Reverse	TGATTCGGCAGGTGAGTTGTTAC
	EEF1A1 Forward	GCCCCAGGACACAGAGACTTTATC
	EEF1A1 Reverse	CAACACCAGCAGCAACAATCAG
	U6 Forward	CGCTTCGGCAGCACATATAC
	U6 Reverse	TTCACGAATTTGCGTGTCAT
	LRP6 Forward	CGGTGAGAGAAGAGAACGCG
	LRP6 Reverse	GCCTCCAACATAATCGTAGC

^a* indicates phosphorothioate bond; /5rApp/ indicates 5' adenylation; /3ddC/ indicates 3' dideoxy-C

^b bold indicates the index sequence

Table 4

Troubleshooting

Problem	Cause	Solution
Low PCR1 yield	Adapter dimers in PCR1 or low starting quantities	Repeat AMPure XP beads clean-up and/or amplify 5–10 additional cycles. Check the AMPure XP bead calibration.
Low PCR2 yield	Adapter dimers in PCR1 and/or PCR2 or too much salt in the DSN reaction	Repeat AMPure XP beads clean-up on PCR1 and/or amplify 5–10 additional cycles. Check the AMPure XP bead calibration.
Poor rRNA reduction	Adapter dimers in PCR1 or mechanical failure during the DSN reaction	Remove adapter dimers with additional AMPure XP bead clean ups. Mix the DSN hybridization and reaction well, keep the temperature at a constant 68°C during these steps.
No mature miRNA in the smRNA library	Loss during RNeasy isolation of RNA or AMPure XP bead clean-up due to size selection or low ethanol concentration in the bead washes	Use RWT buffer with the proper amount of ethanol for the RNeasy column wash. Check the AMPure XP bead calibration. Make 80% ethanol fresh for RNeasy column and bead washes.
Reduction of rRNA qRT-PCR validation inaccurate	Secondary inflection in the amplification curve resulting in high C_T values for the library rRNA amplification	Use the 'Absolute Quantification Analysis Using Fit Points Method' to set the cycle count lower for qRT-PCR on the 480 LightCycler.
Poor PCA for biological replicates	Poor size fractionation of the input RNA due to overloading the RNeasy Minelute column	Use RNeasy mini column for the size fractionation or use less RNA. Typically the data will still be robust.