SOP Title: illumina TruSeq RNA Sample Preparation	Version 1.1, Page 1
Authorized Signature:	Issuing Date: 02.09.11
	Last Revision Date: 11.21.11 - SV

Staff Able to Perform Procedure: Research Technician and higher

Principle of the Method:
This protocol explains how to convert mRNA in total RNA samples into a library of template molecules suitable for subsequent cluster generation using the reagents provided in the illumina reagents kit.

Sample Type: 100ng – 4ug of high quality total RNA

Equipment Requirements:

- Agilent 2100 Bioanalyzer (Agilent)
- PicoTiter Plate Shaker (Fischer)
- Bioanalyzer Chip Vortex (IKA)
- Fluorometer (TBS)
- Thermal cycler (Applied Biosystems)
- Vortex (VWR)
- Microcentrifuge (VWR)
- Magnetic Stand (Ambion)
- Timer (1 hour)

Reagents & Material Requirements:

- TruSeq RNA Sample Preparation Kit (illumina)
- Agencourt AMPure XP 60 ml kit (Beckman Coulter)
- Agilent RNA 6000 Nano Bioanalyzer Chip Kit (Agilent)
- Quant-IT Ribogreen Assay (Invitrogen)
- SuperScript II Reverse Transcriptase (Invitrogen)
- Buffer EB (Qiagen)
- Tween-20 (Sigma)
- 100% Ethanol (VWR)
- 80% Ethanol (VWR)
- Ultra pure water (VWR)
- Plate sealers (VWR)
- 96-well 0.4ml PCR plates (Applied Biosystems)
- RNAse- free centrifuge tubes 1.5ml, 200 ul (USA Scientific)
- Pipette Man 2ul, 20ul, 200ul, 1000ul (Rainin)
- Filter Pipette Tips (Rainin)

Procedural Notes

- For first time use, remove one tube each of Bead Binding Buffer, Bead Washing buffer, Elution Buffer, and Elute, Prime, Fragment Mix from -15 to -25 C storage and thaw them at room temperature
  - Store Bead Binding Buffer, Bead washing Buffer, and Elution Buffer at 4C after use for subsequent experiments
- Remove the RNA Purification Beads tube from storage and let stand to bring to room temperature
- Pre-heat the thermal cycler to 100°C.

- Pre- program the thermal cycler with the following programs:

Program Name	Temp #1	Temp #2	Temp #3	Temp #4
mRNA Denaturation	65 C for 5 minutes	4C hold		
mRNA Elution 1	80C for 2 minutes	25C hold		
Elution 2 – Frag – Prime	94C for 8 minutes	4C hold		
1st strand cDNA	25C for 10 minutes	42C for 50 minutes	70C for 15 minutes	4C hold

Make RNA Bead Plate (RBP)

*****_A 200 ul PCR tube can be used if only a few samples are being processed at one time._

- Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50 µl in a 96-well PCR plate.
- Vortex the thawed RNA purification Beads to completely resuspend.
- Add 50 µl of RNA Purification Beads to each well of the RBP plate.
- Pipette to mix 6 times.
- Seal the RBP plate with strip tube caps.

Incubation #1: RNA Bead Plate (RBP)

- Place the RBP plate in the thermal cycler and select the **mRNA Denaturation** program.
- Remove the RBP plate from the thermal cycler when it reaches 4°C.
- Place the RBP plate on the bench and incubate at room temperature for 5 minutes.

Wash the RNA Bead Plate (RBP)

- Place the RBP plate on the magnetic stand at room temperature for 5 minutes
- Remove the seal from the plate
- Remove and discard all supernatant from each well of the RBP.
- Remove plate from stand and wash beads by adding 200 ul of Bead Washing Buffer in each well of the RBP.
- Pipette to mix 6 times.
- Place the RBP on the magnetic stand at room temperature for 5 minutes.
- Remove and discard all of the supernatant from each well.
- Briefly centrifuge the thawed Elution Buffer in a minifuge for 5 seconds.
- Remove and discard all of the supernatant from each well.
- Remove the RBP plate from the magnetic stand.
- Add 50 ul of Elution Buffer in wash well of the RBP.
- Pipette to mix 6 times.
- Seal the RBP with strip caps
- Store the Elution Buffer at 4°C

Incubation #2: RNA Bead Plate (RBP)

- Place the RBP plate in the thermal cycler and select the **mRNA Elution 1** program.
- Remove the RBP plate from the thermal cycler when it reaches 25°C.
- Place the RBP plate on the bench and remove strip caps

Make RNA Fragmentation Plate (RFP)

- Add 50 ul of Bead Binding Buffer to each well of the RBP plate.
- Pipette to mix 6 times.
- Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 4°C.
- Place the RBP on the magnetic stand at room temperature for 5 minutes.
- Remove and discard all supernatant.
- Remove the RBP plate from the magnetic stand.
- Wash the beads by adding 200 ul of Bead Washing Buffer in each well.

- Pipette to mix 6 times.
- Store the Bead Washing Buffer tube at 4°C
- Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
- Remove and discard all supernatant.
- Remove the RBP plate from the magnetic stand.
- Add 19.5 ul of Elute, Prime, Fragment Mix to each well.
- Pipette to mix 6 times.
- Seal the RBP with strip caps
- Store the Elute, Prime, Fragment Mix tube at -20°C.

Incubate RNA Fragmentation Plate

- Place the RBP plate in the thermal cycler and select the **Elution 2 – Frag - Prime** program.
- Remove one tube of Frist Strand Master mix from -20°C storage and thaw it at room temperature.
  - Aliquot into smaller quantities if more than six freeze thaws is anticipated.
- Remove the RBP plate from the thermal cycler when it reaches 4°C.
- Proceed immediately

Make cDNA Plate (CDP)

**A 200 ul PCR tube can be used if only a few samples are being processed at one time.*

- Place the RPB plate on the magnetic stand at room temperature for 5 minutes.
- Remove the strip caps
- Transfer 17ul of the supernatant (CONTAINS mRNA) and place in new 96-well PCR plate labeled CDP.
- Briefly centrifuge the thawed First Strand Master Mix in a minifuge for 5 seconds.
- Add 50 ul of SuperScript II to the First Strand Master Mix tube (ratio: 1 ul SuperScript II for each 7 ul First Strand Master Mix).
- Mix gently and centrifuge briefly
- Add 8 ul of First Strand Master Mix and SuperScript II mix to the samples in the CDP plate.
- Pipette to mix 6 times.
- Seal the CDP plate with strip caps
- Store the Fist Strand Master Mix tube at -20°C.

Incubate #1: cDNA Plate

- Place the CDP plate in the thermal cycler and select the **cDNA 1st Strand** program.
- Remove one tube each of Second Strand Master Mix and Resuspension Buffer from -20°C and taw it at room temperature
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring to room temperature
- Remove the CDP plate from the thermal cycler when it reaches 4°C.
- Proceed immediately

Add Second Strand Master Mix (SSM)

- Briefly centrifuge the thawed Second Strand Master Mix in a minifuge for 5 seconds.
- Add 25 ul of Second Strand Master mix to each well of the CDP plate.
- Pipette to mix 6 times.
- Seal the CDP plate with strip caps.

Incubate #2: cDNA Plate

- Incubate the CDP plate with the lid closed at 16°C for 1 hour. DO NOT PLACE PLATE IN THERMOCYCLER UNTIL TEMPERATURE HAS REACHED 16C.
- Remove the CDP plate from the thermocycler and let stand to bring the plate to room temperature

Clean Up cDNA Plate

- Vortex the AMPure XP beads until they are well dispersed
- Add 90 ul of AMPure XP beads to each of the CDP plate samples
- Gently pipette to mix 10 times.
- Incubate the CDP plate at room temperature for 15 minutes
- Place the CDP plate on the magnetic stand at room temperature for at least 5 minutes..
- Remove and discard 135 ul of the supernatant from each well.
- **Leave the CDP plate on the magnetic stand while performing the following 80% Ethanol wash steps**
- Add 200 ul of freshly prepared 80% EtOH to each well without disturbing the beads.
- Incubate the CDP plate at room temperature for 30 seconds, then remove and discard all of the supernatant.
- Add 200 ul of freshly prepared 80% EtOH to each well without disturbing the beads.
- Incubate the CDP plate at room temperature for 30 seconds, then remove and discard all of the supernatant.
- Let the plate stand at room temperature for 15 minutes to dry and then remove the CDP plate from the magnetic stand.
- Briefly centrifuge the thawed Resuspension Buffer in a minifuge for 5 seconds.
- Add 52.5 ul Resuspension Buffer to each well of the CDP plate.
- Gently pipette to mix 10 times.
- Incubate the CDP plate at room temperature for 5 minutes
- Place the CDP plate on the magnetic stand at room temperature for 5 minutes.
- Transfer 50 ul of the supernatant from the CDP plate to the new 0.3 ml PCR plate labeled IMP
- SAFE STOPPING POINT. Can store sealed plate at -20C for up to 7 days.

Preparation for End Repair

- Thaw one tube of End Repair Mix and one tube of End Repair Control at room temperature. Aliquot if you are not using all 48 reactions
- Remove the IMP plate from -20°C and thaw at room temperature
- Remove AMPure beads from 4°C and let stand at room temperature for at least 30 minutes.
- Pre-heat thermocycler lid to 30°C

Making IMP

- Centrifuge thawed End Repair Control tube to 600 xg for 5 seconds and dilute the End Repair Control to 1/100 in Resuspension Buffer (1 µl End Repair Control + 99 µl Resuspension Buffer). Discard the diluted End Repair Control after use.
- Add 10 µl of diluted End Repair Control (or 10 µl of Resuspension Buffer) to each well of the IMP plate.
- Add 40 µl of End Repair Mix to each well of the IMP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

- Incubate the IMP plate on the pre-heated thermal cycler, with the lid closed, at 30°C for 30 minutes.
- Remove the IMP plate from the thermal cycler.

Clean Up IMP

- Remove adhesive seal from the IMP plate.
- Vortex the beads until well dispersed, then add 160 µl of well-mixed beads to each well of the IMP plate. Gently pipette up and down 10 times to mix.
- Incubate the IMP plate at room temperature for 15 minutes.
- Place IMP plate on magnetic stand at room temperature for at least 5 minutes, until liquid appears clear.

- Remove and discard 127.5 µl of the supernatant from each well of the IMP plate. Repeat once.
- **Leave the IMP plate on the magnetic stand while performing the following 80% Ethanol wash steps**
- Add 200 µl of freshly prepared 80% EtOH to each well. Incubate the IMP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Repeat once for a total of two 80% EtOH washes.
- Let the IMP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- Resuspend dried pellet in 17.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times.
- Incubate the IMP plate at room temperature for 2 minutes.
- Place the IMP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- Transfer 15 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the new 0.3 ml PCR plate labeled ALP plate.

Preparation of Adenylate 3' Ends

- Remove one tube of A-Tailing Mix and one tube of A-Tailing Control per 48 reactions from storage and thaw them at room temperature.
- Remove the ALP plate from storage, and thaw to room temperature.
- Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute, then remove the adhesive seal from the plate.
- Pre-heat thermal cycler to 37oC.

Add ATL

- Briefly centrifuge the A-Tailing Control tube to 600 xg for 5 seconds and dilute the control to 1/100 in Resuspension Buffer (1 µl A-Tailing Control + 99 µl of Resuspension Buffer) before use. Discard the diluted A-Tailing control after use.
- Add 2.5 µl of diluted A-Tailing Control (or 2.5 µl of Resuspension Buffer) to each well of the ALP plate.
- Adjust pipette to 30 µl and gently pipette the entire volume up and down 10 times.
- Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate.
- Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

- Incubate the ALP plate on the thermal cycler, with the lid closed, at 37°C for 30 minutes.
- Immediately remove the ALP plate from the thermal cycler and proceed immediately.

Preparation of Ligate Adapters

- Remove the appropriate RNA Adapter Index tubes and one tube of Stop Ligase Mix and Ligase Control, per 48 reactions from storage, and thaw at room temperature. Remove the AMPure XP beads from storage and let stand for at least 30 minutes at room temperature.
- Pre-heat the thermal cycler to 30°C.

Add LIG

- Centrifuge the thawed RNA Adapter Index tubes, Ligase Control, and Stop Ligase Mix tubes to 600 xg for 5 seconds.
- Immediately before use, remove the DNA Ligase Mix tube from storage.
- Remove the adhesive seal from the ALP plate.
- Add 2.5 µl of DNA Ligase Mix to each well of the ALP plate.
- Return the DNA Ligase mix tube back to -20°C storage immediately.
- Dilute Ligase Control to 1/100 in Resuspension Buffer (1 µl Ligase Control + 99 µl Resuspension Buffer). Discard diluted Ligase Control after use.

- Add 2.5 µl of diluted Ligase Control (or 2.5 ml of Resuspension Buffer) to each well of the ALP plate.
- Add 2.5 µl of each thawed RNA Adapter Index to each well of the ALP plate.
- Adjust pipette to 40 µl and gently pipette up and down 10 times.
- Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 2 ALP

- Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed, at 30°C for 10 minutes.
- Remove the ALP plate from the thermal cycler.

Add STL

- Remove the adhesive seal from the ALP plate
- Add 5 µl of Stop Ligase Mix to each well of the ALP plate. Gently pipette up and down 10 times.

Clean Up ALP

- Vortex the AMPure XP beads until they are well dispersed, then add 42 µl of mixed AMPure XP Beads to each well of the ALP plate. Gently pipette up and down 10 times.
- Incubate the ALP plate at room temperature for 15 minutes.
- Place the ALP plate on the magnetic stand at room temperature for at least 5 minutes or until liquid appears clear.
- Remove and discard 79.5 µl of the supernatant from each well of the ALP plate. Do not disturb beads.
- **Leave the ALP plate on the magnetic stand while performing the following 80% Ethanol wash steps.**
- Add 200 µl of freshly prepared 80% EtOH to each well. Incubate the ALP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Repeat once for a total of two 80% EtOH washes.
- Let the ALP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- Resuspend dried pellet in 52.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times.
- Incubate the ALP plate at room temperature for 2 minutes.
- Place the ALP plate on the magnetic stand at room temperature for at least 5 minutes or until the liquid appears clear.
- Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled CAP plate.
- Vortex the AMPure XP Beads, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up. Gently pipette up and down 10 times.
- Incubate the CAP plate at room temperature for 15 minutes.
- Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until liquid appears clear.
- Remove and discard 95 µl of the supernatant from each well of the CAP plate.
- **Leave the CAP plate on the magnetic stand while performing the following 80% Ethanol wash steps**
- Add 200 µl of freshly prepared 80% EtOH to each well without disturbing beads. Incubate CAP plate at room temperature for at least 30 seconds, then remove and discard supernatant. Do not disturb beads. Repeat for a total of two 80% ETOH washes.
- Let CAP plate stand at room temperature for 15 minutes to dry and then remove plate from the magnetic stand.
- Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Pipette up and down 10 times.

- Incubate the CAP plate at room temperature for 2 minutes.
- Place the CAP plate on the magnetic stand at room temperature for at least 5 minutes or until the liquid appears clear.
- Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the next 0.3 ml PCR plate labeled PCR plate.
- SAFE STOPPING POINT. Can store sealed plate at -20°C for up to 7 days.

Enrich DNA Fragments

- Remove one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage and let stand to thaw at room temperature. Briefly centrifuge the thawed PCR plate to 280 xg for 1 minute. Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler as follows:
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C
- Pre-heat the thermal cycler lid to 100°C.

Make PCR

- Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate.
- Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Adjust the pipette to 40 µl and pipette the entire volume up and down 10 times to mix.

Amp PCR

- Amplify the PCR plate in the pre-programmed thermal cycler (see above), with the lid closed.

Clean Up PCR

- Vortex the AMPure XP beads, then add 50 µl of the mixed beads to each well of the PCR plate. Pipette entire volume up and down 10 times to mix.
- Incubate the PCR plate at room temperature for 15 minutes.
- Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until liquid appears clear.
- Remove and discard 95 µl of the supernatant from each well.
- **Leave the PCR plate on the magnetic stand while performing the following 80% Ethanol wash steps**
- Add 200 µl of freshly prepared 80% EtOH to each well.
- Incubate the PCR plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well.
- Repeat the last two steps for a total of two 80% EtOH.
- Let the plate stand for 15 minutes to dry and then remove the plate from the magnetic stand.
- Resuspend with 32.5 µl Resuspension Buffer. Pipette the entire volume up and down 10 times.
- Incubate the PCR plate at room temperature for 2 minutes.
- Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

- Transfer 30 µl of the clear supernatant from each well to a new 0.3 ml PCR plate (the TSP1 plate).
- ***Safe Stopping Point; store at -15° to -25°C for up to seven days.

aRNA Quantification using Quant-IT RiboGreen Assay

- Preparing the TE and Dye Mix
  - Remove Ribogreen Dye from -20°C and thaw in dark drawer for 1 hour +
  - Determine the quantity of reagents to produce by adding three samples to the total number of RNA samples to be quantified (ex. If quantifying 8 samples make enough reagents for 11 samples)
  - To prepare 1X TE Solution for 1 sample : Add 0.1 ml of 20X TE to 1.9 ml of Milli-Q Water
  - To prepare Ribogreen Dye Solution for 1 sample: Add 5 ul of Ribogreen to 0.995 ml of 1X TE
  - Label PMMA Cuvettes for total RNA Samples along with a cuvette each for Blank and Control
  - Add 1 ml of 1X TE to each cuvette
  - Add 1 ul of total RNA sample to each sample cuvette and 1 ul of RNA control (yellow) to Control cuvette
  - Add 1 ml of Ribogreen Dye Solution to each cuvette. Pipette up and down 6x and change tips between cuvettes
  - Incubate samples at room temperature for 5 minutes.
- Operating TBS-Fluorometer
  - Turn on fluorometer with ‹ON/OFF› button
  - Press ‹CAL› and ‹ENTER› to begin calibration of samples
  - Shake Blank cuvette and insert into fluorometer. Press ‹ENTER›
  - After Blank has completed, insert Control cuvette into fluorometer. Press ‹ENTER›
  - To accept calibration, press ‹ENTER›
  - To proceed with total RNA quantification, shake sample cuvette and insert into fluorometer. Press ‹READ›
  - Read each sample cuvette twice and record concentration in ng/ul

Evaluation of Fragmented RNA Samples

- Evaluate 1 ul of each fragmented sample on an Agilent 2100 Bioanalyzer RNA 6000 Nano chip. The reaction should produce a distribution of 35-200 nt with a peak at approximately 100-120 nt.
- Loading the Gel-Dye Mix
  - Put a new RNA 6000 Nano chip on the chip priming station
  - Pipette 9.0 ul of gel-dye mix in the well marked Ⓖ.
  - Make sure the plunger is positioned at 1 ml, at the highest position on the chip priming station and then close the chip priming station.
  - Press the plunger until it is held by the clip
  - Wait for exactly 30 seconds then release clip
  - Wait for 5 seconds. Slowly pull back plunger to 1 ml position
  - Open chip priming station and pipette 9.0 ul of gel-dye mix into the wells maker G.
  - Discard the remaining gel-dye mix
- Loading the Agilent RNA 6000 Nano Marker
  - Pipette 5 ul of RNA 6000 Nano marker (green) in all wells sample will be added plus the well marked ladder.
- Loading the Ladder and Samples
  - Pipette 1 ul of prepared ladder(yellow) in well marked ladder
  - Pipette 1 ul of sample in each of the sample wells being used.
  - Pipette 6 ul of RNA 6000 Nano marker (green) in all wells not being analyzed.
  - Put the chip horizontally in the adapter of the IKA vortexer and vortex for 1 min at 2400

rpm.
    - Run the chip on the Agilent 2100 Bioanalyzer within 5 min.
- Saving Results
    - Enter sample names and information on the "Chip Summary" screen
- Press Print key and save the experiment as a .pdf file.

Preparation of Pool Libraries (optional)

- Remove the TSP1 plate from -20oC storage and let stand to thaw at room temperature. Briefly centrifuge the thawed TSP1 plate to 280 xg for 1 minute. Remove the adhesive seal.

Make DCT

- Transfer 10 µl of sample library from each well of the TSP1 plate to the new MIDI plate.
- Normalize the concentration of sample library in each well of DCT plate to 10 nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.
- Pipette entire library volume up and down 10 times to mix.
- Do one of the following:
    - For non-multiplexed libraries: Proceed to cluster generation or seal DCT plate with a Microseal 'B' adhesive and store at -15° to -25°C.
    - For multiplexed libraries: Proceed to Make PDP.

Make PDP

- **Do not make a PDP plate if there is no pooling.**
- Determine the number of samples to be combined in each pool.
- Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to a new 0.3 ml PCR plate (the PDP plate).
- Pipette the entire volume up and down 10 times to mix.
- Do one of the following:
    - Proceed to cluster generation OR
    - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.