SOP Title: SMARTer Ultra Low RNA Sample Preparation	Version 1.1, Page 1
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Staff Able to Perform Procedure: Research Technician and higher

Principle of the Method:
This protocol allows high-quality cDNA synthesis starting from as little as 100 pg of total RNA or cells.

Sample Type: 10pg- 10ng of high quality total RNA (at least 100pg is recommended)

Equipment Requirements:
- Agilent 2100 Bioanalyzer (Agilent)
- PicoTiter Plate Shaker (Fischer)
- Bioanalyzer Chip Vortex (IKA)
- Fluorometer (TBS)
- Thermal cycler (Applied Biosystems)
- Covaris S2 or E210 System (Covaris)
- IsoFreeze Flipper Rack (MIDSCI)
- IsoFreeze PCR Rack (MIDSCI)
- 96-well 0.4ml PCR plates (Applied Biosystems)
- Vortex (VWR)
- Microcentrifuge (VWR)
- Magnetic Stand (Ambion)
- Timer (1 hour)

Reagents & Material Requirements:
- SMARTer Ultra Low RNA Kit for Illumina Sequencing (2 Boxes; Clontech)
- DNA-OFF Solution (Takara)
- Advantage 2 PCR Kit (Clontech)
- High sensitivity DNA Kit (Agilent)
- Agencourt AMPure PCR Purification Kit (Beckman Coulter)
- Covaris 100 ul (6 X 16 mm) round bottom tubes with AFA fiber (KBiosciences)
- Illumina Paired-End DNA Sample Prep Kit (Illumina)
- 80% Ethanol (VWR)
- Plate sealers (VWR)
- RNAse- free centrifuge tubes 1.5ml, 200 ul (USA Scientific)
- Pipette Man 2ul, 20ul, 200ul, 1000ul (Rainin)
- Filter Pipette Tips (Rainin)
- MicroAmp Clean Adhesive Film
- QIAGEN EB Buffer

Procedural Notes
- Prior to cDNA synthesis, make sure that RNA is intact and free of contaminants.

First-Strand cDNA Synthesis
- **Perform in PCR Clean Work Station
- Prepare a stock solution of reaction buffer by mixing 19 µl Dilution buffer and 1 µl RNase Inhibitor.
- Prepare each sample as follows, transferring each whole volume of 3.5 µl to individual 0.2 mL PCR tubes (the control RNA is supplied at a concentration of 1 µg/µl; Control RNA should be diluted in nuclease-free water to match concentration of test sample):

Components	*Negative Control*	*Positive Control*	*Test Sample*
Reaction Buffer	2.5 µl	2.5 µl	2.5 µl
Nuclease-free water	1 µl	-	-
Diluted Control RNA	-	1 µl	-
Sample	-	-	1 µl
Total Volume	3.5 µl	3.5 µl	3.5 µl

- Place samples on a -20°C prechilled IsoFreeze PCR rack in a PCR clean station, and add 1 µl of 3' SMART CDS Primer II A. Mix contents and spin tubes briefly in a microcentrifuge.
- Incubate the tubes at 72°C in the thermal cycler for 3 minutes, then put samples on IsoFreeze PCR rack. Proceed immediately to next steps.
- Prepare a Master Mix for all reactions plus one by combining the following:
  - 2 µl 5X First-Strand Buffer
  - 0.25 µl DTT
  - 1 µl dNTP Mix
  - 1 µl SMARTer II A Oligonucleotide
  - 0.25 µl RNase Inhibitor
  - 1 µl SMARTScribe Reverse Transcriptase (Add to the master mix just prior to use. Mix well by vortexing and spin tubes briefly in a microcentrifuge.)
- Add 5.5 µl of the Master Mix to each reaction tube. Mix contents by pipetting up and down and spin tubes briefly.
- Incubate the tubes at 42°C for 90 minutes.
- Terminate the reaction by heating the tubes at 70°C for 10 minutes.

Purification of First-Strand cDNA using SPRI Ampure Beads

- Remove beads from storage and bring to room temperature and mix well.
- Add 25 µl SPRI Ampure XP beads to each sample. Adjust pipette to 35 µl and pipette entire volume up and down 10 times to mix
- Incubate at room temperature for 8 minutes.
- Briefly spin the sample tubes.
- Place the sample tubes on Magnetic Stand for 5 minutes, until the solution is completely clear.
- Keeping the samples on the Magnetic Stand, pipette out the solution and discard. Briefly spin the tubes.
- Place the tubes back on the Magnetic Stand for 2 minutes.
- Pipette out the residual liquid from the beads and discard. Make sure there is no supernatant remaining in the tube.

ds cDNA Amplification by LD PCR

- Prepare a PCR Master Mix for all reaction plus on additional reaction. Combine in the order shown, then mix well by vortexing and spin the tube briefly in a microcentrifuge.
  - 5 µl 10X Advantage 2 PCR Buffer
  - 2 µl dNTP Mix
  - 2 µl IS PCR Primer
  - 2 µl 50X Advantage 2 Polymerase Mix
  - 39 µl Nuclease-Free Water
- Add 50 µl of PCR Master Mix to each tube containing beads. Mix well and briefly spin down.
- Transfer samples from the PCR Clean Work Station to the general lab.
- Place the tube in a preheated thermal cycler with a heated lid using the following program:
  - 95°C for 1 minute

- X Cycles (see below)
  - 95°C for 15 seconds
  - 65°C for 30 seconds
  - 68°C for 6 minutes
- 72°C for 10 minutes
- Hold at 4°C

- To determine how many cycles, follow this table:

Input Amount, Total RNA	Input Amount, Cells	Typical No. of PCR Cycles
10 ng	1000 cells	12
1 ng	100 cells	12
500 pg	50 cells	13
100 pg	10 cells	15

Purification of ds cDNA using SPRI Ampure Beads

- Vortex beads until even, then add 90 µl of SPRI Ampure XP beads to the wells of a 96-well plate.
- Transfer the entire PCR product to the well of the plate. Pipette entire volume up and down 10 times to mix.
- Incubate at room temperature for 8 minutes.
- Place the 96-well plate on the Magnetic stand for 5 minutes, until the liquid appears completely clear
- With the plate sitting on the magnetic stand, pipette out the supernatant.
- **Keep the plate on the magnetic stand for the EtOH washes.**
- Add 200 µl of freshly made 80% Ethanol to each sample.
- Wait 30 seconds and pipette out the supernatant.
- Repeat the last 2 steps for a total of two 80% EtOH washes.
- Seal the sample wells on the plate and briefly spin down for 10 seconds at 1,000 rpm.
- Place the plate on the magnetic stand for 30 seconds, then remove all the remaining EtOH.
- Place the plate at room temperature for 3-5 minutes until the pellet appears dry.
- Add 12 µl Purification Buffer to cover beads.
- Remove the plate from the magnetic stand and incubate at room temperature for 2 minutes.
- Pipette pellet up and down 10 times to mix.
- Place the plate back on the magnetic stand for 1 minute, until the solution appears clear.
- Transfer clear supernatant from each well to a new tube.
- Store tube at -20°C.

Validation Using the Agilent 2100 BioAnalyzer

- Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit.
- Compare the results for the samples and controls to verify whether the sample is suitable for further processing.

Covaris Shearing of Full-length cDNA

- Turn the covaris system and the main cooler ON.
- Add about 1.9 L of distilled water to water bath. Make sure water goes to the FULL line.
- Close the door and open the Sonolab software. Click ON for the degassed button, and degas the water bath for 30 minutes.
- Add 65 µl of Purification Buffer to the DNA.
- Transfer 75 µl of the Purification Buffer + DNA mixture into the 100 µl Covaris tube. Put sample tubes into appropriate location on sample holder.
- Set up process configuration based on the following:

Duty %	Intensity	Burst cycle	Time (min)	Mode
10	5	200	5 min	Frequency

				Sweeping

- Save file and click return to back to the main page.
- Open the door. Place the tube holder with sample tubes on the transducer positioning system.
- Close the door.
- Click START on the main page to run the process.
- After shearing is complete, transfer 75 µl of sheared DNA to 1.5 ml tubes.
- Proceed to generate an Illumina Sequencing Library with the Illumina Paired-End DNA Sample Prep Kit.

Perform End Repair

- Preheat a thermal cycler to 20°C.
- Prepare the following reaction mix for all reactions plus on additional reaction.
  - 75 µl Covaris Sheared DNA
  - 10 µl T4 DNA Ligase Buffer with 10 mM ATP
  - 4 µl 10 mM dNTP Mix
  - 5 µl T4 DNA Polymerase
  - 1 µl Klenow Enzyme
  - 5 µl T4 PNK
- Incubate the sample on a thermal cycler for 30 minutes at 20°C.
- Transfer 100 µl of the sample to each well of a new 96-well plate.
- Vortex AMPure Beads, then add 180 µl to each well. Pipette entire volume up and down 10 times to mix.
- Incubate the plate at room temperature for 8 minutes.
- Place the plate on the magnetic stand at room temperature for 5 minutes, until the liquid appears clear.
- Remove and discard all of the supernatant from each well of the plate.
- **Keep the plate on the magnetic stand for the EtOH washes.**
- Add 180 µl of freshly prepared 80% EtOH to each well of the plate.
- Incubate the plate at room temperature for 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 washes.
- Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- Resuspend the dried pellet in each well with 32 µl QIAGEN EB. Pipette the entire volume up and down 10 times to mix.
- Incubate the plate at room temperature for 2 minutes.
- Place the plate on the magnetic stand at room temperature for 2 minutes, or until liquid appears clear.
- Transfer 32 µl of each sample to a new 0.2 ml PCR tube.
- SAFE STOPPING POINT: Store at -15° to -25°C for one day.

Adenylate 3' Ends

- Preheat a thermal cycler to 37°C.
- Prepare the following reaction mix for all reactions plus on additional reaction.
  - 32 µl Eluted DNA
  - 5 µl Klenow Buffer
  - 10 µl 1 mM dATP
  - 3 µl Klenow exo-
- Incubate the sample on a thermal cycler for 30 minutes at 37°C.
- Transfer 50 µl of the sample to each well of a new 96-well plate.
- Vortex the AMPure Beads, then add 90 µl of the beads to each sample. Pipette the entire volume

up and down 10 times to mix.
- Incubate the plate at room temperature for 8 minutes.
- Place the plate on the magnetic stand at room temperature for 5 minutes, until the liquid appears clear.
- Remove and discard all of the supernatant from each well of the plate.
- **Keep the plate on the magnetic stand for the EtOH washes.**
- Add 180 µl of freshly prepared 80% EtOH to each well of the plate.
- Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant.
- Repeat the last 2 steps for a total of two 80% EtOH washes.
- Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- Resuspend the dried pellet in each well with 19 µl QIAGEN EB. Pipette the entire volume up and down 10 times to mix.
- Incubate the plate at room temperature for 2 minutes.
- Place the plate on the magnetic stand at room temperature for 2 minutes, until the liquid appears clear.
- Transfer 19 µl of the clear supernatant from each well to a new 0.2 ml PCR tube.
- SAFE STOPPING POINT: Store at -15° to -25°C for one day.

Ligate Adapters

- Prepare a dilution of the paired-end adapter in ultra-pure water in a new 0.2 ml PCR tube:

Covaris Input DNA (ng)	Adapter:Water Dilution
8-50	1:9
4-8	1:14
< 1-4	1:19

- Prepare the following reaction mix for all reactions plus on additional reaction.
  - 19 µl Eluted DNA
  - 25 µl DNA Ligase Buffer 2X
  - 1 µl PE Adapter Oligo Mix (diluted)
  - 5 µl T4 DNA Ligase
- Incubate the sample at room temperature for 15 minutes.
- Transfer 50 µl of the sample to each well of a new 96-well plate.
- Vortex the AMPure beads, then add 80 µl of the mixed beads to each well. Pipette the entire volume up and down 10 times to mix.
- Incubate the plate at room temperature for 8 minutes.
- Place the plate on the magnetic stand at room temperature for 5 minutes, until the liquid appears clear.
- Remove and discard all of the supernatant from each well.
- **Keep the plate on the magnetic stand for the EtOH washes.**
- Add 180 µl of freshly prepared 80% EtOH to each well.
- Incubate the plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant.
- Repeat the last 2 steps for a total of two 80% EtOH washes.
- Remove the plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.
- Resuspend the dried pellet in each well with 23 µl QIAGEN EB. Pipette the entire volume up and down 10 times to mix.
- Incubate the plate at room temperature for 2 minutes.
- Place the plate on the magnetic stand at room temperature for 2 minutes, until the liquid appears clear.

- Transfer 23 µl of the clear supernatant from each well to a new 0.2 ml PCR tube.
- SAFE STOPPING POINT: Store at -15° to -25°C for one day.

Enrich DNA Fragments

- Prepare the following reaction mix for all reactions plus on additional reaction.
  - 23 µl DNA
  - 25 µl Phusion DNA Polymerase
  - 1 µl PCR Primer PE 1.0
  - 1 µl PCR Primer PE 2.0
- Amplify the PCR tube in the thermal cycler, with the lid closed:
  - 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
- Transfer 50 µl of the sample to each well of a new 96-well plate.
- Vortex the AMPure XP beads, then add 80 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 8 minutes.
- Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until liquid appears clear.
- Remove and discard the supernatant from each well.
- **Leave the PCR plate on the magnetic stand while performing the following 80% Ethanol wash steps**
- Add 180 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 15 µl QIAGEN EB. 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 2 minutes or until the liquid appears clear.
- Transfer 15 µl of the clear supernatant from each well to a new 1.5 ml microcentrifuge tube and store it at -20°C.

Validate Library

- Load 1 µl of the resuspended construct on an Agilent 2100 Bioanalyzer using a DNA specific chip such as the Agilent DNA-1000.
- Check the size, purity, and concentration of the sample. The final product should be a distinct band at approximately 250 bp.