Laboratory Operating Procedure

RNA fragmentation, cDNA synthesis and library preparation for NGS applications

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LOP-Version: 1

Attachments:

Introduction

The RNA is the template for cDNA synthesis. For this step, the use of superscript II reverse transcriptase combined with non-ribosomal hexamer tends to decrease cDNA synthesis from ribosomal RNA coming from prokaryote and eukaryote. This strategy aim to select preferentially RNA virus during cDNA synthesis. Covaris M220 Ultrasonicator is use for the fragmentation of cDNA in order to produce a library. Covaris technology use adaptive focused acoustics to precisely break cDNA fragment with a well define range of size. In the present protocol, the size range is 200 bp. Final step is the use of NEBNext Ultra RNA library Prep Kit for Illumina to perform a library with a size distribution centred to 300 bp. The analysis of the library produced will be done by Miseq 2X150bp.

Sample Material

Pure RNA extract.

Equipment and Reagents

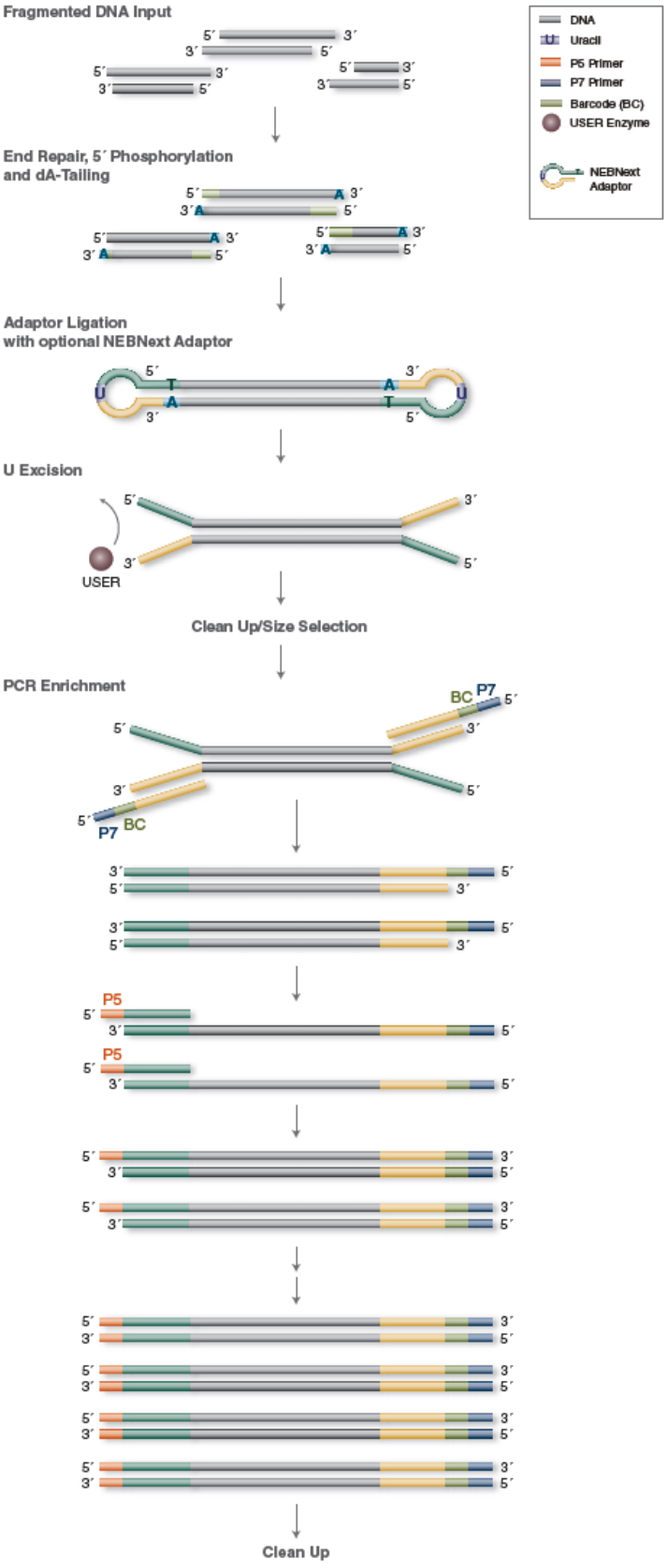
Equipment

- Covaris M220 Ultrasonicator
- M-Series microTUBE Holder
- MicroTube-15 AFA Beads Screw-Cap (Covaris Cat No PN 500420)
- Pipettes
- Disposable gloves
- 1.5 ml and/or 2 ml tubes
- Sterile, RNase-free pipet tips
- Thermocyler apparatus
- PCR tubes
- Thermal cycler
- Magnetic rack (Alpaqua, cat No A001322 or equivalent)

Reagents

- Covaris AFA-grade water PN 520101 or highly purified water
- Non-ribosomal hexamer 100 µM (Endoh et al 2005)
- *E.coli* ligase buffer (Invitrogen cat No . 18052-019)
- $MgCl_2$ 100 mM (Invitrogen cat No . AM9530G)
- Superscript II (SSCII) (Invitrogen cat No . 18064-014)
- dNTPs 25 mM of each (Applied biosystem cat No . N8080261)
- DTT 0.1M (Invitrogen cat No . 18064-014)
- Nuclease-free Water
- NEBNext Ultra RNA Librery Prep Kit for IlLumina (NEB, Cat No E7530)
- NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609 or #E7600) Oligos for Illumina
- 80% Ethanol (freshly prepared)
- Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- 0.1X TE, pH 8.

General remarks: workflow



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Literature

- Current protocols, RNA Fragmentation (Covaris , http://covaris.com/wp-content/uploads/pn_010183.pdf)
- Endoh D, Mizutani T, Kirisawa R, Maki Y, Saito H, et al. (2005) Species independent detection of RNA virus by representational difference analysis usingnon-ribosomal hexanucleotides for reverse transcription. Nucleic Acids Res 33:e65.

Before getting started

- Turn the power switch at the back of M220 instrument to ON position, power on the computer, and start the SonoLab software.
- Open the safety cover and place the M-Series microTUBE Holder insert into the water bath housing.
- Fill the provided wash bottle with the AFA-grade water (use either Covaris AFA-grade water PN 520101 or highly purified water)
- Using the wash bottle, add water in the water bath housing. The water level should reach the top surface of the Tube Holder and water level indicator in SonoLab should turn to green
- Wait until the water temperature in the water bath reaches 7 °C and the water temperature indicator in SonoLab turns green.
- AMPure XP Beads are required throughout the protocol. Allow beads to reach room temperature prior to use.

Procedure

1. - **cDNA synthesis :**
- For each sample, prepare mix I by adding in a PCR tube :
- 4.6µL of non-ribosomal hexamer (100 µM)
- 3 µL of 10X *E.coli* ligase buffer
- 2.4 µL $MgCl_2$ (100 mM)

2. - For each sample, prepare mix II by adding in a microtube :
- 2 µL of 10X *E.coli* ligase buffer.
- 1µL of SuperScript II (200U/µL)
- 1 µL of dNTs (25 mM each)
- 15 µL of Nuclease-free Water
- 1µL DTT (0.1M)

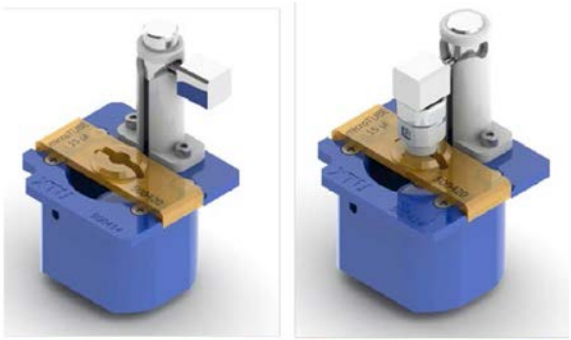
3. - Add 15 µL of purified RNA to 10 µL of the mix I
- Incubate 2 min at room temperature
- Add 20 µL of mix II
- Incubate 90 min at 37°C followed by 20 min at 70°C
- The cDNA can be use immediately or store at -70°C

4. - Check by real-time PCR

5. - Select the method « DNA-200pb» with the setting peak incident power (W) :30 ; duty factor (DF) :20% ; cycles per dust :50 ; time :150 sec

6. - Load 15µL of the sample into microTUBE by pushing the pipette tip through the slit in the cap (Snap-Cap microTUBE) or directly into Screw-Cap microTUBE and carefully dispense the fluid. Make sure that no air bubbles are in the fluid sample, as they will interfere with the acoustic energy.

7. - Open the safety cover, lift the Sliding Weight and rotate it into the loading position.
- Place the microTUBE in the central opening of the microTUBE Holder insert and place the Sliding Weight on the top of microTUBE (as the pictures below).



Place the "Holder XTU Insert microTUBE 15 µl" (PN 500420) in the Holder XTU, you should feel the magnets contact

Load microTUBE-15 into insert and lower the holder weight on top to keep the microTUBE in place

8. - Close the Safety Cover and click "Run" button in the SonoLab software to start the process.
- After the treatment is completed, lift the Sliding Weight and remove the microTUBE from the Tube Holder.

9. - Collect 15µL of fragmented cDNA in a new microtube. If you have, enough quantity (minimum 50pg/µL) perform a High Sensitivity DNA Agilent analysis to check the size range of RNA that should be close to 200pb.
- If the size is too long, repeat the procedure
- if it's too short, discard the treated nucleic acid and restart fragmentation treatment with shorter time.
- The fragmented cDNA can be use immediately or stored at -70°C.

10. - **Perform Second Strand cDNA Synthesis :**
- Add 15 µL of cDNA in a clean PCR tube
- Add 53 µL of nuclease-free water
- Add 8 µL of second strand synthesis reaction 10X buffer (orange cap)
- Add 4 µL of second strand synthesis enzyme Mix (orange cap)
- Mix thoroughly by gentle pipetting (volume 80µL)
- Incubate in a thermal cycler 1h at 16°C

11. - **Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads**
- Vortex AMPure XP Beads to resuspend.
- Add 144 µl (1.8X) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- Incubate for 5 minutes at room temperature.
- Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- Repeat previous step for a total of 2 washing steps.
- Air dry the beads for 5 minutes while the tube is on the magnetic rack with lid open. Caution: Do not over dry the beads. This may result in lower recovery of DNA target.

- Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
- Remove 55.5 µl of the supernatant and transfer to a clean nuclease free PCR tube. Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

12. - **Perform End Prep of cDNA Library**

- Add 6.5 µL NEBNext End Repair Reaction 10X Buffer (green cap) to the purified double-stranded cDNA (55.5 µL)
- Add 3 µL NEBNext End Prep Enzyme Mix
- Incubate the sample in a thermal cycler (with the heated lid set at 75°C) 30 minutes at 20°C fallowed by 30 minutes at 65°C
- Hold at 4°C. Proceed immediately to Adaptor Ligation.

13. - **Perform Adaptor Ligation**

- Add 15 µL Blunt/TA Ligase Master Mix (red) to end prep reaction (65µL)
- Add 1 µL diluted NEBNext Adaptor (10-fold dilution (1:9) with 10 mM Tris-HCl)
- Add 2.5 µL Nuclease-free Water
- Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
- Incubate 15 minutes at 20°C in a thermal cycler.
- Add 3 µl of USER Enzyme (red) to the previous ligation mixture.
- Mix well and incubate at 37°C for 15 minutes.

14. - **Purify the Ligation Reaction Using AMPure XP Beads with sizing (200 bp insert)**

- Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl. It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.
- Add 100 µl (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
- Incubate for 5 minutes at room temperature.
- Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments (Caution: do not discard the beads).
- Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- Repeat previous step for a total of 2 washing steps.
- Briefly spin the tube, and put the tube back in the magnetic rack.
- Completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic rack with the lid open. Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
- Remove the tube from the magnet. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
- Transfer the 50 µl supernatant to a clean PCR tube. (Discard beads).

- To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
- Incubate for 5 minutes at room temperature.
- Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments (Caution: do not discard the beads).
- Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- Repeat previous step for a total of 2 washing steps.
- Briefly spin the tube, and put the tube back in the magnetic rack.
- Completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic rack with the lid open.
- Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
- Remove the tube from the magnet. Elute DNA target from the beads with 21 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
- Without disturbing the bead pellet, transfer 20 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

15. - **PCR Enrichment of Adaptor Ligated DNA**

- Add 2.5 µL Universal PCR Primer/i5 Primer (blue) to purified ligation (20µL)
- Add 2.5 µL Index (X) Primer/i7 Primer (blue), X will be different for each sample
- Add 25 µL NEBNext Q5 Hot Start HiFi PCR Master Mix
- Incubate in a thermal cycler with the fallowing PCR cycling conditions

Initial Denaturation : 98°C 30 sec

20 cycles [Denaturation 98°C 10 sec
Annealing/Extension 65 °C 75 sec

Final Extension 65°C 5 min

Hold 4°C infinity

16. - **Purify the PCR Reaction using Agencourt AMPure XP Beads**

- Vortex Agencourt AMPure XP Beads to resuspend.
- Add 45 µl (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
- Incubate for 5 minutes at room temperature.
- Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
- Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
- Repeat previous step for a total of 2 washing steps.

- Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open. Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
- Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 µl 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
- Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

17. - **Assess library quality on a Bioanalyzer (Agilent High Sensitivity Chip)**
- Dilute 2–3 µl of the library in 10 mM Tris or 0.1X TE.
- Run 1 µl in a DNA High Sensitivity chip
- Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.