FR-AgEncode: a French pilot project to enrich the annotation of livestock genomes

Preparation of TrueSeq stranded mRNA libraries for long mRNA sequencing
Version 1 – 2016-09-08

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This protocol only includes the preparation of TrueSeq libraries for long mRNA sequencing using the Illumina technology.

Long mRNA were previously isolated as described in :
INRA_SOP_RNA_extraction_20160504.pdf available for download :
ftp://ftp.faang.ebi.ac.uk/ftp/protocols/assays/INRA_SOP_RNA_extraction_20160504.pdf

I. Prerequisites

Samples:

Quantity: 200ng to 1µ of total mRNA
Purity (Nanodrop): 260/230 ratio over 1.8 and 260/280 ratio over 1.8
Quality (BioAnalyser): RIN over 8, best over 8.5

Consumables:

TruSeq Stranded mRNA Sample Prep Kit - v2
Magnetic beads AMPure XP (Beckman, A63882)
Standard Sensitivity RNA Analysis Kit, DNF-471
High Sensitivity NGS Fragment Analysis Kit DNF-474
SuperScript II reverse transcriptase Invitrogen (Fisher Scientific, 18064071)
Sterile Water, molecular biology grade
Ethanol, molecular biology grade

Plastics:

- Tube 0.2 ml RNase-free (Dutscher, 038196)
- Tube 0.2 ml (Dutscher, 016139)
- Tube 1.7 ml RNase-free (Dutscher, 038485)
- Tube 1.7 ml (Dutscher, 017040A)
- Falcon 15 ml (StarLab, A1415-0200)
- Falcon 50 ml (StarLab, A1450-0200)
- 5000 µL tips (StarLab, I1009-5000)
- 1250 µL Filter tips (Dutscher, 010380)
- 330 µL Filter tips (Dutscher, 010350)
- 10 µL Filter tips (Dutscher, 010320)
- 96 PCR plate AB-1400L (Dutscher, 016682)
- Kimberly-Clark paper (Dutscher, 002907)

II. Purification and fragmentation of mRNAs

Purification of the mRNAs using poly-T magnetic beads followed by chemical fragmentation of the mRNAs into fragments of about 155 bp (between 120 and 210 bp)

Preparation of handling:

- Defrost Bead Binding Buffer, Bead Wash Buffer, Elution Buffer and Fragment, Prime, Finish mix
- Place the resuspension buffer at room temperature.
- Keep RNA Purification Beads at room temperature (RT) a few minutes before handling.

1. Place each sample (0.2 to 1 µg of RNA in a volume of 50 µL) into a well of the 96-well plate. Note the layout of the samples on a tracking sheet.

   Comment: libraries can also be done in individual tubes
2. Vortex the poly-T beads (RNA Purification beads).
3. Add 50 µL of RNA Purification beads to each well containing 50 µL of sample.

   Mix by pipetting up and down, seal the plate with a PCR adhesive film.
4. Incubate 5 'at 65 ° C in a thermocycler and then place at 4 ° C.

   Comment: this step allows to denature the RNAs and thus facilitate the fixing of the poly-A RNAs on the beads.
5. Remove the plate from the thermocycler and incubate 5 'at RT

   Comment: this step lets mRNAs attach to the beads
6. Place the plate on the magnetic rack, when the beads are pelleted, remove and discard the supernatant.

   Remove the plate from the magnetic holder.
7. Wash the beads with 200µL of Bead Wash Buffer and mix by pipetting up and down several times.
8. Place the plate on the magnetic rack. When the beads are pelleted, remove and discard the supernatant.

   Remove the plate from the magnetic holder.
9. Add 50µL of Elution Buffer to each well of the plate. Mix by pipetting up and down several times to pick up the beads and seal the plate.
10. Incubate 2 'at 80 ° C in a thermocycler, then set to 25 ° C.

    Comment: the mRNA is released from the beads
11. Remove the plate from the thermocycler.
12. Add 50 µL of Bead Binding Buffer to each well and mix by pipetting up and down several times.
13. Incubate 5 'at RT.

    Comment: this step lets mRNAs attach to the beads
14. Place the plate on the magnetic rack, when the beads are pelleted, remove and discard the supernatant.

    Remove the plate from the magnetic holder.

15. Wash the beads with 200µL of Bead Wash Buffer and mix by pipetting up and down several times.

16. Place the plate on the magnetic rack. When the beads are pelleted, remove and discard the supernatant.

Remove the plate from the magnetic holder.

17. Pipet 19.5µL of Fragment, Prime, Finish Mix into each well of the plate.

Comment: this mix contains the random hexamers necessary for the synthesis of the cDNA

Mix by pipetting up and down several times and seal the plate.

18. Incubate 2´at 94ºC in a thermocycler and then at 4ºC for 5´.

Meanwhile store used buffers at 4ºC, Fragment, Prime, Finish Mix at -20ºC and thaw the First Strand Synthesis Act D Mix.

19. Remove the plate from the thermocycler and proceed immediately to the cDNA first strand synthesis.

III First Strand cDNA synthesis

1. Place the plate on the magnetic rack to pellet the beads

2. For each sample/well, transfer 17ul from the supernatant (that contains the fragmented mRNA and the primers) into a new well

3. Add to each well :

7.2µl of First Strand Synthesis Act D Mix

0.8µl of Superscript II RT

Mix by pipetting up and down and seal.

4. Place the plate in a thermocycler and run the following program:

25°C for 10min
42°C for 15min
70°C for 15min
4°C hold

5. Remove the plate from the thermocycler and proceed to the second strand synthesis

IV Second Strand cDNA synthesis

1. Add to each well:

20µl of Second Strand Marking Master Mix

5µl of resuspension buffer

Mix by pipetting up and down and seal.

2. Incubate 1h at 16°C in a thermocycler

3. Remove the plate and keep at room temperature

4. Purification with AMPure XP beads

Warm the beads at Room Temperature

Thaw an aliquot of Resuspension Buffer

Prepare Ethanol 80%

- Vortex the beads' stock solution for 20s.
- Add 40µl of AMPure XP to each well that contains 50µl of cDNA

Mix by pipetting up and down several times

- Incubate 5min at RT
- Place the plate on a magnetic rack

When the beads are pelleted remove the supernatant (85µl) and throw it away.

- Wash the beads twice by :

Adding 200µl of EtOH 80%

Incubate 30s at RT

Remove EtOH and throw it away

- Let dry the beads 3min on the magnetic rack
- Remove the plate from the magnetic rack and resuspend the beads in 20µl of Resuspension Buffer. Mix by pipetting up and down several times
- Incubate 2min at RT
- Place the plate on a magnetic rack

For each well, recover 17.5µl of supernatant and transfer it into a new well

V 3' Adenylation

Thaw on ice the A-Tailing mix

1. Add to each well:

12.5µl of A-Tailing

Mix by pipetting up and down several times and seal

2. Place the plate in a thermocycler and incubate 30min at 37°C then 5min at 70°C

3. Remove the plate and immediately proceed to adapters' ligation

VI Ligation of indexed adapters

Thaw on ice the selected adaptors, the ligase mix, the stop ligation buffer, the resuspension buffer

Warm AMPure XP beads at room temperature

1. Add to each well:

2.5µl of Resuspension Buffer

2.5µl of DNA Ligase Mix

2.5µl of the selected RNA Adapter Index

Mix by pipetting up and down several times and seal

2. Place the plate in a thermocycler and incubate 10min at 30°C

3. Remove the plate from the thermocycler and add to each well 5µl of Stop Ligation Buffer

Mix by pipetting up and down several times.

4. First purification with AMPure XP beads

- Vortex the beads' stock solution for 20s.

- Add 42µl of AMPure XP to each well that contains 50µl of cDNA

Mix by pipetting up and down several times

- Incubate 5min at RT

- Place the plate on a magnetic rack

When the beads are pelleted remove the supernatant and throw it away.

- Wash the beads twice :

Add 200µl of EtOH 80%

Incubate 30s at RT

Remove EtOH and throw it away

- Let dry the beads 3min on the magnetic rack

- Remove the plate from the magnetic rack and resuspend the beads in 52.5µl of Resuspension Buffer. Mix by pipetting up and down several times

- Incubate 2min at RT

- Place the plate on a magnetic rack

For each well, recover 50µl of supernatant and transfer it into a new well

5. Second purification with AMPure XP beads

- Vortex the beads' stock solution for 20s.

- Add 50µl of AMPure XP to each well that contains 50µl of cDNA

Mix by pipetting up and down several times

- Incubate 5min at RT

- Place the plate on a magnetic rack

When the beads are pelleted remove the supernatant and throw it away.

- Wash the beads twice :

Add 200µl of EtOH 80%

Incubate 30s at RT

Remove EtOH and throw it away

- Let dry the beads 3min on the magnetic rack

- Remove the plate from the magnetic rack and resuspend the beads in 22.5µl of Resuspension Buffer. Mix by pipetting up and down several times

- Incubate 2min at RT

- Place the plate on a magnetic rack

For each well, recover 20µl of supernatant and transfer it into a new well.

VII PCR amplification of DNA libraries

This PCR allows to amplify DNA fragments that integrated adapters at both DNA extremities. PCR cycles are determined to minimize PCR bias.

Thaw on ice the PCR Primer Cocktail and the PCR Master Mix

1. Briefly centrifuge the PCR master mix and PCRPrimer Cocktail tubes.

2. Add to each well/tube that contains the cDNA (20µl) :

5µl of PCR Primer Cocktail

25µl of PCR Master Mix

3. Put the plate/tube in a thermocycler and run the following program:

98°C – 30s
11 cycles :
98°C – 10s
60°C - 30s
72°C - 30s
72°C - 5min
4°C hold

If available switch to a Post-PCR room or space

4. Purification with AMPure XP beads

- Vortex the beads' stock solution for 20s.
- Add 40µl of AMPure XP to each well that contains 50µl of cDNA

Mix by pipetting up and down several times

- Incubate 5min at RT
- Place the plate on a magnetic rack

When the beads are pelleted remove the supernatant and throw it away.

- Wash the beads twice :

Add 200µl of EtOH 80%

Incubate 30s at RT

Remove EtOH and throw it away

- Let dry the beads 3min on the magnetic rack
- Remove the plate from the magnetic rack and resuspend the beads in 32.5µl of Resuspension Buffer. Mix by pipetting up and down several times
- Incubate 2min at RT
- Place the plate on a magnetic rack

For each well, recover 30µl of supernatant and transfer it into a new well.

VIII Quality Control : insert size and quantity

With the High Sensitivity NGS Fragment Analysis Kit DNF-474 and the Fragment Analyser system.

Profile should be of this type: