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Protocol: Sample preparation for MiSeq (Illumina Sequencing) using SPIA amplification method

Step 1: Trizol extraction from lung tissues

Prior process: left lung was excised from the animals and snapped frozen with liquid nitrogen. Lungs were stored in -80°C before processing.

1. Weigh left lung to make sure that the weight is less than 100mg
2. Homogenize left lung in Gentle Macs M tubes (Miltenyi Biotec) with 1ml of Trizol (Life technologies) using “M tubes RNA 02-01” protocol settings at room temperature
3. Pulse spin down the M tubes at 20sec to collect all the homogenate
4. Transfer homogenate into a clean 1.5ml microcentrifuge tube (not-autoclaved, clean from the pack which comes with RNase/DNase/Pyrogen free tubes). Incubate at room temperature for 5 mins.

   Stopping point* Homogenate can be stored in -80°C for less than 1 month before proceeding to next step.

5. Add 200µl clean chloroform per tube and shake vigorously for 15 secs. Incubate at room temperature for 2-15minutes.
6. Centrifuge homogenate/lysate for 15mins, 12,000g at 4°C.
7. Collect the top aqueous phase (~500µl) and transfer into another clean tube (1.5ml).

*Note: if extracting RNA from low-RNA samples such as cell culture supernatant or Broncho-alveolar lavage fluid (BALF), add 1µl of 5mg/ml Linear acrylamide (Life technologies, RNase-Free) as carrier. Mix well by inverting tubes a few times

*Note: do not use nucleic-acid based carriers or glycogen (that is from an animal source and may be contaminated with traces nucleic acids.

8. Add 500µl of ice-cold isopropanol (pre chilled at -20°C). Mix well by inverting tubes 20 times.
9. Incubate at room temperature for 10minutes.
10. Short spin (~1-2secs only) to collect all fluid. Aspirate the mixture up and down, and apply to a Qiagen’s RNease mini column (other types of silica-based RNA binding columns may be used as well such as the Invitrogen’s Purelink columns).
11. Centrifuge for 15sec at ≥8000g. Discard flow through
12. Follow RNeasy mini kit instructions from there onwards.
    a. Add 700µl Buffer RW1 to RNeasy spin column. Centrifuge for 15sec at ≥8000g. Discard flow through
    b. Add 500µl Buffer RPE to RNeasy spin column. Centrifuge for 15sec at ≥8000g. Discard flow through
    c. Add 500µl Buffer RPE to RNeasy spin column. Centrifuge for 2mins at ≥8000g. Discard flow through

    d. Replace spin column in a new 2ml collection tube and centrifuge at full speed for 1 min. Dab the base of the spin column dry with a kim wipe to remove all traces of ethanol.
13. Elute RNA with 50µl of Nuclease-free water. Spin down for 1min at ≥8000g to elute the RNA. Repeat this step with the same eluent to increase RNA concentration.
14. Measure nucleic acid content with Nanodrop.

Step 2: DNA removal using TURBO DNA-free kit (Invitrogen)

1. Dilute RNA to 10µg/50µl with nuclease free water before proceeding with Rigorous DNase treatment
   Note* for samples with less than 200µg/ml of RNA (e.g. from BALF or cell culture supernatant), use Routine DNase treatment based on manufacturer's protocol
2. To 50µl of RNA, add 5µl of 10x TURBO DNase buffer, mix well by aspirating up and down.
    a. To BALF and culture supernatant samples, add 1µl of DNase and incubate at 37°C for 30 mins
    b. To lung samples which has slightly more than 200µg/ml or diluted to 200µg/ml, do the same as above, but after 30mins of incubation, add an additional 1µl of DNase and incubate for another 30mins
       (i.e. 1 hour in total)

   Note: Optional to add the inactivation reagent because I found that removal of this reagent is not entirely clean and will affect the quality of the sample

3. Store on ice to stop the reaction and proceed with RNA cleanup with Qiagen RNeasy mini kit

Step 3: RNA cleanup with RNeasy Mini kit

1. To 100µl of sample (dilute with water if the volume is less than 100µl), add 350µl of RLT buffer. Mix well by aspirating the samples a few times.
2. Add 250µl of 100% ethanol

   Note: Fresh 100% ethanol that is purchased should be aliquoted into 15ml tubes all the way to the brim of the tubes and sealed to prevent oxidation of the ethanol. High quality ethanol is very important to maintain the quality of RNA for further sample prep.

3. Apply mixture to column. Centrifuge for 15sec at ≥8000g. Discard flow through
4. Follow manufacturer's instructions from there onwards.
    a. Add 500µl Buffer RPE to RNeasy spin column. Centrifuge for 15sec at ≥8000g. Discard flow through
    b. Add 500µl Buffer RPE to RNeasy spin column. Centrifuge for 2mins at ≥8000g. Discard flow through
    c. Replace spin column in a new 2ml collection tube and centrifuge at full speed for 1 min. Dab the base of the spin column dry with a kim wipe to remove all traces of ethanol.

5. Elute RNA with 50µl of Nuclease-free water. Spin down for 1min at ≥8000g to elute the RNA. Repeat this step on time the same eluent to increase RNA concentration.

Step 3 (Optional): Determine the quality of RNA using Bioanalyzer RNA pico chip

Note: Usually RNA from cell culture supernatant or BALF contain a lot of degraded RNA from dying cells that were infected with influenza, and that probably accounts for the poor RIN score and shift in the RNA peaks to the left. However, the benefit of the Nugen ovation RNA-seq kit is that it can amplify samples with low yield and quality.

Step 4: SPIA amplification of RNA with Nugen's ovation RNA-seq system v2 kit (PART NO. 7102)

1. Perform nanodrop estimation of RNA concentration
2. With a starting material of between 500 pg and 100 ng total RNA, carry out SPIA amplification based on manufacturer's protocol.
   Note: Use fresh ethanol aliquots to make all the ethanol needed for the protocol.
   Note: Use separate benches for pre-PCR and post PCR procedures!
   Note: Use RNA zap to clean and keep benches and magnetic plates clean of remaining nucleic acids. Clean with 70% ethanol to remove remnants of RNA-zap after the entire experiment.

Step 4: SPIA product cleanup

1. Perform SPIA cDNA cleanup using Qiagen Qiaquick PCR Purification Kit following the instructions given in Nugen's protocol

   Note: The protocol is modified from Qiagen Qiaquick PCR purification kit and strict compliance can improve the quality of the SPIA cDNA.

Step 5: Library prep and Miseq run

The SPIA cDNA can be sent to MIT BioMicroCentre for Fragmentation (to 400-500bp), library prep using SPRIworks, barcoding and QC.

If PCR amplification during the library prep stage is not desirable, library preparation can be performed using Encore Complete RNA-Seq DR Multiplex System which eliminates the use of PCR amplification to add adaptors and barcodes to cDNA fragments.

Step 6: Miseq run

1. If library preparation was performed by BioMicroCentre, spike in 15uL of 100uM custom primer into position 13 of the Miseq catridge.

   Custom primer: AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCG (HPLC grade)

2. Otherwise proceed as per manufacturer's protocol