UPitt TriState SenNet TMC Total RNA isolation & quality analysis for bulk RNA-seq

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We use this protocol and it's working

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ABSTRACT

Bulk RNA sequencing (RNA-seq) is a quantitative method used to interrogate the transcriptome of a biological sample at a given point in time. This protocol describes a method for the extraction and quality assessment of total RNA from pulverized tissue.

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GUIDELINES

For RNA isolation

- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

For BioAnalyzer use

- When pipetting samples into the LabChips, insert the pipette tip to the bottom of the well to prevent the formation of bubbles.
- Protect the dye and dye mixtures from light to prevent decomposition.
- RNA gel-dye mix must be made fresh on the day of the run. If unused, throw it away.
- Clean the electrodes before the first run of the day, between each bioanalyzer run, and after the last run of the day.
- Use a new electrode cleaner chip with each new LabChip Kit.
- Sample wells must all contain 5.0 µl, or assay will not run.
- Do not touch the bioanalyzer during an assay.
- Electrode cleaner chips should not be filled more than 350 µl, or electrodes may malfunction.
- RNA ladder and samples must be denatured at 70°C to avoid gel mobility errors from secondary structure.

MATERIALS

For RNA isolation

- RNeasy Plus Mini Kit (50) QIAGEN Cat. No. / ID: 74134
- QIAshredder QIAGEN Cat. No. / ID: 79656

For RNA quality assay

- Agilent RNA 6000Nano LabChip kit (Agilent 5607-1511)
- Nuclease-free water (ThermoFisher AM9932).

SAFETY WARNINGS

Personal Protective Equipment (PPE) should be used at all times while operating this protocol.

BEFORE START INSTRUCTIONS

Remove RNases cleaning the surfaces, pipettes/forceps and gloves using RNaseZAP according to the manufacturer protocol.

For RNA isolation

- Add 4 volumes of ethanol 100 to 4 volume RPE buffer.
- β-mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use. Add 10 µl β-ME per 1 ml Buffer RLT Plus.

Total RNA extraction and purification

1 Use 5 mg of pulverized tissue, and add 350 µl of the RLT Plus buffer prepared initially. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at maximum speed.

2 Carefully remove the liquid from the collection tube of the QIAshredder by pipetting, and transfer it to a gDNA Eliminator spin column placed in a 2 ml collection tube. Centrifuge for 30 s at 8000xg. Discard the column, and save the flow-through.

3 Add 1 volume of 70% ethanol to the flow-through, and mix well by pipetting. Do not centrifuge. Proceed immediately to next step.

4 Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at 8000xg. Discard the flow-through.

Note: Flow-through contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach.

5 Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000xg to wash the spin column membrane. Discard the flowthrough. Reuse collection tube.

Note: Flow-through contains Buffer RLT Plus or Buffer RW1 and is therefore not compatible with bleach.

6 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000xg to wash the spin column membrane. Discard the flowthrough. Reuse collection tube.

7 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 8000xg to wash the spin column membrane.

8 Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.

9 Place the RNeasy spin column in a new collection tube. Add 40 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000xg to elute the RNA.

10 Aliquot the purified sample in 10 µl aliquots and separate 2 µl for quality assessment.

RNA quality assesment (RIN)

11 Setup:

11.1 Prepare gel aliquots (upon first use of Nano 6000 kit):

a. Allow RNA gel matrix (red vial) to equilibrate to room temperature for 30 min.
b. Pipette 550 µl of RNA 6000 Nano gel mix (red vial) into an RNA 6000 spin filter.
c. Centrifuge at 4000 g for 10 minutes.
d. Aliquot 65 µl (enough for two LabChips) of filtered gel into 0.5 ml RNase-free microfuge tubes. Write “gel” and the date on tubes. Use filtered gel within 4 weeks. Store at 4°C.

11.2 Prepare ladder aliquots (upon first use of Nano 6000 kit):

a. Thaw RNA 6000 ladder tube at room temperature for 5 minutes.
b. Vortex and transfer contents (35 µl) into 0.65 ml tube.
c. Heat denature at 70°C for 2 minutes. Immediately transfer to ice bucket for five minutes.
d. Dispense 2.2 µl into 0.65 ml tubes with single-channel P20 repeater.
e. Label with date, as “Ladder, date”.
f. Store at -80°C until ready for use. One aliquot will supply two LabChips.

11.3 Clean the Bioanalyzer electrodes:

a. Pipette 350 µl of nuclease-free water into a labeled electrode cleaner chip.
b. Place the water chip inside the bioanalyzer and close the lid. Check that the Bioanalyzer software acknowledges the presence of a chip. Close the bioanalyzer lid.
c. After one minute, open the lid and remove the chip. Wait 10 seconds for the water on the electrodes to evaporate.

12 Prepare the gel-dye mix:

12.1 Allow the RNA 6000 Nano dye concentrate (blue vial) and RNA gel aliquot to come to room temperature for 30 minutes. Protect dye concentrate from light.

12.2 Vortex the dye concentrate for 10 seconds, spin down.

12.3 Add 1.0 µl dye concentrate to 65 µl aliquot of filtered gel. Using a blue sharpie, write the date on the top of the lid to indicate that dye has been added as it will not be visible.

12.4 Vortex and spin tube at 13000 rcf for 10 minutes at room temperature. Protect from light.

13 Prepare samples for analysis:

13.1 Thaw the sample tubes from -80°C on ice. Spin down.

13.2 Remove plate seal and cover with a 96-well rubber mat.

13.3 Denature for 5 minutes at 70°C in heat block (tubes) or thermocycler with no heated lid (plates). After denaturing, place on ice for a full 5 minutes, then spin down. During bioanalyzer chip loading, the samples should remain on ice.

Note: This step is critical to keep the product 'snapped' in position after the sample has been denatured.

13.4 Remove a ladder aliquot from -80°C and keep on ice until needed.

14 Load the gel-dye mix and samples:

14.1 Make sure the chip priming station base plate is set at position "C" and plunger is in the topmost position.

14.2 Add 9.0 µl gel dye mix to the well marked "G" inside a black circle.

14.3 Place chip in priming station.

14.4 Close the chip priming station and press the plunger until the clip holds it.

14.5 Wait 30 seconds, then release the clip.

14.6 Wait 5 seconds for the plunger to rise, and slowly pull it up to the 1 ml position.

14.7 Open the chip priming station and check the underside of the chip that the capillaries are no longer visible. If there are any visible, repeat steps chip priming steps. If the capillaries remain visible start over with a new chip.

14.8 Add 9.0 µl gel-dye mix to each of the two other wells marked “G.”

14.9 Add 5.0 µl RNA 6000 marker in the ladder well and 12 sample wells.

14.10 Add 1.0 µl RNA 6000 ladder to the well marked by a picture of a ladder.

14.11 Add 1.0 µl of denatured RNA sample into each of the sample wells.

14.12 Place the chip in the Vortex Mixer and vortex at 2200 rpm for one minute.

15 Run the bioanalyzer and software:

15.1 Run the chip in the Agilent 2100 Bioanalyzer within 5 minutes of loading.

15.2 Open the 2100 Expert program on the Bioanalyzer computer.

15.3 Place the loaded chip inside the Bioanalyzer and close the lid.

15.4 If it is not already selected, click on “Instrument” on the left side of the screen.

15.5 On the right of the screen, select “Eukaryote Total RNA Nano Series II” for the Assay Selection (click “Assay…,” then “RNA,” and select “Eukaryote Total RNA Nano Series II.”)

15.6 In the field marked “File Prefix,” after “BA_” type in “BA_YYMMDD_NN_sample” where YY=year, MM=month, DD=day, and NN=the number of the bioanalyzer run of the day (e.g. the fifth run of the day would be “05”). Record run name.

15.7 Click the “Start” button.

15.8 Once the assay starts, click on “Data” on the left side of the screen, then “Chip Summary” tab.

15.9 Enter the sample information for each of the 12 samples in the format “sample_YYMMDD_PP_RCC” where YYMMDD=date of the sample plate, PP=sample plate number, R=sample row from sample plate, and CC=sample column from sample plate.

15.10 Electronic gel pictures and electropherograms of the ladder and samples may be viewed by clicking on the “Gel” and “Electropherogram” tabs, respectively.

16 Take Down:

16.1 Remove the LabChip from the bioanalyzer and dispose of it once the run has completed.

16.2 Clean the electrodes with the electrode cleaner as described in the Setup section.

16.3 Empty the electrode cleaner chips at the end of the day.

Post-Processing

17 All the RNA and genomic DNA samples were sent to a sequencing company (Novogene America, UC Davis Sequencing Center) for library preparation and sequencing.