Standard operating procedure for Milk sampling and RNA extraction from milk cells

AGRICULTURE VICTORIA

Updated 1stof March, 2016 by Amanda Chamberlain

Sampling Milk for RNA

Requirements

- 50ml falcon tubes (2 per sample)
- 250ml container (1 per sample)
- 20cm x 20cm cheese cloth (1 per sample)
- Elastic band (1 per sample)
- Spatula (1 per sample)
- 2ml microcentrifuge tube (1 per sample)
- Kimwipes
- 0.5M EDTA
- PBS solution with 0.5 mM EDTA (10 µl 0.5M EDTA into 10ml PBS)
- PBS solution
- RNAlater

Protocol

NOTE: Dirt and faecal contamination can be a problem under certain environmental conditions. For best results ensure milking machines and cows teats are clean.

1. Label two 50ml falcon tubes and 250ml container with the cow ID.
2. Fix cheese cloth to 250ml container with elastic band
3. Milk cow into a bucket 2 to 3 hours after the AM milking, ensure milking machines and bucket are clean to reduce environmental and cross sample contamination.
4. Pour ~150ml of milk from bucket into 250 ml container through clean cheese cloth, remove cheese cloth, cap and place on ice and transport to laboratory and continue processing within 30 minutes.
5. Add 50 µl of 0.5M EDTA to each 50 ml falcon tube.
6. Fill each 50ml falcon tubes (containing 0.5M EDTA) with 50ml of filtered milk, spin at 1800 rpm at 4°C for 10 min.
7. Scrap off cream with clean spatula and decant skim milk, use kimwipe to wipe excess cream from the falcon tube.
8. Add 10 ml PBS solution with 0.5 mM EDTA, and spin at 1800 rpm for 10 minutes at 4°C.
9. Decant supernatant and wipe off any residual cream with clean kimwipe.
10. Repeat steps 8.
11. Decant supernatant from one falcon tube, then most of the supernatant, leaving about 200 µl of the second
12. Resuspend the pellet in the second falcon tube in the remaining supernatant , and then transfer the volume to the first falcon tube and resuspend pellet. Transfer all to labelled 2 ml microcentrifuge tube.
13. Spin down in microfuge, pipette off supernatant and resuspend in 200 µl PBS.
14. Add 1 ml of RNAlater and place on ice.
15. Store samples at –20°C.

NOTE: Milk cells are stable in RNAlater for up to 3 months, however beyond this RNA will begin to degrade. For best results extract RNA within two weeks.

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RNA extraction

NOTE: This procedure uses Ambion's TRIzol Plus RNA Purification Kit, and is based on the

protocol available from http://www.lifetechnologies.com/order/catalog/product/12183555. The following method produces high quality RNA transcripts greater than 200bp. Should small RNA be required alternative extraction method should be used.

Warnings

This procedure uses the following hazardous chemicals:

- TRIzol Reagent. Contains phenol and guanidine isothiocyanate.
- Wash Buffer I. Contains guanidine isothiocyanate.
- Chloroform.

Phenol is corrosive and harmful by inhalation.

Guanidine isothiocyanate is harmful by inhalation and in contact with skin. Guanidine isothiocyanate reacts with strong oxidising agents and strong acids to form toxic gases.

Chloroform is harmful by inhalation and in contact with skin.

Perform this procedure in a fume hood, and wear a lab coat, gloves and safety glasses throughout the procedure. Phenol and chloroform should not be stored in plastic containers. Dispose of liquid waste in a glass bottle. Dispose of all waste via ChemSal. Do not combine waste from this procedure with bleach or acid.

Requirements

- TRIzol® Plus RNA Purification Kit
- 100% ethanol
- 70% ethanol prepared with RNase-free water
- Chloroform
- RNase-free water
- 1.5ml microcentrifuge tubes

Prepare Wash Buffer II

When starting a new kit, add 60 ml 100% ethanol to the Wash Buffer II bottle.

Lysate preparation

1. Centrifuge samples suspended in RNAlater at 12, 000 g for 1 min at 4ºC. Remove supernatant with a pipette and discard.
2. Suspend cells in 1 ml TRIzol.
3. Centrifuge at 12,000 g for 10 min at 4ºC. Transfer supernatant to a new microcentrifuge tube.

Phase separation

4. Incubate supernatant for 5 min at room temperature.
5. Add 200 µl chloroform. Shake vigorously by hand for 15 seconds.

6. Incubate for 2-3 minutes at room temperature.
7. Centrifuge at 12,000 g for 15 min at 4ºC. After centrifugation, the mixture separates into a lower red phenol-chloroform phase, an interphase, and an upper aqueous phase which contains the RNA.
8. Angle the tube at 45º and, using a pipette, remove the upper aqueous phase without disturbing the interphase. Transfer the upper phase to a new microcentrifuge tube. About 500 µl should be transferred. The lower red phenol-chloroform phase and the interphase may be used for DNA/protein isolation or otherwise discarded.

RNA binding

9. Add an equal volume of 70% ethanol and mix by vortexing.
10. Transfer up to 700 µl of sample to a Spin Cartridge with Collection Tube.
11. Centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same Collection Tube.
12. Repeat steps 11 and 12 to process the entire sample.
13. Add 700 µl Wash Buffer I to the Spin Cartridge and centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into a new Collection Tube.
14. Add 700 µl Wash Buffer II with ethanol to the Spin Cartridge. Centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and reinsert the Spin Cartridge into the same Collection Tube.
15. Repeat step 14 twice.
16. Centrifuge the Spin Column with its emptied Collection Tube at 12,000 g for 1 min at room temperature to dry the membrane. Discard the Collection Tube and insert the Spin Column into a Recovery Tube.

Elution

17. Add 50ul of RNase-free water to each column, incubate for 1 min at room temperature.
18. Centrifuge at 12,000 g for 2 min at room temperature.
19. Place samples on ice.
20. Assess RNA yield and purity on Nanodrop.
21. Assess RNA integrity on Bioanalyzer Tapestation or by agarose gel electrophoresis (1.5% agarose in 0.5× TBE, run at 60V for 1 hour).
22. Store samples at -80ºC.

NOTE: Expect RNA yield of >40ng/ul and RIN >7.5 (Figure 1)

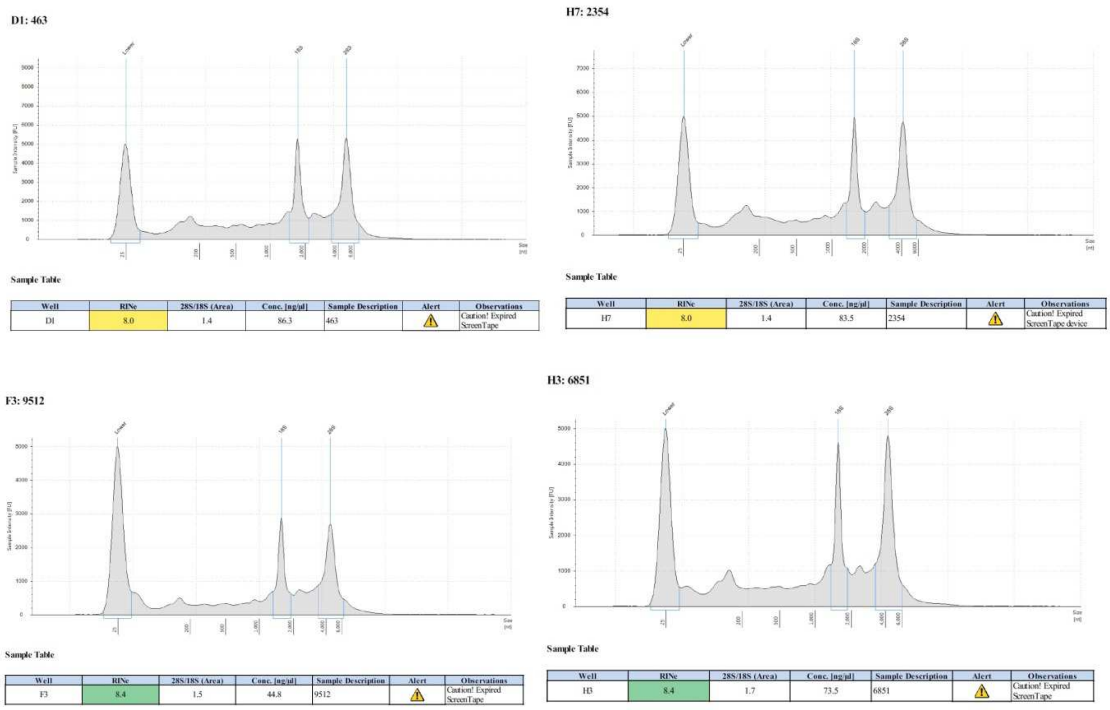

Figure 1. Bioanalyzer electropherograms from 4 RNA extractions from milk cells.