RNA Extraction protocol from vaginal swabs

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Modified from protocol by Jordan Bisanz

Sample Preparation for RNA Isolation

Vaginal swabs

1. Shake/vortex swab in RNAprotect+rifampicin (700ul per swab containing 100ug/mL rifampicin [1.4ul of 50ug/uL])
2. Incubate at room temp for ~5-10 min
3. Centrifuge for 10-20 mins at 5000 x *g* (put the centrifuge in the cold room)
4. Carefully remove all RNAprotect supernatant and store pellet in the -80°C (up to 4 weeks)

Broth cultures (if organism growth is at bottom of tube)

1. Carefully remove supernatant without disturbing bacterial growth
2. Add 2 volumes of RNAprotect reagent (Qiagen) containing 100 ug/mL rifampicin to the remaining culture
3. Incubate culture solutions at room temperature for 5-10 minutes.
4. Centrifuge solutions at 3500 x *g* for 20 minutes at 4°C.
5. Remove and discard supernatant. (optional: Freeze pellets completely with liquid nitrogen and) store at -80 C until required.

RNA Isolation

1. Resuspend swab pellets in 500-700ul lysozyme solution (in DEPC water):
   Lysozyme (20mg/mL = 0.02g/ml))
   50 U/mL mutanolysin
   Vortex to mix very well.
   Note: Mutanolysin is a gram positive-specific enzyme used to augment lysis.
2. Aliquot 500-700ul of the lysate into sample tubes.
3. Incubate samples at 37°C (incubator) for 45 minutes [20 minutes has worked]
4. Centrifuge samples at 5500 x *g* for 15 minutes at 4°C.
   [no more than 5500 x g or the pellet will be too dense to break up]
5. Remove and discard supernatant. Resuspend pellets in 1mL TRIzol reagent by vortexing. Break up pellet really well (use pipetting, vortex, syringe and needle for large samples) Incubate samples at room temperature for 10 minutes.
6. Add 200 uL chloroform to each sample. Vortex samples for 15 seconds each and incubate at room temperature for 10 minutes.
7. Centrifuge mixture at 16 000 x *g* for 20 minutes at 4°C.
8. Transfer 500 uL of the upper aqueous phase to a new RNase-free microcentrifuge tube. Be very careful not to get any interphase layer (contains DNA and RNases).

Precipitation

9. Add 50-60 uL of 3M sodium acetate and mix by pipetting ($1/10^{th}$ of volume)
10. Add 550 uL (1 volume) of isopropanol to each sample. Optionally add 1ul of glycogen as well (if <1ug RNA expected). Mix briefly by pipetting and incubate at room temperature for 10 minutes.

11. Centrifuge samples at 16,000 x *g* for 20 minutes at 4°C.
    Note: use proper centrifugation technique to know location of pellet (ie. take note of position of tube hinge when placing the microcentrifuge tube in the centrifuge).
12. Carefully remove supernatant and discard. Wash (vortex) pellet in approximately 500 uL of 75% ethanol (made from 100% etOH and DEPC H_2O)
    Note: Use a fine-tipped pipette as pellet will probably be loose. Make sure pellet is released
13. Centrifuge samples at 16,000 x *g* for 3-5 minutes.
14. Carefully remove and discard supernatant. Resuspend pellet in 20-30 uL of RNase-free water (Qiagen kit water) and 1 uL RNasin (optional - an RNase inhibitor).
15. Store samples at -80°C.

Option 1:

Perform TURBO DNase if needed (follow manufacturer's protocol)

If not using RNeasy cleanup, do second ethanol precipitation

1. Add 1/10th volume 3M sodium acetate
2. Add 3 volumes ice cold ethanol
3. Leave in -20C overnight
4. Spin 30min at > 10,000 x *g*
5. Do 70% ethanol washes
    a. Add ~250ml ice cold 70% ethanol
    b. Spin at > 10,000 x *g* for 10 min
    c. Repeat wash
6. Resuspend in 15-30 ul H_2O

Option 2:

RNA Cleanup and DNA digest using Qiagen RNeasy Spin Column ("RNA Cleanup" pp. 56-57 in RNeasy Mini Handbook)

Use up to 100uL of RNA from TRIzol prep, and 100ug RNA max

Before starting:

- First make up DNase with RDD buffer (step 6)
- All centrifuge steps should be at 20-25°C. Do not let centrifuge cool below 20°C
- Before using buffer RPE for the first time, add 4 volumes of 96-100% ethanol

1. Adjust volume to 100uL using RNase-free water.
2. Add 350 uL Buffer RLT mix by pipetting.
3. Add 250 uL 100% ethanol. Mix by pipetting.
4. Transfer 700 uL of sample to a spin column in a 2ml collection tube. Centrifuge at ≥ 10 000 rpm (≥ 8000 x *g*) for 15 seconds. Discard flow-through* being careful not to contact column membrane to flow-through
    *Flow through contains Buffer RLT and is not compatible with bleach
5. Add 350 uL Buffer RW1. Centrifuge at ≥ 10 000 rpm for 15 seconds (re-use collection tube). Discard flow-through.
6. Add 80 uL DNase incubation mix (10 uL DNase I stock and 70 uL buffer RDD) directly to spin column membrane. APPLY DIRECTLY TO MEMBRANE or digestion could be incomplete. Incubate at room temperature for 15 minutes.

Note: Aliquots of DNase stock are stored in freezer. Mix 10ul DNase I with 70ul Buffer RDD (fridge) before use. Mix by gently inverting the tube - do not vortex.

7. Add 350 uL Buffer RW1. Centrifuge at ≥ 10 000 rpm for 15 seconds. Discard flow-through.
8. Add 500 uL Buffer RPE to spin column. Centrifuge at ≥ 10 000 rpm for 15 seconds (re-use collection column). Discard flow-through.
9. Add 500 uL Buffer RPE. Centrifuge at ≥ 10 000 rpm for 2 minutes. Discard flow-through and collection tube. Place column in a fresh 1.5 mL collection tube.
10. Add 30-50 uL Rnase-free water to spin column membrane. Centrifuge at ≥ 10 000 rpm for 1 minute.
11. Repeat step 10. Start ethanol precipitation...

After isolation and cleanup, quantify (by spec) and check quality (by Bioanalyzer)

Use MICROBExpress according to manufacturer's directions using 8-9 ug of total RNA

Check for rRNA depletion using Bioanalyzer (gels are not reliable). More than 1 round of MICROBExpress may be needed to deplete all rRNA.