RNA Isolation Protocol – Damla D. Bilgin - IGB, UIUC.

**Materials:**
- Acid Phenol: Chloroform: Ambion Cat# 9722
- Ammonium Thiocyanate: Sigma A7149
- Chloroform (HPLC Grade): Fisher Cat# C606SK-1
- Glycerol: Fisher G33-500
- Guanidine Thiocyanate: Ambion Cat# 9422
- Phenol: Sigma P4557
- 3M Potassium Acetate pH 5.5: Ambion Cat# 9610
- 3M Sodium Acetate pH 5.5: Ambion Cat# 9740

**HMT Preparation Protocol:**

<table>
<thead>
<tr>
<th>Added</th>
<th>Conc.</th>
<th>20mL</th>
<th>40mL</th>
<th>100mL</th>
<th>200mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol Solution</td>
<td>38%</td>
<td>7.6mL</td>
<td>15.2mL</td>
<td>38.0mL</td>
<td>76.0mL</td>
</tr>
<tr>
<td>Guanidine Thiocyanate</td>
<td>0.8M</td>
<td>1.89g</td>
<td>3.78g</td>
<td>9.45g</td>
<td>18.9g</td>
</tr>
<tr>
<td>Ammonium Thiocyanate</td>
<td>0.4M</td>
<td>0.638g</td>
<td>1.276g</td>
<td>3.19g</td>
<td>6.38g</td>
</tr>
<tr>
<td>3M Sodium Acetate pH 5.5</td>
<td>0.1M</td>
<td>0.668mL</td>
<td>1.336mL</td>
<td>3.34mL</td>
<td>6.68mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5%</td>
<td>1.0mL</td>
<td>2.0mL</td>
<td>5.0mL</td>
<td>10.0mL</td>
</tr>
</tbody>
</table>

Add nuclease-free water to complete to volume.

**Protocol**

1. Pre-spin empty 50 mL Phase Lock Gel-Heavy tubes (PLG) briefly to collect gel on tube bottoms (1500g for 1 min @ 4°C is sufficient to collect gel at tube bottoms).
2. Pre-cool mortar with liquid nitrogen. Add excess liquid nitrogen so it does not fully evaporate.
3. Add frozen tissue to mortar containing liquid nitrogen.
4. Grind to a fine powder, carefully adding more nitrogen, if necessary, as it evaporates to ensure tissue does not thaw. *Be careful not to splash tissue out of the mortar*
5. Once ground sufficiently, let nitrogen evaporate off and add 6 mL of HMT and keep on grinding.
6. Cut the end off a pipette tip with a clean razor and transfer the sample to 50 mL PLG tube.
7. Add 2.13 mL HMT to the mortar again and rinse the mortar and transfer the liquid to the same PLG tube. (Total of 8 mL)
8. Add 1/5 volume of chloroform (1/5 * Total vol. of HMT added)
9. Mix by rocking and incubate at room temperature for 5 minutes.
10. Centrifuge swing out rotor program 2 (2000g for 10min @ 4°C)
11. Transfer and measure aqueous phase to a new 50 mL PLG tube add cold 3M Potassium Acetate pH 5.5 (1mL Potassium Acetate for every 3 mL of aqueous layer)
12. Mix by swirling
13. Add equal volume of acid phenol:chloroform (1mL / 1mL aqueous layer)
14. Mix by swirling
15. Incubate on ice for 1hr
16. Centrifuge swing out rotor, program 2 (2000g for 10min @ 4°C)
17. Transfer and measure the aqueous phase to ‘oak ridge’ tube
18. Add 0.25 mL of 0.8 M sodium citrate/1.2 M NaCl per 1 mL of aqueous phase transferred.
19. Mix by swirling
20. Add 0.25mL of absolute EtOH / 1mL aqueous (0.25*aqueous phase transferred)
21. Mix by gentle inversion and incubate at -20 °C for at least 30 minutes (overnight incubation produces higher yields).
22. Centrifuge fixed rotor, program 3 (11,000g for 10min @ 4°C)
23. Decant the supernatant (being careful not to lose pellet)
24. Add 2mL of 70% EtOH, vortex
25. Centrifuge, program 3 (11,000g for 10min @ 4°C)
26. Discard supernatant (being careful not to lose pellet)
27. Repeat 70% EtOH wash
28. Centrifuge, program 3 (11,000g for 10min @ 4°C)
29. Discard supernatant (being careful not to lose pellet)
30. Air dry the pellet
31. Dissolve in RNase free water (200 µL)

**Notes:**
1 – depends on starting material
2 – dark green = thawed
3 – Add appropriate amount of HMT until glue-like consistency
4 – based on amount added of HMT added in step 7
5 – Volume should be equal to amount HMT added
6 – DO NOT SHAKE!
7 – If Phase Lock broke or there seems to be gel in sample then transfer sample to a new PLG tube and add equal amounts of acid phenol:chloroform and centrifuge again with a water a counter balance.
Quick Glance Flow Chart:

- Add HMT to mortar to transfer and wash
- Add 1/5 volume chloroform
- Incubate 5 min while rocking
  - Centrifuge Program 2
  - Transfer and measure aqueous to new PLG
- Add 1/3 volume KAc and swirl
- Add 1 vol. of acid phenol:chloroform - swirl
- Incubate 1 hour on ice
  - Centrifuge Program 2
  - Transfer and measure aqueous to oak ridge
- Add ¼ volume of NaCitrate/NaCl and swirl
- Add ¼ volume of 100% EtOH
  - Gently invert and incubate at -20°C overnight
    - Centrifuge Program 3
    - Decant supernatant
      - Add 2mL 70% EtOH
        - Centrifuge Program 4
        - Discard supernatant
      - Add 2mL 70% EtOH
        - Centrifuge Program 4
        - Discard Supernatant
        - Air Dry pellet
        - Dissolve in 200μL of nuclease-free water

Sample #_____ → Sample #_____ →

Sample #_____ → Sample #_____ →