

Boiling RNA Preparation: for RNA isolation (from cell pellets). Modified by Jiyeon Kim on June 6, 2011. From Sarah Young,'s protocol and RNeasy Mini Handbook.

Media to have:

Lysis Buffer (1.2 mL), make this fresh immediately before starting

1M Tris-HCl, pH 6.8	300 $\mu$ l
0.5 M EDTA, pH 8.0	24 $\mu$ l
20% SDS	600 $\mu$ l
H <sub>2</sub> O	276 $\mu$ l

2 M NaOAc, pH 5.2

Glacial acetic acid

4 M NH<sub>4</sub>OAc

100 % ethanol

Everything must be made RNase free. Use RNA-only phenol, chloroform, and ethanol (you have to use the common acetic acid). Use plastic pipets and tubes as much as possible (the chloroform will melt the long pipets, work quickly). You will be working in 1.5 mL tubes (also RNase free).

Make sure all phenol/chloroform extractions are carried out in the hood. Phenol is toxic and corrosive. Make sure to wear gloves and a lab coat such that all skin surfaces are covered,

Samples are frozen cell pellets stored in the -80°C freezer and contain  $\sim 5 \times 10^8$  cells from growth experiments.

Before opening enzymes, make sure to spin briefly using mini-centrifuge to get contents out of cap.

Never vortex or warm enzymes – they are too unstable! You can flick tube gently with finger.

Protocol

1. Get samples from -80°C and keep on ice until ready to start.
2. Start heating the waterbath so that it will be boiling by the time you are ready to boil the lysis buffer. During that waiting time, prepare the lysis buffer (step 3) and resuspend the cell pellets (step 4).
3. Make the lysis buffer fresh using the volumes listed above. Make sure

that the tube is secured with the brightly colored lid lock caps before putting it in the boiling water bath. Place the tube in the water bath to bring buffer to a boil-one minute will do, it can go longer.

4. While lysis buffer is in the boiling water bath, resuspend your pellet in 250  $\mu$ l RNase-free H<sub>2</sub>O. Complete this by repeatedly drawing volume up and down in pipet tip and vortexing. (The RNase-free H<sub>2</sub>O is on the 2<sup>nd</sup> level of the bench). Keep on sample on ice until ready to add lysis buffer.
5. Take the lysis buffer out of the water bath and add 50  $\mu$ l to each resuspended pellet. (Leave water still boiling, do not turn it off.)
6. Vortex to mix (2 *seconds*), put lid lock back on, and immediately place the tubes back in the water bath to boil for 2 minutes.
7. Turn off the Bunsen burner and let the water stop boiling (*wait 20 seconds*). Take out the tubes and let stand at room temperature for 3 minutes. After cooling, mini-centrifuge for a few seconds to get liquid off the sides.
8. Add 25  $\mu$ l of 2M NaOAc, pH5.2. Vortex for 5 seconds.
9. Move the sample to the chemical fume hood for the following phenol/chloroform extractions.
10. Get the amount of phenol and chloroform you will need for your samples. Remove the amount of phenol you need from the stock bottle using a plastic pipet and putting it in 15 ml plastic test tube. (Phenol is stored in the 4°C refrigerator). Make sure to remove the phenol from the bottom; the top contains buffer you do not want. You will need a total of 600  $\mu$ l of phenol for each sample. For chloroform, you will need 900  $\mu$ l chloroform for each sample. Remove the amount of phenol you need from the stock bottle using a plastic pipet and putting it in 15 ml plastic test tube. (The chloroform stock solution is stored under the hood in the corrosive cabinet in a Styrofoam container). Go quick with chloroform; it burns the plastic. You also need three 1.5 mL tubes for each sample for the extractions.
11. Add 300  $\mu$ l of phenol to the sample, cap tightly, and mix well for 5 minutes. Place the samples in the special attachment for the vortexer that holds 1.5 ml tubes and. The more you shake, the cleaner it is.
12. Turn off vortexer, remove tubes, add 300  $\mu$ l of chloroform to the samples, and cap tightly. Mix well for 5 minutes on vortexer as before.
13. Spin at room temperature (23 °C) for 4 minutes at max rpm (~13,000 rpm) in the microcentrifuge.
14. Extract aqueous layer (the top layer) into a new 1.5 ml tube. DO NOT get too close to interface and avoid taking white gunk.
15. Repeat phenol and chloroform extraction 1 more time as in steps 11-14, removing top layer to a fresh tube.

16. Do one final chloroform only extraction by adding 300  $\mu$ l of chloroform to the sample, mixing as before on vortexer, and removing top layer to a fresh 1.5 ml tube. This is VERY IMPORTANT so that leftover phenol will not inhibit DNase in subsequent steps.
17. Add 37.5  $\mu$ l of glacial acetic acid. Brings pH to 5.0.
18. Add 20  $\mu$ l 4 NH<sub>4</sub>OAc, bringing to a ~ 0.2 M final concentration.
19. Add 100% ethanol (the “good stuff”) to bring volume up to top line on microfuge tube. This should be ~1-1.2 ml.
20. In a rush, precipitate by storing at -80°C for 1 hour. Otherwise, precipitate at -20 °C for 2 hours to overnight (can go longer). **You can stop here if you need to go.**
21. Centrifuge to precipitate RNA at max rpm for 45 minutes in the refrigerated microcentrifuge. Place hinges up so you know the pellet will be on the hinge side of tube. Make sure to remove ethanol from tubes immediately as pellet will soften if left. Let it spin longer if you can't be right there when it stops.
22. Remove ethanol with pipetman and invert tube on kimwipe to drain excess ethanol.
23. Air dry pellets, which normally takes from 15 minutes to 1 hour. If visible, pellet should become clear when dry. It appears as a grainy precipitate along hinge length of tube.
24. Resuspend pellet in 270  $\mu$ l RNase free water by repeatedly drawing volume up and down in pipet tip and vortexing briefly. Do not make bubbles.
25. MEASURE THE CONCENTRATION OF THE RNA using the Nanospec in the CMB to make sure you have RNA.
26. Add 30  $\mu$ l of 10X DNase I salts (our own made from Ambion recipe or provided with DNase).
27. Add 2  $\mu$ l of RNase-free DNaseI (Ambion **recombinant** DNase). Make sure to insert tip into sample to ensure that enzyme went in. DO NOT VORTEX enzyme, but spin down using microcentrifuge before taking cap off.
28. Incubate at 37°C (water bath) for ½ hour.
29. Add another 2  $\mu$ l of RNase-free DNaseI and incubate for ½ hour.
30. Purify the sample using RNeasy columns by the following steps and referring to the protocol provided by Qiagen.
31. Mix with sample in 2 mL (or 2063 5 ml tube) tube:
  - (a) 1050 uL Buffer RLT
  - (b) 750 uL Ethanol

32. Apply the sample (700 uL) an RNeasy Mini spin column (the pink tubes provided by Qiagen at room temperature in red box, not the RNA MinElute columns in fridge ) in fractions (it will take three times), spin and dump flow-through after each fraction. Centrifuge at >10,000 rpm for 15 s for each fraction. (Note: RNAeasy Mini spin columns are placed in the supplied 2ml collection tubes)
33. Add 500 ul Buffer RPE (make sure there's a check or an X that indicates addition of ethanol. If not, add appropriate amount of 100% ethanol to RPE) and centrifuge for 15s at >10,000 rpm (Reuse the collection tube from before). Discard the flow-through.
34. Add 500 uL Buffer RPE and centrifuge for 2 min at >10,000 rpm.
35. Carefully remove the columns after centrifugation such that column does not come in contact with the flow-through.
36. Place the columns in new 2 ml collection tubes (supplied), and discard the old collection tube with flow-through.
37. Centrifuge the columns at full speed (~14000 rpm) for 1 minute. DON'T throw away pink columns – need for repeating elution in next step!
38. Place the columns in a new 1.5 ml collection tubes and add 45  $\mu$ l RNase-free water (from kit) directly to the spin column membrane. Make sure water lands directly on membrane and do not poke membrane with tip.
39. Centrifuge for 1 min at >10,000 rpm to elute the RNA.
40. Repeat elution using an additional 45  $\mu$ l RNase-free water (from kit). The final volume should be in 90  $\mu$ l. Check concentration on Nanospec. Can store in 20°C overnight (or -80°C for longer periods) if you need to go. If stored, incubate samples before Turbo, the next step.
41. DNase using the Turbo DNA free kit (Ambion) by adding 10  $\mu$ L 10x buffer and 1  $\mu$ L DNase turbo. (Again, don't vortex enzyme – spin down using microcentrifuge). Mix gently.
42. Incubate at 37 °C for 30 minutes, vortexing periodically.
43. Add another 1  $\mu$ l of DNase.
44. Incubate at 37°C for 30 minutes, vortexing periodically.
45. Add 10  $\mu$ l inactivation agent and let it sit at room temperature for 5 minutes, vortexing periodically.
46. Centrifuge 90 seconds. Transfer RNA which is in the supernatant to new tube trying NOT to disturb the white inactivation agent pellet at the bottom (approx. 90 uL). If you transfer any by mistake, spin tube for 90 seconds and transfer RNA to a fresh tube.
47. Check concentration of RNA on Nanospec.
48. If completing QRT-PCR, dilute to 16.7 ng/ul using RNase-free water to total volume desired of diluted RNA. Please refer to the Syber Green

QRT-PCR protocols for further directions.

49. If completing microarrays, use the Qiagen RNA MinElute columns to concentrate RNA (these columns are kept in 4°C fridge). Refer to the kit for further directions.

	1. After ppt (270uL)	2. After 1 <sup>st</sup> DNase and column (90uL)	3. After 2 <sup>nd</sup> Dnase (85uL)
Average (ug)	22.6	18.1	12.4
Range (ug)	7.8-41.3	5.5-28.9	3.7-19.2
% yield compared to 1	/	68.8%	47.6%
% yield compared to 2	/	/	69.1%
Average concentration (ng/uL)	98.6	200.9	145.5
Range concentration (ng/uL)	28.9-153.1	60.6-320.9	43.6-225.4