Ketterson / Nolan Research Group Collection

This document is part of a collection that serves two purposes. First it is a public archive for data and documents resulting from evolutionary, ecological, and behavioral research conducted by the Ketterson-Nolan research group. The focus of the research is an abundant North American songbird, the dark-eyed junco, *Junco hyemalis*, and the primary sources of support have been the National Science Foundation and Indiana University. The research was conducted in collaboration with numerous colleagues and students, and the objective of this site is to preserve not only the published products of the research, but also to document the organization and people that led to the published findings. Second it is a repository for the works of Val Nolan Jr., who studied songbirds in addition to the junco: in particular the prairie warbler, *Dendroica discolor*. This site was originally compiled and organized by Eric Snajdr, Nicole Gerlach, and Ellen Ketterson.

Context Statement
This document was generated as part of a long-term biological research project on a songbird, the dark-eyed junco, conducted by the Ketterson/Nolan research group at Indiana University. For more information, please see IUScholarWorks (https://scholarworks.iu.edu/dspace/handle/2022/7911).

RNA EXTRACTION Trizol protocol, modified by CMBB/KAR on 1/20/10, again by KAR 6/17/10

Preparation

RNA extraction will take place under hood.

1. Put on a lab coat and nitrile gloves.
2. Lay a fresh layer of white lab paper in the hood, if necessary (located in the corner of the lab, near the centrifuge)
3. Prepare the centrifuge (the one in the corner, above the flammable liquids cabinet). Turn it on with the switch on the right side. Set it to 12 RCF (not RPM!) and 4 degrees C. Be sure to do this early enough that it will be cold by the time you need to use it. Run a quick test run (say, 10 min), to ensure that its cold enough. Be sure the inner lid is tight enough. If the outer lid doesn't close well, you may need to push back small latches inside.
4. Note the spill kit that is there for emergencies (located in the white bucket on the floor, not far from the sink).
5. Get RNAse away spray.
6. Get a tube rack, 1000uL pipette and 200uL pipette. Wipe them all down with RNAse away.
7. Ensure that you have enough filtered tips to use for today (lots of 1000uL and 200uL)
8. Get out glass culture tubes and lids (14-956-3C) and label them with your sample numbers. Do not reach into the container; re-seal with lab tape. Record the real sample codes in notebook.
9. Get our epi tubes (1.5mL), one for each sample (for now). Pour them onto a kimwipe. Do not reach into the beaker to get them out. Close lids immediately. Label them with your samples numbers.
10. Get dry ice, from the storeroom downstairs. Keep it in a small foam cooler. Weigh it and charge to DNA account (66-273-00)
11. Get regular ice. Bucket is near sink; ice in the first floor autoclave room (A108).
12. Get blade and tweezers (x2)
13. Get timers (on fridge)
14. Prepare waste containers -- Trizol is hazardous and you'll need a solid Trizol trash (a big plastic bag, labeled appropriately as trizol waste) and a liquid Trizol trash (a large jug, also labeled with hazardous waste). Keep Rose up to speed on fullness of the hazardous waste.
15. Get out 3 small beakers (~80 mL each). Clean them with water, then millipore water, then ethanol, then more m-water, then RNAse away before you use them. Millipore turns on by pressing the bottom half of the button on top. The read-out should say "18.2" on it. Remove the clip on the tube and use RNAse away before using.
16. Fill one small beaker with millipore water
17. Fill one small beaker with alcohol (200 proof ethanol, from the chemical cabinet), to use for cleaning blade and tweezers after each tissue sample
18. The 3rd small beaker is for Trizol, which is stored in small black fridge next to the centrifuge. You'll need 1 mL Trizol/sample plus 2 mL extra Trizol in the beaker for washing the homogenizer. To transfer Trizol from its storage container, use a 10mL serological pipette (the long glass pipette that's disposable). They are located in a drawer at the same table as the centrifuge. NOTE: Juli said always pour it out, don't pipette directly from the stock. You will use this pipette with the gun-shaped "accujet", which is stored next to the Millipore. Be sure to plug it in later, to charge it, after you use it. Put the used serological pipette in the solid trizol trash.
19. Get the homogenizer from the shelf near the Millipore water. Clean it by running it in the beaker of ethanol, then in water.
    - The tip can also unscrew for more intense scrubbing, if needed.
    - Homogenizer use: the blue adjusts the speed (use at 4.5 speed).
20. Clean *everything* with RNAse away (or wipe with a kimwipe sprayed with RNAse away) -- pipettes, gloves, vortex, small centrifuge. Also put some RNAse away on your gloves!
21. *Start steps 1 and 2 of the homogenization process before retrieving samples from -80.*
22. Get your samples from the -80. Put them directly in cooler of dry ice. Return to CISAB lab to begin extractions.

Homogenization

The most important thing is to do the extraction immediately after the tissue is out of -80, so be ready!

1. Fill each culture tube with 1mL trizol, and keep them on ice, lids on.
2. Double check temperature on centrifuge (4 degrees) just before you homogenize.
3. Get out one sample from the -80 at a time. Keep it in your cooler of dry ice.
    - Invitrogen suggests 1mL of trizol per 50-100 mg of tissue for best results. Too much tissue will give bad yield at the end.
    - Put any parts of the sample that you're not using immediately back in the -80 (e.g. if you use 1/2 an ovary).
    - If you need to cut your sample, cut it on a block of dry ice with the razor blade. Shield the area with your hand so that your sample will not go flying!
4. Put sample immediately into its culture tube and homogenize on ice for max of 45 seconds (probably less for softer tissue, like ours). Move your hand up and down a little, like you would with a hand blender. If there are still chunks, you can come back and homogenize again in a minute.Try not to mash the sample into the bottom (it can get stuck). Do many small steps of homogenization, so as to not warm the sample. Only homogenize ~10 sec at a time.
5. After homogenizing each sample, wash homogenizer in trizol, then either store the homegenizer in water or rinse in water before next use.
6. Repeat homogenization for each sample.
7. Transfer each sample from the culture tube into an epi tube with a pipette (1000uL)
8. Balance samples in the centrifuge. Put in centrifuge: 12 RCF for 10 min at 4C
    - To operate centrifuge, open it with open button, remove inner lid by turning, put tubes in slots (with hinges facing out), balance them appropriately. Put inner lid back in place. Be sure that it is tight. Then, close outer lid, and start.
    - RNA will be in supernatant

Phase separation

1. While waiting, get out new epi tubes and label them.
2. Get out chloroform from under hood. Transfer with sterile serotological pipette & accujet the amount you need (200 uL/ sample) into epi tube. Label the chloroform epi tube with "C" on top.
3. Transfer supernatant into new tubes, using a small pipette (100 or 200uL) and let them sit for 5 min at room temperature. Put the old tubes in the trizol waste.
4. Add 200 uL chloroform and shake hard for 15 sec, then let sit for 3 min. at room temperature
5. Balance samples in the centrifuge. Put in centrifuge: 12 RCF for 15 min at 4C
    - RNA will stay in aqueous phase

RNA Precipitation

1. While waiting, prepare new epi tubes for your samples; label them properly as final tubes.
2. Get out propenol (a.k.a. isopropyl alcohol or 2-propanol) from chemical cabinet. Transfer appropriate amount (500uL/sample) from storage container into conical tube (if needed).
3. Add 500 uL propenol to each new tube with a pipette. Pipette slowly – propenol is jumpy.
4. Pipette supernatant into propanol using 200 uL pipette. Avoid the white film! Move pipette around surface of liquid. Do many small steps to avoid contaminating supernatant with the white film.
5. Vortex thoroughly, then let sit for 10 min at room temp
6. While waiting, turn up the heat on water bath to ~7, then when it gets to 55-60 C (takes ~15 min), turn it down to ~2.4.
7. Balance samples in the centrifuge
8. Put in centrifuge: 12 RCF for 10 min at 4C
    - RNA will stay in pellet

RNA Wash

1. Pour off all propenol supernatant into beaker without nudging the pellet (ultimately, pour it all into trizol waste)
2. Add 1 ML of 75% ethanol (located in conical tube in chemical cabinet)
3. Vortex gently, just until pellet free floating (don't pipette up and down to mix it). Pellet will "hop" off side of tube. If you vortex for too long, the pellet with break apart and make the next step more difficult.
4. The pellet will be well visible at this point -- note the size
5. (*You can store this product in the fridge overnight, if you'd like*)
6. Balance them in the centrifuge
7. Centrifuge at 7.4 RCF for 5 min at 4C
8. Pour off ethanol supernatant, being careful not to spill ethanol on the tubes and remove the labels
9. Dry with a kimwipe -- shove kimwipe into tube to wick up the ethanol. Be careful not to lose pellet. [Do not overdry!]
10. For 10 min, let tubes dry out in the hood. Sometimes, you will not be able to see the pellet at this point. Do not be afraid.
11. Estimate amount of nuclease-free water needed per sample according to pellet size (30-80 uL) and write it down.
    - For example, we used 35 uL for a small male testes and 50uL for a half ovary and 70 uL for a whole ovary. We used 80 for big brain/gonad samples (like PTR or HYPO), and it was far too concentrated for making cDNA without first diluting.

Redissolving RNA

1. Add appropriate amount of millipore water to each sample according to pellet size
2. Vortex until pellet dissolves (only a few seconds).
3. Spin down your samples VERY briefly, using the spinner on the main bench.
4. Heat samples in water bath, on orange floaty-thing, at 55-60 C for 10 min.
5. Put on ice.

(THIS IS A GOOD TIME TO MAKE A GEL, if you haven't already)

NANODROP at CISAB

1. Turn on the computer in the CISAB lab if its not on already: user: cisab, password: animal! Turn on reader (switch on front, right)
2. Get some millipore water, 10uL pipette and tips, kimwipes, and 70%EtOH spray
3. Remove Take3 holder from James Bond case. Don't drop it. Wipe clean with millipore water and EtOH spray/kimwipes
4. Open Gen 5 icon on the desktop
5. Click Take 3 session (to get 260/280 and concentration of your RNA sample
6. Check the appropriate pull down tabs for what we're working with: Take3/microspots/RNA
7. Highlight the desired wells on the plate (must be contiguous)
8. Load clean water onto the desired wells (can use 2-5uL per sample. we use 3 uL typically). Be careful not to create bubbles or to scratch the bottom of the wells with your pipette tip.
9. Click "read blanks". Load take3 into machine (can push the button to open if it doesn't open for you...) then "OK"
10. If everything is okay, the wells will look green on the screen now.
11. Next, highlight the wells of samples that you'll need (same ones as the blanks)
12. Click read samples... the tray will eject. Wipe off the water with a kimwipe. Then, load your samples. Be sure to write down the order in which you loaded the samples into the wells. When ready, click "ok"
13. When the machine is done reading, click end of batch (unless you have more than 16 samples, in which case you can read more samples)
14. In the summary sheet, create a new row that has the name of your samples (e.g. VAM4HYPO)

15. Save the excel file in the "Rosvall" folder. Save file name as "RNA_VA_date_run1" etc.
16. Print out a copy and paste it into your notebook.
17. Close all programs.
18. If your RNA samples have a 260/280 of 2, that's ideal. as low as 1.8 is okay.
19. If your RNA samples have a concentration of ~1000ng/uL to 100ng/uL, that's okay. Anything more than 2000ng/uL will need to be diluted to make cDNA. Anything less than 90ng/uL is too dilute (i.e. not enough to reliably make cDNA)

GEL ELECTROPHORESIS

1. **Prepare a 1% gel.** You will need:
    - 0.5 g agarose
    - 50 ml 1x TBE
    - 3 ul EtBr
2. Prepare the gel mold. There's a drawer across from the door with all the necessary parts. If not, check the drying rack.
3. Balance the mold on the table by laying it flat on the countertop and adjusting the knobs until the bubble is centered in the black circle.
4. Choose comb appropriate for number of samples to be run, and place at one end of the gel, blue side facing out, knobs facing in.
5. Adjust the height of the comb with the knobs.There should be a pipette tip's worth of space between the comb and the bottom of the tray but otherwise the comb can be pretty close to the tray bottom. Now for the gel!
6. The weighing station is next to the hood fridge and materials can be found in the drawers under the counter.
7. Get a plastic weigh boat and tare the scale (center button).
8. Weigh 0.5g agarose. Agarose is in the cupboard above the scale. Pour into Erlenmeyer specially marked for gel use, which should be at the center table near the pipettes.
9. Measure 50ml 1x TBE in a graduated cylinder; TBE is also at the center table.
10. Mix with agarose in flask.
11. Stuff a kimwipe in the flask mouth. Microwave for 30-50s until the solution is boiled and clear. Remove from microwave - the flask is very hot! Use kimwipes to wrap around the neck of the flask to protect hands long enough to transfer to the table.
12. Add EtBR to beaker while liquid is still very hot - just drop it in
13. EtBr is hazardous: All solid waste involving EtBr goes into appropriate bucket in center of table: gloves, gel, pipette tips etc.
14. Gently swirl beaker to mix in EtBr. Pour solution into gel tray once the flask is cool to touch.
15. Cover with kimwipe, being careful not to touch the gel.
16. Wait ~30 min for gel to harden at room temperature; less if refrigerated
17. Now is a good time to put the flask in water, and brush down the balance with a paintbrush found in weigh boat drawer.
18. More 1x TAE can be made by diluting 10x TAE, as needed: 50ml 10xTAE into 450 millipore.There needs to be about 5mL to run the tray later.

Prepare to load gel

1. Cut parafilm on which to prepare dye solutions; fold upper left corner for orientation
2. Label spots where samples will go a record in notebook.
3. Retrieve dye from the hood fridge now, and allow to thaw. It's in the CISAB box in the freezer.
4. Mix up dye, sample, and millipore water in small beads on parafilm by pipetting one into another
   Dye should always be 1ul dye : 5ul other stuff
    - 2ul dye ("6x loading dye"),
    - 9ul millipore water

- 1ul sample (hand mix and spin samples gently first before adding them, if they've been sitting for a while)

Running the gel

1. Transfer the gel from the mold to the electrophoresis setup, making sure the wells are on the same side as the black leed.
2. Add 1X TBE until wells are covered.
3. Load the samples, being careful not to puncture bottom of wells
4. Run at 97V for about 20 minutes
    - Voltage should run black (bottom with wells; negative) to red (top; positive)
5. Transfer the gel to the UV room. There's a glass plate to help with this in the gel drawer.
6. Slide gel onto the viewing screen and center.
7. Place the triangular viewing hood over the gel and turn the machine on.
8. You should see 2 bands, for 18S and 28S ribosomal RNA.
9. Turn on the camera and printer.
10. Zoom in on the camera until you have a clear view of the gel. Adjust the ISO using the direction pad (80-100 works best) and take the photo.
11. Flip the switch on the black USB control box and follow directions on screen to print.
12. Additional setting can be accessed on the camera screen such as setting date/time and trimming extra black (saves ink!) Adjust the size of the trim rectangle after navigating to the option by using the zoom settings.
13. Print using the camera display.
14. Immediately label well numbers and gel orientation.
15. Turn OFF the machine before disposing of gel in EtBr waste.

Clean up:

1. Make sure samples are in -80, and be sure any leftover dry ice is put back in -80.
2. Note which freezer box you put the RNA samples in. Record this information in your notebook.
3. Wash dissection tools with soap in sink in chemical room, then rinse with water, mp-H20, then put on drying rack.
4. Put the trizol solid waste into the trizol solid waste container.
5. Wash 3 little beakers with soap, then mp-H20
6. Ensure that all electronics are off: centrifuge, waterbath, gel rigs, hood lights, regular lights, etc.
7. Wash all of the glassware used in making the gel. Dry off the UV/gel machine with paper towels and EtOH (EtBr waste).