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).

Quantitative PCR

Protocol provided by J. Barske, modified by KAR/CBB for CISAB/CGB

Days before – Preparation

1. Sign up for qPCR machine (plan on 2-3 hr to prep, then 3.5 hr of machine time)
2. Make sure you have all reagents
3. Use software to organize samples on plate and define the thermocycler program, and add the program file to thumb drive
4. Do the math for the mixes using the google spreadsheet. Mixes will contain the following amounts for each sample (total=22.5ul per sample).
   - 7ul water
   - 1.5ul forward primer (diluted)
   - 1.5ul reverse primer (diluted)
   - 12.5ul syber green enzyme

NOTE: If primers require dilution, this can be done during mix preparation. The spreadsheet calculates total amount of diluted primer needed, but also breaks it down into amounts of fully concentrated primer and water to be added to the mix.

Day of – Preparation

1. Turn on the fan and UV light for >10 minutes in the hood before beginning any work with samples.
2. Get 2 bucket of ice (small one is for mixes), pipettes, tips, kimwipes, wells and caps, 2 plate holders (one for refrigerator), markers… Clean RNase clean bench and all supplies with EtOH and RNAse AWAY. Clean your gloves
3. Get out a tube of SYBR from the freezer, place upright in fridge, in tube rack, covered with foil. Never refreeze the tube once it's been thawed - any extra stays in fridge. One tube is approx enough for one plate. Light sensitive!
4. Make several new 1.5 mL epis of water, directly from the freshly cleaned mpH2O – enough for 1/replicate of NTC + 1 for the mixes + 1 for making cDNA dilutions. Only fill them partway, so that you don't have contamination related to the lids having water on them and it getting on your fingers. For example, if your plate had 4 sets of NTCs and I was running a 2-gene plate, I would make 6 tubes of water. Remember that you will only use 5uL from the NTC water tubes, so these tubes do not have to be full at all! Aliquot all of your waters before you open any other reagents. This will ensure that your waters are clean. Once you use a water, throw it away.
5. Pour out and label small eppendorf tubes for all sample dilutions, and large eppendorf tubes for the mixes.
6. Pipette all water (for mixes and sample dilutions) before opening any cDNA. For mixes, Kim recommends the 200uL pipette (for accuracy), even it if means you have to pipette several times.
7. If it has been 10 minutes since you turned on the UV lamp, **turn it off** and get out your primers.
8. Remove appropriate primers from freezer and allow them to thaw at room temperature. Once thawed, flick and spin down primers thoroughly, store carefully on ice. Do not open them yet!
9. Spin your mixes to ensure that all water goes to the bottom of the tube. If any sample dilutions need spinning to make sure water is at the bottom, do the same. Keep at room temp until you add anything to waters.
10. Now add your stock primers to your mixes using values from spreadsheet. Use extended tips, mix up and down. Put mix on ice.
11. Keep your stock primers on ice (or in the fridge) until you are done loading your whole plate, in case you mess up and need more mix.
12. Clean your gloves again.

Diluting cDNA samples 1:10 (1.1ul sample + 9.9ul H2O)
Two-person job: "loader" and "assistant"

1. Get our your cDNA samples. They will thaw very quickly at room temp. Flick them and spin them once thawed. Put on ice. Kim typically puts them in a small rack in the ice, so she doesn't lose them or get them wet with ice. They will stay cold enough – cDNA is stable.
2. The assistant should sit outside the hood, with the cDNA stock in from of him/her.
3. Assistant should open cDNA tube, making sure not to splash liquid around or get cDNA on his/her fingertips. As long as the lid is open at least 1/3 of the way, the loader can open it the rest of the way with the pipette tip. Announce the sample #, so the loader can open the tube of water.
4. Assistant hands loader open cDNA tube. Loader pipettes appropriate amount into water, mixing up and down with pipette. Loader hands open cDNA to assistant, who closes it, and prepares to open the next cDNA tube. Loader closes the cDNA dilution and puts it on ice, in the hood. ***The key here is that the loader never touches the lids of the cDNA stock, so as to avoid contaminating fingertips. Assistant should periodically clean gloves with RNAse away to remove any trace amounts of cDNA. Assistant should also take care to not get the loaders hands wet with anything (ice, sample, etc.).***
5. Repeat this process for all samples.
6. Flick and spin all cDNA dilutions. Arrange in ice according to plate layout to facilitate loading.
7. Clean your hands again with RNAse away.

Adding SYBR green to mixes

1. Have foil ready to cover your ice bucket of mixes (small red bucket). I also turn off the hood lights at this point, but be sure to leave the fan on.
2. Get SYBR green from refrigerator. Mark date that any new SYBR tube is opened. If you need to use SYBR from two different tubes, be sure to evenly divide it in the two mixes, especially if the tubes are from different lots (they probably aren't, but best to be cautious, this is qPCR!). Transfer from one tube to another, if necessary.
3. Spin your SYBR for 1 -2 seconds only – just enough to get any residual off the lid, so as to avoid contamination of the SYBR or your fingers. Remember, its enzyme, so you don't want to overspin.
4. Add appropriate amount of SYBR green (from spreadsheet) to each mix with 200uL pipette. Mix up and down, slowly each time you add it. If you mix too quickly, your SYBR will stick to the filter and your mix calculations will be off. **If this does happen, make a note of it and proceed – it will not affect your fold changes much, if at all.**
5. If necessary, spin again for 1-2 seconds, to get the last bit out. Tapping the tube on the counter (with lid on) will help to get rid of bubbles (tapping works better than spinning to remove bubbles).
6. Return any remaining to fridge and cover with foil
7. At this point, your mixes should contain appropriate amounts of water, primers, and SYBR green. Make sure your lids are tightly closed on your two mixes, then gently invert them to make sure they are mixed. Spin 1-2 second to remove any liquid from lids.

Loading wells

1. Wriggle out a new plate, or new strips of wells, without touching the others in the pack. Kim typically loads 3 strips at a time, no more. She also makes sure that she has one set of NTC per "loading run" (e.g. one NTC for every 3 strips).
2. While loading, be careful that your arm/sleeve doesn't get near the plate at all.
3. Add 22.5ul of mix to each well. Wet your mix tip first so that you use the exact same mix amount for all wells. If you have to change tips, try to do it after completing a replicate, unless you are worried about contamination. Check volume on pipette every so often.

4. Kim releases the pipette two times only per well: "I put the tip all the way to the bottom of the well, release to second stop as I slowly raise the tip to avoid the level of the liquid (~3mm), then I release again to second stop, while touching the side of the tube ~5mm from the bottom. I tend to move in a slightly circular motion, but I'm not sure if that matters. Then, lift the tip straight up without touching the sides of the well. ***The key thing here is try to get all of your mix at the bottom and avoid too many bubbles (from multiple releases of pipette plunger). Bubbles along sides and near the top of the well will make it more likely for your adjacent wells to contaminate each other when you put on the lids."***
5. Add 2.5ul of water to each NTC first before touching any samples. Use a fresh water each time, then throw it away, even though you only used 5uL of it!
6. Add 2.5ul sample to each duplicate well (new tips between all replicates and samples). The assistant should open the tubes and close the tubes, and the loader should only ever touch the sides of open tubes. Take an open tube from the assistant, move towards the plate, put sample in 1st well, change tips, put sample in 2nd well. Then hand the open tube back to the assistant in his/her left hand, and the assistant will have the next sample open in his/her right hand. ***The key thing is that the loader never touches cDNA at all.***
7. Loader wriggles out a strip of lids. Carefully close wells. Be careful not to get any liquid on the lids when putting them on which could cause contamination. Press down slightly on all wells first before returning to each well to press down more firmly and fully close them.
8. Number your strips at the top, where the "A" is on the lids. Both loader and assistant should check lid closure while transferring to fridge. Store strips in refrigerator while loading next set.
9. Spin plate on speed "3" for 1-2 minutes (less time and you will still have liquid on the sides of the tubes). Check lid closure again.
10. Close all partly used bags of lid/wells with lab tape. Date them.
11. Carry plate in foil on ice to CGB – also bring along thumb drive

At CGB

1. Insert thumb drive, find plate file
2. Press lamp on
3. Check that everything looks good in the thermocycle (60 degrees, then 70 degrees for 2 plateaus)
4. Load plate (checking lid closure, no lint on lids and making sure there are no bubbles as you do)
5. Run plate, after warm-up
6. Check "lamp off at end of run"
7. Return to CGB 15 minutes later to make sure it is actually running.

Finishing Up

1. Go clean up at CISAB
2. After 2.5 hours, go get plate from CGB
3. Throw it away.
4. Save the file onto thumb drive
5. Look at results on your own computer