Day 1 – (mRNA → cDNA)

Assume you have "n" number of samples and "p" number of gene sequences you want to look at

Before getting started, clean all surfaces with RNase Zap

Procedure	Additional notes
1. Make solutions/buffers:	1. You don't have to do this step every time. Check the most recent date on the RLT and if that date is more than a month ago, you have to read the BME. Check to see if you have enough RPE and RW1.
a. RLT: add 450uL BME to RLT	a. RLT i. Always add 10uL BME per 1mL RLT ii. Mixed solution lasts for 1 month (write day solution was made on bottle) iii. After 1 month, need to add more BME (10uL/1mL RLT) iv. Ex. If you used 10mL of solution, have ~35mL left so add 350uL BME
b. RPE (wash buffer): add 44mL 200 proof ethanol to 11mL RPE	b. RPE i. Use "RNA-only" graduated cylinder to measure out 44mL ethanol ii. May see precipitate – just mix until precipitate is no longer visible
c. RW1 (wash buffer)	
2. Let homogenized tissue and DNase thaw completely on ice	2. Homogenized tissue should have been preserved in the -80 freezer and the DNase should have been preserved in the -20 freezer
3. Label "n" microcentrifuge tubes and label "n" QIA shredder tubes (purple)	3. Make it a habit to label the sides of the tubes as well as the top. You will thank yourself later.
4. Pipette out ALL thawed homogenized tissue into the labeled QIA shredder	4. The QIA shredder further homogenizes the tissue and takes care of all the fat of the brain that the tissue homogenizer didn't.
5. Centrifuge sample – max speed for 2 minutes	5. All of the fat will be trapped in the purple tube (toss the purple tube after centrifuging)
6. Recentrifuge sample – max speed for 3 minutes (may be a good time to change gloves at this time)	6. May see very fine yellow ppt./pellet (which is fine)
7. Pipette out 500uL of sample from clear tube and place in new and labeled microcentrifuge tube	7. Try not to pipette the ppt. on the bottom if present. Sample is now mostly RNA at room temperature so you want to work fast and clean

8. Add 70% EtOH to sample. You want to double the total volume when adding ethanol (doesn't have to be exact)

9. Centrifuge all of sample in spin column (pink tubes) for 30 seconds at 13,000rpm (split total volume of sample into two since we have ~1,000uL and only ~700uL fits in the spin column

10. Add 350uL of RW1 to sample and centrifuge (30s, 13,000 rpm)

11. Mix DNase and RDD: 10uL DNase per 70uL RDD per sample (add enzymes last). Mix in RNAse-free tube

12. Pipette out 80uL of DNase + RDD mixture and place right in membrane of each spin column (pink tube) and let it sit for 15 minutes on ice

13. Add another 350uL of RW1 and centrifuge (30s, 13,000rpm)

14. Add 500uL buffer RPE ad centrifuge (30s, 13,000rpm)

15. Add another 500uL buffer RPE and centrifuge (2min., 13,000rpm)

16. Replace clear tube with new one and centrifuge (1min., 13,000rpm)

8. For example, if you eyeball 500uL of sample, add 500uL of EtOH. EtOH is added because it ppts. RNA. You can make 70% EtOH by diluting the 200 proof EtOH.

9. So add ~600uL of sample, centrifuge it, dup out the contents of the clear tube and then add the rest of the sample and recentrifuge (30 seconds at 13,000rpm); dump contents of clear tube when you're done.

10. Dump out content of clear tube after centrifuging

11. Follow "n+1 rule" (n = # of samples):

n	n+1	DNase (uL)	RDD (uL)
1	2	20	140
2	3	30	210
3	4	40	280
4	5	50	350
5	6	60	420
6	7	70	490
7	8	80	560
8	9	90	630
10	11	110	770
12	13	130	910
14	15	150	1050
16	17	170	1190
18	19	190	1330
20	21	210	1470
22	23	230	1610

12. Try to be as precise as you can for this step

13. Dump out contents of clear tube after centrifuging

14. Dump out contents of clear tube after centrifuging

15. This is the final washing step

16. Gets rid of any residual EtOH

17. Transfer pink tube (with the sample in it) into new and labeled microcentrifuge tubes	17. Place pink tube within new tubes
18. Elute in 30uL of RNase-free water directly onto membrane and centrifuge (1min., 13,000rpm)	18. Place a cover in the centrifuge is possible since the caps of the microcentrifuge tubes may break off. RNA will be collected in the new tube and you can toss out the pink tube
19. Get out the iScript reaction mix from the freezer and thaw the buffer and enzymes on ice	19. You will need these later when you put the samples in the thermocycler.
20. Get the microcentrifuge tubes with your RNA sample and figure out the concentration of RNA via a Nanodrop (Keck Center, Nelson Labs)	20. Bring the following with you: - Kimwipes - Gloves - Paper - Pen/pencil - Samples on ice - 30uL of RNase-free water to be used as blank - 0-10uL pipette - 0-10uL pipette tips (bring whole box)
21. To use the Nanodrop: - On the computer, select "nucleic acid," you will be prompted to calibrate the machine. - Pipette 1.7uL RNase-free water onto nanodrop lens and select "enter" - Change setting from measuring DNA to RNA (upper right corner) - Pipette another 1.7uL RNase-free water onto nanodrop lens and select "blank" - Pipette 1.7uL of sample onto nanodrop lens and select "measure" - Record sample name, concentration, and A210 purity	21. Clean the Nanodrop lens between each sample use with a kimwipe. The ideal purity is 2.10 and you want the purity to generally range between 2.05 and 2.15.
22. Make a master mix of buffer and enzyme (from the iScript reaction kit). 4uL of buffer and 1uL of enzyme per sample.	22. Follow the n+1 rule. For example, if you have 3 samples, mix 16uL of buffer and 4uL of enzyme
23. Based on the concentration, and instructions based on the iScript kit, normalize the sample into a 96-well (that can be used in the thermocycler) so that the concentration of the RNA is equal in	23. You will adjust the volume of both the water and the sample to normalize the concentration of the RNA so a different volume of each will be going into each of the wells.

each of the wells. 24. Place in thermocycler and run cycle ONCE (40 minutes) 25. Now you have cDNA which is ready for PCR. Pipette sample into new, labeled microcentrifuge tube, add 80uL nuclease-free H2O to dilute down and store in -20 freezer until ready to perform PCR	24. The thermocycler machine will automatically run two cycles so you have to time out 40 minutes and stop the thermocycler after one cycle

Day 2 – (qPCR)

Procedure	Additional notes
1. Thaw cDNA samples, primers (both forward primer and backward primer), and dye (ex. Cybergreen) 2. Label n number of microcentrifuge tubes and pipette in the following according to the given ratio so that you end up with (67.5*p) uL in each microcentrifuge tube (we will call this batch A)	1. Note: p = the number of gene sequences and not the number of primers (For example, if looking at ARC and GAPDH, p=2, but you would have 4 primers to thaw: ARC forward ARC reverse GAPDH forward GAPDH reverse) Note: If using Cyber Green, it is light sensitive so thaw in the bag that it came in and minimize exposure to light 2. When labeling the microcentrifuge tube, you want to include sample ID and gene sequence (Ex. If you have 3 samples (n=3) and are looking at ARC and GAPDH (p=2), you would label 3 microcentrifuge tubes (n): <PCR 1> <PCR 2> <PCR 3> And each of the 3 microcentrifuge tubes would have (67.5 x (p=2) = 135uL in it) Note: You may want to consider doing all the labeling involved in both steps #2 and #3 before actually pipetting to minimize exposure to light if using Cyber Green. Note: If you are working with many gene sequences, you may have to label more microcentrifuge tubes to accommodate the fluid volume

	Ratio	Ex. If p=2
Ratio	1x	7.5x
Rx (dye)	10	75uL
Template/cDNA	1	7.5uL
Nuclease-free H2O	7	52.5uL

3. In addition, label np number of microcentrifuge tubes, For each sample n, there should be p number of microcentrifuge tubes

4. From batch A (the microcentrifuge tubes that you just pipetted a total of 135uL in), pipette 66uL in

   (we will call this batch B)

5. In each microcentrifuge tube of batch B, add 3uL of forward primer and then add 3uL of the reverse primer for each of the primers

6. Spin the microcentrifuge tubes of batch B briefly (~5 seconds) to make sure that every little bit of primer is in the solution

3. Number of microcentrifuge tubes to be labeled:

	Number of gene sequences (p)						
Number of samples (n)	0	1	2	3	4	5	6
	1	1	2	3	4	5	6
	2	2	4	6	8	10	12
	3	3	6	9	12	15	18
	5	5	10	15	20	25	30
	10	10	20	30	40	50	60
	20	20	40	60	80	100	120

4. Try to be as accurate as possible, you will a narrow margin of error in the coming steps. Work slow and precise. Remember, you are now working with cDNA which is more stable than mRNA so you don't have to rush so much

   (Based on example of previous step, you would now label 6 microcentrifuge tubes:
   <PCR 1 ARC>, <PCR 1 GAPDH>
   <PCR 2 ARC>, <PCR 2 GAPDH>
   <PCR 3 ARC>, <PCR 3 GAPDH>
   And each microcentrifuge tube will contain a total volume of 66uL)

5. 3uL is not a lot and it is VERY easy to make an error in this pipetting step so be very careful and cautious

   (Based on the example of previous step, you would now:
   add 3uL of ARC forward to:
   <PCR 1 ARC>, <PCR 2 ARC>, and <PCR 3 ARC>
   And then you would add 3uL of ARC reverse to:
   <PCR 1 ARC>, <PCR 2 ARC>, and <PCR 3 ARC>
   Now same thing with GAPDH; add 3uL GAPDH forward:
   <PCR 1 GAPDH>, <PCR 2 GAPDH>, <PCR 3 GAPDH>
   And then add 3uL of GAPDH reverse to:
   <PCR 1 GAPDH>, <PCR 2 GAPDH>, <PCR 3 GAPDH>
   So you should end up with 72uL (66+3+3 uL) in each microcentrifuge tube)

6. Since you are working with such a small amount of the primers, you don't want it to adhere to the walls of the microcentrifuge tubes and not enter the solution

7. Finally, the PCR plate…handle the PCR plate with a Kimwipe. Do NOT actually touch the PCR plate and do not place on table directly. Place a Kimwipe on your work surface and place the PCR plate on top of that

8. Fill out the PCR form (found under the folder titled "PCR (JCYK)"

9. Print a blank PCR plate form (also found under the folder titled "PCR (JCYK)" and label the wells so your data can mean something to you after the PCR is done. (Otherwise, you end up with a bunch of useless numbers)

7. Handling the PCR plate with a Kimwipe ensures that the data collected later has a better chance of being accurate since it won't be skewed by any oils from your skin, or dirt from the table

8. The DNA Core facility requires that you request to bring a plate to make sure that they are available to receive it at the time you want to drop it off and they close at 1pm so plan accordingly.

9. The PCR plate is a 96-well (12x8 plate) and each of the microcentrifuge tubes of batch B occupies 3 of the wells (preferably 3 adjacent wells per microcentrifuge tube of batch B)

   Note: the rows of the PCR plate is labeled by letters A-H and the columns of the PCR plate is labeled by numbers 1-12

   (Based on example above, you may want to label the PCR plate form as follows:

   Legend:

	<PCR 1 ARC>
	<PCR 1 GAPDH>
	<PCR 2 ARC>
	<PCR 2 GAPDH>
	<PCR 3 ARC>
	<PCR 3 GAPDH>

   PCR plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

   Make sure you keep the PCR plate form for your records so that you can refer back to it once your data comes in)

10. Wrap the PCR plate in aluminum foil, try to keep the plate on Kimwipes and avoid letting it touch the foil directly and bring to the DNA Core along with the PCR form. The data is generally emailed to you within 1 or 2 business days.	10. If you are not changing the settings that we have been using in Dr. Otto's lab, you can simply tell whoever is at the DNA Core to use the same settings as previous submission.