RT-qPCR Protocol Maden Lab

Check section breaks view for sub-protocols

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**Designing Aco Primers**

* Files are inside the Maden Dropbox
  + https://www.dropbox.com/home/SHARE\_MADEN
* Open ‘Acomys\_RT\_Primers.txt’
  + CHECK TO SEE IF JASON ALREADY MADE PRIMERS for gene of interest
* Open Acomys\_Mus\_ortholog\_only\_table.xlsx
  + Table of Aco orthologous sequences that were annotated with Mus gene sequences
    - Gene symbol in column A
    - Name of Aco transcript in column B
      1. Naming convention used in Trinity (TR)
    - This is the Acomys seq of these Mus genes
      1. Transcriptome assembly
      2. Annotation
    - BLAST searches at the nucleotide level and at the translated protein level
    - Name genes based on genes from other species
      1. Removed all trinity transcripts that DON’T have Mus ortholog
  + Search in Excel file for name of gene
    - HIGHLIGHT ONLY COLUMN A when searching
  + Copy all 3 transcripts for AREG
    - could be a different isoform or could be that gene doesn’t have single transcript
      1. couldn’t assemble transcript to fit whole gene
      2. could be 3’ and 5’ section
* go to UCSC genome browser (<https://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=702807745_0vMvKmS4U6sqixw0WnYq08ZcDyvx&command=start>)
  + Use defaults: Mouse genome Dec. 2011 (GRCm38/mm10)
* Paste copied AREG sequences into BLAST Search Genome
  + If you put multiple sequences in, convert them to fastA format
  + Add > and name
    - >Name
  + every entry in FastA file starts w ‘>Name’ then next line has the sequence
  + at the end of one sequence, press ‘Enter’ then add >Name
    - >AREG1
    - sequence
    - >AREG2
    - sequence
    - >AREG3
    - sequence
* hit ‘Submit’
  + Ordered by score
  + Choose one with highest ‘Score’ (first one in the list)
* click on ‘Browser’
  + zoom out
    - we see entirety of AREG gene
    - seq coverage over whole thing
  + Aco ortholog in red
  + Mus gene in blue
    - Make sure Mus gene matches what you thought it did in Aco
  + For Mus fat bar is exon, skinny bar is UTR, line is intron
    - For Aco, we don’t know what is exon or UTR
      1. Intron is not included because mRNA has introns spliced out
  + Arrows indicate direction of transcription 5’>>>3’ OR 3’<<<5’
    - Arrows will vary depending on whether gene is transcribed right to left or left to right
    - Look at 3’ end of Mus and 5’ end of Aco
      1. Transcriptome is based on cDNA
      2. cDNA is made by complimentary basepairing to Aco mRNA
      3. 5’ end of Aco cDNA = 3’ end of Aco mRNA
* DESIGN PRIMERS IN THE 3’ UTR of Mus (5’ UTR of Aco)
  + Rule of thumb: if you have alt spliced genes that exclude certain exons, typically all of them use the same 3’ UTR
  + If you have a gene and you skip this exon, you’ll still end on same 3’ UTR
  + If tissue type uses diff isoform that skips exon, you won’t amplify that
  + Appear that expression has gone down but not looking at right isoform
  + First preference is 3’ UTR then 5’ UTR
  + Share same 3’ UTR
  + Probably gonna share same 5’ UTR
  + Common to have alt start site leading to 2 diff 5’ UTRs
  + If 3’ UTR of Mus doesn’t align with Aco, we can use the 3’ exon of Mus as long as Aco matches
    - For the most part, 3’ UTR is extension of last exon - no intron in between them
  + **Order of priority: 3’ UTR, 3’ most exon, 5’ UTR, 5’ exon OF MUS**
    - **5’ UTR, 5’ most exon, 3’ UTR, 3’ exon OF ACO**
* Transcript 1 covers the whole thing so we’ll just use transcript 1
  + 2 and 3 are just artifacts of the assembly
* Zoom in on 3’ UTR of Mus/5’ UTR of Aco by **holding command and clicking then dragging to zoom to selected area**
  + DO NOT INCLUDE ANY INTRON SEQUENCE
    - If it is included, the primer might be designed to an intron which is spliced out when mRNA is made
  + See how many bases are in the window (only 185 for Aco)
  + Select the first 185 bases from the Excel sheet
* We want the beginning of the read from the Excel Sheet (5’ UTR Aco corresponds to 3’ UTR Mus)
  + Entire 3’ UTR sequence of AREG is only 185 bases; very short
  + Ideal minimum is 300 bases but the more the better
    - Giving a larger 3’ UTR will make it more likely that you find a good 125 bp amplicon to use
    - Take as much of the Aco sequence that aligns with Mus as possible
* Try and select the first 300 or so bases from the Excel sheet
  + Copy-paste the sequence to Microsoft Word
  + Select the first (5’ UTR/exon of Aco) or last (3’ UTR/exon of Aco) 300+ characters (however many bp are in the window when you select the total area of the Aco 5’ UTR/Mus 3’ UTR)
    - Use the character count tool
  + Wait a few seconds
* Paste seq into Blat
  + Check to make sure that we have enough of the sequence to cover the 3’ UTR
* WE NOW HAVE THE **ACO 5’ UTR SEQ equivalent to the 3’ UTR of Mus**
* Open up window primer3
  + <https://biotools.umassmed.edu/bioapps/primer3_www.cgi>
  + Press ‘Advanced’ then Proceed (unsafe)
* Paste in Aco sequence
* Only parameter we change is ‘Product Size’
  + Min: 100
  + Opt: 125
  + Max: 150
    - These are the parameters for RT-qPCR
    - Short amplicon
    - Want amplicons to be close in length
    - Mix and match to compare later on
* DON’T CHANGE ANY OTHER PARAMETERS
* Click ‘Pick Primers’
  + Len is length of primer
    - Should be at least 20
    - Any shorter you run into problems with specificity
  + Tm is melting temp
    - Needs to be close to 60
  + Product size has to be within our parameters
* Open ‘Acomys\_RT\_Primers.txt’
* Copy from ‘Oligo to Product size’ and put in file
* Area with >>> is fwd primer
* Area with <<< is rev primer
* Copy amplicon sequence between and including >>> and <<< and paste in file
  + Delete carets and numbers
  + The rest of the sequence outside the carets was the template we input
  + Anything not in between the primers is not amplified
* Sequence of primers on top
  + This is what we care about for ordering
* Seq of template strand to which the primers will anneal
  + We have it here just in case we need it down the line
  + DON’T USE THIS FOR ORDERING
* Always click ‘Don’t Save’ when you close Acomys\_Mus\_ortholog\_only\_table.xlsx

**Designing Mus Primers**

* Using the same window that we zoomed in on for Aco primers
  + Make sure not to include any Mus introns
* ‘View’ on the top
* ‘DNA’
* ‘Get DNA’
  + Don’t change any settings
  + Gives you the seq of Mus 3’ UTR
* Copy seq
* Come back to primer3
* Paste in seq
  + Don’t have to edit it
* Pick primers
  + Check to see if Product size is within our parameters and Tm is within our parameters
* Open ‘Mus\_RT\_Primers.txt’ and save all the info

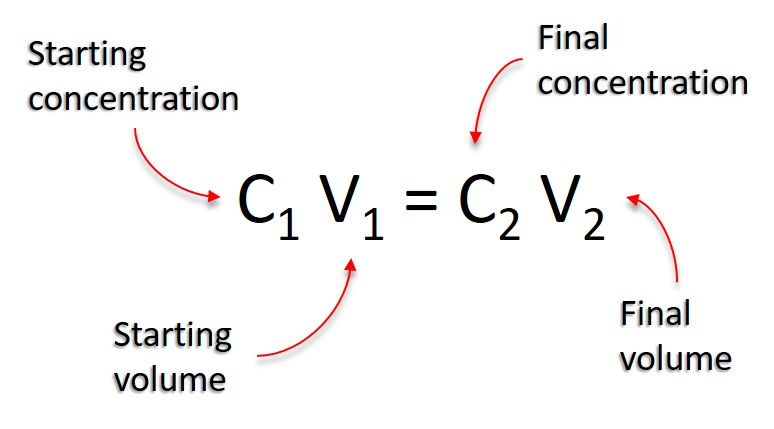
**Checking Primer Design**

* Take the amplicon (not primer) sequences from the .txt files for Aco and Mus
* Blat them together in UCSC genome browser
  + Use FastA formatting (>Name)
* Check to make sure they amplify correct spot

**Ordering Primers**

* Once you have the primer sequences…
* Go to <https://www.sigmaaldrich.com/pc/ui/tube-home/standard>
* Login
  + Username: MALCMADEN
  + Password: madenlab2019
    - Whenever you need a password try madenlab20XX (madenlab2014, 17, etc.)
* Manual
  + Type in name
  + aco\_Gene\_RT\_For
  + Paste in ‘Fwd’ sequence from .txt
    - LEFT PRIMER Seq = FOR
    - RIGHT PRIMER Seq = REV
  + Oligo properties pops up
  + Specify the scale
  + **We want the smallest Synthesis Scale 0.025umole**
    - This is how much we need
    - Good for a few thousand reactions
  + Only purification method is Desalt
  + Format is always Dry
    - In solution costs 4x as much and we can do this ourselves
  + Click ‘Add Oligo’
  + Add other primers
* OR you can use Excel template
  + ALL YOU NEED TO CHANGE IS NAME AND SEQUENCE
    - aco\_Gene\_RT\_For
    - Paste in ‘Fwd’ sequence from .txt
      1. LEFT PRIMER Seq = FOR
      2. RIGHT PRIMER Seq = REV
  + KEEP THE REST THE SAME
    - Make sure to add/delete new rows for each primer
      1. aco\_Sox2\_RT\_For
      2. aco\_Sox2\_RT\_Rev
      3. mus\_Sox2\_RT\_For
      4. mus\_Sox2\_RT\_Rev
         * For and Rev primers for both species (Aco and Mus)
  + Select the file
* Put the primers in the cart then tell Megan and she’ll order it
* Bring to Malcolm and let him pay for it (or do it and just say Malcolm did)
  + Each primer should cost $3.96
    - If not, you’re probably not logged onto the lab account

**Re-Suspending Primers**

* Primers come with Delivery Note (not important), stickers (not important), and Datasheet (IMPORTANT)
  + Put datasheet in the folder
* resuspended them in TE (Tris + EDTA) to **100uM stock solutions**
  + Tris and EDTA pH8 (10 mM Tris and 1 mM EDTA)
    - 
  + **Second to last column on data sheet labeled ‘ul for 100 uM’ tells you how much TE to add to each tube**
  + Spin the tubes in centrifuge to make sure lyophilized product is on the bottom
  + Add appropriate amount of TE to each tube (watch to make sure names match sheet)
    - You can make new TE if we run out:
      * **250 mL of TE = 2.5 mL of 1M TRIS + 250 uL of 1M EDTA then volume up to 250 mL in glass bottle with NanoPure water from machine**
      * TRIS and EDTA are above Jason’s bench
  + Vortex each tube
  + Write number on top of cap and store in in -20C boxes
    - number based on last number in binder of sheets
* diluted to **10uM working solutions** with nuclease free water
  + 10 ul stock + 90ul rnase free water
  + Kept in -20C

**Testing PCR Primers**

* Run melting curve on RT machine
* If you have multiple peaks, it means you amplified multiple bands – bad primers (primer dimer or genomic dna, but gdna should be removed)
* always use positive control of DNA template that you know amplifies with primer that you know works (may need to test multiple positive controls)
* always do a test PCR to make sure primers work

**Harvesting Tissue**

* Sack the animal, harvest immediately, place in RNAlater immediately
  + RNA begins to degrade immediately so be quick
* Fill a 2 mL blue capped tube or an eppendorf depending on tissue size with RNAlater
  + RNAlater is on Jason’s bench in clear bottle
    - Protects cellular RNAs
  + Fill up tube almost to the top
* Harvest the tissue and place in RNAlater
* Leave tube at room temperature for an hour to let RNAlater permeate the tissue
* Move the tube to the -80C freezer for long term storage
  + RNAlater will freeze

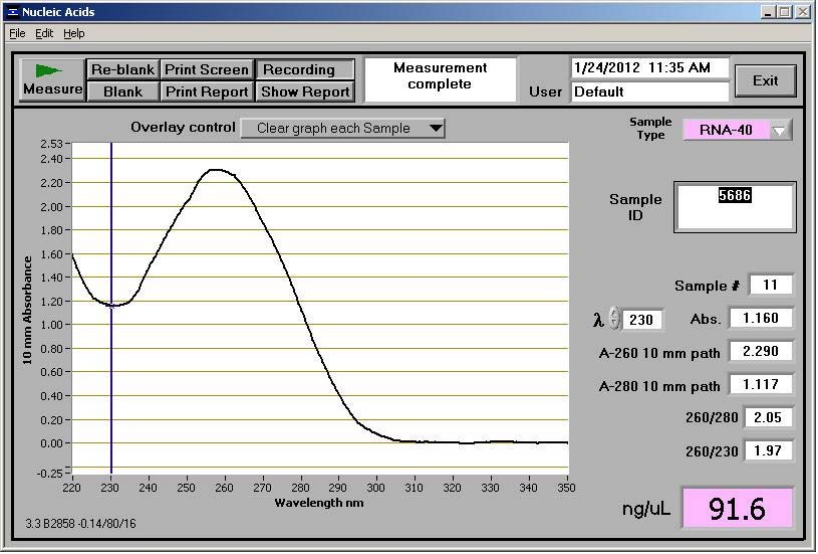
**RNEasy Fibrous Tissue**

* Use Trey’s protocol (includes proteinase K and DNAse separate steps)
* RNAse Zap hood surface, pipets, gloves, etc.
  + Close door next to hood and put sign up
* Set heating block to 55°C
* Weigh tissue first to ensure tissue it is ≤30 mg
* Homogenize ≤30 mg tissue in 300 μL Buffer RLT with 3 uL β-ME
* Add 590 μL RNase-free water
* Add 10 μL proteinase K
* Incubate at 55°C (10 min)
* Centrifuge at maximum setting (3 min)
* Transfer supernatant to new tube
* Add/mix 0.5 volumes 100% EtOH. Discard pellet.
* Centrifuge 650 μL sample in column at 10,000 x g (30 sec).
  + Discard flow-through. Repeat until all sample is used.
* Add 350 μL Buffer RW1 to column.
  + Centrifuge at 10,000 x g (30 sec).
  + Discard flow-through.
* Mix 10 μL DNase stock with 70 μL Buffer RDD. Add to membrane and incubate at room temperature (15 min)
  + Buffer RDD in little blue box in door of 4C
  + RNAse free **DNAse** in freezer in box
    - Good for months reconstituted in freezer
    - Good for 2 weeks in fridge after defrosting
      * Don’t put back in freezer after defrosting!
* Add 350 μL Buffer RW1 to column.
  + Centrifuge at 10,000 x g (30 sec).
  + Discard flow-through.
* Add 500 μL Buffer RPE to column.
  + Centrifuge at 10,000 x g (30 sec).
  + Discard flow-through.
* Add 500 μL Buffer RPE to column.
  + Centrifuge at 10,000 x g (2 min).
  + Discard flow-through.
* Place column in new 2 mL tube and centrifuge at maximum setting (1 min)
* Place column in 1.5 mL tube. Add 30 μL RNase-free water.
  + Centrifuge at 10,000 x g (1 min).
  + Save elution.
* Determine RNA concentration via Nanodrop in Baker lab (1st floor)
* Aliquot into several tubes to avoid freeze-thaw
* Store at -80C

**Using Nanodrop on 1st Floor Baker Lab**

* Ethanol in solution leads to peak at 230 nm
* Clean w di and kim
* Nd 1000 program in bottom left
* Blank with water x2 (re-blank every 30 minutes)
* Switch from dna to rna on sample type dropdown
* Blank with same water in machine
* Wipe off
* Pipet 1 ul of sample
* Edit sample id
* Measure
* 260/280 should be near 2
  + - 280=wavelength for proteins
    - 260=wavelength for salt
* Concentration is ng/ul (if below 10ng/ul it’s unreliable)
* Wipe with kim wipe in between
* Save report on shared drive
* Show report
* **Dilute and aliquot then put in - 80C**

**Jason’s RNA Extraction using RNEasy Plus Mini KIt**

* Get the tissue samples in RNAlater from the -20C or -80C
* Allow vials to thaw
* Clean off the bench surface as well as the hood with RNAse Away and some paper towels
  + RNAse Away and RNAse Zap both destroy RNAse
    1. Use Away for large surfaces bc you can buy in bulk
    2. Use Zap for pipettes and gloves and smaller things
* Clean off all the pipettes with RNAse Zap
  + Move the 1 mL and 100 uL pipettes to the hood
  + Move barrier tips to the hood as well
* Clean some forceps with RNAse Zap
* Move the samples from the RNAlater tube to a new FLATTER BOTTOM 2ML TUBE (2.0ml microtubes, clear)
  + In Jason’s drawer under ‘Tissue Grinder’ drawer
  + This tube allows the rotostat tissue grinder to work better
* Weigh the tissue on the scale with the sliding door
  + Write the weight of the tissue on the top of the tube
  + You can tare the scale with an identical tube then weigh the tissue
  + Or you can use weigh paper
* Close door next to hood to stop air flow
* Get RNeasy Plus Mini Kit (50)
  + Create RLT Plus
    1. **Add 10 ul of B-mercaptoethanol (BME) to 1 mL of RLT Plus to make working solution in an eppendorf**
       - **Use the one that says RNA**
  + Pipet the correct amount of RLT Plus buffer in the FLATTER BOTTOM 2ML TUBE with the tissue
    1. <20 mg tissue in 350ul RLT Plus/BME
       - You can put 40 mg of tissue in 700ul RLT Plus/BME and aliquot in two later
    2. 20-30 mg tissue in 600ul RLT Plus/BME
       - Trim tissue if you need to or just do multiple aliquots later on
  + Get the rotostat tissue homogenizer and plug it in
    1. Set it to the very end of the second speed square
  + Get the probes and insert them into the rotostat
  + Take the flatter bottom tube with the whole tissue and RLT Plus Buffer and insert probe to very bottom
    1. Turn on rotostat
    2. Allow to tissue to be ground into homogeneous mixture
    3. Move up and down very little
       - If you move up too much, bubbles will occur
    4. Check to make sure no tissue is getting stuck in probe
    5. Grind for another 15 or so seconds until the mixture has been homogenized
  + Aliquot the lysate into tubes
    1. ≤30 mg of tissue is used for each reaction so use appropriate amount of lysate
       - If you have a tissue that weighs 44 mg, grind tissue then divide into 2 tubes = 22 mg each
       - If you have tissue that weighs 100 mg grind tissue then divide into 3 tubes = 33 mg each
         * *You can use one tube then freeze the others and use them later*
         * Aliquot the tubes out so you don’t have to freeze-thaw them
  + Centrifuge the lysate for 3 min at max speed
    1. Make sure to point the hinge of the tube cap upwards when putting in the centrifuge
    2. This allows you to know that the pellet will be formed directly across from it on the bottom edge on the same side of the hinge
    3. Be careful to avoid the pellet when obtaining the supernatant
  + Carefully remove the supernatant by pipetting and use it in step 2
  + Follow rest of protocol
    1. Rcf = x g
    2. Make sure to watch steps carefully to see when to discard filter or flow-through
  + Do the first Optional step (Place the RNEasy spin column in a new 2 ml…)
  + Skip the second Optional step (Repeat elution with another volume of water…)
* Once you have the RNA in water, put on ice
* Also put RNAse free water on ice
  + RNAse free water and Nuclease free water are the same thing
* Take RNA, water, and 2 ul pipet to Barbazuk’s lab
  + Nanodrop is in the back room all the way to the left when you enter
* Log into the computer using Gatorlink login
* Wipe Nanodrop with Kimwipe
* Click Nanodrop 2000
* Select ‘Nucleotides’ from the options
* Select ‘RNA’ from dropdown
* Pipet 1ul of RNAse free water onto Nanodrop and blank (top right corner)
* Open up and dry with Kimwipe
* Load sample
* Run
* 260/280 deals with RNA/DNA contamination
  + should be 2.0 for RNA
  + 1.8 for DNA
  + 1.9 is ok for RNA too
* 260/230 deals with organic contamination
  + Should also be 2.0 for RNA
* 
* Curve should look like this
  + The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; **a ratio of ~2.0 is generally accepted as “pure” for RNA.** If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.
  + This ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. **Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.**
* Concentration
  + Anything above 20 ng/uL is good
* Aliquot RNA **4ul into 200ul small tubes**
  + You can either use single tubes or strips depending on how many samples you have

**Sending RNA down to ICBR**

* + Label the side of the tube
    1. Initials and a number
       - Keep track of what each sample is
  + Make a ticket for it online
    1. ICBR website (<http://www.biotech.ufl.edu/>)
    2. Log in to iLab
    3. Core FAcilities list on left side
    4. Request Services
    5. Find list of Cores/Services
       - RNA DNA QC (3)
       - Bioanalyzer/2200 TapeStation option
         * Number of Samples
         * Click TapeStation
         * Nucleic acid type dropdown: Total RNA
         * Select Chip: Eukaryote (NOT high sensitivity)
         * Type of Sample: Total RNA
         * Download Excel file of sample sheet and edit that

Sample Name - AS2

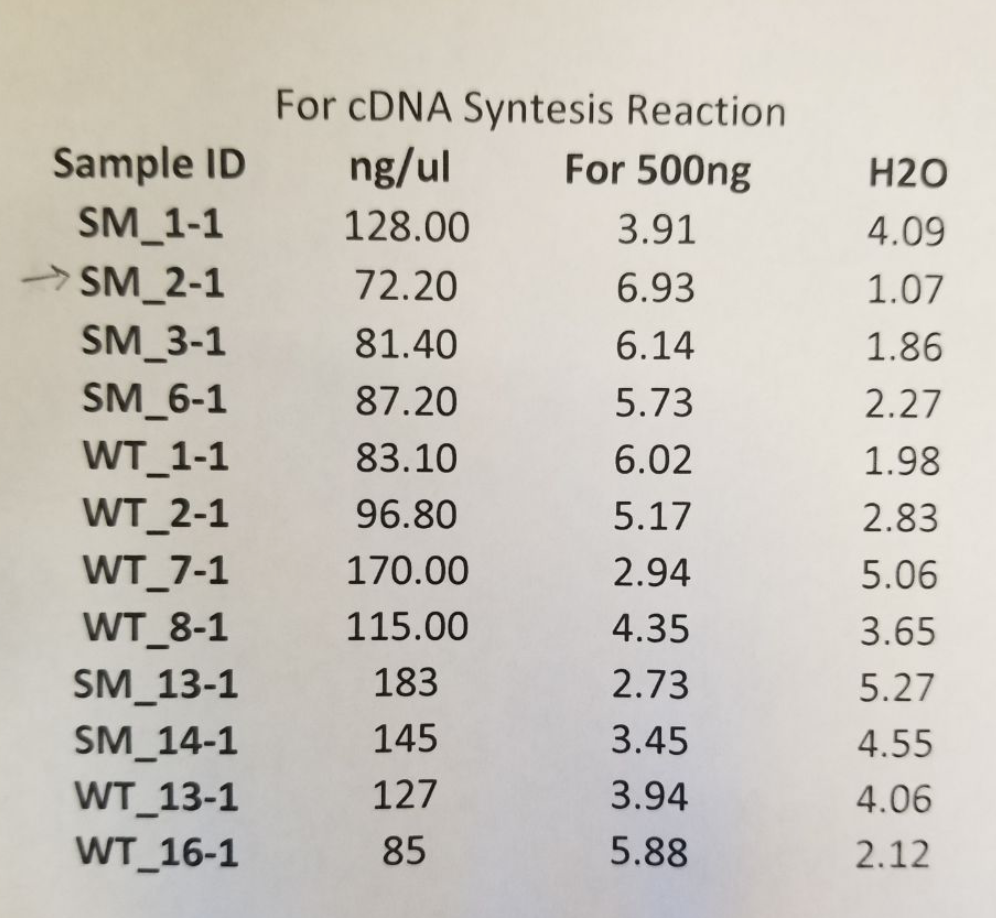
Concentration (ng/ul) - just approximate/round the number

LEAVE A1 EMPTY BC THAT’S WHERE THEY RUN LADDER

* + - * + Attach Excel sheet
        + Agree to services
      * Payment information
        + Check which grant it goes to
        + Costs around $7 per sample
    1. We get a number
       - service id: ICBR-GE-2761
       - Write this down on a piece of paper and give it to them
       - That’s how they know our sample goes with this order
    2. 3 emails
       - They received sample
       - They send us bill and we have to click AGREE
       - They send us notification that it’s done
         * Log back in here and see link to our data
  + Submit job online
  + Get a number
  + Write that number down on post it note
* Bring down samples in ice bucket along with piece of paper
  + Go down stairs to ICBR facility next to little market
  + Tell desk person you’re going to tape station so they open the doors
  + Go down the hall to 178G?
    1. It’s called the Gene Expression Core
    2. Find someone to give it to
       - Don’t give them the ice bucket
* Bring it downstairs for tapestation
  + Tapestation separates by weight due to electrical current
    1. smaller RNAs run further
    2. Little capillary
    3. Runs down on current
    4. 10 minutes to run
  + Will give you RIN score the next day as PDF online at the website
    1. First page show images and give you score
    2. Perfect RIN score is 10
       - For RT anything in the green to yellow range is usable
       - Red means bad you can’t use it
    3. Dont care about area
    4. Tells you concentration - never what you get from NanoDrop
       - We go by the NanoDrop concentration when making cDNA
    5. Graphs for each sample
       - Higher peak means darker staining in tapestation
       - Not super important, RIN is the most important
* Place the rest of the RNA at -20C
  + If it’s late, you can aliquot 4 ul of RNA to send downstairs the next day
* Go back to lab and clean up
* Put pipets away
* Clean probes
  + Rinse in sink with tap water
  + Use sponge and hand soap to scrub
  + Make sure water goes through top hole in probe and out the bottom hole
  + Twist probe to let water get in all crevices
  + Set up 3 50 mL conicals
    1. RNAse Away
    2. Nuclease Free Water
    3. 100% EtOH
  + Fill each one to about 30 mL
  + Put probes into RNAse away and dunk up and down
    1. Twist to make sure probe is cleaned thoroughly
    2. Let sit in RNAse away for 5 minutes
    3. Rinse in the water and 100% EtOH
  + Prop the probes up to dry standing up or leaning on something
    1. The tip that touches the tissue should be facing upwards
* Rotostat goes back in its case and into the drawer below

**Making cDNA from RNA**

* SET UP EXCEL SHEET: Take concentration from NanoDrop and RIN from tapestation
  + Ideally make cDNA with 500ng of RNA per sample but calculate to make sure you have enough RNA
  + If you have a decent yield, make cDNA out of **500 ng of RNA** 
    - Set up calculator on Excel
    - Have concen, how much you need to make 500 ng
      * Make sure this number is less than 8
    - Then **fill to 8 ul with water**
      * In water column have 8 - what you need for 500 ng
  + If you have small tissue or low yield
    - Multiply ng/ul\*8
      * Only have 8 ul to work with
    - Look for smallest number
    - That would be the most amount of cDNA you could make
      * Do the same amount for all the samples
      * Need to use same amount of RNA to make cDNA for ALL SAMPLES

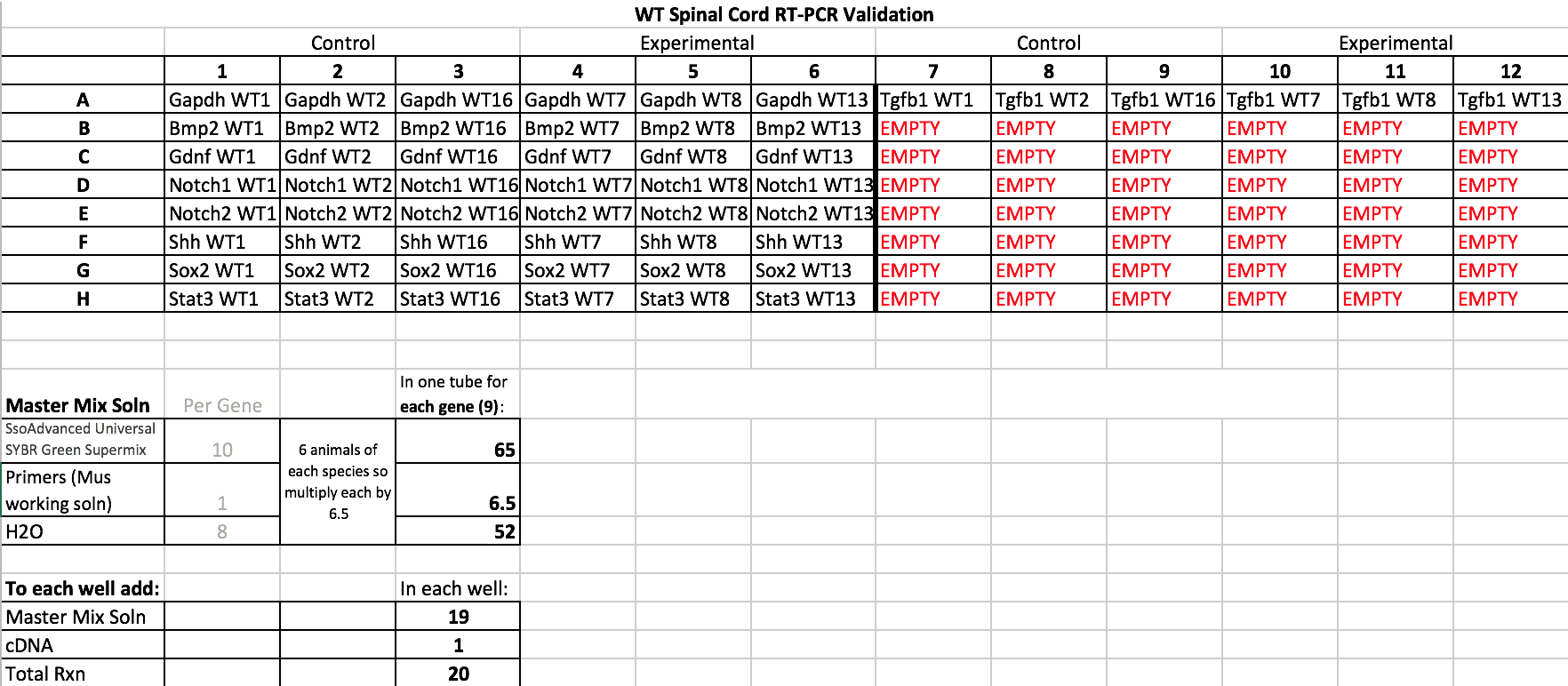


*Example of calculations for 500ng of cDNA* ***volumed up to 8ul*** *using data from Spinal Cord Injury*

* RNA is usually stored in the -80C freezer
  + Slows down RNAse activity
* Concentrate or dilute RNA to achieve optimal concentration
  + If <20 ng/ul according to Nanodrop, use GeneJET RNA Cleanup and Concentration Micro Kit - series of washes and spins
  + If >200 ng/ul according to Nanodrop, dilute 1:10 (1 part RNA and 9 parts nuclease free water)
* Prepare gDNA digestion reaction mix (this step destroys any contaminant DNA)
  + KEEP ON ICE BUCKET throughout
  + Label small PCR tubes found above bench
  + All reagents are found in ‘JOB RT’ Box in -20C (vortex and briefly centrifuge all)
    - Template RNA (1.5 pg to 2.5 ug total RNA)
    - Nuclease free water (CLEAR) up to 8 uL
      * RNA+Water=8uL
    - 1 uL - 10X ezDNase Buffer (BLUE)
    - 1uL - ezDNAse enzyme (YELLOW)
      * Make B+E then add 2 uL to each - **do this then add RNA/water**
    - **Add 10% extra for pipetting errors**
* Go to Barbazuk lab and spin down in mini Fisher Sci centrifuge
* Place PCR tubes into Thermocycler (MJ Research PTC-200 DNA Engine Gradient Cycler - Black)
  + Turn on with switch in back
  + Wait for self testing to finish
  + Add samples
  + Close lid
  + Spin blue circle to the left until resistance is felt
    - Use < and > to move between selections
    - Use ^ to select
    - Use v to cancel
  + **RUN=MAIN=DNASE**
    - 37C for 2 minutes
  + Instead of RUN, select EDIT to check steps
* Add 4 uL of SSIV VILO MM (RED) and 6 uL of nuclease free water (CLEAR) to each PCR tube (vortex and briefly centrifuge)
  + Mix water + MM then just add 10 uL to each tube
* Go to Barbazuk lab and spin down in mini centrifuge
* Place PCR tubes into Thermocycler and close lid
  + RUN=MAIN=SS’IV (use heated lid) = **25 minutes**
    - It will do all of the steps automatically
    - Anneal primers - 25C for 10 min
    - Reverse transcribe RNA - 50C for 10 min
    - Inactivate enzyme - 85C for 5 min
  + After it is finished it will hold ‘for ever at 4C’
    - This makes 20 uL of cDNA
* Dilute 1:4 (add 60 uL) and **note this on tube**
  + If I start with 250 or 500 ng of RNA which makes 20 uL of undiluted cDNA which I can dilute 1:4 to get 80 uL of diluted cDNA and since each reaction requires 2 ul of cDNA, I can test 40 genes
  + **MUST INCLUDE REFERENCE GENE EACH TIME**
* Can be stored indefinitely at -20C

**Using cDNA for RT-qPCR**

* **Sign up for time on the PCR machine on notebook next to machine**
  + From start to finish the run itself takes like 1 hr 10 min
  + Start an hour before to have plate ready to go
  + Reserve machine for about 1.5 hrs
  + Make the effort to use the machine
  + If you finish making plate early, leave it on ice and cover it
  + Could let it sit in fridge overnight as long as it’s covered
    - Polymerase is inactive until you heat it
* SET UP EXCEL SHEET FOR SAMPLES
  + Plan out ahead
  + Do math for master mix for the RT
  + For each rxn use **10 ul of MM, 1 ul primer, 7 ul H2O**
    - Aliquot mastermix in each well first
    - Add cDNA afterwards
* keep reagents on ice
* make a master mix per reaction FOR **EACH GENE** in **each species** + **GAPDH as reference gene**
  + 10 uL SsoAdvanced Universal SYBR Green Supermix BIO-RAD (in -20C JOB RT box)
    - Binds to double stranded DNA, not single stranded
  + 1 uL of primer
    - JOB Primer Box 1 **working dilutions** (in -20C)
      * 5 uM
  + 8 uL of nuclease free water (above Jason’s bench)
* To a 96 well PCR plate add **18 uL of master mix per well**

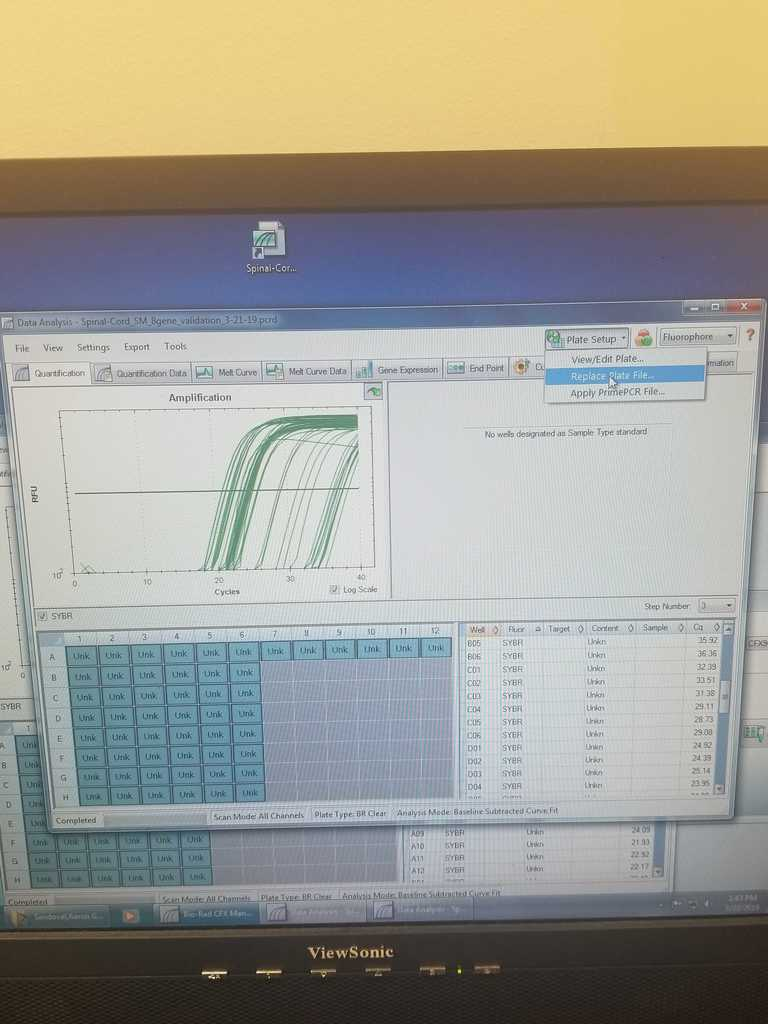
****

* **IF NOT ALREADY DILUTED:** DILUTE cDNA 1:4 with multichannel pipette (Jason already did this for the Spinal Cord stored for months in the -20C)
  + If 20 uL of cDNA, add 60 uL of nuclease free water
  + This might be done already if used before
  + *If you dilute 1:2 (20 uL of cDNA + 20 uL of water), you ONLY use 1 uL of cDNA (harder to pipet) but make sure to change amount of nuclease free water added to MM (8) and amount of MM added to plate (19)*
* Add **2 uL of cDNA per well (if diluted 1:4)**
  + Use channel pipette to do multiple at once
  + Add cDNA to the side-wall of the well, then you know it is there. MM at bottom.
* Put on plastic sticker with yellow/orange backing
  + press down to remove air bubbles and form tight seal
  + Flaps should go to the left of the wells
  + Rip off perforations
* *Place the plate wrapped in aluminum foil in -20C if you have to wait a few minutes*
* **Mix well by flicking whole plate**
* **Go to Barbazuk lab and centrifuge plate with a blank plate as a counterweight**
  + turn on using switch on side
  + press open button
  + Use blank with X as a counterbalance
  + hold up lid bc it's broken
* Centrifuge at 1000 g for 1 min
  + turn off using switch on side
* Go to room next to microtome/cryostat
* Turn on computer and log in with UFL credentials
* Bio-Rad CFX Manager
* Open cycler
* Place plate in
* Wipe plate with kimwipe
* File > Open > Protocol > **‘Aco\_Protocol’** if doing RT-qPCR with SsoAdvanced Universal SYBR Green Supermix > OK > Next
* You should be on the plate screen now
* Edit
  + Conversely you can ‘Select Existing’ if running a similar plate
    - Select Existing... > **RT2\_Profiler\_Array (1/8/2018 3:19 PM)**
    - Edit Selected... > make sure everything is SYBR
* Select all
* Clear wells
* **Choose which wells filled up**
* choose fluorophore
  + uncheck all other types of fluorophores
  + type is 'unknown'
  + load sybr
* It will ask you to save plate layout
  + Save as something
* Close cycler
* Start
* Name something like 'Aco\_CTX\_10-18-18' and save to desktop
* takes 1 hr 15 min to finish
* When finished, ‘Save as’ to **Sandoval, Aaron Gabriel W folder**
* Remember to rename accordingly

After run is finished:

* Set to **Log Scale**
* Right click on horizontal line and select **Show Threshold Values**
* Move the horizontal threshold line down to a bit more than just above where the curves go from curved to linear
  + set to X RFU (relative fluorescent units) as the Ct value
    - **try to set all the plates you’re comparing to this same Ct value**
  + Ct = cycle threshold
  + number of cycles of PCR it took for a gene to amplify to X RFU
    - SYBR chelates into double stranded DNA and fluoresces
    - more double stranded DNA synthesized = more fluorescence
    - cDNA is single stranded so no chelation
* **Save As to Desktop AND Export > All Data Sheets > Excel 2007** 
  + save exported data on flashdrive (white Toshiba) AND desktop of computer
* Open machine
* Take out plate and draw X on it
* close machine
* log out or run next plate

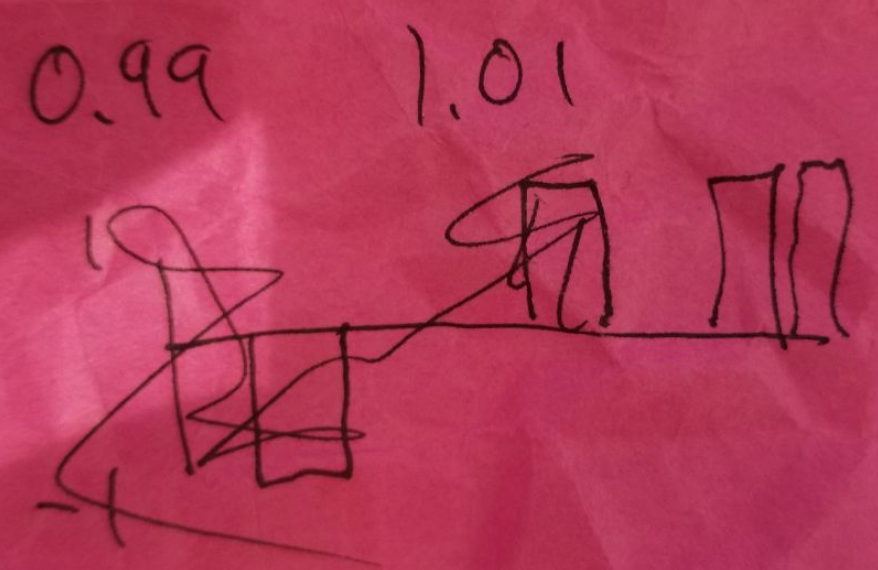
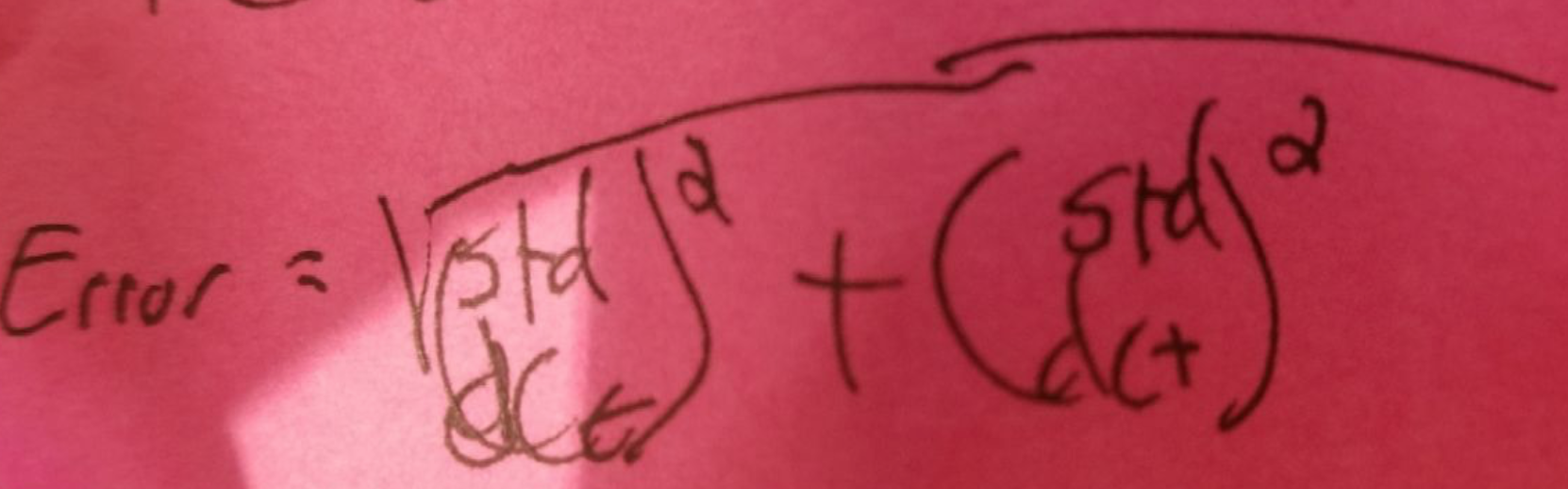
**IF THERE IS NO PCRD OPTICAL FILE**

* Use the touchscreen on the Bio-Rad machine itself
  + Must close out all Bio-Rad programs on the computer before doing this
* Saved files
* Real time data
* Written by date and time
* Plug in usb
* File options
* Export
  + Open this exported file on the computer since it has Bio-Rad software
    - If it has the wrong fluorophores, click ‘Replace Plate File’ in the upper right hand corner (see pic)
    - Select proper plate layout with SYBR
  + Switch to Log
  + Adjust threshold
  + Save As AND Export 2007 Excel files to flashdrive

**Analyzing Data**

* All the formulas are on the Excel sheets
  + Cts then he gets an average deltaCt per time period per gene
  + 1st delta Ct = gene of interest - reference gene (GAPDH)
    - Within sample difference
    - This table will NOT include GAPDH
    - All the values are relative to GAPDH
  + 2nd delta delta Ct = expt condition - control condition
    - Between sample difference
    - List of genes and time points but no row for D0 time point
    - All these values are relative to day 0
  + The individual delta Cts give us values to make standard deviation from
  + Do a delta Ct for the averages
    - Full change data we plot comes from delta delta Ct of averages
      * Stdev for each gene for all 3 time points
      * For the error, logarithmic values
        + When you plot the data you unlog it
        + 2^delta delta Ct
        + =square root (sum of the squares of the stdev)
    - Delta Ct of individual samples used to calculate variance in the data (error bars)
    - **Use this value as up and down error bar**
      * **Custom > Specify Value**
      * **copy/paste same exact thing for positive and negative error**
    - Change Chart Type > Jason RT Template for RT PCR graphs
* quantification plate view
* copy data and paste it into chart with gene names y axis and sample names on x axis
* transpose data
* paste special > transpose
* get avg of Ct values for each replicate at each time point
* deltaCt
* gene of interest - gapdh (reference gene)
* $ right before cell name of gapdh will lock on column but still change by row
* $ before letter locks column
* $ before number locks row
* do the same thing but with the avgs
* ddCt is day X - day 0
* just use avgs
* day4-day0
* NOT good science to compare Aco vs Mus
* transform the data to get FOLD CHANGE
* 2^-(ddCt)
* final formula
* 2nd way to display fold change
* =if X<1,-1/X,X
* if that number less than 1 does the neg reciprocal, if not just keeps value as is
* when graphed, original data is just relative to 1 or 100%
* 2nd way is change relative to 0

**Analyzing RT-qPCR Array Plate Data**

* Did this for neurogenesis
* Created sheet for each species
* Column with names of genes
* Other columns with Ct values of each of the mice
* Calculate average Ct for the 5 reference genes in newly made ‘Reference’ row
* Get rid of bad reference genes
  + Blank space where PCR failed
  + Ct values between control group and experimental group differ depending on group
    - Got rid of Actb for neurogenesis array
* Calculate average and stdev for both control and expt groups
  + Double click bottom right corner to automatically calculate other rows
  + Must be directly next to other values to work
  + DRAG DOWN TO REFERENCE ROW TO CALCULATE THE TOTAL AVERAGE OF THE 5 REF GENES THAT HAVE BEEN AVERAGED
    - Will be used as the Ct Ref Gene when calculating dCt
* New columns for dCt Control and dCt Experimental
  + dCt = Ct Gene of Interest - Ct Reference Gene
    - For Ct Gene of Interest use the Average of the Gene
    - For Ct Reference Gene use the Average of the Reference Genes since there are 5 of them on the array plate
      * Be sure to put a $ in front of the number (not the letter) of the reference gene to **lock the row in place** on the Excel sheet
* New column for ddCt
  + ddCt = dCt Experimental - dCt Control
* New column for Fold Change
  + Fold Change = 2-dCt
  + =2^-(dCt)
  + Undoing the log base 2
* New column for Neg Inverse Fold Change
  + Transforming the data to fold change makes it so that 0.99 and 1.01 look radically different on a bar graph with y axis -1 to 1 when in reality those values are very close except one is slightly over 1 and the other is slightly under 1
  + 
  + Underestimates decimals
  + Need to transform data so that decimals become negative whole numbers
    - =IF(FC<1, -1/FC, FC)
    - =IF(Fold Change is a decimal, if it IS then take negative inverse to turn decimal into neg whole number, if it ISN’T then leave as FC)
      * FC = Fold Change
    - Jason doesn’t like transforming some data (whole numbers) but not other data (decimals) because it exaggerates the difference between numbers that are very close to 1
      * 0.99 and 1.01 fold change are very close yet look very far on the graph
* New column for Error Bars to put on bar graph for publication
  + Error = SQRT((STDEV dCt Control**^2**)+(STDEV dCt Experimental**^2**))
  + 
* New columns for calculating dCt of individual animals
  + 6 animals so 6 columns
  + dCt=Ct Gene of Interest - Ct Reference Gene
    - Use $ in front of number to lock row of gene of interest
    - D$11
* T-test
  + =(dCts of Expt, dCts of Ctrl, 1, 2)
  + 1 tailed
  + Type 2
    - Data not from the same individual
* New Sheet
  + Paste in Neg Inverse Fold Change and P-Values from T Test
    - PASTE ‘VALUES ONLY’

**Making Bar Graphs**

* Make a new Excel sheet
* Copy paste in the Aco/Mus Neg Inv Fold Changes and the Errors calculated
* Make bar graph out of the 2 Fold Change values
  + Or just use old ‘Neurogenesis Validation Array’ as template
  + Might have to switch selected data to get 2 series instead of 1
* Change Chart Type > Jason RT Template for RT PCR graphs
  + Or some other template
    - Make sure to change axis labels since that might get modified
  + You can save any graph format as a template
* Error is used value as up **and** down error bar
  + Chart Design > Error Bars > Custom > Specify Value
  + Use same exact error value for positive and negative error
* Calculate significance p-values between Aco vs. Mus
  + Not really good to compare RT data between different species but we do it anyway
  + Calculate **AVERAGES** for **Control dCt** of Aco and Mus
  + ddCt = dCt Experimental - dCt Control **AVERAGE**
    - Average the dCts of the uninjured controls
    - Subtract each dCt of injured experimentals from that AVERAGE dCt
  + Calculate the p-value using the ddCts
    - when the data are transformed (2^-ddCt). It's more appropriate to use the untransformed ddCt
    - T-test
      * =T.TEST(ddCts of Expt, ddCts of Ctrl, 1, 2)
      * 1 tailed
        + Means are different in one direction only (either up or down)
      * Type 2
        + Data not from the same individual, non-paired data
* \* and line are added in PowerPoint as textbox
  + If a p-value is less than 0.05, it is flagged with one star (\*). If a p-value is less than 0.01, it is flagged with two **stars** (\*\*). If a p-value is less than 0.001, it is flagged with three **stars** (\*\*\*).