Aspergillus fumigatus RNA Preparation for Real Time PCR, cDNA Synthesis

RNA Extraction

1. Harvest mycelia tissue (or conidia) by centrifugation or vacuum filtration. Completely remove any liquid broth (get the sample as dry as possible).
2. Freeze the sample in dry ice, liquid nitrogen, or -80 C. Samples can be stored at -80C. However, samples must be frozen before freeze drying them.
3. Take frozen samples and freeze dry (lyophilize) on the speed vac. The time it takes to freeze dry the tissue depends on the size of the sample and moisture content. To be safe, an overnight incubation is best, but for small samples 3 hours is sufficint.
4. Following lyophilization, samples are ready for RNA extraction. Transfer 20-50 mg of tissue (no more than 50 mg!) to an RNase free **SCREW-CAP 2.0 ml tube**. Add 1mm glass beads, about 1/2 a 0.2 ml PCR tube worth to the sample. Use the mini-bead beater to powder the lyophilized tissue. I use 2 30 seconds runs .
5. Proceed with RNA extraction Method of choice. Either the Qiagen RNeasy Plant mini Kit or a TRIsure/TRIzol extraction work well. **For large amounts of RNA, utilize the TRIsure (Bioline) mixture (same as TriZol).** The protocol you use from here depends on the kit you use. Remember to work quick (but not carelessly!) and keep samples on ice when not using.

Qiagen Kit:

1. Follow the protocol using buffer RLT. Don't forget to add the B-mercaptoethanol prior to extraction.
2. Go ahead and **DO** the optional DNase I treatment steps. This will NOT completely eliminate DNA contamination, but will help.
3. Elute your RNA sample in 50 ul of Rnase Free water.
4. Following elution, use the Nano-drop to get an estimate of the concentration of your RNA.

TRIsure:

1. Any other companies extraction reagent, i.e. Trizol, Trireagent, work just as well.
2. Add 1 ml of the reagent to your powdered tissue and Follow manufacturer's instructions.
3. IT may be necessary to CLEAN the RNA with Qiagen columns.

TRIsure + Qiagen Kit:

**We use this protocol for extracting RNA for microarray experiments. The TRIsure step ensures substantial recovery of total RNA, about 10 fold more then using just the Qiagen kit alone, and the Qiagen Kit ensures clean, salt and organic solvent free RNA. Removing these salts and organic contaminants is important for successful amino-allyl labeling and other downstream applications.

1. Isolate RNA Using TRIsure or TRIzol Reagent
    a. Follow TRIsure protocol above, when you get to the step where you collect the upper clear layer containing your RNA, **STOP**. DO NOT PRECIPITATE THE RNA.
    b. Next, add an equal volume of 70% Ethanol to the TriSURE or Trizol upper layer. Mix well but do not vortex, and apply to an RNeasy Spin Column (The PINK ONES).
    c. Centrifuge as indicatd in Qiagen protocol book, discard flow through, and wash the column 2X with RPE Buffers supplied in the Qiagen Kit.
    d. Dry column as indicated in Qiagen protocol with brief centrifugation, make sure you have ZERO ethanol carry over.
    e. Elute RNA with 50 microliters of RNase Free Water.
    f. Nano-Drop, should have a yield between 1000 to 2000 ng/ul.
    g. For microarrays, examine RNA Integrity with Bioanalyzer

Removing Genomic DNA Contamination:

Removing gDNA is critical for successful, accurate qRT-PCR. We utilize Ambion's Turbo DNA Free kit for gDNA removal.

1. Take an aliquot of your RNA sample into a NEW RNase Free 1.5 ml Tube. The Ambion Turbo-Free DNAase can treat UP TO 10 ug of RNA. **However, to be on the safe side, treat 5 ug or less of your RNA.** Reaction conditions are as follows:

    0.1 Volume of 10X Turbo DNase Buffer
    1 ul of Turbo DNase
    X ul of RNA
    X ul of RNase free water

A typical reaction is 50 ul. So if you digest 5 ug of RNA your final concentration, would be 100 ng/ul of RNA, which is a good concentration for your reverse transcription reaction. I always over estimate and shoot for 150 ng/ul because at least part of your nanodrop reading is coming from gDNA contamination.

2. Incubate the DNase reaction for 30 minutes at 37C.

3. Add 0.1 volumes of DNase inactivation reagent. Incubate at room temp for 2

minutes, gently mixing every 30 seconds.

4. Centrifuge at full speed for 2 minutes and transfer RNA to NEW RNase free 1.5 ml tube WITHOUT getting any of the DNase inactivating reagent carryover. You may need to leave some of the RNA to avoid getting any DIR in your RNA, which can inhbit downstream applications.

5. Use the Nano-drop to get an estimate of the concentration of your RNA. It should be between 100-150 ng/μl.

Reverse Transcriptase Reaction – First Strand cDNA synthesis

1. Protocol depends on RT kit being used. We have found the 2 best kits are Qiagen's Quantitect Reverse Transcription Kit or Invitrogen's SuperScript III Kit. Following manufacturer's instructions.

2. The amount of total RNA you reverse transcribe into cDNA is up to you. Real time PCR is VERY sensitive and thus low amounts of total RNA (as low as 50 ng) can be used. In general, I use 200-500 ng of starting RNA. The kit says you can use up to 5 ug, but this is way overkill. **Use the amount you feel comfortable with and stick with that for ALL your experiments.** If you have a low abundance gene, you may need to adjust your starting RNA concentration.

*****DON'T Forget to do a "NO RT" control with each sample. Typically, I use an aliquot of my DNase treated RNA as the control.**

3. Transfer cDNA reactions to new Nuclease Free 1.5 ml tubes, label appropriately, and store at -20 C until use. **It's a good idea to make your 1:5 cDNA dilutions for RT-PCR at this point before freezing the cDNA for the first time. This includes your "No RT" controls.**

Real-Time PCR Set-Up:

1. Again, protocol depends on what machine, enzyme, etc. you are using. The following protocols are for the Biorad MyIQ cycler in our lab and the Biorad iQSYBR green master mix. **Make sure you are using aerosal tips**.

2. Protocol:

For each gene make a master mix which includes:
- 2X IQ SYBR Green Master MIX
- Nucleese Free Water
- Primers (10 uM working stocks)

Each PCR reaction is based on a final volume of 20 ul so each reaction consists:

10 ul 2X IQSYBR Green Master Mix
0.4 ul Primer 1 (10uM stock)
0.4 ul Primer 2 (10 uM stock)
2 ul 1:5 diltuon first strand cDNA
7.2 ul Nuclease Free Water

**You can use 1-3 ul of the 1:5 diluted cDNA template. Start with 2 ul to see where your CT values fall …if they are greater than 25 cycles, you might want to try 3 ul of template, then 4, etc. etc. But that is up to you.

When setting up your 96 well plate for PCR I use the following template:

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

The various times points I am examining are put ACROSS the plate i.e. wells A1-A12. Then working down is your different replicates and Genes for example if I was examining the expression of gene YFG over a 12 point time course in RPMI media I would have wells A1-A12 as replicate 1 of YFG, wells B1-B12 as replicate 2 of YFG, wells C1-C12 Replicate one of the reference gene Actin, wells D1-D12 replicate 2 of Actin, wells E1-E12 NO RT controls of YFG and F1-F12 NO RT Controls of Actin.

Add appropriate amount of master mix for the appropriate gene to each well making sure master mix gets to the bottom of each well.

Next add template to each well, making sure you mix the template in with the mastermix. Avoid “blowing out” the pipettes as this can create a micro aerosol, which can contaminate other wells. Try not to drag dirty pipette tips over the other wells, come straight out, up and eject over waste disposal which is a good distance away from the plate you are setting up.

Finally, place the plastic film over the plate, and carefully seal it down over the plate. Make sure you have a firm seal, and avoid touching the surface with your hands.

Your plate is ready for the Real Time PCR machine. Keep on ICE as you set-up the qRT machine