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RNA Extraction and Quantification, Reverse Transcription, and Real-time PCR (q-PCR)

Preparation of Samples

- **Cells:**
  - Remove media and wash cells 2X with cold PBS. (2 ml for 6 well plate or 3 ml for 6cm plate)
    - Keep on ice at all times!
  - Add 200-500 ul cold PBS
  - Scrape cells and collect a 1.5 eppendorf tube
  - Spin down to collect pellet at 12000 rpm for 10 min (4 degree)
  - Remove PBS completely
    - store the pellet at -80 freezer if you are not extracting RNA immediately
- **Snap frozen tissue:**
  - Homogenize tissue by placing it in liquid nitrogen-cooled aluminum foil and crushing it into fine pieces (using a hammer) for use in Western blots, RNA extractions etc.
    - Only a small amount of tissue is needed for RNA extraction (20-30 mg)
  - The rest of the homogenized tissue can be aliquoted and stored at -80.

RNA Extraction

RNA extraction is done using different RNA extraction kits specific for certain cells, tissue, blood, etc. You can learn more about different kits for different tissues on the Qiagen website: http://www.qiagen.com.

The RNeasy Mini Kit from Qiagen is the kit used most often in our lab. The kit is supplied with a Quick–Start protocol for RNA extraction. You can find more detailed explanation in the Qiagen handbook on their website:www.qiagen.com/handbooks.

Notes before start using Qiagen kit:

- Add 10ul β –mercacptoethanol (β-ME) to 1ml RLT buffer provided in the kit. Make appropriate volume of this based on the volume that has to be used (see table 1 in the Quick-Start protocol).
- Buffer RPE in the kit is supplied as a concentrate. Make a working solution by adding an amount of ethanol (96-100%) as indicated on the bottle.
- Make 70% Ethanol using RNase free water.

- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Quick-Start protocol.
- Wear gloves all the time and set up and maintain a working environment free of DNA contamination.

Cells: Harvest a maximum of $1X10^7$ cells as a pellet (as mentioned above) or by direct lysis in the vessel. Add the appropriate volume of RLT buffer + β-ME

Tissues: A maximum of 30 mg can be used for extraction. Disrupt the tissue and homogenize the lysate in the appropriate volume of Buffer RLT + β-ME (see table 1 in Quick-Start protocol).

1. Add the appropriate amount of Buffer to cells or tissue and vortex for 1 minute . For pelleted cells, loosen the pellet thoroughly by flicking the tube. Tissue samples have to be hammered into fine pieces as mentioned before. Samples can be homogenized using rotor-stator or QIAshreder from Qiagen.
    - Incomplete homogenation leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column.
    - Homogenization with a rotor-stator or QIAshredder homogenizer results in higher RNA yield.
2. After disrupting and homogenizing, pipet the lysate directly into a QiAshredder spin column placed in a 2 ml collection tube; centrifuge 2 min at full speed.
3. Add the same amount of 70% Ethanol to the lysate, and mix well by pipetting up and down. DO NOT centrifuge.
4. Transfer up to 700 ul of the sample, including any precipitate, to an RNeasy Mini spin column placed in a 2ml collection tube (supplied). Close the lid, centrifuge for 15sec at ≥ 8000 x g (10000 rpm). Discard the flow-through.
    - Save the tube to be used again in step 6
    - If the sample volume exceeds 700ul, centrifuge successive aliquots in the same spin column. Discard the flow-through after each centrifugation.

Optional : On column DNase digestion
If you are using RNase–free set for first time, prepare DNase I stock solution as it says in the quick-start protocol by injecting 550 ul RNase–free water into the DNase I vial. Mix gently by inverting the vial. Do not vortex. It can now be stored at -20 °C for up to 9 months. Do not refreeze aliquots after thawing.

- Add 350ul Buffer RW1 to RNeasy column, close lid, and centrifuge 15s at ≥8000 xg (10000 rpm). Discard flow through.
- Add 10ul DNase stock solution as mentioned above to 70 ul Buffer RDD. Mix gently by inverting tube. Centrifuge briefly.
- Add DNase I incubation mix (80ul) directly to RNeasy column membrane and incubate for 15 min at room temperature.
- Add 350 ul Buffer RW1 to RNeasy column, close lid, centrifuge for 15 s at ≥ 8000 x g. Discard flow through. Continue with step 6.

5. Add 700 ul Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000xg. Discard flow–through.
6. Add 500 ul Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥ 8000 x g . Discard flow-through.
7. Add 500ul Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2min at ≥ 8000 x g.

Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.

8. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied) or 2ml (supplied). Add 30ul-50 ul of RNase–free water directly to the spin column membrane. Close the lid, and centrifuge for 1min at ≥ 8000x g to elute the RNA.
9. If the expected RNA yield is >30ug, repeat step 8 using another 30-50 ul of RNase–free water.

RNA can be stored at -80.

RNA quantification and quality control

In the MUH lab, RNA is measured on the BioTek machine. Turn machine on. Open a GEN 5 Take 3 Session to measure RNA concentration.
Under sample type, select RNA (not DNA).

1. In the drawer under the computer, take the 16 well black plate (Take 3, from BioTek) from box.
2. Pipet 2ul of your blank (RNase free water) or sample onto wells (spots).
3. Put your plate in machine to be read. Do not close!
4. Go back to open take 3 session and under the blank highlight well (wells) that you have blank. Click “read blanks”. Wait for machine to take your plate. Press OK after that window appears on the screen.
5. After the blank is read, highlight sample wells. Press “read samples”. Wait for OK window and click on it.
6. You will get your concentration in ug/ul . Calculate the amount of RNA you need to have for using 1ug of RNA for each sample for next step (Reverse Transcription)
7. A260:A280 ratio of 1.8-2.0 indicates pure RNA.

Reverse Transcription (making cDNA)

Starting amount of RNA is usually 1ug . It can be used as little as 25ng up to 5ug. The optimal amount of starting amount depends on the relative abundance of the transcript of interest. The protocol for making cDNA depends on the Reverse Transcription Mix (Company) you use.

Reaction Setup for a Singe cDNA Synthesis Reaction iScript - Bio Rad Reverse Transcription Supermix for RT-qPCR (total volume 20ul /reaction)

1. Use PCR plates for doing Reverse Transcription.

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General Surgery Labs SOP

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5x iScript reverse transcription supermix	4ul
RNA template (for 1ug)	Variable
RNase free water	Variable
Total Volume	20ul

Keep everything on ice.

For multiple cDNA reactions you can make a master mix of RNase free water + SuperMix. For example: your master mix for one sample can be 15ul (4ul, 5xiScript + 11 ul RNase -free water) per well of total 20ul/reaction/well. Multiply those amounts with the number of RNA samples and add 2-3 more as extra to accommodate loss during pipetting. For 10 samples, multiply by 12 and your master mix will consist of 48 ul of 5x iScript and 132ul of RNase–free water.

2. Add 15ul of master mix to each well
3. Add 5ul RNA to each tube. Adjust volume up to 5uls using RNase free water.
    - Each reverse transcription reaction should have 20ul/well.
4. Along with the Reverse Transcription, make one NRT control by using 5x iScript RT master mix with no RT control (it has a clear cap). Use 4ul of this instead of reverse transcription mix with one of the RNA you used for RT. The total reaction is 20ul.
    - NRT is important control to check if you have any DNA carryover in your RNA samples.
5. Spin your plate for 1min at 1000 rpm and incubate in thermal cycler (PCR machine) using the following protocol that is saved under the protocol express load.

Reaction protocol:

Priming	5 min at 25°C
Reverse Transcription	30 min at 42°C
RT Inactivation	5 min at 85°C

cDNA can be stored at -20 in your plate or you can transfer to tubes.

Real- Time PCR (q-PCR)

Bio Rad iTaq Universal SYBR Green Supermix is 2X concentrated, ready–to–use reaction mix optimized for dye –based quantitative PCR (qPCR).

1. Thaw iTaq Universal SYBR Green supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of the tubes and store on ice (protected from light).
2. Prepare enough master mix for all reactions for EACH primer. For each sample you are running qPCR, you must have a housekeeping gene (usually actin or GAPDH). At the minimum, run each sample in duplicate (for example, have two wells for housekeeping gene and two wells for each gene of interest for each sample). Also, you must have NRT control (to check if you

have any genomic DNA contamination) and NTC control (negative control). It is not necessary to do this in duplicate.

Per sample mixture

iTaq Universal SYBR Green supermix (2x)	10 ul
Forward and Reverse primers	1ul
DNA template	1ul
RNase free water	8 ul
Total Volume	20 ul

You can increase the amount of DNA template per PCR reaction up to 10% (2ul in 20ul reaction) if you need to.

3. For multiple reactions create a master mix of all components except the DNA template. Mix thoroughly and pipet 19uls into the wells of a qPCR plate.
    - Good pipetting is very important!
4. Add 1ul DNA template, NRT control, or RNase free water into each well.
5. Seal the plate with optically transparent film. Spin the plate for 5min at 1000 rpm to remove any air bubbles and collect the reaction mix in the vessel bottom.
6. Programing the thermal cycling protocol on the real-time PCR instrument depends on the SYBR green mix you are using and the machine. You can find this program in the box with SYBR Green.
7. Program for iTaq Universal SYBR Green from Bio Rad is saved under CFX96 program for the PCR machine in MUH.
8. Put your plate in the PCR machine. Open the CFX96 Manager program on the computer desktop. Under user pick up administrator, open and click on "user define". Under the protocol in express load pick up "iTaq SYBR Universal" if you are using SYBR Green from BioRad.
9. Under plate option make your template by labeling your samples, NRT, NTC, targets (your primers), and replicates.
10. Click "Start Run". Change the sample volume in necessary.
    - For assistance with BioRad programming, see the user manual next to the machine or ask Sladjana Stratomirovic for assistance.