cDNA from Muscle RNA

Revised by C. Pham from Manufacturer's Protocols
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Procedure

Prepare Tissue Sample

1. Homogenize muscle tissue and get RNA:
   Always use filter tips.
2. Cut tissue with razor blade on glass plate
3. Add Buffer RLT (pipette 200-1000ul – (040)) to tube then add 4ul BME (pipette 2-20ul – (040)) in the hood. Vortex solution.
4. Add 300 ul of mix (RLT and BME) to mm tissue.
5. Now homogenize tissue in solution.
   Remember to clean in H20 and Ethanol
6. Add 590ul H20 distilled (pipette 200—1000ul – (059)) to homogenized tube and then add 10ul Protease K (pipette 2-20ul -- (100)).
7. Incubate tube at 55 degrees Celsius (10 minutes)
8. Centrifuge tube for 3 min. at 10,000 x g
9. Pipet supernatant into new tube (approximately 900ul) (pipette 200-1000-(090))
10. Add 450ul of Ethanol (96%-100%) to supernatant. Mix well by pipetting….DO NOT centrifuge.

Isolate RNA using Qiagen RNeasy kit.

1. Pipet 700ul (pipette 200-1000ul—(070)) of sample into new 2ml RNAeasy column collection tube (in QIAGEN kit)
2. Centrifuge 15sec. at 8000 x g
3. Dump liquid clear contents in bottom tube

4. Repeat step 9 with the rest of the of the sample (Ethanol and supernatant)

5. Add 350ul Buffer RW1 (pipette 200-1000ul – (035)) to RNAeasy column and re-spin and centrifuge for 15sec.

6. Put pink part into new 2ml clear tube from QIAGEN kit

7. Add 500ul RPE Buffer (pipette 200-1000ul—(050) into new tube set-up and centrifuge for 15 sec. at 8000 x g.

8. Discard liquid in bottom part of tube

9. Add another 500ul (pipette 200-1000ul-(050)) Buffer RPE to RNAeasy column and centrifuge another 2 min. at 8000 x g.

10. Transfer RNAeasy column to new 1.5ml collection tube (from QIAGEN kit).

11. Add 50ul (pipette 50-200ul (050)) RNAse-free H20 (from QIAGEN kit) to new set up (apply to white part and let it sit for 1 min.)

12. Centrifuge for 1min. at 8000 x g

13. Keep bottom part of tube and throw away pink part.
    Now you have your RNA sample

Quantify RNA by Spectroscopy

1. Turn on Spec.
2. Turn on UV (bottom left of screen) → should turn RED
3. Let it warm up
4. 1:50 Dilution (standard)
5. Add 98ul (pipette 50-200ul –(098)) RNAase free H20 (QIAGEN kit) into two little tubes.
6. The H20 acts as a blank
7. Add 2ul RNA (pipette 0.2-2ul-(200)) into one of the tubes with H20.
8. Should have one H20 tube and another with H20 + RNA
9. Get CUVET box (don't put fingers on window), pipette and take to SPEC machine.
10. On computer make sure it says "FIXED WAVELENGTH"

11. On computer make sure it says "Method name: Nucleic"

Wavelength	260	280
Factor	2.0	4.0

12. Put H20 (blank) into cuvet box and put into machine
13. Red dot should face forward
14. Click: Blank
15. Turn on vacum and suck out water from the cuvet box
16. Put tip on plastic hose
17. Computer should read approximately 0.0002
18. Put RNA sample into cuvet and repeat process
19. Click: Read samples (at top of screen)
    Print out specs or write out.
    Now you have determined how much RNA is needed for RT reaction

Prepare RT-PCR Reaction Using Taqman

1. Get out TAQMAN solutions
2. Put RNAse inhibitor and RT purple top tube on ice and thaw the others
3. Let thaw
4. Make Master Mix:

5. EXAMPLE (see example sheet):

RT RxN Master Mix

10xRT Buffer	5ul	x	3.5= 17.5 (pipette 2-20ul (175))
MgCl2	11ul	x	3.5=38.5 (pipette 50-200ul (038))
dNTPs	10ul	x	3.5=35 (pipette 50-200ul (035))
Random Hexamer	2.5ul	x	3.5=8.75 (pipette 2-20ul (087))
RNase inhibitor	1.0ul	x	3.5=3.5 (pipette 2-20ul (035))
= MASTER MIX			
DEPC H20 & RNA	19.25 – 4.03	=	15.22 H20
RNA from Sample	500/124ng	=	4.03 ul RNA

6. Add 31 ul Master Mix (everything but H20 & RNA) into 3 different tubes

7. Add 15.22ul H20 (pipette 2-20ul—(152)) and add 4.00ul RNA (pipette 2-20ul—(040)) to each tube with Master Mix

8. NOTE: 31ul Master Mix is constant
9. NOTE: Left hand #s are constant in master mix solution
10. NOTE: 124 is from the spec absorbance

11. REGULAR PCR machine (in Judd's Lab):
    11.1. Press Standby (turns machine ON)
    11.2. Press protocol library (F1)
    11.3. Press ABI-RT and then ENTER
    11.4. Run setup 50 sample volume
    11.5. Begin RUN (F5).

12. PRIMERS:
13. Determine quantity of Master Mix (SybrGreen, cDNA, H20)

EXAMPLE (see below):

20ul RxN:

10ul SYBER Green	x	3	= 30 ul SYBER Green
1ul cDNA (long)/H20	x	3	= 3 ul cDNA
2ul primer	x	3	= 2 ul Primer
20 – 13 = 7	x	3	= 21 ul H20

Label tubes (H20, Hela, Sample X, Sample Y) to make Master Mix

20 – 2 = 18ul Master Mix/well

14. Write down plate set up on sheet of paper

	2	3	4	5
B.Myostatin→	H20	Hela	Sample X	Sample Y
C. MyoD →	H20	Hela	Sample X	Sample Y

15. Dilute Primers (MyoD, Myostatin) if they haven't already been diluted:

80ul H20 (pipette 50-200—(080))
10 Forward Primer (pipette 2-20—(100))
10 Reverse Primer (pipette 2-20—(100))

2ul primer in each well (pipette 0.2-2ul—(200))

16. Perform RT-PCR (using e.g. "Real-Time PCR with the iCycler5" protocol)