Vezina Lab RT-PCR Protocol

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1 *Works for me* This protocol is published without a DOI.

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RNA Isolation

1 Obtain Supplies:
- Tinfoil
- Microfuge-sized Pestles and Molecular Grinding Resin (G-Biosciences, Cat. No. 786-138PR).The Pestles can be washed, autoclaved, and reused.
- RNase-Free microfuge tubes
- Beta-mercaptoethanol
- 70% Ethanol
- 55C incubator
- Liquid Nitrogen
- Tube decapper
- Illustra RNAspin Mini kit (GE Healthcare #25-0500-17)

2 Suspend molecular grinding resin in 1 mL buffer RA1 (with no beta-mercaptoethanol).

3 Place tinfoil on benchtop. Clean microfuge with RNase-away. Obtain RNase-free microfuge tubes and label them appropriately. You will need 2 microfuge tubes, one RNAspin Mini Filter, and 1 RNAspin Mini Column per tissue sample. You will also need one plastic pestle per sample.

4 Place 1ml of H20 in 55°C incubator.

5 Place a cardboard microfuge tube storage box inside of a Styrofoam freezer box and fill the storage box with liquid nitrogen.

6 Remove tissue samples from -80°C freezer and place inside them inside the microfuge tube storage box containing liquid N2.

RNA Purification

7 Remove tubes one at a time and use a tube decapper tool to uncap the tubes.

8 Work on one sample at a time until all are homogenized

9 Cut the tip off the bottom of a p200 pipette. Use the cut tip to add 10 µl molecular grinding resin to the sample. Use the pestle to grind the tissue. Add the appropriate amount of lysis buffer to each sample. Store the homogenates at room temperature until all samples have been homogenized.

10 Follow the kit directions to pass homogenates through the filters, bind RNA to the column and wash the column.

11 Carefully remove column from collection tube after it has been washed and dried. Place column in a clean, labeled microfuge tube. Add 50 µl of 55°C H20. Incubate for 10 min at 55°C.

12 Centrifuge at room temp for 1 min at 10,000 rpm.

13 Repeat step 5, using eluate from Step 6.

14 Centrifuge at room temp for 1 min at 10,000 rpm.

15 Place RNA on ice and spectrophotometrically determine concentration by diluting 3 µl sample into 57 µl H20.

Reverse Transcription

16 Reverse transcription should be conducted in PCR tubes. Number each tube with an indelible pen so that the sample can be identified in future PCR runs.

17 Synthesize cDNA with oligo d(T) primers, according to the instructions of the SuperScript® III First-Strand Synthesis System (Invitrogen # 18080-051).

Combine the following items into PCR tube:
x µl (500ng total RNA)
1 µl of 50 µM oligo (dT)20
1 µl of 10 mM dNTP
make up final volume to 10 µl with RNase free (molecular grade) water.

18 Incubate at 65°C for 5 min and then place on ice for at least 1 min

19 To each tube then add:
2 µl 10X RT buffer
4 µl 25mM MgCl2
2 µl 0.1M DTT
1 µl RNaseOUT (40U/µl) 1 µl Superscript III RT (200 U/ µl).

20 Mix gently then incubate at 50°C for 50 min

21 Terminate the reaction at 85°C for 5 min. Chill on ice.

22 Add 1 µl of RNase H to each reaction, mix and then incubate at 37°C for 20 min

23 Dilute cDNA with 80 µl H20 to a final volume of 100 µl. Print the form on the next page, fill in the details, and add it to the box containing the samples. store at -20°C.

Primer design for real-time PCR

24 Extract mRNA sequence for gene in Entrez Database (http://www.ncbi.nih.gov/entrez
Use the RefSeq sequence in Entrez (starts with NM_)

25 Identify PCR primers for gene
Go to primer3 website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

Input mRNA sequence

Configure Primer 3 Settings as follows:
Mispriming Library (repeat library): RODENT_AND_SIMPLE
Product Size Ranges: 80-150.
Max Mispriming: 10
Primer Size Min:18, Opt: 21, Max: 23
Primer Tm Min:59, Opt: 62, Max: 64
Max Tm Difference: 1.
Primer GC% Min:20, Opt:50, Max: 80.
Max 3' self-complimentarity: 1.
Max Poly X: 3.
GC Clamp: 1.
Leave all other parameters the same and order primers from www.idt.dna.com.

26 Ordering Primers
For RTPCR name primers by the following convention: species+“genesymbol”, followed by “QRTPCR” for reverse transcription, followed by “F” or“R” for the direction of the primer (If for MeDIP also include in name). For example, the forward primer for mouse beta-catenin (ctnnb1) RT-PCR would be named “mctnnb1RTF”.

Order primers on a 25 nmol scale from integrated DNA technologies (idtdna.com).

Standard desalting is all you need for purification

Shipment will arrive in about two days, if you order primers in the morning.

Real-Time PCR (Hernandez Lab)

27 Obtain Supplies:
96-well PCR plates from USA scientific (#1402-9890)
PCR plate sealers from Fisher Scientific (#08-408-240)
2X SsoFast EvaGreen Supermix 200x20uL from Bio-Rad (#172-5200)

28 Assemble master mix (shown is the amount needed per sample)

(µl) Rxn Components (Initial Concentration)
6.25µl SsoFast EvaGreen Supermix (2x)
0.5µl Primer mix (10 µM each, Integrated DNA Technologies)

=6.75 µl/ well + 3.75µl cDNA = final reaction volume is 10.5µl

29 Add PCR master mix to each well of 96 well plate

30 Add cDNA to each well

31 Include at least one water blank for each gene run.

32 Place plastic seal on top of plate

33 Keep plate on ice to take to **BioRad CFX96 real-time PCR machine**

34 Centrifuge plate (lab across the hall): 3500rpm for 1 min at 4C

35 In computer room turn PCR machine on (button on right back side)

36 Open BioRad CFX manager on desktop

37 Create a new run, select OK

38 Select run- existing (run 60) or new run. (I pick existing then edit selected- and change the annealing temp, add or subtract a melting curve or change the # of cycles I did 49 for first run)

39 Conditions are: 95˚C for 3:00min, 95˚C for 10 sec, 64˚C for 30 sec x 49. Can add melting curve 65˚C for 5 sec, 95˚C to 0.5˚C. (Takes 1hr and 18 min)

40 Click Next

41 Select plate-existing (select) or new plate: (change code samples to unknown, negative control for water blanks, click on Sybr for all samples, change replicate size to 3)

42 Start Run: Save as “gene-date-user” on desktop

43 When finished copy data to excel, save on desktop and email spreadsheet to yourself

44 Can then delete your run from the computer’s desktop

45 Hit the large button on front of machine to open lid, remove plate, hit button again to close lid

46 If no one else is using the machine switch it off with the back right button.

47 Analyze results using the spreadsheet created “Template for RT-PCR results” in the RNA protocols folder of the L drive.

48 Run stats using R Version 2.13.1

Real-time PCR (Peterson Lab)

49 Turn on the computer.

50 Turn on the light cycler (toggle switch is located on the back right side of machine).

51 Login to computer. Username: Set Up with Tien-Min Lin. Password: Set up with Tien-Min

52 Double click on “lightCycler3 front screen” icon.

53 Click on run.

54 Set up protocol of your choice.

55 If the program asks to do self-test, click “ok” (optional).

56 There is an “experiment” window in the upper left part of the screen that indicates the Experiment method that you opened.Each experiment method contains 5 components: Denaturation, Amplification, Melting, and Cooling.You can single click any of these steps on the right side of the screen to bring up a detailed method window for each.For optimizing new primers, these are the details for each step:

Denaturation.You can probably leave this as its default, which is 0 seconds, unless you are using a hot-start Taq, then follow manufacture’s instructions

Amplification.Change the Annealing temp to match the value listed on the RT-PCR gene list on the chest freezer.If using primers for the first time, start with an annealing temperature about 2°C less than the optimal temperature for which the primers were designed (i.e. start with 58°C for primers that were designed to have a 60°C annealing temp).The annealing time should be left at its default.The extension time depends on the length of the PCR product.Extensions should be [1 second per 25 bp product] + 1 second.For example, a 250 bp product would have an extension time of 11 seconds.The transition rates for each step should be left as default (20°C /second for denaturation/annealing and 2°C/second for extension.

57 Open the “edit samples” window. You can type in the name of the samples. If you have a no cDNA control sample, highlight “unknown” from the drop down menu next to the sample name. If the sample is an internal standard, highlight “standard” from the dropdown menu and type in the concentration (you can also control the units for your samples by typing in your desired concentration units in the window at the bottom-right of the screen). If your sample is an unknown, select “unknown” from the dropdown menu.

58 Take your samples out of the ice box and place them in the carousel to the right of the lightcycler work station. The carousel with the brownish colored gasket (and a small blue dot in the punched out region in the side) works the best, followed by the carousel without the notch on its side. Push the capillaries down gently into their respective positions. If one breaks inside the carousel, use the Christmas tree-shaped brush to clean out the glass shards and replace with a new capillary.

59 Place the carousel containing samples into the centrifuge to the left of the lightcycler. Press “on.” It will spin down the samples for a predetermined period of time.

60 After its done spinning, remove the carousel and examine the contents of the capillaries. Each tube should contain nearly the same volume. If not, you pipetted poorly. Go back to pipetting school. Throw that capillary away and use your leftover master mix and mix with cDNA to make a new sample.

61 Put the carousel in the PCR machine. Line up the carousel notch with the white dot on the lightcycler. Click the carousel into place. GENTLY push down the cover part way, the machine will automatically pull it down the rest of the way.

62 Push “run” on the pcr machine. It will prompt you for a file name and you should designate the folder for it to be stored in. The hard drive is partitioned and we have a folder called ‘mouse lab’ in the E:// driver. You can set up your own private folder within this drive. Name your file, click done, and the run will begin. The results of the run will be saved automatically. You can print off your data immediately after the run, or save it for a later date. Sometimes you might receive an error message after the run that states something about INF. If this is true, go to the folder where you saved your data, open the associated .FLO file in notepad, then use the search function to find “INF”. Replace the INF text with the number that immediately proceeds it in the same column. Repeat the search for “INF” until it cannot be found again in the file, then save the file.

63 Collect your capillaries, uncap them, and turn them upside down into a clean microfuge tube. Spin at 1000 rpm in a microfuge for a few seconds to extract the reaction contents from the tube. Run the contents on a gel to visualize the PCR products.

64 Turn on the computer

65 Double click on the “LightCycler3 Front Screen” icon on the desktop.

66 Double click on the “data analysis” option.

67 Select the folder containing file to be analyzed from the drop down menu on the left.

68 Double click on the file name on the right hand side of the window.

69 To analyze the melting curve:
Choose “Melting anaylsis” on the left drop-down menu.
Click on “melting curve.” On the right drop-down menu
Single click on the sample names on the left side of the screen that you want to view.To view more than one sample at a time, single click on each sample while holding the shift key down.
To save a picture of the melt curve, you can either print the screen to .pdf, or you can hit the “Print Screen/SysRq” key on the keyboard, which will copy a picture of the screen to the clipboard.
Paste the picture from the clipboard into powerpoint.

70 To perform quantification:
Click on quantification in the left drop-down menu..
Click on “quantification” on the right drop-down menu.
Select the “second path derivative maximum” under the Analysis option at the top of the screen.
Select “arithmetic” under the baseline adjustment option at the top of the screen.
Highlight all samples on the left.
Under the Quantification window at the top of the screen, select Export à Baseline adjustment.
It will prompt you to name your file.Note the location of the folder for which you are saving your file.The file is saved as a tab-delimited text file that can be opened with excel.