Medina Lab, Penn State University

RT-qPCR protocol (with aRNA), prepared by M. DeSalvo

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1. cDNA synthesis

a. Place 1ug aRNA into a PCR tube (volume can't exceed 11uL).
b. Add 1uL random hexamers.
c. Add 1uL 10mM dNTPs.
d. Bring final volume to 13uL with dH_2O.
e. Place @ 65°C for 5min.
f. Chill on ice for at least 1min.
g. Prepare the RT master mix.

Reagent	**1x (uL)**	**e.g. 11x (uL)**
5x RT buffer	4	44
0.1M DTT	1	11
RNaseOUT	1	11
SuperScript III	1	11

h. Add 7uL RT master mix to each tube (mix by pipetting).
i. Place @ 25°C for 5min.
j. Place @ 50°C for 50min.
k. Place @ 70°C for 15min.
l. Chill on ice.
m. Add 1uL RNaseH to each tube.
n. Place @ 37°C for 20min. Test PCRs
a. Place RT reactions into new 1.5mL tubes.
b. Add 180uL dH_2O.
c. Use 2uL of cDNA in a PCR reaction using good qPCR primers.
d. PCR master mix (25uL reaction volumes):
    i. This will change depending on Taq being used.

Reagent	**1x (uL)**	**e.g. 11x (uL)**
10x ExTaq PCR buffer	2.5	27.5
ExTaq dNTPs	2.0	22.0
10uM forward primer	0.5	5.5
10uM reverse primer	0.5	5.5

Takara ExTaq	0.125	1.375
dH_2O	17.375	191.125
cDNA	2.0	

2. <u>PCR Program</u>

e. PCR program as follows:

i. 94°C for 30sec

ii. 94°C for 15sec

iii. 60°C for 15sec

iv. 72°C for 15sec

v. go to step ii. – repeat 35x

vi. 72°C for 10min

vii. 4°C for ever.

f. Run 5uL of each PCR reaction on a 2% agarose gel.
g. You should get a single band ~100bp.

RT-qPCR

a. Use 12.5uL reaction volumes.
b. Use 96-well Optical Reaction Plates (ABI Part #4306737).
c. Use Power SYBR Green PCR Master Mix (ABI Part #4367659).
d. Prepare a separate master mix (in a 1.5mL tube) for each gene that you will be testing.

Reagent	**1x (uL)**
SYBR Green	6.25
Forward primer (10uM)	0.25
Reverse primer (10uM)	0.25
dH_2O	3.75

3.

e. Calculate the # of reactions needed for each gene:

i. (# of samples) X (3 technical rep's per sample)

ii. e.g. (9 samples) X (3) = 27 total rxns.

3.

f. Overshoot by a few reactions to allow for slop, and to have left over master mix for your 3 non-template controls (NTCs).

i. Thus, the total # of actual qPCR reactions cannot exceed 93.

3.
g. Bring the following to the Choi lab:
   i. Sample tubes.
   ii. Master mixes.
   iii. 96-well plate.
   iv. Plate sealers.
   v. Tube with PCR-grade water.
   vi. P200 and P2 tips.

3.
h. Start software.
i. Choose “Absolute quantification” à “Next”.
j. Click “SYBR Green” à “Add” à “Next”.
k. Drag the mouse over (thus highlighting) the wells you plan to fill with reactions à click “SYBR Green” à “Finish”.
l. Click on the “Instrument” tab.
m. Highlight “Stage 1” (50^{o}C for 2min) à “Delete”.
n. Click “Add Dissociation Stage”.
o. Enter “13” in the “Sample Volume (uL)” field.
p. Make sure “Data Collection” takes place at “Stage 2, Step 2”.
q. Click on the “Setup” tab.
r. Enter names for all reactions.
s. Choose “NTC” for 3 wells.
t. Fill appropriate wells with 10.5uL of the appropriate Master Mix.
u. Add 2uL of cDNA to each well.
v. Add 2uL of dH_2O to the NTC wells.
w. Seal the plate with a plate sealer and use the plastic tool to ensure that each well is sealed.
x. Spin the plate for 30sec.
y. Insert the plate into the machine.
Go to the “Instrument” tab and click “Start”.