qRT-PCR Protocol

Reagents/Notes

- Use Luna® Universal Probe One-Step RT-qPCR Kit (NEB, E3006L)

-Use IDT Tool (https://www.idtdna.com/scitools/Applications/RealTimePCR/Default.aspx) to design primers and probes—desired amplicon size: 75-200 bp

-Primers => resuspend to 200 uM in RNase-free ddH20

-Probes => 5' 6-FAM, 3' TAMRA, HPLC purified, resuspend to 100 uM in RNase-free ddH20

-Use RNase free technique: separate filter tips, spray work area down with RNase Zap, RNase-free ddH20

1. Isolate RNA—See RNA Isolation Protocol
    a. Run Taq PCR to determine if DNA contamination is present in RNA
        i. Can use any PCR amplicon that amplifies easily
    b. If no amplification present—continue with RT-PCR protocol

2. Nanodrop RNA
    a. Dilute RNA samples to 5 ng/ul in RNase-free ddH20

3. Make RNA standard curve
    a. Start with 5 ng/ul WT control RNA ($1x10^{-9}$)
    b. Make 4 succesive 1:1 dilutions in RNase-free ddH20
    c. Final concentrations: $1 x10^{-9}$, $5x10^{-10}$, $2.5x10^{-10}$, $1.25x10^{-10}$, $6.25x10^{-11}$

4. Set up qRT-PCR Mastermix (on ice!)
    a. Always include wells for Act1 control
    b. Construct separate mastermix for each primer/probe combination:

Reaction Mixture (18 ul)

2x One-Step Reaction mix:	10 ul
2x Warmstart RT mix:	1 ul
Primer 1 (20 uM):	0.4 ul
Primer 2 (20 uM):	0.4 ul
Probe (10 uM):	0.4 ul
RNase-free ddH20:	5.8 ul

5. Aliquot Mastermix (in chilled PCR rack)—see plate layout on next page
    a. 18 ul of mastermix per well (in triplicate

6. Add 2 uL of 5 ng/ul RNA to corresponding wells (10 ng total RNA/reaction)

7. Run on “NEB Luna” qRT-PCR cycle (see NEB Luna manual for cycling conditions)

8. Analyze data using Biorad CFX software, using Act1 as reference control

Plate Layout (use alternate standard curve on previous page)

1	2	3	4	5	6	7	8	9	10	11	12
Act-1 10-9	Act -1 10-9	Act 1 10-9									
Act-1 10-10	Act -1 10-10	Act 1 10-10									
Act-1 10-11	Act -1 10-11	Act 1 10-11									
Act-1 10-12	Act -1 10-12	Act 1 10-12									
Act-1 10-13	Act -1 10-13	Act 1 10-13									
Pos	Pos	Pos									
NTC	NTC	NTC									
Blank	Blank	Blank									