Singleplex Quantitative RT-PCR Andes lab protocol

A.) Determine exon/intron regions in genes of interest.
1.) Ideally design intron flanking primers.
2.) If gene is a single exon, use Primer Express™ 1.5 as described below.
a.) Open primer express.
b.) File-> New-> TaqMan MGB Probe & Primer design-> Paste in nucleotide sequence.
c.) Options->Find Primers/Probes Now
d.) Examine probe sequence.
1.) If G>C, then use reverse complement as probe.
e.) Make sure rev comp does not have 5' G.
1.) If 5'G then select primers in a different region of the gene. Note: Scroll down the primer list further than expected because the program designs primers in the same region, varying primer selection by only 1 bp. Remember to examine probe sequence again.

B.) Order forward and reverse primers from IDT.
1.) If genome is not sequenced design and order another primer ~100 bp upstream from Primer Express™ 1.5 generated forward primer. PCR sequencing primer with reverse primer; ~100uL rxn. Clean Qiaquick and elute w/30 uL H_2O. Use 11 uL as template in 20 uL sequencing rxn.

n=1
11 uL PCR template
1 uL 0.8 uM sequencing primer
4 uL big dye enzyme
4 uL big dye buffer
20 uL total volume

a.) Add mineral oil. Pulse Spin.

94 °C 1m, 54 °C 15s, 60 °C 4m, 30 cycles
72 °C 7m
4 °C hold

b.) Clean up w/G50. Process at BTC. Verify primer/probe sequences.
2.) Reconstitute primers 20 uM, or 20 pmol/uL and aliquot into ~10 tubes. Keep track of freeze/thaws.
a.) PCR using yeast gDNA and mouse cDNA and water as template. Expect no product with water or mouse template and band of interest, ~70-80 bp, in yeast template.
b.) If primer dimers or ~70-80 bp mouse product are apparent, go back to Primer Express ™ 1.5 and select primers in a different region of the gene.

C.) Order probe primer with 5' 6-FAM flourophore and 3' TAMRA quencher from IDT.

1.) Reconstitute probe 10 uM, or 10pmol/uL and aliquot into ~15 tubes. Keep track of freeze/thaws. Use probe at 0.2 uM.

2.) Use Qiagen Quantitach RT-PCR kit cat # 204443, 1-800-426-8157.

n = 1	
25 uL	QuantiTect Probe RT-PCR Master Mix
variable	Forward Primer 20uM
variable	Reverse Primer 20uM
1 uL	Probe 10uM
0.5 uL	QuantiTect Probe RT Mix
variable	RNase-free H_2O
1.0 uL	heat-labile UNG (1U/uL)
variable	template RNA, 500 ng
50 uL	total volume

a.) Always add template last to cocktail.

b.) Heated lid; do not add mineral oil.

3.) Optimize primers with 0.4 uM, 0.7 uM, and 1.0 uM concentrations, and use template with high expected mRNA copy number.

a.) Use primer concentration that yields lowest C_t value and highest Δ rxn value; Aubrey uses 1 uM.

4.) Use 1 u = 1 uL heat-labile UNG, uracil-N-glycosylase, per rxn. Roche cat # 775367, 1-800-428-5433.

5.) Use both NTC, not template controls, and NAC, no amplification controls.

a.) Do UNKs, unknowns, and NTCs in duplicate, NAC can be single rxn.

6.) Spin plate before running rxns at no more than 1K rpm, and keep on ice while programming run.

D.) Use ABI Sequence Detection System 7700, sign up 1d in advance.

1.)Shut down Mac computer.

2.)Turn off ABI Sequence Detection System 7700.

3.)Wait 1m.

4.)Turn on ABI Sequence Detection System 7700.

5.)Turn on Mac.

6.)File->New Plate

a.) Highlight wells and select sample type

b.) Select thermal cycler conditions.

1.) stage 1: 30m 50^{o}C

2.) stage 2: 15m 95 ^{o}C

3.) stage 3: 15s 94 ^{o}C, 1m 60 ^{o}C 40 cycles

4.) 50 uL rxn

c.) Dye layer should be FAM; TAMRA is default quencher.

7.)Click Show analysis.

i. Put gray box around all wells.

8.)Click Run.

9.)Verify time to complete cycle is 2h19m.

10.)Verify comm. button on ABI SDS machine is lit or blinking.

11.)When run is completed, Analysis-> Analyze.

12.)Save SDS file and export data.report and data.results on zip disk.

b. Delete run from SDS folder.

c. Remove samples immediately when run is complete; do not let sit ON.