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## I. Primer design and validation

1. Identify the gene of interest by its mRNA RefSeq identification (NM\_) and paste the sequence into Primer3 (<http://frodo.wi.mit.edu/primer3/>).
2. Change the following parameters in Primer3:
  - Restrict the size of the amplicon to 150-200 bp — *this size is small enough to amplify easily in a rapid RT-qPCR format but large enough to be readily separated from primer dimers by melt-curve analysis*
  - Restrict the position of the amplicon to within 400 bp on the 3' end of the gene — *this ensures that the Superscript III has reverse transcribed through the region of the gene that will be amplified*
  - Increase the number of primer pairs returned to 10 — *sometimes, the first five primer pairs are not sufficiently different to allow two distinct pairs to be selected*
3. Pick the top-scoring primer pair and BLAST the sequence (fwd\_primer NNNN rev\_primer) against the appropriate genome. The only perfect matches should be the mRNA and the two genomic sequences from the reference and alternative assemblies.
  - *This BLAST search will identify pseudogenes or other homologous regions that could confound results*
4. Repeat Step #3 with the next best-scoring primer that is substantially different from the first one.
  - *One primer pair will often work substantially better than the other, so it is cheap enough to order two pairs at the same time*
5. Order both pairs of primers at the minimum synthesis scale with standard desalting and dissolve in H<sub>2</sub>O at 50 µM.
  - *Use barrier tips to avoid contaminating the primer stocks with PCR amplicons*
6. Check primer pairs by RT-qPCR (see below) with a blank sample (to check for primer dimers), a no RT sample (to check for pseudogenes or genomic DNA), and a first-strand cDNA sample (to check for amplification).
7. Good primers show a strong exponential amplification that appears at least five cycles before the blank sample (ideally, the blank should not amplify at all) and also give a single peak by melt-curve analysis that is clearly separable from any low T<sub>m</sub> peaks in the blank or no RT controls.
  - *Primers that give high levels of primer dimers in the blank sample can often be salvaged by decreasing the primer concentration twofold in the RT-qPCR reaction*
8. Save the completed RT-qPCR reaction from the cDNA sample (referred to as the “primer amplicon” hereafter) of each candidate primer pair to check for quantitative accuracy of the primer pair and to use for a standard curve with unknown samples if validated.
9. Dilute 1 µl of the primer amplicon in 449 µl of H<sub>2</sub>O to form a 1× stock. Serially dilute the 1× stock 10<sup>2</sup>-, 10<sup>3</sup>-, 10<sup>4</sup>-, 10<sup>5</sup>-, 10<sup>6</sup>-, and 10<sup>7</sup>-fold in H<sub>2</sub>O to assemble a six-log standard curve of each primer amplicon.
  - *It is essential to use barrier tips for all liquid-handling steps involved in RT-qPCR; after an amplification is done the first time, the amplicon becomes a contaminant that will ruin future amplifications*
  - *Change tips after each tenfold serial dilution, because carryover on the tips will hurt the linearity of the standard curve*
  - *When mixing the different serial dilutions, be sure not to vortex the tubes so vigorously that the liquid hits the cap of the tube; a “wet” cap (even after centrifugation) will contaminate the entire plate with amplicon when it is opened*

10. Analyze the blank sample, the amplicon dilution series, and the no RT control by RT-qPCR (see below).
11. Good primers should have amplification slopes in the range of  $-2.9$  to  $-3.9$ , corresponding to amplification efficiencies of 80–120%.
  - *Primers with exceedingly “high” efficiencies have competing reactions taking place during RT-qPCR that flatten the amplification slope; often, these primers can be salvaged by decreasing the primer concentration twofold in the RT-qPCR reaction*
  - *Primers with exceedingly low efficiencies are not effectively priming the elongation step during annealing; often, these primers can be salvaged by increasing the primer concentration twofold in the RT-qPCR reaction*
12. Validated primers can now be used to quantify relative gene-expression changes in unknown samples.

## II. RT-qPCR on the CFX96 instrument

1. Dilute 1  $\mu\text{l}$  of the blank sample, the no RT sample, and the unknown cDNA samples in 44  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . For each gene, prepare a fresh amplicon dilution series as described in Step #9 above.
  - *It is essential to use barrier tips for all liquid-handling steps involved in RT-qPCR; after an amplification is done the first time, the amplicon becomes a contaminant that will ruin future amplifications*
  - *P2 barrier tips do not work well, so just pipet very carefully with regular tips to avoid aerosol generation*
  - *Primer amplicons are stable in their concentrated forms at  $-20^\circ\text{C}$  for at least one year; dilutions should be prepared fresh each day*
  - *For small-sample cDNA amplifications, 1  $\mu\text{l}$  of the cDNA samples should be diluted in 449  $\mu\text{l}$   $\text{H}_2\text{O}$*
  - *Dilute cDNA samples can be stored for several days at  $4^\circ\text{C}$  if a series of genes is being analyzed*
2. Add 4.5  $\mu\text{l}$  of each sample (blank, dilution series, no RT, and unknowns) to the base of Hard-Shell 96well PCR plate (Biorad #HSP9601) or strip tube (Biorad #TLS0801) on ice.
  - *To reduce cost, the RT-qPCR reaction does not use a "hot-start" Taq polymerase, so the reaction must be assembled on ice to avoid background mispriming and elongation of the Taq that would occur at room temperature*
3. Add 3  $\mu\text{l}$  of primer master mix to the side of the wells on ice. Tap the plate gently on the lab bench to mix the primer into the sample.
  - *Keeping the samples and primers physically separated enables you to use one barrier tip to dispense the primer to the samples in the different wells*
4. Add 7.5  $\mu\text{l}$  of 2 $\times$  RT-qPCR master mix to the side of the wells on ice. Tap the plate gently on the lab bench to mix the primer into the sample-primer mix. • *Note that this dilutes the RT-qPCR master mix to 1 $\times$* 
  - *It is not necessary to mix the individual reactions by pipetting*
5. Seal the Hard-Shell plate completely with optically clear Microseal 'B' film (Biorad #MSB1001) or the strip tube with Flat 8-Cap Strips (Biorad #TCS0803)
  - *Pay particularly close attention to the edges and corners of the 96-well plate; the reaction will evaporate if not completely sealed and give artifactual results*
6. Run the following cycling program on the CFX96 instrument (105 $^\circ\text{C}$  hotlid)
  - 95 $^\circ\text{C}$  denaturation, 1.5 min
  - 40 cycles of 95 $^\circ\text{C}$  denaturation (10 sec), 60 $^\circ\text{C}$  annealing (10 sec), 72 $^\circ\text{C}$  elongation (12 sec) with a fluorescence read at the end of the elongation
  - 65 $^\circ\text{C}$   $\rightarrow$  95 $^\circ\text{C}$  touchup in 0.5 $^\circ\text{C}$  increments with a fluorescence read after each increment (meltcurve analysis)
7. Compressed .zpcr files can then be imported into the CFX96 reader software for analysis

**Buffer recipes**

- **2× RT-qPCR master mix** (store at –20°C in 788 µl aliquots) (revised 1/13/22-Piotr Przanowski)

	1X	X63 (50ml tube)	X250
100 mM Tris-HCl pH8.3, 500 mM KCl	160 ul	10080	40000
100 mM MgCl <sub>2</sub>	64 ul	4032	16000
10 mM dNTP	32 ul	2016	8000
10 mg/ml BCA	24 ul	1512	6000
50% Glycerol	160 ul	10080	40000
Nuclease-free H <sub>2</sub> O	348 ul	21924	87000
Total Volume (ul)	788	49644	197000

After thawing, add 8µl 5U/µl Taq polymerase (NEB M0267X) and 4 µl 100× SYBR Green (Invitrogen #S7563 diluted 100-fold in DMSO to 100X) Store thawed 2X mix at 4°C for 1–2 weeks

We typically make 50 aliquots at a time.

- **Primer master mix** Prepare on ice
  - 0.2 µl 50 µM forward primer (10 pmol)
  - 0.2 µl 50 µM reverse primer (10 pmol)
  - 2.6 µl H<sub>2</sub>O

The amount of forward and reverse primer should be balanced but can range from 0.1–0.4 µl (5–20 pmol) per 3 µl depending on the efficiency of the amplification and the existence of primer dimers.