Helpful suggestions and top considerations for good qPCR

1.) RNA quality/integrity assessments, e.g. achieving an "RNA Integrity Number" (RIN) of 7 and above for all RNA samples (e.g. using an Agilent Bioanalyzer 2100) is an absolute necessity preceding downstream activities including reverse transcription and subsequent qPCR using the resulting cDNA. See http://dna.biotech.iastate.edu/nextgen.html for more info on required RNA and DNA quality and quantity assessments.

2.) Splice variants exist for >60% of all known mRNA transcripts in most species, so, during primer-probe design, it is of utmost importance to acknowledge this. Know which splice variant(s) you are targeting with each of your primer-probe designs (or target them all at the same time). Check all designs against all available data bases including BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat). In the event that splice variants are a non-issue, pseudo-genes and other homologous sequences must be screened for as well in order to be certain that chosen primer-probe(s) are not inadvertently sequestered away from the intended final target PCR/qPCR reactions - rendering them unacceptably less efficient.

3.) Choice of Reverse Transcriptase enzyme for cDNA synthesis is a major decision as some RT enzymes are very poor (e.g. Multiscribe™ RT) at yielding plentiful and full-length cDNAs. Suggested RTases are: Quanta BioScience' QuantaScript™, Invitrogen's SuperScript III™ and Takara's PrimeScript™ for first strand synthesis (e.g. for Two-Step qPCR).

4.) Ample dilution of RNA samples beyond their RT-inhibitory range, and dilution from there, to the same ng/µl preceding their addition to RT reactions is an essential practice for generating cDNA intended for subsequent use in qPCR.

5.) Priming of RT reactions. A mixture of oligo d[T] and random hexamers, octamers or nonamers has shown to be the most effective, although sole use of random pentadecamers has been lauded as even more effective by some. Oligo d[T] priming is not suggested for RNA extracted from formalin-fixed tissue, as many RNAs lose their poly A tails after such tissue treatment. If oligo d[T] priming is used, 3'-bias is a concern, therefore, primer-probes should be designed to target sequences nearer the 3' end rather than the 5' end of the known mRNA transcripts. 18S rRNA and histones mRNAs do not contain poly A tails from the start, so oligo d[T] priming is not appropriate for those targets – although mis-priming by some RT enzymes can yield varying amounts of 18S rRNA cDNA when reverse transcription is carried out using oligo d[T] for first-strand priming.

6.) Using the "PREXCEL-Q Method" is suggested for all qPCR approaches. All dilution-related complications of qPCR are dealt with using this tool. And this tool has been endorsed by some of the world's foremost qPCR experts (Bustin, Vandesompele).

7.) Removal of inhibitors from DNA samples (e.g. using MO Bio Labs reagents) is highly suggested.