

Quantitative Real Time PCR workflow

3. Reverse Transcription

Synthesis of cDNA from purified poly(A)+ or total RNA is performed by the action of a reverse transcriptase, typically isolated from retrovirus. The reverse transcriptase has three biochemical activities: as a RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase and ribonuclease H. Many commercially available kits, such as Super Script® III (Invitrogen/Life Technologies) have specifically engineered enzymes that possess reduced RNase H activity and provide increased thermal stability. The enzyme is used to synthesize cDNA at a temperature range of 42–55°C, providing increased specificity, higher yields of cDNA, and more full-length product (potentially greater abundance of your target gene). Because SuperScript® III RT is not significantly inhibited by ribosomal and transfer RNA, it may be used to synthesize first-strand cDNA from a total RNA preparation.

It is important to perform the reverse transcription of total RNA samples immediately following Bioanalysis to avoid exposure to conditions potentially compromising the integrity of the RNA (freeze/thaw, inconsistent storage, RNase contamination). RNA transcripts from 100 bp to >12 kb can typically be detected although the efficiency of transcription of larger genes is greatly reduced if priming solely with an oligo(dT) primer (the RT primer binding to the poly(A) tail of mRNA). Thus, to ensure maximal efficiency of coverage, an oligo(dT) primer should be used in combination with a random hexamer (that will prime at random locations in the target mRNA). The amount of starting material can vary from 1 pg–5 µg of total RNA and it is **recommended that equal amounts of RNA between treatment groups/experimental animals be used in the RT reaction** (with quantitation from the **Bioanalyzer**) for accuracy and minimizing the variability in downstream qPCR results.

The reverse transcription reaction mix should include the primer mix (oligo(dT) and random hexamer), the template (in equal concentration across biological replicates), RNase H (to degrade the RNA from the RNA/cDNA complex), dNTPs, DTT (to minimize secondary structure and maximize RT efficiency), buffer, MgCl₂ and a high efficiency reverse transcriptase. Most commercially available kits will contain all these, in addition to positive controls. It is also recommended that you have a “no RT” control in your experimental design (i.e. all the ingredients of an RT reaction, including your RNA template but without the reverse transcriptase) so you can include this sample in your qPCR reaction to control for possible genomic contamination (for further discussion see qPCR set up). Following RT, your cDNA samples can be stored at -20°C or -80°C.