3.3. cDNA synthesis

Before pyrosequencing can be used to quantify RNA abundance, the RNA must be converted into (single- or double-stranded) cDNA. Any cDNA synthesis protocol can be used for this purpose. Furthermore, "poly T" primer, which will allow cDNA to be synthesized from all polyadenylated mRNA, or one or more gene-specific primers can be used depending on the number of genes that will be examined. A DNAse treatment of the RNA template immediately prior to cDNA synthesis is strongly recommended to remove any genomic DNA that survived the DNAse treatment administered during RNA extraction. The protocol that my laboratory typically uses to synthesize cDNA from total RNA extracted from *Drosophila melanogaster* follows:

1. Combine 8.0 µl (~1.5 µg) total RNA extracted as described in section **3.2**, 8.4 µl 5x First-Strand Buffer, and 2.0 µl DNAse. Incubate at 37°C for 1 hour.
2. Heat to 65°C for 15 minutes to inactivate the DNAse.
3. Add 5.4 µl (500 µg / ml) primer for reverse transcription and slowly cool to 37°C over 10 minutes.
4. Add 4.2 µl dNTP mix (10mM per nucleotide), 1 µl RNAsin and 1.0 µl SSII-RT and incubate at 37°C for 1 hour.
5. Dilute as appropriate. We typically add 69.8 µl of nuclease free water to dilute the 30.2 µl cDNA synthesis reaction to 100 µl total. For most genes, 1 - 2 µl of this diluted single-stranded cDNA is sufficient to produce a strong PCR product suitable for pyrosequencing (see below). For lowly expressed genes, increasing the amount of cDNA used may help to achieve a strong PCR product.