determine yields of control and target template by scanning ethidium bromide-stained gels using a suitable densitometer, or by using computer imaging of the Polaroid film. Plot the increase of the log of the yields of both templates in each tube during the PCR. Equal amplification efficiencies will generate graph lines of the same gradient, and identical product yields, if identical amounts of template were originally added.

3.5.3. Construction of Control RNA Template

1. Following a similar procedure to **steps 1–3** in **Subheading 3.5.2.**, incorporate a RNA polymerase recognition sequence, such as 5'-AATTTAATACGACTCA-CTATAGGGAT-3', for T7 polymerase binding and transcription initiation *(11)* at the 5' end of the forward primer, and carry out in vitro transcription.
2. Perform in vitro transcription in a 50-μL reaction vol using 25 μL of above template prepared with modified primers, and 25 μL transcription reaction mix, according to manufacturer's instructions. For T7 polymerase (Promega), the final reaction buffer should be 40 m*M* Tris-HCl, pH 7.9, 6 m*M* MgCl$_2$, 2 m*M* spermidine, 10 m*M* NaCl, 10 m*M* DTT, 0.5 m*M* of each ribonucleotide (e.g., Promega P1221), 10 U of T7 RNA polymerase, and 20 U of RNasin (e.g., Promega N2511). Incubate at 37ºC for 2 h.
3. Quantitate yield of RNA product by measuring the OD$_{260}$ of an aliquot, and using the approximate conversion factor of 1 OD$_{260}$ unit represents 40 μg of RNA/mL.

For an alternative method, *see* **Note 20**.

3.5.4. Competitive (RT-)PCR

1. Estimate the approximate range of target template present in the sample of interest. For each sample, carry out replicate (about five) extractions, to which a series of known amounts of competitor RNA or DNA template, covering the estimated range of target template, have been added.
2. Perform (RT-)PCR on all samples, and electrophorese 10–15 μL of PCR product on an agarose gel, as described in **Subheading 3.4.1.** Measure the yields of target and competitor products.
3. Plot the yields of target and competitor products for each PCR reaction in the dilution series, after correcting the values to allow for the size difference of the products. Extrapolate from the graph the amount of competitor DNA that gives rise to an equal molar amount of product to that of the target. Depending on the accuracy required, a couple of runs may be necessary to determine the range of competitor template to add to exactly quantify the copies of template.

3.6. Internal Controls

An alternative to the optimized controls discussed in **Subheading 3.5.** is to use an endogeneous gene transcript as a control for RT-PCR. This is especially useful when PCR is being used to check the presence or absence of viral sequences, because all that is needed is a second set of primers which give a