3. Methods

3.1. In Vitro Transcription

Once the gene of interest has been cloned into the appropriate vector, template DNA must be linearized at a restriction site downstream from the insert that is to be transcribed. After digestion, all traces of RNases and other proteins should be removed. This is achieved by extracting with phenol and chloroform, and precipitating with ethanol. Treatment with proteinase K prior to phenol extraction is optional (*see* **Note 4**). The DNA pellet obtained is resuspended in DEPC-treated water and generally a small sample is checked on an agarose gel to ensure digestion has been successful.

Detailed instructions regarding assembly of the in vitro transcription reaction are supplied with the various kits that are available, but a summary will be provided here.

1. The following components are mixed in a 1.5-mL microcentrifuge tube: 1–2 μg of linearized DNA; 2 μL of RNA transcription buffer (10X); 20–40 U RNase inhibitor; 1 μL each of rATP, rCTP, rGTP, rUTP (10 m*M* stock); 1–10 U RNA polymerase (T7, T3, SP6) (*see* **Note 5**); DEPC-treated water to a final volume of 20 μL.
2. Reactions are incubated at 37°C for 30–60 min.
3. DNA template is removed by adding 2 U of RNase free DNase and incubating at 37°C for a further 15 min.
4. Transcripts are then purified by phenol:chloroform extraction and ethanol precipitation.
5. Purified RNA can be quantified spectrophotomically or visually on an ethidium bromide-stained agarose gel. mRNA molecules produced may now be used in translation reactions.

Capping increases the stability of a transcript molecule. Capped transcripts can be readily synthesized in vitro by the addition of cap analog to the transcription reaction. The level of normal GTP in the reaction is reduced to a concentration of 50 μ*M*, and cap analog (m7G[5']ppp[5']G) is added at a concentration of 10 m*M*. The reaction is carried out according to **Subheading 3.1.**

3.2. In Vitro Translation

Translation reactions are carried out according to manufacturer's instructions provided with RRL or WGL. Transcript should be heated to 65°C before use, to increase the efficiency of translation by removing secondary structure. Components of the reaction are assembled in a 1.5-mL microcentrifuge tube. Rabbit reticulocyte lysate reactions are incubated at 30°C, and wheat-germ lysate reactions at 25°C, for 60 min. Results may be analyzed by incorporation assays (**Subheadings 2.3.** and **3.3.**) or by SDS-PAGE (**Subheadings 2.4.** and **3.4.**).