Chapter 1

Preparation of Total RNA

Introduction

The purity and integrity of isolated RNA are essential prerequisites for a successful analysis of differential gene expression by DDRT-PCR. Thus extreme care should be taken during all the steps of RNA preparation.

Regardless of the procedure used, isolation of intact and pure RNA requires four important steps:

- Effective disruption of cells or tissue
- Inactivation of endogenous ribonucleases (RNase) activity
- Denaturation of nucleoprotein complexes
- Purification of RNA from contaminating DNA and proteins

The most crucial step is the immediate inactivation of endogenous cellular RNase, which is released from membrane-bound organelles upon cell disruption. Samples should be immediately processed or quickly frozen or stored in the presence of RNase inhibitors to avoid intracellular RNA degradation. Even minimal degradation of RNA samples will result, after PCR amplification, in a considerable number of false-positive bands on denaturing gel.

The majority of RNA purification procedures makes use of the most potent known inhibitors of RNase, i.e., guanidine thiocyanate and β-mercaptoethanol (Chirgwin et al. 1979). In addition, all purification steps must be performed on ice to further avoid RNA degradation.

However, RNA samples can be accidentally contaminated with RNase from external sources. Thus special care should be taken to avoid undesirable RNase contamination during or after RNA isolation if the starting material was obtained with difficulty and may not be easily purified.
Note

- Two of the most common exogenous sources of RNase contamination are user’s hands, bacteria, and moulds present in dust particles. To prevent any of these forms of contamination, a proper microbiologically sterile technique should be applied when handling reagents and solutions and, whenever possible, sterile and disposable plastic should be used.

- Glassware should be baked at 220°C for at least 6 h. Plastic should be soaked with 3 % H₂O₂ or 0.1 N NaOH, 1 mM ethylenediaminetetraacetate (EDTA) followed by rinsing with RNase-free water.

- Unless otherwise stated, all solutions, including distilled water, should be treated with 0.1 % diethyl pyrocarbonate (DEPC) for at least 12 h at 37°C and then autoclaved for 30 min to remove any trace of DEPC.

- Disposable plastic gloves should be worn during all the time during handling, because active RNases are present on the skin.

Once extracted, total RNA samples should be quantitated by absorbance at 260 nm, and absence of degradation should be evaluated by visualizing ribosomal RNAs (rRNAs) using ethidium bromide staining on denaturing or nondenaturing agarose gels. Intact total RNA should be stored either as water or EtOH suspension at −80°C in small aliquots to avoid repeated freezing and thawing. Moreover, although polyadenylated (poly-A⁺)-mRNAs can be easily isolated from total RNA by traditional methods (Liang and Pardee 1995), this step is not necessary or desirable for DDRT-PCR. In fact, potential oligo-dT contamination may cause smearing on DNA display gel, even when magnetic beads have been used for purification. In addition, degradation of poly-A⁺ RNA is difficult to check by agarose gel.

Basically, any kind of procedure can be used to prepare total RNA to be processed in DDRT-PCR, provided that it is reliable method of isolating intact and pure RNA.

In the following, different protocols of RNA purification from different cell sources are reported, based on phenol or guanidine thiocyanate extractions. Readers should refer to any other available protocol for specific requirements.