

Isolation of RNA

Protocols are provided for preparing labeled cRNA from either total RNA or purified poly-A mRNA. We have found that results obtained from samples prepared by both of these methods are similar, but not identical. Therefore, to get the best results we suggest only comparing samples prepared using the same type of RNA material.

Please review precautions and interfering conditions in Section 1.

IMPORTANT

The quality of the RNA is essential to the overall success of the analysis. Since the most appropriate protocol for the isolation of RNA can be source dependent, we recommend using a protocol that has been established for the tissues or cells being used. In the absence of an established protocol, we suggest using one of the commercially available kits designed for RNA isolation.

When using a commercial kit, follow the manufacturer's instructions for RNA isolation.

Isolation of RNA from Yeast

Total RNA

We have successfully isolated good quality total RNA from yeast cells using a hot phenol protocol described by Schmitt, *et al. Nucl Acids Res*, **18**:3091-3092 (1990).

Poly-A mRNA

Affymetrix recommends first purifying total RNA from yeast cells before isolating poly-A mRNA from total RNA. Good-quality mRNA has been successfully isolated from total RNA using QIAGEN's Oligotex mRNA Kit. A single round of poly-A mRNA selection provides mRNA of sufficient purity and yield to use as a template for cDNA synthesis. Two rounds of poly-A mRNA selection will result in significantly reduced yield and is not generally recommended.

Isolation of RNA from Arabidopsis

Total RNA

We have been using TRIzol Reagent from Invitrogen Life Technologies to isolate total RNA from Arabidopsis. Please follow the instructions provided by the supplier and, when necessary, use the steps outlined specifically for samples with high starch and/or high lipid content.

Poly-A mRNA

We have successfully isolated Arabidopsis poly-A mRNA using QIAGEN Oligotex products. However, other standard isolation products are likely to be adequate.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

We have successfully isolated high-quality total RNA from mammalian **cells** (such as cultured cells and lymphocytes) using the RNeasy Mini Kit from QIAGEN.

If mammalian **tissue** is used as the source of RNA, we recommend isolating total RNA with a commercial reagent, such as TRIzol.

IMPORTANT

If going directly from TRIzol-isolated total RNA to cDNA synthesis, it may be beneficial to perform a second cleanup on the total RNA before starting. After the ethanol precipitation step in the TRIzol extraction procedure, perform a cleanup using QIAGEN RNeasy Mini Kit. Much better yields of labeled cRNA are obtained from the in vitro transcription-labeling reaction when this second cleanup is performed.

Poly-A mRNA

Good-quality mRNA has been successfully isolated from mammalian **cells** (such as cultured cells and lymphocytes) using QIAGEN's Oligotex Direct mRNA kit and from total RNA using the Oligotex mRNA kit. If mammalian **tissue** is used as the source of mRNA, total RNA should be first purified using a commercial reagent, such as TRIzol, and then using a poly-A mRNA isolation procedure or a commercial kit.

Precipitation of RNA

Total RNA

It is not necessary to precipitate total RNA following isolation or cleanup with RNeasy Mini Kit. Adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment. Ethanol precipitation is required following TRIzol isolation and hot phenol extraction methods; see methods on page 2.1.9.

✓ Note

Affymetrix recommends starting the cDNA synthesis protocol with a minimum of 0.2 µg poly-A mRNA at a minimum concentration of 0.02 µg/µL, or 5 µg of total RNA at a minimum concentration of 0.5 µg/µL, in order to obtain sufficient quantity of labeled cRNA for target assessment and hybridization to GeneChip expression probe arrays. There are two major advantages to starting with at least the recommended amount of material:

- 1. Enough material to check sample yield and quality at the various steps of this protocol.*
- 2. Production of enough cRNA for hybridization of the target to multiple probe arrays.*

For smaller amounts of starting material, please refer to the alternative protocol for target labeling described in *Small Sample Target Labeling Assay Version II*, available at www.affymetrix.com.

Poly-A mRNA

Most poly-A mRNA isolation procedures will result in dilution of RNA. It is necessary to concentrate mRNA prior to the cDNA synthesis.

Precipitation Procedure

1. Add 1/10 volume 3M NaOAc, pH 5.2, and 2.5 volumes ethanol.*
2. Mix and incubate at -20°C for at least 1 hour.
3. Centrifuge at $\geq 12,000 \times g$ in a microcentrifuge for 20 minutes at 4°C.
4. Wash pellet twice with 80% ethanol.
5. Air dry pellet. Check for dryness before proceeding.
6. Resuspend pellet in DEPC-treated H₂O. The appropriate volume for resuspension depends on the expected yield and the amount of RNA required for the cDNA synthesis. Please read ahead to the cDNA synthesis protocol in order to determine the appropriate resuspension volume at this step.

***Addition of Carrier to Ethanol Precipitations**

Adding carrier material has been shown to improve the RNA yield of precipitation reactions.

■ Pellet Paint

Addition of 0.5 μL of Pellet Paint per tube to nucleic acid precipitations makes the nucleic acid pellet easier to visualize and helps reduce the chance of losing the pellet during washing steps. The pellet paint does not appear to affect the outcome of subsequent steps in this protocol; however, it can contribute to the absorbance at 260 nm when quantifying the mRNA.

■ Glycogen

Addition of 0.5 to 1 μL of glycogen (5 mg/mL) to nucleic acid precipitations aids in visualization of the pellet and may increase recovery. The glycogen does not appear to affect the outcome of subsequent steps in this protocol.

Quantification of RNA

Quantify RNA yield by spectrophotometric analysis using the convention that 1 absorbance unit at 260 nm equals 40 μg RNA per mL.

- The absorbance should be checked at 260 and 280 nm for determination of sample concentration and purity.
- The A_{260}/A_{280} ratio should be close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).