

Eukaryotic Target Preparation *from Affymetrix*

Reagents and Materials Required

Isolation of RNA

- Isolation of RNA From Yeast
- Isolation of RNA from Arabidopsis
- Isolation of RNA from Mammalian Cells or Tissues
- Precipitation of RNA
- Quantitation of RNA

Reagents and Materials Required

The following reagents and materials are recommendations and have been tested and evaluated by Affymetrix scientists. For supplier phone numbers in the U.S. and Europe, please refer to the Supplier and Reagent Reference List, Appendix A of this manual. Information and part numbers listed are based on U.S. catalog information. Additional reagents needed for the complete analysis are listed in the appropriate chapters. Appendix A contains a master list of all reagents used in this manual.

Do not store enzymes in a frost-free freezer.

Total RNA Isolation

- TRIzol Reagent, Invitrogen Life Technologies,P/N 15596-018
- RNeasy Mini Kit, QIAGEN,P/N 74104

Poly (A) + mRNA Isolation

- Oligotex Direct mRNA Kit (isolation of mRNA from whole cells),QIAGEN,P/N 72012, 72022,or 72041
- Oligotex mRNA Kit (isolation of mRNA from total RNA),QIAGEN,P/N 70022,70042,or 70061
- Qiashredder, QIAGEN,P/N 79654 (Required only for use with QIAGEN Oligotex Direct Kit)
- DEPC-Treated Water, Ambion,P/N 9920

Miscellaneous Reagents

- 7.5 M Ammonium Acetate (NH₄ OAc), Sigma-Aldrich,P/N A2706
- Absolute ethanol (stored at -20 ° C)
- 80%ethanol (stored at -20 ° C)
- SYBR Green II,FMC Bioproducts,P/N 50523;or Molecular Probes,P/N S7586 (optional)
- Water,Molecular Biology Grade,BioWhittaker,P/N 16-001Y
- Pellet Paint,Novagen,P/N 69049-3 (optional)
- Glycogen,Ambion,P/N 9510 (optional)
- 3 M Sodium Acetate (NaOAc),Sigma-Aldrich,P/N S7899
- Ethidium Bromide,Sigma-Aldrich,P/N E8751
- NaOH (1 N)
- HCl (1 N)
- 50 mM MgCl₂
- 0.5 M EDTA

Miscellaneous Supplies

- Sterile, RNase-free, microcentrifuge tubes, 1.5 mL, USA Scientific, P/N 1415-2600 (or equivalent)
- Micropipettors, (P-2, P-20, P-200, P-1000), Rainin Pipetman or equivalent
- Sterile-barrier, RNase-free pipette tips (Tips must be pointed, not rounded, for efficient use with the probe arrays)
- Mini agarose gel electrophoresis unit with appropriate buffers
- Vacuum filter units (1 liter capacity, 0.22 μm or 0.45 μm), VWR Scientific Products, P/N 28199-730
- UV spectrophotometer
- Heatblock
- Cooling waterbath

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.

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.* (1990) *Nucl Acids Res*, **18**:3091-3092.

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 yields of yeast mRNA 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) + RNA using QIAGEN Oligotex product. However, other standard isolation products are likely to be adequate.

Isolation of RNA from Mammalian Cells or Tissues

Total RNA

We have successfully isolated good quality total RNA from mammalian **cells** (such as cultured cells and lymphocytes) using QIAGEN's RNeasy Total RNA Isolation kit. If mammalian **tissue** is used as the source of RNA, we recommend isolating total RNA with a commercial reagent such as TRIzol.

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 Total RNA isolation 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 Total RNA Isolation kit. Please adjust elution volumes from the RNeasy column to prepare for cDNA synthesis based upon expected RNA yields from your experiment.

Affymetrix recommends starting the cDNA synthesis protocol with a minimum of 0.2 µg poly(A)⁺ RNA 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.*

Ethanol precipitation is required following TRIzol isolation and hot phenol extraction methods; see methods below.

Poly(A) + mRNA

Most poly(A) + mRNA isolation procedures will result in dilute solutions of RNA. It is necessary to concentrate mRNA prior to the cDNA synthesis.

Precipitation Procedure

1. Add 1/10 volume 3 M 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 H_2O . 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

Affymetrix has found that adding 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.

Quantitation 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).