TOTAL RNA ISOLATION: Quantify Gene Expression Level using Real-Time PCR method

Development of PCR technique to amplify DNA has greatly accelerated the rate of genetic discovery in the area of molecular biology. Reverse Transcriptase (RT)-based PCR method (in class discussion) evolved for a need to measure differences in gene expression between samples. The RT-based PCR method has proven to be more sensitive and efficient than the conventional Northern and in-situ hybridization techniques to quantitate gene expression level in samples. However, several inherent limitations in RT-based PCR have left this method to be used for only semi-quantitative measure in gene-expression differences. In this lab, we will use Real-Time PCR technique that will help us in quantifying differences in transcript amplifications in 'Real Time'. The lab will entail an elaborate lecture.

Flow Chart:

Tissue→ Isolate total RNA (includes mRNA) → Synthesize cDNA from the mRNA→ Real Time PCR with Gene specific and house-keeping gene specific primers → results analysis to discern fold differences (if any) in the gene expressions within the assayed samples.

Over the last several years, the development of novel chemistries and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time RT-PCR as the method of choice for quantitating changes in gene expression. We will briefly discuss the real-time RT-PCR method in class.

During RNA isolation care should be taken as RNA is both chemically and biologically far more labile than DNA. From a practical viewpoint the extreme sensitivity of RNA to RNases and the wide prevalence and stability of these enzymes means that in order to prepare intact molecules of RNA (particularly large RNA), one must pay careful attention to eliminating RNases.

ISOLATION OF TOTAL RNA USING TRIZOL

SOURCE: GIBCO/BRL/INVITOGEN TRIZOL TECHNICAL MANUAL.

INTRODUCTION:

TRIzol (Gibco/BRL) is an acid phenol extraction reagent containing detergents and guandine thiocyanate to denature RNAses (see Chomczynski, BioTechniques 15:532-537). The technique is quick and gives good yields of high quality RNA.

1.

A. Tissues: Your TA will use the liquid nitrogen to grind leaf tissues from WT and mutant plants. You will be given a centrifuge tube with 1 ml of TRIzol Reagent (use caution handling the reagent). Add ~50 mg of ground tissue powder to the TRIzol reagent and mix well.

- To remove insoluble material from the homogenate, centrifuge the mix at full speed (~13000 RPM) 10 minutes at 4°C.
- **transfer the cleared homogenate solution** to a fresh tube and proceed with chloroform addition and phase separation as described.

Phase separation

2. Incubate the homogenized samples or lysed tissue culture cells for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

3. Add 0.2 ml of chloroform per 1 ml of TRIzol Reagent.

4. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds

5. Incubate tubes at room temperature for 2 to 3 minutes.

6. Centrifuge the samples at no more than 12,000 x g (14K in centrifuge) for 15 minutes at 4°C.

7. Following centrifugation, the mixture separates into a lower, red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol Reagent used for homogenization.

RNA Precipitation

8. Transfer the aqueous phase to a fresh tube.

9 Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIzol Reagent used for the initial homogenization. Once you add the isopropanol, you may or may not see a cloudy suspension of RNA. Do not panic if you fail to see the cloudy suspension, just keep going. Incubate samples at room temperature for 10 minutes and centrifuge at no more than 12,000 x g (14K in microfuge) for 10 minutes at 4°C. The RNA precipitate, forms a pellet on the side and bottom of the tube.

RNA Wash

10. Remove the supernatant.

11. Wash the RNA pellet once with 75% RNAse-free ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol Reagent used for the initial homogenization.

12. Mix the sample by vortexing and centrifuge at no more than 7,500 x g (~8 K in microfuge) for 5 minutes at 4°C.

Redissolving the RNA

13. At the end of the procedure, briefly air dry the RNA pellet for 5-10 minutes. Dissolve RNA in RNAse-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55°C - 60°C.

Proceed to RT-PCR reactions:
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

2X RT buffer 5 µl
RT Enzyme mix 1 ul
RNA (10 ng) 2 ul from 5 ng/ul stock
DEPC-water to 10 ul

At 25C for 10 min
At 42C for 50 min
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C.
8. cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.

Proceed to set up the Real Time PCR mix:

Platinum® Quantitative PCR SuperMix 12.5 µl
Forward primer, 30 pmol (3 µl)
Reverse primer, 30 pmol (3 µl)
cDNA 5 µl (From step 8)
Water to 25 µl

PCR Machine Cycling parameters:

Standard Cycling Program
50°C for 2 minutes hold (UDG incubation)
95°C for 2 minutes hold
40 cycles of:
95°C, 15 seconds
60°C, 30 seconds

Appropriate program will be added to develop a Melting Curve