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Estimation of Relative mRNA Content by Filter Hybridization to a Polyuridylic Probe

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Accurate measurement of changes in the expression of specific genes requires reliable normalization to total mRNA levels. While normalization to the level of expression of a specific "housekeeping" gene can give a reasonable estimate in some cases, expression of such housekeeping genes may not remain constant after exposure to stress and often varies between different cell types (2). In our experience, a more reliable approach has been the normalization of the relative polyadenylated (polyA) tract content of the compared samples. We have developed an

Table 1. Relative Hybridization of cDNA and polyU Probes to Cellular RNA Samples[a]

Sample[b]	RNA[c]	RNA Level with Given Probe[d] GAPD	GADD45	MDM2	polyU	Normalized to polyU[e] GAPD	GADD45	MDM2
Control	2.4 µg	1.99	2.13	1.76	2.19	0.91	0.97	0.80
Control	1.2 µg	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Irradiated	2.4 µg	1.60	35.2	14.4	1.77	0.90	19.9	8.15
Irradiated	1.2 µg	0.89	17.2	6.29	0.82	1.04	20.9	7.64

[a] Relative hybridization of treated samples was determined by quantitative RNA dot-blot hybridization using the RNAThink program.

[b] Whole cell RNA was prepared from untreated ML1 cells (designated “Control”) and cells harvested 4 h after 5 Gy γ-irradiation.

[c] Value represents the starting amount of RNA used in the first dot blot of each series; seven subsequent samples were reduced sequentially by 50% (twofold dilutions).

[d] Values represent relative expression uncorrected for the amount of starting RNA or poly A tract content.

[e] Relative hybridization was normalized to the relative poly A tract content for each sample by dividing the relative hybridization of the given cDNA probe by the relative hybridization of the polyU probe.

improved and more convenient method for determining the relative mRNA content of RNA samples that allows accurate quantitation of relative transcript abundance in different samples. This new method, involving polyuridylic acid (polyU) as a probe, updates a previous method developed in our laboratory (3) that used a polythymidylate (polyT) probe. We have found the polyU method to be less expensive, less time consuming and more specific.

The previously published polyT protocol involved the use of ^{35}S-thymidine 5′-(α-thio) triphosphate in a reverse transcriptase reaction. The amount of ^{35}S used was 250 µCi compared to only 10 µCi adenosine 5′-γ ^{32}P-triphosphate (Amersham Pharmacia Biotech, Piscataway, NJ, USA), which is needed for the polyU method of labeling, rendering the newer method less expensive. The amount of time required for the preparation of polyU probe is approximately half that required for the preparation of the polyT probe, making it a more efficient method as well. Finally, the ^{32}P end-labeling reaction for polyU is a more desirable method for labeling than the ^{35}S oligo dT- labeling reaction for polyT because ^{32}P offers higher specific activity than ^{35}S and the higher energy of ^{32}P gives improved sensitivity. The integrity of the labeled polyU probe is also maintained because, as the label decays, the probe degrades only at the 5′ end, while with the labeled polyT, degradation occurs throughout the probe.

The polyU used in this protocol is a synthetic RNA homopolymer made up of approximately 234 nucleotides (Amersham Pharmacia Biotech). It is 5′ end-labeled with adenosine 5′-γ ^{32}P-triphosphate and hybridized to whole cell RNA samples that have been immobilized on nylon membranes; the method also works well with polyA-selected RNA. PolyU is used as a probe because it hybridizes to the polyA tails of cellular transcripts, rather than to a specific transcript such as a housekeeping-gene mRNA. We have consistently found that normalization to polyA tract RNA gives reliable results in a wide variety of cellular conditions and in different cell types (2). Therefore, it can be used to standardize the amount of mRNA in RNA samples based on the polyA tract content of mRNA. The polyU method is a better method for standardizing the amount of mRNA in RNA samples than other methods, for example, relative hybridization to cDNA probes such as actin and β2-microglobulin because even housekeeping gene transcript levels can vary according to cell type and cell treatment (3).

Preparation of the polyU probe involved a phosphatase reaction followed by a kinase ^{32}P end-labeling reaction and purification. Here, we describe the simplified method for generating the polyU probe. PolyU was dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, USA) for 25 min at 37ºC. The reaction was stopped with 0.5 M EDTA and heated at greater than 90ºC for 10 min. PolyU was then phosphorylated with ^{32}P in a kinase (Promega) reaction for 30 min. This reaction was stopped by adding 20% SDS and 0.5 M EDTA, and the probe was purified from unincorporated nucleotides on a Centricon 30™ microconcentrator (Amicon, Danvers, MA, USA). In a typical reaction, the incorporation of ^{32}P into the probe was calculated to be between 10% and 40%. The probe was aliquoted and stored at -20ºC for up to 30 days without substantial loss of performance.

The polyU protocol was optimized by dot blotting whole cell RNA from RKO cells (1) onto Nytran® nylon filters (Schleicher & Schuell, Keene, NH, USA) using a Hybri-dot® blot manifold (Life Technologies, Rockville, MD, USA). To determine optimal hybridization with polyU, RNA was diluted in 50 mM phosphate buffer from 1.5 M stock solution of 0.75 M monobasic sodium

phosphate and 0.75 M dibasic heptahydrate sodium phosphate. Twofold serial dilutions of RNA (800–6.25 ng) were blotted (Figure 1). The concentration of ^{32}P-labeled polyU hybridized to the filters was 6.6 ng/mL. The amount of cold competitor polyU was varied to determine the optimal conditions for hybridization without reaching saturation. Amounts tested were 0, 75, 200 and 250 ng/mL. Filters were prehybridized for 10 min at 44ºC in a solution of 1% BSA, 7% SDS, 0.5 M phosphate buffer (0.25 M dibasic heptahydrate sodium phosphate and 0.25 M monobasic sodium phosphate) and 1 mM EDTA in a Hybridiser HB-1D oven (Techne, Princeton, NJ, USA). ^{32}P-labeled and cold polyU were then added, and filters were hybridized for 3 h at the same temperature. The filters were then washed in a solution of 0.5% BSA, 5% SDS, 40 mM phosphate buffer (0.20 mM dibasic heptahydrate sodium phosphate and 0.20 mM monobasic sodium phosphate) and 1 mM EDTA twice at room temperature for 10 min, followed by three washes in the same buffer at 44ºC for 10 min. Filters were then exposed overnight to a PhosphorImager® screen (Molecular Dynamics, Sunnyvale, CA, USA), then scanned on a PhosphorImager (Molecular Dynamics). As shown in Figure 1, it was determined that 75 ng/mL cold polyU was the most sensitive condition used without reaching saturation.

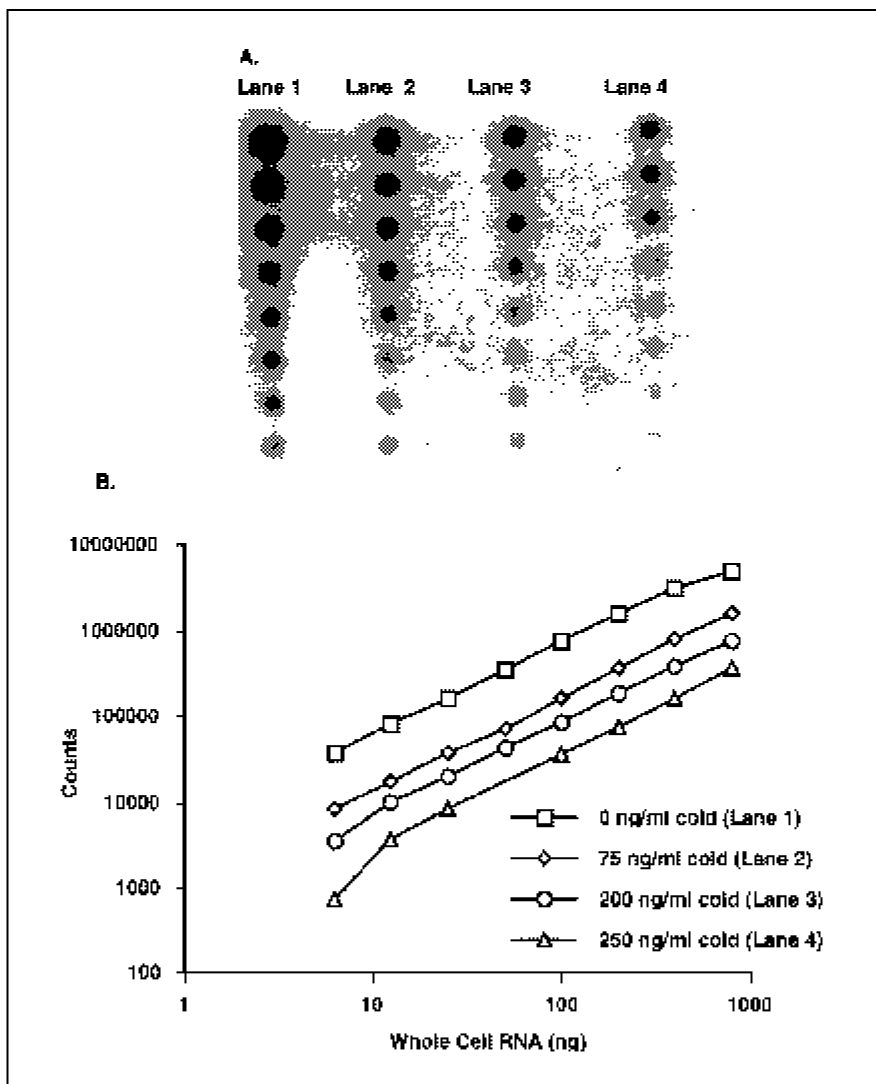

Figure 1. PolyU probe hybridization to cellular RNA. (A) For hybridization to polyU probe, whole cell RNA from RKO cells was blotted at 800, 400, 200, 100, 50, 25, 12.5 and 6.25 ng. All lanes were hybridized with 6.6 ng/mL ^{32}P-labeled polyU at 10^5 cpm/mL. Lane 1, 2, 3 and 4 were hybridized with 0, 75, 200 and 250 ng/mL cold polyU, respectively. (B) PhosphorImager counts for hybridization of ^{32}P-labeled polyU to RNA immobilized on the nylon filters in panel A.

Since filter hybridization follows only pseudo-first-order kinetics (4), a program has been written to make an accurate estimation of the relative mRNA levels between samples. In addition, the program facilitates higher throughput analysis compared to manual data-processing methods by automating much of the data analysis. The program, called RNAThink (http://rex.nci.nih.gov/RESEARCH/basic/lbc/fornace.htm) has been developed in Java and provides accurate estimates of the relative mRNA levels between samples. This program takes the PhosphorImager counts from different dilutions of the control RNA and creates a standard curve of hybridization versus the amount of RNA; individual values for each experimental sample (dot) are then compared to this standard curve, and an average is derived for the different dilutions of each experimental RNA sample. Results are equivalent to those obtained by RNase protection (2,5) and are in a tab-delimited format that can be easily exported to spreadsheet programs. The program can be compiled to run on most computer platforms and can be downloaded from http://rex.nci.nih.gov/RESEARCH/basic/lbc/fornace.htm.

To demonstrate the utility of our approach, RNA samples from untreated and stress-treated cells were analyzed in Table 1. Total RNA was prepared from control cells and cells 4 h after exposure to 5 Gy γ irradiation. The RNA was isolated as before and dot blotted onto filters in twofold serial dilutions. Eight dilutions of each RNA sample were blotted, the first four dilutions were hybridized to the cDNA probes and the last four dilutions to the polyU probe. To demonstrate how this approach can correct for RNA misloading or other variables in which the level of mRNA is not constant, duplicate samples were included when the amount of RNA employed was reduced by 50%. As shown in Table 1, normalization to polyU content corrected for loading differences between the duplicate samples. In the case of *GADD45* and *MDM2*, these genes were induced by

radiation, while expression of the housekeeping gene *GAPD* was not appreciably affected. This approach has given useful and consistent results in our laboratory in a wide variety of stress-gene expression experiments. In addition, it has been effective in comparing the expression of genes between different cell lines (Amundson et al., manuscript submitted) in which the expression of housekeeping genes often varied. In these studies, there was a good correlation between relative expression of specific transcripts and their expression at the protein level.

In an era when high-throughput expression screening techniques such as microarray analysis are becoming increasingly widespread, there is a pressing need for rapid and accurate methods of confirming the results of such studies. Our single-probe hybridization with normalization to polyU is one such technique. With the current RNA-Think program, up to 50 RNA samples can be analyzed in one run and ten to twenty different probes can be used in one day, allowing the analysis of hundreds of samples. Unlike techniques such as RNase protection and real-time PCR, no specialized probes need to be developed before a new gene can be analyzed with this technique. Finally, this single-probe hybridization technique is more sensitive and more quantitative than hybridizations using complex probes, such as those used in microarray analysis, thus providing an important complement to such high-throughput techniques.

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Reliable and Reproducible Method to Extract High-Quality RNA from Plant Tissues Rich in Secondary Metabolites

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The extraction of high-quality RNA for Northern blot and enzymatic reactions is an important prerequisite for studies of molecular responses to stress and developmental processes in trees. In the same way, ideally, the efforts to achieve high-quality RNA should not substantially restrict its yield. In most plants, this is not a major problem and reliable methods have been developed (1,17,18). Also, commercial kits are available for a range of applications. However, many plants, such as most gymnosperms and many woody angiosperm species, are rich in polyphenols, terpenes, carbohydrates and other secondary metabolites that severely restrict the use of common methods developed for RNA extraction. The available RNA extraction kits are also of limited use.

The need for a method to extract RNA from these particular species is evident and is mirrored in several papers published on tree molecular biology (2–6,10–16,19). However, none of these methods has performed satisfactorily in our hands, and we developed a novel method.

The materials used for our RNA extraction method were roots, needles and bark of Norway spruce (*Picea abies*). These tissues contain high amounts of different secondary metabolites. The roots are high in polyphenols and other phenolic compounds; the needles are high in carbohydrates, and the bark is high in both polyphenols and terpenes. Here, we present a method for RNA extraction that has worked reliably in our laboratory and results in high-quality RNA that is useful for both Northern blot hybridization and enzymatic reactions such as reverse transcription and RT-PCR. No ultracentrifugation is required, and the yield is sufficient for subsequent analysis. The Protocol presents the method that is modified from previous publications, primarily those