Challenges of small-RNA purification

. . . and a strategy to meet them

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In the past few years, several discoveries have underlined the importance of small RNAs (20–24 nucleotides) in various cellular functions. Small double-stranded RNAs (dsRNAs), called small interfering RNAs (siRNAs), have been used to silence the expression of specific genes at the post-transcriptional level by a pathway known as RNA interference (RNAi) (*1*). Many researchers are exploiting siRNAs in gene function studies or pathways analyses. Others are using RNAi in their drug development processes or are evaluating siRNAs as therapeutic agents (*2*).

Other small regulatory RNA molecules, referred to as microRNAs (miRNAs), have been shown to regulate target gene expression (*3*, *4*). Early studies of miRNA processing and expression offer great promise for the use of miRNAs as a tool to improve understanding of the ways gene expression can direct development, differentiation, and proliferation. This area of research could also provide more insight into the causes of many diseases and lead to the discovery of novel therapeutic or diagnostic targets.

It is also now becoming apparent that siRNAs and miRNAs are related molecules, sharing common processing pathways and potentially even functional mechanisms. In response to this research, interest in the identification, detection, and use of small RNA molecules has exploded. However, the standard RNA isolation and quantitation techniques have been optimized for larger molecules and are not always favorable for analyzing smaller RNA species. Here, we will describe methods for isolating and detecting small RNAs such as siRNA and miRNA.

Small-RNA isolation

Obtaining high-quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments. There are two main methods for isolating RNA: phenol-based extraction and silica matrix or glass fiber filter (GFF)-based binding.

Phenol-based reagents contain a combination of denaturants and RNase inhibitors for cell and tissue disruption and subsequent separation of RNA from contaminants. The tissue or cell sample is homogenized or disrupted in the reagent, chloroform is mixed with the lysate, and the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase using isopropanol.

In the GFF procedure, RNA is bound to a GFF housed in a microcentrifuge tube-fitted assembly. Solutions are driven through the filter by microcentrifugation or by using a vacuum manifold. Cells or tissues are disrupted in a chaotropic agent (e.g., guanidinium thiocyanate solution), which lyses cells and inactivates endogenous RNases.

The lysate is then diluted with an ethanol solution and applied to an RNA-binding GFF. Proteins, DNA, and other contaminants are removed by washing steps, and the bound RNA is then eluted in concentrated form. No phenol:chloroform extraction, protease digestion, or alcohol precipitation steps are required, making the procedure ideal for processing multiple samples.

Most RNA isolation procedures, however, were developed and optimized to recover messenger RNA (mRNA) while ignoring smaller molecules. Unfortunately, these procedures can result in the loss of substantial amounts of small RNA from samples. Figure 1 demonstrates that phenol-based isolation procedures can recover RNA species in the 10–200-nucleotide range (e.g., the miRNAs, 5S rRNA, 5.8S rRNA, and U1 snRNA, as labeled in the figure). But phenol-based RNA isolation is tedious, requires the use of noxious chemicals, and is not easily amenable to high throughput.

The standard GFF methods, on the other hand, are less hazardous, less laborious, and adaptable to high throughput. However, they are much less efficient at recovering small RNAs. In the GFF lanes in Figure 1, even though the 5.8S rRNA is efficiently recovered, other small

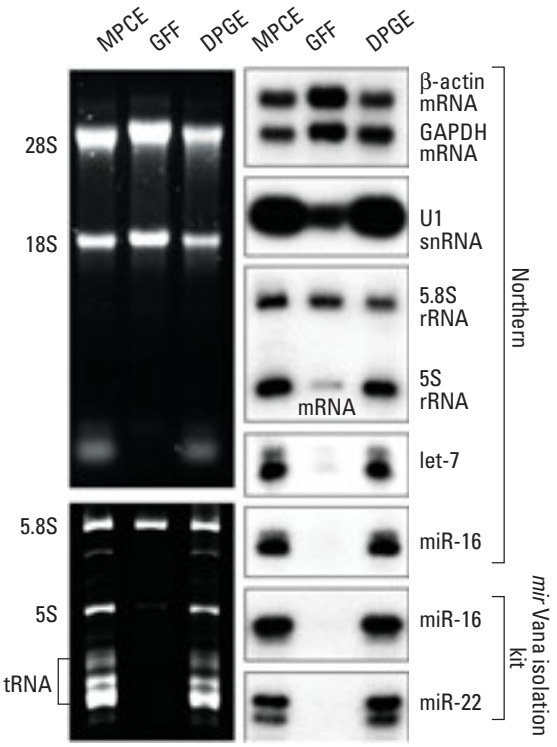

Figure 1. Differential recovery of small RNAs. Total RNA was isolated from 1×10^6 HeLa cells using three different techniques: monophasic phenol–chaotropic extraction (MPCE), binding on glass fiber filter (GFF) in guanidinium thiocyanate solution, or double phenol–guanidinium extraction (DPGE). The purified RNA (1 µg) was resolved on a 1.2% denaturing agarose gel (top left panel) or polyacrylamide gel (bottom left panel). The indicated mRNAs or small RNAs were detected by Northern blot or using a detection kit (right panels). (Courtesy of Ambion, Inc.)

RNA species, such as U1 snRNA, 5S rRNA, tRNA, and various miRNAs, are partially or completely depleted.

Detection–quantitation

The size, and sometimes the low expression levels, of small RNAs can make analysis difficult. The small size of these molecules alone precludes the use of reverse transcriptase-PCR as a detection method. Most researchers analyze expression patterns of small RNA molecules by Northern blot analysis with polyacrylamide gels, a technique that is relatively insensitive and labor-intensive. A few researchers performing gene-silencing experiments also use this technique to analyze siRNA levels after RNAi induction, although most scientists performing these experiments do not monitor siRNA levels at all.

In Northern blot analysis, RNA samples are first separated by size via electrophoresis in a polyacrylamide gel under denaturing conditions. The RNA is then transferred to a membrane and cross-linked, and the membrane is hybridized with a labeled probe. If the RNA sample is even slightly degraded, the quality of the data and the ability to quantitate expression can be severely compromised. Multiple-probe analysis is difficult, and it is usually necessary to strip the initial probe off the membrane before hybridizing with a second probe.

Small-volume solution hybridization is far more efficient than membrane-based hybridization (Northern blot analysis). After hybridization, any remaining unhybridized probe and sample RNA are removed by digestion with a mixture of ribonucleases. Then, in a single step, the nucleases are inactivated and the remaining probe–target hybrids are precipitated. These products are separated on a denaturing polyacrylamide gel and visualized by autoradiography. This technique, known as a ribonuclease protection assay (RPA), is the method of choice for simultaneously detecting several RNA species. During solution hybridization and subsequent analysis, individual probe–target interactions are completely independent of one another. Thus, several RNA targets and internal controls can be assayed simultaneously, provided that the protected fragments of individual probes are of different lengths.

Optimized procedures

Scientists at Ambion (*www.ambion.com*) adapted the solution hybridization assay specifically for detecting small RNAs. In this way, the molecules affecting target mRNA expression as well as the target mRNA itself can be simultaneously monitored. Relatively abundant miRNA can be detected in as little as 10 ng of total RNA. Furthermore, this detection approach has been paired with a GFF-based isolation kit with the ability to provide quantitative yield of small RNAs from virtually any biological sample. RNA is bound to the GFF in an optimized lysis-binding solution at a relatively high ethanol concentration, and impurities are then washed from the filter. Finally, RNA is eluted from the filter in a nuclease-free solution to recover RNA species of all sizes, from large mRNA and rRNA to 10-mers.

Small-RNA analysis often requires extremely large amounts of input total RNA to detect a specific species of the low-abundance small-RNA fraction. However, this can lead to nonspecific hybridization or smearing of gels. The isolation kit includes an enrichment procedure for the preparation of fractions containing only RNA molecules smaller than ~200 nucleotides. The lysate is first passed through a GFF at low ethanol concentration to capture the large RNA species. The filtrate is then bound at higher ethanol concentration on a second GFF. Large or small RNA species can then be eluted from each filter (Figure 2).

Outlook

As the field of small RNA molecules continues to develop, the need for fast, robust, and sensitive analysis tools will grow. High-throughput detection of miRNA will help to better decipher their complex spatial and temporal expression patterns, as well as variations in miRNA levels during tissue development or in disease states. Novel sample preparation methods, allowing simultaneous isolation of mRNA, small RNA, and protein from the same experimental sample, will make it possible to monitor siRNA or miRNA expression levels in conjunction with assessing their target mRNA and protein knockdown levels. The development of these improved isolation and detection methods will undoubtedly contribute to harnessing the power of RNA-mediated gene silencing for applications to basic, applied, and therapeutic research efforts.

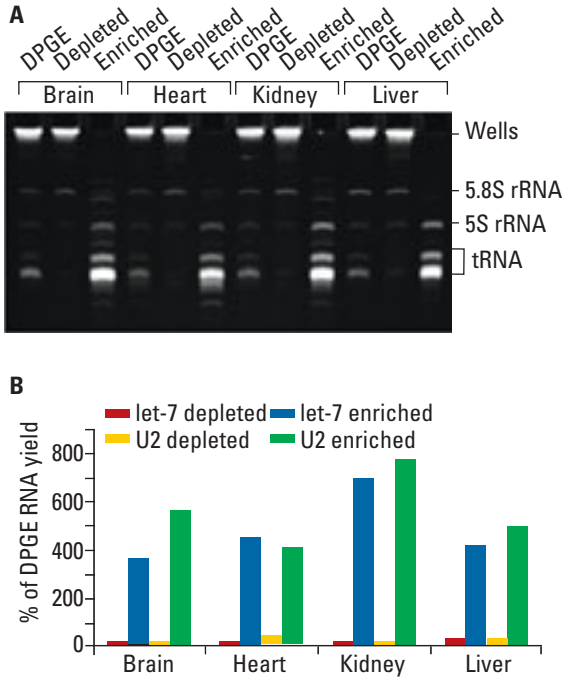

Figure 2. Preparation of fractions enriched for small RNAs. (A) Total RNA was isolated using a double phenol–guanidinium extraction (DPGE) procedure from half of four different lysates prepared from mouse brain, heart, kidney, or liver. Long (depleted) or small (enriched) RNA species were isolated from the other half of each lysate. (B) RNA from the same gel was transferred to a membrane and hybridized with probes specific for U2 snRNA and let-7 miRNA. The graph shows the percentage of recovery with respect to the DPGE prep. (Courtesy of Ambion, Inc.)

References

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