Short Technical Report

Increased Yield of Total RNA from Fine-Needle Aspirates for Use in Expression Microarray Analysis

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ABSTRACT

Fine-needle aspirate samples hold the potential for gaining valuable insight into the molecular details and prognostic indicators for certain types of cancer in a limited volume of relatively pure tumor cells. Although limited, such clinical samples can be used with high efficiency when analyzed in conjunction with gene-dense expression microarrays. For this reason, it is essential to retrieve as much high-quality genetic material as possible from each fine-needle aspirate sample. We have conducted a study to improve the efficiency of extracting high quality total RNA to use in microarray analysis from single ex vivo fine-needle aspirate samples of 11 breast cancers added to RNeasyTM RNA Stabilization Reagent immediately upon collection. Approximately half the total RNA from fine-needle aspirate samples of breast cancers was isolated from the supernatant, and that RNA had similar quality and gene expression profile to the RNA that was isolated from the corresponding cell pellet. We recommend that the supernatant not be discarded when extracting RNA from fine-needle aspirate samples stored in RNeasy.

INTRODUCTION

Fine-needle aspirate samples from tumors can be manipulated with molecular biology protocols to help to reveal the molecular mechanisms of cancer (3,8,9). In addition, they can be used to monitor the course of the disease and the tumor's response to treatment (2,4,10,11,13,15,16).

For this study we have extracted RNA from fine-needle aspirate samples to use in conjunction with gene-dense cDNA expression microarrays (1). The fine-needle aspirate samples used in this study were collected from breast cancer patients and were immediately added to RNeasyTM RNA Stabilization Reagent (Ambion, Austin, TX, USA) (7). In general, the manufacturer's suggested protocol instructs the user to sediment the cells in the RNeasy by centrifugation and to discard the supernatant, treating only the cell pellet for RNA isolation. We reasoned that membrane lysis of some tumor cells would occur as a result of the shearing needle movement during the fine-needle aspirate sampling procedure, rinsing, mixing, and lysing of the cells during freeze-and-thaw as a cellular suspension in RNeasy. Here we separated the cell pellet and the supernatant and attempted to extract RNA from each fraction. RNA from each fraction was then amplified, labeled, and hybridized onto cDNA microarrays, comparing each fine-needle aspirate's cell pellet to its supernatant.

MATERIALS AND METHODS

RNA Isolation from Fine-Needle Aspirates

Tumor samples were obtained in a standard clinical manner by fine-needle aspirate using a 25-gauge needle attached to a 10-cm³ syringe. A single ex vivo fine-needle aspirate sample was obtained from each of 11 breast cancer specimens immediately following surgical resection. The tumor samples were anonymous. Immediately upon collection, fine-needle aspirates within the needle hub were rinsed in 200 μ L RNeasy. The time delay between devascularization and entry into RNeasy varied from 10 to 30 min. The tumor samples were kept at room temperature for 30 min and then stored at -80°C, usually for less than one month. RNA was isolated using the RNeasy[®] Mini kit (Qiagen, Valencia, CA, USA). Tumor samples were thawed on ice, and cells were pelleted by centrifugation for 5 min at 9500 $\times$ g in a refrigerated model Z 233 MK centrifuge (Hermle Labnet, Wehingen, Germany). The RNeasy solution was removed from the cell pellet and placed into a fresh, nuclease-free, 1.5-mL Eppendorf[®] tube. Both the cell pellet and RNeasy supernatant were independently subjected to the RNA isolation procedure as separate samples, as described in the manufacturer's suggested protocol, except that each lysate was homogenized by passing it through a

Table 1. Yield of RNA from Cell Pellet and Supernatant Fractions of Fine-Needle Aspirate Samples

Sample	RNA Extracted (μ g)		Percent of Total (%)		260/280 Ratio		28s/18s Ratio	
	Average	Range	Average	Range	Average	Range	Average	Range
Cell Pellet	1.76	0.06–8.16	53	7–90	1.97	1.08–2.5	0.79	0–1.60
Supernatant	1.21	0.14–5.82	47	10–93	1.99	1.39–2.12	0.97	0–1.93
Combined	2.97	0.23–13.98	—	—	1.98	1.08–2.5	0.88	0–1.93

28-gauge needle 10–20 times. RNA was eluted twice in 40 μ L nuclease-free water (Invitrogen, Carlsbad, CA, USA) by centrifuging for 1 min at 20 000 $\times$ *g* after applying the water directly to the membrane.

RNA Analysis and Quantitation

Each sample was analyzed with a spectrophotometer at 260 and 280 nm for RNA concentration and protein contamination and then evaluated for degradation and genomic DNA contamination on a 1% denaturing agarose gel containing 1 $\times$ MOPS buffer and 2% formaldehyde. In addition, each sample was also loaded into an RNA LabChip[®] and analyzed on the model 2100 Bioanalyzer using the RNA 6000 Nano Assay (Caliper Technologies, Mountain View, CA, USA).

Microarray Hybridization

Based on the RNA yield and quality for the cell pellet and supernatant portions, five fine-needle aspirates were chosen to be hybridized to a cDNA microarray containing 4800 features

produced in-house. RNA samples were reverse-transcribed and made double-stranded using a second-strand synthesis protocol that was followed a single round of in vitro transcription with T7 RNA polymerase as previously described (12,14,17). For each FNA sample chosen, the cell pellet's amplified RNA was labeled with Cy5 dCTP while the supernatant's amplified RNA was labeled with Cy3 dCTP. Labeled fractions from individual fine-needle aspirate samples were co-hybridized, and the slides were then scanned on an LS-IV laser scanner (Genomic Solutions, Ann Arbor, MI, USA). For each fine-needle aspirate sample, the results from the cell pellet were compared directly to those from the supernatant using the GeneSpring Version 4.1.5 software package (Silicon Genetics, Redwood City, CA, USA).

RESULTS

RNA was isolated from fine-needle aspirate samples from 11 breast cancers, first separating the cell pellet from the RNA_{later} supernatant and isolating RNA from each portion independently. Paired RNA samples were quantitated spectrophotometrically and analyzed for protein contamination. All samples yielded sufficient RNA to be detectable by spectrophotometer (Table 1). On average, samples yielded a total of 3 μ g total RNA with 53% of the total RNA coming from the cell pellet. The 260/280 ratios ranged from 1.1 to 2.5 with an average of 2.0, indicating most

Figure 1. Denaturing agarose gel electrophoresis of RNA from four FNA samples. M, 3 μ g 0.24–9.5 Kb RNA Ladder (Invitrogen); lane 1, 1 μ g RNA from Sample 4 cell pellet; lane 2, 1 μ g RNA from Sample 4 supernatant; lane 3, 1 μ g RNA from Sample 10 cell pellet; lane 4, 0.26 μ g RNA from Sample 10 supernatant; lane 5, 1 μ g RNA from Sample 11 cell pellet; lane 6, 0.5 μ g RNA from Sample 11 supernatant; lane 7, 1 μ g RNA from Sample 3 cell pellet; lane 8, 0.84 μ g RNA from Sample 3 supernatant.

MICROARRAY *Technologies*

RNA samples to be relatively free from significant protein contamination.

Each RNA sample was evaluated for degradation by denaturing agarose gel electrophoresis. RNA that is not degraded should have two clear bands, representing the 28S and 18S rRNA species, with the larger 28S band appearing twice as bright as the 18S band. The sample should also be free of contaminating genomic DNA, which would appear on the gel as a band larger than the 28S rRNA band. Most of the RNA samples isolated from the fine-needle aspirate samples have detectable 28S and 18S rRNA bands, but they show some signs of degradation, possibly because of variable delay between surgical excision and ex vivo sample collection. The quality of RNA was the same in matched pairs of cell pellet and supernatant from the same tumor fine-needle aspirate sample (Figure 1). In addition, samples in Figure 1, lanes 5, 6, and 8, show possible contamination with genomic DNA.

An aliquot from each RNA sample was also loaded on the model 2100 Bioanalyzer. The Bioanalyzer is an automated system that uses both microfluidics and gel electrophoresis technologies to enable analysis of DNA, RNA, and protein samples. The Bioanalyzer uses microelectrodes to electrophorese samples through capillary channels in the chip onto which the samples are loaded. As nucleic acid samples move through the gel matrix, they incorporate dye that the machine detects and displays as peaks. The software uses a ladder and internal markers to quantitate and size fragments in each sample. Using this technique, the Bioanalyzer system conducts a rapid, highly sensitive assay that has better resolution than denaturing gel electrophoresis.

For a Bioanalyzer run designated for RNA analysis, the software recognizes the peaks representing 28S and 18S rRNA, assigns empirical values for the area of each, and calculates the corresponding 28S/18S ratio for each sample. Ratios in the range of 1.5–2.5 indicate high-quality RNA that is free of degradation. The majority of the RNA samples from FNA showed detectable 28S and 18S rRNA peaks, but they also showed significant degradation (Figure 2 and Table 1). A small number of sam-

ples actually had 28S/18S rRNA ratios of 0.0 because the software did not identify any peaks, perhaps because of underloading the sample on the chip.

Five FNA samples were chosen to be hybridized on cDNA microarrays, comparing cell pellets to their respective supernatants. In general, the microarrays had good images after scanning. Because each FNA sample was divided into two fractions and those fractions were hybridized together on one slide, we could directly compare the data from the cell pellet to that from the supernatant. The signals from each channel were normalized by dividing each signal by the median of all measurements from

Table 2. Correlation Values for Microarray Data from Five Fine-Needle Aspirate Cell Pellets Compared to Their Supernatants

Sample	Standard Correlation
3	0.977
4	0.972
7	0.957
10	0.938
11	0.935

this channel. The comparison between the data from the cell pellet and that from the supernatant showed high correlation for each fine-needle aspirate sample (Table 2 and Figure 3).

Figure 2. Representative paired Bioanalyzer results of relatively intact RNA compared to significantly degraded RNA. (A) RNA from Sample 4 cell pellet. (B) RNA from Sample 4 supernatant. (C) RNA from Sample 3 cell pellet. (D) RNA from Sample 3 supernatant.

Figure 3. Representative scatter plot of microarray data from cell pellet versus supernatant for Sample 3. Each "+" indicates one gene on the microarray. The middle diagonal line in the graph indicates identical expression levels in the two samples, and the two flanking lines indicate 2-fold changes in expression level.

DISCUSSION

As an expression microarray core facility, we have determined that the best way to get good, reliable results from a microarray experiment is to start with RNA of exceptionally high quality. Isolating such RNA poses a significant problem, especially when the samples are taken directly from patients. However, this obstacle must be overcome because patient samples tell the most physiologically relevant story, as opposed to cell lines or animal models. Although there was degradation of RNA in some of the FNA samples, that might be explained by variable delay between surgical excision and ex vivo FNA sampling of tumors. Of note, there was similar quality of RNA isolated from matched cell pellet and supernatant samples.

Fine-needle aspirate samples from tumors are particularly useful because

they represent a relatively pure population of tumor cells, relatively free from the contaminating normal cells that would be expected in a surgical resection. This purity is particularly useful when we consider the subtle changes that may occur at the beginning of tumorigenesis (6). Contaminating normal cells could potentially dampen a small but important change in a tumor's gene expression pattern. In addition, obtaining fine-needle aspirates is a much safer and less invasive procedure than surgery. In fact, it is conceivable that repeated aspirates could be collected from the same tumor to monitor the progression of the disease and the tumor's response to therapy (4,5,15,16). Finally, fine-needle aspirates provide samples with sufficient RNA yield and adequate quality for microarray analysis (1). These qualities together allow for valuable high-density analysis.

For the present study, we isolated to-

tal RNA from a set of single, ex vivo fine-needle aspirate samples of breast cancer that were added to *RNAlater* RNA Stabilization Reagent at collection. *RNAlater* is an aqueous solution formulated to protect RNA from degradation in tissues. The solution has also been tested successfully with *E. coli*, *Drosophila*, tissue culture cells, white blood cells, and plant cells, but it is not recommended for whole blood, plasma, or serum. The manufacturer's instructions say that cellular integrity should be maintained, even after freezing samples at -80°C. However, we have found a significant portion of cellular RNA in the *RNAlater* reagent, as opposed to the cell pellet, which is consistent with a traditional method for lysing cells by freezing and thawing.

RNAlater is undoubtedly easy to use and valuable for preserving RNA in clinical samples. To maximize the overall RNA yield, we suggest treating the

entire sample for RNA isolation, not just the cell pellet. Our microarray results demonstrated no significant differential expression in the fine-needle aspirate cell pellets compared to their corresponding supernatants. Isolating the maximum possible amount of RNA by using both cell pellet and supernatant could allow for better detection of low expressing genes or allow for additional experiments.

Even when we isolated the maximum possible amount of RNA, fine-needle aspirate samples did not typically yield more than 5 µg total RNA. This quantity is sufficient for microarray analysis but is not optimal for the traditional denaturing agarose gel electrophoresis that needs to be done to determine RNA quality before the microarray hybridization protocol is started. Usually 0.5–1 µg RNA needs to be loaded on a gel to visualize with UV light. This amount could conceivably be most of the RNA yield from a fine-needle aspirate sample. For such samples with low yield or any other precious samples, the model 2100 Bioanalyzer can remove the need for running a standard gel. The acceptable loading range for an RNA LabChip is 5–500 ng total RNA. This range is dramatically lower than the amount needed to determine RNA quality on a gel, leaving a much larger proportion of the sample available for experimental work.

After completing this preliminary feasibility study, we plan to hybridize additional expression microarrays with RNA isolated from additional FNA samples, combining cell pellet and supernatant, to screen for differentially expressed genes that may serve as prognostic indicators or therapeutic targets.

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