Short Technical Report

Microarray RNA Quantification Assay for Large-Scale Nanosamples Analysis

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ABSTRACT

We have developed a convenient and sensitive method for the quantification of RNA in samples from microbiopsies. This procedure is especially suitable for quantifying very small amounts of RNA in large-scale biological samples. This method, using a microarray-spotting facility for the study of multigenic expression, entails the hybridization of a DNA probe with RNA spotted at high density on nylon membrane. Furthermore, with this procedure, the reproducibility, sensitivity, and accuracy of the assay are notably improved as compared to current methods.

INTRODUCTION

Nucleic acid isolation and quantification are essential steps in many analytical procedures in molecular biology. The accurate, reproducible, and sensitive estimation of the quantity of a particular nucleic acid is of critical importance when dealing with biological materials originating from clinical samples.

Various methods are currently available for detecting and quantifying RNA. Spectrophotometric measurements and northern blotting, dot blotting, RNase protection assay, and quantitative, competitive RT-PCR (3–5,8, 10–12) are frequently used. The translation of these techniques to the clinical area has been difficult because of the development of micro-invasive procedures for the clinical diagnostic.

The accurate determination of RNA concentration may be difficult, especially when only a limited amount of biological material is available. This problem is notably encountered in the case of clinical investigation in which recently introduced micro-invasive technologies, such as microbiopsies or laser microdissection, provide a minimal amount of tissue sample. Actually, several methods are emerging in response to sensitivity requirements, such as real-time PCR and competitive PCR.

In this context, we have developed a convenient and sensitive method for the quantification of total RNA extracts from microbiopsies. This approach is based on the hybridization of DNA probes (6) with RNA high-density filters. The advantage of the microarray RNA quantification, contrary to the other methods, consists in the possibili-

ty to determine the concentration of all RNA samples of the study (eventually several hundreds) in the same analysis, limiting relative variations. The present report describes this technical approach and discusses the corresponding data.

MATERIALS AND METHODS

RNA Isolation

Total RNA was isolated from 14 different frozen human tumoral tissues (breast, brain, and lung) using RNA-gents® Total RNA Isolation System (Promega, Madison, WI, USA) based on mechanical harvest (i.e., electrical polytron) in denaturing solution and phenol-chloroform extraction (2). The integrity of the extracted RNA was assessed by 28 S northern blot analysis.

Calibration Samples for Reference Values

RNA standard concentration was determined by absorbance measurements at 260 and 280 nm. The range concentration was between 0.5 and 2.8 µg/µL.

RNA Membrane Preparation

Determination of correlation between hybridization level and concentration of RNA samples. For this experiment, we used several dilutions of 14 RNA samples (1:10, 1:50, and 1:100). Forty drops of each sample were spotted onto membranes, which were then treated as described below. As a negative control, a panel of PCR product was also spotted.

Measurement of concentration of RNA samples. Samples and standard RNA were diluted in 96-well microplates (Corning Costar, Acton, MA, USA) in denaturing buffer (1× MOPS, 50% formamide, 17% formaldehyde, 10% glycerol, and bromophenol blue). RNA samples were further denatured at 65°C for 10 min and stored on ice until spotting. Finally, 1 µL RNAsin® (10 U/µL) (Promega) was added to the samples. The minimum volume assay recommended was 10 µL.

RNA filters were then prepared by spotting RNA onto Nytran® Plus nylon membranes (Schleicher & Schuell, Keene, NH, USA) using an Affymetrix® 417 Microarrayer (MWG, Ebersberg, Germany). This robot had four pinheads, and the spacing between each spot (or dot) was 375 µm.

Different quantities of each sample were arrayed by modulating the number of drops/dot (20, 50, 80, 100, and 200 drops). Dots were spotted at least in triplicate on each membrane. Moreover, the reproducibility was verified by spotting 10 filters, which were then hybridized in 10 independent experiments. Finally, RNA were cross-linked to membranes upon UV radiation at

254 nm (700 J/m²), and the filters were stored, if necessary, at room temperature until hybridization.

Oligonucleotide Probe Labeling

Oligonucleotide 28 S or poly(dT) (OligoExpress™; Genome Express, Grenoble, France) was labeled (1 µg) upon incubation with 30 µCi $\gamma^{33}\text{P}$ -ATP (3000 Ci/mM; ICN Biomedicals, Costa Mesa, CA, USA) using 10 U T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) according to the standard method (9). The sequences used were 5'-TGAATCCTCCGGGCG-GACT-3' for 28 S and dT25 for poly(dT).

Membrane Hybridization

Membranes were prehybridized in 5× sodium citrate solution (SSC), 5× Denhart's reagent, 0.5% SDS, with 100 µg/mL denaturated salmon sperm DNA at 42°C for 3 h. The prehybridization solution was then replaced with fresh solution containing labeled probes, and hybridization was carried out at 42°C for 12 h. The probe must be present in the reaction in large molar excess over

Figure 1. Correlation between hybridization signal and concentration of RNA samples. Fourteen RNA samples were quantified by absorbance at 260 nm, then diluted 1:10, 1:50, and 1:100 (resulting concentrations reported in abscissa), and spotted with 40 drops on filters for 28 S radiolabeled oligonucleotide hybridization. The signal intensity was detected using the high-resolution phosphor imager BAS-5000 and reported on the Y-axis.

the RNA target. Furthermore, unbound probes were removed by washing the membrane three times in 2× SSC and 0.1% SDS buffer for 5 min at room temperature. As a negative control, a labeled bacterial probe without human homology was hybridized onto the RNA microarray.

Detection and Quantification of Hybridization Signals

Membranes were exposed for 12 h at room temperature and scanned in a phosphor screen high-resolution system (BAS-5000, Fuji-Film™; Raytest, Paris la Defense, France). The signal intensity was quantified using analyzer software (Fuji Image Reader™; Raytest), and the means and standard variations were calculated.

RESULTS

Correlation between Hybridization Signal and Concentration of RNA Samples

Fourteen RNA samples were quantified by measuring absorbance (ranging between 0.5 and 2.8 µg/µL), diluted 1:10, 1:50, and 1:100, and spotted onto membranes. We observed good linearity between RNA densitometric measurement and 28 S probe hybridization level, as described in Figure 1.

Quantification and RNA Integrity

Five RNA extracts that were analyzed by 28 S northern blot appeared to be very damaged, showing a smear pattern without 28 S bands. Nine RNA appeared to be intact, with only one distinct 28 S band in northern. The correlation between 28 S hybridization and RNA amount was verified for these five degraded RNA samples. The results demonstrated that the proportional response remained linear, independent of RNA degradation (Figure 2).

Optimization of Assay

We confirmed that the number of drops spotted by our microarrayer remained proportional to the hybridization signal intensity (the RNA quantity

in the assay also remained proportional) even after using hundreds of drops (Figure 3). Usually, that permits us to prepare only one dilution for unknown and standard samples (using a minimal quantity of RNA) and to spot from 10 to 200 drops/dot. Then, 200 drops/dot in the assay, the minimum concentration detectable was about 0.002 ng/nL. Furthermore, using standard samples

for the calibration curve, we determined the concentration of seven unknown samples in 10 independent tests. The cv of the different samples was between 14% and 22%.

The same experiment was run simultaneously with a poly(dT) probe. The results that we obtained were similar to those obtained with the 28 S hybridization probe. The detection limit

Figure 2. RNA degradation effect on assay. Three dilutions (1:10, 1:50, and 1:100) of 14 RNA samples were analyzed (40 drops/dot); nine of them were intact (white circles), and five were damaged (black squares).

Figure 3. Correlation between hybridization signal and number of spotted drops. The RNA samples were spotted with 40, 60, 80, and 100 drops/dot in triplicate, and 28 S oligonucleotide hybridization was performed. The signal intensity detected was reported on the Y-axis.

and linear response range were identical (data not shown).

DISCUSSION

We report a new technique for RNA quantification using radioactive 28 S probe hybridization on high-density RNA nylon membrane. Alternatively, with an adapted protocol, it should be possible to use glass slides and fluorescently labeled probes, but it would be less sensitive.

This method seems particularly well adapted to microarray study. Indeed, the technique requires neither specific material nor new expertise because it makes use of the instrumentation setup for large-scale gene expression studies (1). Such an instrumental facility is highly suited to rapidly and reproducibly treating thousands of nanosamples inexpensively, without supplementary investment. This technique is especially useful for investigators with a microarray facility.

The reproducibility and linearity of this quantitative assay have been demonstrated. Indeed, nylon has the capacity to linearly absorb a high amount of nucleic acid material. Furthermore, this assay is a quantification method that is independent from RNA integrity as a spectrophotometric method. To our knowledge, for RNA quality control, no high-throughput technologies are available for nanosamples. Alternatively, microelectrophoresis methods could be used for limited sample series.

Moreover, the assay sensibility (0.002 ng/nL) of our RNA quantification technique is suitable for the analysis of the very low quantities of RNA provided by the new sampling methods such as laser microdissection and biopsies, both before and after amplification, commonly used in microarray analyses (7).

In addition, the volume of the diluted RNA extracts (10 μ L) required to feed the spotting system limits the material RNA consumption for quantification, unlike the commonly used spectrophotometric method, which wastes more material. Moreover, with the spotting using about only 1 of 10 μ L available, 90% of the RNA assay could be recovered.

Microarray technology is being introduced in the clinical area devoted to the diagnostic, prognostic, and therapeutic analyses of pathological tissue samples. To be statistically significant, these studies will impose retrospective and prospective studies dealing with hundreds of samples. In this perspective, this procedure is especially suitable for the dosage of numerous RNA samples (up to several hundred in the same experiment). This allows one to limit inter-assay variations. In conclusion, this study presents a sensitive and useful technique of RNA quantification that could interest many users of microarray systems in the area of clinical applications.

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