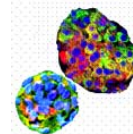


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Title:	RNA quality: Basics of purity determination and RNA related techniques				
Protocol #:	1.1	Submitted:	050510	Approved:	200610
Category:	MB	Author(s): ¹	SNS,MVJ	Checked by:	AAH

Reagents:

1. RNase-free water
2. TrisCl
3. Trizol (Invitrogen, Carlsbad, CA)
4. Primers and probes were Assay-on-Demand (Applied Biosystems, Foster City, CA).
5. cDNA archive kit' (Applied Biosystems, Foster City, CA)
6. AmpliTaq Gold (Applied Biosystems, Foster City, CA)
7. Complete mouse miRNA panel (Applied Biosystems, Foster city, CA)
8. Mature miRNA-specific primer sets (Applied Biosystems, Foster City, CA)
9. microRNA reverse transcription kit (Applied Biosystems, Foster City, CA)
10. miRNA-specific taqman-based probe-primer sets (Applied Biosystems, Foster City, CA)
11. siPORT NeoFX, a lipid based reagent (Ambion, Austin, TX)

Equipment

1. Cuvettes
2. ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE)
3. 7500 FAST real time PCR cycler (Applied Biosystems, Foster City, CA).

Reagent Setup

1. 10 mM Tris-Cl, pH 7.5.

Procedure:

Quantitation of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A₂₆₀) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A₂₆₀ = 1 => 40 µg/ml). This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be done in water. As discussed below, the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity. When measuring RNA samples using cuvettes, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1M

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NaOH, 1 mM EDTA followed by washing with RNase-free water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. (However, this may not be necessary for most of the present spectrophotometers that require very low volumes of diluted RNA). An example of the calculation involved in RNA quantitation is shown below:

Volume of RNA sample = 2.2 ml

Dilution = 10 μ l of RNA sample + 490 μ l distilled water (1/50 dilution).

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free).

$$A_{260} = 0.75$$

Concentration of RNA sample = 40 x A_{260} x dilution factor

$$= 40 \times 0.75 \times 50$$

$$= 1500 \mu\text{g/ml}$$

Total yield = concentration x volume of sample in milliliters

$$= 1500 \mu\text{g/ml} \times 2.2 \text{ ml}$$

$$= 3300 \mu\text{g} = 3.3 \text{ mg RNA}$$

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution. For determination of RNA concentration, dilution of the sample in water is recommended since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 $\mu\text{g/ml}$ RNA) is based on an extinction coefficient calculated for RNA in water.

RNA isolation and quantitative real-time PCR

Tissue samples should be homogenized and frozen in Trizol after they are completely solubilized in Tri reagent / Trizol. RNA should be isolated as per the manufacturers' instructions, measured on ND-1000 spectrophotometer and taken for reverse transcription / quantitative real-time pcr.

First strand cDNA synthesis should be carried out using 'high capacity cDNA archive kit' (Applied Biosystems, Foster City, CA). PCR should be performed in 5 μ l total volume in 96-well plates using cDNA prepared from 100ng of total RNA. Generally, use 100ng RNA in 10 μ l of reverse transcription reaction and then make up this volume to 100 μ l by adding 90 μ l of nuclease-free water. Then, use 1 μ l of this for real-time PCR in a 5 μ l total reaction volume for 7500 FAST real time PCR cyler. Primers and probes used here are Assay-on-Demand (Applied Biosystems / Life Technologies, Foster City, CA). For estimation of fold-changes by qRT-PCR when the initial transcript levels were undetectable, the initial Ct value is assigned to be 38, which would lead to a possible underestimation of the actual fold-change. All qRT-PCR results are normalized to 18S (VIC-labeled) ribosomal RNA carried out in duplex reaction

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(with FAM labeled target gene probes) to correct for any differences in RNA input. Alternatively, you can use 18s / Gapdh (FAM-labelled) in separate reaction.

For transfection of antisense miRNAs or microRNA duplexes, siPORT *NeoFX*, a lipid based reagent (Ambion, Austin, TX) is used for transfection as per the manufacturers recommendations. Briefly, siPORT *NeoFX* is diluted in Opti-MEM 1 medium and incubated at room temperature for 10 min. MicroRNA inhibitors or mutant-anti-miRNA are diluted in Opti-MEM to a final concentration of 30 nM. Diluted RNA and diluted siPORT *NeoFX* complexes are mixed by gentle pipetting and incubated at room temperature for 10 min. The RNA/siPORT *NeoFX* complexes are then distributed to each well and overlaid with cell suspension. Cells are harvested after 4 or 6 days of transfection and taken for transcript analysis and immunostaining.

Target prediction and cluster analysis

Since mammalian miRNAs are generally thought to recognize 3'UTR of target mRNA via partial complementarity, we used carefully designed computational approaches to predict mRNA targets for mammalian miRNAs. Two target search engines from Memorial Sloan-Kettering Cancer Center (<http://www.microrna.org/>), miRanda software and target analysis by PicTar (<http://pictar.bio.nyu.edu/>) are mainly used to confirm targets for specific miRNAs. Normalized data sets from realtime pcr analysis of miRNA expression profiles are taken as input data for bi-directional clustering. Bi-directional clustering is one of the most widely used algorithms to recognize patterns in datasets with similar expression profiles. Since functional modules of genes are generally regulated together, such modules can be identified from the similarity of their expression patterns in a bi-directional analysis. Two-way clustering is performed in MatLab™, using the Bioinformatics Toolbox (MatLab™ v 7.0, R 14), which basically groups the samples with similar matching gene profiles together across the X-axis. Genes within these grouped samples that show similar expression patterns are grouped together along the Y-axis. Bi-directional clustering thus offers an important tool to assess closely related samples as well as similar gene expression pattern within these sample groups.

Representative image / picture

N/A