Chapter 5

mRNA and microRNA Purity and Integrity: The Key to Success in Expression Profiling

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Abstract

RNA quality control is a crucial step in guaranteeing integer nondegraded RNA and receiving meaningful results in gene expression profiling experiments, using micro-array, RT-qPCR (Reverse-Transcription quantitative PCR), or Next-Generation-Sequencing by RNA-Seq or small-RNA Seq. Therefore, assessment of RNA integrity and purity is very essential prior to gene expression analysis of sample RNA to ensure the accuracy of any downstream applications. RNA samples should be nondegraded or fragmented and free of protein, genomic DNA, nucleases, and enzymatic inhibitors. Herein we describe the current state-of-the-art RNA quality assessment by combining UV/Vis spectrophotometry and microfluidic capillary electrophoresis.

Key words RNA purity, RNA integrity, microRNA (miRNA), mRNA, Microfluidic capillary electrophoresis, UV/Vis spectrophotometer, Denaturing gel electrophoresis, qPCR, MIQE

1 Introduction

Quantification of RNA expression levels serves as a prime indicator of the physiological status of a cell or tissue and plays a central role in a wide variety of life science studies. The purity and integrity of RNA samples were shown to have a direct influence on the outcome of gene expression experiments and may strongly compromise the accuracy of any RNA profile, irrelevant of the method by which it was obtained [1–3]. RNAs are very sensitive molecules, especially compared to DNAs, and are easily fragmented by heat, UV, or the ubiquitous occurring nucleases. In addition contaminants introduced through sloppy lab handling and ineffective sampling or extraction procedures, like salts, phenol, or heparin, were proven to impair downstream reactions and overall affect quantitative gene expression results [4]. Therefore, special care should be taken during each step of RNA preparation (e.g., tissue sampling and storage, RNA extraction, stabilization, and storage)

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to avoid any contamination or degradation [5–7]. Especially in clinical application, where diagnostic, therapeutic, and prognostic conclusions are drawn and sampling tissues tend to be unique and limited, a reliable and standardized RNA quality control is essential. The necessity of RNA quality control is also highlighted by the MIQE (Minimal Information for Publication of Quantitative Real-Time PCR Experiments) guidelines where it is listed as an essential required element of sample preparation prior to qPCR analysis [8].

RNA quality is defined by a set of criteria that all samples must fulfill in order to obtain comparable and reproducible results. At first, RNA preparations should be free of protein and any enzymatic inhibitors or complexing substances of RT and PCR. Secondly, samples should be nondegraded and free of nucleases. Lastly, contamination with genomic DNA should be excluded [8, 9]. While DNA contamination can be determined easily by negative reverse transcription controls during qPCR analysis, all other parameters should be evaluated prior to that. RNA purity can be assessed most conveniently on a spectrophotometer by measuring the optical density (OD) and comparing the absorption at different wavelengths. Nucleic acids (RNA as well as DNA) have their absorption maxima at 260 nm whereas proteins have their maxima at 280 nm. Additionally, contaminant and background absorption can be measured at 230 and/or 320 nm. An OD260/280 ratio higher than 1.8 is generally viewed as suitable for gene expression profiling [10]. On the other hand the OD260/230 and OD260/320 ratios should be maximized, as no fixed values exist for them since they depend mostly on the used sample tissues and extraction protocols. Preferably, spectrophotometer instruments should be used that do not rely on a cuvette format to avoid positioning errors and excess sample consumption (e.g., NanoDrop, Thermo Fischer Scientific; NanoVue, GE Healthcare; NanoPhotometer, Implen).

To check for enzyme inhibitors a dilution series of the sample in question is quantified via RT-qPCR and correlated against their respective Cq values in a semilogarithmic plot. Noninhibited reactions should exhibit a high linearity (determined by the coefficient of determination, R2) and qPCR efficiency (determined by slope of the linear regression). Optionally, if using only very small amounts of RNA (samples from, e.g., single cells, laser capture microdissection, or biopsies) a universal inhibition assay such as SPUD can be performed. By measuring a positive qPCR control that lacks homology to any known sequence, in and without the presence of nucleic acid samples any inhibition will be clearly shown in a rise of the corresponding Cq [11].

Various methods have been proposed for the measurement of RNA integrity, but over the last decade microfluidic capillary

electrophoresis has emerged as the preferred technology. By combining easy handling even for large numbers of samples and offering the most objective way of assessing the RNA degradation level, instruments such as Agilent Technologies' 2100 Bioanalyzer or Bio-Rad Laboratories' Experion has become the standard for RNA quality control [12]. RNA samples are separated electrophoretically on a microfabricated chip, and fragments are detected via laser-induced fluorescence measurement. Estimation of RNA band sizes and total concentration is achieved by using an RNA ladder as a mass and size standard. Comparable to old fashioned denaturing agarose gel electrophoresis, RNA integrity is mostly determined by the ratio of 28S to 18S rRNA (ribosomal RNA of eukaryotic samples) bands. Ideally, the ratio should be around 2.0, since 28S rRNA has approximately twice the quantity of 18S rRNA, but this is rarely accomplished in practice. Elevated levels of degradation appear as an increased threshold baseline as well as a decreased 28S/18S ratio in the electropherogram [13] (Fig. 1). In addition the instrument's software calculates an objective numerical value based on the rRNA ratio and the occurring RNA bands, ranging from one (almost completely degraded) to ten (intact and nonfragmented). For reliable PCR results a RIN (RNA Integrity Number; Agilent Technologies) or RQI (RNA Quality Index; Bio-Rad Laboratories) of higher than five has been proposed [12]. Although there is conflicting literature data about the correlation of mRNA integrity with 18S or 28S rRNA [14, 15], it is generally believed that mRNA degradation closely resembles that of 28S rRNA. Evaluation of miRNA integrity remains difficult and little is known about the accessibility of miRNAs to degradation processes. However as for mRNA, a significant correlation between miRNA expression data and RIN values was demonstrated [1]. Supplementary information can be gained by the small RNA assay from Agilent Technologies, enabling the separation and analysis of RNA fragments with less than 200 nt and therefore 91 quantifying the absolute concentration of these small RNA fractions and the respective percentage of miRNAs (Fig. 2). Since ongoing RNA degradation, especially longer mRNA species, causes the formation of smaller degraded RNA fragments, it will also be shown as an overrepresentation of the miRNA amount [1].

Thus by combining UV/Vis spectrophotometry and microfluidic capillary electrophoresis we are able to reliably and reproducibly assess the quality of mRNA and miRNA samples with minimal effort and sample consumption. By choosing only biological samples of adequate RNA purity and integrity for gene expression profiling we can now guarantee the correctness and validity of our quantitative results.

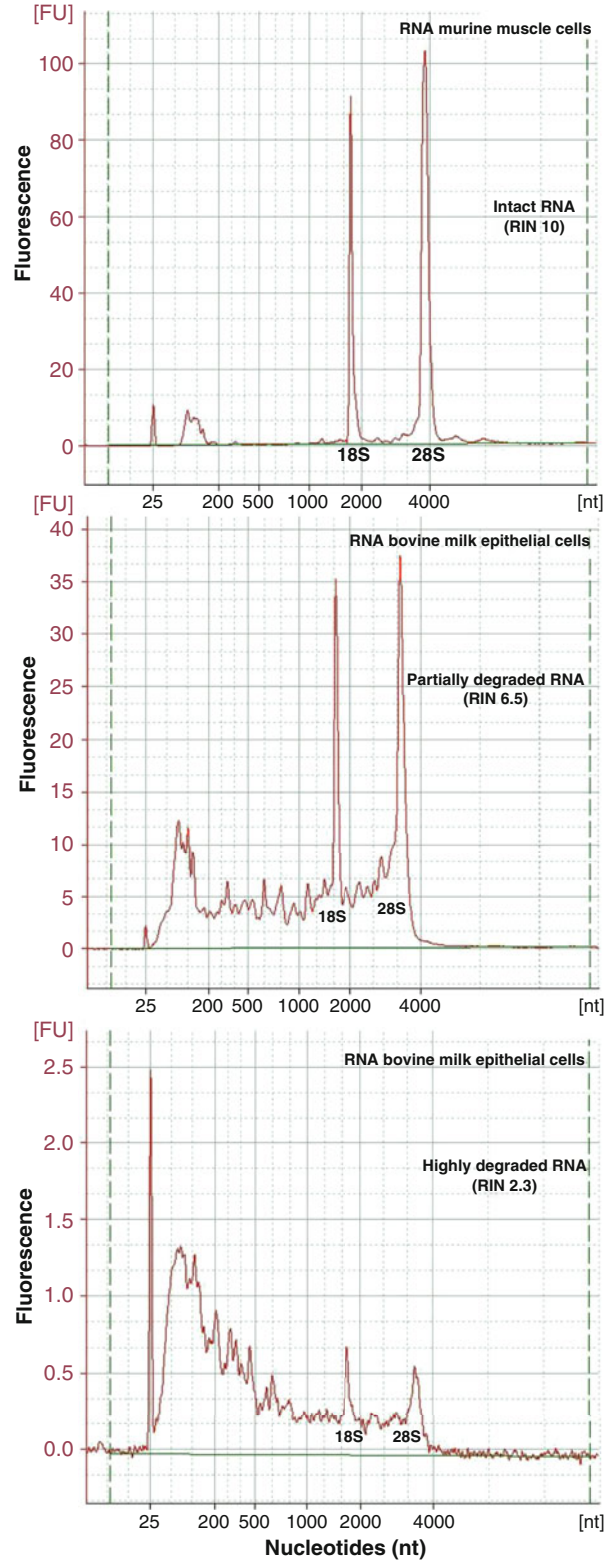

Fig. 1 Electropherograms of total RNA with varying degradation levels (perfect and integer total RNA = RIN 10; intermediate RNA quality with partial degradation = RIN 6.5; and highly degraded RNA = RIN 2.3) using the 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies)

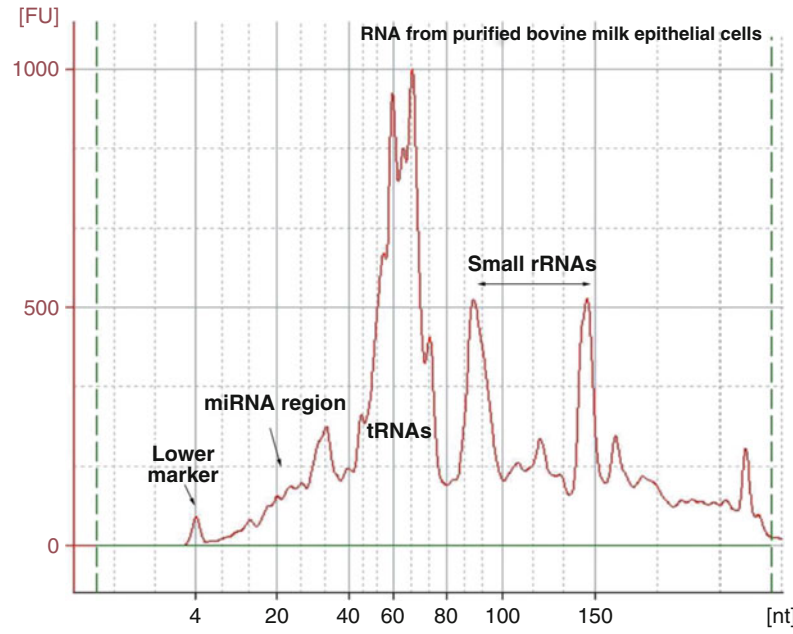

Fig. 2 Electropherogram of a small RNA integrity analysis using the 2100 Bioanalyzer and the Small RNA Kit (Agilent Technologies)

2 Materials

Use only PCR-grade water (DEPC-treated, double distilled, deionized, autoclaved, and free of nucleases) for all preparations and solutions. Prepare and store all reagents at room temperature (unless indicated otherwise). RNA samples for quality control should be kept on ice during all procedures. Diligently follow all waste disposal regulations.

2.1 UV/Vis Spectrophotometry Components

1. NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA).
2. NanoDrop 2000 operating software, version 1.4.2.
3. Ethanol for washing, 70 %.
4. Lint-free lab wipes.

2.2 2100 Bioanalyzer Components

All components except **item 1** are manufactured by Agilent Technologies, Santa Clara, USA.

1. Heating block or water bath.
2. 2100 Bioanalyzer.
3. 2100 Expert software for instrument control and data analysis.
4. Chip priming station.
5. Chip vortexer.

6. Agilent RNA 6000 Nano Kit containing
   - RNA Nano chips.
   - Electrode cleaners.
   - Syringe kit.
   - RNA 6000 Nano ladder (store in aliquots at –70 °C).
   - RNA 6000 Nano dye concentrate (store at 4 °C).
   - RNA 6000 Nano gel matrix (store at 4 °C).
   - RNA 6000 Nano marker (store at 4 °C).
   - Spin filters.
   - PCR clean safe lock tubes.
7. Agilent Small RNA Kit containing
   - Small RNA chips.
   - Electrode cleaners.
   - Syringe kit.
   - Small RNA ladder (store in aliquots at –70 °C).
   - Small RNA dye concentrate (store at 4 °C).
   - Small RNA gel matrix (store at 4 °C).
   - Small RNA marker (store at 4 °C).
   - Small RNA conditioning solution (store at 4 °C).
   - Spin filters.
   - PCR clean safe lock tubes.

2.3 Denaturing Agarose Gel Electrophoresis

1. 10× MOPS buffer: 0.2 M 3-(N-morpholino) propane sulfonic acid (MOPS), 50 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA). Add nuclease-free water to respective final volume and adjust pH to 7.0 with acetic acid or NaOH (prepared in nuclease-free water).
2. 1 % denaturing agarose gel: For 50 ml solution; cook 0.5 g agarose with 5 ml 10× MOPS buffer and 45 ml water until agarose is completely dissolved (*see* **Note 1**). Wait till agarose solution is cooled down to around 40 °C and add 2 ml formaldehyde (*see* **Note 2**) and let it solidify in a gel chamber.
3. Sample buffer (*see* **Note 3**): For 300 µl; add 150 µl formamide, 50 µl 37 % formaldehyde, 30 µl MOPS buffer, 55 µl bromophenol blue in water mixed with 50 % glycerol, 15 µl ethidium bromide stock solution (*see* **Note 4**).
4. RNA marker ranging from 200 to 6,000 nt.
5. Sodium hydroxide 0.1 M.
6. Electrophoresis chamber.
7. UV transilluminator.

2.4 SPUD Assay

1. SPUD amplicon: 5′-AACTTGGCTTTAATGGACCTCCAATTTTGAGTGTGCACAAGCTATGGAACACCACGTAAGACATAAAACGGCCACATATGGTGCCATGTAAGGATGAATGT-3′.
2. SPUD Forward Primer: 5′-AACTTGGCTTTAATGGACCTCCA-3′.
3. SPUD Reverse Primer: 5′-ACATTCATCCTTACATGGCACCA-3′ 164.
4. SPUD Taqman probe: 5′-FAM-TGCACAAGCTATGGAACACCACGT-TAMRA-3′.
5. Reverse transcription and qPCR kit of user's choice.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 RNA Purity Control: Optical Density Measurement on Nanodrop

1. Clean the sensor plate carefully with 70 % ethanol and dry it with a lint-free wipe.
2. Start the software and choose nucleic acids measurements.
3. Let the instrument initialize itself by pipetting 1.5 μl of water on the sensor plate and measuring it.
4. Wipe the sensor plate clean and apply 1.5 μl of the solution in which your RNA is dissolved to blank the background photospectrum and remove it from the analysis.
5. Once again wipe the sensor plate clean and proceed to measure your RNA samples.
6. Analyze the obtained spectra concerning its absorption at 260, 280, and 230/320 nm with their respective ratios (*see* **Note 5**).

3.2 Total RNA Integrity Control: Microfluidic Capillary Electrophoresis on Agilent 2100 Bioanalyser

1. Denature all RNA samples as well as the RNA 6000 Nano ladder at 68 °C for 2 min and immediately cool them on ice (*see* **Note 6**). For best readouts dilute all RNA samples to a final concentration of 100 ng/μl (*see* **Note 7**).
2. Let the RNA 6000 Nano kit reagents including dye concentrate to equilibrate at room temperature for 30 min in dark and protected from light.
3. Clean the electrodes by pipetting approximately 350 μl of water in the electrode-cleaning chip and putting it in the instrument for 60 s. Repeat it once.
4. Prepare the gel by pipetting 550 μl of RNA 6000 Nano gel matrix into a spin filter and centrifuging it at 1,500 × *g* for 10 min (*see* **Note 8**).

5. Add 1 μl of dye concentrate to 65 μl of filtered gel in a fresh microcentrifuge tube, vortex it briefly, and centrifuge it at 13,000 × *g* for 10 min (*see* **Note 9**).
6. Put a RNA 6000 Nano chip on the chip priming station and pipette 9 μl of gel-dye mix into the well marked with a white G in a black circle (*see* **Note 10**).
7. Lift the plunger to the 1 ml position, adjust the lever of the clip to top position, and close the priming station.
8. Press down plunger until it is arrested by clip and wait for exactly 30 s.
9. Release the clip and after 5 s slowly pull the plunger to its starting position (*see* **Note 11**).
10. Open the priming station and pipette 9 μl of gel-dye mix in each of the wells marked with a black bold G.
11. Load 5 μl of RNA 6000 Nano marker in all 12 sample wells and the ladder well.
12. Pipette 1 μl of respective RNA sample in each of the 12 sample wells and 1 μl of ladder into the ladder well. Pipette 1 μl of marker in every unused sample well.
13. Vortex the chip for 1 min at 2,400 rpm in the chip vortexer and start the run within 5 min on Agilent 2100 bioanalyser.
14. Analyze the run by checking rRNA ratios, general electropherogram progression, and RIN values.

3.3 Small RNA Integrity Control: Microfluidic Capillary Electrophoresis on Agilent 2100 Bioanalyser

1. Denature all RNA samples as well as the Small RNA ladder at 68 °C for 2 min and immediately cool them down on ice (*see* **Note 6**). For best readouts dilute all RNA samples to a final concentration of 50 ng/μl total RNA.
2. Let all other reagents equilibrate at room temperature for 30 min while protected from light.
3. Clean the electrodes by pipetting approximately 350 μl of water in the electrode cleaning chip and putting it in the instrument for 60 s. Repeat it once.
4. Prepare the gel by pipetting 650 μl (complete volume of one tube) of small RNA gel matrix into a spin filter and centrifuging it at 10,000 × *g* for 15 min (*see* **Note 8**).
5. Add 40 μl of filtered gel to 2 μl of vortexed Small RNA dye concentrate in a new tube, vortex it briefly and centrifuge it at 13,000 × *g* for 10 min (*see* **Note 12**).
6. Put a Small RNA chip on the chip priming station and pipette 9 μl of gel-dye mix into the well marked with a white G in a black circle (*see* **Note 10**).
7. Lift the plunger to the 1 ml position, adjust the lever of the clip to the lowest position and close the priming station.

8. Press down plunger until it is arrested by clip and wait for exactly 60 s.
9. Release the clip and after 5 s slowly pull the plunger to its starting position (*see* **Note 11**).
10. Open the priming station and pipette 9 µl gel-dye mix in each of the wells marked with a black bold G.
11. Slowly pipette 9 µl of small RNA conditioning solution in the well marked with CS.
12. Load 5 µl of small RNA marker in all 11 sample wells and the ladder well.
13. Pipette 1 µl of RNA sample in each of the 11 sample wells and 1 µl of ladder into the ladder well. Pipette 1 µl of marker in every unused sample well.
14. Vortex the chip for 1 min at 2,400 rpm in the chip vortexer and start the run within 5 min on 2100 bioanalyser.
15. Analyze the run by checking relative miRNA content and general electropherogram progression.

3.4 Optional Test for qPCR Inhibitors: SPUD Assay

1. Prepare cDNA solution from 500 ng of total RNA according to the manufacturer instructions.
2. Prepare qPCR master mix according to the manufacturers instruction with 240 nM of each forward and reverse SPUD primer and 200 nM SPUD TaqMan probe.
3. Perform qPCR SPUD assay by measuring the SPUD amplicon in the presence of water (negative control with no inhibitors) and in the presence of your RNA samples. SPUD amplicon should be in the range of 20,000 copies per reaction to ensure high reproducibility and reliability of the fluorescence signal.
4. Analyze amplification plots and obtained Cq's. A higher Cq and lower amplification curve compared to the control sample indicates qPCR inhibitors in your RNA extractions.

3.5 Alternative Method for Total RNA Integrity Measurement: 18S/28S Ratio Agarose Gel Electrophoresis

1. Before preparing the gel, wash the gel chamber for 2 min in 0.1 M NaOH to destroy RNases. Thoroughly flush the chamber with water and let it dry.
2. Prepare the 1 % denaturing agarose gel.
3. Dilute 5 µg of each RNA sample and an appropriate volume of RNA marker with the same volume of sample buffer and incubate it at 65 °C for 10 min to completely denature all RNA.
4. Position the prepared gel in the electrophoresis chamber and fill it with 1× MOPS buffer (1:10 dilution of 10× MOPS buffer with nuclease-free water).
5. Load 10–20 µl (depending of the depth of the gel) of denatured samples in the pockets of the gel and let it run at 60 V

for approximately 1–2 h. Check the progress of the separation via the unspecific bromophenol blue band.

6. After separation RNA is visualized with the UV transilluminator at 256 nm. Determine RNA integrity by comparing rRNA band sizes and fluorescence levels as well as any possible RNA fragments that will appear as an unspecific smear with lower nt sizes.

4 Notes

1. Agarose dissolves at around 36 °C. If you use a microwave for heating, pay special attention to possible boiling retardation.
2. Adding the formaldehyde too early can cause it to evaporate and create toxic fumes.
3. Prepare shortly before loading the samples on the gel to guarantee optimal results.
4. If you are concerned with ethidium bromide toxicity, you could also use a replacement like GelRed (Biotium, Hayward, USA).
5. Pay special attention to any shifts of the maximum absorption caused by impurities. Contaminated samples sometimes appear to have a normal spectrum but at a closer look have their maximum absorption at for example 270 nm.
6. If not denatured, secondary structures of RNA will compromise the size separation during electrophoresis.
7. If you are interested in RNA concentration measurement via 2100 Bioanalyzer do not dilute your samples. Be aware that higher concentrations than 500 ng/μl can impair the performance of the chip and are not recommended by the manufacturer.
8. Filtered gel can be stored at 4 °C for approximately 4 weeks.
9. Gel-dye mix lasts for two chips but should be used on the day of preparation.
10. When pipetting on the chip make sure to pipette directly to the bottom of each well and avoid air bubble formation at all costs.
11. If the plunger is not rising on its own after releasing the clip, check if the priming station was closed properly and if the sealing ring inside the station is undamaged. Incompletely primed chips can sometimes be salvaged by priming them a second time.
12. Careful pipetting is strongly recommended due to high viscosity of the gel.

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