Romanian Reports in Physics, Vol. 68, No. 3, P. 1178–1188, 2016

A COMPARATIVE ANALYSIS OF THREE METHODS USED FOR RNA QUANTITATION

SORINA SCHIPOR[1], SUZANA VLADOIU[1], ANCUȚA ELENA BACIU[1,2], ANA MARIA NICULESCU[3], ANDRA CARAGHEORGHEOPOL[1], IULIA IANCU[4], ADRIANA PLESA[4], A. I. POPESCU[2], DANA MANDA[1]

[1] "C. I. Parhon" National Institute of Endocrinology, Bucharest, Romania
[2] University of Bucharest, Faculty of Physics, P.O. Box MG-11, Magurele, Romania
[3] Agilrom Scientific, Bucharest, Romania
[4] "Stefan S. Nicolau" Institute of Virology, Bucharest, Romania

Received July 27, 2014

Abstract. The high RNA sample quality is essential for downstream molecular biology applications. Two simultaneous conditions should be accomplished by the RNA samples: the structural integrity of the molecules and an adequate concentration. The objective of this study is to do a comparative analysis between three different methods of measuring RNA concentration. The three methods considered here are: UV spectrophotometry, spectrophotofluorimetry, and microfluidic capillary electrophoresis. Sixteen RNA samples were assayed by these three methods. The principles and the results of each method as well as advantages, disadvantages and perturbations factors are analyzed and discussed. Fluorescent labelling of RNA gives more accurate results even in the presence of the frequent contaminants like DNA and proteins. Knowing the strength and the limits of each method required for RNA quantitation, the scientist can choose the most cost-effective protocol.

Key words: RNA quantitation, UV spectrophotometry, spectrophotofluorimetry, microfluidic capillary electrophoresis.

1. INTRODUCTION

RNA quantification is an essential step for downstream molecular biology applications, RNA-based like gene expression analysis, RNA-sequencing (transcriptome profiling using deep-sequencing technologies), and RNA interference (an endogenous post-transcriptional gene regulatory mechanism mediated by non-coding RNA molecules known as microRNAs). There are several techniques used to determine RNA concentration, purity and integrity: ultraviolet spectroscopy, fluorescence, agarose and acrylamide gel electrophoresis, on-chip electrophoresis, reverse transcription coupled with real-time polymerase chain reaction (PCR) or inductively coupled plasma-optical emission spectroscopy (ICP-

OES). The difference between them is the concentration range, the sample volume needed for analysis, and the cost.

Laboratory infrastructure requirements need to be evaluated depending on types of samples, concentration range and further applications, as well as available financial resources for RNA quantification.

Here we evaluated and compared three of most popular RNA quality control methods: ultraviolet spectrophotometry, fluorescence-based quantification, and on-chip electrophoresis. Ultraviolet absorbance measurement was carried out on a Nanoquant Infinite Pro-M200, the fluorescence-based technique was performed using Qubit™ RNA Assay Kits on Qubit® 2.0 Fluorometer, and on-chip microfluidic capillary electrophoresis was carried out on the Agilent 2100 Bioanalyzer associated with the RNA 6000 Nano kit.

The principle of the **UV absorbance method** (*i.e.*, spectrophotometry) consists in the existence of a specific absorbtion peak (at 260 nm) of conjugated double bonds both in purine and pyrimidine rings of nucleic acids (DNA or RNA). The intensity of peak is proportional to the concentration of nucleic acid [1]. The extinction coefficients of nucleic acids are the sum of the extinctions of each of their constituent nucleotides. For large molecules it is impossible to sum up the coefficients of all nucleotides and an average extinction coefficient is used. For double-stranded DNA (dsDNA), the average extinction coefficient is 50 $(\mu g/mL)^{-1}$ cm^{-1}; for single-stranded DNA (ssDNA) and RNA, the values 37 and 40 $(\mu g/mL)^{-1}$ cm^{-1}, respectively, are used [2], although these values are under debate [3].

Infinite® M200 PRO NanoQuant is a microplate reader developed for absorbance applications with small sample volumes. This spectrometer uses Quad4 Monochromators technology and can detect DNA concentrations as low as 1 ng/μL and is compatible with Tecan's patented NanoQuant Plate™.

The **fluorescence method** uses a fluorometer and RNA-binding fluorescent dye that specifically binds to single-stranded RNA (ssRNA) [4]. The fluorescence intensity is proportional to the amount of binding dye, so the RNA concentration of samples can be calculated by comparison with a standard curve generated by a reference solution of known RNA concentration.

The Qubit® 2.0 Fluorometer is a benchtop fluorometer for the quantitation of DNA, RNA, and proteins, using the Qubit™ assays that contain advanced dyes specific for DNA, RNA, or proteins. This specificity allows getting very accurate results because Qubit™ technology only reports the concentration of the molecule of interest and not of the contaminants. Detection technologies used in the Qubit® 2.0 Fluorometer help attaining the highest sensitivity using 1 μL of sample still achieving high levels of accuracy, even in the case of very diluted samples [5].

On-chip microfluidic gel electrophoresis with the 2100 Bioanalyzer system enables rapid, accurate and reproducible analysis of the integrity and quality of RNA, DNA and protein samples and an estimation of sample concentration, using

small volume sample. It is the recommended method for quality control of RNA and/or DNA prior to downstream analysis like quantitative Real Time PCR, microarray, reverse transcription, next generation sequencing, etc. On-chip microfluidic electrophoresis is based on the traditional gel electrophoresis principles transferred to a chip format. Each micro fabricated chip contains separate wells (for samples, gel and the external standard – ladder) and an interconnected set of micro-channels used for separation of nucleic acid fragments based on their size as they are driven electrophoretically through it. During chip preparation, the micro-channels are filled with a sieving polymer and a fluorescence dye, the chip becoming an integrated electrical circuit. The 16-pin electrodes of the electrophoresis cartridge are arranged so that they fit into each wells of the chip. Charged RNA biomolecules are electrophoretically driven by a voltage gradient. Depending on the constant mass-to-charge ratio and the presence of polymer matrix, the molecules are separated by size. Dye molecules intercalate into RNA strands and these complexes are detected by laser-induced fluorescence (LIF). The data are translated by the *Bioanalyzer Expert Software* into gel-like images (bands) and electropherograms (peaks). With the help of a ladder that contains fragments of known sizes and concentrations, a standard curve of the migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the fragment sizes are calculated.

For RNA assays, the estimation of RNA concentration is done based on the area under the peaks of the ladder. The area under the ladder peaks is compared with the sum of the sample peak areas. For total RNA assays, the ribosomal peaks are identified. The ribosomal ratio is calculated and the integrity and the quality of the RNA sample are determined.

The *2100 expert software* plots fluorescence intensity *versus* migration time and produces an electropherogram for each sample. The data can also be displayed as a densitometry plot, creating a gel-like image.

2. MATERIALS AND METHODS

TISSUE SAMPLING. Thyroid samples were collected from patients who underwent a surgery in the Surgery Department of the National Institute of Endocrinology "C. I. Parhon". Tumoral and peri-tumoral pieces of 50–100 mg were put in RNA*later* ®(Sigma Aldrich), stored over night at 4 ºC and then at –80 °C until RNA extraction will be performed.

RNA EXTRACTION. Total RNA from 8 patients (2 samples for each patient from tumoral and peri-tumoral tissue, respectively) was extracted using the TRIzol (Invitrogen) reagent according to the manufacturer's instructions. Briefly, tissue was homogenized in 1 mL TRIzol Reagent, incubated 5 min at room temperature.

For phase separation, 0.2 mL chloroform was added, incubated 2 min and centrifuged at 12,000 g, 15 min at 4 ºC. The aqueous phase that contains RNA was transferred into a new tube for the RNA isolation procedure. RNA was precipitated with isopropanol, incubated 10 min at room temperature and centrifuged at 12,000 g, 10 min at 4 ºC. The pellet containing RNA was washed with 1 mL ethanol 75 %. After a centrifugation at 7,500 g, 5 min, at 4 ºC, the pellet was air dried. RNA was resuspended in RNase free water (Roche).

RNA PURIFICATION. For microarray protocol we purified total RNA with RNeasy Mini kit (Qiagen) following manufacturer's protocol. Briefly, 100 µL of RNA solution are mixed with buffer and with 250 µL ethanol. The whole solution is transferred on RNeasy Mini spin column and centrifuged at 10,000 g, 15 min, at room temperature. RNA was retained on the column. The column is twice washed with 500 µL buffer. The RNA is eluted from the column in 30 µL RNase free water.

RNA CONCENTRATION MEASUREMENT. After RNA purification we used the three quantification methods to measure RNA concentration. We also used Bioanalyzer 2100 Agilent to determine RNA quality (RIN = RNA integrity number) a factor important in the microarray protocol.

The UV absorbance was measured on a Infinite Pro-M200 Nanoquant (Tecan) according to the manufacturer's instructions. Reference (or blank) was set with RNase free water in which the RNA was eluted in the final step of RNA purification. Then, 1 or 2 µL of each sample were taken for measurements. The software instrument was set to measure RNA. Absorbances (A) at 260 and 280 nm were collected. Concentration values and A260/A280 ratios were calculated by the Infinite software (i-control™).

The same RNA samples quality was checked by on-chip electrophoresis on *Agilent 2100 Bioanalyzer* following manufacturer's protocol. Agilent RNA 6000 Nano kits contain chips, spin filters and reagents (RNA 6000 nano gel matrix, RNA 6000 nano dye concentrate, RNA 6000 nano marker and RNA 6000 nano ladder) designed for analysis of total or messenger RNA fragments. All reagents were allowed to equilibrate to room temperature for 30 min before use and cautions were taken to prevent RNase contamination and all sources of dust or other contaminants. The RNA gel matrix was filtered by centrifugation at 1,500 g for 10 minutes at room temperature, using the spin filters supplied with the kit. 65 µL of filtered gel were mixed well with 1 µL of dye concentrate and centrifuged at 13,000 g for 10 min at room temperature. The prepared gel-dye mixture must be used within one day. The gel-dye mixture was loaded on the chip following manufacturer's protocol, using the chip priming station. 5 µL of the 6,000 nano marker were pipetted in all sample wells as well as in the ladder well. The marker contains two RNA fragments of known sizes and concentrations (upper and lower

marker) and serves for the alignment at RNA fragments. Before loading the samples and ladder on the chip, they are heat denatured for 2 min at 70 °C. At the end, 1 μL of ladder and 1 μL of each sample were pipetted in the appropriate wells. The prepared chip was vortexed for 1 min using the IKA vortex mixer equipped with the chip adaptor and loaded on the 2100 Bioanalyzer for analysis.

RNA concentration measurements by fluorimetry were done on *Qubit 2.0* fluorometer using Qubit® RNA Assay Kit. Briefly, 10 μL standards or diluted RNA were mixed with Qubit® Working Solution and incubated for 2 min. The two standards have 0 ng/μL and 10 ng/μL, respectively. The relative fluorescence is plotted against the two concentrations in order to obtain the calibration curve. Concentration values were calculated by the fluorometer built-in software.

STATISTICAL ANALYSIS. The linear dependence between each pair of methods was evaluated by a Pearson correlation and the 95 % confidence intervals were calculated. The concordance between methods (methods agreement) was evaluated by Passing-Bablok regression using MedCalc. vers. 13.2.2. *P* values below 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSIONS

The concentrations of solutions containing purified RNA measured by UV absorbance, spectrophotofluorimetry and on-chip microfluidic electrophoresis are presented in Fig. 1.

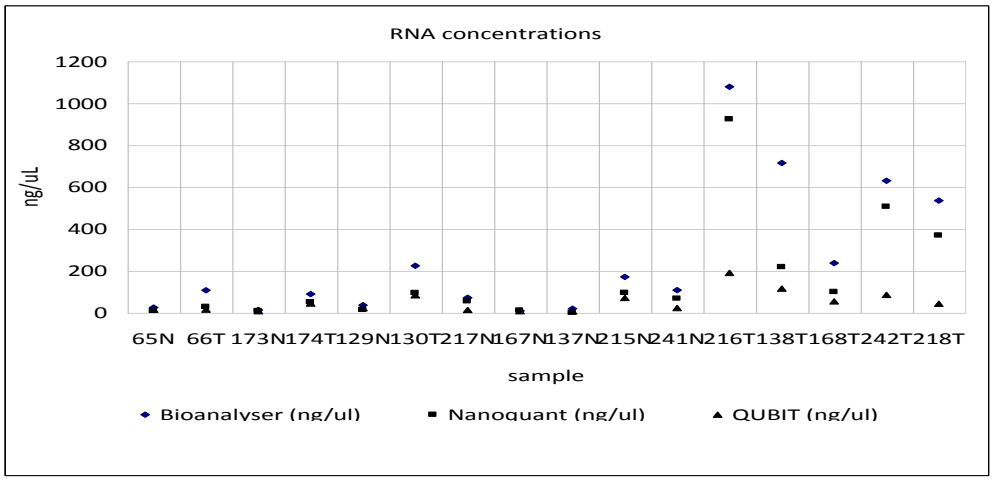

Fig. 1 – RNA concentration (ng/μL) values for every sample using Bioanalyzer, NanoQuant, and Qubit.

Scatter diagrams for regression analysis performed between NanoQuant and Qubit, Bioanalyzer and Qubit, NanoQuant and Bioanalyzer, are presented in Figs. 2A, 2B and 2C, respectively.

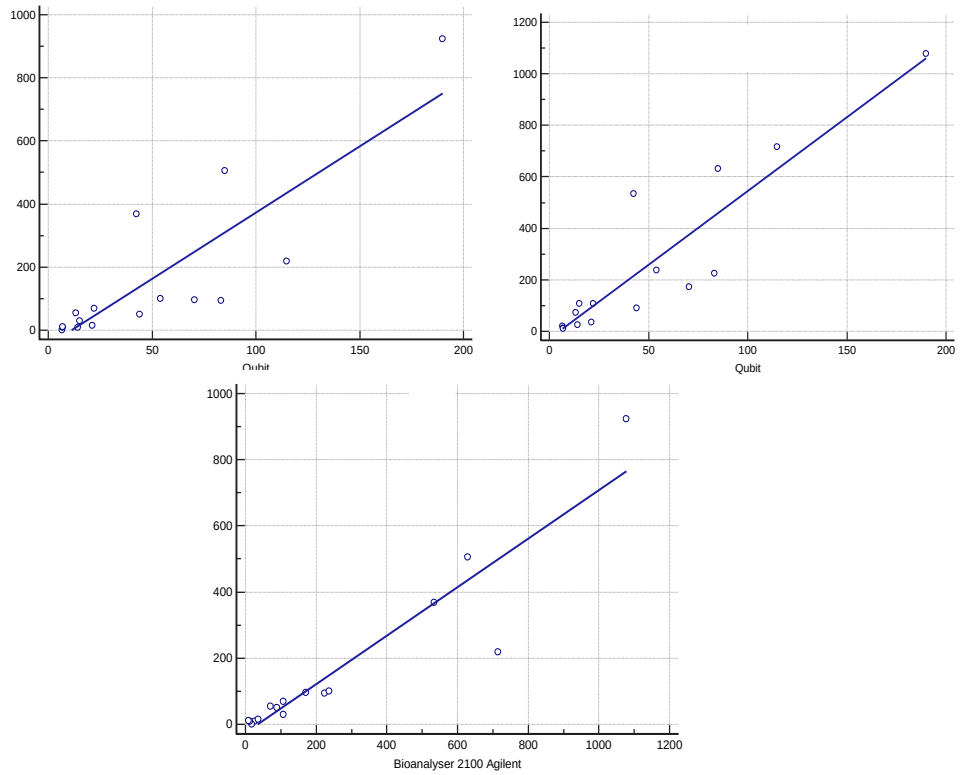

Fig. 2 – Scatter diagram of regression analysis performed between (A) NanoQuant and Qubit, (B) Bioanalyzer and Qubit, (C) NanoQuant and Bioanalyzer.

The results of simple regression analysis can be interpreted as a good correlation between measurements with important statistical significance (Table 1), but the correlation coefficient (*r*) and regression technique are inadequate and can be very misleading when assessing agreement between methods because they evaluate only the linear association of two sets of observations. Similarly R^2, the coefficient of determination, tells us only the proportion of variance that the two variables have in common.

Table 1

Comparison of the three methods by linear regression analysis

	R^2	r	95 % *C I* for r	Regression equation	*P* value
Bioanalyzer vs. Qubit	0.8271	0.9095	0.7534–0.9685	$y = -29.4690 + 5.7303x$	< 0.0001
NanoQuant vs. Qubit	0.7209	0.8490	0.6100–0.9464	$y = -48.9344 + 4.2064x$	< 0.0001
NanoQuant vs. Bioanalyzer	0.8740	0.9348	0.8183–0.9775	$y = -27.5510 + 0.7350x$	< 0.0001

R^2 = the square of the correlation coefficient, r = correlation coefficient, CI = confidence interval, *P*-value < 0.05 is considered significant

To overcome this issue we performed Passing-Bablok regression analysis to evaluate the method agreement. The results of Passing-Bablok analysis for measurement of agreement between Qubit, Nanoquant and Bioanalyzer for RNA are shown in Table 2 and Fig. 3 (A, B, C).

Table 2

Methods comparison by Passing-Bablok analysis

	Equation	95 % CI of intercept	95 % CI of slope
Qubit vs. Bioanalyzer 2100	$y = -32.079250 + 6.154179\ x$	–96.0000 to 11.7532	3.3248 to 8.5211
Qubit vs. NanoQuant	$y = -24.855414 + 3.552093\ x$	–83.86400 to 1.6844	1.6177 to 6.7200
NanoQuant vs. Bioanalyzer	$y = 9.844078 + 1.571864\ x$	–13.44437 to 22.6860	1.2075 to 2.4648

These results indicate that there are important differences between all three RNA quantification methods. According to Passing and Bablok (1985) if 95 % CI for slope has a value of 1 and for intercept a value of 0, then there is no significant difference between the two methods [6]. Results of Passing-Bablok analysis for comparability of Qubit, NanoQuant and Bioanalyzer indicate that this assumption cannot be applied for RNA. Differences between these 3 methods for RNA quantification arise from instruments' features, methods principles and sample quality (in terms of purity, integrity and DNA contamination). Thus, these three

methods for determination of RNA concentration in biological samples cannot be used interchangeably.

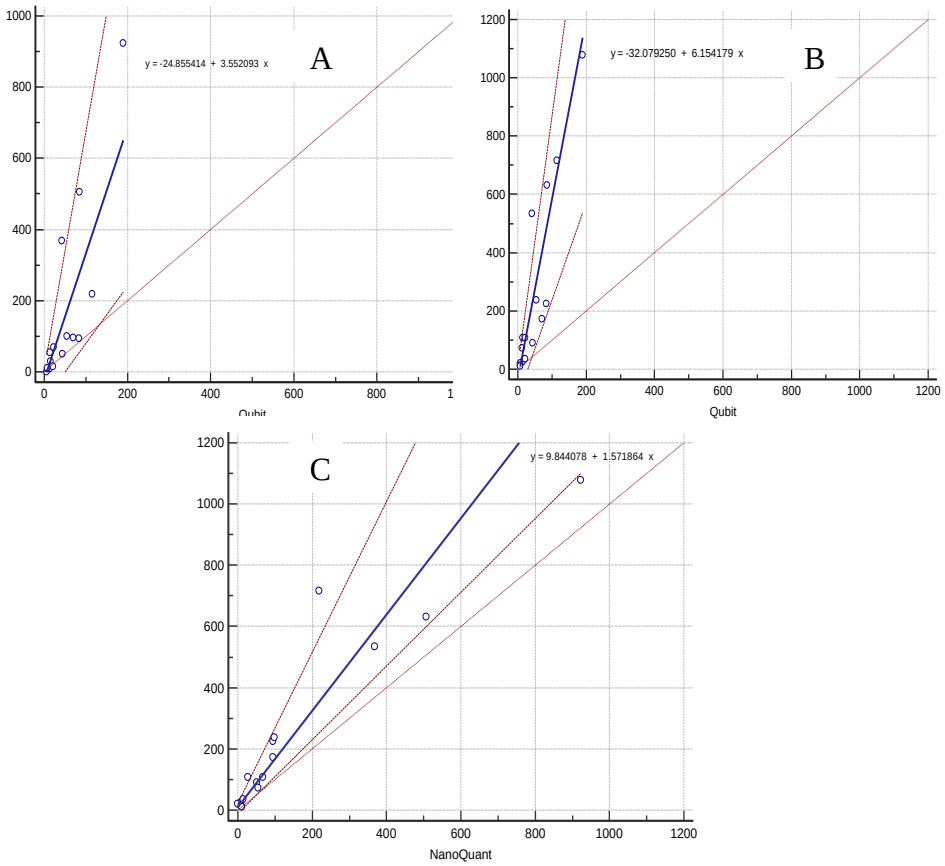

Fig. 3 – Passing-Bablok regression performed between (A) NanoQuant and Qubit, (B) Bioanalyzer and Qubit, (C) NanoQuant and Bioanalyzer.

The NanoQuant instrument can measure absorbance in the wavelength range of 230 and 1,000 nm and requires only 1–2 µL of sample for an accurate measurement. For RNA, the NanoQuant instrument detects a minimum of 2 ng/µL up to 3,000 ng/µL. Considering the wide range of concentration and small sample volume, there is no need for sample dilution and the analysis time can be less than 30 seconds. A great advantage of this technique is the total independence from reagents or accessories. Although UV absorbance at 260 nm is one of the most popular methods for nucleic acid quantification, it requiring extremely pure

samples of nucleic acids due to the interfering absorbance of contaminating molecules. Low-level nucleic acid samples are evaluated with a poor specificity and a lack of sensitivity because all nucleic acids (dsDNA, RNA and ssDNA) absorb at 260 nm, and the method is not capable of distinguishing between the various forms of nucleic acids. The A260/A230 ratio is used to estimate nucleic acid purity, but the amount of genomic DNA present in RNA preparation cannot be determined by absorbance. Also, if significant amounts of contaminants that absorb around 260 nm are present in a sample, they can contribute to the absorbance value, resulting in an overestimation of nucleic acid concentration [7]. For RNA samples with a certain degree of degradation due to the sample nature or sample handling and preparation the measurement is not accurate because single nucleotides also will contribute to the 260 nm reading.

The 2100 Bioanalyzer (Agilent Technologies) uses kits that are specific for RNA analysis in the range of 5–500 ng/μL (RNA 6000 Nano Kit) and 50–5,000 pg/μL (RNA 6000 Pico Kit).

This bio-analytical device is based on a combination of microfluidic chips, voltage-induced size separation in gel filled channels and laser-induced fluorescence (LIF) detection on a miniaturized scale. Twelve samples can be processed sequentially while consuming only very small amounts of each sample (1 μL). RNA molecules are stained with an intercalating dye and detected by means of LIF. Data are archived automatically and available as electropherograms, gel-like images, as well as in tabular format.

The most important feature of this instrument is its ability to measure RNA integrity or RNA Integrity number (RIN) [8]. To determine the RIN, the instrument software uses an algorithm that allows calculation of RNA integrity using a trained artificial neural network based on the determination of the most informative features that can be extracted from the electrophoretic traces out of 100 features identified by signal analysis. The selected features which collectively catch the most information about the integrity levels include the total RNA ratio (ratio of area of ribosomal bands to total area of the electropherogram), the height of the 18S peak, the fast area ratio (ratio of the area in the fast region to the total area of the electropherogram) and the height of the lower marker [9]. There is a scale for RIN ranging from 0 to 10 (maximum RNA integrity). The software also estimates RNA concentration by comparing peak areas of the ladder with RNA fragments of known concentration and peak areas of the unknown samples. Also, contamination with LW (low molecular weight) and HW (high molecular weight) genomic DNA can be checked with the 6000 nano assay on the Bioanalyzer.

The main disadvantage includes the lack of information on sample purity, especially protein contamination. If information about the amount of DNA or protein contamination is required, separate samples on specific DNA or protein chips would need to be run and analyzed. These unique and powerful features

come with a prohibitive instrument price to some labs, as well as reagents and new chip with every run.

Fluorescent dye-based RNA quantification [10] is based on specific dye binding to the nucleic acids that generates a conformational change resulting in increased fluorescence at a wavelength specific to the dye used. By measuring fluorescence against a reference standard, the value is converted in nucleic acid concentration using linear regression equation that best describes the standard curve. The great advantage of this technique is the sensitivity (as low as 1 pg/μL), a very important feature for RNA application in low-starting material. Qubit™ RNA Assay uses an RNA-specific dye and there is no need for a DNase treatment before measurement.

RNA molecule is highly exposed to degradation by RNase and all the extraction, preparation and handling steps alter RNA characteristics [11]. RNA concentration, purity, integrity and contamination by DNA are very important for downstream applications.

4. CONCLUSION

Identification of the main advantages and disadvantages of the analyzed methods for RNA quantitation is very useful in RNA analysis.

The results of this work confirmed that every used instrument has strengths and limits. Therefore the availability of all of them in a laboratory is an ideal situation (NanoQuant is frequently more used in routine measurement of DNA concentration, Bioanalyzer is widely recognized as a reference method for RIN measurement and Qubit is increasingly becoming more used for its specificity in DNA and RNA measurement analysis).

The comparison of the three RNA measurement methods demonstrated that they are not interchangeable and their use has to be correlated with downstream applications.

Acknowledgements. This work was supported by a grant PNII of CNDI – UEFISCDI, Project no. 135/2012 and strategic grant POSDRU/159/1.5/S/137750, "Project Doctoral and Postdoctoral programs support for increased competitiveness in Exact Sciences research" co-financed by the European Social Fund within the Sectoral Operational Program Human Resources Development, 2007–2013.

REFERENCES

1. M. Ogur, G. Rosen, Arch. Biochem. **25**, 262 (1950).
2. M. De Mey, G. Lequeux, J. Maertens, S. W. De Maeseneire, W. Soetaert, E. Vandamme, Anal. Biochem. **353**, 198 (2006).
3. R. K. Hartmann, A. Bindereif, A. Schon, E. Westhof (Eds.), *Handbook of RNA Biochemistry*, 2008.

4. L. J. Jones, S.T. Yue, C.Y. Cheung, V. L. Singer, Anal. Biochem. **265**, 368 (1998).
5. Qubit® 2.0, Fluorometer user manual, Catalog no. Q32866 (2010).
6. W. Bablok, H. Passing, J. Automat. Chem. **7**, 74 (1985).
7. T. Okamoto, S. Okabe, Int. J. Mol. Med. **5**, 657 (2000).
8. A. Schroeder, O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, T. Ragg, BMC Mol. Biol. **7**, 3 (2006).
9. S. Imbeaud, E. Graudens, V. Boulanger, X. Barlet, P. Zaborski, E. Eveno, O. Mueller, A. Schroeder, C. Auffray, Nucleic Acids Res. **30**, e56 (2005).
10. L. J. Jones, S. T. Yue, C. Y. Cheung, V. L. Singer, Anal. Biochem. **265**, 368 (1998).
11. S. M. Hewitt, S. S. Badve, L. D. True, Clin. Cancer Res. **18**, 1524 (2012).