Volume : 3 | Issue : 3 | March 2014 • ISSN No 2277 - 8179

Evaluation of Three Approaches For The Measurement of Rna Integrity, Concentration and Purity in Tissues of Apricot Flower Buds

Horticulture

KEYWORDS : RNA measurement, spectrophotometry, fluorometry, bioanalyzer, mRNA

Aleš Eichmeier — Mendeleum - Institute of Genetics and Plant Breeding, Faculty of Horticulture in Lednice, MENDELU, Valtická 337, 691 44 Lednice, Czech Republic.

Tomáš Kiss — Department of Fruit Growing, Faculty of Horticulture in Lednice, MENDELU, Valtická 337, 691 44 Lednice, Czech Republic

Jana Čechová — Mendeleum - Institute of Genetics and Plant Breeding, Faculty of Horticulture in Lednice, MENDELU, Valtická 337, 691 44 Lednice, Czech Republic.

ABSTRACT

The RNA quantity and quality are basic factors for successful studies of gene expression. On absorbance and fluorescence based measurements are routinely used techniques for RNA measurement. A new technique for measurement of RNA is available with Agilent 2100 bioanalyzer in combination with the RNA 6000 Nano LabChip® kit. This method offers in addition the advantage of assessing RNA integrity. Agilent 2100 Bioanalyzer is compared with UV spectrophotometry and fluorometry quantitation systems assessing the quantity, purity and RNA integrity. This study was focused on comparison of RNA isolated from apricot flower buds which were evaluated by mentioned methods. Obtained results show important differences in RNA which was isolated in 2008 (stored at -80 oC) and RNA which was newly isolated from the same stored tissues in 2013. Study also compares techniques of mRNA quantity, quality and integrity evaluation.

INTRODUCTION

Initial quantity and quality of RNA are essential parameters for the success of further RNA analysis such as Real-Time PCR, RT-PCR, microarray analysis, sequencing, etc.. This requires precisely measured input material, particularly its integrity and quantity.

More methods can be used to quantify the RNA. The most common method is the measurement of absorbance at the wavelength of 260 nm (A260) using an UV spectrophotometer. It is a method where the sample is very easily prepared and for measurement does not need any additional reagents. The method has very good reproducibility. One of the main disadvantages is the inability of RNA/DNA discrimination because both of them absorb light at 260 nm, thus contamination of RNA with DNA and vice versa is not detectable. Another disadvantage is low accuracy when samples with concentration lower than 1 ng/µl are measured [4]. Sample contaminants such as phenol and proteins can be reveald by low A260/A280 ratio (<1,8).

To achieve higher levels of sensitivity and specifity of RNA quantification, metohods based on intercalation of fluorescent dyes to RNA are used. Fluorescence is measured using a number of fluorescence detectors such as microplate readers or spectrofluorometers. However, the data collected by fluorometry are not as reproducible as data generated by spectrophotometry, which is mainly caused by stirring mixes for flourometric analysis. Different kits for measurement of RNA and DNA are used. Contaminants such as phenol or proteins may cause imprecise and unreproducible data.

Agilent 2100 Bioanalyzer is a device based on fluorescent staining of nucleic acids. The system achieves separation of traveling nucleic molecules or proteins on the gel by microfluidic technology. Bioanalyzer uses for excitation of fluorescent dye lasers, thus achieving a high degree of sensitivity. Data is exported in the form of electrophoreograms such as gel-like image or spreadsheet format. The system exhibits slightly less reproducible data than UV spectrophotometry. Bioanalyzer is able to accurately quantify and detect contaminants and the consumption of the sample is only 1 µl.

This study compares three different approaches to determine the starting quality and quantity of RNA, which was isolated in two diferent terms from apricot flower buds tissue.

MATERIALS AND METHODS

Sample collection and storage

Apricot shoots with flower buds were collected weekly on experimental fields of Deparment of Fruit Growing, Faculty of Horticulture in Lednice, Czech Republic. For testing 3 apricot varieties – 'Stark Early Orange'-SEO, 'Vestar' and 'Betinka' were chosen. Sampling period covered the time before and after exit from endogenous dormancy [1]. Four samples from each cultivar were tested in this study.

Total RNA was isolated from 100 mg of flower buds using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO) in spring on 10th april 2008 for the first time. For the second time the isolation was done on 27th august 2013 from the same tissues which were stored in RNAlater® (Ambion, Life Technologies, Darmstadt, Germany) at -80 °C until RNA isolation. In first isolation RNA was diluted in 100 µl and in second second in 50 µl of buffer.

Table 1. Signature of collected samples and dates of sampling

sign	SEO	sign	Vestar	sign	Betinka
SEO5	3/1/2008	VESTAR2	13/12/2007	BETINKA4	27/12/2007
SEO6	10/1/2008	VESTAR3	20/12/2007	BETINKA5	3/1/2008
SEO7	17/1/2008	VESTAR4	27/12/2007	BETINKA6	10/1/2008
SEO8	25/1/2008	VESTAR5	3/1/2008	BETINKA7	17/1/2008

Reagents

Quant-iT™ RiboGreen RNA quantitation kit and reagents were supplied by Molecular Probes Inc., Eugene, OR. RNA 6000 Nano LabChip kits were provided by Agilent Technologies (Palo Alto, CA).

Instruments and software

Measurement of absobrance was performed by using BioTek PowerWave™ XS Microplate Reader (BioTek, Winooski, VT, USA). UV transparent 96 well microplates were used. Absobrance was measured at 260 and 280 nm. Data were analyzed by Gen5™ 1.0 Software (BioTek, Winooski, VT, USA).

Analysis of fluorescence was provided by Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA). All samples with added dye were incubated for 5 - 10 minutes. Evaluation of RNA concentration of samples was based on the extrapolation of obtained values to calibration curve.

For microfluidic measurements, Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) was used in combination with the RNA 6000 Nano LabChip® Kit (Agilent Technologies, Inc., Santa Clara, CA). Assays set in Agilent 2100 bioanalyzer were Plant total RNA Nano and mRNA Nano. Data analysis was performed in accordance with the Agilent protocol.

Measurement

All samples were premixed prior the measurement. All dilutions were made with TE buffer pH 8.0. Absorbance at 260 nm was assessed by UV spectrophotometer. Ribogreen analysis was performed by one cuvette system where into 100 µl volume 1 µl of sample was added, which was diluted 10 or 100 times in advance. Samples and RNA standards were prepared according to Molecular Probes Inc. instructions. For the analysis on Bioanalyzer, samples were run on 4 chips.

Formulas for calculation of RNA quantity (results in ng/µl):

1) *Spectrophotometry*
   Concentration = X/0.025*100
   X..... OD(260) – resulting measurement
   0.025....coeficient for RNA measurement
   100..... TE buffer pH 8.0 dilution of RNA
2) *Fluorometry*
   Concentration = X/1000*100*(1/10/100)
   X.....resulting measurement (ng/ml)
   1000....conversion from ml to µl
   100..... TE buffer pH 8.0 dilution of RNA
   1/10/100..additional TE buffer pH 8.0 dilution of RNA
3) *Agilent 2100 Bioanalyzer*
   Data are in required format, moreover electrophoreograms are provided by software.

RESULTS AND DISCUSSION

RNA Quantity

Results of RNA quantitification of all methods are denoted in Table 3. First measurement (samples from the first isolation) show significantly lower concentration than second measurement which is in accordance with the fact that RNA from first isolation was diluted in 100 µl and from second in 50 µl. Comparison of measurement methods provides surprisingly very various results. Average spectrophotometric measurement values of samples from the first isolation were 241 ng/µl, which is the lowest value of all measurements (Table 2), spectrophotometric measurement of samples from first isolation also shows the lowest standard deviation – 71, which confirms the balance of the method. When medians of measured values from first RNA isolation are compared, their range is between 242 – 406 ng/µl, where Bioanalyzer shows value in the middle – 311 ng/µl, between spectrophotometry (242 ng/µl) and fluorometry (406 ng/µl).

In the second measurement fluorometry showed the lowest standard deviation – 306 ng/µl, whilst Bioanalyzer showed the highest – 1329 ng/µl Medians of measured values ranged from 334 ng/µl (fluorometry) to 724 ng/µl (Bioanalyzer), spectrophotometry median lied in between – 624 ng/µl. The higher concentration of second measurement was expected due to dilution of total isolated RNA in 50 µl amount of TE buffer pH 8.0, whereas in first isolation total isolated RNA was diluted in 100 µl of TE buffer pH 8.0.

Table 2. Statistical evaluation of used methods in two measurements

	Measurement 1			Measurement 2		
	Spectro photometry	Fluoro metry	Bioa nalyzer	Spectro photometry	Fluoro metry	Bioanalyzer
Number of values:	12	12	12	12	12	12
Minimum:	137	143	166	192	113	106
Maximum:	428	716	527	2165	1060	3993
Average:	241	407	325	796	490	1329
Median:	242	406	311	624	334	724
Scatter:	5003	27 130	11 331	274542	93919	1417063
Standard deviation:	71	165	106	524	306	1190
Skewness:	1	0	0	1	0	1
Spikiness:	5	2	2	4	2	3

Table 3. Comparison of three techniques for RNA concentration measurement

	Sample	Spectro photometry (ng/µl)	Fluorometry (ng/µl)	Bioanalyzer (ng/µl) PLANT RNA nano
1. isolation	1SEO5	218	529	224
	1SEO6	197	246	221
	1SEO7	246	383	305
	1SEO8	428	619	527
	1VESTAR2	205	398	317
	1VESTAR3	155	183	213
	1VESTAR4	137	143	166
	1VESTAR5	259	501	367
	1BETINKA4	260	296	402
	1BETINKA5	237	458	459
	1BETINKA6	287	716	415
	1BETINKA7	257	414	279
2. isolation	2SEO5	773	700	1801
	2SEO6	1030	690	1705
	2SEO7	898	890	1840
	2SEO8	310	162	106
	2VESTAR2	264	177	158
	2VESTAR3	532	258	490
	2VESTAR4	594	450	957
	2VESTAR5	192	113	113
	2BETINKA4	761	410	1342
	2BETINKA5	654	215	343
	2BETINKA6	1378	760	3096
	2BETINKA7	2165	1060	3993

RNA Purity

The purity measured by spectrophotometer (A260/A280 ratio) [2, 4, 5, 6] is different than purity measured by Bioanalyzer (25S/18S rRNA ratio). A260/A280 ratio deals with presence of contaminants such as phenol and proteins, while 25S/18S ratio deals with quality of rRNA. Pure RNA has A260/A280 ratio of 2.1 and it depends on pH of used water for dilution. This may cause, that A260/A280 ratio is 1.5 – 1.8 when water with pH 5.4 is used but with TE buffer pH 8.0 will give a ratio of 2.0 [6]. Ratio under 1.8 indicates contamination by phenol and proteins mostly.

Purity from spectrophotometry of samples from first isolation ranged from 2.3 (sample 1SEO8) to 2.9 (1VESTAR4). Purity of samples is suitable for subsequent sensitive downstream applications, except maybe for 1VESTAR4 with ratio of 2.91 and 1VESTAR4 with ratio of 2.67, which ratios are too high. However protocols and literature do not explain the cause of higher than 2.1 ratios and all RNA samples are therefore considered as pure above this ratio. Concentration measurement in fluorometry is provided by fluorescence of highly specific fluorescent dye, which binds only to RNA, therefore it is not possible to evaluate the purity of RNA by fluorometry.

Purity and also the integrity of RNA (Figure 1) can be evaluated by Bioanalyzer. It measures rRNA ratio (25S/18S) as well. Samples from the first isolation had values of rRNA ratio from 2.0 (1VESTAR2) to 2.4 (1VESTAR4). The suitable range is 1.9/3.7 for plant cytoplasmatic RNA but for very sentitive techniques the ratio 25S/18S should be close to 2 [7]. RNA integrity number (RIN) is another parameter for RNA quality evaluation. RIN is a software tool designed to help scientists estimate the integrity of total RNA samples. This parameter forms a scale of 0 to 10 where the value 10 stands for RNA of excellent conditions and zero stands for totally degraded RNA. RIN of 5 might not

Volume : 3 | Issue : 3 | March 2014 • ISSN No 2277 - 8179

work for microarray experiments, but might work well for an appropriate RT-PCR experiment. Obtained RINs in the first isolation measurement were from 8.7 (1VESTAR4) to 9.1 (1SEO6).

Spectrophotometric purity measurement of samples from second isolation was in range from 1.8 (2VESTAR5) to 2.6 (2VESTAR6). The Bioanalyzer rRNA ratio 25S/18S was in range from 1.7 (2SEO8) to 2.1 (2BETINKA4). Isolates 2SEO8, 2VESTAR2, 2VESTAR5 2BETINKA7 had little bit lower values of 25S/18S ratio than is usuall. But RNA integrity number ranged from 8.6 (2BETINKA7) to 9.9 (1VESTAR2).

mRNA measurement

For evaluation of mRNA purity and contamination only Bioanalyzer is suitable with its specific assay - mRNA Nano. Samples from first isolation show almost no mRNA and very high level of rRNA contamination, from 44.7 to 50.7 %. Additional purification or next isolation is necessary for mRNA exploration.

Samples from second isolation show higher levels of mRNA than samples from the first isolation, with values ranging from 19.7 to 38.1 % of rRNA conatmination. Samples from second isolation confirmed better quality of RNA for downstream mRNA analysis but it is still not enough. [3] consider 10 % the highest level of rRNA contamination suitable for sensitive mRNA downstream applications and the curve must look similarly like in Figure 1. Comparing the two pictures of Figure 3 is clearly evident that RNA from the first isolation in 2008 contains almost no mRNA. This RNA, after more than 5 years, is not suitable for further analysis. In addition to gel electrophoresis, very high contamination of rRNA suggests to this result.

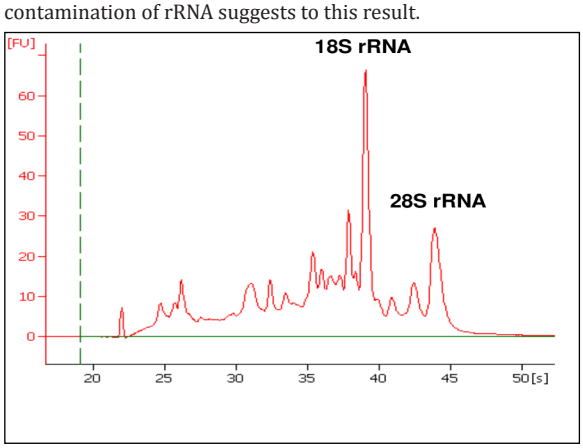

Figure 1. Agilent 2100 Bioanalyzer electrophoreogram, mRNA isolated from total RNA profile showing 18S rRNA and 28S rRNA peaks, there is about 10.0 % rRNA contamination mainly due to the presence of 18S and 28S rRNA [3].

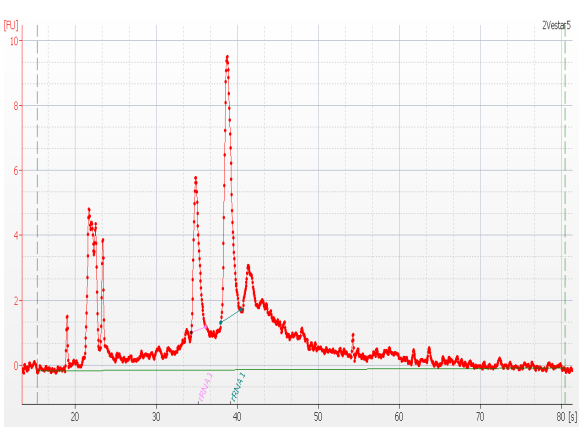

Figure 2. Agilent 2100 Bioanalyzer detailed electrophoreogram of 2VESTAR5. Contamination by rRNA was 19,7 %, which is the second lowest contamination of all samples.

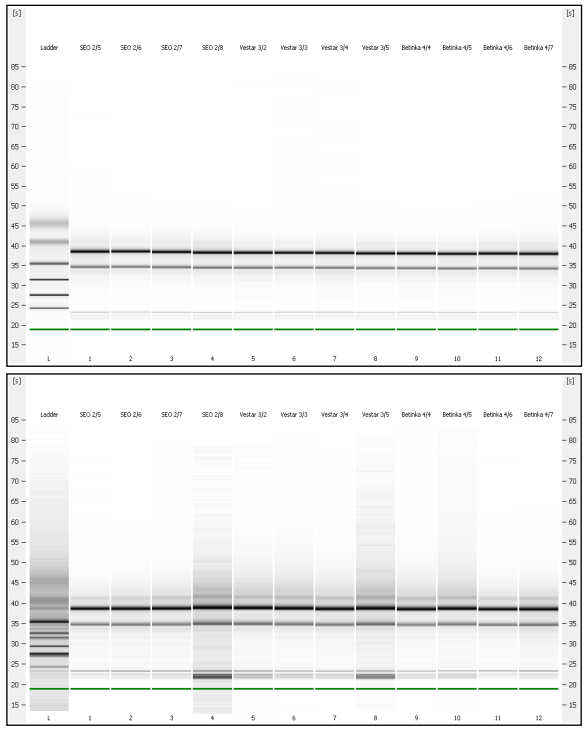

Figure 3. Gel electrophoresis on chip, mRNA assay nano, A –isolates from 1. isolation in 2008, B – isolates from 2. isolation in 2013

Conclusion

All three investigated methods allow quantification of RNA with certain level of accuracy and reproducibility. Surprisingly, very wide range of values of concentration was obtained. Interestingly, concentrations of samples from second isolation were significantly higher using Bioanalyzer than of other two established methods. Nevertheless, for assessments of RNA, like for example approximate concentration of RNA, any of the three methods is suitable.

But not all methods are suitable for evaluation of RNA purity or integrity. These characters of RNA are the most important for sensitive downstream applications, for example gene expression analysis. As a conclusion it can be stated that for total apricot flower buds RNA evaluation for assesing the purity the best solution is Bioanalyzer, because it provides all required parameters. Purity measured by spectrophotometry is the only method which shows contamination (phenol, protein) of RNA, which is essential for further analysis, because some chemicals can interfere and even inhibit these analysis. Best results of purity might show the combination of analysis on Bioanalyzer and on spectrophotometer.

The mRNA from the first isolation was degradated which was influenced by long term storage in -80 °C even when it was treated by RNaseOUT™ (Life Technologies, Darmstadt, Germany). Better quality data of total RNA and also mRNA was achieved in measurement of newly isolated (second isolation) RNA samples from tissues stored in RNAlater® (Ambion, Life Technologies, Darmstadt, Germany). Based on results from this study, if downstream analysis wouldn't be planned right after RNA isolation, better approach would be the storage of plant tissues treated by RNAlater® at -80 °C than storage of isolated RNA in the same temperature.

DEDICATION

To Mgr. Miroslav Baránek, Ph.D. for his valuable advices. Thanks for the provided plant material from Department of Fruit Growing.

REFERENCE

[1]Čechová, J., Baránek, M., Krška, B., Pidra, M. (2012) "Screening of differentially expressed genes during the end of endogenous dormancy of flower buds in Prunus armeniaca L.", Plant Growth Regulation, 67(2), 141-150. | [2]Glasel, J. (1995) "Validity of nucleic acid purities monitored by 260/280 absorbance ratios.", Biotechniques, 18(1), 62–63. | [3]Kumar, V., Katudia, K., Kumar, V. M., Srivastava, N., Kanak Vaidya, K., Chaudhary, S., Kumar Chikara, S. (2011), "An effective method for obtaining high quality messenger ribonucleic acid (mRNA) with minimal ribosomal ribonucleic acid (rRNA) contents.", International Journal for Biotechnology and Molecular Biology Research Vol. 2(8), 143-145. | [4]Manchester, K. L. (1995), "Value of A260/A280 ratios for measurement of purity of nucleic acids." Biotechniques,19(2),208-10. | [5]Warburg, O., Christian, W. (1942), "Isolation and crystallization of enolase.", Biochemische Zeitschrift, 310,384-421. | [6]Wilfinger, W. W., Mackey, K., Chomczynski, P. (1997), "Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity.", Biotechniques, 22(3), 474-6, 478-81. | [7]Xiang, C., and Brownstein, M. J.(2004), "Methods in Molecular Biology.", Vol 224, Functional Genomics: Methods and Protocols, Totowa, NJ: Humana Press, Inc. |