COMPARISON OF TWO AVAILABLE PLATFORMS FOR DETERMINATION OF RNA QUALITY

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ABSTRACT

The integrity of RNA is a very critical aspect regarding downstream RNA based quantitative analysis like RT-qPCR. Low-quality RNA can compromise the results of such experiments. Today automated lab-on-chip capillary electrophoresis allows rapid RNA quality and quantity determination, e.g. 2100 Bioanalyzer (Agilent Technologies) and the Experion (Bio-Rad). Both platforms determine RNA quality using a numerical system which represents the integrity of RNA. The Bioanalyzer offers the RIN algorithm (RNA Integrity Number) on the Bioanalyzer 2100 and Bio-Rad developed a new Experion software version that offers an algorithm for calculating the RNA Quality Index (RQI).

The aim of this study was to compare both systems regarding sensitivity, reproducibility, linearity and the influence of individual tissue extractions and different chip runs on RNA quality and quantity determination.

Overall it was confirmed that both algorithms are very comparable and beneficial for the determination of RNA quality for downstream applications. The Experion showed slightly better results regarding reproducibility and absolute sensitivity, whereas the 2100 Bioanalyzer showed a higher linearity.

Introduction

The integrity of RNA is a very critical aspect regarding downstream RNA based analysis like microarray technology and real time RT-qPCR (1, 3, 5, 6, 8). Low-quality RNA can compromise the results of such experiments. To determine the purity of RNA, the OD_{260nm}/OD_{280nm} ratio can be taken into account (2) whereas this parameter only provides information about protein or phenol contamination and does not give appropriate and full information according RNA integrity. For decades the only way to determine the degradation level of RNA was the use of agarose gel-based electrophoresis, but this method is variable, inaccurate, time consuming and cost intensive.

To save time, costs and material, automated platforms for the determination of RNA quality play an important role. Currently two automated systems are available for the analysis of RNA quality and quantity, the Experion (Bio-Rad Laboratories, Hercules, CA, USA), and the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Both systems are based on an automated and miniaturized electrophoresis system, realized by Lab-on-chip technology. This includes a microfluidic system, combined with computer-controlled instruments and measurements and a software based report and analysis (4).

Both platforms determine RNA quality either by using the ribosomal 28S/18S ratio, or a numerical system which represents the integrity of RNA. Agilent Technologies offers the RIN algorithm (RNA Integrity Number) on the 2100 Bioanalyzer and Bio-Rad developed a new Experion software version that offers an algorithm for calculating the RNA Quality Index (RQI) (4, 7). The RIN and the RQI are based on a numbering system from 1 to 10, whereas 1 being the most degraded RNA profile and 10 being the most intact.

The aim of these experiments was to compare the 2100 Bioanalyzer and the Experion systems regarding sensitivity, reproducibility, linearity, and the influence of individual tissue extractions on RNA quality and quantity. Focus is on the comparative study concerning the RQI and RIN numbering system determination.

Materials and Methods

Tissue sampling

Samples of bovine kidney, muscle, blood, heart, intestine and liver were collected at the slaughterhouse. Pieces of 1g were put into RNA later (Ambion, California, USA), stored over night at room temperature and then stored at -80°C until RNA extraction.

Total RNA extraction

Total RNA from six bovine tissues (kidney, muscle, blood, heart, small intestine, liver) was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To show the influence of different extractions on RNA quality and quantity measurements RNA from each tissue was extracted six times. Thus 6 tissues by

6 extraction replicates (n=36 samples) were available for the later experiments. RNA samples were stored at -80°C until quality analysis.

Hardware

For microcapillary electrophoresis measurement, the Experion system was used in conjunction with the Experion RNA StdSens kit (Bio-Rad) and the Experion Software Version 3.0 and the 2100 Bioanalyzer with the RNA 6000 Nano Reagent kit (Agilent Technologies) and the Agilent Technologies 2100 Expert Software according to the manufacturer's instructions. Data Analysis was done according to the presetting of the software. No manual settings were used.

To prevent systematic handling errors, the assays on both platforms were performed in parallel at the same time and always by the same person.

Influence of repeated extractions and different chip runs

The first aim of this study was to show the influence of repeated extractions on RNA quality and quantity determination by the two platforms and to compare the results of the Experion and the 2100 Bioanalyzer with regard to the influence of different chip runs on the RQI and RIN algorithm output. Therefore the six samples of each tissue were measured four times to verify the influence of different chip runs on RNA quality and quantity results (in total n=4). Thawing of RNA samples occurred on ice and sample were stored on ice between the different runs on one day. Every chip composition was made and measured simultaneously in the Experion and the 2100 Bioanalyzer.

To verify the RNA quantity results that were obtained from both platforms, RNA concentration was simultaneously determined by UV measurement using the NanoDrop 1000 (Peqlab, Erlangen, Germany).

Sensitivity in various RNA quality ranges

The second aim of this study was to analyze and compare the sensitivity of the two platforms in various RNA quality ranges.

Therefore total cellular RNA of each tissue was degraded artificially by UV irradiation and a dilution series of degraded and intact RNA of the same RNA pool with 6 dilution steps (degradation 1, **Table 1**) was prepared in order to get RNA samples with different degradation levels, started from the identical transcriptome and mRNA distribution.

TABLE 1

Dilution series of degradation sub study 1

| High quality RNA (%) | 100 | 80 | 60 | 40 | 20 | 0 |
| Low quality RNA (%) | 0 | 20 | 40 | 60 | 80 | 100 |

For quality determination in RIN/RQI levels in the medial range a second dilution series with 11 dilution steps of heart and intestine samples was created (degradation 2, **Table 2**). The dilution series of each tissue was quantified four times for degradation 1 and two times for degradation 2. Every chip composition was made and measured simultaneously in the Experion and the 2100 Bioanalyzer.

To verify the RNA quantity results obtained from both platforms, RNA concentration was simultaneously determined by UV measurement using the NanoDrop 1000 (Peqlab).

Statistics

To find the level of influence of different extractions and different chip runs on RNA quality and quantity determination Two Way ANOVA calculation in Sigma Stat 3.0 (Systat Software GmbH, Erkrath, Germany) was employed. To determine the influence of degradation level on RNA quantity measurements Two Way ANOVA calculation in Sigma Stat 3.0 (Systat Software GmbH, Erkrath, Germany) was employed. Results showing $p < 0.05$ were regarded as statistically significant.

TABLE 2

Dilution series of degradation sub study 2

| High quality RNA (%) | 100 | 90 | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 | 0 |
| Low quality RNA (%) | 0 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |

TABLE 3

Influence of repeated extraction, chip runs and performance day on RNA quality results

	RIN			RQI		
	Extraction	Chip run	day	Extraction	Chip run	day
Kidney	**<0.001**	0.930	0.172	**<0.001**	0.452	0.236
Muscle	0.308	0.218	0.724	0.123	0.407	0.689
Blood	0.115	**0.037**	0.145	0.080	0.376	0.264
Heart	0.352	**0.008**	0.055	0.434	0.383	0.777
intestine	0.060	**0.008**	0.327	0.307	0.372	0.091
Liver	0.088	**<0.001**	0.470	**<0.001**	0.054	0.907

P-values of the Two Way Annova Analyses. Results with p<0.05 are marked in bold

To correlate the degradation level and the quality determination of the two platforms and the corresponding RIN and RQI values, linear regression analysis in Sigma Stat (Systat Software GmbH) was employed. Results showing $p<0.05$ were regarded as statistically significant. The coefficient of determination (R^2) describes how well the regression model describes the data. R^2 values near 1 indicate that the straight line is a good description of the relation between the independent and dependent variable.

Results and Discussion

Influence of repeated extractions and different chip runs

The influence of different extractions on RNA quality determination was significant ($p<0.05$) for kidney in both platforms and for liver in the Experion. The influence of different chip runs on RNA quality determination was significant for blood, heart, intestine and liver in the Bioanalyzer and for none of the tissues in the Experion. There is no significant influence on the results obtained on different days. P-values of the Two Way ANOVA analysis are listed in **Table 3**.

The mean RIN and RQI values for each single tissue including standard deviations are shown at **Fig. 1**. It can be observed that all data points are located near the expected grey ideal line (ideal line: RIN=RQI). Data points of tissues with lower RNA quality (kidney, liver, intestine) are located under that line and those of tissues with higher RNA quality (muscle, blood, heart) are located above the ideal line. Further it is shown, that the standard deviations related to the RIN are higher than those related to the RQI.
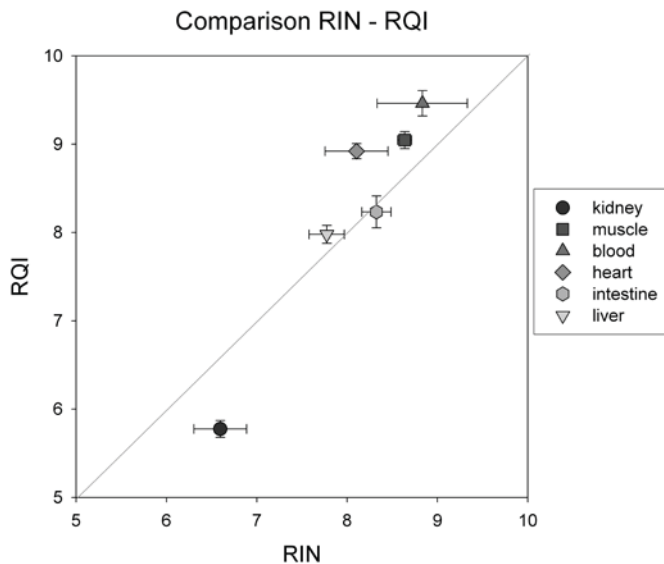

Fig. 1. Comparison of the corresponding RIN and RQI values

Mean values for each tissue ±SD are shown. The grey line (y=x) represents the actually expected situation, that the RQI is equivalent to the RIN

Sensitivity in various RNA quality ranges

The purpose of the second project was to analyze and compare the sensitivity of the two platforms in various RNA quality

ranges. Therefore, two dilution series with 6 or 11 linear dilution steps respectively were established to get total RNA in different degradation steps from degraded to intact total RNA.

The results of the experiment with six degradation steps in all 6 tissues are shown at **Fig. 2**. Each spot represents the correlation between RIN and RQI for each measurement. The black line represents the ideal situation that RIN=RQI. Linear equations for each tissue are included. The slope of the graph for all tissues except for kidney is nearly 1, representing an optimal relation. In most cases, kidney samples show lower RIN than RQI levels. Kidney samples also show the worst R^2 value (**Fig. 2**).
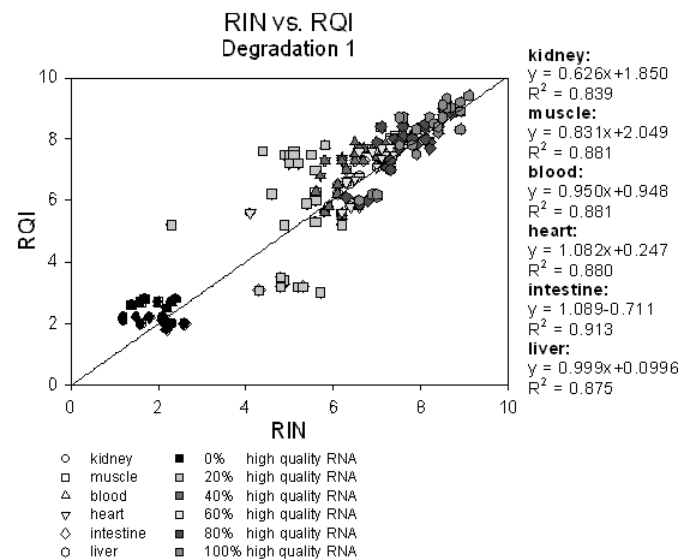

Fig. 2. Comparison of the corresponding RIN and RQI values of degradation sub study 1 using 6 dilution steps

RIN values were plotted against the associated RQI values for all RNA quality measurements of six degradation steps for six tissues
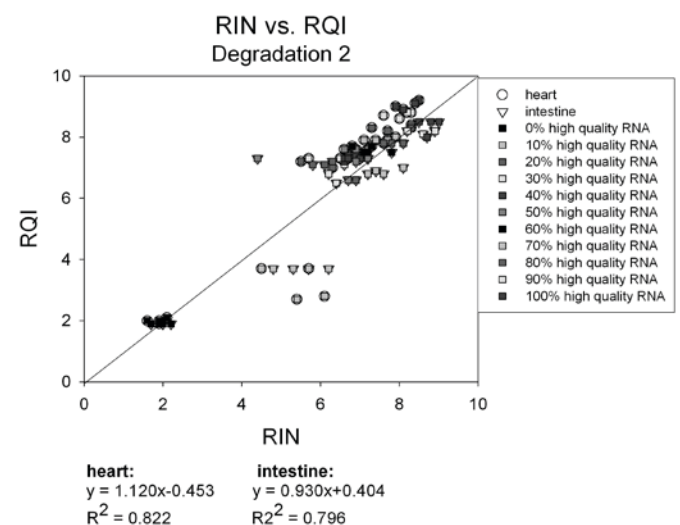

Fig. 3. Comparison of the corresponding RIN and RQI values of degradation sub study 2 using 11 dilution steps

RIN values were plotted against the associated RQI values for all RNA quality measurements of eleven degradation steps for heart and intestine

Another observation was made, that both platforms clearly show a lack of any RIN or RQI values in the area between 3 and 5.

To get a more differentiated picture of the degradation and to fill the missing data of the previous degradation sub-study, a second degradation experiment with eleven linear degradation steps was performed in heart and intestine total-RNA. The aim was to close the lack of RNA quality values in the middle RIN/RQI area by this way. The results presented in **Fig. 3** show that there are still values missing in the low area.
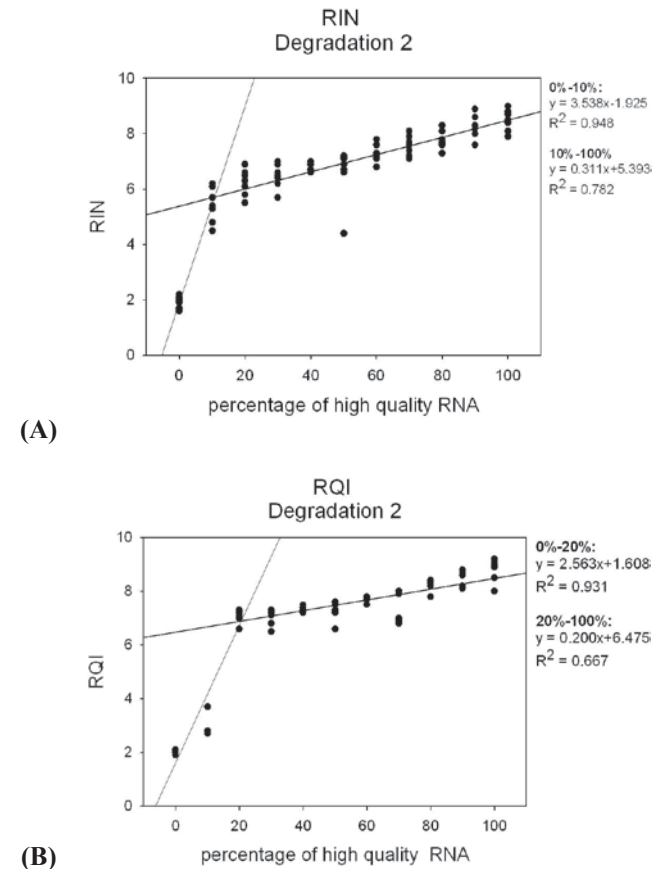

RIN
Degradation 2

0%-10%:
$y = 3.538x-1.925$
$R^2 = 0.948$

10% -100%
$y = 0.311x+5.393$
$R^2 = 0.782$

(A)

RQI
Degradation 2

0%-20%:
$y = 2.563x+1.608$
$R^2 = 0.931$

20%-100%:
$y = 0.200x+6.475$
$R^2 = 0.667$

(B)

Fig. 4. Linear regression of degradation sub study 2

RIN (A) or RQI (B) values were plotted against the percentage of high quality RNA and linear regression lines were added. Linear equations of both regression lines are shown

Quality data obtained in the second degradation experiment are shown in **Fig. 4**. Regarding the RIN and RQI values it can be observed, that the RIN shows a constant slope between 100% and 10% of high quality RNA content and with 0% high quality RNA the values clearly drop down. The RQI shows a constant slope between 100% and 20% of good RNA content and with 10% high quality RNA the values clearly drop down. The shown linear regression analysis was done in two steps. Between 10% and 100% and 0% and 10% of high quality RNA for the RIN and between 20% and 100% and 0% and 20% of high quality RNA for the RQI (**Fig. 4**). Both platforms show a linear but low dynamic and flat slope in the higher quality

area and a more rapid slope and very high dynamic only in the lowest small RNA quality area. A full linear behavior through all degradation steps was not given.
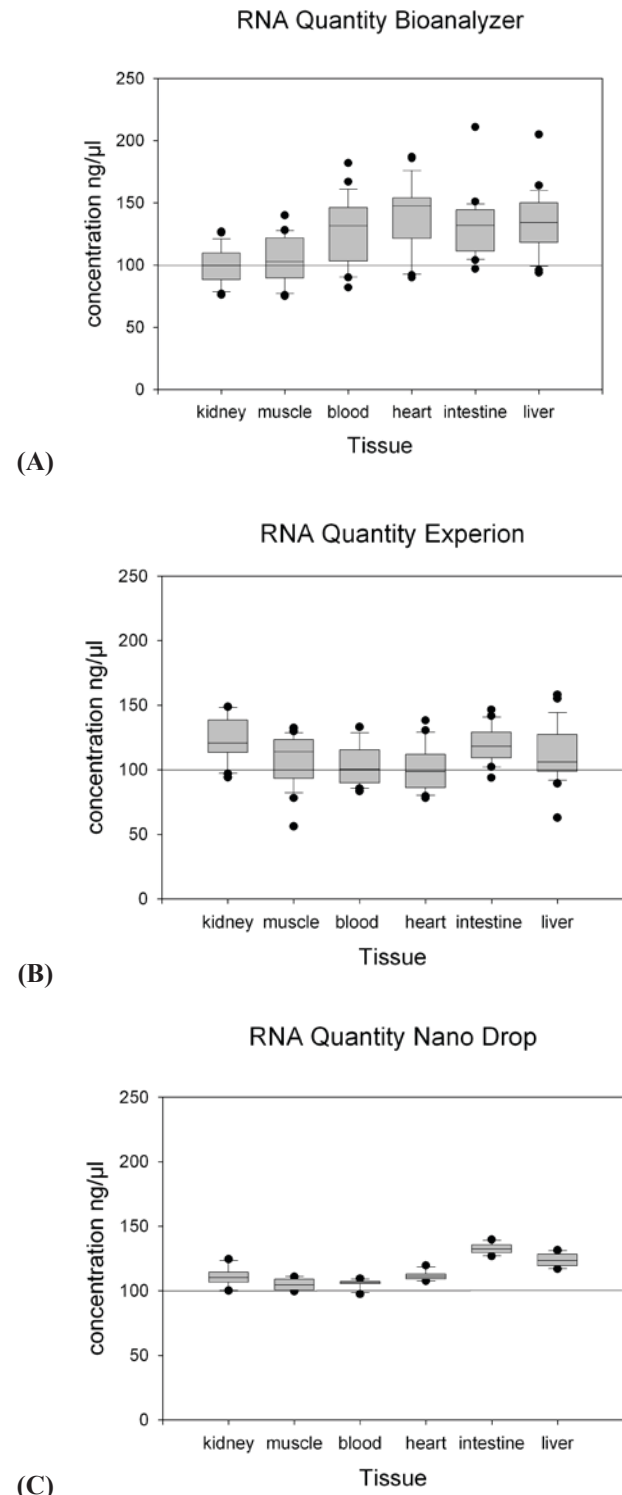

RNA Quantity Bioanalyzer

(A)

RNA Quantity Experion

(B)

RNA Quantity Nano Drop

(C)

Fig. 5. Reproducibility of RNA quantity measurement

Box plots of RNA quantity results of each tissue obtained by the Bioanalyzer (A), Experion (B), and the NanoDrop 1000 (C)
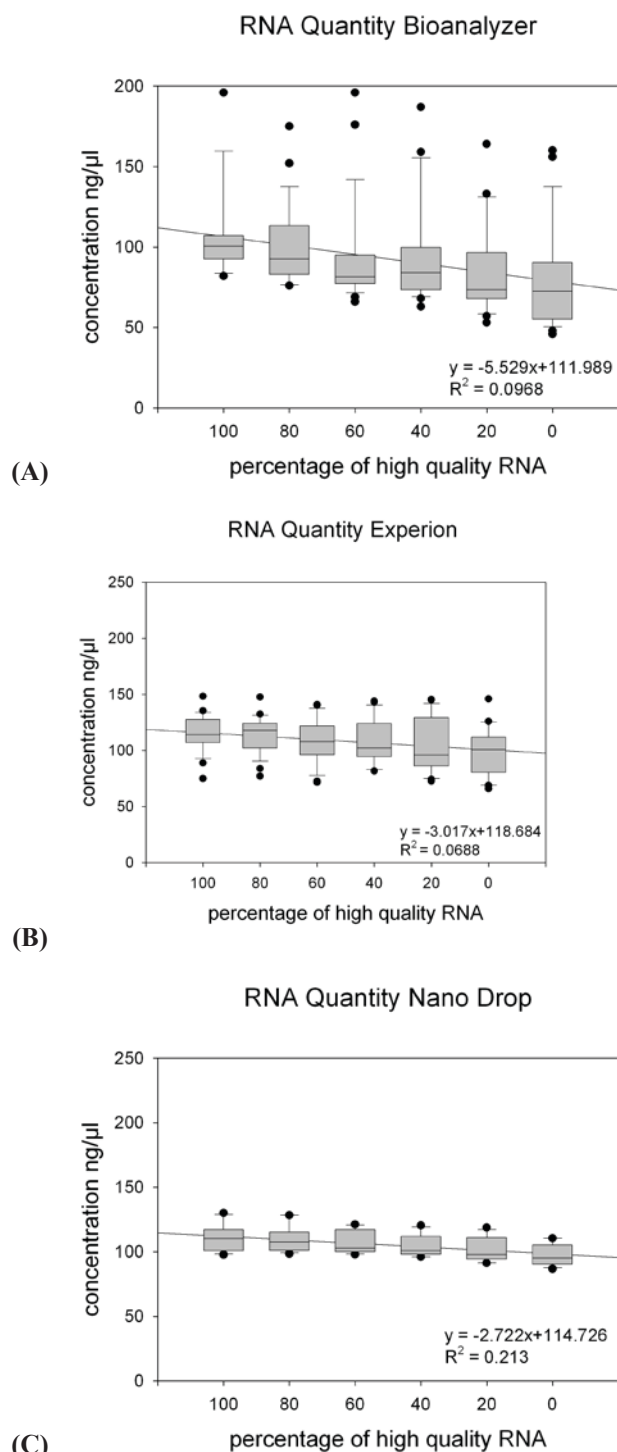
RNA Quantity Bioanalyzer

(A)

$$y = -5.529x + 111.989$$
$$R^2 = 0.0968$$

RNA Quantity Experion

(B)

$$y = -3.017x + 118.684$$
$$R^2 = 0.0688$$

RNA Quantity Nano Drop

(C)

$$y = -2.722x + 114.726$$
$$R^2 = 0.213$$

Fig. 6. Sensibility of RNA quantity measurement

Box plots of RNA quantity results including linear regression lines and linear equations of each degradation step obtained by the Bioanalyzer (A), Experion (B), and the NanoDrop 1000 (C)

Determination of RNA Quantity

Another feature of both platforms is the determination of RNA Quantity. For the verification of the RNA quantity results that were obtained by the Experion or the 2100 Bioanalyzer, RNA

concentration was in parallel determined by UV measurement using the NanoDrop 1000 (PeqLab). The influence of different extractions on RNA quantity determination was not significant in all three used platforms, whereas the influence of repeated measurements was significant in the Experion ($p < 0.001$) and the Bioanalyzer ($p < 0.001$) and showed no influence on UV measurements done by the NanoDrop 1000 ($p = 0.30$). RNA quantity determination by all three platforms was significantly influenced by degradation level (Experion: $p = 0.008$; Bioanalyzer: $p = 0.04$; NanoDrop 1000: $p = 0.009$). **Fig. 5** and **Fig. 6** show box whisker plots with outliers of RNA quantity results obtained by the Bioanalyzer, the Experion and the Nano Drop 1000. **Fig. 5** shows that the variance of the results obtained by the NanoDrop 1000 is smaller than those of the other two platforms and that the Bioanalyzer mostly overestimates the real concentration. With increasing degradation levels lower RNA concentrations were measured by both capillary electrophoresis platforms. **Fig. 6** shows box plots of RNA quantity results of all three platforms including linear equations. Same results were retrieved for the RNA degradation sub study 2 (data not shown).

This study was designed to compare two lab on a chip platforms- the 2100 Bioanalyzer (Agilent Technologies) and the Experion (Bio-Rad), regarding sensitivity, reproducibility, linearity, and the influence of individual tissue extractions on RNA quality and quantity determination.

It could be shown that there was a significant influence of repeated extractions on RNA quality results in kidney for both platforms and for liver samples in the Experion. Kidney samples show the worst quality results (RIN: 5.2-8.1, RQI: 4-7.5) followed by liver samples (RIN: 7.4-8.2, RQI: 7.6-8.4). This indicates that the RNA quality is dependent on the extraction performance, and varies significantly between them. Regarding the influence of different chip runs it could be observed that significant influence was only present in the 2100 Bioanalyzer system and documented by higher standard deviations (**Fig. 1**). This indicates that the quality determination of the Experion platform seems more reproducible than that of the 2100 Bioanalyzer. This may be a consequence of the more automated chip setup and preparation system of the Experion which also includes an automated priming station.

Regarding the sensitivity it could be observed that RQI and RIN data are missing in the low RNA quality range. Therefore the second degradation experiment with 11 degradation steps was performed to close the lack of RNA values in the middle RIN/RQI range, but the lack was still present. Quality data were not linear and RNA quality data are missing as well in the range between 2.5-6.0 for RQI and 2.5-4.0 for RIN (**Fig. 3**). Regarding the RIN, it could be observed that there is a constant slope of the values between 10 and 100% of high quality RNA and only samples with highly degraded RNA show RIN values around 2. Between 20 and 100% of high quality RNA the RQI values show a lower slope than the RIN. RQI values already drop down at the two lowest quality RNA samples.

These results indicate sensitivity and linearity problems in both platforms on the used RIN and RQI algorithms, mainly in the lower and medial areas of the quality determination. The Experion overestimates RNA with medial quality.

In terms of RNA quantification it could be shown, that the quantity results showed high variability compared to the spectrometric measurement in the NanoDrop 1000. RNA concentrations that were determined in the degradation experiments showed that with lower RNA quality the calculated RNA concentration dropped down. These results show, as described earlier (8), that RNA quantification by the Experion and the Bioanalyzer is less accurate than by using UV measurement.

Conclusions

In conclusion, data obtained by the Experion show better results regarding reproducibility and absolute sensitivity, whereas the 2100 Bioanalyzer shows a higher linearity in the lower RNA quality range (RIN/RQI 3 to 5). Overall it was shown that both algorithms are very comparable and beneficial for the determination of RNA quality for downstream applications, like RT-qPCR or hybridization arrays.

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